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**THE ROLE OF THE INSULIN-LIKE GROWTH FACTOR 1
RECEPTOR (IGF-1R) IN NON-SMALL CELL LUNG CANCER
(NSCLC)**



*A thesis submitted to the University of Dublin, Trinity College
for the degree of*

DOCTOR OF PHILOSOPHY (Ph.D.)

by

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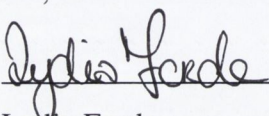
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Lydia Forde



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Summary

Lung cancer is the most common cause of cancer related death in Ireland and worldwide. Despite modest advances in traditional cytotoxic chemotherapy, survival rates remain disappointing at approximately 15%. Targeted therapies such as EGFR tyrosine kinase inhibitors (TKIs) provide some improvement in clinical outcomes. However the development of resistance to these drugs remains a major problem in the treatment of cancer. The rationale for investigating new targeted agents and the use of combination therapies is clear.

After the recent success of EGFR TKIs the IGF-1R has emerged as an attractive therapeutic target. Both receptors play an important role in cancer biology. When activated, together they promote tumour growth by inhibition of apoptosis, transformation, metastasis and induction of angiogenesis through the PI3K and MAPK signalling pathways. They are commonly overexpressed in many cancers including lung cancer and have been the focus of many studies over the last two decades. This study will provide us with a better understanding of the role of the IGF-1R in lung cancer and focuses on the crosstalk between the EGFR and IGF-1R signalling pathways.

Initially exons 16-20 of the IGF-1R tyrosine kinase domain were screened by Sanger sequencing for the presence of mutations or deletions, in the DNA of a cohort of 100 lung cancer patients and 100 controls. A synonymous SNP (rs2229765) was identified in exon 16. A further 100 lung cancer patients and 766 controls were analysed and the SNP was present in 53.5% and 49.4% respectively. There was no significant correlation between this SNP and patient survival, tumour histology or IGF-1R expression

Using immunohistochemical analysis the expression of IGF-1R and EGFR were examined in a cohort of 184 lung cancer patients who had undergone lung tumour resection surgery. The expression of either receptor alone is not prognostic. However overexpression of both receptors correlates to a poorer overall survival in lung cancer patients. The percentage of patient tissue samples with IGF-1R and EGFR expression was found to be 76.4% and 87.5% respectively. High coexpression of EGFR and IGF-1R was identified in 35% of the cohort of NSCLC patients and was associated with a poorer overall survival on univariate and multivariate analysis ($p=0.048$;

$p=0.05$ respectively). In a separate cohort of 40 lung cancer patients IGF-1R and EGFR expression was quantified in matched tumour and normal tissue by Western blot analysis. Tumour tissue expressed significantly higher levels of IGF-1R and EGFR when compared to normal tissue.

The IGF-1R has been previously shown to be a mechanism of resistance to EGFR TKIs. Targeting both the IGF-1R and EGFR, particularly in patients with high expression of both receptors, may prove to be an effective treatment strategy in overcoming resistance to either therapy alone. This study examined the effect of erlotinib (EGFR TKI) and R1507 (IGF-1R monoclonal antibody), alone and in combination in a panel of lung cancer cell lines. Treatment with both inhibitors reduced cell proliferation and induced apoptosis compared to treatment with either drug alone. Silencing of the IGF-1R through RNA interference also increased sensitivity to R1507 and erlotinib and reduced downstream signalling through the MAPK signalling pathway resulting in reduced cell proliferation.

Finally, the expression of the Insulin-Like Growth Factor-Binding Proteins 1-6 (IGFBPs 1-6) were examined in a panel of lung cancer cell lines and human bronchial epithelial cells (HBECs) under normoxia and hypoxia as well as in matched tumour and normal tissue from NSCLC patients. A549 cells had significantly higher expression of IGFBP1-6 than HCC827, H1819, H1299 and SKMES-1 cells. IGFBP-3 expression increased significantly in A549 and HBEC4 cell lines under hypoxia over 24 h ($P<0.05$). A decrease in IGFBP-5 expression was also observed in A549 cells under hypoxia over 24 h. There was a significant increase in mRNA expression of IGFBP-3 ($P=0.0009$), IGFBP-5 ($P=0.0189$) and IGF-1R ($P=0.0001$) in tumour compared to matched normal tissue. IGF-1 expression was significantly reduced in tumour compared to matched normal tissue ($P=0.0001$). IGFBP-3 protein expression was also found to be significantly increased in tumour tissue compared to matched normal tissue in NSCLC patients ($P=0.0001$).

In conclusion, this project demonstrated that lung cancer patients with high co-expression of both IGF-1R and EGFR have a poorer survival. Both the IGF-1R and EGFR are overexpressed in lung tumour tissue when compared to normal tissue. These data highlights the importance of targeting both the EGFR and IGF-1R

signalling pathways in the treatment of lung cancer patients. Dual receptor targeting may be an effective measure in overcoming resistance to EGFR TKIs.

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Abbreviations

Abs	absorbance
ADC	Adenocarcinoma
ANOVA	analysis of variance
APS	ammonium persulfate
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCP	1-bromo-3-chloro-propane
BrdU	bromo-deoxy uridine
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CO ₂	carbon dioxide
DAC	5-Aza-2'-Deoxycytidine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DH ₂ O	distilled water
DMEM	dulbecco's modified Eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
ECACC	European Culture and Tissue Collection
ECM	extra-cellular matrix
EDTA	ethylenediamine tetra acetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked ImmunoSorbent Assay
EMEM	Minimum essential medium eagle with earle's balanced salt solution
ETS	environmental tobacco smoke
EtOH	ethanol
FBS	foetal bovine serum
Fig	Figure
FS	first strand

H ₂ SO ₄	sulphuric acid
HBEC	human bronchial epithelial cell line
HCl	hydrochloric acid
IGFBP	Insulin-like growth factor binding protein
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor
LCC	Large cell carcinoma
MeOH	Methanol
Mg	Magnesium
mRNA	messenger RNA
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NaI	Sodium iodide
NSCLC	Non Small Cell Lung Cancer
NS	Non Signifiant
o/n	overnight
O ₂	Oxygen
P/S	penicillin streptomycin
PAGE	Polyacrylamide gel electrophoresis
PB	Phenylbutyrate
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PIC	protease inhibitor cocktail
PMSF	phenylmethysulfonyl fluoride
pO ₂	dissolved partial pressure of oxygen
PVDF	polyvinylidene fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
s/n	supernatant
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate

SDW	sterile distilled water
SFM	serum free media
SNP	single nucleotide polymorphism
TAE	Tris-Acetate-EDTA
TBS-T	Tris buffered saline-containing 0.1% Tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TNS	trypsin neutralising solution
TSA	Trichostatin A
UV	Ultra violet

Units

bp	base pairs
°C	degrees Celsius
g	grams
h	hour(s)
IU	International Units
μg	microgram
μL	microlitre
μM	micrometre
μM	micromolar
kB	kilobases
kDa	kilodaltons
kg	kilograms
hz	hertz
L	litre(s)
M	molar
mA	miliamp
mg	milligram
min	minutes
mL	millilitre
mM	millimolar
mm	millimetre
n	number (sample size)
nM	nanometres
pg	pictogram
rpm	revolutions per minute
s	second(s)
U	unit(s)
V	Volt
w/v	weight per volume
v/v	volume per volume

Presentations and Publications

Poster Presentations

IMM 2011. '*Expression, regulation and function of IGF-1, IGF-1R, IGFBPs in Non-Small Cell Lung Cancer (NSCLC)*'. Lydia Forde, Kathy Gately, Stephen Gray and Ken J O'Byrne. Dublin, Ireland.

Cancer 2011. '*Expression, regulation and function of IGF-1, IGF-1R, IGFBPs in Non-Small Cell Lung Cancer (NSCLC)*'. Lydia Forde, Kathy Gately, Stephen Gray and Ken J O'Byrne. Dublin, Ireland.

9th Annual British Thoracic Oncology Group conference 2010. '*Targeting the IGF-1R signalling pathway in Non-Small Cell Lung Cancer (NSCLC)*'. Lydia Forde, Kathy Gately, Stephen Gray and Ken J O'Byrne. Dublin, Ireland.

IMM 2010. '*Targeting the IGF-1R signalling pathway in Non-Small Cell Lung Cancer (NSCLC)*'. Lydia Forde, Kathy Gately, Stephen Gray and Ken J O'Byrne. Dublin, Ireland.

8th Annual British Thoracic Oncology Group conference 2010. '*The IGF-1R pathway in NSCLC. Expression, Mutational Analysis & Activation by Hypoxia*'. Lydia Forde, Kathy Gately, Stephen Gray and Ken J O'Byrne. Dublin, Ireland.

13th World Conference on Lung Cancer 2009. '*IGF-1R Expression and Mutational Status in Non-Small Cell Lung Cancer (NSCLC)*'. Lydia Forde, Kathy Gately, Sinead Cuffe, E. Kay and Ken J O'Byrne. San Francisco, USA.

Cancer 2009. '*IGF-1R Expression and Mutational Status in Non-Small Cell Lung Cancer (NSCLC)*'. Lydia Forde, Kathy Gately, Stephen Gray, Sinead Cuffe, David Easty and Ken J O'Byrne. Dublin, Ireland.

Papers

Accepted Clinical Lung Cancer

Brief Report: A role for IGF-1R targeted therapies in SCLC?

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Chapter 1:

General Introduction

1.0 Introduction

1.1 Lung Cancer

1.1.1 Epidemiology

Lung cancer is one of the most common types of cancer in Ireland and worldwide, and is responsible for 12.5% of all new cancer diagnosis [1] [2]. It is also one of the highest in terms of incidence (Table 1.1) and mortality with 1.6 million cases and 1.4 million deaths worldwide in 2008 [3]. It is the main cause of death in men and is the second most common cause of death (after breast cancer) in women [4], [5].

Tobacco plays a significant role in the development of lung cancer (Fig 1.1). The carcinogens found in tobacco smoke are the main cause of lung cancer, accountable for around 90% of lung cancer cases. In Ireland, around 25% of the population smoke which explains the high number of cases per year. Non-smokers who are diagnosed with the disease account for 5 to 15% of cases, and their disease is thought to be caused by factors such as radon, occupational carcinogens, diet, ionizing radiation or a pre-existing non-malignant lung disease [3].

The overall 5 year survival rate for lung cancer is 13.7% in the United States, 7.8% in developing countries, 7% in Eastern Europe, 7.9% in China, and 6.7% in India. Outcome depends on stage, overall health and other clinico-pathological parameters at diagnosis. Deaths from lung cancer were estimated at 90% in men and over 80% in women in the United States [6].

The National Cancer Registry Ireland (NCRI) report in 2011 stated that on average 29,745 cancer cases were registered annually during the three year period 2007-2009. This figure is a 12% increase from the annual average over the previous three years (2004-2006). (<http://www.ncri.ie/pubs/pubfiles/AnnualReport2011.pdf>). Incidence was higher in men than in women with a risk factor of approximately 1 in 3 for men and 1 in 4 for women (excluding non-melanoma skin cancer). Since the mid 1990s, the number of all cancer cases diagnosed annually has risen by almost 50% according to the NCRI. Ireland has a higher overall cancer incidence compared with the rest of Europe, however Irish cancer mortality rates are similar to the EU average. The NCRI also reported that lung cancer 5 year survival has increased from 8% to 11% between 1994 and 1997 and 2003 to 2007

(<http://www.ncri.ie/pubs/pubfiles/AnnualReport2011.pdf>). In Ireland, almost 7,500 people die from lung cancer each year. Recent reports have revealed that lung cancer has now overtaken breast cancer as the main cause of cancer deaths in women.

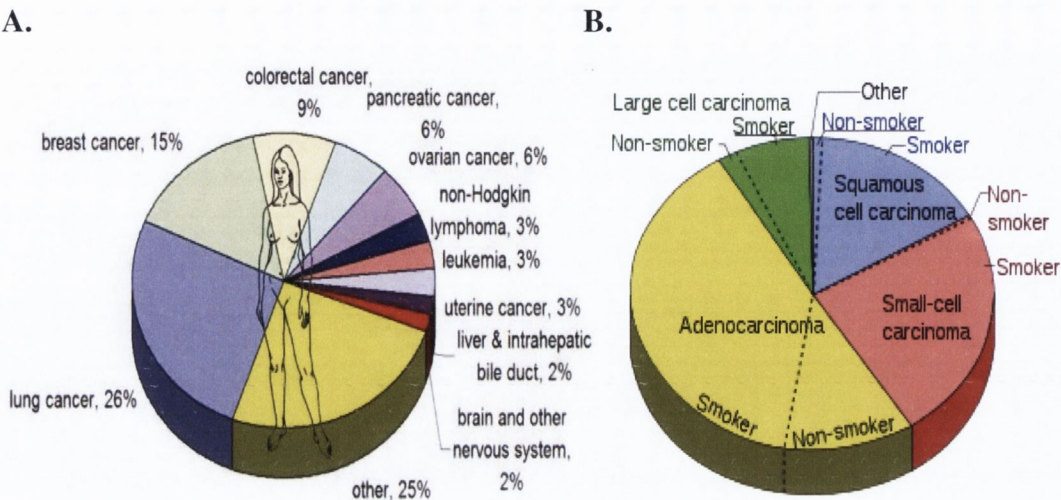


Fig 1.1 Cancer statistics 2008. Pie chart displaying the incidence of (a) lung cancer compared to other cancers and (b) histological subtypes [7].

1.1.2 Etiology

Over the past century, lung cancer has changed from being an epidemic disease among men in industrialized regions in the world, to affecting both men and women in developed and developing countries [8] [9]. While other agents have also been confirmed as lung carcinogens, tobacco smoking was identified as the main cause of lung cancer at least 60 years ago [10]. Lung cancer incidence has always mirrored smoking prevalence among the population. There is also evidence of a genetic association to the disease as molecular biology studies have identified several important genetic factors that contribute to an individual’s risk for lung cancer [11] [12].

Table 1.1: Annual average incidence for the main cancers diagnosed in Ireland, 2007-2009. Taken from National Cancer Registry Ireland (<http://www.ncri.ie/pubs/pubfiles/AnnualReport2011.pdf>).

cancer	cases			rate			risk %			% of all invasive cancers ^f		
	females	males	total	females	males	total	females	males	total	females	males	total
invasive cancers:												
head & neck	98	206	304	4.3	10.5	7.3	0.4	0.9	0.6	1.2	2.2	1.7
oesophagus	133	255	388	5.4	12.8	8.9	0.4	1.0	0.7	1.6	2.7	2.2
stomach	184	296	480	7.4	14.8	10.8	0.5	1.1	0.8	2.2	3.2	2.7
colorectal	943	1327	2270	40.0	66.6	52.2	3.1	5.1	4.1	11.4	14.3	12.9
pancreas	214	230	444	8.6	11.7	10.1	0.6	0.9	0.8	2.6	2.5	2.5
lung	782	1128	1910	33.8	56.6	44.1	2.7	4.3	3.5	9.5	12.2	10.9
melanoma	392	327	720	17.0	16.1	16.4	1.3	1.2	1.3	4.7	3.5	4.1
breast	2673	20	2692	125.4	1.0	64.6	10.0	0.1	5.2	32.3	0.2	15.4
cervix	287	—	—	12.9	—	—	1.0	—	—	3.5	—	—
corpus uteri	356	—	—	17.0	—	—	1.5	—	—	4.3	—	—
ovary	315	—	—	14.4	—	—	1.2	—	—	3.8	—	—
other gynaecological cancers ^g	99	—	—	4.2	—	—	0.3	—	—	1.2	—	—
prostate	—	2748	—	—	140.1	—	—	11.9	—	—	29.7	—
testis	—	166	—	—	6.8	—	—	0.5	—	—	1.8	—
kidney	156	278	434	7.0	13.8	10.3	0.6	1.2	0.9	1.9	3.0	2.5
bladder	134	309	443	5.4	15.5	9.9	0.4	1.0	0.7	1.6	3.3	2.5
brain & other CNS	139	192	331	6.2	9.4	7.8	0.5	0.8	0.6	1.7	2.1	1.9
all lymphomas	327	384	711	14.4	18.7	16.4	1.1	1.5	1.3	4.0	4.1	4.1
Hodgkin's disease	49	64	113	2.2	2.9	2.5	0.2	0.2	0.2	0.6	0.7	0.6
non-Hodgkin's lymphoma	279	320	598	12.2	15.7	13.9	1.0	1.2	1.1	3.4	3.5	3.4
multiple myeloma	87	117	203	3.6	5.9	4.7	0.3	0.5	0.4	1.0	1.3	1.2
leukaemia	150	252	402	6.5	12.4	9.2	0.5	1.0	0.7	1.8	2.7	2.3
non-melanoma skin (NMSC)	3345	3987	7333	140.7	199.5	167.0	10.2	14.2	12.2	—	—	—
other invasive cancers not listed	808	1027	1836	33.5	51.2	41.7	2.4	3.8	3.1	9.8	11.1	10.5
all invasive cancers, excluding NMSC	8278	9261	17538	367.0	463.8	408.8	25.6	31.6	28.5	100	100	100
non-invasive cancers	3798	1076	4874	157.7	53.7	105.5	11.4	4.2	7.8	—	—	—
all registered cancers	15421	14324	29745	665.5	716.9	681.3	40.8	43.8	42.2	—	—	—

rate: number of cases per 100,000 population per year (European age standardised)
risk: cumulative lifetime risk (risk of cancer diagnosis to age 75), expressed as a percentage
^f refers to all invasive cancers, excluding non-melanoma skin cancer
^g includes cancers of vulva, vagina, uterus (NOS), other female genital & placenta

Anti-smoking laws, increasing cigarette taxes and smoking cessation campaigns have been implemented which have been responsible in decreasing respiratory disease and may influence lung cancer rates in a few years time. Smoking bans in all public areas including the workplace gives protection to employees and the public who are exposed to the harmful and toxic effects of passive tobacco smoke inhalation. A study carried out in the United States to examine if reduced smoking rates have contributed to a decrease in cancer rates in the american population indicated that there was a 40% decrease in overall male cancer deaths between the period 1991 to 2003 [13]. However tobacco smoking is alarmingly on the increase in developing countries. It is predicted based on current smoking statistics that lung cancer will continue to be the leading cause of death worldwide for the foreseeable future [14].

1.1.3 Clinical presentation

At the time of presentation or diagnosis, approximately 30% of patients are candidates for curative surgery, an additional 30% of patients have inoperable disease (locally advanced) and 40% have metastatic disease [15]. Most of the patients who are symptomatic have advanced disease, this is sometimes discovered during screening for other illnesses. Lung cancer symptoms are usually non-specific but can include; a persistent cough, dyspnea (shortness of breath), and haemoptysis (sputum containing blood) an ache or pain when breathing or coughing, loss of appetite and fatigue. Breathing difficulty usually occurs when the cancer has spread to the airway which can lead to pneumonia [16].

A chest radiograph or computed tomography (CT) scans are often used to detect an obvious mass in the lungs. A more definitive diagnosis is made by taking tissue samples either during a bronchoscopy or a CT-guided biopsy. The biopsy is stained by hematoxylin and eosin (H & E) in addition to a panel of lung specific markers (e.g. thyroid transcription factor 1 (TTF-1)), to determine the histology of the tumour. TTF1 is an important diagnostic marker as it encodes a transcription factor that is expressed in adenocarcinomas of the lung and is rarely expressed in squamous cell carcinomas. The treatment of lung cancer depends on the histology and stage of the tumour, as well as the patient's overall health at time of diagnosis. Options for treatment include surgery, chemotherapy and radiotherapy. Early stage (I-IIIa) NSCLC is usually treated with surgery. Patients diagnosed with stage IA lung cancer have a five year survival rate of 82% compared to 68% for those who are diagnosed at stage IB [17]. These survival rates decline dramatically as tumour stage increases. SCLC is treated with chemotherapy and radiation therapy as this type of cancer spreads early to other parts of the body [18].

A CT screening programme for the early detection of lung cancer has been tested in a trial population with varying degrees of success [19]. This is due to a high number of false-positive CT scans which occur due to the detection of non-calcified nodules as small as 2-3 mm. These are found at various percentages in different nationalities [20]. This process may lead to anxiety for the patient due to the high number of further investigations required and unnecessary exposure to irradiation. However, according to another study, a trial CT screening undertaken in a cohort of 484

participants, detected 85% of participants who had stage I lung cancer. The overall 10 year survival for these patients who underwent surgical resection within one month of diagnosis was 92% [21]. CT screening may be of great benefit for people with a high risk for lung cancer. Cytological examinations of patient sputum may be useful in the early detection of lung cancer as sputum atypia is associated with an increased risk of lung cancer [22].

1.1.4 Classification and staging

Lung cancers are classified according to histological examination under a microscope by a pathologist. Tumour histology dictates patient prognosis and which course of treatment should be given [23]. The majority of lung cancers are carcinomas which arise from epithelial cells. There are two main types of lung cancer, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC is the most common form representing over 80% of lung cancers [24]. There are three main sub-types of NSCLC; squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC) [25].

Squamous cell carcinoma accounts for 25% of lung cancers and usually arise near a central bronchus [26] (Fig 1.2). The centres of these tumours are usually hollow with necrosis present. Adenocarcinoma tumours are the most dominant subtype, responsible for over 40% of NSCLC and usually arise in peripheral lung tissue [26] (Fig 1.1). Bronchioloalveolar carcinoma (BAC) is a subtype of adenocarcinoma and accounts for 4% of NSCLC. It is mostly found in female never-smokers and displays a different response to therapy and prognosis compared to other NSCLC tumours [27]. SCLC tumours are initially more sensitive to chemotherapy and radiation, but patients often present with metastatic disease and therefore have a worse prognosis. SCLC is sub-divided into limited and extensive stage disease. Patients who have SCLC are usually smokers [28]. It is common for tumours to contain more than one subtype as they are very heterogeneous.

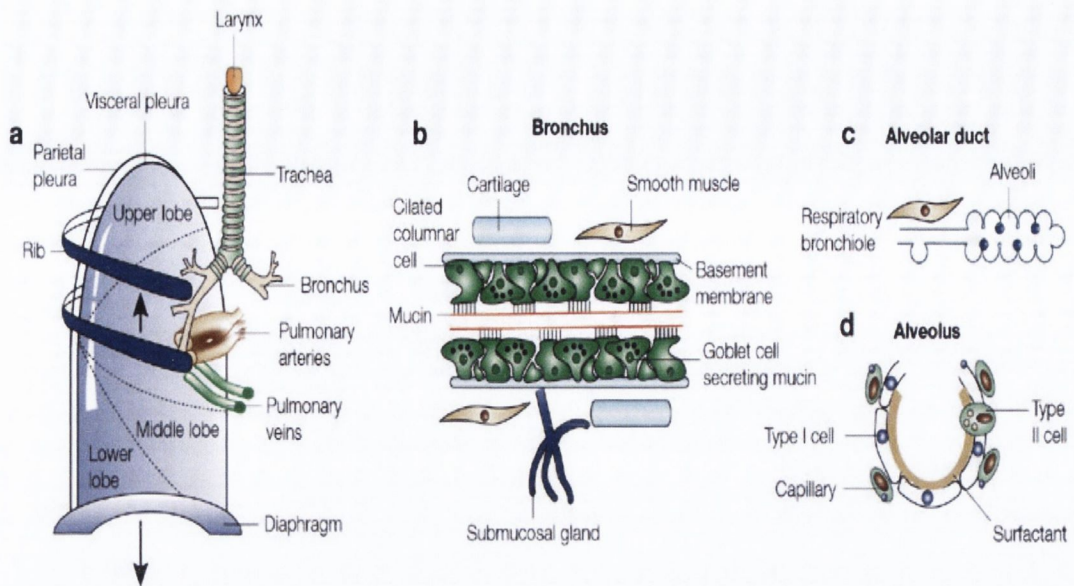


Fig 1.2. The anatomy of the lung with cross-sections of bronchi, bronchioles, alveolar ducts, and alveoli. Gross anatomy of lung and thorax (a). Microscopic anatomy of bronchial wall (b). View of terminal airway and alveoli (c). Alveolar structure (d). The proximal airway and bronchus are the main sites for SCLC and SCC, while the peripheral sites, such as bronchioles and alveoli are associated with AC. Taken from http://www.nature.com/gimo/contents/pt1/fig_tab/gimo73_F3.html

Tumour staging is usually done before treatment is given to patients and varies for NSCLC and SCLC. It is assessed through physical examination, laboratory tests, imaging, and/or biopsies. NSCLC is usually staged from IA for best prognosis to IV for worst prognosis [29]. SCLC is either limited stage which is when the cancer is confined to one half of the chest or extensive stage which is when the disease is more widespread. Both NSCLC and SCLC have two general types of staging evaluations: Clinical Staging: done prior to surgery and is based on the results of the physical examination, imaging, and laboratory analysis. Pathological Staging: usually evaluated during or after surgery and is based on the combined results of surgical and clinical findings [30].

The Tumour, Node, Metastasis (TNM) system for classifying lung cancer is the cornerstone of modern lung cancer treatment and underpins comparative research. The TNM system has evolved through several updated revisions. Tumour classification systems for tumour diagnosis are used to ensure reproducibility, consistency, clinical significance and simplicity in order to reduce the number of unclassifiable samples. The WHO 1981 classification system was used for tumour

classification but due to an increased understanding of the molecular biology of lung cancer, this has been revised to include more categories [31].

The main changes to the updated classification systems included the addition of two pre-invasive lesions to squamous dysplasia and carcinoma in situ; atypical adenomatous hyperplasia and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia [32]. Several new NSCLC subtypes have been added such as sarcomatoid carcinoma, salivary gland tumours, carcinoid tumour, and adenosquamous carcinoma. Adenosquamous carcinoma tumours contain at least 10% each of adenocarcinoma and squamous cell carcinoma. Combined small-cell carcinoma is a variant of small-cell carcinoma which contains a mixture of both small-cell carcinoma and non-small-cell carcinoma. The most common form of lung tumours in infants and children include pleuropulmonary blastoma and carcinoid tumours [33]. Lung cancer commonly metastasises to other parts of the body such as the adrenal glands, liver, brain and bone. Secondary tumours that have metastasised to other organs are still called lung cancer.

Recently the Union Internationale Contre le Cancer (UICC) and the International Association for the Study of Lung Cancer (IASLC) published the 7th Edition TNM Classification for lung cancer (Table 1.2). This latest staging system addresses many of its predecessor's shortcomings and has been subject to rigorous evidence-based methodology. This system is based on retrospective analysis of over 80,000 lung cancer patients treated between 1990 and 2000. An international dataset was used which included patients treated by all modalities. For the first time, a single classification system has been shown to be applicable not only to NSCLC, but also to be of prognostic significance in SCLC and bronchopulmonary carcinoid tumours.

Table 1.2. The TNM classification (7th edition). This classification system is used to stage NSCLC [34]

Descriptors	Definitions
<u>T</u>	<u>Primary tumor</u>
T0	No primary tumor
T1	Tumor ≤ 3 cm, surrounded by lung or visceral pleura, not more proximal than the lobar bronchus
T1a	Tumor ≤ 2 cm
T1b	Tumor > 2 but ≤ 3 cm
T2	Tumor > 3 but ≤ 7 cm or tumor with any of the following : Invades visceral pleura, involves main bronchus ≥ 2 cm distal to the carina, atelectasis/obstructive pneumonia extending to hilum but not involving the entire lung
T2a	Tumor > 3 but ≤ 5 cm
T2b	Tumor > 5 but ≤ 7 cm
T3	Tumor > 7 cm; or directly invading chest wall, diaphragm, phrenic nerve, mediastinal pleura, or parietal pericardium; or tumor in the main bronchus < 2 cm distal to the carina ; or atelectasis/obstructive pneumonitis of entire lung; or separate tumor nodules in the same lobe
T4	Tumor of any size with invasion of heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, or carina; or separate tumor nodules in a different ipsilateral lobe
<u>N</u>	<u>Regional lymph nodes</u>
N0	No regional node metastasis
N1	Metastasis in ipsilateral peribronchial and/or perihilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph nodes
<u>M</u>	<u>Distant metastasis</u>
M0	No distant metastasis
M1a	Separate tumor nodules in a contralateral lobe; or tumor with pleural nodules or malignant pleural dissemination
M1b	Distant metastasis
<u>Special situations</u>	
TX, NX, MX	T, N, or M status not able to be assessed
Tis	Focus of <i>in situ</i> cancer
T1	Superficial spreading tumor of any size but confined to the wall of the trachea or mainstem bronchus

1.1.5 The genetic basis of lung cancer

There is evidence that a genetic predisposition to lung cancer can be inherited as a mendelian autosomal trait which may explain why only 5- 10% of smokers develop the disease [35]. Polymorphisms in genes encoding enzymes involved in the activation and detoxification of carcinogens in the environment may be an important cause of the disease [36] [37]. Inherited polymorphisms in proto-oncogenes, tumour suppressor genes or genes involved in DNA repair and cell growth, may also increase susceptibility to lung cancer [38] [39]. As with most cancers, lung cancer is initiated by the activation of oncogenes or the inactivation of tumour suppressor genes. In lung adenocarcinomas, 26 different genes were found to be significantly mutated such as TP53, KRAS, EGFR, STK11, CDKN2A, PTEN, NRAS, ERBB2, BRAF, P16 and PIK3CA [40]. Overexpression of genes such as EGFR and HER2 is also associated with lung cancer (Table 1.3).

The tumour suppressor gene p53, is the most frequently mutated gene in smoking related cancers [41]. Small cell lung cancer (SCLC) tumours have the highest percentage of p53 mutations compared with other lung cancers [42]. Around 70% of SCLC tumours harbour p53 mutations, followed by 65% of squamous cell carcinoma tumours, 60% of large cell tumours and 33% of adenocarcinoma tumours [43]. The p53 gene plays an important role in cell cycle either initiating or inhibiting growth arrest and cell death [44].

KRAS mutations were identified in non-small cell lung cancer (NSCLC) tumours over 20 years ago. KRAS mutations are usually found in adenocarcinoma tumours from smokers and are less frequently detected in squamous cell carcinoma or SCLC [45]. Activated KRAS genes account for around 30% of lung cancer adenocarcinomas and belong to the family of RAS genes [46]. RAS genes encode a family of membrane-bound 21-kD guanosine triphosphate (GTP)-binding proteins that control cell proliferation, differentiation, and apoptosis by interacting with molecular molecules, including those in the MAPK (mitogen-activated protein kinase), STAT (signal transducer and activator of transcription), and PI3K (Phosphatidylinositol 3-kinase) signalling pathways [47]. KRAS mutations are a predictor of resistance to certain chemotherapy drugs and patients harbouring this

mutation are more likely to be resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) [48] [49] [50].

EGFR regulates cell proliferation, apoptosis, angiogenesis and tumour metastasis [51]. Mutations of EGFR are more common in adenocarcinomas than in other subtypes of NSCLC and are also more frequent in women and never smokers [52] [53]. Japanese patients display the highest number of EGFR mutations especially among Japanese women with adenocarcinomas [54].

Table 1.3: The molecular genetics of lung cancer. Some of the common genetic alterations in lung cancer.

Gene	Percentage	References
Overexpression		
EGFR	70%	[55]
HER2	30%	[56]
c-KIT	40-70%	[57]
c-myc	8-10%	[58]
Bcl-2	10-35%	[55]
Telomerase	80%	[59]
Mutation		
K-ras	20-30%	[55]
P53	50%	[60]
P16	15-30%	[61]

1.1.6 Current treatments

Upon diagnosis of lung cancer, a CT scan and position emission tomography (PET) scan are used to assess if the tumour is confined to one location and suitable for surgical resection. Blood tests and lung function tests will also be carried out to measure the patient’s health and determine their suitability for surgery. In NSCLC, surgery is used only if the tumour is confined to one lung and is between stage IA and IIIA. Different procedures are used in surgery for resecting the tumour. Anything from part of a lobe (wedge resection), one lobe (lobectomy), two lobes (bilobectomy)

or a whole lung (pneumonectomy) can be removed from patients. Studies have shown that SCLC patients have a better chance of survival when treated with chemotherapy and/or radiation than with surgery [62]. However, studies examining surgical options for early stage SCLC patients are ongoing [63].

Radiotherapy is usually given with chemotherapy to SCLC patients who are not suitable for surgery [64]. Different Gy radiotherapy is given based on each individual case. Radical radiotherapy is a high intensity radiotherapy or continuous hyperfractionated accelerated radiotherapy (CHART) is when a high dose of radiotherapy is given in a short time period [65]. Radiotherapy is not usually given to NSCLC patients but may benefit patients where there is metastasis to adjoining lymph nodes [66].

As the majority of patients have advanced stage lung cancer by the time they are diagnosed it is important that they receive a personalised chemotherapy plan that is matched to the type of disease they have [67]. The choice of chemotherapy given to patients depends on the histology of the tumour [68]. Advanced NSCLC is usually treated with cisplatin or carboplatin in combination with gemcitabine, paclitaxel, docetaxel, etoposide, Bevacizumab or vinorelbine [69] [70] [71]. However an increased understanding of cancer biology and oncogenesis has led to the development of targeted therapies which target molecular markers such as vascular growth factor (VEGF) and its receptors and epidermal growth factor receptor (EGFR) [72] [73]. Cisplatin and etoposide are commonly used for treatment of SCLC, along with combinations of carboplatin, gemcitabine, paclitaxel, vinorelbine, topotecan and irinotecan [74] [75] [76].

Molecular biomarkers and mutations are used to determine the optimal chemotherapy agent for patients [77]. Patients with EGFR mutations in exon 19 and L858R are known to respond better to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib [78] [79] [80]. Whereas KRAS mutational status in patients is important in predicting resistance to EGFR TKIs [48] [81] [82].

1.2 EGFR signalling pathway

The epidermal growth factor receptor (EGFR) gene encodes a 170 kDa transmembrane protein consisting of an extracellular EGF-binding domain, a short transmembrane region, and an intracellular domain with ligand-activated tyrosine kinase activity [83] (Fig 1.3). The EGFR belongs to a subfamily of four closely related proteins: EGFR (also called ErbB-1), HER-2/neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4) [84].

The HER-2/neu gene codes for a 185-kDa transmembrane glycoprotein which is related to EGFR. Activation of HER-2/neu is associated with its ability to transform cells and occurs when a point mutation in the HER/neu gene results in the change of amino acid residue 664 from valine to glutamic acid. The HER/neu gene is associated with a variety of cancers including NSCLC [85].

The mammalian ligands that bind EGFR include EGF, transforming growth factor- α (TGF- α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR) and epigen [86]. Each of the mature peptide growth factors are characterised by a consensus sequence known as the EGF motif which is crucial for binding members of the HER receptor tyrosine kinase family [87].

EGFR regulates the intracellular effects of ligands such as EGF and TGF- α . Upon ligand binding to the EGFR extracellular domain there is an increase in the proportion of dimerised receptor and the enzymatic activity of its intracellular tyrosine kinase domain increases. The EGFR interacts with its three known homologues ErbB2, ErbB3 and ErbB4 to form heterodimers. When the tyrosine kinase intracellular domain of the receptor is activated it undergoes autophosphorylation, which initiates a cascade of intracellular signalling events leading to increased cell proliferation and inhibition of apoptosis [88]. The binding of the ligand and activation of the EGFR kinase also induces the clustering of EGFR complexes into clathrin-coated pits that are subsequently internalised in a kinase dependent manner [89].

EGFR signalling plays an important role in cancer progression by regulating cell proliferation, angiogenesis, metastasis, and inhibition of apoptosis [83] [88] [90]. EGFR is overexpressed in over 60% of NSCLC cases and is associated with advanced disease and poor prognosis [91] [92] [93]. EGFR overexpression is also associated with resistance to cytotoxic drugs, including cisplatin [94]. High expression of HER-2/neu and combined EGFR-HER-2/neu were found to have a negative effect on survival and patient outcome [95]. Examination of EGFR and HER-2/neu gene expression and survival in matched tumour and normal tissues from 83 patients with NSCLC demonstrated that EGFR and HER-2/neu mRNA were expressed in all specimens analyzed [95]. High HER-2/neu and EGFR expression was detected in 34.9% and 33.7% of patients, respectively, while high HER-2/neu and EGFR coexpression was detectable in 16.9% of patients. Up-regulation of HER-2/neu was associated with shorter survival ($P = 0.004$), whereas high EGFR expression showed a trend toward reduced survival.

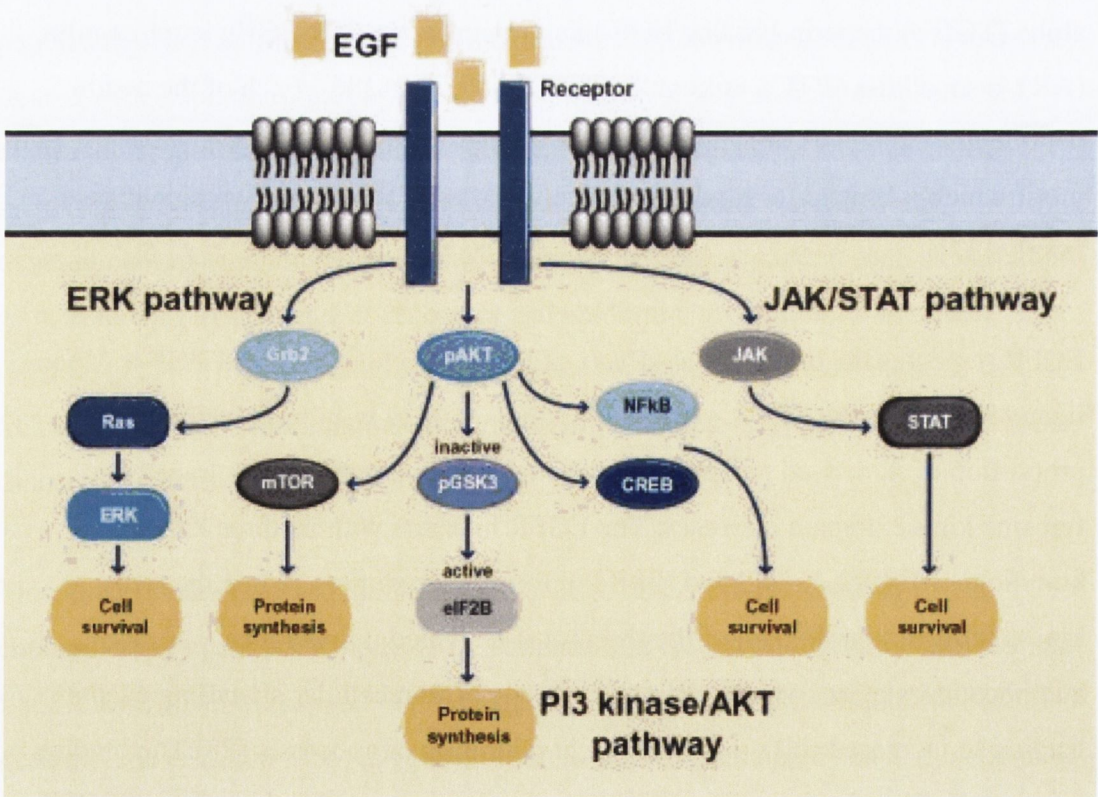


Fig 1.3: Epidermal growth factor receptor (EGFR) signalling pathway. Taken from (<http://www.abcam.com/index.html?pageconfig=resource&rid=10723&pid=10628>)

1.2.1 Targeting the EGFR pathway

The RAS–MAPK and PI3K–AKT pathways are downstream signalling pathways that are triggered upon EGFR activation to induce cell proliferation and survival. Activation of EGFR is cell or tissue-specific and depends on the binding ligand, the dimer formed, the activated downstream signalling cascade and the cross-talk interactions which occur between other intracellular pathways [96].

During the last decade there has been great advancement in the treatment of NSCLC due to the development of new targeted therapies. Targeted therapies are specific to the oncogenic tyrosine kinase pathways activated in tumour cells e.g. EGFR. Two EGFR tyrosine kinase inhibitors (TKI), Gefitinib (Iressa) and Erlotinib (Tarceva) are used as firstline treatment in chemo-naïve patients with tumours harbouring EGFR mutations [97].

EGFR TKIs compete with ATP for the ATP-binding site on the EGFR tyrosine kinase domain preventing phosphorylation and the activation of downstream signalling molecules involved in cell proliferation and tumour growth [98]. In contrast to gefitinib, erlotinib has shown anti-tumour activity in patients with advanced NSCLC and prolongs survival in those who have failed all prior treatment regimens [99]. It has been shown that certain mutations within exons 18-21 of the EGFR TK domain can increase drug sensitivity and patients harbouring these mutations respond better to treatment with erlotinib treatment (10). Activating EGFR mutations are found in 10–15% of Caucasian and 30–40% of Asian NSCLC patients [100]. This subset of patients have a 65-90% response rate to EGFR TKIs compared to only 10% in the non-mutation patients [101]. EGFR copy number and mutation status are currently used to predict response to erlotinib in patients [102]. Patients should be stratified for erlotinib treatment based on their mutation status as not everyone is a candidate for this treatment. Patients who initially respond to EGFR TKIs develop resistance to the targeted therapy due to the emergence of an acquired secondary mutation e.g. T790M [103][104], or the activation of another signalling pathway e.g. IGF-1R [105].

1.3 IGF-1R signalling pathway

The insulin-like growth factor receptor (IGF-1R) has emerged as an attractive target for anti-cancer drug development. It is over-expressed in several tumour types where it plays a role in promoting tumour growth by inhibition of apoptosis, transformation, metastasis, and induction of angiogenesis through vascular endothelial growth factor (VEGF) [106] [107] [108]. The IGF-1R is found in various tissues in which it plays a role in tissue growth, generally through the growth hormone, which releases IGF-1 to activate the IGF-1R [109]. IGF-1R activation protects cells from apoptosis induced by osmotic stress, hypoxia and anti-cancer drugs depending on the level of receptor expression. IGF-1R activation is also capable of protecting cancer cells in the hypoxic and nutrient-deprived microenvironment that can occur in large tumours [110]. IGFs and insulin can increase the hypoxic response by activation of AKT and MAPK signalling, resulting in stabilisation of hypoxia-inducible factors HIF-1 α and HIF-2 α , and upregulation of VEGF [111].

The role of the insulin-like growth factor receptor 1 (IGF-1R) in the pathogenesis of malignant epithelial tumours including lung cancer has been well characterised [112] [113]. The IGF-1R is a member of a large class of tyrosine kinase (TK) receptors which includes the insulin receptor (IR) and the IGF-2R. The IGF-1R controls the activity of two ligands IGF-1 and IGF-2, three cell surface receptors, six IGF-binding proteins (IGFBP1-6) and binding protein proteases [114] (Fig 1.4). The IGF-1R is a phylogenetically conserved transmembrane receptor TK made up of two extracellular α -subunits and two β -subunits which pass through the cellular membrane and are linked by disulfide bonds. IGF-1R signalling occurs mainly through the phosphatidylinositol 3-kinase (PI3K) and Ras–Raf–MAPK pathways [115].

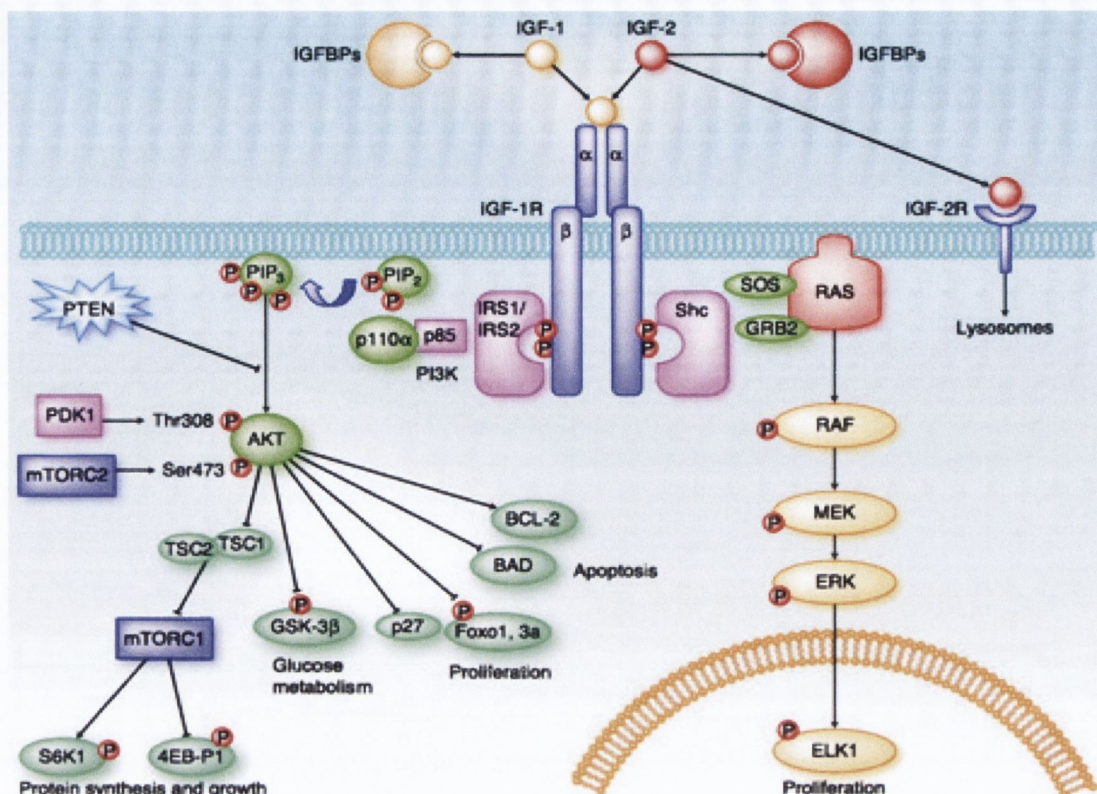


Fig 1.4: Insulin-like growth factor 1 receptor (IGF-1R) signalling pathway [116]

1.3.1 Targeting the IGF-1R pathway

Identifying the complex molecular events that underlie carcinogenesis is essential in the design of new targeted therapeutics. In recent years the tyrosine kinases receptors have become well documented targets for the treatment of cancer. Most of the strategies to target the IGF-1R have been at the receptor itself [117]. The importance of the IGF-1R in cancer biology and the fact that the IGF axis could be blocked at several different levels makes this receptor an attractive drug target. It is not clear what factors influence sensitivity to IGF-1R inhibitors but it is likely that tumours that respond well to treatment will be those where IGF-1R over-expression results in a poor patient prognosis [118] [119].

Currently, several different approaches are being investigated for targeting the IGF-1R, including small-molecule kinase inhibitors, IGF-1R antibodies, antisense oligonucleotides and RNA interference. All of these approaches hinder cell growth and proliferation [117] [106]. While high IGF-1R expression has been shown to influence sensitivity in only some NSCLC cell lines other molecular biomarkers that may predict for benefit from anti-IGF-1R directed therapy need to be sought [118] [120].

The development of IGF-1R TKIs has been difficult as the kinase domain of the IGF-1R shares 85% homology with that of the IR [106]. Inhibitors that bind to the ATP binding cleft are not likely to show complete specificity, as the ATP binding cleft is 100% conserved, but recently selective IGF-1R inhibitors have been created. These new therapeutic interventions are doing well in pre-clinical and early phase clinical trials. Several groups are now manufacturing agents that show potential as IGF-1R inhibitors. NVP-AEW541 and NVPADW742 (Novartis) were the first selective IGF-1R kinase inhibitors, capable of distinguishing between the IGF-1R and the closely related IR in cells and inhibiting the growth of cells that depend on IGF-I signalling [121] [122].

The microenvironment of the tumour has an impact on tumour growth and progression. Highly aggressive tumours such as those which occur in the pancreas are able to survive in nutrient deprived and hypoxic conditions. IGF-1R inhibitors have shown promising results particularly in hypoxic tumours. A specific inhibitor of IGF-1R kinase, AG1024, exhibited specific cytotoxicity to human pancreatic cancer PANC-1 cells grown in a nutrient-deprived medium [123]. IGF-1R kinase inhibitors such as AG1024 block phosphorylation of IGF-1R by IGF-1 in cells cultured in nutrient-deprived medium compared to those grown in medium with ample nutrients. IGF-1R kinase inhibitors also block phosphorylation of Akt and Erk, so that activation of pathways downstream of the IGF-1R are also blocked in nutrient-starved conditions.

Monoclonal antibodies and single chain antibodies can down-regulate IGF-1R levels and block ligand binding, therefore inhibiting tumour growth. These antibodies do not interfere with insulin receptor signalling or interaction between insulin and its receptor. R1507 (RO4858696; Roche) is a fully humanized IgG1 monoclonal antibody directed against the extracellular portion of IGF-1R. It binds with high selectivity to the extracellular domain of IGF-1R (and not to IR), leading to displacement of IGF-1 ligand binding and loss of receptor at the cell surface due to receptor internalization and degradation [118]. R1507 showed promising results in a phase I clinical trial where it was reported that the drug was safe, efficacious, and with few adverse side effects in patients [124]. Disease stabilization was also observed in 9 out of 34 patients with solid tumours following treatment with R1507.

R1507 is currently being investigated in phase II clinical trials and has shown mixed results to date (<http://meeting.ascopubs.org/cgi/content/abstract/27/15S/10503>) (http://www.asco.org/ascov2/Meetings/Abstracts?&vmview=abst_detail_view&confID=65&abstractID=34060). A phase II clinical trial (NO21160) investigating the effect of R1507 in combination with Tarceva (Erlotinib) on progression-free survival (PFS) in patients with stage IIIb/IV Non-Small Cell Lung Cancer (NSCLC) having received Tarceva monotherapy was recently prematurely terminated in August 2011 after almost 3 years due to discontinuation of the drug (not for safety reasons). There is an urgent need for a better understanding of the mechanisms involved in IGF-1R inhibition and the identification of biomarkers that will predict response to IGF-1R inhibitors so that correlative investigations can be implemented at phase II studies.

In vitro analysis investigating the cellular response to a humanized anti-IGF-1R antibody h7C10 showed the link between high IGF-1R levels and the antiproliferative effect of the antibody in rhabdomyosarcoma tissue samples and cell lines [125]. Data from this study revealed that there is a strong dependence on IGF-1R for AKT signalling in cells with high IGF-1R levels. Active AKT blocked the sensitivity of the high IGF-1R-expressing cells. This could be used as a possible predictive biomarker for anti-IGF-1R treatment in cancer.

An antisense approach has also been shown to be an effective strategy in cancer gene therapy by inhibition of IGF-1R expression with antisense or small interfering RNAs (siRNAs) [126]. Another approach is the use of an adenoviral vector-based short hair-pin(sh) RNA system [127]. The ad-shIGF-1R system blocked downstream cell signalling and induced apoptosis in NSCLC cell lines. Another study found that when A549 cells were transfected with IGF-1R shNA there was increased sensitivity to cisplatin *in vitro* and *in vivo* and decreased the IC₅₀ of cisplatin in A549 cells up to 72 h [128]. This shNA-mediated silencing of IGF-1R together with cisplatin treatment inhibited cell growth and increased apoptosis.

Due to the homology between the IGF-1R and IR, specific IGF-1R inhibitors are essential, as the regulation of insulin can be disrupted causing type II diabetes. Further studies are needed to identify patients who will benefit from targeting the IGF-1R pathway. It is important to understand biomarkers of response and pathways involved in resistance to targeted therapy.

1.3.2 Mutations in IGF-1R

To date there have been few reports of mutations in the IGF-1R tyrosine kinase domain. Single nucleotide polymorphisms (SNPs) are variations which can occur in a DNA sequence when a single nucleotide (A, T, C, or G) in the genome is changed. Around 90% of human genetic variations are caused by SNPs. These occur every 100–300 bases along the 3-billion-base human genome [129]. SNPs can occur in both coding and non-coding regions of the genome. The presence of a SNP in an individual's genome may play a role in disease or influence drug sensitivity or it may have no effect on the function of a cell. SNPs present outside gene promoter or coding regions may still effect gene expression and DNA repair. There have been numerous studies that have linked IGF-1R gene polymorphisms to certain diseases such as in dementia and ischemic stroke [130] [131] but not much is known about the functional consequences of SNPs in this gene and their link to cancer [132]. Somatic mutations in the kinase domain of a receptor could impact on the ability of a therapeutic agent to bind efficiently or can cause the cell to become highly dependent on the constitutively active receptor signalling pathway, a state referred to as “oncogenic addiction” [133] [134].

IGF-1 and IGF-1R polymorphisms and increased IGF-1 plasma levels have been associated with cancer risk [135] [136] [137]. Given the important role of the IGF-1R signalling pathway in tumour cell survival and growth, it is possible that genetic variation may play a role in the activation of this pathway in cancer [138] [139]. Identifying functional polymorphisms in the IGF-1R pathway could be used to select patients who would benefit from IGF-1R inhibitors resulting in improved treatment for individuals with reduced toxicities.

A study by Deming et al. [140] on genetic variation in patients with breast cancer found SNP rs951715 within the IGF-1R gene was associated with breast cancer survival in postmenopausal women. Another polymorphism, SNP rs2229765 was found to have no association with survival in breast cancer patients. SNP rs2229765 appears to be a silent mutation and so far has not been associated with any epidemiological traits. Nineteen SNPs with known functionality or that were located in functional regions of the gene were evaluated in the study [140]. It was found that polymorphisms in the IGF-1R and IGFBP-3 genes may be associated with altered survival among subgroups of breast cancer patients defined by menopausal status

[141]. In another study, single SNP analysis revealed significant association of SNP rs2229765 with both percent and absolute mammographic density, increased numbers of the G allele increased the least squares means of mammographic density [142].

A recent study investigated whether germ line polymorphisms in the IGF-1-pathway are associated with response to cetuximab in wild-type KRAS drug- refractory metastatic colorectal cancer patients (mCRC) [139]. Tissue samples of 130 drug-refractory mCRC patients enrolled in a phase II clinical trial of cetuximab monotherapy (IMC-0144) were used for the study. Analyses revealed five IGF-pathway SNPs were significantly associated with progression-free-survival (PFS) and/or overall survival (OS). Patients harbouring IGF-1 rs2946834 A/A genotype had a 50 % overall response rate (ORR) while patients with A/G genotype had 0%. This indicates that IGF-1-pathway polymorphisms can predict sensitivity to cetuximab in wt KRAS mCRC patients.

A study by Gong et al. used SNP array analysis of IGF-1R gene copy number status in R1507 resistant and sensitive NSCLC cell lines [118]. Cell lines with copy number gain of IGF-1R were sensitive to R1507 however none of the sensitive lines had any mutations in IGF-1R.

1.3.3 IGFBPs

The activities of IGFs in regulating cell proliferation, differentiation, and apoptosis are controlled by a family of high-affinity specific IGFBPs. The IGFBP family is made up of seven members, IGFBP-1 to IGFBP-7, which transport IGF-I and IGF-II and regulate their availability and activity (Fig 1.5). They are found in the circulation either in free form or in binary complexes with IGFs. IGFBPs' genes have a common structural arrangement made up of four conserved exons located within genes ranging from 5 kb (IGFBP-1) to more than 30 kb (IGFBP-2 and IGFBP-5) [143]. The highly conserved protein structure consists of three domains of approximately equal size, the amino-terminal domain, carboxyl-terminal domain and the central domain. Mature IGFBPs have between 216 and 289 amino acids and a core molecular mass ranging between 22.8 and 31.3 kDa, depending upon

posttranslational modifications such as glycosylation and phosphorylation. IGFBP-3 has molecular masses of 43–45 kDa and is usually bound to IGF-1 [144].

Binding affinities of IGFBPs for IGFs are determined by substantial post-translational modification [145]. IGFBPs can bind to certain cell membrane receptors, the cell surface or to the extracellular matrix [145]. Hormones, including estrogens, glucocorticoids, parathyroid hormone, FSH, GH, thyroid hormone, insulin, vitamin D, and cortisol, can be involved in the complex regulation of IGFBP gene transcription [146] [147] [148] [149] [150] [151] [152]. IGFBP expression is also regulated by growth factors including EGF, TGF- β and IGFs [153] [154] [155].

Through IGF dependent mechanisms, IGFBPs can control cell proliferation by binding to IGFs and blocking IGF activation of the IGF-1R. They are involved in transporting IGFs, protecting IGFs from proteolytic degradation therefore increasing their bioavailability in local tissue and regulating binding of IGFs to the IGF-1R. The effect of IGFBPs on IGFs depends on post-translational modification of IGFBPs by phosphorylation and proteolysis. The binding affinity of IGFBPs to IGFs may also be affected by their relationship with the cell membrane or extracellular matrix. IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-5 have been found to regulate IGFs by either suppressing or enhancing their actions [143]. Proteolysis of IGFBP-2, IGFBP-3, and IGFBP-5 effects the regulation of IGFs, while phosphorylation controls the effect of IGFBP-1 on IGFs. IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-5 also bind to specific cell membrane receptors or attach to the cell surface. This reduces their binding affinities for IGFs and results in the release of free IGFs. IGFBP-5 binding affinity for IGFs reduces when IGFBP-5 is associated with the extracellular matrix. IGFBP-1, IGFBP-2, and IGFBP-5 effect cell adhesion and migration by IGF-independent mechanisms [156].

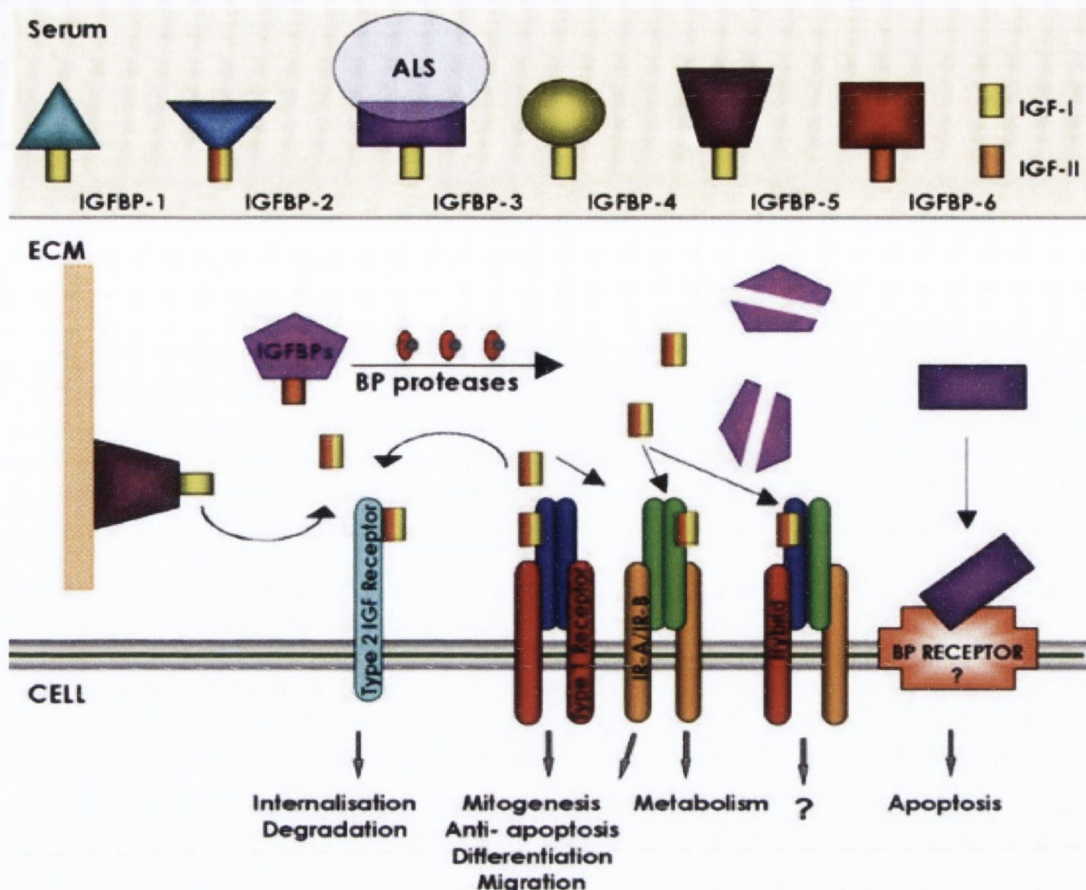


Fig 1.5: IGFBPs and receptors. Taken from www.igf-society.org

1.3.3.1 IGFBP-3 and its role in cancer

IGFBP-3 is the most abundant IGFBP in the serum followed by IGFBP-2 [157]. More than 75% of IGF-I and IGF-II and 90% of IGFBP-3 in the serum circulate as heterotrimeric complexes in healthy adults [158]. IGFs have a higher affinity for IGFBPs than IGF-1R. IGFBPs sequester IGFs away from the IGF-1R inhibiting the possibility of mitogenesis, differentiation, survival and other IGF-stimulated events. Degradation of IGFBP by proteolysis can reverse this inhibition and create IGFBP fragments with novel functions. IGFBP-3 is produced by cells, such as fibroblasts, endothelial cells and epithelial cells [159] [160] [161]. IGFBP-3 functions through IGF-dependent or IGF-independent mechanisms *in vitro* resulting in either an anti-apoptotic or pro-apoptotic environment [143] [162].

In addition to its IGF-dependent function, IGFBP-3 has IGF-independent antitumour activities through cell surface or intracellular protein interaction, its nuclear translocation, or its transcriptional regulation [163] [164] [165] [166]. The biochemical/molecular mechanisms mediating IGFBP-3's IGF independent

antitumour activity are not yet clear. This effect may be controlled through a cell membrane receptor that is specific for IGFBP-3.

The IGFBP-3R has been identified as a novel death receptor in a recent study [167]. This is a single-span membrane protein which binds specifically to IGFBP-3 and not other IGFBPs. Expression analysis of IGFBP-3 and IGFBP-3R indicates that the IGFBP-3/IGFBP-3R axis is decreased in breast and prostate cancer. *In vitro* studies demonstrated that IGFBP-3R mediates IGFBP-3-induced caspase-8-dependent apoptosis in various cancer cells which was prevented by IGFBP-3R knockdown. However overexpression of IGFBP-3R enhanced IGFBP-3 biological activities. Knockdown of caspase-8 expression also inhibited IGFBP-3/IGFBP-3R-induced apoptosis. The IGFBP-3/IGFBP-3R may be used as a therapeutic and prognostic indicator for the treatment of cancer.

Although it is known that IGFBP-3 plays a significant role in stimulating cell proliferation and inducing apoptosis in cancer cells, IGFBP-3 expression levels and its importance in NSCLC is not clearly defined. A study using a transgenic mouse model of prostate cancer, found IGFBP-3 inhibited prostate tumour growth [168]. Studies using tumour cell xenografts also displayed growth inhibition from IGFBP-3 in NSCLC. Hochscheid *et al.* demonstrated that overexpression of IGFBP-3 in an NSCLC cell line inhibits cell proliferation *in vitro* and resulted in decreased tumour growth *in vivo*. This effect could not be reversed by stimulation with IGF-I and IGF-II or insulin [169]. Lee *et al.* demonstrated that injection of Ad5CMV-BP3 into H1299 NSCLC xenografts in nude mice decreased growth of the tumours significantly while no effect was observed in normal bronchial epithelial cells [170]. IGFBP-3 overexpression was found to inhibit phosphorylation of Akt and glycogen synthase kinase-3 β and the activity of MAPK. The addition of IGF-I was able to rescue the NSCLC cells from serum depletion-induced apoptosis but was blocked in Ad5CMV-BP-3-infected H1299 NSCLC cells.

However reports on the cellular function of IGFBP-3 are conflicting as IGFBP-3 has been shown to have growth stimulatory effects in other studies [171]. It is not known whether the growth stimulatory effects of IGFBP-3 are by IGF-dependent or IGF-independent mechanisms. A high level of serum IGFBP-3 has been shown to have a beneficial effect in protecting against cancers such as prostate and lung cancers,

while there is also evidence that IGFBP-3 may serve as a biomarker for increased risk for cancers such as breast and colon cancer [172].

Many studies have investigated the relationship between the plasma levels of IGF-1 and IGFBP-3. Elevated plasma levels of IGF-1 have been shown to be associated with an increased risk of lung cancer [173] and high plasma levels of IGFBP-3 are found to be associated with a reduced risk of lung cancer [174]. However, there are many conflicting results found in other studies where the opposite was observed [175] [176].

IGFBP-3 is frequently overexpressed in esophageal cancer along with overexpression of EGFR [177]. It was found that EGFR tyrosine kinase activity was involved in regulating IGFBP-3 expression *in vitro* but in some esophageal cancer cell lines EGF inhibited IGFBP-3 expression. This EGF-mediated inhibition of IGFBP-3 occurs through the Ras-MAPK signalling pathway, resulting in activation of IGF-IR due to increase in IGF-I in human esophageal cell lines [178]. Therefore, EGF inhibition of IGFBP-3 may positively regulate the IGF signalling pathway.

High IGFBP-3 expression inhibits IGF-mediated survival and proliferation of lung cancer cells [170]. Decreased IGFBP-3 expression is associated with poor prognosis in patients with stage 1 NSCLC [179] [180]. IGFBP-3 expression was examined in tumour samples from patients with stage I NSCLC to determine if IGFBP-3 expression influences the prognosis of patients with NSCLC. Reduced IGFBP-3 expression was found in 56.8% of samples, and was higher in large cell carcinoma than in squamous cell carcinoma and adenocarcinoma. This was not linked to clinicopathological parameters, such as age, sex, histological grade, and smoking history. There was a correlation between IGFBP-3 expression and disease specific survival as patients with decreased IGFBP-3 expression had shorter overall, disease free, and event-free survival rates than patients with normal IGFBP-3 expression. IGFBP-3 may therefore act as a tumour suppressor and be indicative of tumour aggressiveness in early stage NSCLC.

The finding that IGFBP-3 overexpression inhibits phosphorylation of IGF-1R in NSCLC cells to suppress cancer cell growth has led to the development of ligand binding inhibitors. Ligand-binding inhibition can be carried out by agents that

interfere with ligand binding to IGF-1R such as IGFBPs, peptides or small-molecule competitive-binding antagonists, and blocking anti-receptor antibodies [170]. Many studies have focused on IGFBP-3 expression, therefore there is little known on the other unexplored members of the IGFBP family.

1.3.3.2 Role of IGFBP-5 in cancer

IGFBP-5 interacts with several extracellular matrix protein including plasminogen activator inhibitor-1 [181], thrombospondin-1 [182], osteopontin [182] and vitronectin [183]. This interaction causes internalisation into the cytosol and translocation to the nucleus. IGFBP-5 is involved in cell growth by either inducing or suppressing cell proliferation. [184]. It can also act as a tumour suppressor by inhibiting angiogenesis [184]. It was observed that overexpression of IGFBP-5 inhibited VEGF-induced proliferation and invasion by interfering with VEGF-induced phosphorylation of Akt [184]. Tumour growth and tumour vascularity were also found to be decreased in the presence of IGFBP-5 expression in a xenograft model of human ovarian cancer [184].

In another study, overexpression of IGFBP-5 induced apoptosis in cancerous cells such as head and neck squamous cell carcinomas and breast cancer cells [185] [186]. A study which examined IGFBP-5 expression in normal and tumour tissue in breast cancer patients found that IGFBP5 mRNA expression correlated positively with the invasion of axillary lymph nodes and the status of hormonal receptor [187]. IGFBP-5 overexpression was associated with poorer survival in breast cancer patients with positive lymph nodes and negative ER [187]. Therefore, the expression level of IGFBP-5 may be involved in the development of breast cancer and is a prognostic factor for breast cancer. A recent study by Shersher *et al.* examined the potential of IGFBP's as biomarkers in NSCLC and observed that low serum IGFBP-5 levels correlated with a positive nodal status and disease recurrence. Patients with low serum levels of IGFBP5 were also found to have poor recurrence-free survivals in the overall cohort and in patients with no nodal metastases [188]. Results from these studies demonstrate the use of IGFBP-5 as a potential biomarker in predicting patient outcome.

1.3.3.3 Role of IGFBP-6 in cancer

IGFBP-6 is an exception in that it binds to IGF-II with higher affinity than it does to IGF-I, unlike the other IGFBPs which bind with higher affinity to IGF-I [189]. The role of IGFBP-6 in NSCLC was investigated by Sueoka *et al.* in a panel of NSCLC cell lines expressing low endogenous levels of IGFBP-6. The cell lines were transfected with an adenovirus expressing human IGFBP-6 under the control of a CMV promoter (Ad5CMV-BP6) and resulted in a decrease in NSCLC cell number though activation of programmed cell death [190]. This effect was examined *in vivo* by intratumoural injection of Ad5CMV-BP6 in NSCLC xenografts established in nu/nu mice. One injection of Ad5CMV-BP6 reduced the size of NSCLC xenografts by 45%. This elucidates the importance of IGFBP-6 in inducing programmed cell death in cancer cells and indicates that IGFBP-6 overexpression may correlate to a good prognosis.

1.3.3.4 IGFBPs and Resistance to cancer therapies

Manipulations of IGFBP-regulated pathways are being investigated for the treatment of cancer [191]. The mechanisms involved in acquired resistance to gefitinib were examined by Guix *et al.* who created gefitinib-resistant (GR) human squamous carcinoma A431 cells by incubation of A431 cells with increasing amounts of the inhibitor over a prolonged period of time [94]. In the GR cells, treatment with gefitinib resulted in a decrease in the phosphorylation levels of EGFR, ErbB3, and Erk, but not Akt. This occurred along with activation of signalling molecules mediated by the IGF-1R, such as phosphorylation of IRS-1 and the interaction of IRS-1 with PI3K. It was found that when IGF-1R was inhibited, the IRS-1 and PI3K association was reversed and sensitivity to gefitinib was restored through reduced Akt phosphorylation.

Guix *et al.* also found that the expression levels of IGFBP-3 and IGFBP-4, were reduced in GR cells. When the GR cells were incubated with recombinant IGFBP-3 and gefitinib, Akt signalling was reduced and inhibited cell proliferation [94]. When gefitinib was combined with an anti-IGF-IR antibody in nude mice, this was also successful in inhibiting tumour growth. Therefore it was concluded that resistance to EGFR-targeted TKIs may arise from loss of IGFBPs and activation of IGF-IR

signalling and that dual inhibition of EGFR and IGF-IR would benefit cancers characterized by overexpression of EGFR. The GR cells were also found to be resistant to the EGFR TKI erlotinib and the EGFR-targeted monoclonal antibody cetuximab, hence the loss of IGFBPs may be involved in resistance to other ErbB-targeting TKIs and antibodies (Fig 1.6).

The most effective systemic chemotherapy for NSCLC is cisplatin-based combination treatment. Chemoresistance is a major therapeutic problem and understanding the mechanisms involved is critical to the development of new therapeutic intervention strategies including novel biological targeted agents with different mechanisms of action. DNA-hypermethylation, is a process that is thought to be involved in the development of drug-resistance by inactivating genes required for drug-cytotoxicity.

Expression microarray analysis was used to identify genes in two sets of cisplatin resistant and sensitive NSCLC cell lines after epigenetic treatment [192]. Results showed that there was silencing by promoter hypermethylation of IGFBP-3 in cisplatin resistant cells, whereas IGFBP-3 siRNA, induced resistance to cisplatin in sensitive cells. There was a correlation between methylation status and cisplatin response in tumour samples.

Stage I patients, whose tumours had an unmethylated promoter had increased disease-free survival (DFS). Therefore the loss of IGFBP-3 expression through promoter-hypermethylation leads to reduced tumour cell sensitivity to cisplatin in NSCLC. Measuring the basal methylation status of IGFBP-3 in patients prior to treatment may be used to predict response to chemotherapy. Inactivation of IGFBP-3 by methylation in NSCLC is associated with poor prognosis [180].

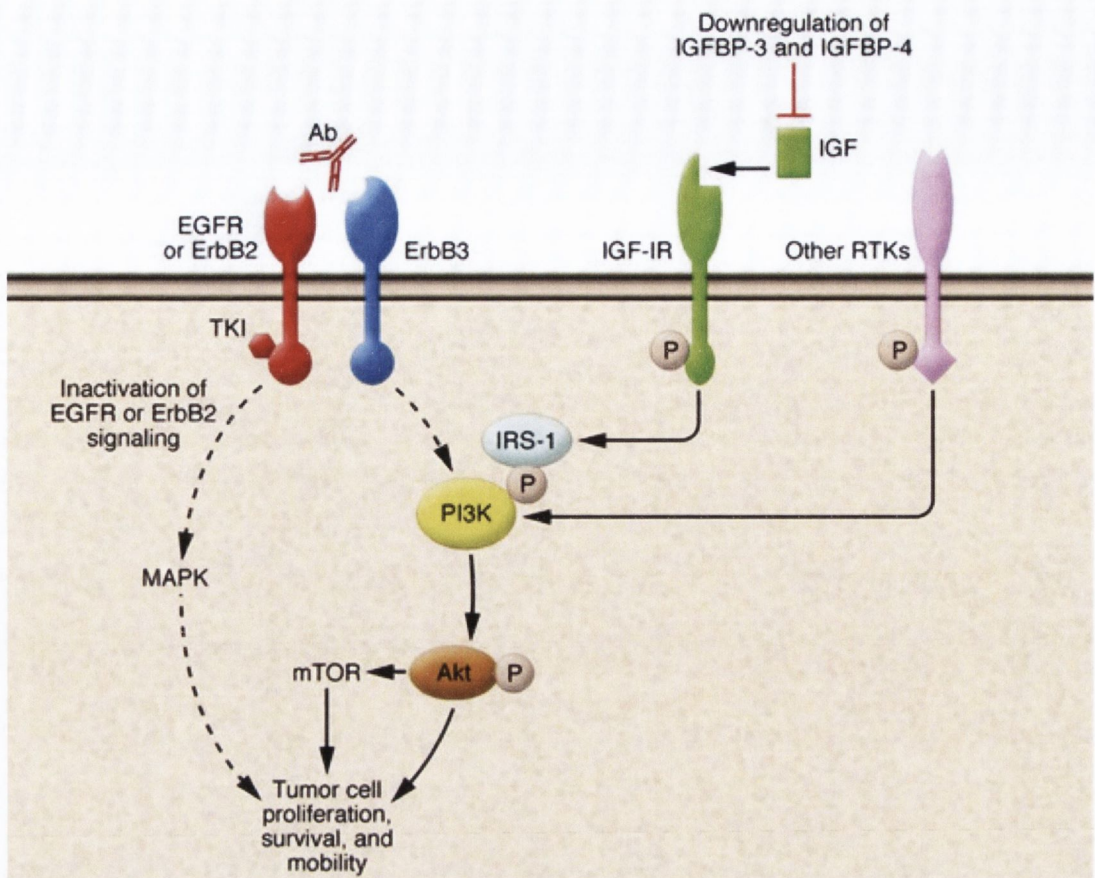


Fig 1.6: Mechanisms of resistance to EGFR targeted therapy.

1.4 Crosstalk between EGFR and IGF-1R

The IGF-1R and EGFR share common downstream signalling molecules, and receptor crosstalk occurs between these two powerful signalling pathways [193] (Fig 1.7). This functional cross-talk during lung carcinogenesis affects the majority of the downstream cascades in both pathways and may affect current anti-EGF receptor treatments in lung-cancer patients. Results from both *in vitro* and *in vivo* studies indicate that cross-talk between EGFR and IGF-1R can lead to acquired resistance against EGFR-targeted drugs [193] [194] [195]. The interaction between EGFR and IGF-1R occurs directly by mediating the availability of their ligands, or indirectly, via common interaction partners or downstream signalling molecules [196]. This has led the way for the novel therapeutic approach of combination therapy targeting both EGFR and IGF-1R [197] [198]. To date, this dual targeting has had promising results however further *in vitro* and *in vivo* studies are necessary to further characterise the inhibition of both pathways. It is hypothesised that erlotinib induces EGFR/IGF-1R heterodimerization on the cell membrane. This interaction initiates signalling through

the IGF-1R pathway through PI3K/Akt and p44/42 MAPK to stimulate mammalian target of rapamycin (mTOR)-mediated synthesis of EGFR and antiapoptotic survivin proteins. Therefore, inactivation of IGF-1R, suppression of mTOR-mediated protein synthesis, or knockdown of survivin protein should increase sensitivity to erlotinib in EGFR-overexpressing NSCLC cells [196].

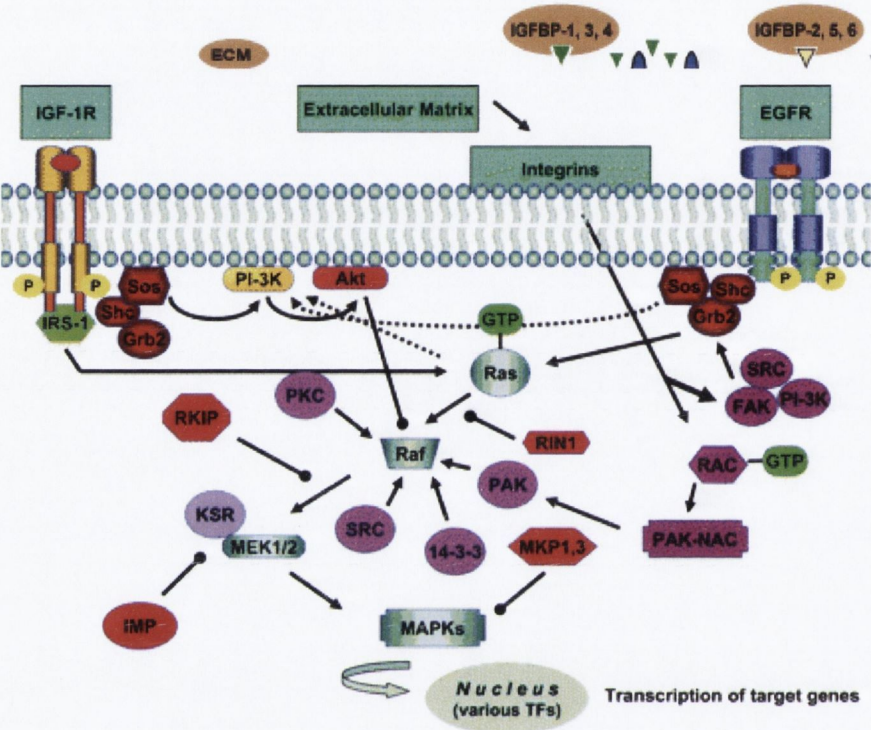


Fig 1.7: Crosstalk between IGF-1R and EGFR through Ras/ MAPK signalling network [199].

1.5 Hypoxia and cancer

Cancer cells go through complex genetic and adaptive changes which enable them to survive and proliferate in hypoxic conditions (Fig 1.8). The molecular mechanisms behind these changes involve the transcription of numerous genes that increase oxygen availability in tissues or reduce oxygen consumption in cells through the activation of glycolysis [200]. The high rate of glycolysis which occurs in hypoxic cells is known as the Warburg effect [201]. Cancer cells produce high levels of lactate and pyruvate which are the end products of glycolysis [202]. Disease progression is known to be associated with glycolysis in several cancers [203].

Most solid human tumours contain regions of hypoxia which are thought to arise during early tumour development [204]. Tissue homeostasis is controlled by complex oxygen-sensing pathways in cells. Regulation of these pathways is critical for cell function, proliferation and survival. When tumours increase in size, isolated regions of the tumour develop hypoxic regions due to an insufficient supply of blood vessels. Genetic and metabolic changes allow the tumour cells to adapt and survive in low oxygen environments. These changes promote metastasis, angiogenesis and selection of cells with reduced apoptotic potential [205]. Hypoxic tumours are more resistant to chemotherapy and radiotherapy as the cancer cells have adapted to environmental conditions that favour survival [206] [207] [208].

Ionizing radiation kills cells by causing DNA damage (double strand breaks) as a result of free radicals. If the cell has adequate oxygen, the oxygen quickly reacts with the free radical, making the detrimental changes in the DNA molecule permanent thus promoting apoptosis. However, hypoxic cells have a sulfhydryl group that can donate hydrogen to the free radical, thereby restoring the DNA molecule. DNA damage is therefore decreased in the absence of oxygen [209] [210]. Systemically delivered therapies are also affected by resistance in hypoxic tumours as their delivery to the hypoxic regions of the tumour may be impeded due to lack of blood vessels. Some anticancer agents need oxygen to increase the cytotoxicity of the DNA lesions they cause. Also some chemotherapy drugs target proliferating cells and in hypoxic regions of the tumour cell proliferation is often reduced resulting in resistance [211]. Drug resistance as a result of high tumour hypoxia in patients leads to a poorer overall survival [212] [213].

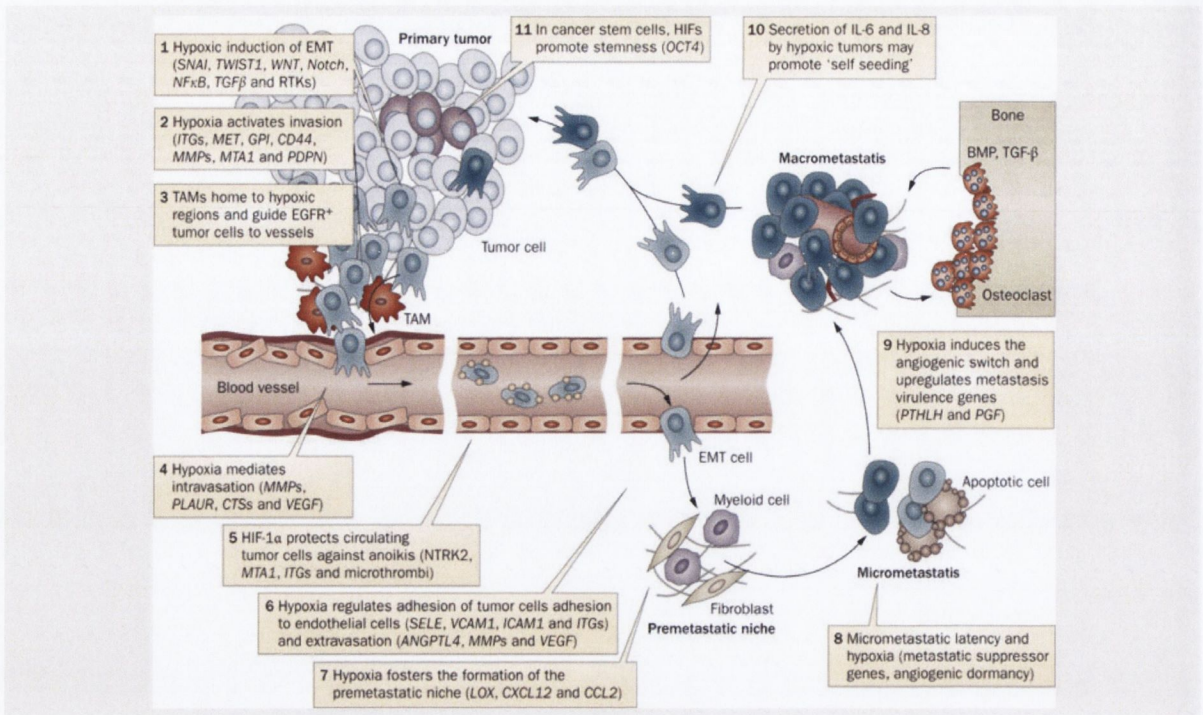


Fig 1.8: Diagram of metastatic cancer cells and their regulation by hypoxia. BMP, bone-morphogenic protein; EMT, epithelial–mesenchymal transition; HIF-1 α , hypoxia-inducible factor 1 α ; IL, interleukin; MMP, matrix metalloproteinase; RTK, receptor tyrosine kinase; TAM, tumour-associated macrophages; TGF- β , transforming growth factor β [214].

1.5.1 Hypoxia regulated signalling pathways

The adaptation of tumour cells to low nutrient and oxygen environments occurs through three molecular mechanisms, the activation of p53 and apoptosis, the activation of hypoxia inducible factor-1 (HIF-1) and the inhibition of mRNA translation. The p53 pathway plays a critical role in the hypoxic response [215]. When activated it results in the death of hypoxic oncogenically transformed cells. Within min of exposure to hypoxia cell growth arrests in the G1/S phase independent of functional p53 tumour suppressor protein inhibiting DNA synthesis. In unstressed cells p53 is present in low levels and during hypoxic exposure p53 is phosphorylated and up-regulated on SER15 through ATR resulting in apoptosis [216]. It is hypothesized that hypoxia may provide an environment which selects tumours cells through clonal selection that tolerate hypoxia and resist apoptosis. This mechanism is achieved through a loss of function or mutation of p53 which induces this tolerant

phenotype [217]. Restoration of wildtype p53 function through gene therapy may be used to decrease hypoxia tolerance in cells.

The adaptation of cancer cells to hypoxia is controlled by hypoxia-inducible factor 1 (HIF-1) which is a key transcription factor [218]. HIF-1 up-regulates the expression of genes involved in cancer progression such as glycolytic energy metabolism, angiogenesis, cell survival, and erythropoiesis. These genes include vascular endothelial growth factor (VEGF), erythropoietin (EPO), glucose transporters (GLUT) and several glycolytic enzymes [201]. HIF-1 plays a major role in tumour progression and metastasis through activation of genes associated with regulation of angiogenesis, cell survival, energy metabolism, apoptosis and proliferation. HIF-1 is composed of an α and a β subunit and is important in the cellular response to hypoxia [219]. The oxygen-regulated expression of the HIF-1 α subunit which is hydroxylated and degraded under normoxia, is induced in response to hypoxia. HIF-1 α is overexpressed in human breast, colon, prostate and lung cancer [220] [221]. High HIF-1 expression is also associated with increased mortality in many cancer types [222]. In prostate cancer, expression of HIF-1 α protein is linked with cell growth rates and metastatic potential [223].

In low oxygen environments HIF-1 is stabilized leading to the formation of a functional transcription factor complex with ARNT. HIF-1 binds to a hypoxia response element (HRE), containing a core 5'-ACGTG-3' sequence, in several genes involved in anaerobic metabolism and oxygen delivery. Activation of these genes results in increased glycolysis, angiogenesis and metastasis allowing the cancer cell to survive and grow in the hostile hypoxic tumour environment [224]. A study in murine models showed that tumours from HIF-1 knock-out cells were considerably smaller than wild type cells and ATP levels and glycolysis were reduced at low oxygen levels [225].

Protection from apoptosis in cancer cells is also associated with activation of the pro-survival PI3K/Akt pathway by hypoxia. This was shown in a study using the oxygen sensitive PC12 rat pheochromocytoma cell line derived from a tumour of adrenal medulla chromaffin tissue [226]. Under hypoxic conditions PC12 cells are protected from apoptosis induced by low oxygen levels or by chemotherapeutic drugs. It is thought that the activation of the PI3K/Akt pathway by hypoxia is responsible for

this antiapoptotic effect as inhibition of PI3K prevents Akt activation and protection against apoptosis in hypoxic conditions.

The role of the PI3K/Akt and extracellular signal-regulated protein kinase (ERK) pathways in hypoxia-induced protection against apoptosis has also been investigated in lung cancer cells [227]. Activation of the PI3K/Akt and ERK pathways increased the synthesis of HIF-1 α and transcriptional activity to create a signalling cascade via HIF-1 α . A549 and NCI-H157 cell lines were treated with either ultraviolet (UV) or etoposide and were placed under hypoxic conditions. Results obtained showed that hypoxia suppressed apoptosis induced by UV or etoposide in both cell lines and activated the PI3K/Akt and ERK pathways. Therefore these pathways may be involved in acquired resistance to apoptosis by hypoxia in lung cancer cells.

The third mechanism of adaption which leads to hypoxia tolerance is through the inhibition of mRNA translation [228]. This is a highly regulated process that is sensitive to a variety of cellular stresses. One hour of hypoxic exposure can reduce protein synthesis by 60–70% which is quickly restored following reintroduction of normal oxygen levels again [229]. Maximum inhibition of mRNA translation through analysis of polysomal RNA occurs approximately 2 h after hypoxic exposure followed by a small recovery by 4 h. However, mRNA translation can remain significantly suppressed for up to 24 h under hypoxia. This appears to be a general reaction to hypoxia as observed in a large panel of cell lines. The reduction in protein synthesis occurs through a HIF-independent, oxygen-sensing pathway which results in direct inhibition of the initiation step of mRNA translation [230]. Inhibition of mRNA translation acts as a cellular survival mechanism by conserving energy demand during hypoxia. Under severe hypoxia, the ATP demand for protein synthesis drops to about 7% of that of normoxic cells [231]. The hypoxic control of mRNA translation can also be used as a mechanism of controlling protein expression in the cell. Inhibiting overall mRNA translation induces the expression of certain proteins that are essential for adaptation to certain conditions.

1.5.2 Regulation of IGFBPs by hypoxia

Induced expression of IGF-1R increases survival of cancer cells in hypoxic and nutrient-deprived microenvironments that can occur in large tumours [110]. IGFs and insulin respond to hypoxia by activation of Akt and MAPK signalling, resulting in stabilisation of hypoxia-inducible factors HIF-1 α and HIF-2 α , and upregulation of vascular endothelial growth factor [111]. HIF-1 α is necessary for maximum expression of genes encoding IGF-2, IGFBP-2 and IGFBP-3 indicating that cross-talk between the HIF-1 and IGF pathway exists [232].

Despite a decrease in protein synthesis under hypoxic conditions, the IGFBP family members IGFBP-1 and IGFBP-3 are hypoxia inducible [233] [234]. IGFBPs which are induced under hypoxic conditions are thought to act as death-promoting factors [235]. IGFBP-3 inhibits IGF signalling under extreme hypoxic conditions [236]. Proteins in the conditioned medium of the human adenocarcinoma pancreatic cancer cell line AsPC-1, which is resistant to hypoxia, were analyzed. It was found that some IGFBPs were increased in culture medium under hypoxic conditions, and that IGFBPs play an important role in cell survival by buffering IGF signalling under severely low oxygen levels. IGFBP-1 and IGFBP-3 protein expression increased which was also seen in the mRNA levels of IGFBP-1 and IGFBP-3. IGFBP-4 and IGFBP-6 were expressed in the AsPC-1 cells but expression remained unchanged under hypoxia. The role of IGFBPs in the survival of AsPC-1 cells was examined by exposure to IGFs under low oxygen tension. Under moderate hypoxic conditions (1% O₂), treatment with IGF-I did not increase cell death. The role of IGFBP-3 in IGF induced cell death under anoxic conditions was analyzed by treating them with wild-type IGF-I. Upregulation of IGFBP-1 was prominent under hypoxic conditions but expression of IGFBP-1 was suppressed by IGF-I under hypoxia. Although multiple IGFBPs were expressed in AsPC-1 cells, IGFBP-3 was critical for the survival of AsPC-1 cells with excessive IGF signalling under anoxic conditions. IGFBP-3 overexpression alone did not change the rate of cell death by IGF-I.

Hypoxia-induced genes could be relevant prognostic targets for therapeutic intervention in cancers. Identifying hypoxic markers will give a better understanding of tumour angiogenesis and for the optimization of anti-angiogenic therapeutic strategy. In the process of generating an adaptive response to hypoxic conditions,

endothelial cells contribute to the onset of angiogenic responses involved in tumour growth. The hypoxia-induced expression of hypoxia-induced transcripts (HITs) such as IGFBP-3, thioredoxin-interacting protein (txnip), neuritin (nrn1) on several types of human tumours provided evidence for nrn1 and txnip as hypoxic perinecrotic markers and for IGFBP-3 as a tumour endothelial marker [237]. These HITs could be targeted as future prognostic markers in cancer therapy. Many studies have provided evidence for the *in vitro* induction of IGFBP-3 mRNA by hypoxia in different cell types [233]. IGFBP-3 is a proapoptotic gene that is transcriptionally up-regulated during hypoxia. When induced, there was no significant increase in apoptosis, which makes the function of this gene during hypoxia unclear suggesting it could play a different role in growth regulation under hypoxic conditions.

1.6 Aims and objectives

1.6.1 General aims

IGF-1R plays an essential role in cancer progression and tumour cell survival suggesting the importance of targeting this molecule in the treatment of NSCLC. To strengthen this hypothesis the role of IGF-1R and its interactions with other signalling molecules and receptors was investigated to elucidate the importance of IGF-1R as a therapeutic target in the treatment of NSCLC. Validating the functional role of the IGF-1R through inhibition of IGF-1R signalling may be an effective strategy of decreasing tumour growth, overcoming resistance to EGFR TKI's and prolong survival in patients with high expression of IGF-1R and EGFR.

1.6.2 Specific objectives

The specific objectives are to (a) identify mutations in the tyrosine kinase domain of the IGF-1R, (b) examine IGF-1R and EGFR expression in matched normal and tumour tissue and to correlate the results to patients survival/pathological data, (c) investigate the effects of dual targeted therapy and IGF-1R gene silencing and (d) elucidate the role of IGFBPs in normal and lung cancer cell lines.

Chapter 2:

Material and Methods

2.0 Materials and Methods

2.1 Preparation and handling of materials

Reagents and chemicals used in the laboratory were of analytical grade and stored in accordance with the manufacturers' instructions. All chemicals were purchased from Merck (Darmstadt, Germany), the Sigma Chemical Company (St. Louis, MO, USA) and Fluka (Buchs, Switzerland), unless stated otherwise. Cell culture reagents were purchased from Lonza (Walkersville, MD, USA) with exceptions noted.

2.2 Chemical and Biological reagents

2.2.1 Drugs

5-Aza-2'-Deoxycytidine (DAC) from Calbiochem (San Diego, CA, USA) was dissolved in 100% methanol (MeOH) and stored at -20°C. Erlotinib from Santa Cruz Biotech (Santa Cruz, CA, USA) was dissolved in DMSO (<0.1%) and stored at -20°C. R1507 from Roche (Roche Diagnostics Ltd., Sussex, UK) was dissolved in sterile water and stored at -80°C.

2.3 Hypoxia chamber

In order to study the effect of low oxygen conditions, cells were placed in a hypoxia chamber (Invivo₂ 400 Hypoxia Workstation with Ruskin hypoxia gas mixer, Biotrace Int Plc, Glamorgan, UK) as shown in Fig 2.1. Oxygen was set to a specific concentration of 0.5% with 5% CO₂ in a humidified atmosphere.



Fig 2.1 The hypoxia chamber. The Invivo₂ 400 Hypoxia Workstation with Ruskin hypoxia gas mixer. The Machine provides accurate control over O₂ (0.1%- 20.9% in 0.1% increments) and allows the transfer of samples and access to the environment without affecting stability.

2.4 Cell culture

Cell culture work was performed aseptically in accordance with good laboratory practice in a Class IIB laminar air flow unit (LAF) (CleanAir Techniek bv, Woerden, The Netherlands). The LAF was allowed to run for at least 20 min before use. The LAF was sanitized using 70% (v/v) EtOH in d H₂O. All cell culture reagents were placed in a water bath at 37°C (Clifton unstirred thermostatic bath, Somerset, UK) for approximately 30 min before use unless stated otherwise.

2.4.1 Lung cancer cell lines

Five lung cancer cell lines were used during the course of this study; bronchioalveolar carcinoma cells (A549, H1299, H1819 and HCC827) and a squamous carcinoma cell line (SKMES-1). Cells (Fig 2.2) were obtained from the European Culture and Tissue Collection (ECACC, Wiltshire, UK). A549 cells were maintained in F-12 (Ham's) medium, which was supplemented with 10% (v/v) foetal bovine serum (FBS), penicillin streptomycin (P/S – 5000 U/mL penicillin, 5000 U/mL, streptomycin), and 2 mM L-glutamine (in 0.85% NaCl). SKMES-1 cells were maintained in minimum essential medium eagle with Earle's balanced salt solution (EMEM) with the addition of 10% FBS, P/S (5000 U/mL penicillin, 5000 U/mL streptomycin), 2 mM L-glutamine (in 0.85% NaCl), and 0.1 M non- essential amino

acids (L-Alanine, L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, Glycine, L-Proline and L-Serine). H1819, H1299 and HCC827 cells were maintained in Roswell Park Memorial Institute (RPMI) -1640 medium which was supplemented with 10% (v/v) foetal bovine serum (FBS), penicillin streptomycin (P/S – 5000 U/mL penicillin, 5000 U/mL, streptomycin), and 2 mM L-glutamine (in 0.85% NaCl). All cell lines are adherent and were incubated in vented flasks at 37°C in a 5 % CO₂ humidified atmosphere (Steri-Cycle CO₂ incubator, ThermoForma, Marietta, OH, USA). The histology and mutation status used in this thesis are outlined in Table 2.1.

Table 2.1: NSCLC cell lines histology and mutation status.

Cell line	Histology	Mutation status
HCC827	Adenocarcinoma	Exon 19 EGFR deletion
H1819	Adenocarcinoma	EGFR wild type
H1299	Adeoncarcinoma	EGFR wild type
A549	Adenocarcinoma	KRAS mutated
SKMES-1	Squamous cell carcinoma	KRAS mutated

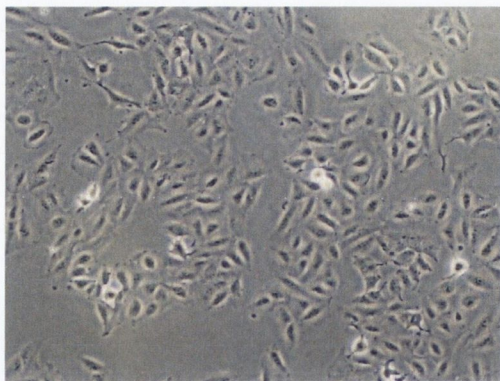
2.4.2 Normal cell lines

Three normal human bronchial epithelial cell lines (HBEC3, HBEC4 and HBEC5), as shown in Fig 2.3, were a gift from Prof. John D. Minna (Hamon Centre for Therapeutic Oncology Research, Dallas, TX, USA). Two of these cell lines were taken from donors who did not develop cancer (HBEC3 and HBEC5), whereas HBEC4 was isolated from a patient who went on to develop lung cancer. Cells were immortalized by the overexpression of telomerase (hTERT) and cyclin-dependent kinase 4 (CDK4) to bypass the effects of telomere shortening and the p16 cell cycle checkpoint respectively [238]. This technique did not utilise viral onco-proteins, which indicate that the HBEC lines are a superior ‘normal cell line’ standard. Cell lines were maintained in keratinocyte serum-free media (SFM), with L-glutamine (GIBCO Invitrogen, Paisley, Scotland) and supplemented with 2.5 µg human recombinant epidermal growth factor (rEGF), and 25 µg bovine pituitary extract (GIBCO Invitrogen). All tissue culture plastics used with the HBEC lines were coated with sterile 1X collagen (0.5 g/L dH₂O gelatine from porcine skin-type A) before use. These cells lines are adherent and were grown in vented flasks, as above.

2.4.3 Cell subculture

Cells were visually examined using an inverted phase-contrasted Nikon microscope (Nikon Corp., Tokyo, Japan). Sub-culturing was performed when cell cultures reached 80-90% confluency. Cells were detached for sub-culturing by trypsinisation.

A.



B.

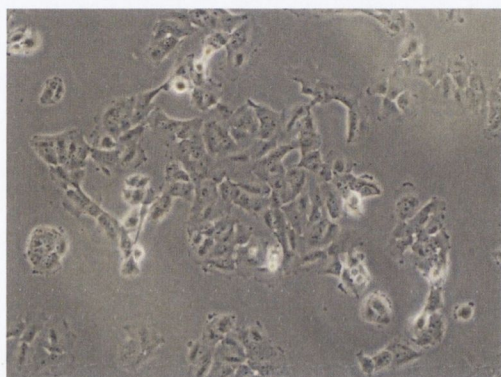


Fig 2.2: Monolayer cultures of lung cancer cell lines. Lung cancer cell lines used in this project included, A549 (A) SKMES-1 (B) at 60% confluency ($\times$ 40 magnification).

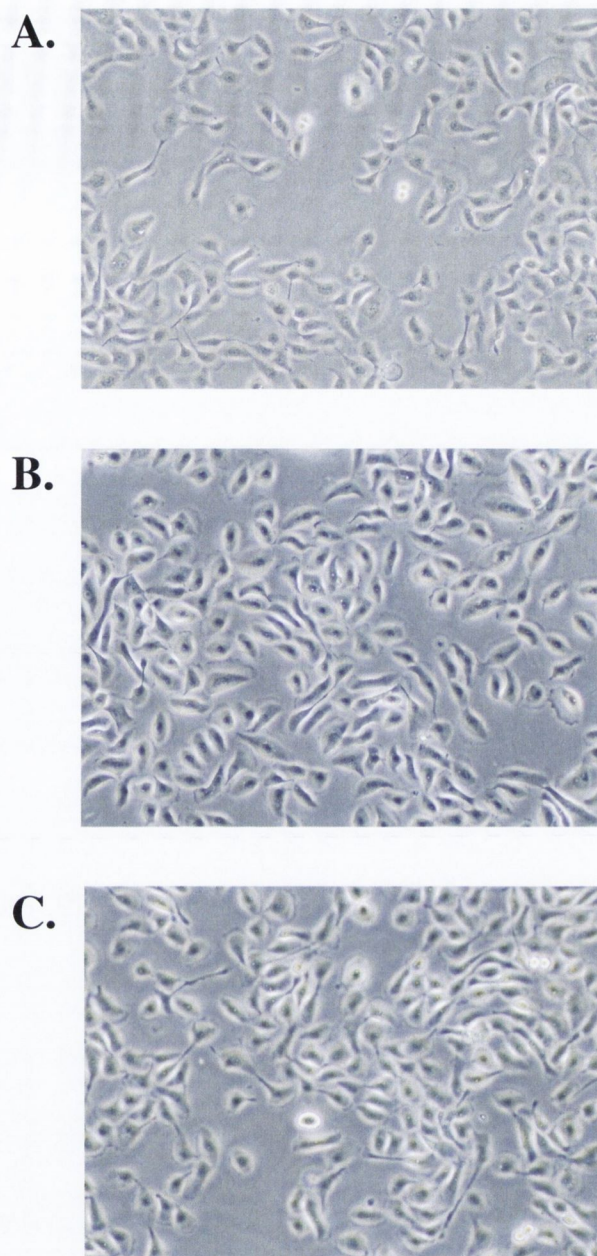


Fig 2.3: Monolayer cultures of normal bronchial epithelial cell lines. Three normal cell lines were used in this project, HBEC3 (A), HBEC4 (B), and HBEC5 (C) ($\times 40$ magnification).

2.4.3.1 Lung cancer cell lines

Cell culture medium was decanted and the cells were washed with 5 mL 0.01M PBS (0.0067M (PO_4), without Ca^{2+} and Mg) to remove residual FBS. Either, 1 mL (25 cm^3 flasks) or 2 mL (75 cm^3) of trypsin ethylene-diamine tetra-acetic acid (EDTA) (200mg/mL Versene/ EDTA), was added to the flasks. Flasks were incubated at 37°C for approximately 5 min to allow the cells to detach from the surface of the

flask. Eight mL complete medium was then added to the flasks to inactivate the trypsin. Cells were transferred to a sterile 15 mL tube and pelleted by centrifugation at 1300 χ g for 3 min (Centra GP8R, Thermo IEC). The supernatant (s/n) was discarded and the cell pellet re-suspended in 10 mL complete medium. This cell suspension was used to seed fresh flasks at a number of different ratios.

2.4.3.2 Normal HBEC cell lines

Cell culture s/n was removed from the flasks and discarded. The cells were then washed in 5 mL PBS and 2 mL of room temperature (RT) trypsin/EDTA added (0.25 mg/mL Versene/ EDTA) to the cells. Cells were allowed to detach in the LAF at RT. HBEC cell lines are strongly adherent and need to be repeatedly agitated and examined under the microscope to confirm cell detachment. When complete, 6 mL of RT trypsin neutralising solution (TNS) was added to the flask and the cell suspension mixed. Cells were transferred to a sterile 15 mL tube and pelleted by centrifugation at 1300 χ g for 3 min. The s/n was discarded and the cell pellet re-suspended in 10 mL complete medium. Representative growth curves for HBEC3-5 is shown in Appendix. A cell count was performed (Methods 2.4.6). Population doubling is calculated as:

$$\frac{\text{Log (No. of cells counted/ No. of cells plated)}}{\text{Log 2}} = \text{PD}$$

These cells are considered immortalized when they have reached a PD of 100. Therefore, these cell lines are tracked by PD instead of passage number (as in the case of lung cancer cell lines).

2.4.4 Preparation of frozen stocks

Stocks were prepared from cells in the exponential growth phase at less than 80% confluency. To prepare frozen stocks, cells were washed in 5 mL PBS, trypsinised and pelleted as above (Methods 2.4.3.1 and 2.4.3.2). Following centrifugation, the s/n was decanted and the lung cancer cells were re-suspended in 3 mL of 10% (v/v) DMSO in complete medium and normal cells in 3 mL of 10% DMSO, 10% FBS in complete media. Aliquots of the cell suspension were transferred to sterile cryovials,

which were placed at -20°C overnight (o/n) and then transferred to -80°C (-85°C ultra low freezer, Nuaire Corp., Plymouth, MN, USA) for short-term storage. Vials were removed to liquid nitrogen (NuAire Corp.) for long term storage.

2.4.5 Retrieval of frozen stocks

Cryovials were removed from liquid nitrogen and placed in a waterbath at 37°C for 5 min or until thawed. Cells were transferred to a 25 cm³ flask to which 5 mL complete medium had been added. After 24 h incubation at 37°C, the media was decanted and cells washed with 2 mL PBS. Cells were fed with 5 mL of fresh media. Flasks were maintained and passaged as previously described. Cells were passaged at least twice (for removal of residual DMSO and FBS (HBEC)) before use in any experiments.

2.4.6 Cell counting

Cells were seeded at specific densities depending on experimental setup. A bright line haemocytometer (Hausser Scientific, Horsham, PA, USA), was used in conjunction with Trypan Blue (0.4% v/v), for cell counting and viability. The strain is based on a dye exclusion principle where live cells do not take up the dye, however dead cells do due to compromised cell membrane integrity. Cells were re-suspended in a specific volume of complete medium after pelleting (Methods 2.4.3.1 and 2.4.3.2) until a single cell suspension was obtained. A 20 µL aliquot of this suspension was added to 180 µL of Trypan Blue and mixed. A cover slip was placed on the haemocytometer. The edge of the cover slip was touched gently by a pipette tip and the chamber filled by capillary action. The cells in the four large squares (each with sixteen smaller squares) were counted and an average cell number obtained. The number of cells per mL was then calculated using the following equation:

$$\underline{\text{Average no. of cells counted} \times 10,000 \times 10 = \text{no. of cells/mL}}$$

Where, 10,000 equals the volume under the cover slip (µL) and 10 equals the dilution factor.

2.4.7 Mycoplasma testing

Cells used in this project were tested for mycoplasma upon receipt and every three months thereafter using the MycoAlert® Mycoplasma detection kit (Lonza, Rockland, ME, USA). This biochemical assay exploits the activity of certain mycoplasma enzymes. Mycoplasma specific enzymes react with the Mycoalert™ substrate catalyzing the conversion of ADP to ATP. Prior to testing, cell lines were grown for at least two passages in complete media without P/S (except for HBEC cell lines). Following this 2 mL of s/n was centrifuged at 200 $\times$ g for 5 min to pellet floating cells. One hundred μ L of the cleared s/n was transferred into a luminescence compatible plate (Costar, New York, NY, USA). The luminometer (Wallac Victor2 1420 multilabel counter, Perkin Elmer Lifesciences, Boston, MA, USA) was programmed for a 1 s integrated reading. A 100 μ L of the MycoAlert® reagent was added to each sample and incubated for 5 min at RT. The first luminometer reading was taken (reading A). Next, 100 μ L MycoAlert™ substrate was added to each sample and incubated at RT for 10 min before a second reading was taken (reading B). The ratio of reading B to reading A determined the presence or absence of mycoplasma in the cell culture medium. A ratio greater than 1 indicates mycoplasma positive cells.

2.5 Growth assays

2.5.1 MTT assay

The MTT assay allows for the measurement of metabolically active cells in response to various conditions. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2),-2,5-diphenyltetrazolium bromide), is reduced to purple formazan crystals by metabolically active cells. These intracellular crystals are solubilised by the addition of DMSO. Cells were seeded at 5×10^3 /well in complete media in triplicate; in a 96 well plate and incubated under normoxic (21% O₂) conditions and treated with erlotinib and R1507. Following specified incubation times, 40 μ L of MTT solution (0.0125 g in 50 mL PBS) was added to all wells. Cells were examined periodically until purple crystals were visible microscopically, taking approximately two h for all cell lines. One hundred μ L DMSO added to the plates, which were quantified by spectrophotometric means (Vesamax tunable microplate reader, Molecular Devices,

CA, USA) at 595nm. The percentage of metabolically active cells was expressed relative to control cells (untreated), which were set as 100%.

2.5.2 BrdU proliferation assay

Cell proliferation was measured using a Cell Proliferation ELISA, BrdU (Roche Diagnostics Ltd). This is a colorimetric method to quantify cell proliferation based on the measurement of BrdU (5-bromo-2'-deoxyuridine – a pyrimidine analogue) incorporation (instead of thymidine) during DNA synthesis in proliferating cells. Cells were seeded at 2×10^3 /well in a 96-well plate and adhered o/n. Subsequently, the complete media was removed and the cells washed with 100 μ L PBS. Serum depleted media (0.5% FBS) was added to the lung cancer cells. Following o/n incubation cells were treated with appropriate drugs for a specific period of time. Following treatment, 10 μ L of a 1:1000 dilution of BrdU labelling solution (final concentration -10 μ M) was added to each well and plates incubated for 4 h at 37°C. Following incubation, the media was removed and the cells fixed and denatured with 200 μ L of a fixative solution for 30 min at RT. One hundred μ L anti-BrdU-POD (mouse monoclonal antibody, peroxidase-conjugated) working solution was added to each well for 90 min at RT. Cells were washed three times with wash buffer and 100 μ L of substrate solution was added for 5-10 min (or until colour change was sufficient for photometric detection). Twenty-five μ L 1 μ M H_2SO_4 was added to each well to stop the reaction. Absorbance was measured on a plate reader at 490 nm with a reference wavelength set to 690 nm.

2.5.3 Cell count using Hoechst staining and IN Cell 1000 Analyser

The IN Cell 1000 Analyser is an automated imaging device used for High Content Screening (HCS) for high throughput microscopic analysis of cells. It can be used for comparative expression analysis of different molecular markers in fixed and live cells under different conditions by quantitatively measuring fluorescent intensity within the cell.

The cell culture medium was removed and 50-75 μ L of pre-warmed fixing solution (4% (v/v) paraformaldehyde) was added to each well and the plates incubated at 37°C for 30 min. The fixing solution was removed and the plates were washed once

with PBS (50 μ L per well). Fifty μ L of PBS and 50 μ L of Hoechst stain (diluted 1:500) were added to each well and incubated at 37°C for 30 min. The plates were washed again with PBS (50 μ L per well). Hoechst stain is a fluorescent dye that binds to the nuclei of a cell. The number of stained cells were then counted using the IN Cell 1000 Analyser workstation.

2.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.6.1 Preparation of cell line samples

On day one cell lines were seeded at 1×10^6 in vented T75 cm^3 flasks. The following day lung cancer cells were washed with PBS and complete medium replaced with serum-depleted medium (0.5% FBS). On day three cells were subjected to specific treatments.

2.6.2 RNA isolation from cell lines

RNA was isolated using TRI reagent[®] (Molecular Research Centre, OH, USA). Media was decanted from the flasks and 1 mL of TRI reagent[®] added. It was incubated with agitation for 5 min, after which time cells were scraped into a tube and stored immediately at -80°C. Samples were thawed at RT and RNA isolated as follows: 100 μ L 1-bromo-3-chloro-propane (BCP), was added to the samples, which were then inverted for 15 s and incubated at RT for 10 min. The tubes were centrifuged at 13500 χ g at 4°C for 15min. Following centrifugation, the upper aqueous phase (containing RNA), was removed to a fresh 1.5 mL eppendorf tube. Five hundred μ L of isopropanol was added and the samples were mixed and incubated at RT for 10 min. Following centrifugation at 13500 χ g at 4°C for 8 min, the s/n was decanted and the pellet washed with 70% EtOH for 5 min. The samples were centrifuged again at 13500 χ g at 4°C for 5 min, and the EtOH wash decanted. The RNA pellet was allowed to air dry for 5 min and re-suspended in 50 μ L molecular grade H₂O (AccuGENE, Cambrex Bioscience, IA, USA). RNA was quantified by the Nanodrop spectrophotometer (Stion 2.6.4) and stored at -80°C.

2.6.3 RNA isolation from tissues

RNA was isolated from human tissue samples using Qiagen RNeasy kit (Qiagen Inc. CA, USA). Tissue samples, stored at -80°C in RNAlater, were removed into a 2 mL tube for homogenization. Six hundred μL of lysis buffer containing 2- β -mercaptoethanol (10 $\mu\text{L}/\text{mL}$) was pipetted into a 2 mL collection tube. One 5 mm stainless steel bead (Qiagen Inc.) was added to each tube. Up to 30 mg of the tissue sample (which was previously stabilized in RNAlater stabilization reagent), was added to each tube. The samples were homogenised on the Mixer Mill rack 300 (Retsch, Haan, Germany) at 20 Hz for 2 min. The Mixer Mill rack was rotated to allow even homogenization, and the samples were homogenised for a further 2 min at 20 Hz. Samples were centrifuged at $13500 \times g$ for 3 min (including the bead). The s/n was transferred to a new centrifuge tube. One volume of EtOH was added to the cleared lysate and mixed immediately by pipetting. The sample was then applied (700 μL) to an RNeasy mini column (in a 2 mL collection tube). The tube was centrifuged for 15 s at $10000 \times g$ and the flow-through discarded. A DNase digestion step was carried out using an RNase-Free DNase Set (Qiagen Inc.). Three hundred and fifty μL RW1 (supplied by the kit) was added to the column and centrifuged at $10000 \times g$ for 15 s and the flow-through discarded. Ten μL DNase I stock solution (1500 kunitz units) was mixed with 70 μL RDD buffer and added to the column. This was incubated for 15 min at RT, 350 μL of buffer RW1 was applied to the column and centrifuged for 15 s at $10000 \times g$. The RNeasy column was then transferred into a fresh 2 mL collection tube and 500 μL RPE buffer was pipetted onto the column. The column was centrifuged for 15 s at $10000 \times g$ and the flow-through discarded. A further 500 μL of buffer RPE was added to the column and the tube centrifuged for 2 min at $10000 \times g$. The RNeasy column was transferred into a new 1.5 mL eppendorf and 50 μL RNase-free water was pipetted directly onto the membrane. The tube was centrifuged for 1 min at $10000 \times g$ to elute the RNA.

2.6.4 RNA/DNA quantification using the nanodrop

RNA/DNA was spectrophotometrically quantified using a Nanodrop 1000 spectrophotometer (version 3.1.0, Nanodrop Technologies, DE, USA). The instrument was initialised and blanked with 1 μL of sterile distilled water (SDW). Then 1 μL of each sample was loaded in turn onto the Nanodrop (wiping after each).

The Nanodrop gives the nucleic acid concentration in ng/μL and also the 260:280 and 260:230 purity ratios.

2.6.5 Assessment of RNA quality

Each sample was run on a 1% agarose gel (see stion 2.6.9), to determine RNA quality. Clear bands for the 18S and 28S ribosomal subunits are a good indication of RNA integrity.

2.6.6 RQ1 DNase treatment

RNA samples (from cell lines only), were diluted in dH₂O to a stock concentration of 0.5 μg/μL. 10 μg RNA was used for RQ1 DNase treatment with the reaction prepared as follows: 20μL RNA sample, 5μL 10X RQ1 buffer (Promega, WI, USA), 1μL RQ1 DNase (Promega) and 24μL SDW. The samples were mixed gently with a pipette and placed at 37°C for 1.5 h. Fifty μL phenol-chloroform was added to the tube, vortexed and centrifuged at 13500 χ g for 5 min at 4°C (Miko 200, Hettich zentifugen, Germany). The upper layer containing the RNA was transferred to a new tube and the following added: 1μL polyacrly carrier (Molecular Research Center), 50μL 5 M Ammonium acetate and 300μL 96% EtOH. The mix was vortexed, incubated at RT for 15 min and centrifuged for 20 min at 4°C at 13500 χ g. The s/n was discarded and the pellet washed with 1 mL of 70% EtOH. The sample was centrifuged at 13500 χ g for 5 min, the EtOH removed, and the pellet re-suspended in 10μL SDW.

2.6.7 cDNA synthesis

For first strand cDNA synthesis, 1 μL 50 μM Oligo(dT)₂₀ (Operon Biotechnologies GmbH, Cologne, Germany), 1μL 10 mM dNTPs (dATP, dGTP, dCTP, dTTP), (Promega) and 14 μL SDW was added to each RNA sample. Tubes were heated to 65 °C for 5 min and transferred to ice for 1 min. The second mix was then added to the tube. This consisted of 4 μL 5X FS Buffer (Invitrogen Corp., CA, USA), 1 μL 0.1 M DTT (Invitrogen), 1 μL recombinant RNasin[®] ribonuclease inhibitor (Promega), 1 μL Superscript[™] III reverse transcriptase (200 U/μL) (Invitrogen) and 7 μL SDW. The sample was mixed gently with a pipette and placed into a G Storm

thermal cycler (GSI, Braintree, Essex, UK) at 50 °C for 3 h, followed by heat inactivation at 70 °C for 15 min.

2.6.8 Polymerase Chain Reaction

cDNA generated by the above protocol was used as a template for the polymerase chain reaction (PCR). The following PCR master-mix was prepared for each sample to be amplified: 10 µL 2 x GoTaq[®] Green Master Mix (400 uM dATP, dGTP, dCTP, dTTP, 3mM MgCl₂, GoTAQ, pH 8.5) (Promega), 2 µL 5 µM forward primer (F), 2 µL 5 µM reverse primer (R), and 6µL SDW. One µL of cDNA template was used per reaction. A negative control was also included, using 1 µL SDW in place of cDNA. Template DNA was initially denatured at 94°C for 5 min, followed by 35-40 amplification cycles, in the G storm thermal cycler. Each cycle consisted of template denaturation, primer annealing (optimised for each target), and extension. This was followed by an elongation step to complete the amplification cycle. The primers and annealing temperatures used in each PCR are outlined in Table 2.2.

Table 2.2: Primer sequences and annealing temperatures

Primer	Sequence F= Forward R= Reverse	Annealing temp
IGFBP-1	F:GGATCATTCCATCCTTTGGGAC R:TCCCATTCCTCAAGGGTAGACG	60°C
IGFBP-2	F:GAGCAGGTTGCAGACAATGG R:CGAGCCTCCTGCTGCTCATT	60°C
IGFBP-3	F:ATGCTAGTGAGTCGGAGGAAGA R:CTTCCCCTTGGTGGTGTAGC	60°C
IGFBP-4	F:CACCCCAACAACAGCTTCAG R:GGATGGGGATGATGATGTAGAGGTC	60°C
IGFBP-5	F:AGGAGACCTACTCCCCCAAG R:ACATGAAAGTCCCCGTCAAC	60°C
IGFBP-6	F:CCCCGAGAGAACGAAG R:CACAGTTTGGCACATAGAG	60°C
IGF-1R	F:GCCCCGAAGGTCTGTGCGGAAGAA R:GGTACCGGTGCCAGGTTATGA	60°C
IGF-1	F:ATGCACACCATGTCCTCCTGGCAT R:CTACATCCTGTAGTTCTTGTT	60°C

2.6.9 Agarose gel electrophoresis

All PCR products were visualised on 1% agarose gels. The agarose (1%) was dissolved in Tris-Acetate-EDTA (TAE) buffer (40 mM Trizma base, 20 mM acetic acid, 1 mM EDTA) by boiling in a microwave for 2-3 min. The solution was cooled to 55-60°C, before the addition of Ethidium Bromide, to final concentration of 1 µg/mL. It was poured into a gel tray with well forming combs to a depth of 3-5 mm and allowed to set. The samples needed no loading buffer as the 2 x GoTaq® Green Master Mix contained a blue and yellow loading dye. One µL 1Kb Plus DNA Ladder (Invitrogen), was mixed with 5 µL loading dye (40% sucrose, 0.25% w/v bromophenol blue) and loaded onto the gel with the samples. Electrophoresis of the DNA samples was carried out in a Maxi Horizontal Electrophoresis Unit Set, model SH413 (Sigma) using 1X TAE as a running buffer. The voltage was kept constant at 120 V, and gels ran for approximately 30-40 min. The bands were visualised and

photographed under UV light using a Biospectrum Imaging System (Ultra Violet Products, Cambridge, UK).

2.7 Western Blotting

2.7.1 Patient cohorts

Matched tumour/ normal tissues from a series of NSCLC patients, included in a European Biomarker study, were collected immediately after resection. Patient consent was obtained prior to surgery. A pathologist identified the normal and tumour tissues. The tissues were immediately snap frozen in liquid nitrogen.

2.7.2 Protein and RNA isolation from tissue using TRI Reagent

Tissue samples were homogenized in TRI Reagent (1 mL/50- 100 mg tissue) using a glass- Teflon or Polytron homogenizer. The homogenate was stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Next, the homogenate was supplemented with 0.1 mL BCP per 1 mL of TRI Reagent, and the samples were covered tightly and shaken vigorously for 15 s. The resulting mixture was stored at room temperature for 2-15 min and then centrifuged at 12,000 xg for 15 min at 4 °C. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase. RNA remained exclusively in the aqueous phase whereas DNA and proteins were in the interphase and organic phase.

The aqueous phase was transferred to a fresh tube and the interphase and organic phase was stored at 4 °C for subsequent isolation of proteins. RNA was precipitated from the aqueous phase by mixing with isopropanol, (0.5 mL of isopropanol per 1 mL of TRI Reagent used for the initial homogenization). Samples were stored at room temperature for 5-10 min and centrifuged at 12,000 xg for 8 min at 4 - 25 °C.

The supernatant was removed and the RNA pellet was washed (by vortexing) with 75% ethanol and subsequent centrifugation at 7,500 xg for 5 min at 4 - 25 °C (1 mL of 75% ethanol per 1 mL TRI Reagent used for the initial homogenization). The ethanol wash was removed and the RNA pellet was briefly air-dried for 3 - 5 min.

The remaining aqueous phase overlying the interphase was removed. DNA was precipitated from the interphase and organic phase with ethanol, 0.3 mL of 100% ethanol per 1 mL of TRI Reagent used for the initial homogenization, and samples were mixed by inversion. The samples were then stored at room temperature for 2-3 min and DNA was sedimented by centrifugation at 2,000 g for 5 min at 4 °C. The phenol-ethanol supernatant was used for the subsequent protein isolation.

A portion of the phenol-ethanol supernatant (0.2 - 0.5 mL, 1 volume) was aliquoted into a microfuge tube. Proteins were precipitated by adding 3 volumes of acetone. Samples were mixed by inversion for 10-15 s to obtain a homogeneous solution then stored for 10 min at room temperature. The protein precipitate was sedimented at 12,000 g for 10 min at 4 °C.

The phenol-ethanol supernatant was decanted and the protein pellet was dispersed in 0.5 mL of 0.3 M guanidine hydrochloride in 95 % ethanol + 2.5 % glycerol (v:v). The pellet was dispersed using a pipet tip. After dispersing the pellet, another 0.5 mL aliquot of the guanidine hydrochloride/ethanol/glycerol wash solution was added to the sample and stored for 10 min at RT. The protein was sedimented at 8,000 g for 5 min. The wash solution was decanted and two more washes in 1 mL each of the guanidine/ethanol/glycerol wash solution were performed. The pellet was dispersed by vortexing after each wash to efficiently remove residual phenol. The final wash was performed in 1 mL of ethanol containing 2.5 % glycerol (v: v). At the end of the 10 min ethanol wash, the protein was sedimented at 8,000 g for 5 min. The alcohol was decanted and the pellet was air-dried for 7-10 min at RT. The protein was solubilized in cold RIPA lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton-X 100, 0.1% (w/v) SDS), supplemented with 10 µL phenylmethylsulfonyl fluoride (PMSF)(87 mg/mL 96% EtOH), and 100 µL protease inhibitor cocktail (PIC) (2 mM AEBSF, 1 mM EDTA, 130 µM Bestatin, 14 µM E-64, 1 µM Leupepin, 0.3 µM Aprotinin) and stored at -20°C.

2.7.3 Preparation of cell lysates

Cell culture s/n was removed from culture flasks and centrifuged at 1300 $\times$ g for 3 min, and the supernatant decanted. The resulting pellet was stored on ice and 250 – 500 µL cold RIPA lysis buffer was added to the flasks and incubated for 5-10 min on ice. The cells were removed from the surface of the flask using a cell scraper and

transferred to the appropriate tube containing the s/n derived pellet. The lysate was mixed and the DNA sheared by repeatedly passing the lysate through a 1 mL U-100 syringe (Becton Dickinson, NJ, USA). Protein samples were stored at -80 °C.

2.7.4 Protein determination

Protein concentrations were determined using the bicinchoninic acid (BCA) (Pierce, IL, USA). This assay utilises the principle that Cu^{2+} is oxidised to Cu^+ in the presence of protein in an alkaline medium. The BCA reagent reacts with the Cu^+ , yielding a coloured product. Prior to use, the kit components were mixed in a 50:1 ratio (alkaline bicarbonate solution to copper sulphate solution, respectively). Two hundred μL of this working reagent was added to 10 μL of protein standard or 2 μL of sample in a 96-well plate, which was then incubated at 37°C for 30 min. Following incubation, the absorbance was measured at 595 nm on the plate reader. Protein concentrations were determined by interpolation from a standard curve of known concentrations of BSA, ranging from 0 to 1000 $\mu\text{g/mL}$ (Appendix).

2.7.5 SDS PAGE

Glass plates (Atto Corp., Tokyo, Japan) were cleaned with EtOH and assembled in an upright position with a rubber seal in place. A 12% (v/v) resolving gel was made using 9.2 mL 30% acrylamide:bisacrylamide, 4.5 mL 1.875 M Tris HCl-pH 8.8, 8.3 mL SDW, 176 μL 10% (w/v) SDS, 120 μL 10% (w/v) fresh ammonium persulfate (APS) and 10 μL TEMED (N,N,N',N'-tetramethylethylenediamine). The solution was mixed gently to avoid bubble formation and pipetted into the upright glass plates. Seventy % EtOH was gently layered on top of the gels to aid polymerization. A 5% (w/v) stacking gel was prepared containing 1.7 mL 30% (w/v) acrylamide:bisacrylamide, 2 mL 0.6 M Tris HCl-pH 6.8, 6 mL SDW, 100 μL 10% (w/v) SDS, 150 μL 10% (w/v) APS, and 10 μL TEMED. Once the resolving gel had fully set, the EtOH was removed from the resolving gel and the stacking gel poured on top. A 12-well comb was inserted into the stacking gel and the gel left to set. Protein samples were diluted (1:1) with 2X Laemmli buffer (2 mL 0.6M Tris HCl, 5 mL 10% (w/v) SDS, 1 mL 2- β -Mercaptoethanol, 2 mL glycerol, 0.05 g (w/v) bromophenol blue), and denatured by incubating at 95°C for 10 min. Ten μL of a trichom pre-stained protein marker (Pierce) was also loaded onto each gel. Samples were separated by electrophoresis (Atto) at 25 mA per gel in electrode buffer (50

mM Trizma base, 384 mM Glycine, 0.1% (w/v) SDS, for 90 min, or until the dye front reached the end of the gel.

2.7.6 Protein transfer

Gels were electrophoresed in duplicate, one for transfer to PVDF and the other for coomassie staining. Separated proteins were transferred onto a 0.45 μ M polyvinylidene flouride (PVDF) membrane (Pall Corp., FL, USA) using a wet Mini-Trans Blot cell (Bio-Rad Laboratories, CA, USA). A pre-activated (1 min in 100% MeOH) PVDF membrane and Whatman filter paper (Whatman Laboratory Division, Kent, UK) were soaked in cold transfer buffer (0.15 M glycine, 20 mM Trizma base, 0.1% (w/v) SDS, 20% (v/v) MeOH), prior to use. The layers in the transfer cassette are outlined in Fig 2.4. The cassette was then placed in a cooled electrode tank and topped up with cold transfer buffer and run at 100 V and 400 mA for 1 h.

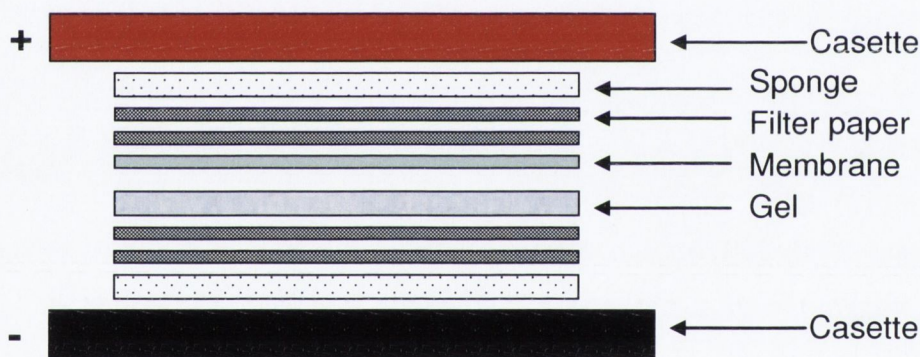


Fig 2.4: Wet transfer layout. Current ran from the anode to the cathode.

2.7.7 Ponceau S staining

Protein transfer to PVDF membrane was confirmed by staining the unblocked membranes in Ponceau S solution (Pierce), for 5 min on a shaker. The membrane was destained in SDW and rinsed in 1 X Tris Buffered Saline containing 0.1% Tween20 (TBST) (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% Tween20), before commencing with immuno-blotting.

2.7.8 Antibody probing of membranes

Following the transfer of proteins, membranes were blocked with between 3-5% (w/v) non-fat dried milk (Marvel), which was reconstituted in 1X TBST. The membrane was incubated in the primary antibody (Table 2.3) in 5 % Marvel TBST on a shaker. Membranes were washed six times for 5 min each in TBST, before incubation in the appropriate species-specific horseradish peroxidase (HP)-conjugated secondary antibody (1:2,000 dilution in 5 % Marvel TBST) (Dako, Glostrup, Denmark) for 1 h. The membrane was washed with 1X TBST over a period of six five min washes. The Supersignal West Pico Chemiluminescent substrate kit (Pierce), was used to detect bound antibody complexes. Working reagent was prepared just prior to use and was applied to the membrane for 5 min. Membranes were then exposed to scientific imaging X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan), which was developed using a medical film processor (Agfa-Gevaert, Mortsels, Belgium). Exposure times ranged from 10 s to 30 min, depending on the signal intensity.

Table 2.3: Antibodies used for Western blotting. Following transfer of the proteins to PVDF membranes, blots were probed with the relevant primary antibody using the conditions shown. All secondary antibodies were HRP labelled. Bound antibody complexes were detected by chemiluminescence.

Antibody	Supplier	Type	Isotype	Blocking	1°
IGF-1R	R & D Systems	Monoclonal	Goat	1 h 5% Milk	2 µg/mL o/n at 4°C
EGFR	Cell Signalling	Monoclonal	Rabbit	1 h 5% Milk	1:1000 o/n at 4°C
IGFBP-3	Millipore	Polyclonal	Rabbit	1 h 3% Milk	0.4 µg/mL o/n at 4°C
Erk 1/2	Cell Signalling	Monoclonal	Rabbit	1 h 5% Milk	2 µg/mL o/n at 4°C
α-β-tubulin	Cell Signalling	Polyclonal	Rabbit	1 h 5% Milk	1:1000 o/n at 4°C

2.7.9 Membrane stripping

In order for the membranes to be re-probed with a different antibody the blots were stripped. The membrane was incubated in 10 mL of RestoreTM western blot stripping buffer (Pierce) at RT on a shaker. This was removed after 20 min and the membrane washed twice for 10 min in TBST and stored at 4°C. Stripped membranes were re-probed as described in section 2.7.7.

2.8 IGF-1R Sequencing analysis

Sequencing was carried out on exons 1-6 of the tyrosine kinase domain of the IGF-1R (primers outlined in Table 2.3).

2.8.1 DNA extraction

DNA was extracted from formalin fixed paraffin embedded (FFPE) tissue from patients with NSCLC using the QIAamp® DNA FFPE Tissue Kit. Using a scalpel the excess paraffin was trimmed off the sample block and cut into 5–10 µm thick sections. Sections were immediately placed in 1.5 mL microcentrifuge tubes and were vortexed vigorously for 10 s twice in 160 µL xylene followed by two washes in 160 µL ethanol solution. One hundred and eighty µL Buffer ATL was added and mixed by vortexing. The samples were centrifuged for 1 min at 11000 χ g, 20 µL proteinase K was added to the lower phase and mixed gently. Samples were incubated at 56°C for 24-48 h (or until the sample has completely lysed). The tubes were briefly centrifuged and the lower phase was transferred into a new 2 mL microcentrifuge tube. Two hundred µL of both Buffer AL and 96% EtOH were added to the tubes and vortexed. The lysate was then transferred in to a QIAamp MinElute column (in a 2 mL collection tube) and centrifuged at 6000 χ g for 1 min. Subsequently, the column was placed in a new collection tube, 500 µL Buffer AW1 was added to the column and centrifuged as above. This was followed by 500 µL Buffer AW2 and again centrifuged as above. The tubes were centrifuged for an additional 3 min at 20000 χ g to dry the column membrane. The column was placed in a clean 1.5 mL microcentrifuge tube, 50 µL Buffer ATE was applied to the centre of the membrane and incubated for 1 min at RT. DNA was eluted by centrifugation (20000 χ g for 1 min).

Control DNA was extracted from the buffycoat cells of patients who donated blood to the blood bank (IBTS) using the QIAamp DNA blood mini kit.

2.8.2 PCR

The following PCR conditions were used: 1X GoTaq green master mix (Promega), 5 μ M of forward and reverse primer and 100 ng of DNA made up to a volume of 50 μ L with dH₂O. A pre-PCR heat step of 94°C for 5 min was carried out to activate the enzyme and the DNA was amplified for 35 cycles at 94°C (1 min), 56°C (1 min) and 72°C (1 min) and at 72°C (10 min) after the last cycle. A portion of the PCR product was electrophoresed on 1.4% agarose gel to verify product integrity. PCR products were purified using Qiagen QIAquick PCR purification kit. Briefly, 5 volumes of buffer PB was added to the PCR samples, and pipetted onto the spin column (placed in a 2 mL collection tube). The column was centrifuged for 1 min at 13000 χ g and flow-through discarded. Seven hundred and fifty μ L buffer PE was added to the column and centrifuged as above. The column was then centrifuged for an additional 1 min at 1300 χ g to ensure complete removal of any residual buffer. The column was then placed in a clean 1.5 mL eppendorf tube and 30 μ L buffer EB added to the column and incubated for 1 min at RT. The DNA was eluted by centrifuging at 13000 χ g for 1 min. The DNA was measured using the Nanodrop (Section 2.6.4). The primer sequences used for PCR are outlined in Table 2.4.

Table 2.4: Primer sequences for IGF-1R receptor tyrosine kinase (RTK).

Exon	Primer Sequence (5'-3')
RTK1	F: GGCTTGTTTCTGTACCTGCT
	R:AGCCAAGAACATACTGGGAG
RTK2	F: ACAACACAGGCATCAGCAAG
	R: GACACAGCATTTTCCTTGCAG
RTK3	F: CTCGAAAGAAATTGGCATGG
	R:TCTCCAGGGGCAGACTAATG
RTK4	F: CTGCTCCAGCGTGTGACTCT
	R:GAGCTAAAGCTGGCAACGGG
RTK5	F: CTGCTCGGGATGTAAGAAGT
	R: CTCCTAATCTCCTGTGACCC
RTK6	F: CGTACGAGGTAAACAGGAG
	R:AGCTTGTTCTCCTCGCTGTA

2.8.3 Sequence set up

The following sequencing reaction was used: 2 µL of BigDye Terminator Mix v3.1, 50 ng DNA and 5 µM of either forward or reverse primer. The positive control, pGem was used in place of the template, to ensure the efficiency of the sequencing reaction (1 µL pGem, 2 µl M13 primer and 2 µL of BigDye® Terminator Mix v3.1) The pGem and BigDye® Terminator v3.1 mix were both sourced from Applied Biosystems (Warrington, UK). Sequencing reactions were set-up in thin wall tubes according to Table 2.5.

Table 2.5: Components of the sequencing reaction.

Reagent	Sample	Control
DNA Template	50 ng	1µL pGem
Primer	5 µM/µL	2µL M13 primer
BigDye® Terminator Mix v3.1	2µL	2µL
dH ₂ O	Made up to 20µL	15µL
Total Volume:	20µL	20µL

The reactions were placed on a G Storm thermal cycler (96°C for 1 min, followed by 35 cycles of 96°C for 10 s, 51°C for 10 s and 60°C for 4 min, lastly 60°C for 10 s). Reaction clean up was required after DNA amplification. This was performed using a Qiagen DyeEx spin kit (Qiagen Operon). The spin columns were vortexed to re-suspend the resin, the cap loosened slightly and the bottom stopper of the column removed. They were placed in a collection tube and centrifuged at 3000 χ g for 3 min. The collection tube was discarded and the spin column was placed into a 1.5 mL eppendorf tube. The sequencing reaction was gently pipetted into the centre of the resin bed surface and centrifuged at 3000 χ g for 3 min. A speedyvac (Savant Instruments Incorporated, NY, USA) was used to dry the recovered reactions (approximately 20 min). The lyophilised reactions were re-suspended in 10µL of HiDi formamide (Applied Biosystems), just before use. Sequencing was performed on a 3130xl genetic analyser (ABI Biosystems, CA, USA), and sequencing files were analysed using the BioEdit v 7.0.8 (Tom Hall, Ibis Biosciences, CA, USA).

2.8.4 SNP genotyping assay

A TaqMan® SNP genotyping assay (Applied Biosystems) which detected the SNP at codon 3129A>G in exon 16 of the TK domain by real-time PCR was also used to screen patients. TaqMan® SNP Genotyping Assay 5' nuclease technology uses two allele-specific TaqMan® MGB probes and a PCR primer pair to detect the specific SNP target. The probes and primers uniquely align with the genome, enabling TaqMan® genotyping technology to provide unmatched specificity.

2.9 Immunohistochemistry

2.9.1 Paraffin-embedded tissue studies

This is a retrospective study in which patients with stage I-III NSCLC were randomly selected from a database of all individuals who underwent a curative-intent surgical resection at St. James Hospital between February 2001 and February 2005. Information on baseline demographics, clinicopathological characteristics and surgical approach was collected after review of clinical notes and histopathology reports. Outcome data, including peri-operative mortality and long-term survival, was updated prospectively. This study was approved by the St. James's Hospital Ethics Committee.

2.9.2 Generation of Tissue Microarrays (TMAs)

A 4 µm section was cut from formalin-fixed, paraffin-embedded tissue blocks from a cohort of 200 patients. This section was stained with haematoxylin-eosin for light microscopic examination by a pathologist to confirm areas of tumour. Three to four areas of tumour were marked on each section. Three cores, each 2 mm in diameter, were removed from each patient "donor" block and inserted into a "recipient" TMA block. A series of sections were cut from each TMA block, the first and last sections being stained with haematoxylin-eosin and subsequently tumour content confirmed by a pathologist.

2.9.3 Immunohistochemistry staining

2.9.3.1 IGF-1R

IGF-1R immunohistochemical (IHC) analysis was performed on 4 µm sections from 22 slides of a Tissue Micro Arrays (TMA) and mounted on Superfrost Plus glass slides (Thermo Scientific). The entire staining procedure was performed on an automated immunohistochemistry device (BenchMark XT, Ventana Medical Systems [VMS], Tucson, AZ, USA). In brief, slides were deparaffinized on thermopads using the EzPrep reagent (VMS) and epitope recovery performed using CC1 buffer, a citric-acid- based antigen retrieval solution (VMS). All subsequent washing and blocking steps followed standard protocols. Slides were incubated with the pre-diluted primary antibody (monoclonal rabbit anti-IGF-1R, clone G11, VMS) for 16 min at 37°C. Negative controls included identically processed slides in which the primary antibody was replaced by accordingly diluted non-immune rabbit IgG (Abcam, Cambridge, UK). Positive controls included identically treated paraffin slides of a H322M xenograft, an IGF-1R over-expressing NSCLC cell line. Detection of primary antibody binding was performed using the UltraView Universal DAB Detection Kit (VMS). After the diaminobenzidine (DAB) reaction was developed, slides were counterstained with Haematoxylin II (VMS) dehydrated in a serial dilution of EtOH, transferred into xylene and mounted in Eukitt (Kindler GmbH, Freiburg, Germany).

2.9.3.2 EGFR staining

EGFR IHC was carried out on a BondMax automated immunostainer from Vision BioSystemsTM, Newcastle, UK. Slides were loaded onto the system which initially de-waxed the sections, followed by a 25 min pre-treatment with BondMax Antigen Epitope Retrieval Solution 2 (the antigen retrieval technique previously optimised for the EGFR antibody in the Thoracic Oncology Research Group). The diluted primary antibody (1:100 diluted, NCL-EGFR-384 Clone, Novocastra) was added to the sections and incubated for 20 min. Detection and visualization of stained cells was achieved using the Bond Polymer Refine Kit (Vision BioSystemsTM) with DAB as the chromagen. Tissues were counterstained with Harris's haematoxylin and processed to coverslip. Appropriate isotype positive (EGF activated NSCLC cell line) and negative controls (omission of primary antibodies) were used in all assays.

2.9.4 Image acquisition

The Aperio ScanScope CS Slide Scanner (Aperio Technologies, Vista, Ca., USA) system was used to acquire whole-slide digitized images with a 20 $\times$ objective. IHC staining was evaluated by two independent pathologists. IHC was considered positive only when distinct membranous staining was observed in >10% of cells. The intensity of IHC membrane staining was evaluated using 4 distinct categories: absent (score 0), weak (score 1), intermediate (score 2), and intense (score 3). In cases with heterogeneous staining patterns, the final scores reflected the more intensely stained areas.

2.9.5 Statistical analysis

The software package SPSS v16.0 (SPSS Inc., Chicago, IL) was used to perform the statistical analysis. Chi-square test, Cox regression analysis, Kaplan–Meier analysis and the log-rank test were used to illustrate the significance of various clinical characteristics. Assumption of proportional hazard was tested for all covariates. P-values were considered statistically significant if <0.05.

2.10 Transfection of lung cancer cell lines

2.10.1 siRNA transfection with DharmaFECT

DharmaFECT 1 (Thermo Scientific) transfection reagent is a low toxicity transfection reagent which maintains cell viability with lipids specially formulated for the successful delivery of siRNA into cells. Cells were seeded at 5×10^4 /well in a 6-well plate in antibiotic-free complete media until 80% confluency was reached. A final concentration of 50 nm ON-TARGET *plus* SMARTpool siRNA reagent (Thermo Scientific) was used for effective IGF-1R knockdown. In separate tubes, 20 μ L of 5 μ M siRNA (tube 1) and 6.6 μ L of DharmaFECT 1 transfection reagent (tube 2) were diluted in 180 μ L and 193.4 μ L of serum-free medium, respectively. The contents of each tube were gently mixed by pipetting and incubated for 5 min at RT. The contents of tube 1 was added to tube 2, mixed by pipetting and incubated for 20 min at RT. Antibiotic-free complete medium was added to the transfection medium for a total volume of 2 mL per well in a 6-well plate. Culture medium was removed from the wells of the 6-well plate and the transfection medium was added to each well. Cells were incubated at 37°C with transfection medium replaced with complete medium after 24 h to reduce cytotoxicity and incubation continued for a further 24 h.

RNA and protein were obtained from the transfected cells as described previously (Methods 2.6.2 and 2.7.3).

2.11 Apoptosis assay

2.11.1 Preparation of cells

Cells were seeded at 4×10^3 /well in an opaque-walled tissue culture plate with solid bottom and left to adhere overnight. The following day, cells were serum starved in media containing 0.5% FBS for 24 h. After 24 h of serum starvation, cells were treated with erlotinib and R1507 with a total volume of 100 μ L per well and incubated for a further 24 h in the presence of the inhibitors before cell viability and apoptosis were measured. Staurosporine was used as a positive control for apoptosis at a concentration of 10 μ M for 6 h.

2.11.2 ApoLive-Glo™ Multiplex Assay

The ApoLive-Glo™ Multiplex Assay uses two assays to assess cell viability and apoptosis within the same well on a plate. Cell viability is measured in the first part of the assay by measuring the activity of a protease marker. The protease activity of intact viable cells is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenylalanyl-amino fluorocoumarin; GF-AFC). The GF-AFC enters intact cells, where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. Twenty μ L of Viability Reagent is added to all wells and mixed briefly by orbital shaking (300–500rpm for ~30 s). The plate was then incubated for 1 h at 37°C. After 1 h fluorescence was measured at the following wavelength set: 400Ex/505Em on a microplate reader (Wallac Victor² 1420 multilabel counter).

The second part of the assay measures apoptosis by measuring caspase-3/7 activation using the Caspase-Glo® Assay. A luminogenic caspase-3/7 substrate, Caspase-Glo® 3/7 Reagent is added to the cells, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Addition of the Caspase-Glo® 3/7 Reagent results in cell lysis, followed by caspase cleavage of

the substrate and generation of a luminescent signal produced by luciferase (Ultra-Glo™ Recombinant Luciferase). The luminescence produced is proportional to the amount of caspase activity present in the cells. One hundred µL of Caspase-Glo® 3/7 Reagent is added to all wells and mixed briefly by orbital shaking (300–500 rpm for ~30 s). The plate was then incubated for 1 h at room temperature and the luminescence was measured on a microplate reader (Wallac Victor² 1420 multilabel counter).

2.12 Statistics

Standard deviation (SD) describes the dispersion of observed values in a data set and is calculated by determining the square root of the variance. Alternatively, the standard error of the mean (SEM) is the standard deviation of the distribution of sample mean. As sample size increases, the SEM decreases. When the SEM is small, it indicates that the distribution of sample means has less error estimating the true mean. SEM is calculated as the SD of the original sample divided by the square root of the sample size. Here, the data are expressed as the mean $\pm$ SEM, which improves the appearance of the data when graphed, as opposed to the mean $\pm$ SD. Expressing the data as either the mean $\pm$ SEM or mean $\pm$ SD does not affect statistical significance in any way. Significance was determined via one way analysis of variance (ANOVA), where the number of groups in the experiment was three or more, a paired student t test or by using a box and whisker plot. A probability of (p) ≤ 0.05 was considered to represent a significant difference between the groups. A *post hoc* test was necessary after using ANOVA to determine which groups were significantly different to each other post test analysis was by Tukey multiple comparisons test. Statistical analysis was computed using InStat version 3.0 and GraphPad prism 5.

Chapter 3:

Mutational analysis of the IGF-1R TK domain

3.0 Introduction

Somatic mutations in the kinase domain of a receptor can impact on the ability of a therapeutic agent to bind efficiently or can cause the cell to become highly dependent on the constitutively active receptor signalling pathway, a state referred to as “oncogenic addiction” [133] [134]. The finding that NSCLC patients with EGFR mutations have increased sensitivity to EGFR targeted therapies such as erlotinib has made an incredible impact on survival in this subset of NSCLC patients. To date there are no reports of a link between mutations in the IGF-1R tyrosine kinase domain with response to IGF-1R targeted therapies. There are studies that have linked IGF-1R gene polymorphisms to certain diseases such as in dementia and ischemic stroke [130] [131] but not much is known about the functional consequences of SNPs in this gene [132].

IGF-1 and IGF-1R polymorphisms and increased IGF-1 plasma levels have been associated with cancer risk [135] [136] [137]. Identifying functional polymorphisms in the IGF-1R pathway could be used to select patients that would benefit from combined EGFR and IGF-1R inhibitors resulting in improved treatment for individuals with reduced toxicities. Given the important role of the IGF-1R signalling pathway in tumour cell survival and growth, it is possible that genetic variation may play a role in the activation of this pathway in cancer [138] [139].

A study by Deming *et al.*, [140] on genetic variation in patients with breast cancer found SNP rs951715 within the IGF-1R gene was associated with breast cancer survival in postmenopausal women. Whereas another polymorphism, SNP rs2229765 was found to have no association with breast cancer survival. SNP rs2229765 appears to be a silent mutation and so far has not been found to be associated with any epidemiological traits. 19 SNPs with known functionality or that were located in functional regions of the gene were evaluated in the study.

A recent study investigated whether germline polymorphisms the IGF-1-pathway are associated response to cetuximab in wild-type KRAS drug- refractory metastatic colorectal cancer patients (mCRC) [139]. Tissue samples of 130 drug-refractory mCRC patients enrolled in a phase II clinical trial of cetuximab monotherapy (IMC-0144) were used for the study. Analyses revealed five IGF-pathway SNPs were

significantly associated with progression-free-survival (PFS) and/or overall survival (OS). Patients harbouring IGF-1 rs2946834 A/A genotype had a 50 % ORR while patients with A/G genotype had 0%. This indicates that IGF-1-pathway polymorphisms are biomarkers of cetuximab efficacy in wt KRAS mCRC patients. A study by Gong *et al.*, used SNP array analysis of IGF-1R gene copy number status in R1507 resistant and sensitive NSCLC cell lines [118]. Cell lines with copy number gain of IGF-1R were significantly enriched for R1507-sensitive cell lines. None of the sensitive lines had any mutations in IGF-1R.

In this chapter 100 NSCLC patients were initially screened for the presence of SNPs in exons 16-20 of the IGF-1R TK domain. The only SNP that was discovered was located in exon 16. A further 98 patients were then screened for the presence of this SNP in exon 16. The polymorphism (rs2229765) located on exon 16 of the IGF-1R gene [GenBank:NM_000875] consisting of a G to A transition at nucleotide 3174 leading to no amino acid change Glu->Glu at position 1043 (E1043E) [GenBank:NP_000876] was the only one found in the screening. A further 98 NSCLC patients were screened for the presence of the polymorphism (rs2229765) and compared with control disease-free individuals (n=866). These results were then correlated to patients survival/pathological data.

The aims of this chapter were to (a) screen 100 NSCLC patients who had undergone a lung tumour resection for mutations in exon 16-20 of the TK domain of the IGF-1R, (b) compare the results to 866 control disease free individuals and (d) correlate the results to patients survival/pathological data.

3.1 Results

3.1.1 Patients

A cohort of 198 stage I-III NSCLC patients, staged according to the International System of Staging for Lung Cancer [239], were randomly selected from the database of all patients who underwent curative-intent surgical resection of a primary tumour, at St. James's Hospital, Dublin between February 2001 and February 2005.

Information on baseline demographics, clinicopathological characteristics and surgical approach was collected after review of clinical notes and histopathology reports. Outcome data, including peri-operative mortality and long-term survival, was updated prospectively. Patients' characteristics are detailed in Table 3.1. (Not all information was available for the total 198 patients). This study was approved by the St. James's Hospital Ethics Committee.

100 NSCLC patients and control patients were initially screened for the presence of SNPs in exons 16-20 of the IGF-1R TK domain. A SNP was found in the coding region of exon 16 (rs2229765) (Table 3.2). No other SNPs were found in exons 17-20 (data not shown). A further 98 NSCLC patients were screened for the presence of the same SNP (rs2229765) and compared with control disease-free individuals (n=866). The extra control patients used for the study came from the Trinity Biobank in the Institute of Molecular Medicine, TCD. An example sequence trace from a homozygous G/G patient and a heterozygous A/G patient is shown in Fig 3.1.

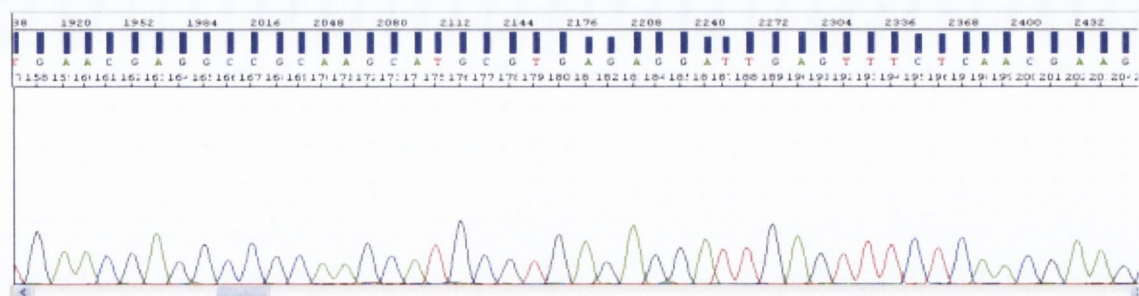
Table 3.1: Patient characteristics.
(Not all information was available for the total 198 patients).

Cases	N
Total	198
Gender	
Male	100
Female	61
Smoking History	
Former	104
Current	48
Never	11
Histology	
SCC	61
ADC	80
Other	20
Age	
< 50	28
>50	133
Controls	
Total	866
Gender	
Male	296
Female	570
Age	
< 50	698
> 50	168

Table 3.2 SNP rs2229765 evaluated in the present study. Polymorphism data on SNP rs2229765 was retrieved from the NCBI SNP reference database.

Gene name	Alleles (Major> minor)	SNP reference ID	Position in gene	Codon
IGF-1R	G> A	rs2229765	Exon 16	Glu1013Glu

A.



B.

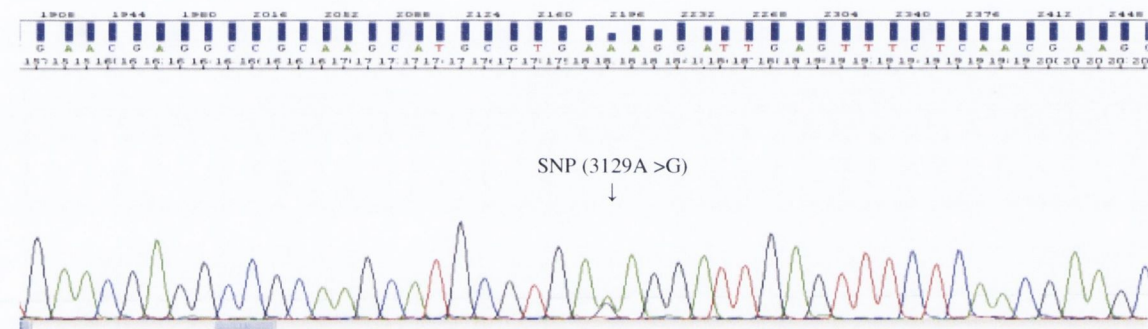


Fig 3.1: Forward sequence traces from a NSCLC patient with (A) no SNP (homozygous G/G) and (B) a heterozygous SNP (SNP 3129A >G) in exon 16.

3.1.2 Genotypic and Allelic distribution

The heterozygous GA genotype was found in 53.5% and 49.4% of NSCLC patients and control individuals respectively (Table 3.3). The dominant GG genotype was found in 28.8% and 30.3% of NSCLC patients and control individuals respectively while the recessive AA genotype was found in 17.7% and 20.2% of NSCLC patients and control individuals respectively. No significance was found in the genotype ($p=0.5487$) frequency. The A allele was found in 44.4% and 44.9% of NSCLC patients and control individuals respectively while the G allele was found in 55.5% and 55% of NSCLC patients and control individuals respectively. No significance was found in the allelic ($p=0.9082$) frequency.

The genotypic and allelic frequencies were then examined in 95 NSCLC patients and 95 control individuals of similar age and sex. Patients over the age of 70 were excluded as there were no matched controls available from this group. Matching ensures that any difference between cases and controls cannot be a result of differences in the matching variables. When patients were age and sex matched the GA genotype was found in 56.8% and 48.4% of NSCLC and control patients respectively (Table 3.4). The dominant GG genotype was found in 22.1% and 29.4% of NSCLC patients and control individuals respectively while the recessive AA genotype was found in 21% and 22.1% of NSCLC patients and control individuals respectively. No significance was found in the genotype ($p=0.4351$) frequency. The A allele was found in 49.4% and 46.3% of NSCLC patients and control individuals respectively while the G allele was found in 50.5% and 53.6% of NSCLC patients and control individuals respectively. No significance was found in the allelic ($p=0.2636$) frequency.

Table 3.3: Genotypic and Allelic frequency in exon 16 of the IGF-1R TK domain in 198 NSCLC (n=198) and control patients (n=866).

Genotype	n (%) NSCLC patients	n (%) control patients	P value	Allele	n (%) NSCLC patients	n (%) control patients	P value
AA	35 (17.7%)	175 (20.2%)	0.5487	A	176 (44.4%)	778 (44.9%)	0.9082
GA	106 (53.5%)	428 (49.4%)		G	220 (55.5%)	954 (55%)	
GG	57 (28.8%)	263 (30.3%)					

Table 3.4: Genotypic and Allelic frequency in exon 16 of the IGF-1R TK domain of NSCLC and control patients (Age and sex matched).

Genotype	n (%) NSCLC patients	n (%) control patients	P value	Allele	n (%) NSCLC patients	n (%) control patients	P value
AA	20 (21.0%)	21 (22.1%)	0.4351	A	94 (49.4%)	88 (46.3%)	0.2636
GA	54 (56.8%)	46 (48.4%)		G	96 (50.5%)	102 (53.6%)	
GG	21 (22.1%)	28 (29.4%)					

3.1.3 Survival analysis

IGF-1R expression was compared to the results of the genotype distribution in NSCLC patients using Kaplan-Meier plots. IGF-1R expression and survival data was available for 100 NSCLC patients which were scored by a pathologist. Results showed no significant difference in genotype distribution (Fig 3.2) or IGF-1R expression (Fig 3.3) in NSCLC patients. Analysis of genotype distribution in NSCLC patients showed that p value was 0.3564 while the p value for trend 0.1514 (Fig 3.2). Analysis correlating IGF-1R expression to genotype distribution in NSCLC patients showed that p value was 0.3005 while the p value for trend was 0.7099 in patients with heterozygous genotype (Fig 3.3 A) and that p value was 0.1164 while the p value for trend was 0.0277 for patients with homozygous dominant and homozygous recessive genotype (Fig 3.3 B).

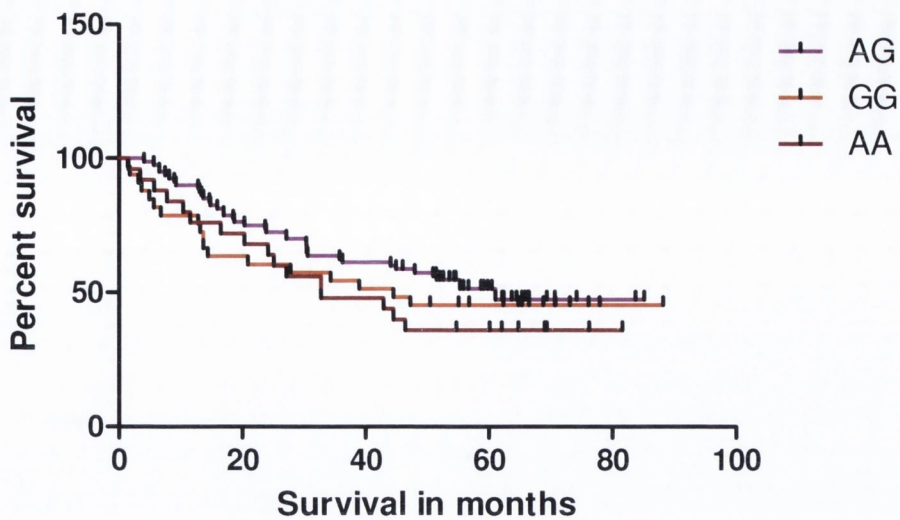
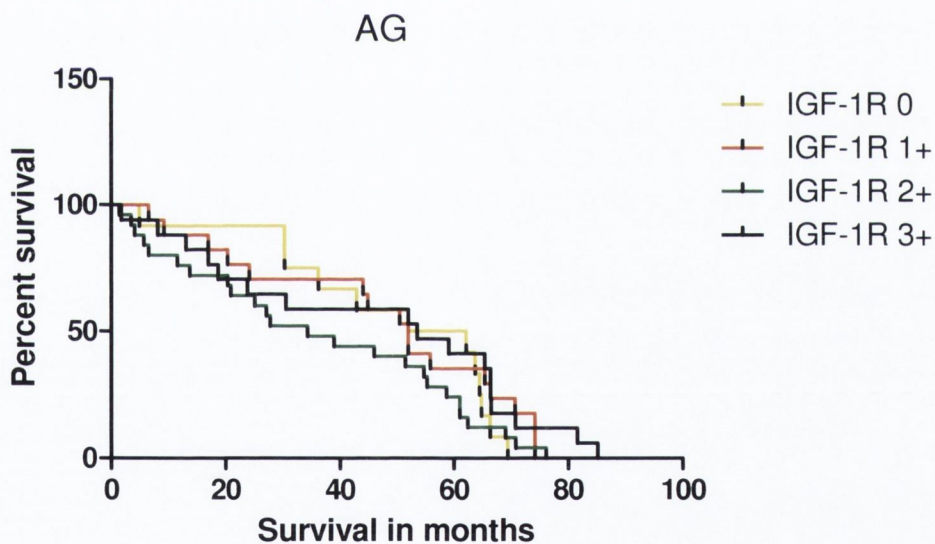


Fig 3.2 Kaplan-Meier plot of genotype distribution in NSCLC patients. Kaplan-Meier results showed that p value was 0.3564 while the p value for trend 0.1514.

A.



B.

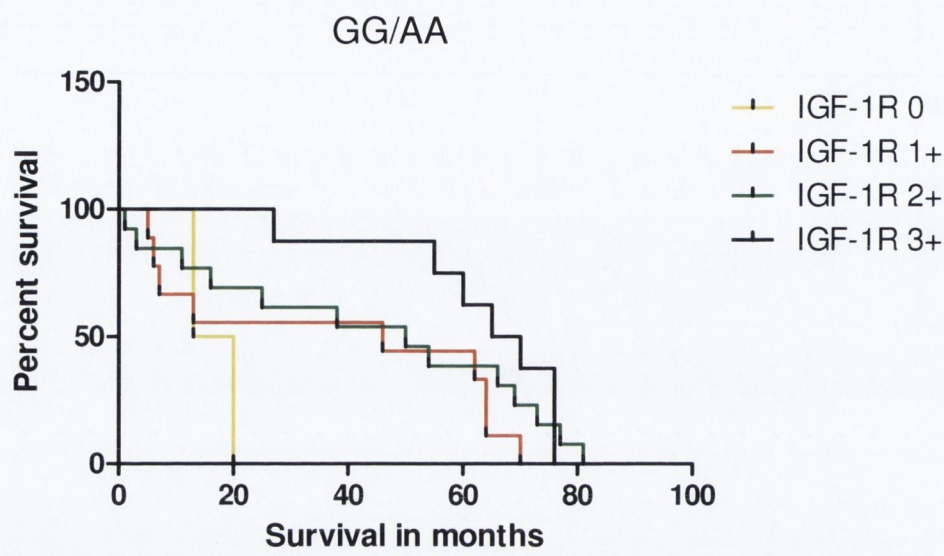


Fig 3.3: Kaplan-Meier plot correlating IGF-1R expression to genotype distribution in NSCLC patients. (A) Heterozygous A/G, (B) Homozygous dominant GG and Homozygous recessive AA. Kaplan-Meier results (A) showed that p value was 0.3005 while the p value for trend was 0.7099. Kaplan-Meier results (B) showed that p value was 0.1164 while the p value for trend was 0.0277.

3.2 Discussion

The discovery that point mutations and deletions within exons 18-21 of the EGFR TK domain can increase drug sensitivity to EGFR TKIs has been a great success in treating patients harbouring these mutations. To date there is limited knowledge regarding mutations in the IGF-1R tyrosine kinase domain. The presence of a mutation in the tyrosine kinase domain of the IGF-1R may help with targeting this receptor with IGF-1R TKIs in NSCLC patients. In this study, we initially screened 100 NSCLC patients for the presence of SNPs in exons 16-20 of the IGF-1R TK domain. The only SNP found in the coding region was in exon 16 (rs2229765). A further 98 NSCLC patients were screened for the presence of the SNP (rs2229765) and compared with control disease-free individuals (n=866). The heterozygous GA genotype was found in 53.5% and 49.4% of NSCLC patients and control individuals respectively (Table 3.3). The dominant GG genotype was found in 28.8% and 30.3% of NSCLC patients and control individuals respectively while the recessive AA genotype was found in 17.7% and 20.2% of NSCLC patients and control individuals respectively (Table 3.3). No significance was found in the genotype ($p=0.5487$) frequency (Table 3.3). The A allele was found in 44.4% and 44.9% of NSCLC patients and control individuals respectively while the G allele was found in 55.5% and 55% of NSCLC patients and control individuals respectively. No significance was found in the allelic ($p=0.9082$) frequency (Table 3.3).

The genotypic and allelic frequencies were then examined in NSCLC patients and control individuals of similar age and sex. Matching ensures that any difference between cases and controls cannot be a result of differences in the matching variables. When patients were age and sex matched the GA genotype was found in 56.8% and 48.4% of NSCLC and control patients respectively (Table 3.4). The dominant GG genotype was found in 22.1% and 29.4% of NSCLC patients and control individuals respectively while the recessive AA genotype was found in 21% and 22.1% of NSCLC patients and control individuals respectively (Table 3.4). No significance was found in the genotype ($p=0.4351$) frequency (Table 3.4). The A allele was found in 49.4% and 46.3% of NSCLC patients and control individuals respectively while the G allele was found in 50.5% and 53.6% of NSCLC patients and control individuals respectively (Table 3.4). No significance was found in the allelic ($p=0.2636$) frequency (Table 3.4). The Kaplan-Meier survival plot showed no significant difference in genotype distribution in NSCLC patients (Fig 3.2). There

was also no significant difference when IGF-1R expression was correlated to heterozygous genotype distribution in NSCLC patients. A significant difference for trend ($P < 0.05$) was observed when IGF-1R expression was correlated to combined homozygous dominant and homozygous recessive genotype distribution in NSCLC patients.

The role of SNP (rs2229765) has been examined in several diseases such as stroke breast cancer and Type II diabetes. It is a synonymous mutation that encodes a change in the DNA sequence without altering the resultant protein sequence. These silent SNPs are presumed to be unimportant but they may represent genetic markers for functional molecular alterations as recent studies have revealed that through various mechanisms these synonymous SNPs may affect gene function and phenotype. Silent SNPs have been linked to over 40 diseases that are a result of a genetic abnormality [240].

The possible role of this SNP (rs2229765) has been investigated in many studies. According to FASTSNP, it is predicted that SNP (rs2229765) may affect splicing regulation. It has been shown to affect the susceptibility to ischemic stroke in Chinese population [241] and to be associated with higher plasma concentrations of circulating IGF-1R [242]. In a study by Bonafe et al. polymorphic variants of IGF-1 response pathway genes including IGF-IR (IGF-I receptor; G/A, codon 1013), PI3KCB (phosphoinositol 3-kinase; T/C, _359 bp; A/G, _303 bp), IRS-1 (insulin receptor substrate-1; G/A, codon 972), and FOXO1A (T/C, _97347 bp), were examined to see if they are involved in systemic IGF-I regulation and human longevity. It was found that subjects carrying at least an A allele at IGF-IR have low free plasma IGF-I levels and live longer. A study done in breast cancer found that SNP rs2229765 had no association with breast cancer survival and that it appears to be a silent mutation [140]. So far it has not been found to be associated with any epidemiological traits. In another study, single SNP analysis revealed significant association of SNP rs2229765 with both percent and absolute mammographic density, increased numbers of the G allele increased the least squares means of mammographic density [142]. The possible role of this polymorphism has also been examined in Type II diabetes which revealed no association with reduced birth weight, insulin sensitivity index, or type II diabetes in a Danish population [243].

These results also suggest that the rs2229765 polymorphism leads to a silent mutation.

Identifying functional polymorphisms in the IGF-1R and its pathway could be used to select patients that would benefit from combined EGFR and IGF-1R treatment resulting in more accurate treatment for individuals with improved effectiveness and reduced toxicities. We did not find any significant association of SNP rs2229765 with NSCLC. Plasma levels of IGF-1R from NSCLC patients could be measured and correlated with patients data and SNP analysis to possibly find a link between this SNP and the IGF-1R. The possible role of this SNP in drug resistance could be investigated further however in the case of IGF-1R polymorphisms, it seems unlikely that there are any mutations in the tyrosine kinase domain that may be used as biomarkers in patient drug targeted therapy.

Chapter 4

IGF-1R and EGFR expression in NSCLC

4.0 Introduction

The epidermal growth factor receptor (EGFR) gene encodes a 170 kDa transmembrane protein consisting of an extracellular EGF-binding domain, a short transmembrane region, and an intracellular domain with ligand-activated tyrosine kinase activity [244]. The EGFR pathway is activated in many epithelial tumours leading to enhanced proliferation, invasion, metastasis and a poor prognosis [245] [246] [247] [248] and is expressed in ~40-85% of primary NSCLC [249] [250]. EGFR targeted therapies are now established as the first and second line treatment of NSCLC [251] [252] [253].

The insulin-like growth factor 1 receptor (IGF-1R) is a member of a large class of tyrosine kinase (TK) receptors which includes the insulin receptor (IR) and the IGF-2R. The IGF-1R controls the activity of two ligands IGF-1 and IGF-2, three cell surface receptors, six IGF-binding proteins (IGFBP1-6) and binding protein proteases [254]. The IGF-1R which is structurally homologous to the insulin receptor (IR) is a phylogenetically conserved transmembrane receptor TK made up of two extracellular α -subunits and two β -subunits which pass through the cellular membrane and are linked by disulfide bonds [255]. The role of the IGF-1R in the pathogenesis of malignant epithelial tumours including lung cancer has been well characterised [256] [257] [258]. The IGF-1R has emerged as an attractive target for anti-cancer drug development [259] [260]. It is over-expressed in several tumour types where it plays a role in promoting tumour growth by inhibition of apoptosis, transformation, metastasis, and angiogenesis [261] [262] [263]. IGF-1R activation protects cells from apoptosis induced by osmotic stress, hypoxia and anti-cancer drugs depending on the level of receptor expression [264] [265]. This protective effect occurs mainly through the phosphatidylinositol 3- kinase (PI3K) and Ras–Raf–MAPK pathways [198] [266].

As the IGF-1R and EGFR share common downstream signalling molecules, receptor crosstalk occurs between these two powerful signalling pathways [267] [193]. This complex cross-talk between EGFR and IGF-1R activates the downstream signalling cascades in both pathways and this may have an adverse affect on current anti-EGF receptor treatments in lung-cancer patients [268] [269]. Results from both *in vitro* and *in vivo* studies indicate that IGF-1R activation and cross-talk with EGFR can

lead to acquired resistance against EGFR-targeted drugs [194] [267] [193]. This has led the way for the novel therapeutic approach of combination therapy targeting both EGFR and IGF-1R [270] [198] [271]. To date, this dual targeting has had promising results however further *in vitro* and *in vivo* studies are necessary to further characterise the inhibition of both pathways.

There are conflicting reports of the significance of IGF-1R expression in outcome and survival of NSCLC patients. A study by Cappuzzo *et al.*, found that there was no correlation between IGF-1R expression and survival in early stage surgically resected NSCLC patients [272]. Another study by Ludovini *et al.*, has reported that high coexpression of both IGF-1R and EGFR is associated with shorter disease-free survival (DFS) in surgically resected NSCLC patients and a trend toward a poorer overall survival (OS) [273]. Finally a study conducted by Lee *et al.*, found that IGF-1R expression did not affect survival of NSCLC patients with surgically resected stage I disease [274].

The aims of this chapter are to (a) examine IGF-1R and EGFR expression in 184 resected NSCLC patients by immunohistochemistry and (b) correlate this expression to patient clinico-pathological and overall survival data, (c) examine IGF-1R and EGFR protein expression in 40 resected NSCLC patients' matched normal and tumour tissue by Western blot analysis.

4.1 Results

4.1.1 Immunohistochemistry analysis of IGF-1R and EGFR expression in NSCLC patients.

IGF-1R and EGFR expression were quantified in a cohort of 201 NSCLC patients by immunohistochemical analysis. Patient characteristics are shown in Table 4.1. The following resections were carried out: 144 lobectomies (71.6%), 21 bilobectomies (10.4%), 35 pneumonectomies (17.4%) (Table 4.1). The median age of the patients at time of surgery was 64 years (range 41-86). Of the patients 62.7% were male, 93.5% were former or current smokers and 54.2% had stage I disease (Table 4.1). The histological subtypes included were; ninety-three (46.3%) squamous cell carcinoma (SCC); eighty-eight (43.8%) adenocarcinomas (ADC); one (0.5%) bronchioloalveolar (BAC); five (2.5%) large-cell; seven (3.5%) pleiomorphic; seven (3.5%) mixed (Table 4.1). Ten patients who died within 30 days of surgery were excluded from the survival analysis. IGF-1R and/or EGFR expression were not determined on seven patients due to damage or loss of tissue cores from the arrays and these were also excluded. Therefore results from 184 patients were used for Immunohistochemistry and survival analysis.

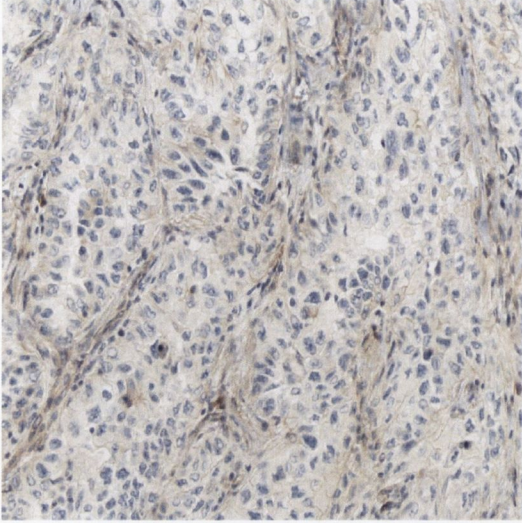
IGF-1R expression was seen in 76.4% of the patient tumours (Fig 4.1). A scoring system to quantify IGF-1R expression is shown in Fig 4.1. Tissue with less than 10% membranous staining was regarded as negative and given a score of zero. Tissues with greater than 10% membranous staining and an intensity of weak, moderate or strong were given scores of 1+, 2+ or 3+ respectively. IGF-1R overexpression, defined as 3+ or 2+ staining, was observed in 53.8% of NSCLC with squamous cell carcinoma (SCC) having higher overexpression than non-SCC (62.6% versus 37.3%, $\chi^2 12.59$, $p=0.0004$) (Table 4.2). No significant association was seen between IGF-1R protein expression and gender, smoking status, stage or grade. EGFR expression was detected in 87.5% of cases in keeping with that observed in recent studies (Fig 4.2). A scoring system to quantify EGFR expression is shown in Fig 4.2. EGFR overexpression, defined as 3+ expression, was detected in 51% of NSCLC and SCC had higher EGFR expression than non-SCC (57.4% versus 42.5%, $\chi^2 4.8$, $p=0.028$). No significant association was seen between EGFR expression and any other clinicopathological characteristic examined (Table 4.2). A significant association between IGF-1R and EGFR protein expression was seen ($\chi^2 3.69$, $p < 0.0052$) (Table

4.3). High co-expression of both receptors was seen in 65 patients (35%). SCC patients had higher co-expression of IGF-1R and EGFR than non-SCC (χ^2 13.59, $p=0.0002$) (Table 4.2). This group was not significantly associated with any other clinico-pathological characteristic.

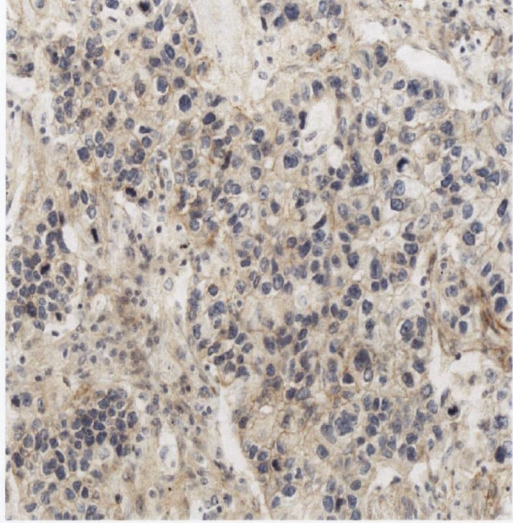
Table 4.1. Patient characteristics (n=201)

Characteristics	N (%)
Total	201 (100%)
Median Age (range)	64 (41-86)
Gender	
Male	126 (62.7%)
Female	75 (37.3%)
Smoking History	
Former/Current	188 (93.5%)
Never	13 (6.5%)
Histology	
ADC	88 (43.8%)
SCC	93 (46.3%)
BAC	1 (0.5%)
LCC	5 (2.5%)
PLEO	7 (3.5%)
Mixed	7 (3.5%)
pTNM stage	
I	109 (54.2%)
II	48 (23.9%)
III	44 (21.9%)
Tumour Size	
<=2cm	22 (10.9%)
>2-3cm	31 (15.4%)
>3-5cm	85 (42.3%)
>5-7cm	27 (13.4%)
>7cm	21 (10.4%)
Type of Restion	
Lobectomy	144 (71.6%)
Bilobectomy	21 (10.4%)
Pneumonectomy	35 (17.4%)
Tumour Grade	
G1	13 (6.5%)
G2	118 (58.7%)
G3	70 (34.8%)

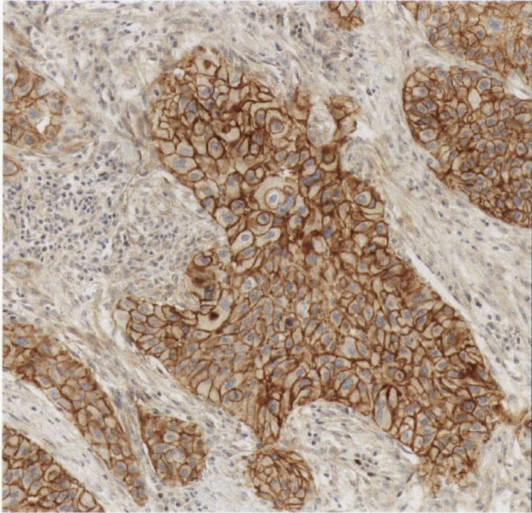
A.



B.



C.



D.

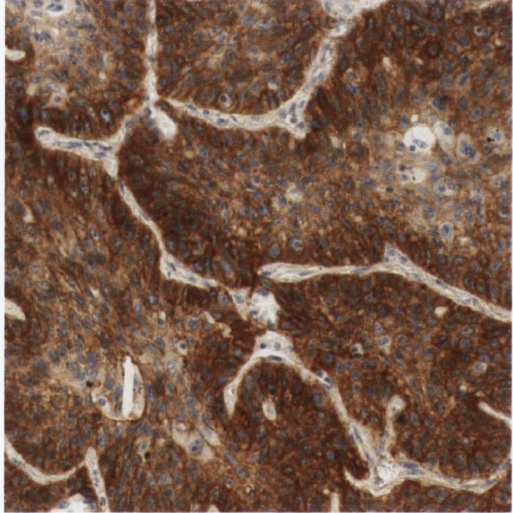


Fig 4.1. IGF-1R Immunohistochemistry (IHC) in NSCLC cells showing cytoplasmic and membraneous staining (x40), (A) negative (0), (B) weak (1+), (C) moderate (2+) and (D) Strong (3+).

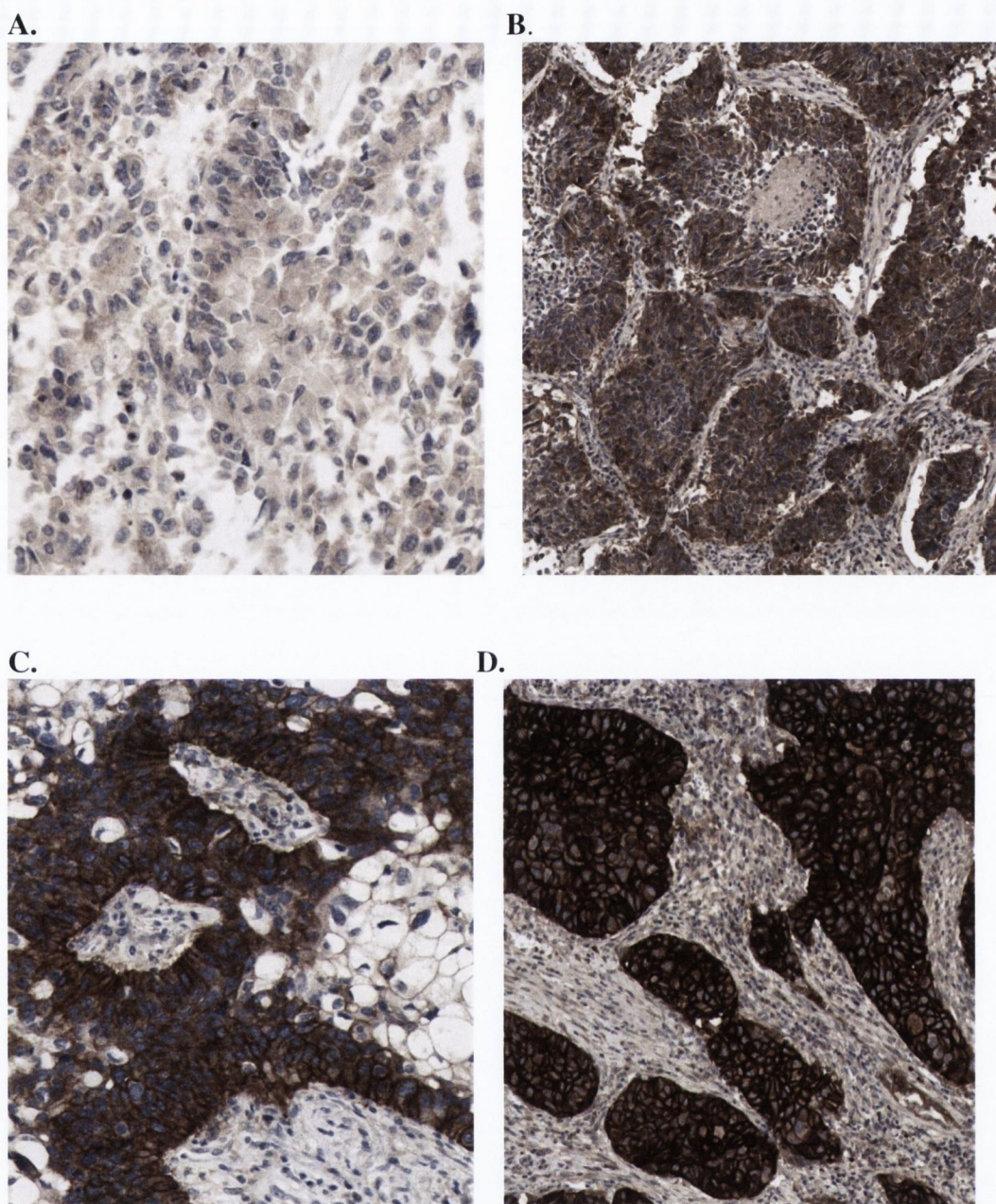


Fig 4.2. EGFR Immunohistochemistry (IHC) in NSCLC cells showing cytoplasmic and membraneous staining (x40), (A) negative (0), (B) weak (1+), (C) moderate (2+) and (D) Strong (3+).

Table 4.2 Clinicopathological characteristics in relation to IGF-1R, EGFR expression and coexpression of IGF-1R and EGFR.

Variable	Patient #	IGF-1R 0,+1(%) +2,+3(%)		P	EGFR 0,+1,+2(%) +3(%)		P	IGF-1R/EGFR (+2, +3/+3) Other Grps		P
Total	184									
Gender										
Male	112	26.1	34.2	0.45	27.2	33.7	0.19	23.4	37.5	0.52
Female	72	19.6	19.6		21.7	17.4		11.9	27.2	
Smoking History										
Former/Current	171	41.8	51.1	0.39	44.6	48.4	0.51	33.7	59.2	1.0
Never	13	4.4	2.7		4.3	2.7		1.6	5.4	
pTNM										
I	99	22.3	31.5	0.44	26.6	27.2	0.82	20.1	33.7	0.85
II	43	10.9	12.5		12.0	11.4		8.2	15.2	
III	42	13	9.8		10.3	12.5		7.0	15.8	
pT status										
T1	39	13	8.2	0.25	12.0	9.2	0.67	5.4	15.8	0.42
T2	115	27.2	35.3		28.8	33.7		22.8	39.7	
T3&T4	30	6.0	10.3		8.2	8.2		7.1	9.2	
pN status										
N0	111	25	35.3	0.06	30.4	29.9	0.91	22.8	37.5	0.74
N1	40	8.7	13.0		10.9	10.9		7.6	14.1	
N2	33	12.5	5.4		7.6	10.3		4.9	13.0	
Histology										
ADC	83	30.4	14.7	0.0004	27.7	17.4	0.028	6.5	38.6	0.0002
SCC	83	11.4	33.7		15.8	29.3		25	20.1	
Other	18	4.3	5.4		5.4	4.3		3.8	6.0	

Table 4.3 Association between IGF-1R and EGFR expression

	IGF-1R 0, +1 (%)	IGF-1R +2, +3 (%)	P	χ ²
EGFR (0,+1, +2) %	56/184 (30.4)	34/184 (18.5)	0.0052	7.80
EGFR (+3)%	29/184 (15.8)	65/184 (35.3)		

4.1.2 Survival analysis

There was a median follow-up time of 65.09 months, overall 51.6% (95/184) of the study population died and the median survival was 48.6 months. There was no significant difference in OS between patients with negative or different intensities of EGFR (Fig 4.3) or of IGF-1R (Fig 4.4) expression ($p=0.357$ and $p=0.729$ respectively). Patients were then split into two groups according to differences in expression of IGF-1R and EGFR. Group I included patients with high co-expression of both receptors [IGF-1R (2+/3+) and EGFR (3+)]; group II included all other combinations of IGF-1R and EGFR expression, 65 and 119 patients respectively. A difference in survival amongst the two groups was shown by univariate analysis, with group I having a significantly poorer OS (HR=0.668, 95% CI 0.444-1.004, $p=0.048$) than group II (Table 4.4). This subset of patients also showed a poorer OS by Kaplan-Meier (39.5 months versus median has not been reached, $p=0.04$) Fig 4.5.

On univariate analysis, tumour size (<5cm versus ≥ 5 cm), pT status (T1&T2 versus T3), pN status (N0 & N1 vs N2) and stage (I-II vs III) showed a significantly poorer OS ($p<0.001$, $p=0.001$, $p=0.010$ and $p<0.0001$) respectively. On multivariate analysis stage (HR 0.480, 95% CI 0.309 - 0.745, $p=0.001$), tumour size (HR 0.550, 95% CI 0.362 - 0.836, $p=0.005$) and high coexpression of both receptors (HR 1.48, 95% CI 0.982 - 2.228, $p=0.05$) were significantly associated with a poorer OS (Table 4.4).

Table 4.4 Univariate and multivariate analyses for clinicopathological characteristics considered for overall survival

Variable	N	HR	95% CI		P-value
Age (years)	184				
< 65	88	0.786	0.522	1.182	0.247
> 65	96	1.0			
Sex	184				
Female	72	0.843	0.553	1.286	0.428
Male	112	1.0			
Smoking Status	184				
Current & former smokers	171	0.521	0.262	1.038	0.064
Never smokers	13	1.0			
Type of Surgery	183				
Pneumonectomy	32	0.920	0.525	1.611	0.770
Bilobectomy	130	0.942	0.497	1.784	0.855
Lobectomy	21	1.0			
Tumour Size	184				
< 5 cm	122	0.454	0.302	0.684	0.000
≥ 5 cm	62	1.0			
pT status	184				
T1-T2	154	0.437	0.274	0.698	0.001
T3	30	1.0			
pN status	184				
N0	111	0.587	0.390	0.882	0.010
N1&N2	73	1.0			
pTNM stage	184				
I-II	142	0.437	0.283	0.674	0.000
III	42	1.0			
Tumour Grade	184				
1	11	1.282	0.594	2.767	0.527
2	111	0.731	0.474	1.126	0.155
3	62	1.0			
Histological subtype	184				
Adenocarcinoma	83	0.839	0.408	1.723	0.632
Squamous	83	0.739	0.359	1.523	0.412
Others	18	1.0			
EGFR+	184				
0	23	0.905	0.472	1.734	0.764
1+	13	0.859	0.390	1.890	0.706
2+	54	0.623	0.379	1.025	0.062
3+	94	1.0			
IGF-1R+	184				
0	31	0.721	0.364	1.429	0.349
1+	54	0.703	0.390	1.267	0.241
2+	66	0.812	0.467	1.413	0.462
3+	33	1.0			
EGFR & IGF-1R	184				
All Other Groups	119	0.668	0.444	1.004	0.048
EGFR (3+)/IGF-1R (2+ & 3+)	65	1.0			
Multivariate Analysis					
EGFR (3+)/IGF-1R (2+ & 3+)		1.48	0.982	2.228	0.05
Tumour Size (<5cm versus ≥5cm)		0.550	0.362	0.836	0.005
pTNM Stage (I&II versus III)		0.480	0.309	0.745	0.001

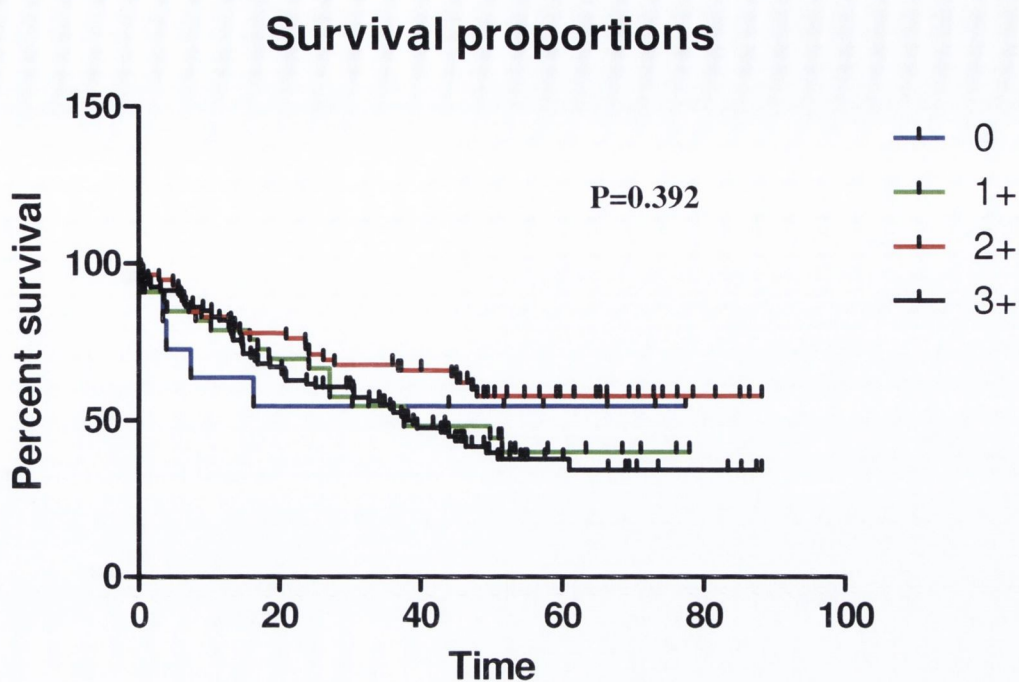


Fig 4.3 Kaplan-Meier survival curve showing overall survival (OS) according to EGFR expression.

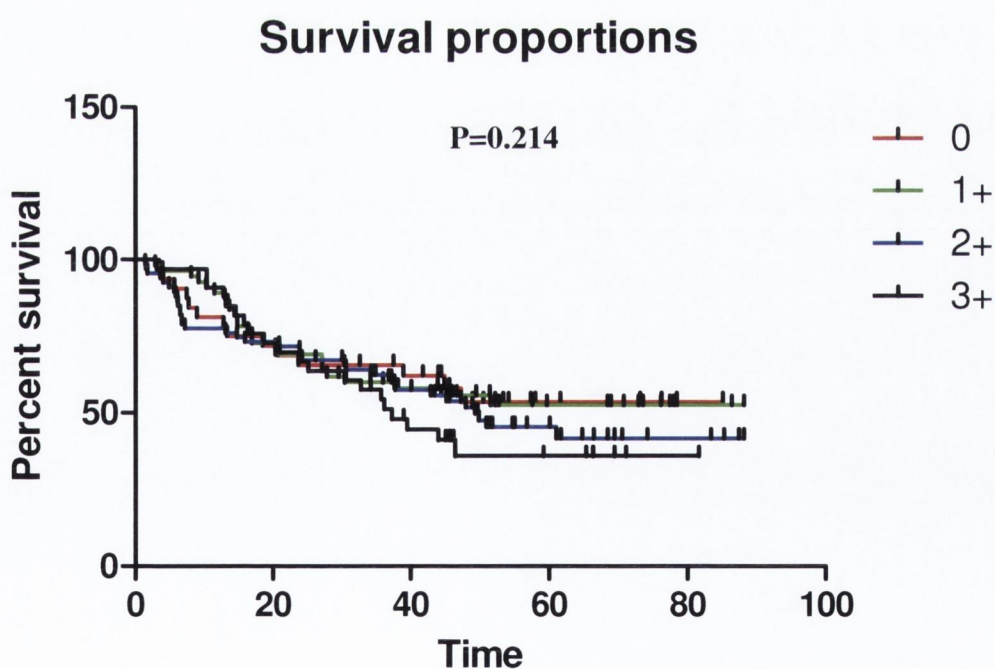


Fig 4.4 Kaplan-Meier survival curve showing overall survival (OS) according to IGF-1R expression.

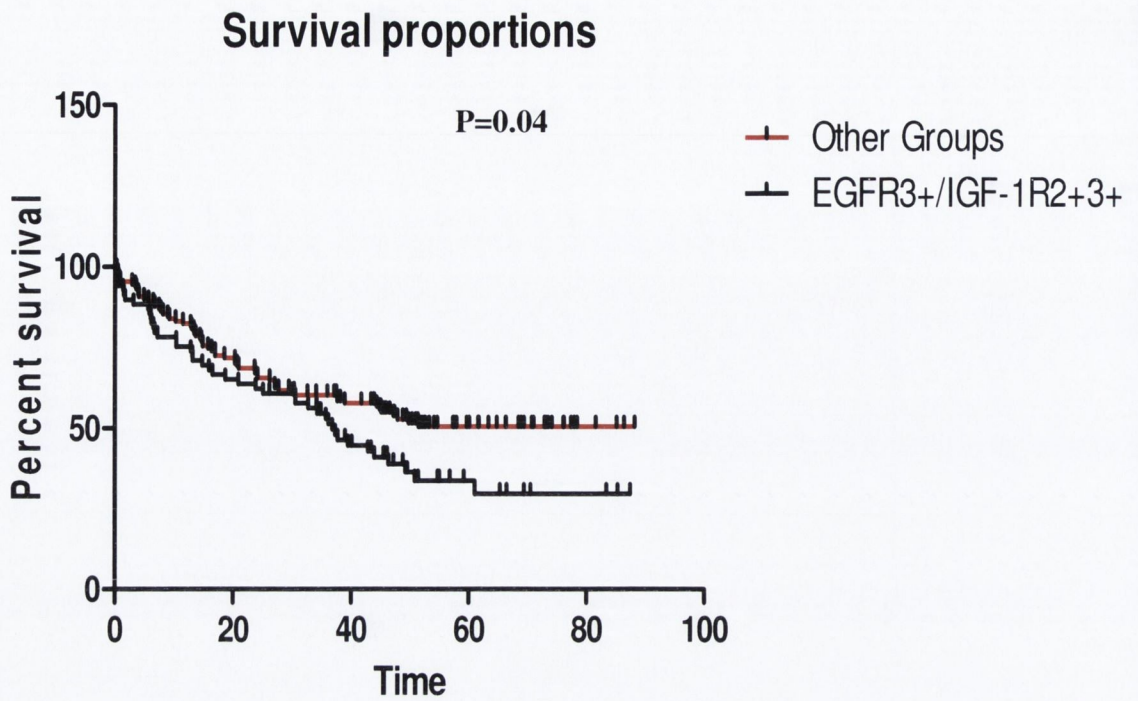


Fig 4.5 Kaplan Meier survival curve showing overall survival (OS) according to EGFR and IGF-1R overexpression.

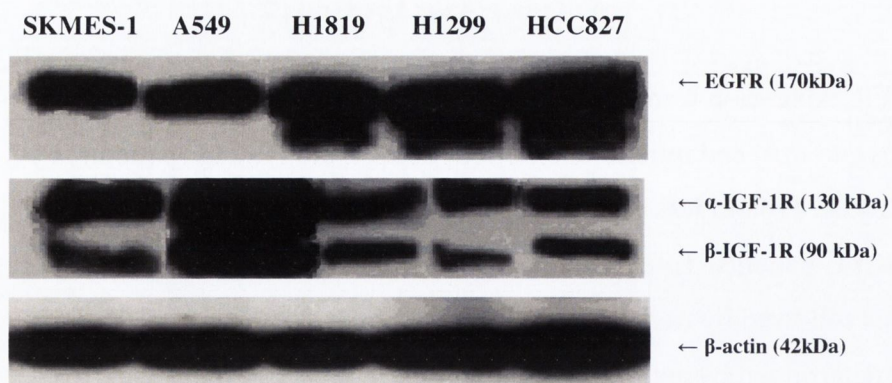
4.1.3 IGF-1R and EGFR expression in NSCLC cell lines and matched tumour and normal tissue

IGF-1R and EGFR expression were examined at the protein level, in a panel of NSCLC cell lines and matched tumour and normal tissue from NSCLC patients, by Western blot analysis. Protein was extracted from a panel of NSCLC cell lines and also from 40 NSCLC patients. Total protein was loaded on an 8% gel and separated by electrophoresis followed by transfer to PVDF membrane. Expression of IGF-1R and EGFR were determined by antibody probing of membranes. A representative gel is shown in Fig 4.6. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using ANOVA.

HCC827 cells had the highest level of EGFR expression out of the 5 NSCLC cell lines. HCC827 cells had significantly higher EGFR expression than A549 cells ($P<0.05$) and SKMES-1 ($P<0.05$) (Fig 4.7). HCC827 cells did not have significantly higher expression than H1819 and H1299 cell lines. A549 cells had the highest level of IGF-1R expression. A549 cells had significantly higher IGF-1R expression than H1819 ($P<0.001$), H1299 ($P<0.001$) and HCC827 ($P<0.001$) cells.

In the fresh frozen resected tumours IGF-1R and EGFR were significantly overexpressed relative to matched normal tissues in NSCLC patients, $P=0.004$ and $P=0.0015$ respectively. When this was examined in terms of histology in NSCLC patients, SCC patients had significantly higher expression of IGF-1R ($P=0.0021$) and EGFR ($P=0.0039$). However in ADC patients IGF-1R ($P=0.1994$) and EGFR ($P=0.0913$) expression were not found to be significantly expressed.

A.



B.

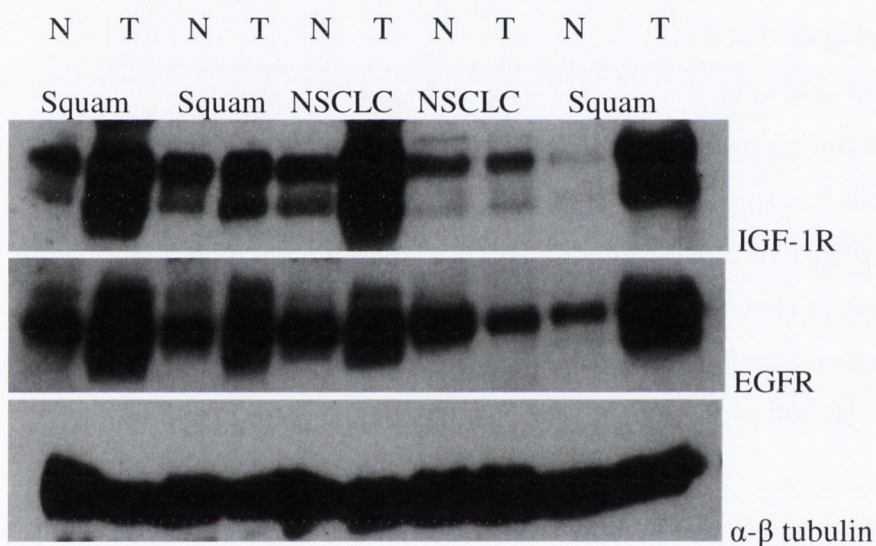
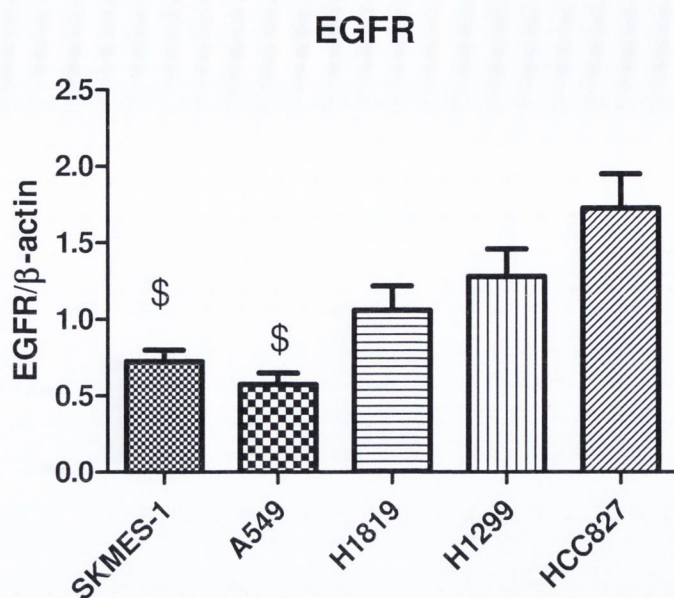


Fig 4.6 Western Blots showing EGFR, IGF-1R & β-actin expression in (a) NSCLC cell lines and (b) matched NSCLC normal (N) and tumour (T) patient tissues.

A.



B.

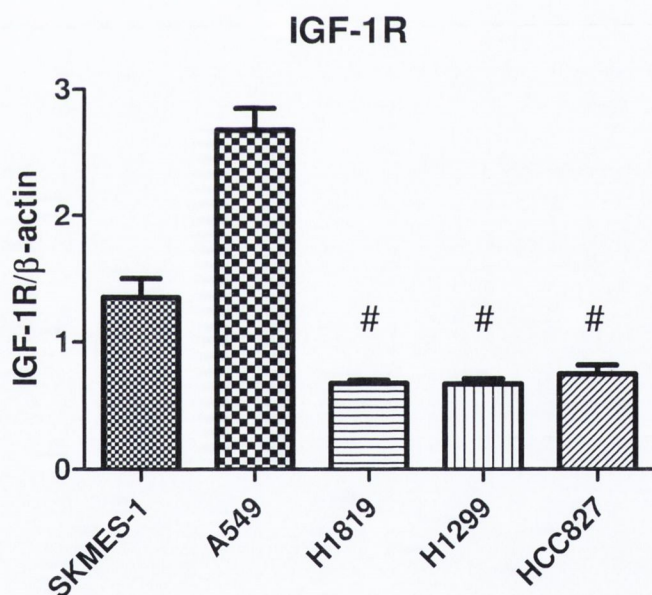
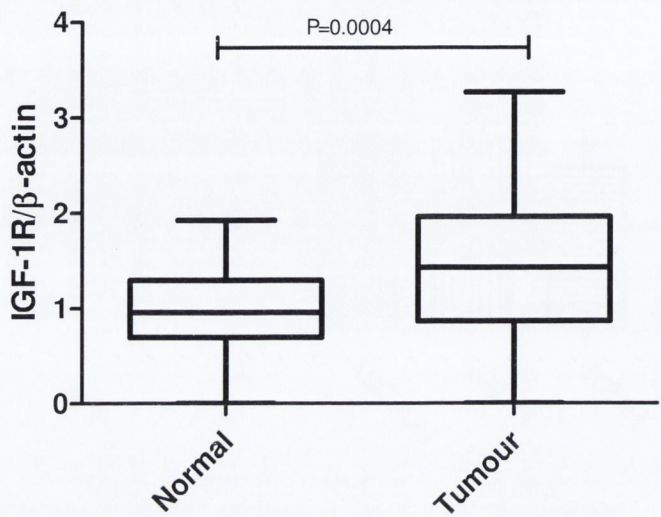


Fig 4.7 Densitometry analysis of the relative expression of (a) EGFR and (b) IGF-1R in NSCLC cell lines. Results are expressed as mean $\pm$ SEM (n=3). Statistical analysis was carried out using ANOVA.

(\$ = significant compared to HCC827, # = significant compared to A549)

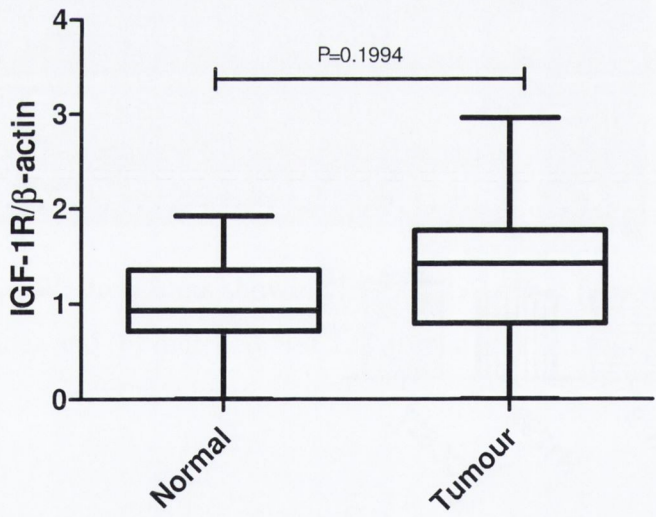
A.

Overall IGF-1R expression in NSCLC patients



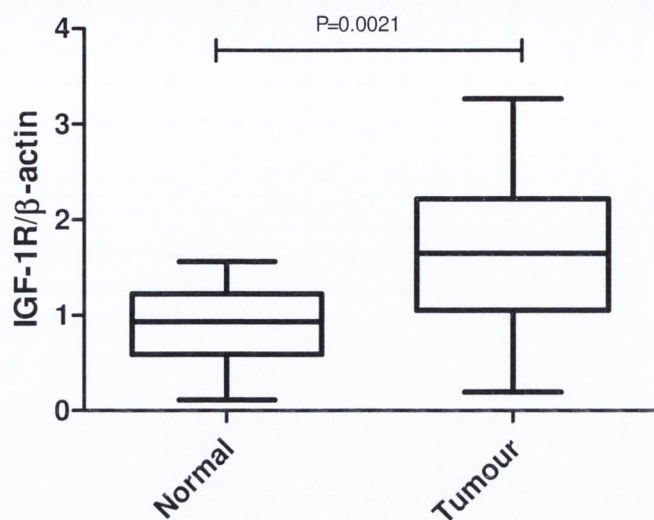
B.

Overall IGF-1R expression in ADC patients



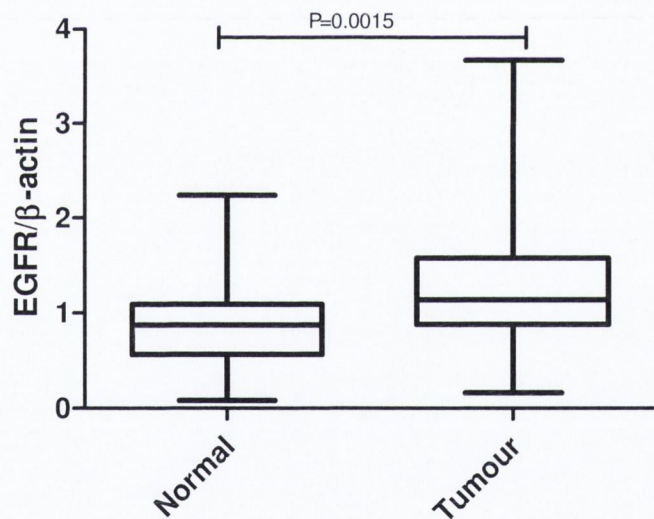
C.

Overall IGF-1R expression in SCC patients



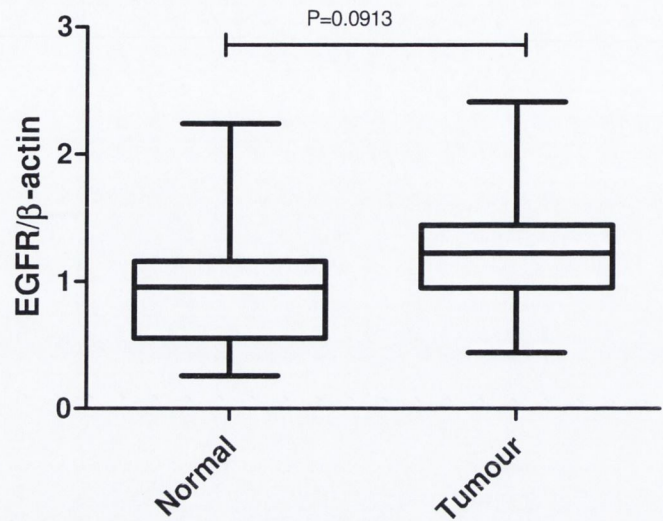
D.

Overall EGFR expression in NSCLC patients



E.

Overall EGFR expression in ADC patients



F.

Overall EGFR expression in SCC patients

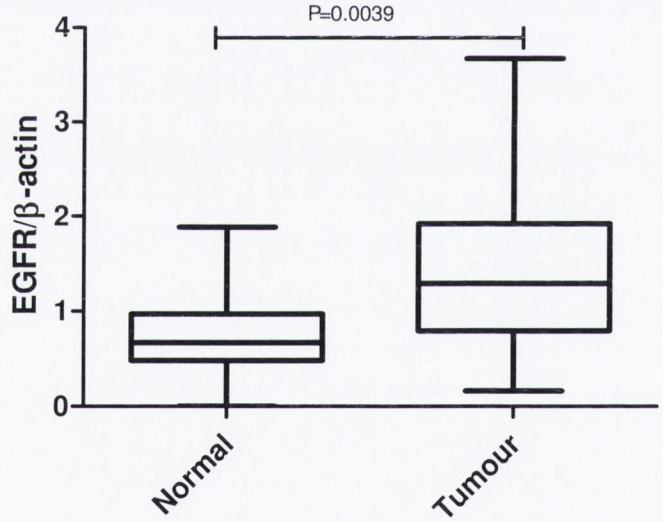


Fig 4.8 Densitometry analysis of the relative expression of (A-C) IGF-1R and (D-F) EGFR in matched normal and tumour tissues. Results are expressed as mean $\pm$ SEM (n=3).

4.2 Discussion

In this chapter it was shown that IGF-1R expression alone is not a prognostic marker in NSCLC. This was shown in 3 previous studies examining IGF-1R expression [275][273][272]. In the first of these 2 studies Ludovini *et al.*, examined IGF-1R expression in 125 patients using the Lab Vision Neomarkers antibody and reported overexpression in 36% of patients. In the second Capuzzo *et al.*, evaluated IGF-1R expression in 369 patients using the Novus Biologicals antibody and found positive IHC staining in 76.4% of cases. The proportion of cases having expression in these reports is similar to that found in our study (83.2%). Furthermore both these studies demonstrated an association between IGF-1R expression and SCC. These findings are in contrast to the initial report by Merrick *et al* which demonstrated that high IGF-1R expression was associated with ADC histology and a poorer survival in 191 resected NSCLC patients [276].

EGFR expression was examined in NSCLC in several previous studies and was found that when taken alone the growth factor is not a robust prognostic factor. One review showed that only 10% of studies published between 1985 and 2000 associated increased EGFR with OS [277] and an extensive review of 16 studies (1989-2001) by Meert *et al.* [278] demonstrated that 12 of those found EGFR expression not to be prognostic. EGFR expression does not predict response to chemotherapy in NSCLC [279]. Again our findings showed no cut-off of EGFR expression was associated with prognosis, which is in keeping with previous observations.

However, high coexpression of EGFR and IGF-1R were identified in 35% of our cohort of NSCLC patients and was associated with a poorer overall survival on univariate and multivariate analysis ($p=0.048$; $p=0.05$) indicating that these two biomarkers taken together are prognostic in resected NSCLC. This observation is supported by the recent study by Ludovini *et al.*, [273] which showed that high coexpression of both IGF-1R and EGFR is associated with shorter disease-free survival and a trend toward a poorer OS ($p=0.09$) in patients with resected NSCLC.

There are a number of explanations as to why co-expression of IGF-1R and EGFR may be associated with a poor prognosis. Both receptors share common signal transduction pathways activating either the Ras/Raf/MAPK or the PI3K/Akt/BAD

(Bcl-xL/Bcl2) pathways resulting in tumour cell survival and proliferation. Several studies have highlighted the importance of crosstalk between receptors including EGFR and IGF-1R [280]. Activation of the IGF-1R pathway is known to cause resistance to EGFR targeted therapies [281][282][283][284]. The abundance of both receptors in a subset of NSCLC patients, in particular SCC, indicates that both pathways could potentially be activated to cause an aggressive tumour phenotype. Treating this subset of patients with a combination of IGF-1R and EGFR targeted therapies may prove to be an effective strategy for the future [285].

When a panel of NSCLC cell lines were screened for EGFR protein expression, HCC827 cells had the highest level of EGFR expression out of the 5 NSCLC cell lines. When IGF-1R expression was examined, A549 cells had the highest level of IGF-1R expression. HCC827 and A549 cell lines have been shown to express high levels of EGFR and IGF-1R respectively in previous studies [94] [286]. In the fresh frozen resected tumours IGF-1R and EGFR were significantly overexpressed relative to matched normal tissues in NSCLC patients, $P=0.004$ and $P=0.0015$ respectively. When this was examined in terms of histology in NSCLC patients, SCC patients had significantly higher expression of IGF-1R ($P=0.0021$) and EGFR ($P=0.0039$). However in ADC patients IGF-1R ($P=0.1994$) and EGFR ($P=0.0913$) expression were not found to be significantly expressed. Overexpression of IGF-1R and EGFR in tumour compared to matched normal tissue has been demonstrated in previous studies [279] [287]. High expression of IGF-1R and EGFR have also been shown to be significantly higher in squamous cell carcinoma compared to adenocarcinoma in previous studies [120] [288]. Squamous cell carcinoma patients have a poorer survival compared to other histologies which is thought to be as a result of high expression of both IGF-1R and EGFR [287].

This chapter highlights the importance of the interaction between the IGF-1R and EGFR in NSCLC, particularly SCC. Targeting both receptors simultaneously may prove a more effective anti-tumour strategy than using EGFR targeted therapies alone in the management of NSCLC. This is supported by pre-clinical studies and a number of ongoing clinical trials are addressing this issue, particularly in the second-line treatment of NSCLC.

Chapter 5:

Targeting the IGF-1R and EGFR in NSCLC

5.0 Introduction

Recent advances in the development of targeted therapies such as the EGFR TKIs and the ALK inhibitor Crizotinib have greatly improved the treatment for a proportion of NSCLC patients who carry an EGFR tyrosine kinase mutation or the EML4-ALK fusion protein respectively [289] [290] [291] [292]. These targeted therapies are specific to the oncogenic tyrosine kinase pathways activated in tumour cells and can therefore selectively kill cancer cells [293]. However once the main “driver” of the tumour is switched off by targeted therapy, cancer cells then activate a network of pro-growth proteins that can bypass this targeted molecule and resistance emerges. To overcome this resistance it may be necessary to target a biological network and not just a single molecule.

EGFR and IGF-1R are overexpressed in many cancers including NSCLC and are associated with advanced disease and poor prognosis which make them ideal therapeutic targets [85] [93] [197]. EGFR and IGF-1R play important roles in cancer progression by regulating cell proliferation, angiogenesis, metastasis, and inhibition of apoptosis [287] [83] [106].

The most commonly used EGFR TKIs for treating locally advanced or metastatic NSCLC include, Gefitinib (Iressa) and Erlotinib (Tarceva) [102] [97] [294] . Compared to gefitinib erlotinib is the only TKI to prolong survival in locally advanced or metastatic NSCLC in those who have failed at least one prior treatment regimen [295] [99]. Early studies showed that the largest population that benefited from EGFR TKIs were Asian, female, never-smoker patients with adenocarcinoma [54]. Later it was discovered that point mutations and deletions within exons 18-21 of the EGFR TK domain can increase drug sensitivity and patients harbouring these mutations respond better to treatment with erlotinib. Another EGFR inhibitor cetuximab, is a monoclonal antibody that has been successful in improving overall and progression-free survival when combined with other chemotherapy agents in advanced NSCLC patients [296] or given alone to colorectal patients that have not responded to chemotherapy [297]. Patients with KRAS mutations do not benefit from cetuximab treatment.

Activating EGFR mutations are found in 10–15% of Caucasian and 30–40% of Asian NSCLC patients [100]. This subset of patients have a 65-90% response rate to EGFR TKIs compared to only 10% in the non-mutation patients [298]. EGFR copy number and mutation status are currently used as a way to select patients for EGFR TKI treatment as not everyone is a candidate [102]. Four recent trials (two with gefitinib and two with erlotinib) investigated the efficacy of front-line treatment with an EGFR TKI compared with standard chemotherapy in patients with NSCLC with proven *EGFR* mutations. The WJTOG3405 and NEJ002 trials showed gefitinib was superior to chemotherapy according to response rate and PFS, with a more-favorable toxicity profile [299] [300]. The EURTAC and OPTIMAL trials showed similar results with erlotinib versus platinum based chemotherapy. As with other targeted therapies several mechanisms of acquired resistance to EGFR TKIs have been identified from resistant cell line models including a secondary mutation in *EGFR* (T790M) and amplification of the *MET* oncogene [104] [301] [302].

Identifying the complex molecular events that underlie carcinogenesis is essential for the design of new therapeutics. Most of the strategies to target the IGF-1R have been at the receptor itself [117]. Currently, several different approaches are being investigated for targeting the IGF-1R, including small-molecule kinase inhibitors, IGF-1R antibodies, antisense oligonucleotides and RNA interference all hinder cell growth and proliferation [117] [106] [121]. These aim to block IGF-1R activity by receptor blockade, kinase inhibition and ligand sequestration. Blocking the receptor though the use of monoclonal antibody therapies have been the most clinically investigated approach to date.

It is not clear what factors influence sensitivity to IGF-1R inhibitors but it is likely that tumours that respond well to treatment will be those where IGF-1R overexpression results in a poor patient prognosis [272] [198]. Creating IGF-1R tyrosine kinase inhibitors has been difficult as the kinase domain of the IGF-1R shares approximately 85% homology with that of the IR [106]. However, recently selective IGF-1R inhibitors have been developed and these new therapeutic interventions have shown promising results in pre-clinical and early phase clinical trials [303]. The immunoglobulin G1 (IgG1) antibody AMG 479 (ganitumab) is starting a randomized phase II trial for the treatment of advanced pancreatic cancer in combination with gemcitabine (<http://clinicaltrials.gov/ct/show/NCT01298401>).

OSI-906 is a small molecule, dual kinase inhibitor of both IGF-1R and IR. It is currently in a Phase III clinical trial in adrenocortical carcinoma (ACC) (<http://clinicaltrials.gov/ct2/show/NCT00924989>) and in a Phase I/II clinical trial in ovarian cancer (<http://clinicaltrials.gov/ct2/show/NCT00889382>).

Figitumumab (CP-751871) was being investigated in a phase III clinical trial until recently but the study was terminated due to no improved survival following treatment and increased the risk of severe toxicities in NSCLC patients.

(<http://clinicaltrials.gov/ct2/show/NCT00673049?term=A4021018&rank=1>). Data collected from these clinical trials and others will help to determine the molecular profile of patients who respond to the IGF-1R blockade, and the best way of combining this new approach with standard therapeutic protocols.

AG1024 (a specific inhibitor of IGF-1R kinase), exhibited specific cytotoxicity to human pancreatic cancer PANC-1 cells grown in a nutrient-deprived medium [123]. IGF-1R kinase inhibitors such as AG1024 block phosphorylation of IGF-1R by IGF-1 in cells cultured in nutrient-deprived medium compared to those grown in medium with ample nutrients. IGF-1R kinase inhibitors also block phosphorylation of Akt and Erk, so that activation of pathways downstream of the IGF-1R are also blocked in nutrient-starved conditions [123].

Monoclonal antibodies and single chain antibodies directed against the receptor have been developed which down-regulate IGF-1R levels and block ligand binding, therefore inhibiting tumour growth. These antibodies do not interfere with insulin receptor signalling and the interaction between insulin and its receptor.

<http://www.discoverymedicine.com/Mario-Scartozzi/2011/02/20/state-of-the-art-and-future-perspectives-for-the-use-of-insulin-like-growth-factor-receptor-1-igf-1r-targeted-treatment-strategies-in-solid-tumors/>. Monoclonal antibody therapies can block the binding of IGF-I and IGF-II to the IGF-1R and down regulate the expression of IGF-1R and IR/IGF-1R hybrid. However not all IGF-1R monoclonal antibodies in the development stages are successful in blocking both IGF-I and IGF-II binding or in down regulating IGF-1R homoreceptor pairs and hybrid receptor pairs. IGF-1R monoclonal antibodies are very specific and are less likely to bind to the insulin receptor. Binding to the IR-B receptor is detrimental and leads to insulin resistance and hyperglycemia in patients [304].

A novel IGF-1R targeted drug, R1507 (RO4858696; Roche) is a fully humanized IgG1 monoclonal antibody directed against the extracellular portion of IGF-1R. It binds with high selectivity to the extracellular domain of IGF-1R (and not to IR), leading to displacement of IGF-1 ligand binding and loss of receptor at the cell surface due to receptor internalization and degradation [124]. R1507 had positive results in phase I clinical trial in patients with solid tumours where disease stabilization was observed in 9 out of 34 patients following treatment. R1507 is currently being investigated in phase II clinical trials and has shown promising results to date (<http://meeting.ascopubs.org/cgi/content/abstract/27/15S/10503>) (http://www.asco.org/ascov2/Meetings/Abstracts?&vmview=abst_detail_view&confID=65&abstractID=34060). Reports indicate the drug to be safe, efficacious, and with few adverse side effects in patients [124]. A phase II clinical trial (NO21160) investigating the effect of R1507 in combination with Tarceva (Erlotinib) on progression-free survival (PFS) in patients with stage IIIb/IV Non-Small Cell Lung Cancer (NSCLC) having received Tarceva monotherapy was recently prematurely terminated in August 2011 after almost 3 years due to discontinuation of the drug (not for safety reasons). These data demonstrate the need for (i) a better understanding of the mechanisms involved in IGF-1R inhibition and (ii) the identification of biomarkers that will predict response to IGF-1R inhibitors.

Cancer cell proliferation can be targeted by blocking the mRNA activity of genes which produce IGF-IR and IGF-II. An antisense approach may be effective in cancer gene therapy strategy by inhibition of IGF-1R expression with antisense or small interfering RNAs (siRNAs) [126]. Another approach is the use of an adenoviral vector-based short hair-pin(sh)RNA system. A study by Lee et al., showed RNA interference of IGF-1R induced IGF-1R silencing in lung cancer cell lines by blocking the downstream pathway of IGF-1R [127].

As the IGF-1R and EGFR share common downstream signaling molecules, receptor crosstalk occurs between these two powerful signaling pathways [305][106][306]. Stimulation of IGF-1R activates a network of intracellular signalling pathways, unaffected by the presence of EGF receptor-targeted therapeutics. Results from both *in vitro* and *in vivo* studies indicate that cross-talk between EGFR and IGF-1R can lead to acquired resistance against EGFR-targeted drugs [307].

The use of combination therapy in the treatment of cancer involves targeting more than one major signalling pathway. This approach may provide the way forward to treating cancer effectively. Recent clinical trials investigating the effects of EGFR and IGF-1R combination therapy have had less success than what was initially expected. In two large-phase III trials, figitumumab an IGF-1R mAb (CP-751,871, Pfizer) was combined with carboplatin/paclitaxel (ADVIGO 1016) and to the EGFR TKI erlotinib (ADVIGO 1018), in patients with advanced NSCLC. These trials were suspended however after results revealed there to be no increased benefit to patients [308]. This case highlights the need for earlier phase studies and the identification of predictive biomarkers. A recent study has reported that patients with high free IGF-1 pre-treatment are more sensitive to figitumumab [309]. There are also contrasting results whether adenocarcinoma or squamous patients are more likely to respond to figitumumab [310].

Patients with high expression of both IGF-1R and EGFR which have poorer survival (as shown previously in chapter 4) would possibly benefit from a combined therapy approach. To date, this dual targeting has had promising results however further *in vitro* and *in vivo* studies are necessary to further characterise the inhibition of both pathways.

The aims of this chapter were to (a) investigate the effects of R1507 in NSCLC cell lines and (b) in combination with erlotinib, (c) examine the effects of ligand stimulation following treatment with both inhibitors and (d) to investigate if silencing the IGF-1R sensitizes the NSCLC cells to erlotinib and influences the downstream MAPK signalling pathway.

5.1 Results

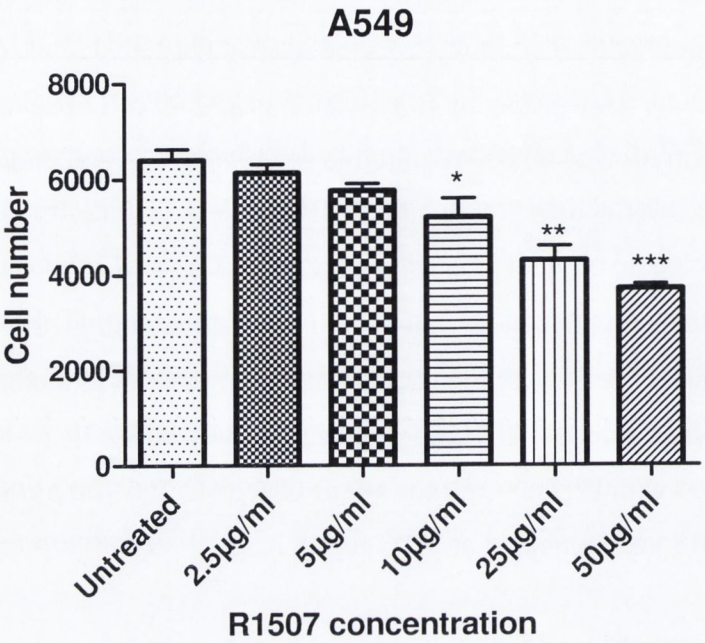
5.1.1 Effects of R1507 on cell proliferation and cell survival in NSCLC cell lines

R1507 is a fully humanised monoclonal antibody directed against the IGF-1R inhibitor. Dose concentration curves were set up in HCC827 and A549 cell lines to establish the optimum concentration of R1507 required to inhibit cell survival and/or proliferation within 48 h.

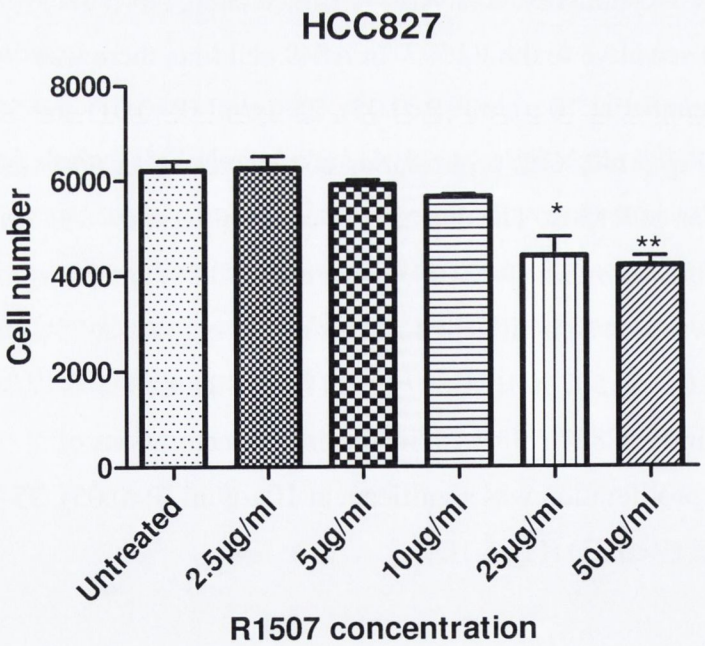
Cells were seeded at 2×10^3 cells/ well in a 96-well plate in media containing 10% FBS and were allowed to adhere. Cells were then serum starved overnight in media containing 0.5% FBS and were treated with R1507 at approximately 80% confluency. Cells were treated with various concentrations of R1507 (2.5– 50 $\mu\text{g/ml}$) in media containing 0.5% FBS and incubated at 37°C for 48 h.

After 48 h treatment with R1507, the effect of the antibody on cell proliferation and cell number were measured using a cell proliferation ELISA BrdU kit and Hoechst staining with the IN Cell 1000 Analyser, respectively. Three independent experiments were carried out and statistical analysis performed using ANOVA. Both NSCLC cell lines were sensitive to the R1507. In A549 cell line, there was significant decrease in cell number at 10 $\mu\text{g/ml}$ ($P<0.05$), 25 $\mu\text{g/ml}$ ($P<0.01$) and 50 $\mu\text{g/ml}$ ($P<0.001$) of R1507 (Fig 5.1A). Cell proliferation also decreased in A549 cells with increasing concentrations of R1507. The decrease of cell proliferation was significant at 5 $\mu\text{g/ml}$ ($P<0.05$), 10 $\mu\text{g/ml}$ ($P<0.05$), 25 $\mu\text{g/ml}$ ($P<0.01$) and 50 $\mu\text{g/ml}$ ($P<0.01$) (Fig 5.1C). There was also a significant decrease in cell number at 25 $\mu\text{g/ml}$ ($P<0.05$) and 50 $\mu\text{g/ml}$ ($P<0.01$) R1507 in HCC827 cells (Fig 5.1B). Cell proliferation also decreased in HCC827 cells with increasing concentrations of R1507. The decrease of cell proliferation was significant at 10 $\mu\text{g/ml}$ ($P<0.05$), 25 $\mu\text{g/ml}$ ($P<0.01$) and 50 $\mu\text{g/ml}$ ($P<0.01$) (Fig 5.1D).

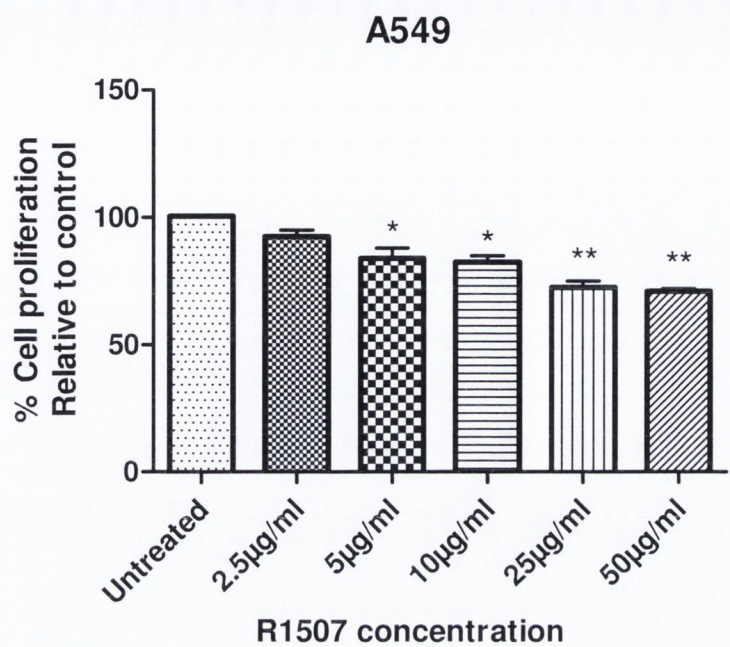
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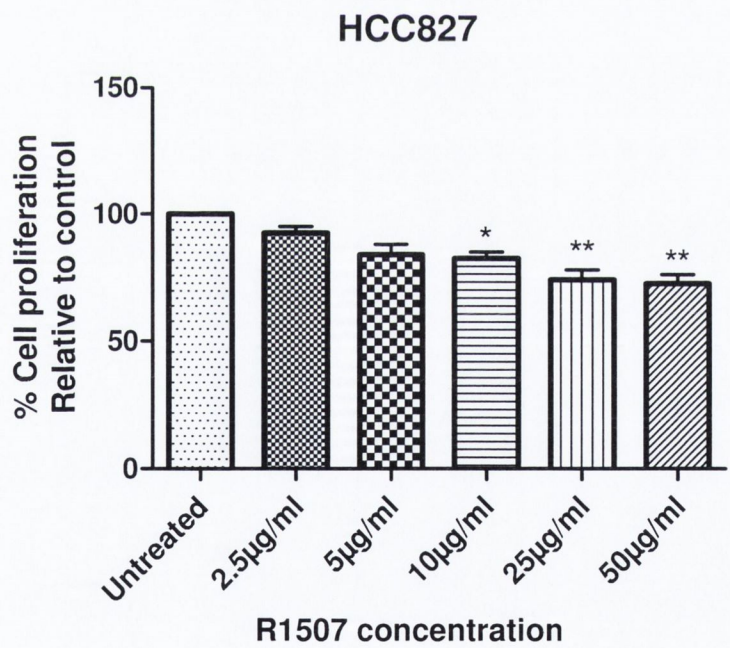


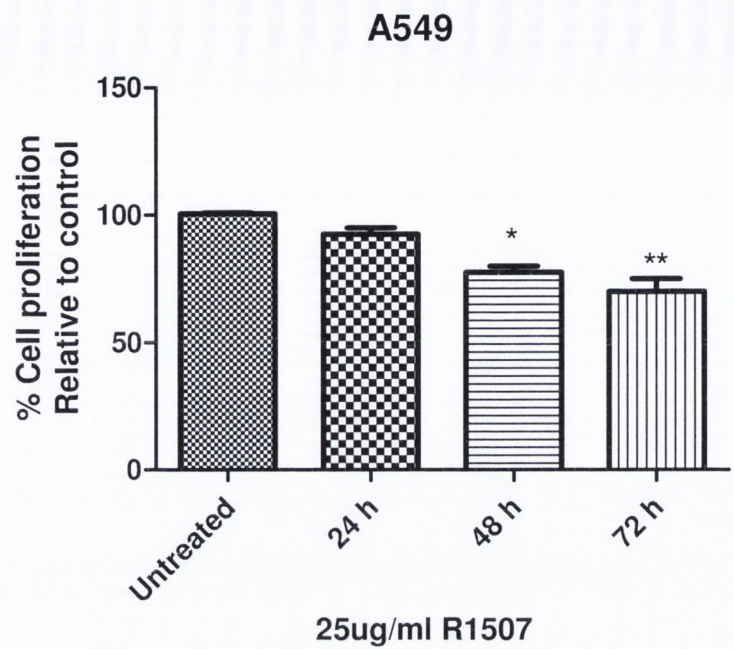
Fig 5.1: The anti-proliferative effect of R1507. Cell number and cell proliferation were examined by BrdU assay and Cell count assay following 48 h treatment with R1507 in A549 (A and C) and HCC827 (B and D) cells. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.

5.1.2 The effect of R1507 on cell proliferation in NSCLC cells lines over 72 h

HCC827 and A549 cell lines were treated with the selected concentration of 25 µg/ml R1507 at 24, 48 and 72 h. This concentration was chosen as it significantly reduced cell proliferation ($P<0.01$) of A549 and HCC827 cells in the previous section (5.1.1). Cells were seeded at 2×10^3 cells/ well in a 96-well plate in media containing 10% FBS and were allowed to adhere. Cells were then serum starved overnight in 0.5% FBS and were treated with R1507 when approximately 80% confluency was reached. Cells were treated with 25µg/ml R1507 and incubated at 37°C for 24, 48 and 72 h. After 24, 48 and 72 h treatment with R1507, the effect of the antibody on cell proliferation and cell number were measured using a cell proliferation ELISA BrdU kit and Hoechst staining on IN Cell 1000 Analyser. Statistical analysis was carried out using ANOVA.

The A549 cell line showed no significant change in cell proliferation at 24 h (Fig 5.2 A). However, at 48 and 72 h there was a significant decrease in proliferation relative to untreated cells which was $P<0.05$ and $P<0.01$, respectively. There was no significant change in cell proliferation at 24 h in the HCC827 cell line but a significant decrease was seen at 48 h ($P<0.05$) and 72 h ($P<0.01$) (Fig 5.2 B).

A.



B.

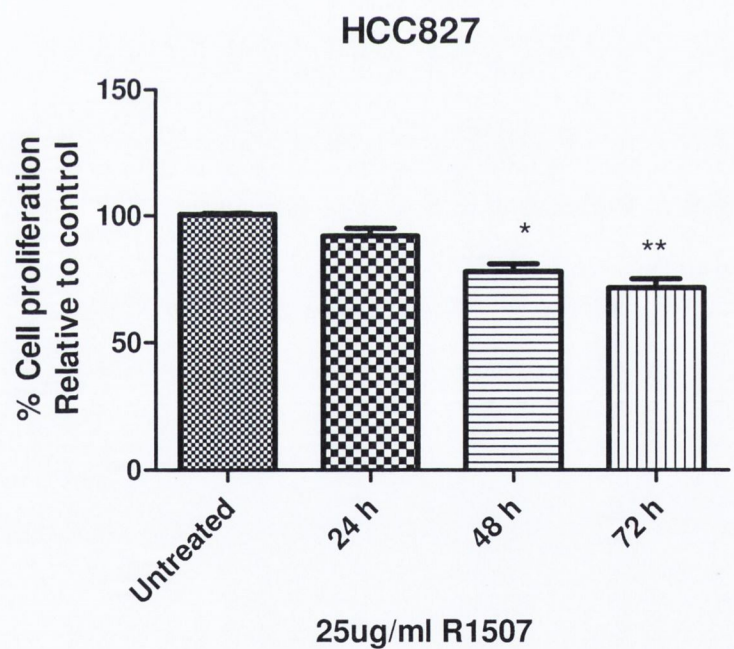


Fig 5.2: The anti-proliferative effect of R1507 over 72 h. Cell proliferation was examined by BrdU assay following 24, 48 and 72 h treatment with 25 μ g/ml R1507 in A549 (A) and HCC827 (B) cells. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.

5.1.3 Effect of erlotinib on cell proliferation and cell survival in NSCLC cell lines

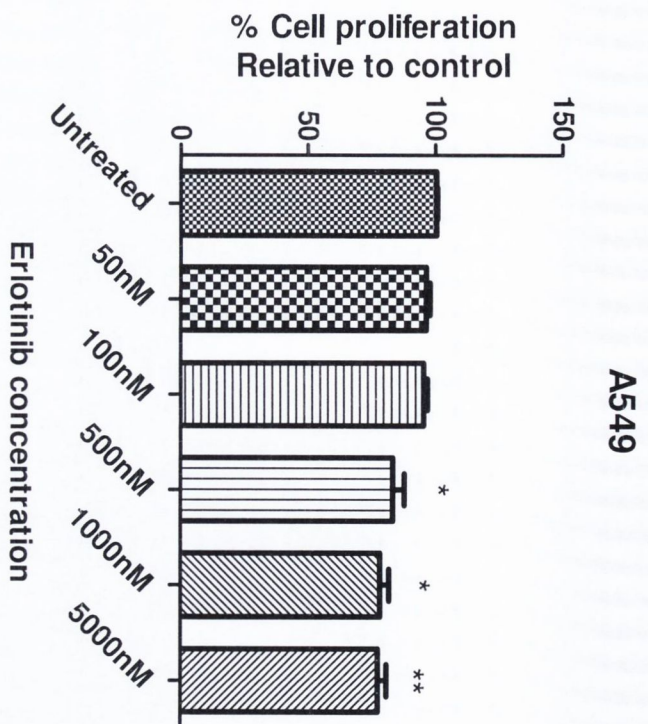
Erlotinib is an EGFR tyrosine kinase inhibitor. Dose concentration curves were set up in HCC827 and A549 cell lines using erlotinib to establish the optimum concentration of erlotinib required to inhibit cell survival and/or cell proliferation within 48 h.

Cells were seeded at 2×10^3 cells/ well in a 96-well plate in media containing 10% FBS and were allowed to adhere. Cells were then serum starved overnight in media containing 0.5% FBS and were treated with erlotinib at approximately 80% confluency. Cells were treated with various concentrations of erlotinib in serum depleted medium (0.5% FBS) and incubated at 37°C for 48 h.

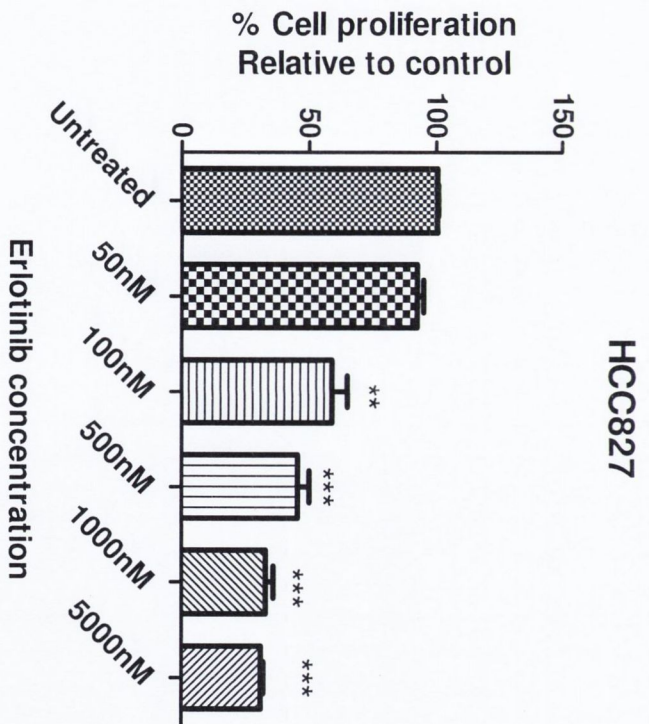
After 48 h treatment with erlotinib, the effect of the TKI on cell proliferation and cell number was measured using a cell proliferation ELISA BrdU kit and Hoechst staining using the IN Cell 1000 Analyser respectively. Statistical analysis was carried out using ANOVA.

The A549 cells showed significant changes in cell proliferation at high concentrations of erlotinib (Fig 5.3 A). A significant decrease in cell proliferation occurred at 500nM ($P<0.05$), 1000nM ($P<0.05$) and 5000nM ($P<0.01$). HCC827 cells showed no significant change in cell proliferation at a concentration of 50nM erlotinib. However there was a decrease in proliferation at concentrations over 100nM. A significant decrease in cell proliferation was seen at 100nM ($P<0.01$), 500nM ($P<0.001$), 1000nM ($P<0.001$) and 5000nM ($P<0.001$) (Fig 5.3 B). No significant difference in cell number was seen in A549 cells until 1000nM ($P<0.01$) and 5000nM ($P<0.001$) (Fig 5.3 C). HCC827 cells showed a significant decrease in cell number at 100nM ($P<0.05$), 500nM ($P<0.01$), 1000nM ($P<0.001$) and 5000nM ($P<0.001$) (Fig 5.3 D).

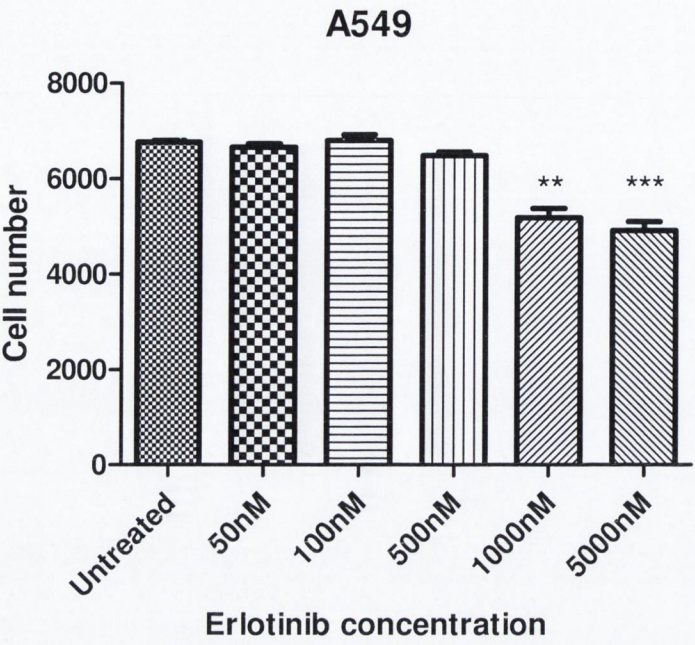
A.



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C.



D.

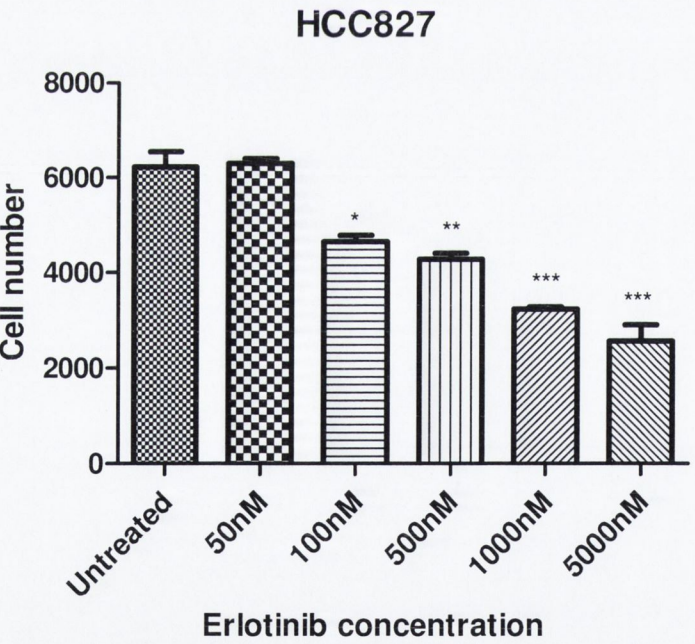


Fig 5.3: The anti-proliferative effect of erlotinib. Cell proliferation and cell survival were examined by BrdU assay and Cell count assay following 48 h treatment with 50-5000nM erlotinib in A549 (A and C) and HCC827 (B and D) cells. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.

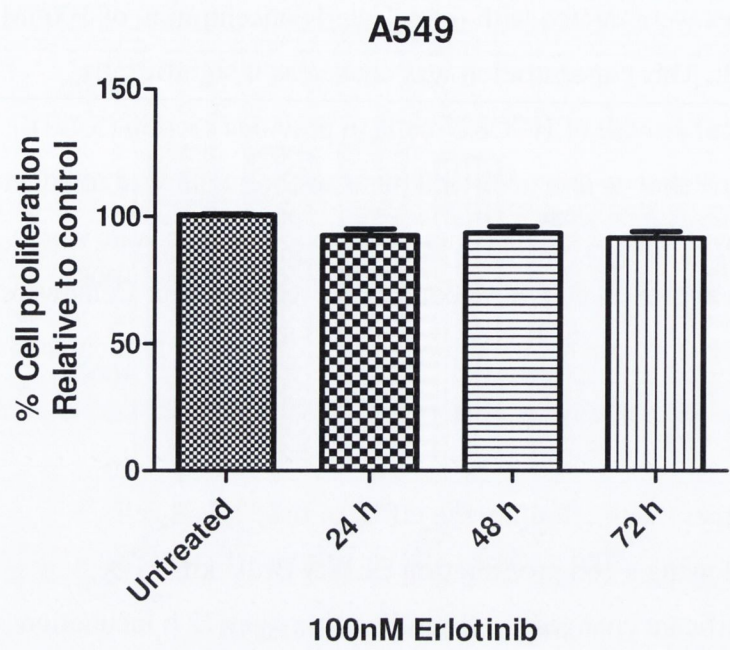
5.1.4 Effects of erlotinib on cell proliferation in NSCLC cell lines over 72 h

HCC827 and A549 cell lines were treated with the selected concentration of 100nM erlotinib for 24, 48 and 72 h. This concentration was chosen as it significantly reduced cell proliferation and growth of HCC827 cells in previous section (5.1.3). Cells were seeded at 2×10^3 cells/ well in a 96-well plate and were allowed to adhere. Cells were serum starved overnight in media containing 0.5% FBS and cells were treated with erlotinib when approximately 80% confluency was reached. Cells were treated with 100nM erlotinib in serum depleted medium (0.5% FBS) and incubated at 37°C for 24, 48 and 72 h.

After 24, 48 and 72 h treatment with erlotinib, the effect of the TKI on cell proliferation was measured using a cell proliferation ELISA BrdU kit.

A549 cells showed no significant change in cell proliferation over 72 h incubation with 100nM erlotinib (Fig 5.4A). HCC827 cells showed a decrease in cell proliferation over time reaching significance at 48 h ($P < 0.01$) and 72 h ($P < 0.001$) (Fig 5.4 B).

A.



B.

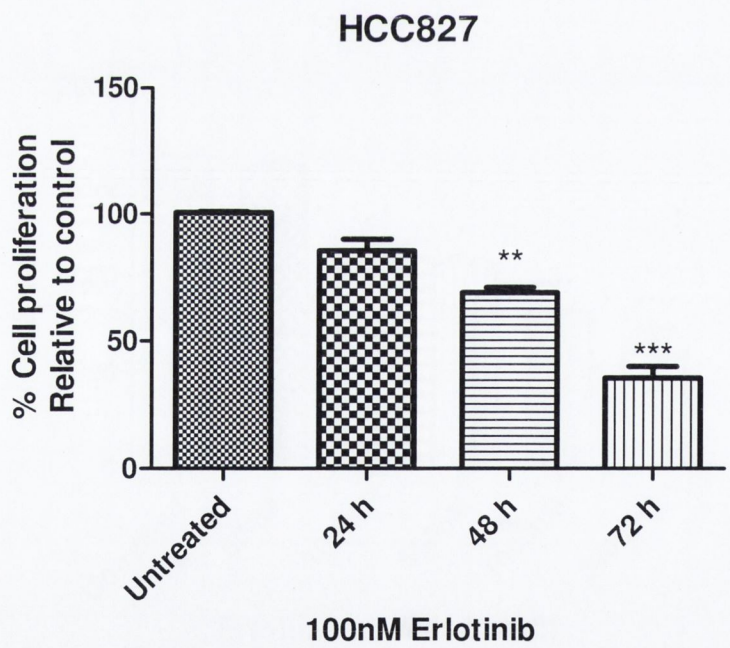


Fig 5.4: The anti-proliferative effect of erlotinib over 72 h. Cell proliferation was examined by BrdU assay following 24, 48 and 72 h treatment with 100nM erlotinib in A549 (A) and HCC827 (B) cells. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.

5.1.5 Effect of IGF-I and IGF-II on NSCLC cells lines treated with R1507

HCC827 and A549 cell lines were treated with the selected concentration of 25 µg/ml R1507 48 h along with IGF-I and IGF-II to examine the ability of these ligands to rescue the cells from the antibody treatment. Cells were seeded at 2×10^3 cells/ well in a 96-well plate in media containing 10% FBS and were allowed to adhere. Cells were serum starved overnight in 0.5% FBS and were treated at approximately 80% confluency. Ten nM of IGF-I and IGF-II were combined together with R1507 to cells and incubated at 37°C for 48 h. Ten nM of IGF-I and IGF-II were also added to cells after they had been treated with R1507 alone for 24 h. These were incubated at 37°C for 48 h.

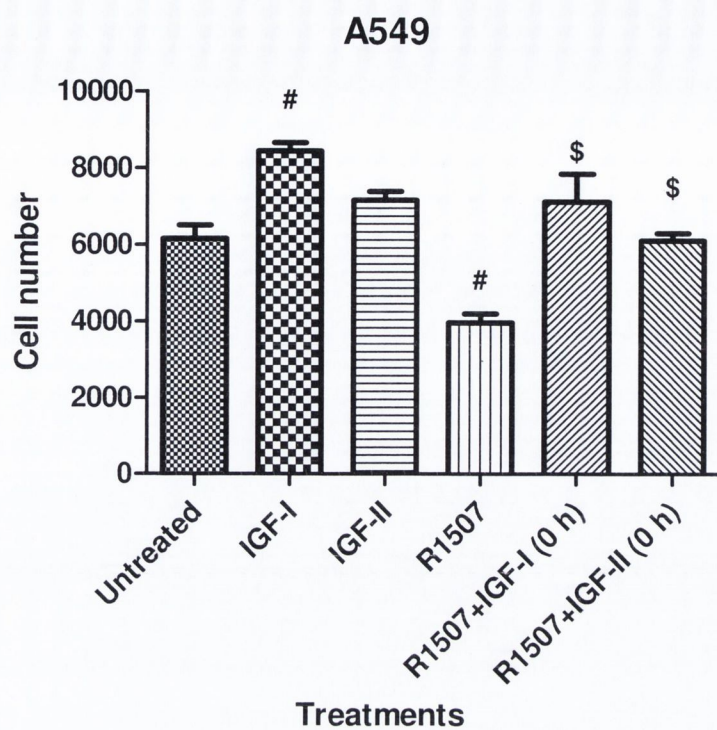
After 48 h treatment with R1507, the effect of the antibody on cell proliferation was measured using a cell proliferation ELISA BrdU kit or cell number by Hoechst staining using the IN Cell 1000 Analyser. Statistical analysis was carried out using ANOVA.

The addition of IGF-I to A549 cells resulted in a significant increase in cell number compared to untreated cells ($P < 0.05$) (Fig 5.5 A). When IGF-I was added at the time of treatment with R1507 for 48 h, the inhibitory effect of R1507 on cell growth was reduced. IGF-II had no significant effect on cell number in A549 cells treated with R1507 although an increase was seen in cells treated with IGF-II alone but this was not significant. IGF-I significantly increased cell growth of R1507 treated A549 cells compared to R1507 treated A549 cells alone ($P < 0.01$). IGF-II significantly increased the growth of R1507 treated A549 cells compared to R1507 treated cells alone ($P < 0.05$). The effect of IGF-I and IGF-II stimulation was then examined in A549 cells which had been treated with R1507 for 24 h before addition of IGF-I and IGF-II for a further 48 h. This did not inhibit the effect of R1507 compared to IGF-I and IGF-II added at the time of treatment with R1507 (Fig 5.5 B). IGF-I ($P < 0.05$) and IGF-II ($P < 0.05$) significantly increased the growth of R1507 treated A549 cells compared to R1507 treated cells alone 24 h after treatment.

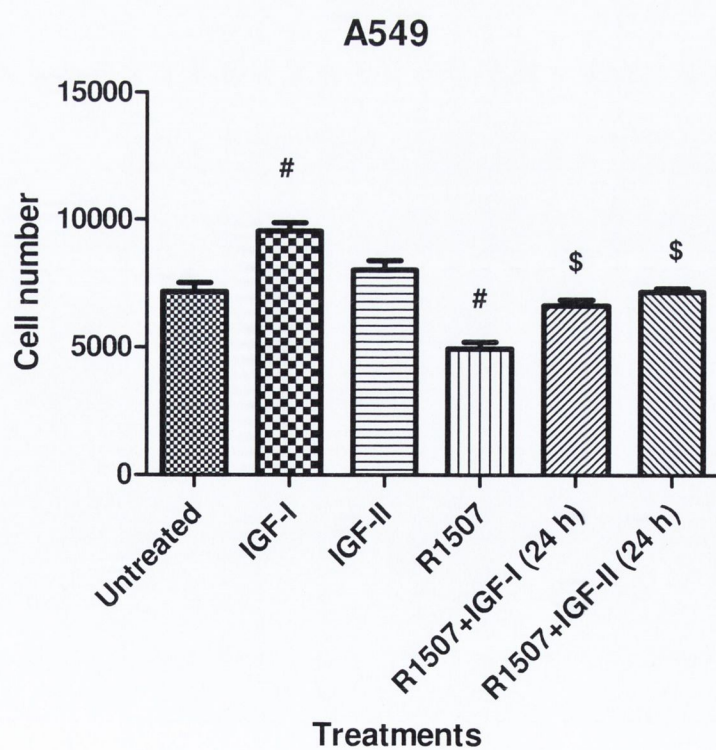
In HCC827 cells addition of IGF-I also significantly increased cell number compared to untreated cells ($P < 0.001$) (Fig 5.5 C). When IGF-I was added at the time of treatment with R1507 for 48 h, the inhibitory effect of the antibody on cell growth

was reduced. IGF-II also increased cell number but this was not significant. IGF-I significantly increased the growth of R1507 treated HCC827 cells ($P<0.05$) whereas IGF-II did not significantly increase cell growth. The effect of IGF-I and IGF-II stimulation was then examined in HCC827 cells which had been treated with R1507 for 24 h before addition of IGF-I and IGF-II for a further 48 h. This did not inhibit the effect of R1507 compared to IGF-I and IGF-II added at the time of treatment with R1507 (Fig 5.5 D). IGF-I and IGF-II did not significantly increase the growth of R1507 treated HCC827 cells compared to R1507 treated cells alone when added 24 h after treatment.

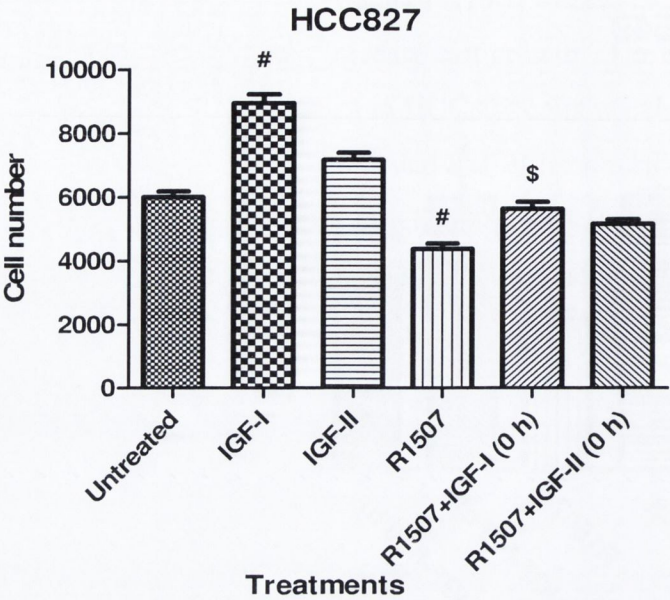
A.



B.



C.



D.

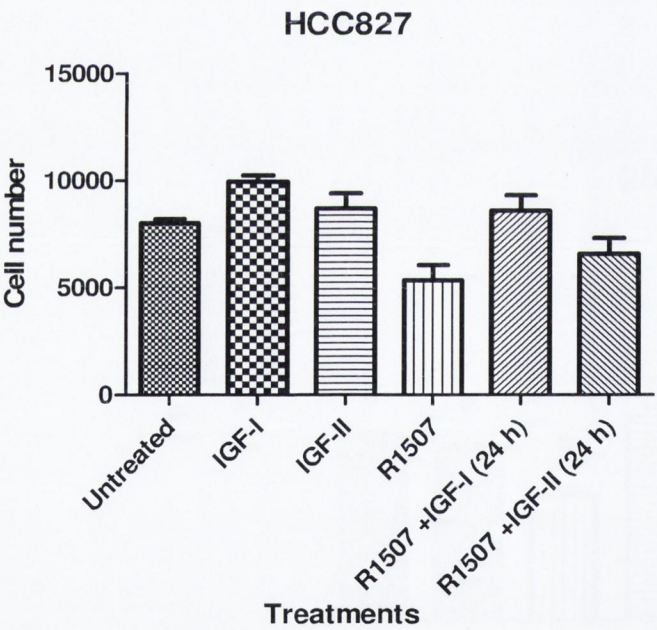


Fig 5.5: The effect of IGF-I and IGF-II on R1507 treated NSCLC cells. Cell number was examined by Hoechst staining followed by analysis using IN Cell 1000 Analyser post 48 h treatment with 25µg/ml R1507 in A549 (A and B) and HCC827 (C and D) cells. IGF-I (10nM) was added to the R1507 treated cells at time of antibody treatment (0 h) and 24 h after treatment (24 h). Data is expressed as mean of triplicate experiments ± SEM. Statistical analysis was carried out using ANOVA. (# = significant compared to Untreated, \$ = significant compared to R1507)

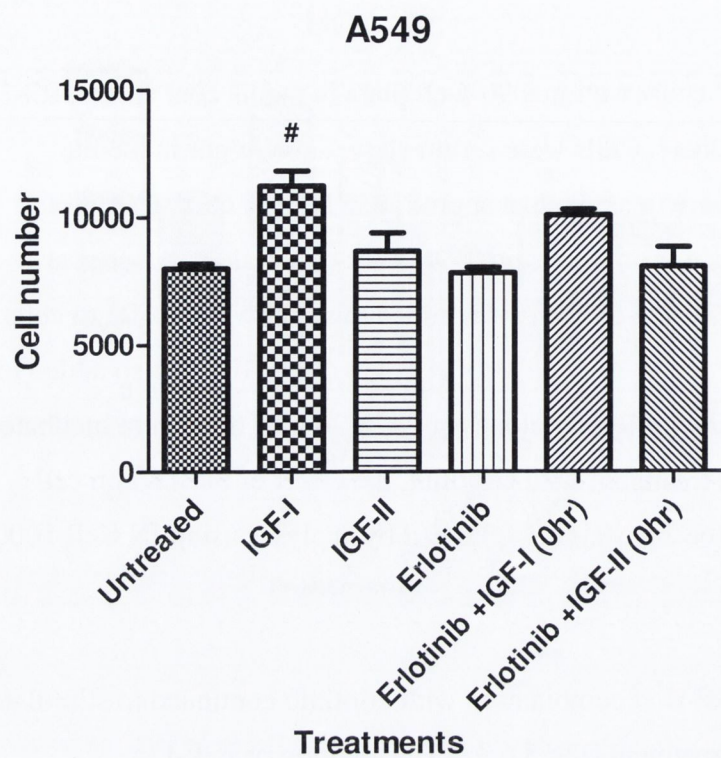
5.1.6 Effect of IGF-I and IGF-II on cell number in NSCLC cell lines treated with erlotinib

Cells were seeded at 2×10^3 cells/ well in a 96-well plate in media containing 10% FBS and were allowed to adhere. Cells were serum starved overnight in media containing 0.5% FBS and were treated when approximately 80% confluency was reached. Subsequently, cells were treated with 100nM erlotinib and incubated at 37°C for 48 h. Ten nM of IGF-I and IGF-II was added together with R1507 to cells (0 h) and incubated at 37°C for 48 h. Ten nM of IGF-I and IGF-II was also added to cells which had been treated with R1507 alone for 24 h (24 h). These were incubated at 37°C for 48 h. After 48 h treatment with erlotinib, the effect of the TKI on cell number was measured by Hoechst staining followed by analyses using IN Cell 1000 Analyser.

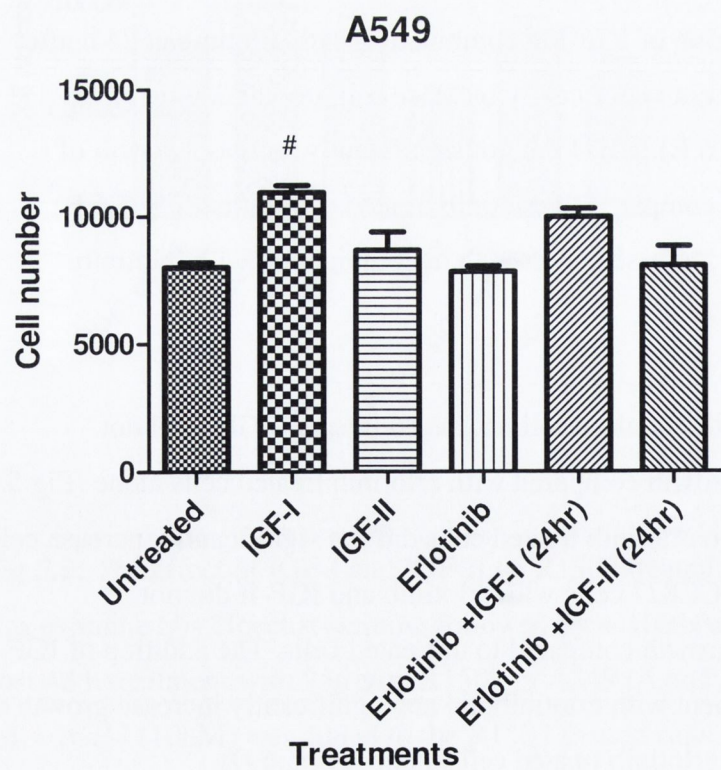
In A549 cells, IGF-I and IGF-II in combination with erlotinib continued to stimulate growth unaffected by TKI treatment (Fig 5.6 A). The addition of IGF-I in combination with erlotinib at 0 h after treatment with erlotinib did not significantly increase cell growth compared to untreated A549 cells. IGF-I did not significantly increase growth of erlotinib treated A549 cells compared to erlotinib treated cells alone (Fig 5.6 A). The addition of IGF-I in combination with erlotinib at 24 h after treatment with erlotinib did not significantly increase cell growth compared to untreated A549 cells (Fig 5.6 B). IGF-I did not significantly increase growth of erlotinib treated A549 cells compared to erlotinib treated cells alone (Fig 5.6 B). IGF-II did not significantly increase cell growth in combination with erlotinib compared to untreated A549 cells.

In HCC827 cells treated with 100nM erlotinib, the addition of IGF-I did not significantly increase cell growth compared with erlotinib treated cells alone (Fig 5.6 C). The addition of IGF-II to erlotinib treated cells did not significantly increase cell growth. The treatment of HCC827 cells with erlotinib and IGF-II did not significantly decrease cell growth compared to untreated cells. The addition of IGF-I and IGF-II 24 h after treatment with erlotinib did not significantly increase growth of HCC827 cells compared to erlotinib treated cells alone (Fig 5.6 D).

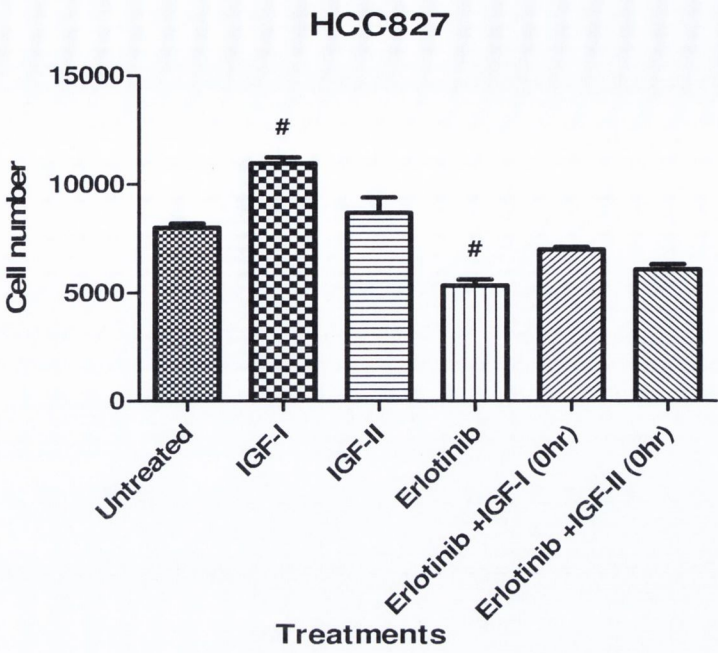
A.



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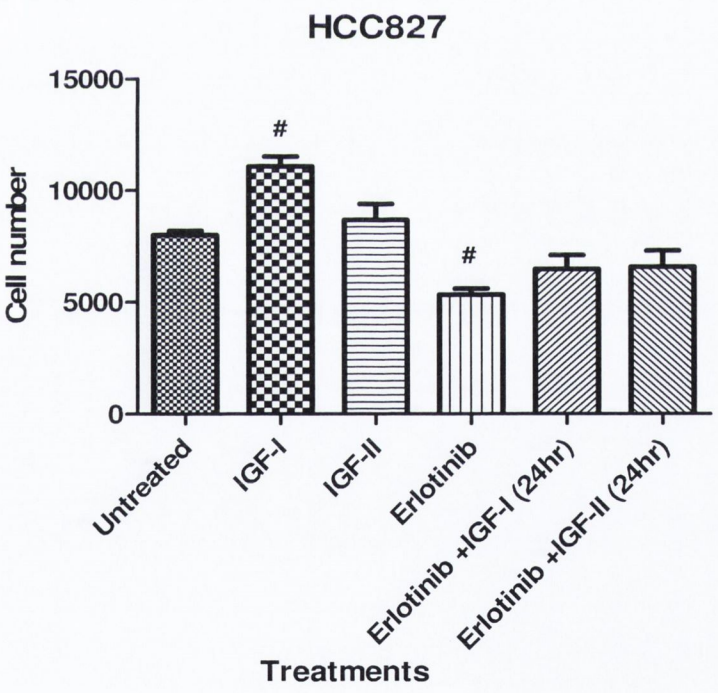


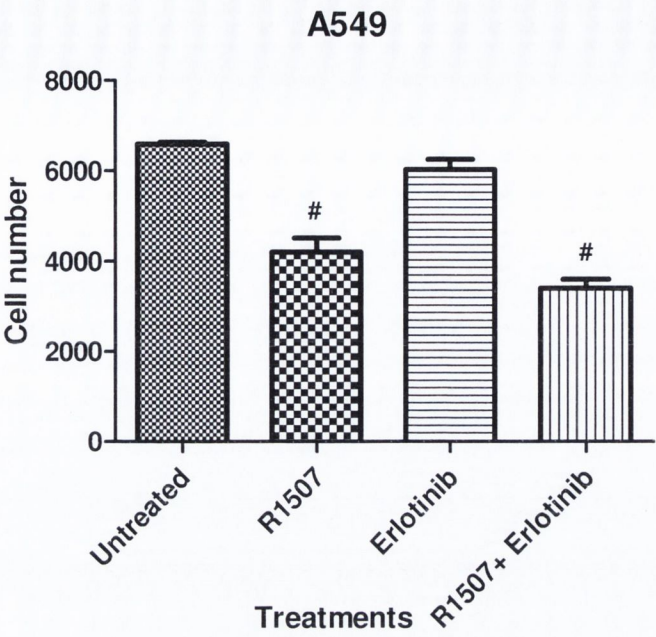
Fig 5.6: The effect of IGF-I and IGF-II on erlotinib treated NSCLC cells. Cell number was examined by Hoechst staining followed by analysis using IN Cell 1000 Analyzser following 48 h treatment with 100nM erlotinib in A549 (A and B) and HCC827 (C and D) cells. IGF-I (10nM) was added to the erlotinib treated cells at time of antibody treatment (0 h) and 24 h after treatment (24 h). Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA. (# = significant compared to Untreated)

5.1.7 Effect of R1507 and erlotinib co-treatment in HCC827 and A549 cell lines

HCC827 and A549 cell lines were treated with the selected concentrations of 25µg/ml R1507 and 100 nM erlotinib for 48 h. Cells were seeded at 2×10^3 cells/well in a 96-well plate in media containing 10% FBS and were allowed to adhere. Cells were serum starved overnight in media containing 0.5% FBS and were treated at approximately 80% confluency. Subsequently, cells were treated with 25µg/ml R1507 and 100nM erlotinib in serum and incubated at 37°C for 48 h. After 48 h treatment with R1507 and erlotinib, the effect of the antibody on cell number was analysed by Hoechst staining followed by cell counting using the IN Cell 1000 Analyser.

R1507 significantly reduced the growth of A549 cells ($P < 0.01$) (Fig 5.7 A). Erlotinib had no effect on the growth of A549 cells. A549 cells co-treated with R1507 and erlotinib ($P < 0.01$) had significantly reduced cell growth when compared with untreated cells (Fig 5.7 A). There was no significant difference when R1507 treated A549 cells were compared with R1507 and erlotinib treated A549 cells. When R1507 was added in combination with erlotinib there was a decrease in cell growth but this was not significant. R1507 significantly reduced the growth of HCC827 cells (Fig 5.7 B). Erlotinib significantly reduced the growth of HCC827 cells ($P < 0.01$). When R1507 was added in combination with erlotinib there was no increased reduction in cell growth compared to R1507 treated cells alone.

A.



B.

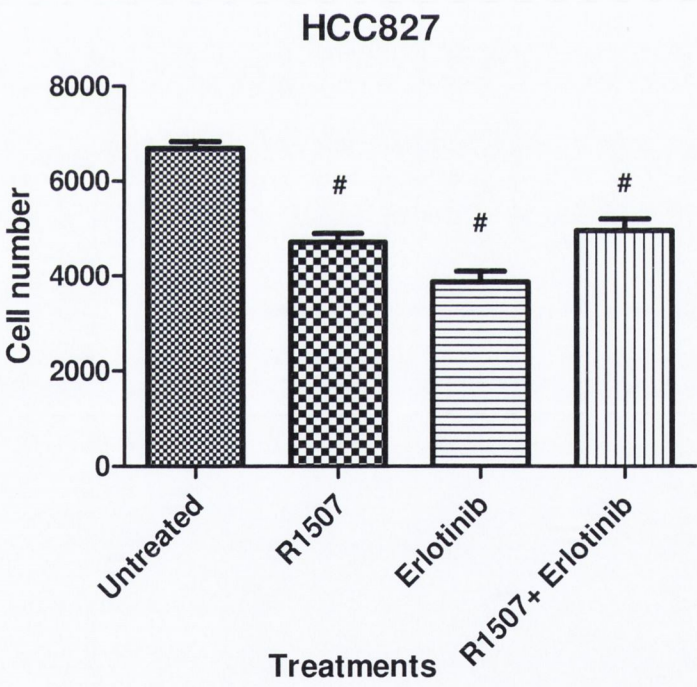


Fig 5.7: The anti-proliferative effect of combined R1507 and erlotinib treatment. Cell number was examined by Hoechst staining followed by analysis using IN Cell 1000 Analyser following 48 h treatment with 25 μ g/ml R1507 and 100nM erlotinib in A549 (A) and HCC827 (B) cells. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA. (# = significant compared to untreated cells)

5.1.8 Effect of IGF-1R siRNA on cell number in NSCLC cell lines

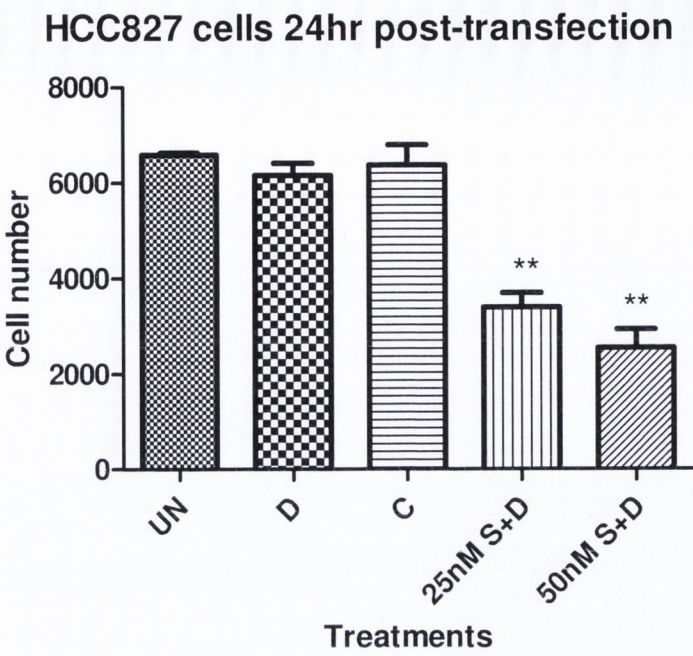
IGF-1R siRNA was used to examine the effect of IGF-1R knockdown in NSCLC cell lines. Optimal transfection reagent and siRNA concentration were first assessed by using different concentrations of each. Initially cells were transiently transfected with green fluorescent (GFP) DNA to optimise transfection efficiency and to determine the optimal concentration of transfection reagent DharmaFECT 1. Cells were seeded in antibiotic-free complete medium and grown to 80% confluency. The cells were treated with various concentrations of DharmaFECT 1 for 24 h and GFP DNA. Images of the cells were taken using a fluorescent microscope 24 h post-transfection. The optimum transfection efficiency was achieved using 0.2 μ L of DharmaFECT 1 and 50nM siRNA ON-TARGET *plus* SMARTpool siRNA reagent which was confirmed by examining IGF-1R protein expression post transfection. Statistical analysis was carried out using a two tailed student t-test.

A549 and HCC827 cells were seeded at 3×10^3 /well in a 96-well plate in antibiotic-free complete media until 80% confluency was reached. The transfection medium was added to antibiotic-free complete medium for a total volume of 200 μ L per well of a 96-well plate. Cells were incubated at 37°C in 5% CO₂ for 24 and 48 h. Media was replaced with complete medium after 24 h to reduce cytotoxicity, with incubation continued for a further 24 h for the 48 h time point.

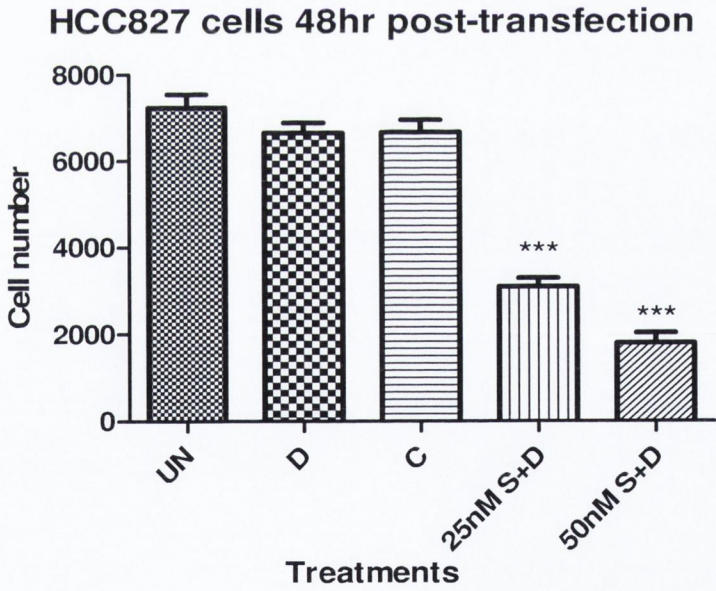
After 48 h treatment with 25nM and 50nM siRNA, the effect of IGF-1R knockdown on cell number was analysed by Hoechst staining followed by cell counting using the IN Cell 1000 Analyser.

In HCC827 cells there was a significant decrease in cell growth when treated with 25nM siRNA ($P < 0.01$) and 50nM siRNA ($P < 0.01$) for 24 h (Fig 5.8 A). Cell growth was reduced further when treated with 25nM siRNA ($P < 0.001$) and 50nM siRNA ($P < 0.001$) for 48 h (Fig 5.8 B). In A549 cells there was a significant decrease in cell growth when treated with 25nM siRNA ($P < 0.001$) and 50nM siRNA ($P < 0.001$) for 24 h (Fig 5.8 C). Cell growth was also significant when treated with 25nM siRNA ($P < 0.001$) and 50nM siRNA ($P < 0.001$) for 48 h (Fig 5.8 D).

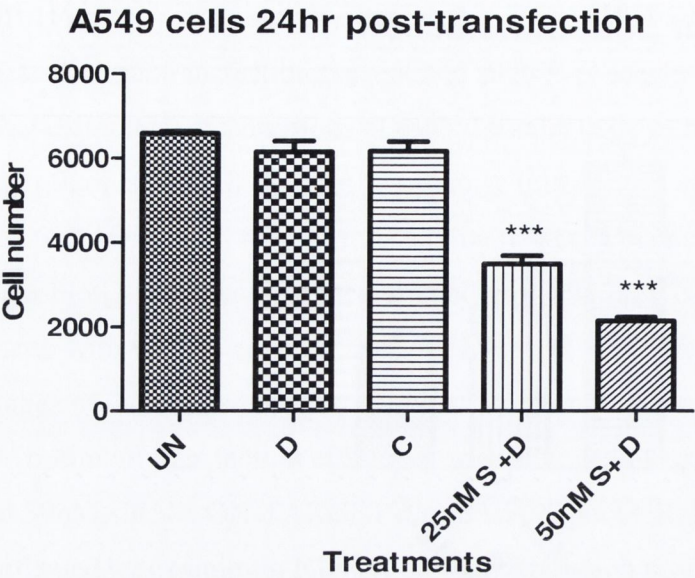
A.



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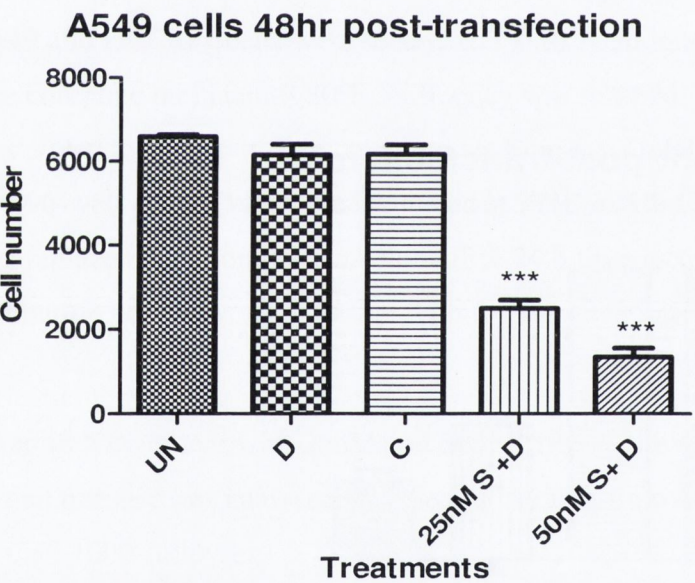


Fig 5.8: Effect of IGF-1R siRNA in NSCLC cells. Cell number was examined by Hoechst staining followed by analysis using IN Cell 1000 Analyzser following 24 h and 48 h treatment with 25nM and 50nM siRNA in A549 (A and B) and HCC827 (C and D) cells. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.
(UN = untreated, D= dharmaFECT-1, C= scramble control, S= siRNA)

5.1.9 Effect of IGF-1R knockdown on cell proliferation in NSCLC cell lines and sensitivity to R1507 and erlotinib

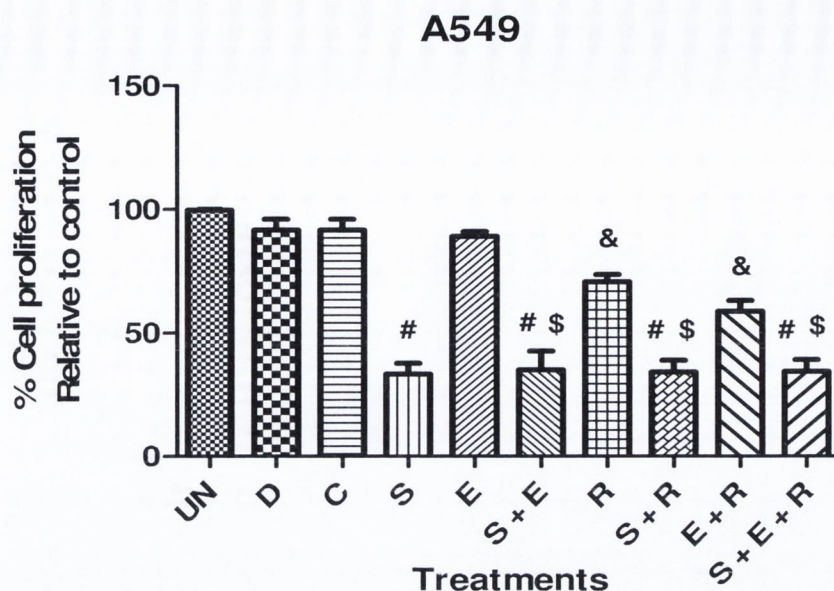
A549 and HCC827 cells were seeded at 3×10^3 /well in a 96-well plate in antibiotic-free complete media and were allowed to adhere. Cells were then serum starved in media containing 0.5% FBS overnight until 80% confluency was reached. Cells were treated with 25µg/mL R1507 and 100nM erlotinib in combination with 50nM IGF-1R siRNA or alone to examine sensitivity of NSCLC cells to R1507 and erlotinib upon IGF-1R knockdown. Cells were incubated at 37°C in 5% CO₂ for 48 h. Media was replaced with complete medium after 24 h to reduce cytotoxicity, and incubation continued for a further 24 h. After 48 h treatment with 50nM IGF-1R siRNA, the effect of IGF-1R knockdown on cell proliferation was measured using a cell proliferation ELISA BrdU kit. Statistical analysis was carried out using a two tailed student t-test.

A549 cells had significantly reduced cell proliferation when treated with 50nM IGF-1R siRNA for 48 h compared to untreated cells ($P < 0.001$) (Fig 5.9 A). When IGF-1R siRNA A549 treated cells were combined with 100nM erlotinib ($P < 0.001$), 25µg/mL R1507 ($P < 0.001$) and both 25µg/mL R1507 and 100nM erlotinib ($P < 0.001$), cell proliferation was significantly reduced compared to untreated cells. A549 cells treated with erlotinib and IGF-1R siRNA had significantly reduced cell proliferation compared to cells treated with erlotinib alone ($P < 0.001$). A549 cells treated with R1507 and IGF-1R siRNA had significantly reduced cell proliferation compared to cells treated with R1507 alone ($P < 0.001$). A549 cells treated with erlotinib, R1507 and IGF-1R siRNA had significantly reduced cell proliferation compared to cells treated with erlotinib and R1507 alone ($P < 0.05$).

HCC827 cells had significantly reduced cell proliferation when treated with 50nM IGF-1R siRNA for 48 h compared to untreated cells ($P < 0.001$) (Fig 5.9 B). When IGF-1R siRNA HCC827 treated cells were combined with 100nM erlotinib ($P < 0.001$), 25µg/mL R1507 ($P < 0.001$) and both 25µg/mL R1507 and 100nM erlotinib ($P < 0.001$), cell proliferation was significantly reduced compared to untreated cells. HCC827 cells treated with erlotinib and IGF-1R siRNA had significantly reduced cell proliferation compared to cells treated with erlotinib alone

($P<0.05$). HCC827 cells treated with R1507 and IGF-1R siRNA had significantly reduced cell proliferation compared to cells treated with R1507 alone ($P<0.001$). HCC827 cells treated with erlotinib, R1507 and IGF-1R siRNA had significantly reduced cell proliferation compared to cells treated with erlotinib and R1507 alone ($P<0.05$).

A.



B.

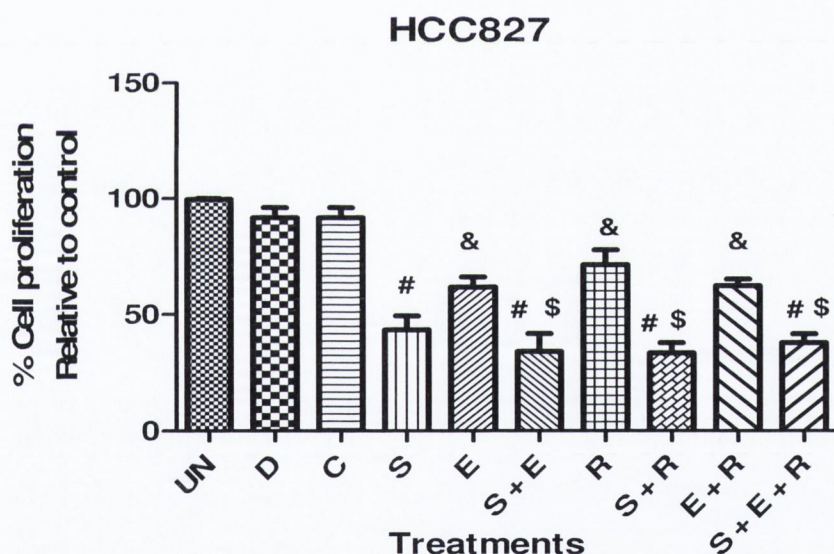


Fig 5.9: Effect of IGF-1R siRNA on R1507 and erlotinib treated NSCLC cell lines. Treatment of (A) A549 cells and (B) HCC827 cells with 50nM IGF-1R siRNA for 48 h to examine the effect of IGF-1R knockdown on cell proliferation and drug sensitivity. Cell proliferation was measured by using a cell proliferation ELISA BrdU kit. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.

(UT – untreated, D- DharmaFECT-1, C- scramble control ,S- siRNA, R- R1507, E- erlotinib, E+R- erlotinib + R1507).

(# = significant compared to untreated cells, & = significant compared to untreated cells, \$ = significant compared to E, R and E/R treated cells)

5.1.10 Effect of IGF-1R siRNA on protein expression in NSCLC cell lines treated with R1507 and erlotinib and the effect on the MAPK signalling pathway

To determine the role of IGF-1R in drug sensitivity to R1507 and erlotinib, an IGF-1R siRNA was used to knockdown the receptor to examine the MAPK pathway. A549 and HCC827 cells were seeded at 1×10^5 /well in 6-well plates in antibiotic-free complete media and were allowed to adhere. Cells were then serum starved in media containing 0.5% FBS overnight until 80% confluency was reached. Cells were treated with 25 μ g/mL R1507 and 100nM erlotinib in combination with 50nM siRNA. Cells were incubated at 37°C in 5% CO₂ for 72 h. Media was replaced with complete medium after 24 h to reduce cytotoxicity, and cultured for an additional 48 h. After 72 h, protein was collected from the flasks using protein lysis buffer containing protease inhibitors.

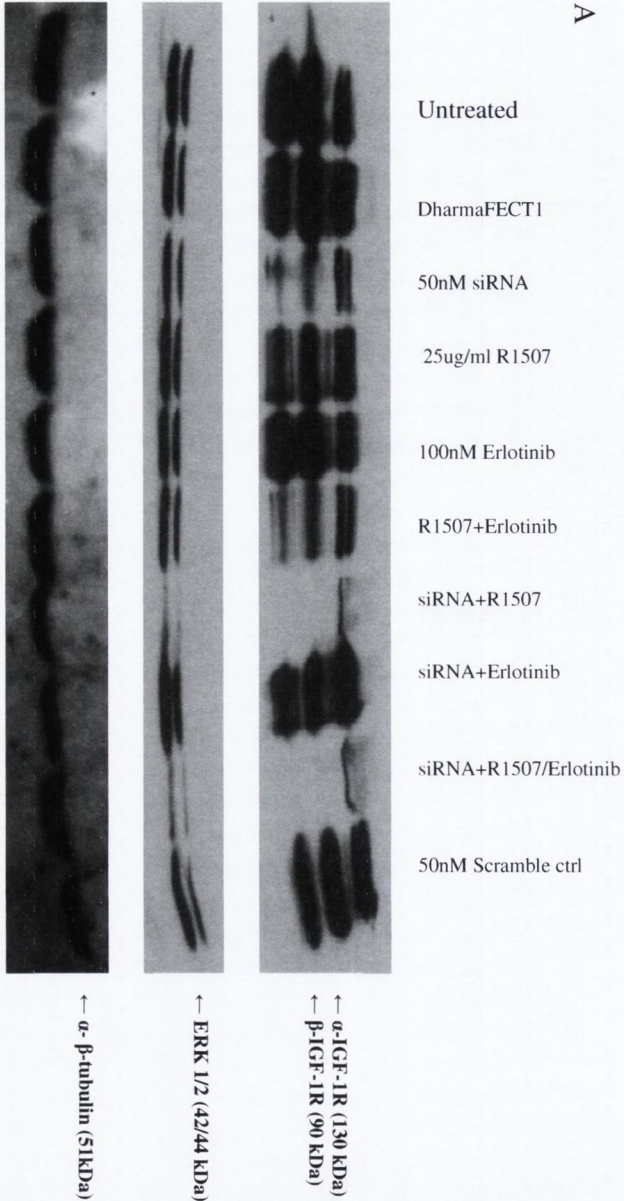
Total protein was loaded on a gel and separated by electrophoresis followed by transfer to PVDF membrane. Expression of IGF-1R, and ERK were determined by antibody probing of membranes. A representative gel is shown in Fig 5.10. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using a two tailed student t-test.

In HCC827 cells IGF-1R siRNA significantly reduced IGF-1R protein expression compared to untreated cells ($P < 0.001$) (Fig 5.10 A). R1507 also decreased IGF-1R protein expression significantly ($P < 0.001$) (Fig 5.10 C). When HCC827 cells were treated with combined R1507 and erlotinib IGF-1R protein expression was significantly reduced compared to untreated cells ($P < 0.001$). The combination of siRNA with R1507 +/- erlotinib in HCC827 cells completely eliminated IGF-1R expression. ERK 1/2 expression was reduced in siRNA treated cells but this was not significant. ERK 1/2 expression was significantly reduced in HCC827 cells treated with siRNA and R1507 ($P < 0.001$) and siRNA, R1507 and erlotinib, ($P < 0.001$) (Fig 5.10 D).

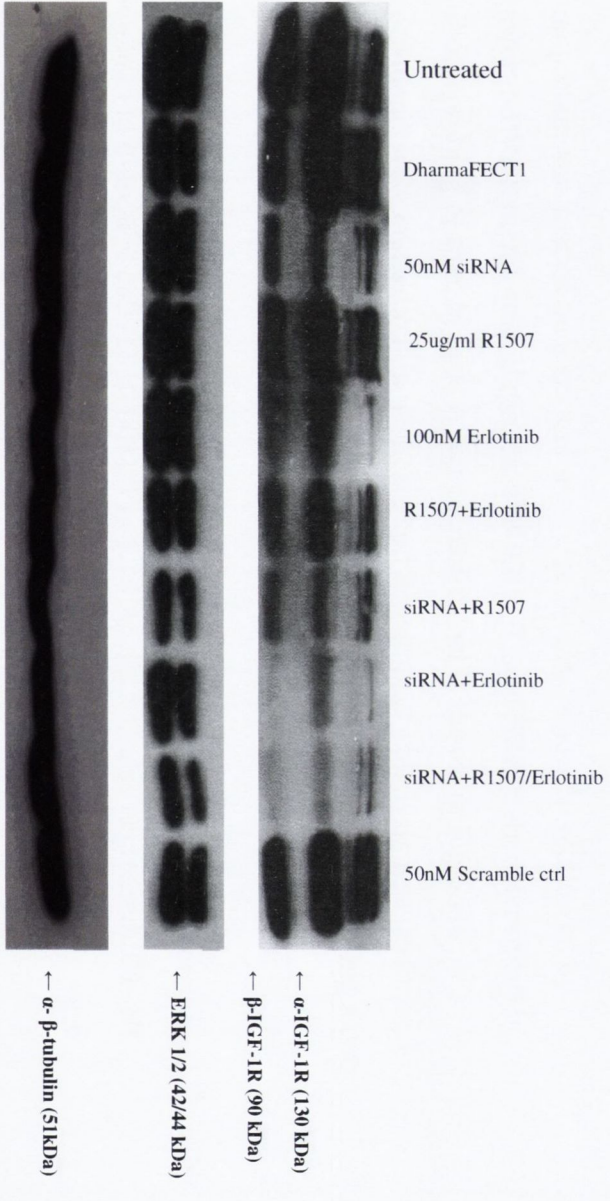
In A549 cells, IGF-1R siRNA significantly reduced IGF-1R protein expression compared to untreated cells ($P < 0.001$) (Fig 5.10 E). R1507 significantly decreased

IGF-1R protein expression compared to untreated cells ($P<0.01$). IGF-1R protein expression was also significantly reduced in siRNA treated cells with erlotinib +/- R1507. A549 cells showed reduced ERK 1/2 expression in siRNA treated cells with R1507 alone ($P<0.01$) and R1507 and erlotinib ($P<0.001$) (Fig 5.10 F).

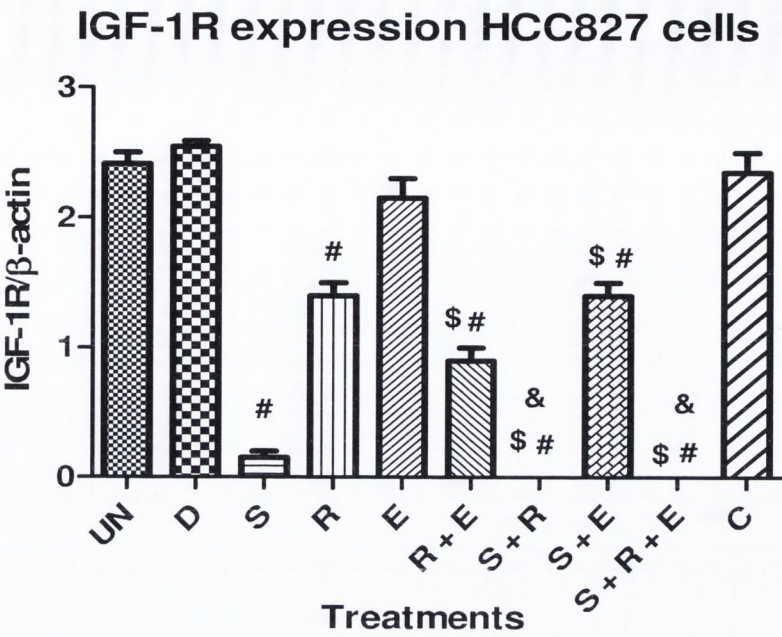
A



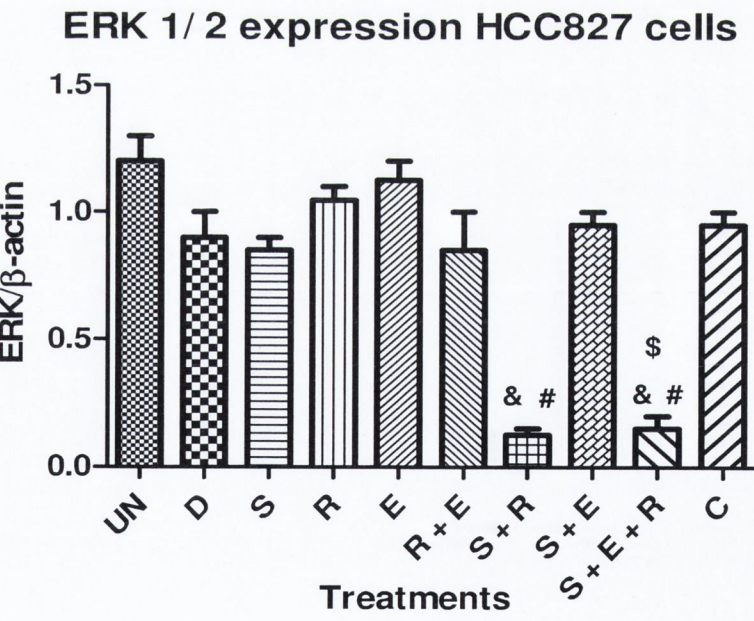
B.



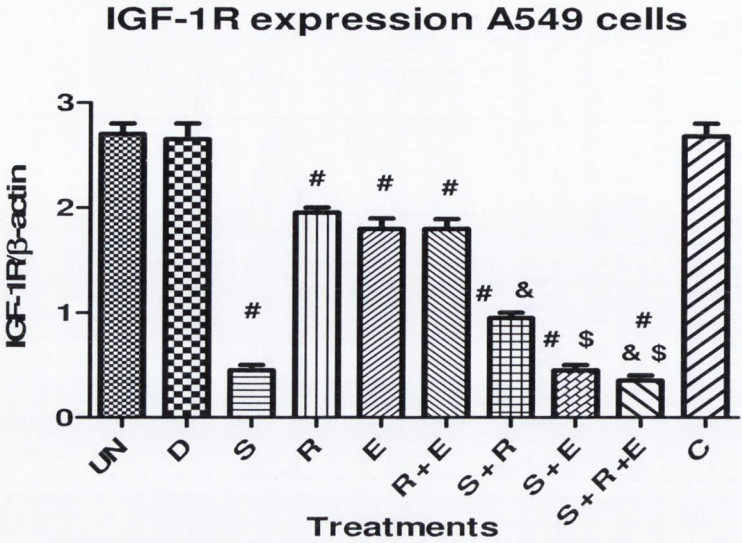
C.



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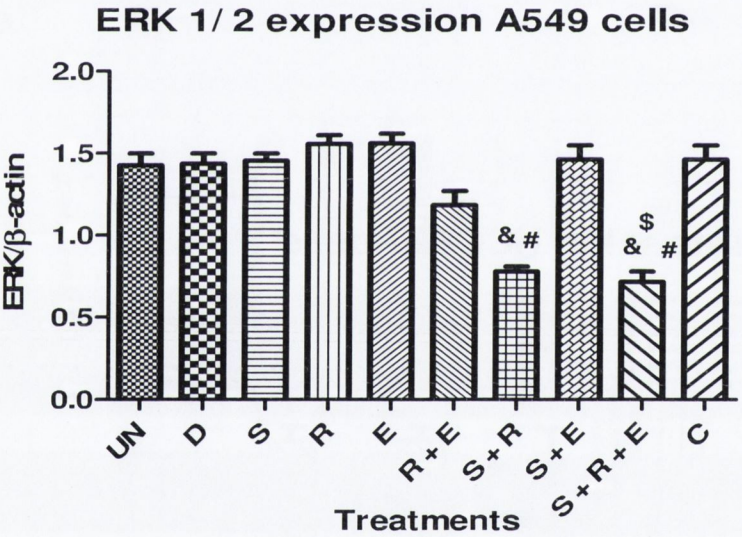


Fig 5.10. Effect of IGF-1R siRNA on IGF-1R and ERK 1/2 protein expression in R1507 and erlotinib treated NSCLC cell lines. Treatment of (A) HCC827 cells and (B) A549 cells with 50nM IGF-1R siRNA for 72 h to examine the effect of IGF-1R knockdown on protein expression using western blot analysis. Densitometry analysis for IGF-1R and ERK protein expression in (C and D) HCC827 cells and (E and F) A549 cells respectively. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.

(UT – untreated, D- DharmaFECT-1, S- siRNA, R- R1507, E- erlotinib, R/E- R1507 + erlotinib, C- scramble control)

(# = significant compared to untreated cells, \$ = significant compared to erlotinib, & = significant compared to R1507)

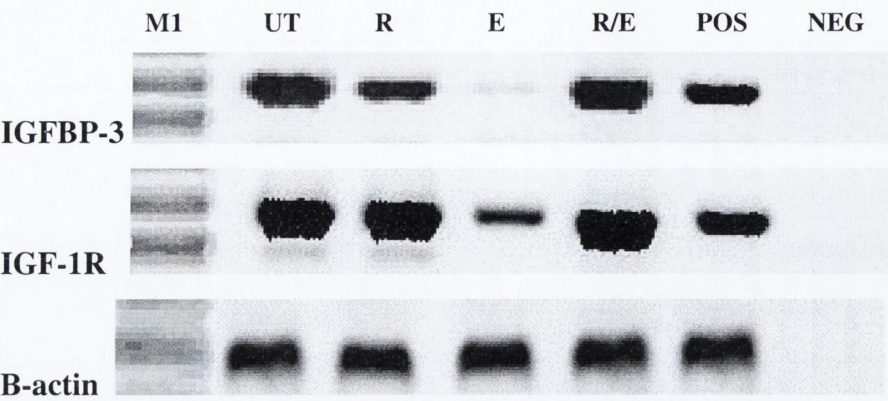
5.1.11 Effect of R1507 and Erlotinib on mRNA expression of IGFBP-3 and IGF-1R in NSCLC cell lines

The effect of an IGF-1R inhibitor R1507 and EGFR inhibitor erlotinib on IGFBP-3 and IGF-1R in HCC827 and A549 cell lines was examined. Cells were seeded at appropriate densities overnight in complete media. The following day when cells reached 80% confluency, R1507 and erlotinib were added to the appropriate wells at concentrations of 25 μ g/ml and 100nM respectively. RNA was isolated from the NSCLC cell lines using TRI Reagent. Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGFBP-3 and IGF-1R mRNA were examined by PCR analysis. A representative gel is shown in Fig 5.11. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software.

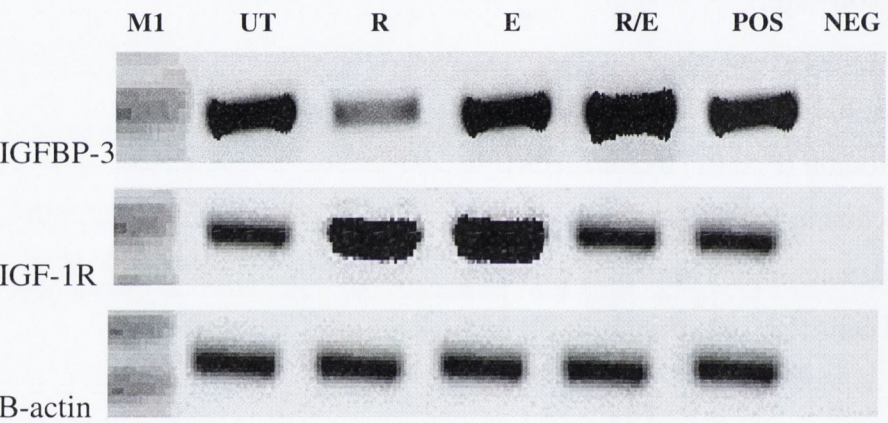
In HCC827 cells, IGFBP-3 mRNA expression was significantly reduced in R1507 ($P<0.05$) and erlotinib ($P<0.01$) treated cells (Fig 5.11 A) (Fig 5.11 C). No significant reduction in IGFBP-3 mRNA expression was found in combined R1507 and erlotinib treatment. IGF-1R mRNA expression was significantly reduced in erlotinib treated cells ($P<0.01$) (Fig 5.11 D).

In A549 cells, IGFBP-3 mRNA expression was significantly reduced in R1507 treated cells ($P<0.01$) while a significant increase in IGF-1R mRNA expression was found in cells treated with R1507 ($P<0.05$) and erlotinib ($P<0.05$) (Fig 5.11 B) (Fig 5.11 E). Combined R1507 and erlotinib treated cells reduced IGF-1R expression compared to A549 cells treated with R1507 or erlotinib alone ($P<0.05$) (Fig 5.11 F).

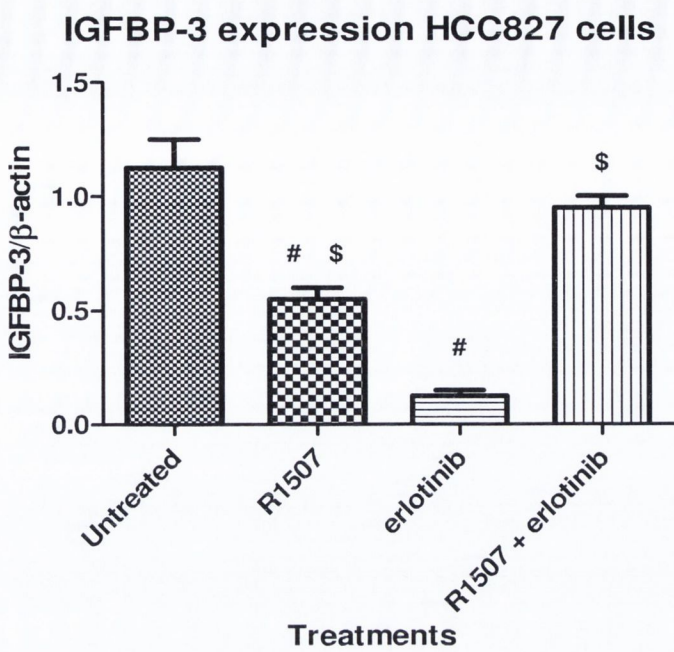
A.



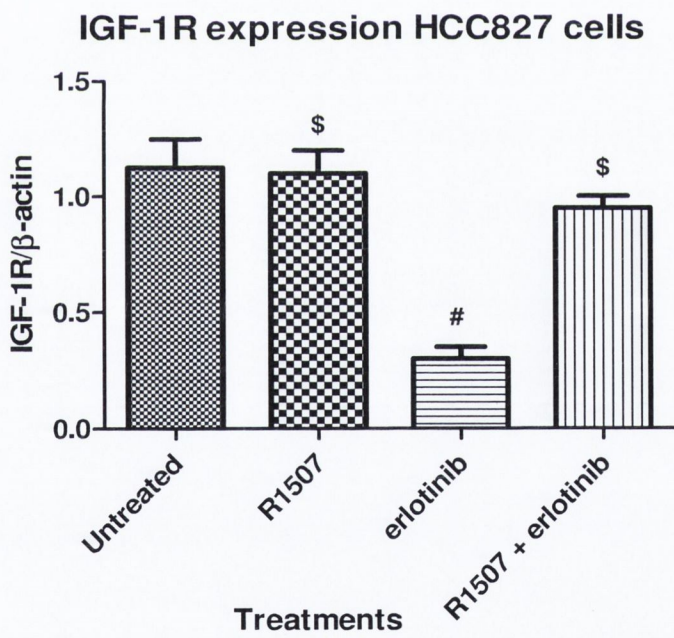
B.



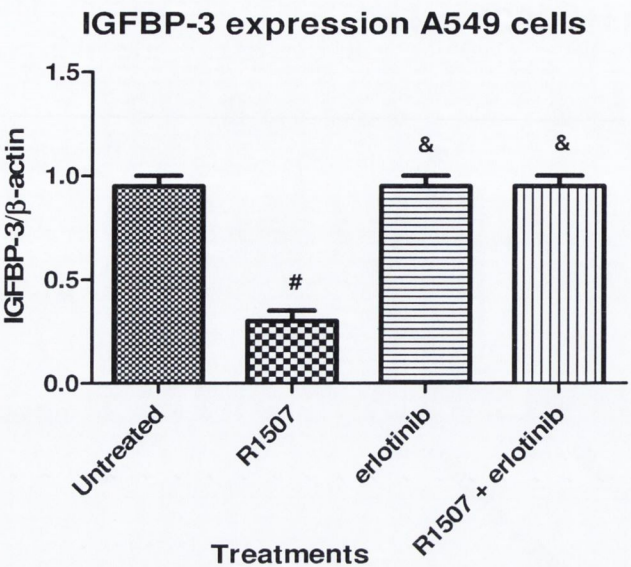
C.



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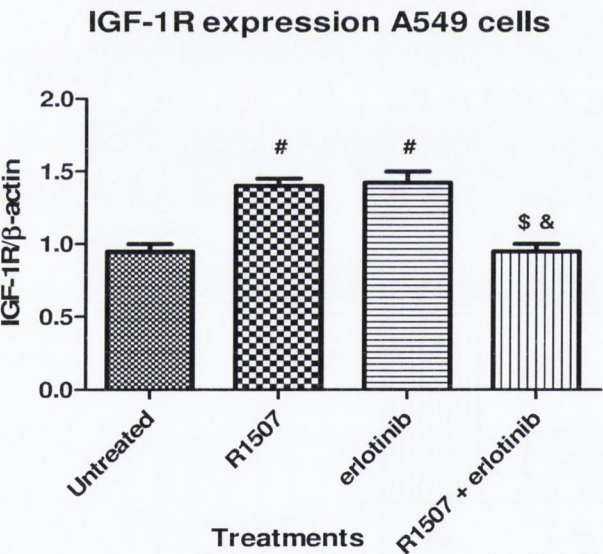


Fig 5.11. Effect of R1507 and erlotinib on IGFBP-3 and IGF-1R mRNA expression in NSCLC cell lines. Treatment of (A) HCC827 cells and (B) A549 cells with 25 μ g/mL R1507 and 100nM erlotinib for 72 h to examine the effect on IGFBP-3 and IGF-1R mRNA expression by RT-PCR. Densitometry analysis for IGFBP-3 and IGF-1R in (C and D) HCC827 cells and (E and F) A549 cells respectively. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA.

(UT- untreated, R- R1507, E- erlotinib, R/E- R1507 and erlotinib)

(# = significant compared to untreated, & = significant compared to R1507, \$ = significant compared to ertlotinib)

5.1.12 Effect of IGF-1R siRNA on IGFBP-3 and IGF-1R mRNA expression in NSCLC cell lines and sensitivity to R1507 and erlotinib

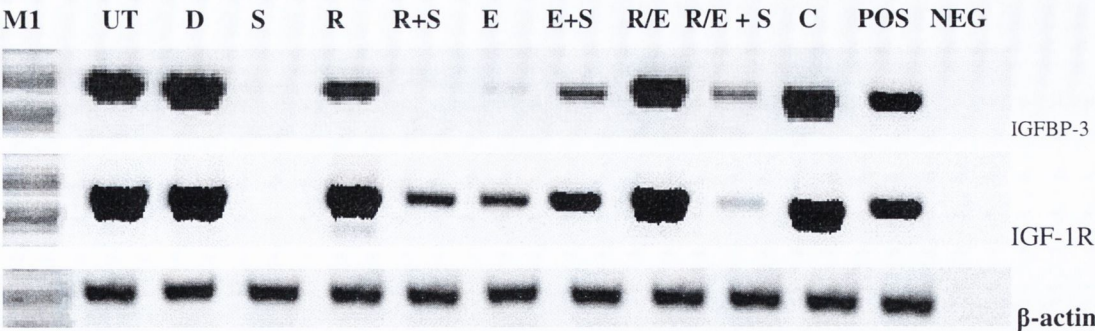
The effect of IGF-1R siRNA on IGFBP-3 and IGF-1R mRNA expression was examined in A549 and HCC827 cell lines treated with R1507 and erlotinib. Cells were transfected with 50nM IGF-1R siRNA and treated with R1507 and erlotinib as described previously in 5.1.10. RNA was isolated from the NSCLC cell lines using TRI Reagent. Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGFBP-3 and IGF-1R mRNA were examined by PCR analysis. A representative gel is shown in Fig 5.12. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using ANOVA.

In HCC827 cells, IGFBP-3 mRNA expression decreased significantly when treated with R1507 ($P<0.001$) and erlotinib ($P<0.001$) compared to untreated cells (Fig 5.12 A). When HCC827 cells were treated with IGF-1R siRNA +/- R1507 there was no IGFBP-3 mRNA expression (Fig 5.12 C). IGFBP-3 mRNA expression was significantly decreased when HCC827 cells were treated with IGF-1R siRNA and R1507 and erlotinib ($P<0.001$) compared to cells treated with R1507 and erlotinib without IGF-1R siRNA. IGF-1R mRNA expression was significantly decreased in IGF-1R siRNA and R1507 treated HCC827 cells ($P<0.001$) compared to cells treated with R1507 alone (Fig 5.12 D). IGF-1R mRNA expression was also significantly reduced in HCC827 cells treated with IGF-1R siRNA and R1507 and erlotinib ($P<0.001$) compared to cells treated with R1507 and erlotinib alone.

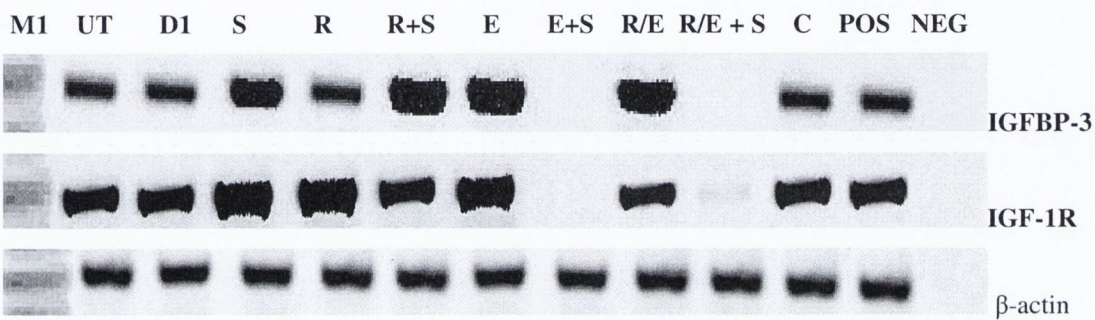
In A549 cells, IGFBP-3 mRNA expression was significantly increased when treated with R1507 and IGF-1R siRNA compared to untreated cells ($P<0.05$) and R1507 treated cells ($P<0.01$) (Fig 5.12 B). There was no IGFBP-3 mRNA expression in A549 cells treated with erlotinib and IGF-1R siRNA compared to cells treated with erlotinib alone (Fig 5.12 E). There was also no IGFBP-3 mRNA expression in A549 cells treated with combined R1507, erlotinib and IGF-1R siRNA compared to cells treated with combined R1507 and erlotinib alone. IGF-1R mRNA expression was significantly decreased in A549 cells treated with combined R1507 and IGF-1R siRNA ($P<0.05$) compared to R1507 treated cells alone (Fig 5.12 F). There was no

IGF-1R mRNA expression in A549 cells treated with erlotinib and IGF-1R siRNA compared to erlotinib alone.

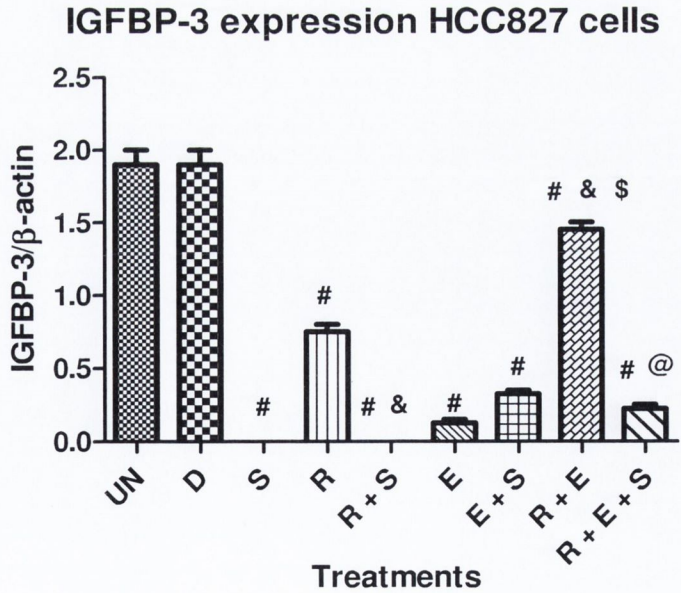
A.



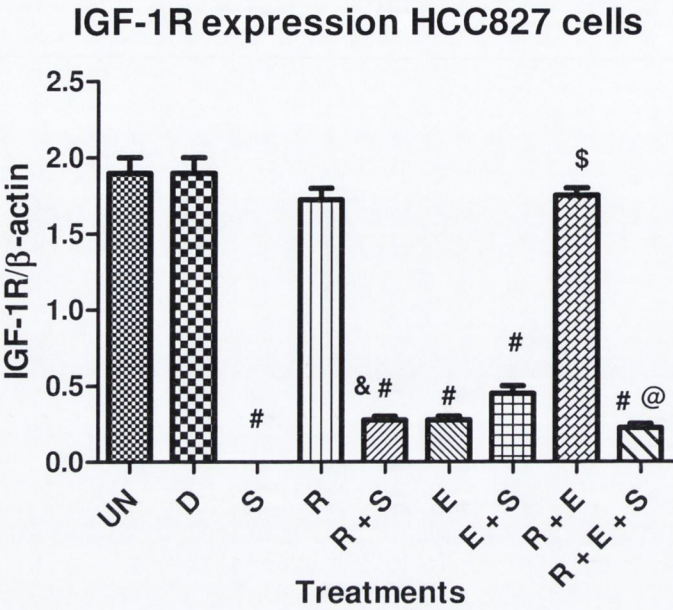
B.



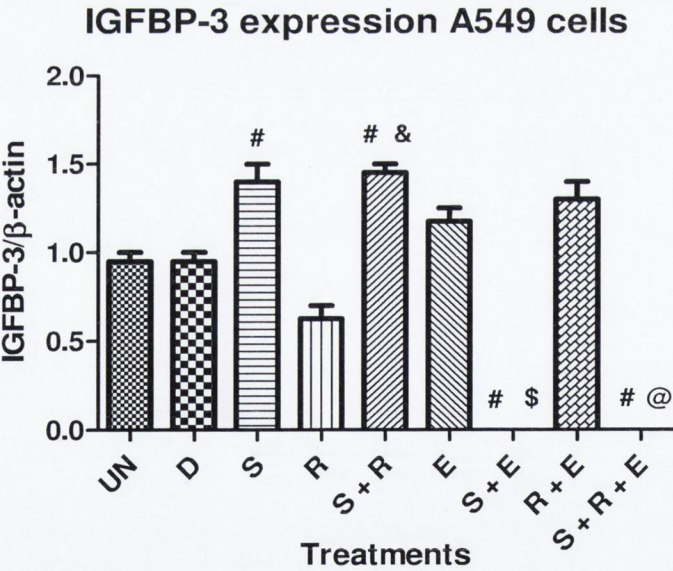
C.



D.



E.



F.

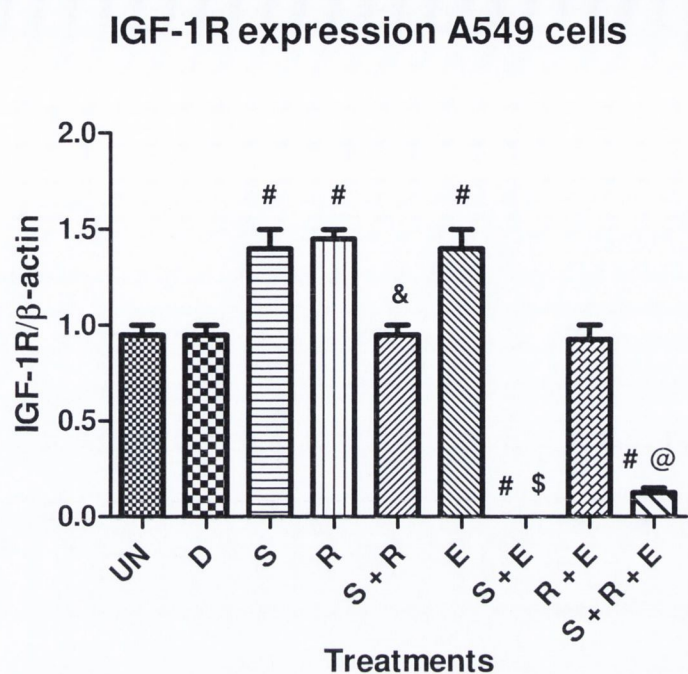


Fig 5.12. Effect of IGF-1R siRNA on R1507 and erlotinib treated NSCLC cell lines. Treatment of (A) HCC827 cells and (B) A549 cells with 50nM IGF-1R siRNA for 72 h to examine the effect of IGF-1R knockdown on mRNA expression using RT-PCR. Densitometry analysis for IGFBP-3 and IGF-1R mRNA expression in (C and D) HCC827 cells and (E and F) A549 cells. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using ANOVA. (UT – untreated, D- DharmaFECT-1, S- siRNA, R- R1507, E- erlotinib, R/E- R1507 + erlotinib, C- scramble control)

(# = significant compared to untreated, \$ = significant compared to erlotinib, & = significant compared to R1507, @ = significant compared to R + E)

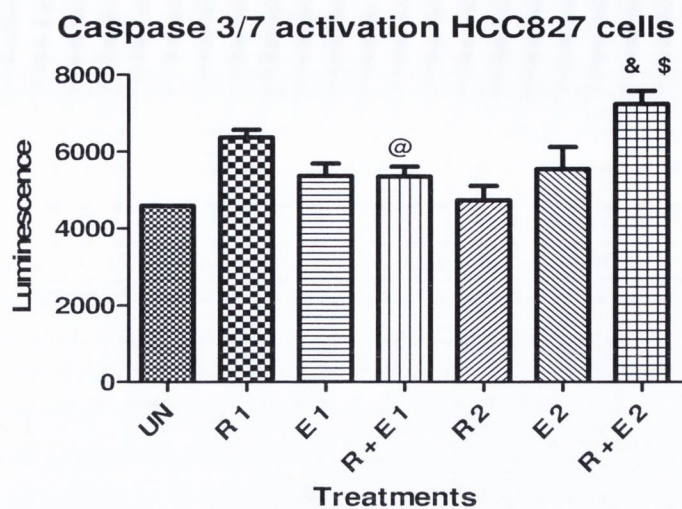
5.1.13 Effect of R1507 and erlotinib on caspase 3/7 activation in NSCLC cells.

The effect of R1507 in combination with erlotinib was examined by measuring caspase 3/7 activation in A549 and HCC827 cells. Activation of caspase 3 and 7 which are early indicators of cell apoptosis within a cell were measured using a ApoLive-Glo™ Multiplex Assay.

Cells were seeded at 4×10^3 /well in an opaque-walled tissue culture plate with solid bottom and left to adhere overnight. The following day, cells were serum starved in media containing 0.5% FBS for 24 h. After 24 h of serum starvation, cells were treated with erlotinib and R1507 with a total volume of 100µL per well and incubated for a further 24 h in the presence of the inhibitors before cell viability and apoptosis were measured. Staurosporine was used as a positive control for apoptosis at a concentration of 10µM for 6 h.

One hundred µL of Caspase-Glo® 3/7 Reagent was added to all wells and mixed briefly by orbital shaking (300–500rpm for ~30 s). The plate was then incubated for 1 h at room temperature and luminescence was measured on a microplate reader. In HCC827 cells, caspase 3/7 activation were significantly increased in cells treated with 25µg/mL R1507 ($P < 0.05$) and cells treated with combined 100µg/mL R1507 and 1000nM erlotinib ($P < 0.01$) (Fig 5.13). In A549 cells, caspase 3/7 activation was significantly increased in cells treated with combined 25µg/mL R1507 and 100nM erlotinib ($P < 0.01$). A549 cells treated with 100 µg/mL R1507 ($P < 0.05$) and 1000nM erlotinib ($P < 0.05$) also had significant increases in caspase 3/7 activation. Combined treatment of 100 µg/mL R1507 and 1000nM erlotinib had the highest significant increase in caspase activation ($P < 0.001$). Statistical analysis was carried out using ANOVA.

A.



B.

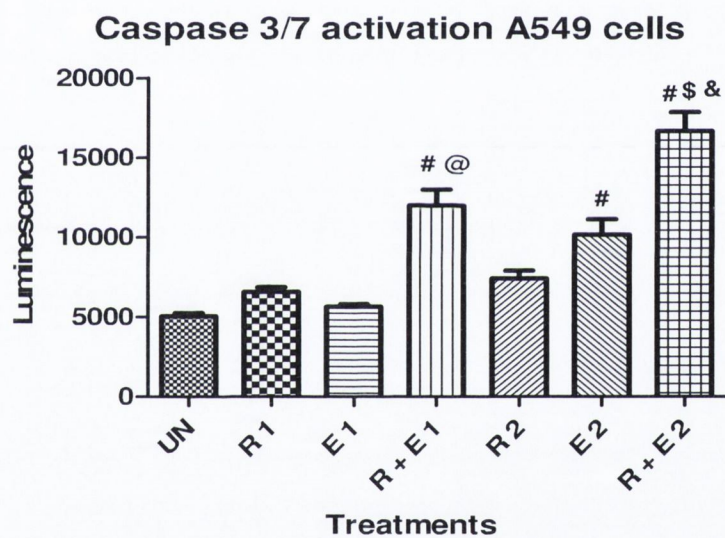


Fig: 5.13 Caspase 3/7 activation in (a) A549 and (b) HCC827 cell lines treated with 25 μ L and 100 μ L R1507 and 100 nM and 1000 nM erlotinib. A549 and HCC827 cells were treated with a higher and lower dose of R1507 and erlotinib, in combination and alone. After 24 h, caspase 3/7 activation was measured by addition of caspase 3/7 Glo Reagent to each well and measurement of luminescence on a microplate reader. Statistical analysis was carried out using ANOVA. (UN- untreated, R 1- 25 μ L R1507, E 1- 100 nM erlotinib, R + E 1- 25 μ L R1507 + 100 nM erlotinib, R 2- 100 μ L R1507, E 2- 1000 nM erlotinib, R + E 2- 100 μ L R1507 + 1000 nM erlotinib) (# = significant compared to untreated, @ = significant compared to R + E2, \$ = significant compared to erlotinib, & = significant compared to R1507)

5.2 Discussion

The aim of this chapter was to examine the effects of R1507 in NSCLC cell lines and also in combination with the EGFR TKI erlotinib. It is not yet known what subset of NSCLC patients would benefit from treatment with the R1507 antibody. As R1507 is still in early clinical trials it is important to determine the biomarkers of response to the antibody. A study by Gong *et al.*, which examined this, showed that NSCLC cell lines with high total IGF-1R expression only had moderate sensitivity to R1507 [311]. Erlotinib (Tarceva) is a TKI that has been used in the clinical setting for a number of years and is the main targeted therapy given to NSCLC patients. The response rate to erlotinib is 60-85% in EGFR mutated patients [298] but resistance usually develops in patients after a few months of treatment [312]. One of the reasons for this is through phosphorylation of IGF-1R which shares downstream signalling molecules with EGFR [94]. Recently studies are focusing on targeting both receptors as a mechanism of overcoming resistance to EGFR TKIs [311] [306].

As EGFR and IGF-1R are two powerful pathways involved in NSCLC targeting both receptors may be an important strategy in preventing signalling through pro-survival pathways. It has been shown that IGF-1R is involved in resistance to EGFR TKIs such as gefitinib as these receptors share common downstream signalling molecules involved in tumourigenesis, proliferation, angiogenesis, and metastasis [283] [313]. PI3K signalling is maintained in the resistant cells through activation of the IGF-1R pathway [94]. EGFR and IGF-1R are over-expressed in many types of cancer including lung [261] [314] and it has been shown that patients who have increased expression of either receptor or both have a poorer prognosis (http://meeting.ascopubs.org/cgi/content/abstract/25/18_suppl/7550). Therefore studies are examining the effects of targeting both receptors in a bid to overcome resistance and increase drug sensitivity.

Two NSCLC cell lines HCC827 and A549 were used in this chapter based on their mutation status and IGF-1R and EGFR expression levels. HCC827 cells have mutated as well as amplified EGFR which makes these cells highly sensitive to erlotinib at nanomolar concentrations [315]. A549 cells contain a KRAS mutation and high IGF-1R expression and are known to be resistant to erlotinib [316].

Firstly, A549 and HCC827 cells were subjected to increasing concentrations of R1507 and erlotinib to examine the effect of dose and time on cell number and proliferation. Both NSCLC cell lines were sensitive to the R1507. In A549 cell line, cell proliferation and cell number decreased with increasing concentrations of R1507 (Fig 5.1). Cell proliferation was significantly decreased with 5 µg/ml treatment ($P<0.05$) whereas cell number was significantly decreased from 10 µg/ml ($P<0.05$) of R1507 (Fig 5.1 A and Fig 5.1 C). In HCC827 cells, there was also a significant decrease in cell number and cell proliferation from concentrations of 10 µg/ml ($P<0.05$) of R1507 and 25 µg/ml ($P<0.05$) respectively (Fig 5.1 B and Fig 5.1 D). In the study by Gong *et al.* which also looked at drug sensitivity to R1507 in NSCLC cell lines, out of 22 cell lines they examined, only 5 cell lines were sensitive to the antibody. Of the sensitive cell lines they found that none of them underwent more than 25-50% growth inhibition [118]. This may be due to the hybridisation which occurs between these two receptors and it is not yet known how targeted anti-IGF-1R therapies are and whether or not they target hybrid receptors. Also R1507 may be more sensitive in *in vivo* studies as studies have focused on the effects of this antibody in cell lines to date.

HCC827 and A549 cell lines were then treated with the selected concentration of 25µg/ml R1507 as cell proliferation was very significantly reduced ($P<0.01$) at this concentration. Cells were treated with R1507 for 24, 48 and 72 h to examine the effects of the antibody at a single concentration over time. In the A549 and HCC827 cells, there was no significant change in cell proliferation at 24 h (Fig 5.2 A and Fig 5.2 B) but a significant decrease was seen at 48 h ($P<0.05$) and 72 h ($P<0.01$) proliferation relative to untreated cells in both cell lines. The results of these experiments were unexpected as A549 cells which have high IGF-1R expression should be more sensitive to treatment with R1507. It has been previously demonstrated by Gong *et al.*, that high expression levels of total IGF-1R may serve as a biomarker for sensitivity to R1507. However these results showed that HCC827 cells were mildly sensitive to the antibody unlike what was observed by Gong *et al.* These experiments suggest that IGF-1R expression alone does not determine response to R1507.

As expected, HCC827 cells were more sensitive to treatment with erlotinib. There was a decrease in cell proliferation ($P<0.01$) and cell number ($P<0.05$) at a

concentration of erlotinib of 100nM (Fig 5.3 B and Fig 5.3 D). No significant decrease in cell number was seen in A549 cells until 1000nM ($P<0.01$) and in cell proliferation 500nM ($P<0.05$) (Fig 5.3 C).

HCC827 and A549 cell lines were then treated with the selected concentration of 100nM erlotinib for 24, 48 and 72 h as this concentration significantly decreased cell proliferation and cell number (Fig 5.4). In A549 cells there was no significant change in cell proliferation over 72 h incubation with 100nM erlotinib (Fig 5.4 A). A higher concentration of erlotinib was not used for A549 cells as the concentration needed would be too high. In HCC827 cells proliferation decreased over time with a significant decrease in cell proliferation at 48 h ($P<0.01$) and 72 h ($P<0.001$) (Fig 5.4 B).

HCC827 and A549 cell lines were treated with the selected concentration of 25 μ g/ml R1507 along with IGF-I and IGF-II for 48 h to examine the ability of these ligands to rescue the cells from the antibody treatment (Fig 5.5). The addition of IGF-I to A549 cells resulted in a significant increase in cell number compared to untreated cells ($P<0.05$) (Fig 5.5 A). When IGF-I was added at the time of treatment with R1507 for 48 h, the inhibitory effect of R1507 on cell growth was reduced. This may be because when the antibody and ligand are added together there is competition for the receptor which may effect antibody binding. IGF-I significantly increased cell growth of R1507 treated A549 cells compared to R1507 treated A549 cells alone ($P<0.01$). IGF-II increased cell number in A549 cells but this was not significant. However IGF-II significantly increased the growth of R1507 treated A549 cells compared to R1507 treated cells alone ($P<0.05$).

The effect of IGF-I and IGF-II stimulation was then examined in A549 cells which had been treated with R1507 for 24 h before addition of IGF-I and IGF-II for a further 48 h. This did not inhibit the effect of R1507 compared to IGF-I and IGF-II added at the time of treatment with R1507 (Fig 5.5 B). IGF-I ($P<0.05$) and IGF-II ($P<0.05$) significantly increased the growth of R1507 treated A549 cells compared to R1507 treated cells alone 24 h after treatment. This demonstrates that when the IGF-1R is targeted with R1507, the receptor is still capable of being stimulated by IGFs and reverses the effects of the antibody.

In HCC827 cells addition of IGF-I also significantly increased cell number compared to untreated cells ($P < 0.001$) (Fig 5.5 C). When IGF-I was added at the time of treatment with R1507 for 48 h, the inhibitory effect of the antibody on cell growth was reduced. IGF-II also increased cell number but this was not significant. IGF-I significantly increased the growth of R1507 treated HCC827 cells ($P < 0.05$) whereas IGF-II did not significantly increase cell growth. The effect of IGF-I and IGF-II stimulation was then examined in HCC827 cells which had been treated with R1507 for 24 h before addition of IGF-I and IGF-II for a further 48 h. This did not inhibit the effect of R1507 compared to IGF-I and IGF-II added at the time of treatment with R1507 (Fig 5.5 D). IGF-I and IGF-II did not significantly increase the growth of R1507 treated HCC827 cells compared to R1507 treated cells alone when added 24 h after treatment. This may be because the inhibitory effect of the antibody is greater in HCC827 cells making IGFs less able to bind to the receptor and stimulate IGF-1R signalling.

HCC827 and A549 cell lines were then treated with the selected concentration of 100 nM erlotinib for 48 h along with the addition of IGF-I and IGF-II to examine the effect of stimulating the IGF-1R on sensitivity to erlotinib (Fig 5.6). In A549 cells, the addition of IGF-I and IGF-II in combination with 100nM erlotinib at 0 h (Fig 5.6 A) and at 24 h (Fig 5.6 B) after treatment with erlotinib increased cell growth however this was not significant when compared to untreated cells or to erlotinib treated cells alone. In HCC827 cells, the addition of IGF-I and IGF-II in combination with 100nM erlotinib at 0 h (Fig 5.6 C) and at 24 h (Fig 5.6 D) after treatment with erlotinib increased cell growth however this was also not significant when compared to untreated cells or to erlotinib treated cells alone.

These results show that IGF-I and IGF-II are able to stimulate IGF-1R activation and maintain cell growth and proliferation even in the presence of specific inhibitors. When IGF-I and IGF-II were added to HCC827 and A549 cells after 24 h treatment with erlotinib and R1507, cells were less sensitive to both inhibitors and cell number was similar to cells treated with IGF-I and IGF-II alone after 48 h. The addition of IGF-I and IGF-II to erlotinib treated cells should mean that the IGF-1R is free for the ligands to bind however cell growth was not increased as much as when each ligand was added alone to the cells. Other factors may be involved here such as the IGFBP-3 which may have bound to the ligands preventing cell growth.

IGF-1 has been shown to be implicated in resistance to EGFR TKI's. It was found that long term addition of IGF-I to a human squamous carcinoma cell line A431 created a phenotype resistant to treatment by EGFR TKIs. This is due to maintenance of Akt signalling by stimulating and activating the IGF-1R pathway. In A549 cells addition of IGF-I had a significant effect on cell number [94]. R1507 has been shown to prevent binding of IGF-1 and the activation of PI3K/AKT survival pathway (<http://www.gistsupport.org/treatments-for-gist/emerging-treatments/insulin-like-growth-factor-pathway-inhibitors/r1507.php>). Preventing binding of IGF-1 to the IGF-1R by treatment with R1507 may prevent resistance to EGFR TKIs such as erlotinib. This may induce apoptosis, decrease cellular proliferation and Akt signalling. It has been demonstrated that treatment with an EGFR TKI and an IGF-IR inhibitor is sufficient to reverse the EGFR resistant phenotype [94].

HCC827 and A549 cell lines were treated with both 25µg/ml R1507 and 100 nM erlotinib for 48 h to examine if treatment with both inhibitors has a greater effect on inhibiting cell growth than treatment with either inhibitor alone. R1507 significantly reduced the growth of A549 cells compared to untreated cells ($P<0.01$) (Fig 5.7 A). Treatment with erlotinib had no effect on A549 cells. When A549 cells were co-treated with R1507 and erlotinib there was significantly reduced cell growth ($P<0.01$) when compared with untreated cells but not when compared to R1507 treated cells alone (Fig 5.7 A). However there was a significant decrease in cell growth when erlotinib treated cells were compared to R1507 treated cells ($P<0.05$) and combined R1507 and erlotinib treated cells ($P<0.01$). R1507 had no effect on the growth of HCC827 cells (Fig 5.7 B). Erlotinib significantly reduced the growth of HCC827 cells ($P<0.01$). When R1507 was added in combination with erlotinib there was no increased reduction in cell growth compared to R1507 treated cells alone. These results indicate that combined R1507 and erlotinib treatment is more effective in decreasing cell growth in A549 cells than treatment with either inhibitor alone.

Treating patients with combined EGFR and IGF-1R therapy immediately after diagnosis may prevent resistance to EGFR TKIs. Results from these experiments and those reported by Gong *et al.* suggest that patients with high IGF-1R and EGFR co-expression may benefit from combined R1507 and erlotinib therapy [118]. Gong *et al.*, found that the combination of R1507 and erlotinib induced apoptosis in the NSCLC cell line 11-18 when treated with 100 or 500 nM erlotinib in the absence or

presence of 25 µg/ml R1507 for 24 h. Results from the discontinued phase II trial (NO21160) investigating the effect of R1507 in combination with erlotinib in advanced lung cancer after first line chemotherapy found that patients with high free serum IGF-1 levels had higher PFS rate at 12 weeks with 16 mg/kg dose of R1507 [317]. Also patients with KRAS mutations were more likely to benefit from R1507 and erlotinib rather than erlotinib alone [317]. However further investigation is needed to identify other biomarkers of sensitivity to R1507.

The effect of IGF-1R siRNA on cell proliferation was examined in A549 cells and HCC827 cells. This was examined at 24 h and 48 h with 25nM and 50nM siRNA. The most significant reduction of cell growth was achieved at 48 h with 50nM siRNA in HCC827 ($P<0.001$) and A549 ($P<0.001$) cells (Fig 5.8 B and Fig 5.8 D). The effect of IGF-1R siRNA in combination with R1507 +/- erlotinib on cell proliferation in A549 and HCC827 cells was then explored. In A549 and HCC827 cells, cell proliferation was significantly reduced in siRNA treated cells alone, $P<0.001$ and $P<0.001$ respectively and in combination with R1507 +/- erlotinib (Fig 5.9 A and Fig 5.9 B). The combination of IGF-1R siRNA and R1507 +/- erlotinib however did not decrease cell proliferation more than treatment with IGF-1R siRNA alone.

The effect of IGF-1R siRNA was then examined at the protein level to examine the effect of R1507 and erlotinib on IGF-1R protein expression and ERK protein expression, the downstream signalling molecule in the MAPK pathway which controls the expression of genes involved in cell proliferation. In HCC827 cells IGF-1R siRNA significantly reduced IGF-1R protein expression compared to untreated cells ($P<0.001$) (Fig 5.10 C). R1507 also decreased IGF-1R protein expression significantly ($P<0.001$). When HCC827 cells were treated with combined R1507 and erlotinib IGF-1R protein expression was further reduced than when cells were treated with either inhibitor alone ($P<0.001$). The combination of siRNA with R1507 +/- erlotinib in HCC827 cells completely eliminated IGF-1R expression. Interestingly IGF-1R siRNA in combination with erlotinib had no effect on IGF-1R protein expression. ERK 1/2 expression was also significantly reduced in HCC827 cells treated with siRNA and R1507 ($P<0.001$) and siRNA with R1507 +/- erlotinib, ($P<0.001$) (Fig 5.10 D).

In A549 cells, IGF-1R siRNA also significantly reduced IGF-1R protein expression compared to untreated cells ($P<0.001$) (Fig 5.10 E). R1507 significantly decreased IGF-1R protein expression compared to untreated cells ($P<0.01$). IGF-1R protein expression was also significantly reduced in IGF-1R siRNA treated cells with either erlotinib ($P<0.001$) or R1507 ($P<0.001$) or combined erlotinib and R1507 ($P<0.001$). Like HCC827 cells, ERK 1/2 expression was reduced in siRNA treated cells with R1507 alone ($P<0.01$) and combined R1507 and erlotinib in A549 cells ($P<0.001$) (Fig 5.10 F). The effect of IGF-1R siRNA on reducing ERK 1/2 expression in A549 and HCC827 cells has not been reported before in the literature. The study by Gong *et al.*, found there was no change in ERK 1/2 expression in 11-18 cells treated with IGF-1R siRNA. REF. The effect of IGF-1R knockdown on reducing total ERK 1/2 expression needs to be examined in additional NSCLC cell lines and also compared to phosphor ERK 1/2 expression in the same cell lines. These results suggest that the optimum way of targeting the IGF-1R and reducing cell proliferation is with using combined IGF-1R siRNA and R1507 treatment. The effects of combined IGF-1R siRNA and R1507 treatment on downstream signalling molecules such as Akt expression need to be investigated.

The effect of R1507 and erlotinib on IGFBP-3 and IGF-1R mRNA expression in HCC827 and A549 cell lines was examined. IGFBP-3 is the most abundant IGFBP in the body and controls activation of the IGF-1R through the binding of IGF-I. It has been reported that loss of IGFBP-3 expression is associated with resistance to EGFR TKIs [94]. In HCC827 cells, IGFBP-3 mRNA expression was significantly reduced in R1507 ($P<0.05$) and erlotinib ($P<0.01$) treated cells (Fig 5.11 A) whereas no significant reduction in IGFBP-3 mRNA expression was found in combined R1507 and erlotinib treatment. The effect of combined R1507 and erlotinib treatment on IGFBP-3 expression has not been reported in the literature. As reduced expression of IGFBP-3 is associated with poorer survival in patients REF, combined treatment with R1507 and erlotinib may prevent loss of expression of IGFBP-3 and increase survival in patients following dual targeted therapy and may also prevent resistance to erlotinib. This would need to be examined further in additional NSCLC cell lines. IGF-1R mRNA expression was significantly reduced in erlotinib treated cells ($P<0.01$). This reduced IGF-1R mRNA expression was not seen at the protein level where IGF-1R protein expression in erlotinib treated HCC827 cells was not significantly reduced (Fig 5.10 A). The reduced IGF-1R expression at the mRNA

level and not the protein level following treatment with erlotinib may be due to post-transcriptional mechanisms which would need to be investigated further as this has not been reported before.

In A549 cells, IGFBP-3 mRNA expression was significantly reduced in R1507 treated cells ($P<0.01$) while a significant increase in IGF-1R mRNA expression was found in cells treated with R1507 ($P<0.05$) and erlotinib ($P<0.05$) (Fig 5.11 E and Fig 5.11 F). Decreased IGFBP-3 expression would mean IGF-I is free to bind to the IGF-1R leading to increased IGF-1R expression. This would explain why an increase in IGF-1R mRNA was observed in A549 cells and suggests that IGFBP-3 expression levels may interfere with the effect of the R1507 inhibitor. No decrease was found in IGF-1R expression in R1507 treated A549 cells at the protein level also (Fig 5.10 B). This would need to be investigated further with higher concentrations of R1507. Combined R1507 and erlotinib treated cells reduced IGF-1R expression compared to treatment with either inhibitor alone ($P<0.05$).

It has been demonstrated in previous studies that IGF-1R mRNA and protein expression levels do not always correlate. A study by Chong *et al.* observed different results when comparing IGF-1R expression levels in matched normal and tumour breast tissue by RT-PCR and immunohistochemistry and concluded that there was no positive correlation between the results for IGF-1R mRNA and protein expression [318]. This may be due to post-translational modifications within the cell. Different results for IGF-1R expression using various scientific methods have often been observed in the literature. This has raised questions as to whether IGF-1R is a potential prognostic biomarker. In a study by Happerfields *et al.* they hypothesised that the IGF-1R may be altered between the cytosol and the cell membrane with the cytoplasmic IGF-1R representing bound, internalised receptors or else other signalling pathways may effect IGF-1R protein levels [319].

The effect of IGF-1R siRNA was examined in A549 and HCC827 cell lines treated with R1507 and erlotinib and IGFBP-3 and IGF-1R mRNA expression was measured (Fig 5.12). In HCC827 cells, IGFBP-3 mRNA expression decreased significantly when treated with R1507 ($P<0.001$) and erlotinib ($P<0.001$) compared to untreated cells (Fig 5.12 A). When HCC827 cells were treated with IGF-1R siRNA +/- R1507 there was no IGFBP-3 mRNA expression (Fig 5.12 C). IGF-1R siRNA expression

was significantly decreased when HCC827 cells were treated with IGF-1R siRNA and R1507 and erlotinib ($P<0.001$) compared to cells treated with R1507 and erlotinib without IGF-1R siRNA. The results of IGF-1R mRNA expression in HCC827 cells were similar to what was seen at the protein level. There was no IGF-1R mRNA expression in siRNA treated HCC827 cells. IGF-1R mRNA expression was significantly decreased in IGF-1R siRNA and R1507 treated HCC827 cells ($P<0.001$) compared to cells treated with R1507 alone (Fig 5.12 D). IGF-1R mRNA expression was also significantly reduced in HCC827 cells treated with IGF-1R siRNA and R1507 and erlotinib ($P<0.001$) compared to cells treated with R1507 and erlotinib alone. Treatment of HCC827 cells with R1507 and erlotinib did not significantly decrease IGF-1R mRNA expression compared to treatment with either R1507 or erlotinib alone which was seen at the protein level. A decrease in IGF-1R expression using IGF-1R siRNA alone and in combination with R1507 would allow free IGF-1 to bind to IGFBP-3. However as a decrease in IGFBP-3 expression was also observed, free IGF-1 would then be degraded with the cell.

In A549 cells, IGFBP-3 mRNA expression was significantly increased when treated with IGF-1R siRNA alone and in combination with R1507 compared to untreated cells ($P<0.05$) and R1507 treated cells ($P<0.01$) (Fig 5.12 B). This is the opposite of what was observed in HCC827 cells. There was no IGFBP-3 mRNA expression in A549 cells treated with erlotinib and IGF-1R siRNA compared to cells treated with erlotinib alone (Fig 5.12 E). There was also no IGFBP-3 mRNA expression in A549 cells treated with combined R1507, erlotinib and IGF-1R siRNA compared to cells treated with combined R1507 and erlotinib alone. There were similar results observed for IGF-1R mRNA and protein expression in A549 cells as IGF-1R mRNA expression was significantly decreased in A549 cells treated with combined R1507 and IGF-1R siRNA ($P<0.05$) with or without erlotinib compared to treatment with R1507 or erlotinib alone (Fig 5.12 F). There was no IGF-1R mRNA expression in A549 cells treated with erlotinib and IGF-1R siRNA. IGF-1R mRNA expression was significantly reduced in A549 cells treated with R1507, erlotinib and siRNA compared to cells treated with R1507 and erlotinib ($P<0.001$). The effect of IGF-1R siRNA in combination with R1507 and erlotinib on IGFBP-3 and IGF-1R expression would need to be investigated further by examining expression using real-time PCR. Also the expression of molecules such as AKT and ERK need to be examined to determine the effect of IGF-1R blockade on these downstream pathways.

The effect of R1507 on apoptosis in combination with erlotinib was examined by measuring caspase 3/7 activation in A549 and HCC827 cells to investigate if apoptosis is induced when both receptors are targeted. In HCC827 cells, caspase 3/7 activation were significantly increased in cells treated with 25 μ g/mL R1507 ($P<0.05$) and cells treated with combined 100 μ g/mL R1507 and 1000nM erlotinib ($P<0.01$) (Fig 5.13). In A549 cells, caspase 3/7 activation was significantly increased in cells treated with combined 25 μ g/mL R1507 and 100nM erlotinib ($P<0.01$). A549 cells treated with 100 μ g/mL R1507 ($P<0.05$) and 1000nM erlotinib ($P<0.05$) also had significant increases in caspase 3/7 activation. Combined treatment of 100 μ g/mL R1507 and 1000nM erlotinib had the highest significant increase in caspase activation ($P<0.001$).

The results observed in this chapter suggest that IGF-1R and EGFR expression levels or mutation status are not indicators of sensitivity to R1507 in NSCLC cells as A549 and HCC827 cell lines did not display high sensitivity to the antibody. However A549 cells which have high IGF-1R protein expression and contain a KRAS mutation were more sensitive than HCC827 cells with low IGF-1R expression and an EGFR mutation. The results of the apoptosis assay correlate with what was previously seen in the proliferation assay Fig 5.7 where combined treatment of 25 μ g/mL R1507 and 100nM erlotinib in HCC827 cells with did not decrease growth further. However IGF-1R protein expression was significantly reduced ($P<0.001$) at concentrations of combined 25 μ g/mL R1507 and 100nM erlotinib in HCC827 cells. Only high concentrations of combined 100 μ g/mL R1507 and 1000nM erlotinib increased caspase 3/7 activation more than either drug alone. The apoptosis assay and the BrdU assay (Fig 5.2) confirms that HCC827 cells are sensitive to treatment with R1507 which has not been shown before in the literature. This suggests that patients with low IGF-1R expression should not be excluded from treatment with the IGF-1R targeting antibody R1507. Results in this chapter confirm that NSCLC patients may benefit from combined R1507 and erlotinib treatment. Further investigation is needed to determine what biomarkers predict response to R1507 in NSCLC cell lines and if dual targeting of the IGF-1R and EGFR with R1507 and erlotinib treatment may prevent resistance to erlotinib in NSCLC patients. However results from this chapter indicate that the additional inhibition of IGF-1R by either siRNA or R1507 treatment augments the proapoptotic effects of EGFR inhibition in

NSCLC cells. This would need to be investigated further in more NSCLC cell lines to demonstrate that inhibiting IGF-IR expression is a promising strategy to prevent resistance and thus enhance the effect of EGFR inhibition. More pre-clinical investigations are needed to determine the effect of R1507 in NSCLC alone and in combination with erlotinib to help identify what subset of patients will benefit from this targeted therapy in the clinical trial stages.

Chapter 6:
The Regulation of IGF-1R pathway
genes by hypoxia

6.0 Introduction

Hypoxia in the microenvironment of large solid tumours leads to activation of the IGF-1R signalling pathway. Induced expression of IGF-1R promotes survival of cancer cells under hypoxic stress [110]. IGFs and insulin responded to hypoxia by activation of Akt and MAPK signalling, resulting in stabilisation of hypoxia-inducible factors HIF-1 α and HIF-2 α , and upregulation of vascular endothelial growth factor [111]. HIF-1 α is necessary for maximum expression of genes encoding IGF-2, IGFBP-2 and IGFBP-3 indicating that cross-talk between the HIF-1 and IGF pathway exists [232].

The IGFBP family is made up of seven members, IGFBP-1 to IGFBP-7, which transport IGF-I and IGF-II and regulate their availability and activity [145]. IGFBPs sequester IGFs away from IGF-1R, inhibiting IGF-stimulated events such as mitogenesis, differentiation and survival. Proteolysis can break down IGFBPs and reverse this inhibition to create IGFBPs fragments with novel functions [158]. IGFBPs regulate cell functions by various mechanisms and have been shown to exert antiproliferative, proapoptotic, antiangiogenic, and antimetastatic effects in different cancer cells [143].

IGFBPs can function through IGF-dependent or IGF-independent mechanisms *in vitro* resulting in either anti-apoptotic or pro-apoptotic events [162] [143]. Through IGF dependent mechanisms, IGFBPs can control cell proliferation by regulating binding of IGFs to the IGF-1R. IGFBPs are involved in transporting IGFs, protecting IGFs from proteolytic degradation therefore increasing their bioavailability in local tissue. Post-translational modification of IGFBPs by phosphorylation and proteolysis can influence the effect of IGFBPs on IGFs. The binding affinity of IGFBPs to IGFs may also be effected by their relationship with the cell membrane or extracellular matrix. IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-5 have been found to regulate IGFs by either suppressing or enhancing their actions [143]. Proteolysis of IGFBP-2, IGFBP-3, and IGFBP-5 effects the regulation of IGFs, while phosphorylation controls the effect of IGFBP-1 on IGFs. IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-5 also bind to specific cell membrane receptors or attach to the cell surface. This reduces their binding affinities for IGFs and results in the release of free IGFs.

IGFBP-5 binding affinity for IGFs reduces when IGFBP-5 is associated with the extracellular matrix [143].

IGFBP-1, IGFBP-2, and IGFBP-5 have been reported to have different effects on cell adhesion and migration of cancer cells. Through complex signalling events they either stimulate or block cell adhesion and migration by IGF-independent mechanisms [158] [320]. Cell surface or intracellular protein interactions, nuclear translocation and transcriptional regulation of IGFBPs are also influenced by the IGF-independent antitumour activities [321] [322] [165] [163] [164]. The biochemical/molecular mechanisms mediating IGFBPs IGF independent antitumour activity have been extensively studied but have not yet been fully elucidated.

IGFBP-3 is the most abundant binding protein in the circulation and has been the focus of the majority of studies. IGFBP-3 inhibits cell proliferation and promotes apoptosis by binding free IGFs in the circulation [323]. Although it is known that IGFBP-3 plays a significant role in cell proliferation in cancer cells, IGFBP-3 expression levels and its role in NSCLC needs to be clearly defined.

Studies using tumour cell xenografts demonstrated growth inhibition by overexpressing IGFBP-3 in NSCLC and prostate cancer cell lines [169] [171] [170]. However there are conflicting reports as IGFBP-3 has been shown to have growth stimulatory effects in other studies. In a study by Martin *et al.* IGFBP-3 stimulated growth in MCF-10A human breast epithelial cells via increased EGFR phosphorylation and activation of p44/42 and p38 MAPK signalling pathways [324].

There is evidence that IGFBP-3 may serve as a biomarker for predicting risk of cancers such as lung, breast and colon cancer. High plasma levels of IGFBP-3 have been found to be associated with a reduced risk of cancer [174]. However, other studies have found no correlation between serum levels of IGFBP-3 and cancer risk [176] [175]. A high level of serum IGFBP-3 has been shown to have a beneficial effect in protecting against cancers such as prostate and lung cancers [173] [325].

IGFBP-3 is frequently overexpressed in esophageal cancer along with overexpression of EGFR [153] [177]. Takaoka *et al.* found that EGFR tyrosine kinase activity was involved in regulating IGFBP-3 expression *in vitro* but in some

esophageal cancer cell lines EGF inhibited IGFBP-3 expression. This EGF-mediated inhibition of IGFBP-3 occurs through the Ras-MAPK signalling pathway which results in activation of IGF-IR due to increased IGF-I in human esophageal cell lines [161]. Therefore, EGF inhibition of IGFBP-3 may be involved in positively regulating the IGF signalling pathway. It has been reported that promoter hypermethylation of IGFBP-3 leads to silencing of IGFBP-3 expression epigenetically which is associated with poor prognosis in NSCLC [179].

This suppression of IGFBP-3 due to DNA methylation in NSCLC is shown to be restored with 5'-aza deoxycytidine, a DNA methyltransferase inhibitor [326] [180].

Guix *et al.* demonstrated that the mRNA expression levels of IGFBP-3 and IGFBP-4, were reduced in gefitinib resistant (GR) cells [94]. When the GR cells were incubated with recombinant IGFBP-3 and gefitinib, Akt signalling was reduced and cell proliferation was inhibited. When gefitinib was combined with an anti-IGF-IR antibody in nude mice, this was also successful in inhibiting tumour growth.

Therefore it was concluded that resistance to EGFR-targeted TKIs may arise from loss of IGFBPs and activation of IGF-IR signalling and that dual inhibition of EGFR and IGF-IR would benefit cancers characterized by overexpression of EGFR. The GR cells were also found to be resistant to the EGFR TKI erlotinib and the EGFR-targeted monoclonal antibody cetuximab, hence the loss of IGFBPs may be involved in resistance to other ErbB-targeting TKIs and antibodies.

Not much is known about the regulation and expression of IGFBPs in NSCLC under hypoxia. The IGFBP family members IGFBP-1 and IGFBP-3 were shown to be induced by hypoxia in vascular endothelial cells, squamous cell carcinoma cell and lung carcinoma cell lines [327] [328] [234]. IGFBP-3 was shown to inhibit IGF signalling under extreme hypoxic conditions in pancreatic cancer cells [236]. Proteins in the conditioned medium of the human pancreatic cancer cell line, AsPC-1, which is resistant to hypoxia were analysed. It was found that some IGFBPs were increased in culture medium under hypoxic conditions, and that IGFBPs play an important role in cell survival by buffering IGF signalling under severely low oxygen levels [236]. Despite a decrease in protein synthesis under hypoxic conditions, IGFBP-1 and IGFBP-3 protein expression increased which was also seen in the mRNA levels of IGFBP-1 and IGFBP-3. IGFBP-4 and IGFBP-6 were expressed in the AsPC-1 cells but expression remained unchanged under hypoxia.

Hypoxia-induced genes could be potential targets for therapeutic intervention in cancers. Many studies have provided evidence for the *in vitro* induction of IGFBP-3 mRNA by hypoxia in different cell types [233]. IGFBP-3 was transcriptionally up-regulated during hypoxia. When induced, there was no significant increase in apoptosis, which makes the function of this gene during hypoxia unclear. The induction of IGFBP-3 under hypoxia may be a protective effect as it results in a decrease in IGF-1R signalling and cell proliferation so that hypoxic cells conserve energy and avoid apoptosis.

Many studies have examined the role of IGFBP-3 in cancer however the role of other members of the IGFBP family are not well characterised. Studies have shown that IGFBP-6 is able to induce apoptosis in cancer cells including NSCLC [190]. NSCLC cell lines infected with an adenovirus expressing human IGFBP-6 under the control of a CMV promoter (Ad5CMV-BP6) reduced NSCLC cell number through activation of programmed cell death. The growth regulatory effect of IGFBP-6 was also investigated *in vivo* by intratumoral injection of Ad5CMV-BP6 in NSCLC xenografts established in nu/nu mice [189]. IGFBP-5 has also been shown to act both as a tumour suppressor and as an oncogene by promoting metastasis when overexpressed in ovarian cancer [184], breast cancer [329] and head and neck squamous cell carcinoma cell lines (HNSCC) [330].

The aims of this chapter is to examine the expression levels of IGFBPs in a panel of normal and lung cancer cell lines in response to (a) hypoxia, (b) IGF-1R knockdown (c) IGF-1R, EGFR and DAC inhibitors, and (d) expression in a number of normal/tumour matched patient samples. Together this will elucidate the functional role of IGFBPs in response to hypoxia and IGF-1R/ EGFR expression as well as their involvement in IGF-1R/EGFR crosstalk.

6.1 Results

6.1.1 Expression of IGFBP1-6 in a panel of cell lines

IGFBP1-6 mRNA expression was examined in a panel of normal human bronchial epithelial cell lines (HBEC3, 4 and 5) and lung cancer cell lines- A549, HCC827 and H1819 (adenocarcinoma), SKMES-1 (squamous cell carcinoma) and H1299 (large cell carcinoma).

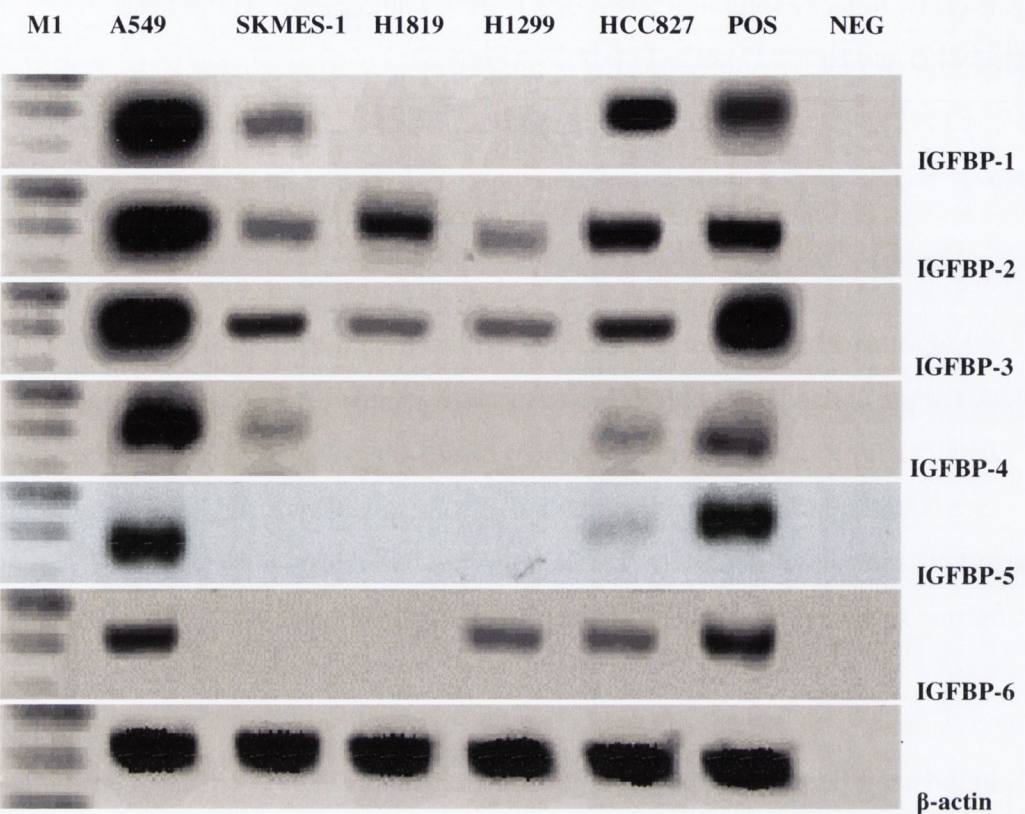
Total RNA was isolated from cells, which had been serum starved overnight in media containing 0.5% FBS (lung cancer cells only), and had reached approximately 80% confluency. cDNA was synthesised by reverse transcription of the isolated RNA samples. mRNA expression was then examined by PCR analysis of these samples. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using a two tailed student t-test.

Expression of IGFBP-1 was present in three out of five NSCLC cell lines but not in HBEC cell lines (Fig 6.1). A549 cells had significantly higher expression of IGFBP-1 compared to SKMES-1 ($P=0.0010$) and HCC827 ($P=0.0068$) (Fig 6.2). IGFBP-2 was expressed in all NSCLC and HBEC cell lines. A549 cells had significantly higher expression compared to SKMES-1 ($P=0.0229$), H1819 ($P=0.0155$) and H1299 ($P=0.0094$) but not HCC827 cells ($P=0.0520$) (Fig 6.2). HBEC 3 had significantly higher IGFBP-2 expression compared to HBEC 4 ($P=0.0125$) and HBEC 5 ($P=0.0499$) (Fig 6.3). IGFBP-3 was expressed in all NSCLC and HBEC cell lines. A549 cells again had significantly higher expression of IGFBP-3 compared to SKMES-1 ($P=0.0128$), H1819 ($P=0.0075$), H1299 ($P=0.0080$) and HCC827 cells ($P=0.0213$) (Fig 6.2).

There was no significant difference in IGFBP-3 expression between the HBEC cell lines (Fig 6.3). The expression of IGFBP-4 was identified in three out of five NSCLC cell lines but not in HBEC cell lines. Expression of IGFBP-4 was significantly higher in A549 cells compared to SKMES-1 cells ($P=0.0022$) and HCC827 cells ($P=0.0040$) (Fig 6.2). IGFBP-5 was expressed in two NSCLC cell lines and one HBEC cell line. A549 cells had significantly higher expression of IGFBP-5 compared to HCC827 cell ($P=0.0018$) (Fig 6.2). Expression of IGFBP-6

was observed in three NSCLC cell lines and one HBEC cell line. A549 cells had significantly higher expression levels of IGFBP-6 compared to H1299 cells ($P=0.0212$) and HCC827 cells ($P=0.0023$) (Fig 6.2).

(A)



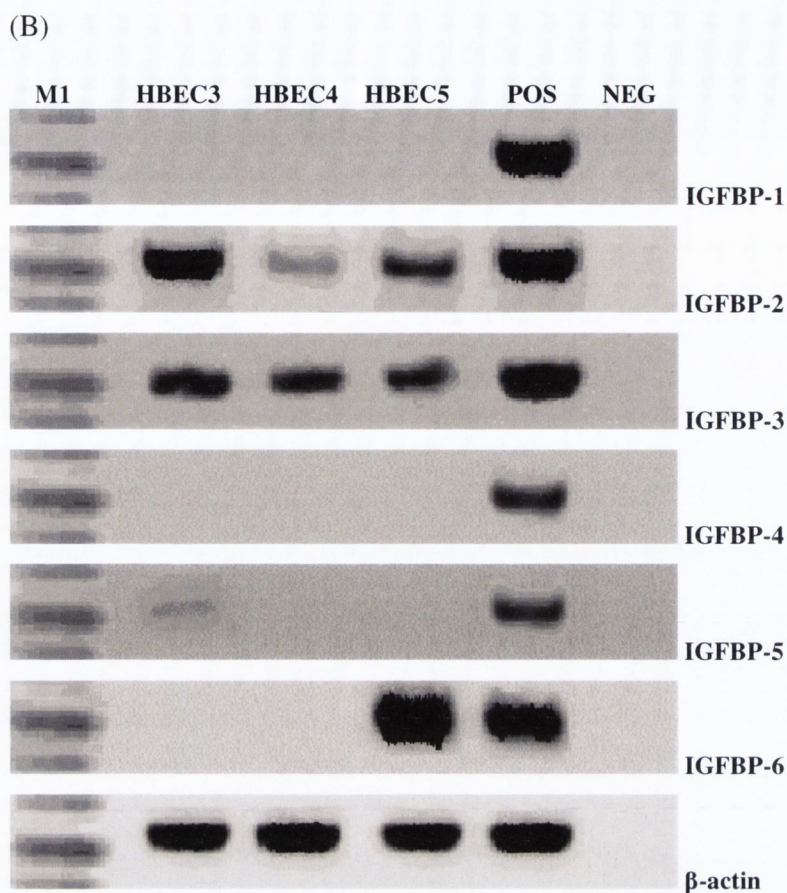


Fig 6.1: Expression of IGFBP1-6 mRNA in a panel of (A) lung cancer and (B) normal bronchial epithelial cell lines. This image is representative of three independent experiments. A PCR was carried out for β -actin in all samples to determine loading efficiency. A 1Kb-plus DNA ladder was loaded in lane 1 (M). A negative sample was set-up with sterile water to control for contamination (NEG). A positive sample was also set-up using Hep3B cDNA to control for positive expression (POS). PCR reactions were loaded in the remaining lanes.

(IGFBP-1 -272bp, IGFBP-2 - 518bp, IGFBP-3 - 434bp, IGFBP-4 – 239bp, IGFBP-5 - 403bp, IGFBP-6 - 345bp, β -actin 294bp)

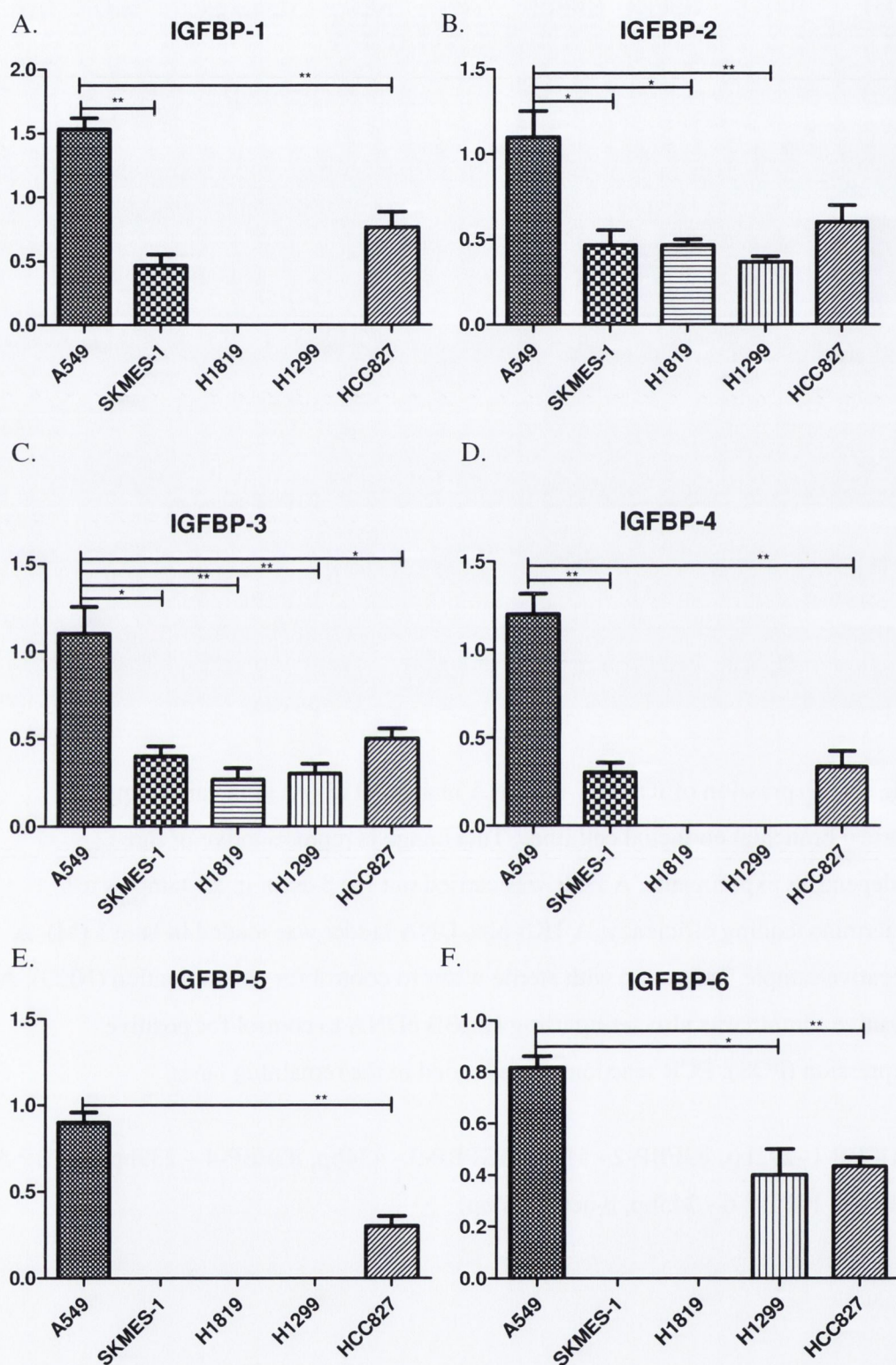


Fig 6.2 Densitometry results of IGFBP1-6 mRNA in NSCLC cells lines. (A) IGFBP-1, (B) IGFBP-2, (C) IGFBP-3, (D) IGFBP-4, (E) IGFBP-5 and (F) IGFBP-6. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test.

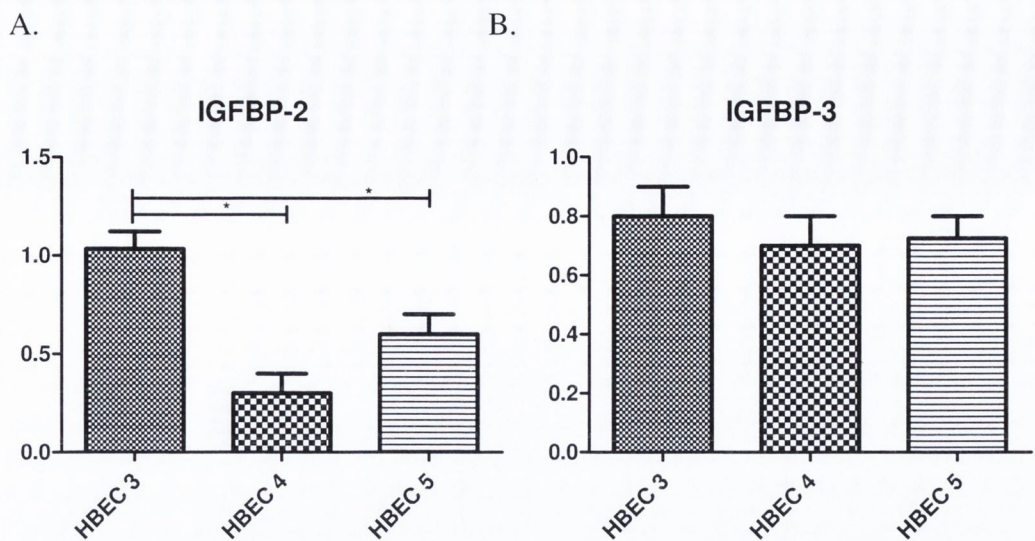


Fig 6.3 Densitometry results of IGFBP-2 and IGFBP-3 mRNA in HBEC cells lines. (A) IGFBP-2 and (B) IGFBP-3 (only significantly different results shown). Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test.

6.1.2 Effect of hypoxia on IGFBP1-6 mRNA expression

To examine whether IGFBP1-6 mRNA expression was changed under hypoxic (0.5% O₂) conditions, cells were seeded at 1×10^6 /T75 cm³ flask and left to adhere overnight. The subsequent morning, cells were incubated in normoxia or hypoxia for 4 h. The lung cancer cells were serum starved overnight in media containing 0.5% FBS prior to insertion into the hypoxia chamber.

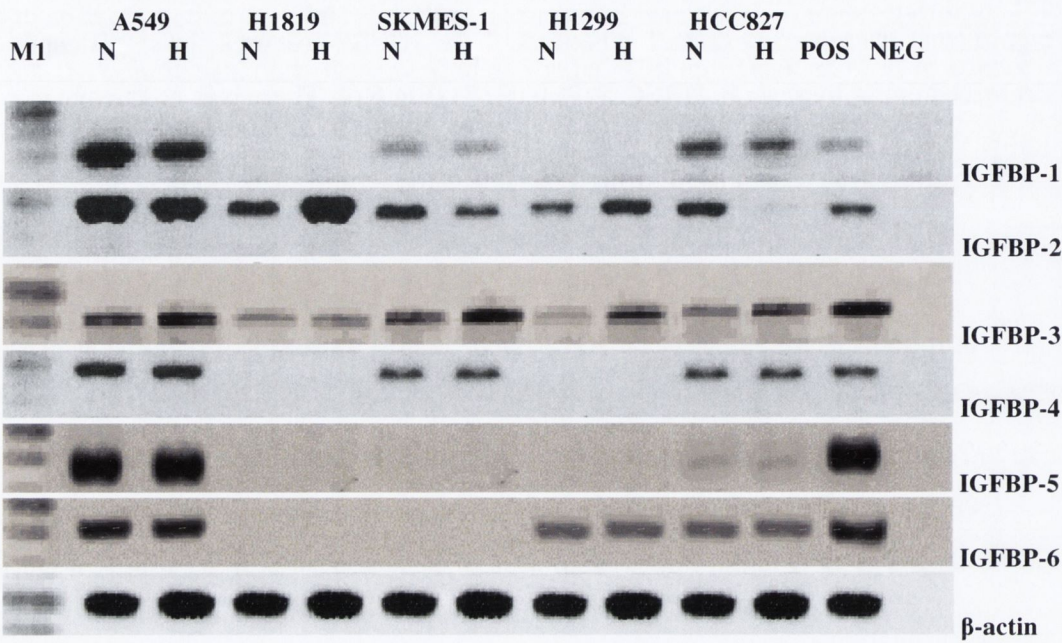
RNA was isolated from the cell lines. Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGFBP1-6 mRNA was examined by PCR analysis. A representative gel is shown in Fig 6.4. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using a two tailed student t-test.

Hypoxic values were compared to normoxic densitometry values. There was no change in IGFBP-1 mRNA expression under hypoxia in A549, SKMES-1 and HCC827 cell lines (Fig 6.4 A). In HBEC5 cells hypoxia upregulated expression of IGFBP-1 under hypoxia (Fig 6.4 B). IGFBP-2 expression increased in H1819 (P=0.0017) or H1299 (P=0.0486) cell lines under hypoxia but decreased in A549, SKMES-1 and HCC827 (P=0.0276) cell lines and decreased expression HCC827 cells (Fig 6.5). There was no significant difference between IGFBP-2 expression between normoxic and hypoxic HBEC 3 and HBEC 4 cell lines but expression was significantly decreased in HBEC 5 cells under hypoxia (P=0.0034) (Fig 6.6).

IGFBP-3 expression increased (not significant) in H1299, A549, SKMES-1 and HCC827 cells under hypoxia. There was no change in IGFBP-3 expression in H1819 cells under normoxia or hypoxia. IGFBP-3 expression increased in all normal cell lines under hypoxia. There was a significant increase in IGFBP-3 expression in HBEC 4 (P=0.0072) and HBEC 5 (P=0.0006) cell lines under hypoxia (Fig 6.6). This increase in expression of IGFBP-3 under hypoxia in HBEC 3 (P=0.0808) cells was not quite significant. IGFBP-4 expression did not change under hypoxia in A549, SKMES-1 and HCC827 cells. There was no expression of IGFBP-4 in any of the HBEC cell lines under normoxia or hypoxia.

There was no change in IGFBP-5 expression in A549 and HCC827 cell lines under hypoxia. IGFBP-5 was expressed in HBEC 3 under normoxia and hypoxia with no significant difference in expression between them. IGFBP-6 expression significantly decreased under hypoxia in HBEC 5 ($P=0.0115$) (Fig 6.6). There was no change in IGFBP-6 expression in A549, H1299 and HCC827 cell lines under hypoxia.

A.



B.

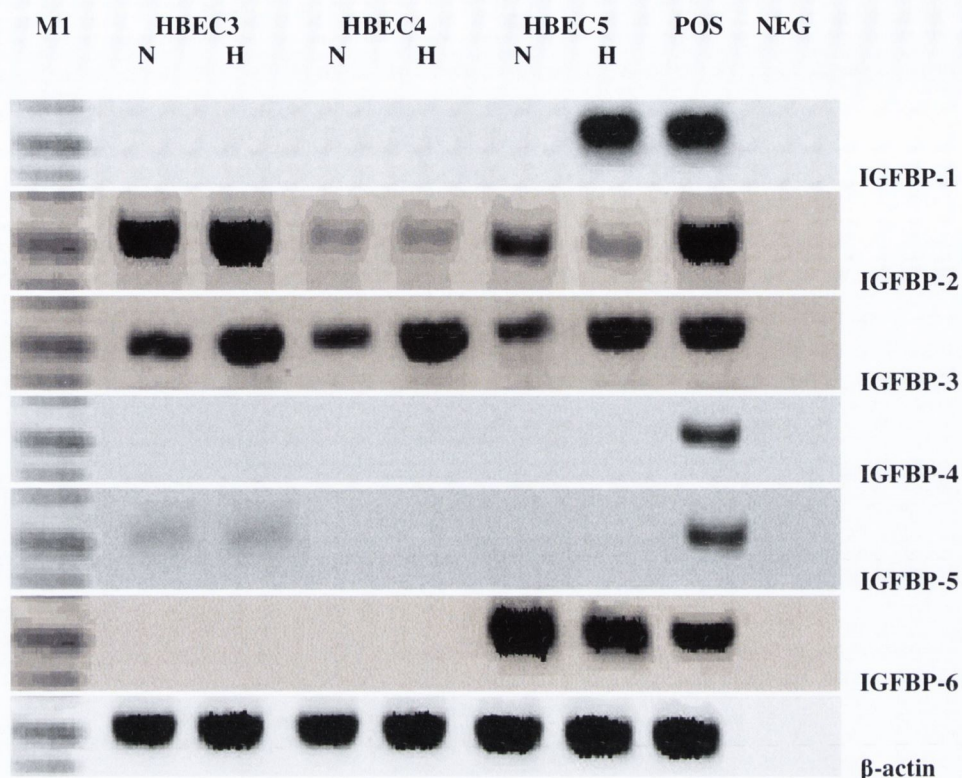


Fig 6.4: The mRNA expression of IGFBP1-6 under normoxia and hypoxia in (A) lung cancer cell lines and (B) human bronchial epithelial cell lines. These images are representative of three independent experiments. A PCR was carried out for β -actin in all samples to normalise for any loading discrepancies. A 1Kb-plus DNA ladder was loaded in lane 1 (M). A negative sample was set-up with sterile water to control for contamination (NEG). A positive sample was also set-up using Hep3B cDNA to control for positive expression (POS). PCR reactions were loaded in the remaining lanes. (N – Normoxia, H – Hypoxia)

(IGFBP-1 -272bp, IGFBP-2 - 518bp, IGFBP-3 - 434bp, IGFBP-4 – 239bp, IGFBP-5 - 403bp, IGFBP-6 - 345bp, β -actin 294bp)

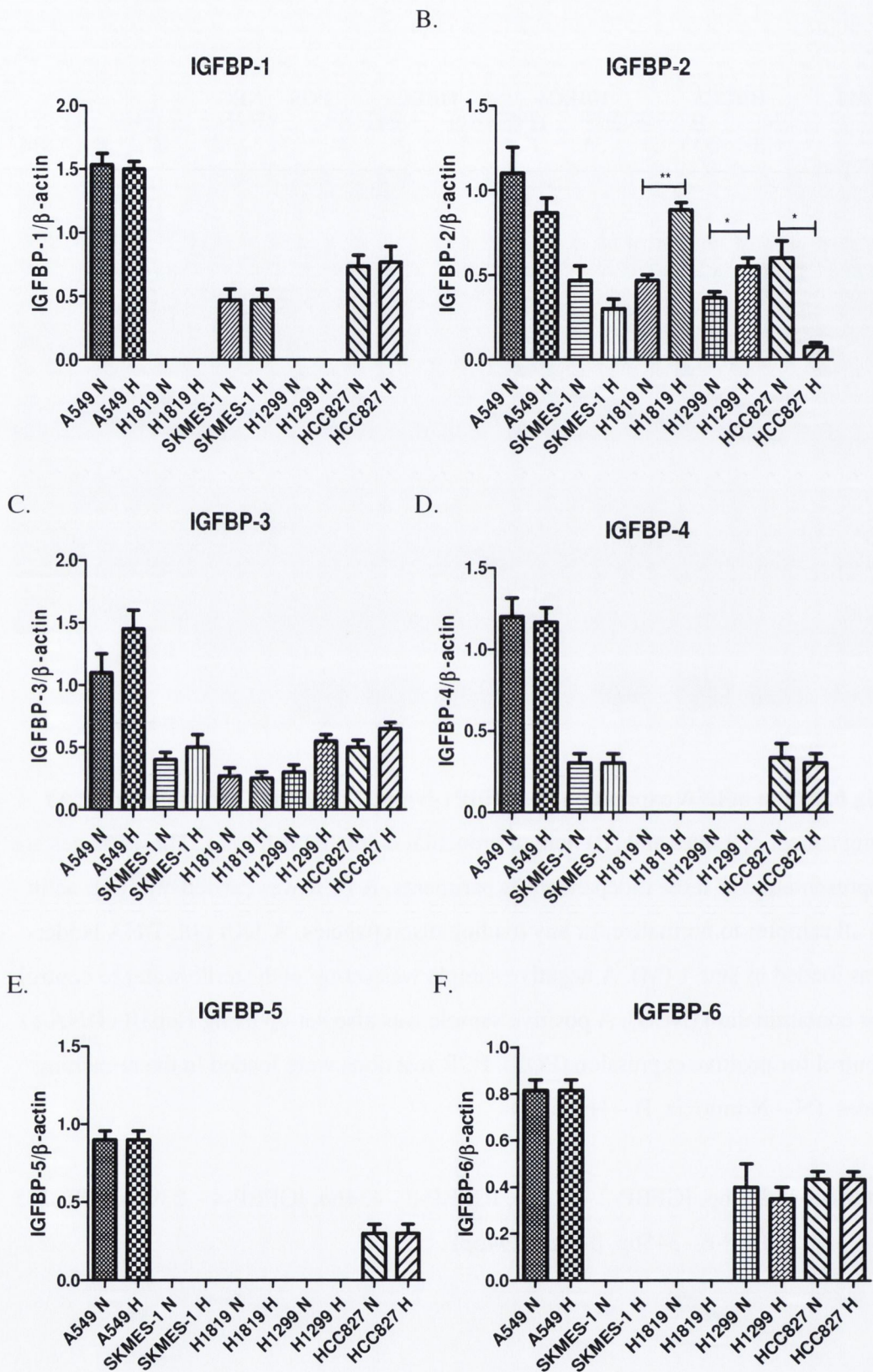


Fig 6.5 Densitometry results of IGFBP1-6 mRNA in NSCLC cells lines under normoxia and hypoxia. (A) IGFBP-1, (B) IGFBP-2, (C) IGFBP-3, (D) IGFBP-4, (E) IGFBP-5 and (F) IGFBP-6. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test.

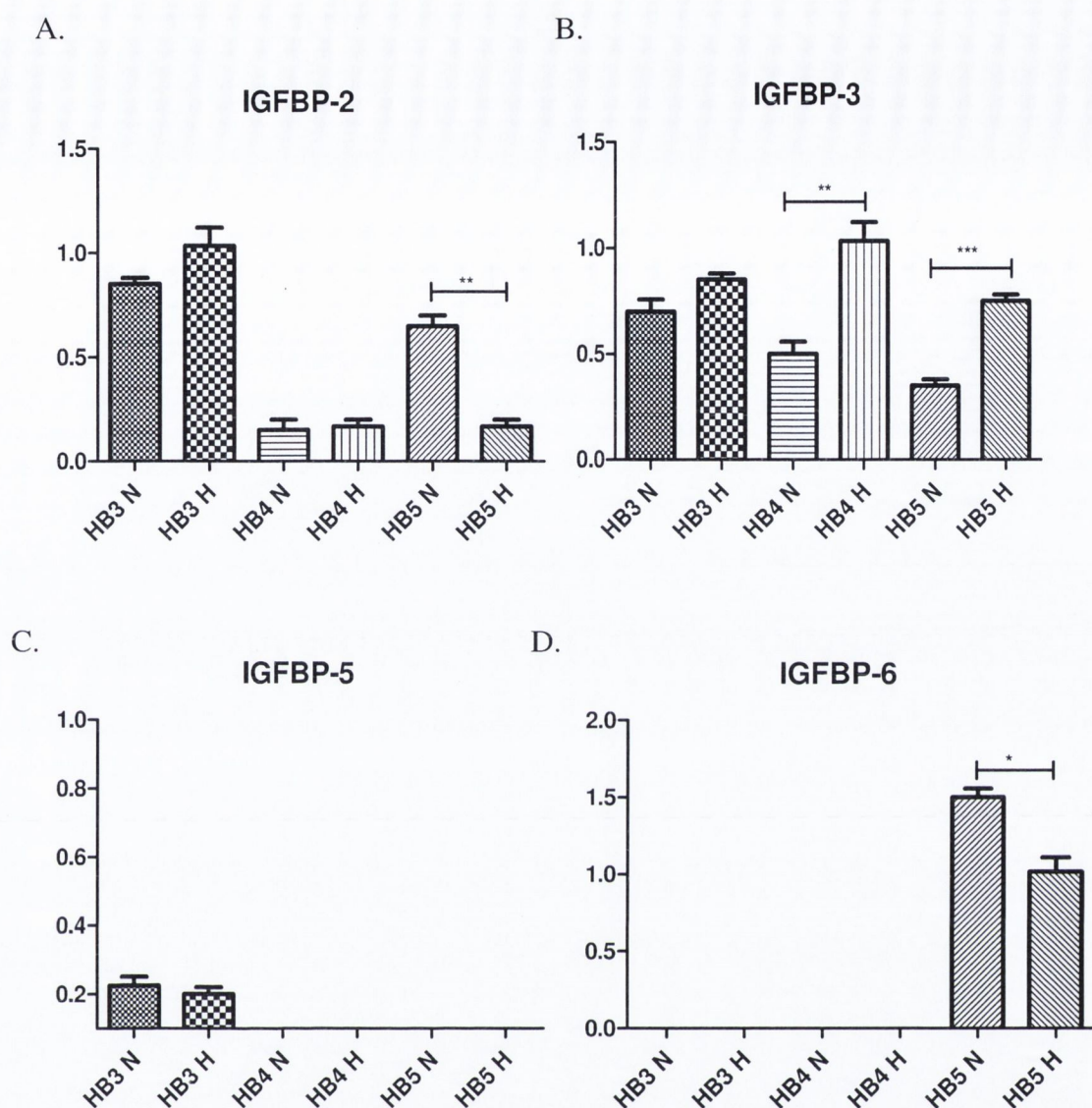


Fig 6.6 Densitometry results of IGFBP1-6 mRNA in HBEC cells lines under normoxia and hypoxia. (A) IGFBP-2, (B) IGFBP-3, (C) IGFBP-5, (D) IGFBP-6 (only significantly different results shown). Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test.

6.1.3 Effect of hypoxia on IGFBP1-5 mRNA expression at different time intervals in NSCLC cell lines

To examine whether IGFBP mRNA and protein expression was changed under hypoxic (0.5% O₂) conditions a time course was performed. A549 and H1299 cells were chosen as a comparison of cells which express high and low levels of IGFBPs respectively. The HBEC 4 cell line was selected out of the three HBEC cell lines as the donor of this normal lung cell line went on to develop lung cancer. Results of this experiment were based on IGFBP expression found in 6.1.1 in A549, H1299 and HBEC 4 cell lines.

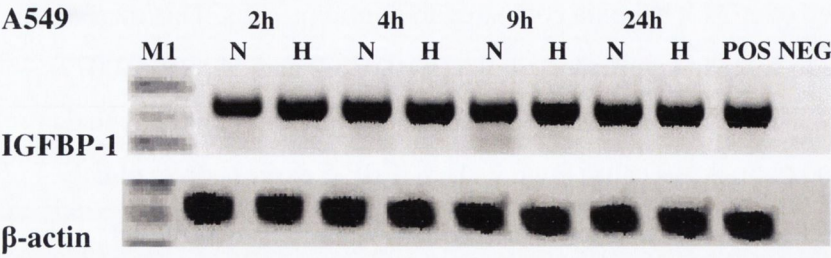
Cells were seeded at 1×10^6 /T75 cm³ flask and left to adhere overnight. The lung cancer cells were serum starved overnight in media containing 0.5% FBS prior to insertion into the hypoxia chamber. Cells were incubated in normoxia or hypoxia for periods between 2 and 24 h. RNA was isolated from the cell lines. Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGFBP1-6 mRNA was examined by PCR analysis. Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGFBP1-6 mRNA was examined by PCR analysis. Representative gels are shown in Fig 6.7 (A-E). Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using a two tailed student t-test. Results that were statistically significant are shown in Fig 6.8 (A-D).

In A549 cells, IGFBP-1 expression was increased (not significant) under hypoxia after 2 h but no difference in expression was seen when cells were subjected to increased hypoxia exposure (Fig 6.7 A). In H1299 cells, IGFBP-2 expression increased at 2 h and 9 h hypoxia (not significant) whereas IGFBP-2 expression significantly decreased at 2 h hypoxia ($P=0.0401$) and 24 h hypoxia ($P=0.0455$) in A549 cells (Fig 6.8 A). There was no change in IGFBP-2 expression in HBEC 4 cells over 24 h hypoxia compared to normoxic cells (Fig 6.7 B).

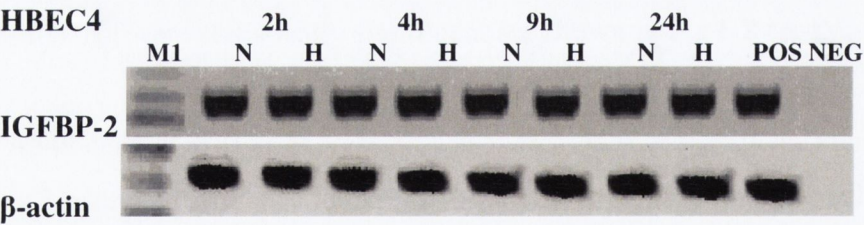
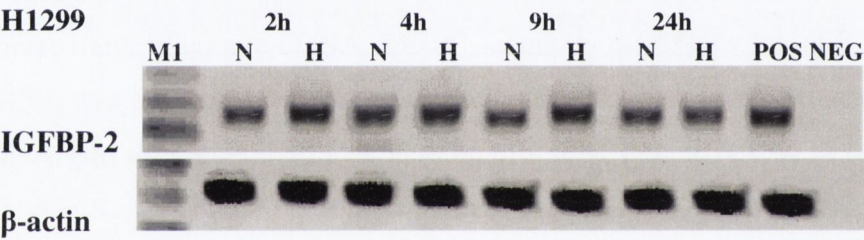
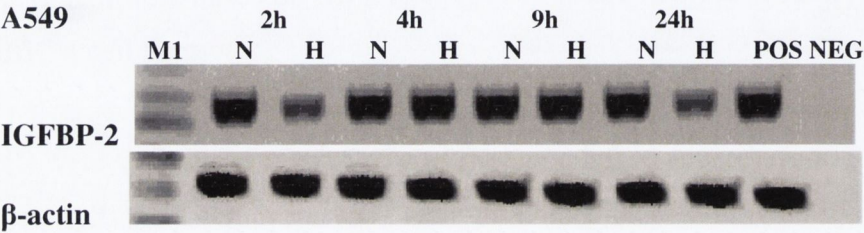
IGFBP-3 expression was increased at 2 h and 4 h in hypoxic compared to normoxic A549 cells. At 9 h ($P=0.0468$) and 24 h ($P=0.0498$) IGFBP-3 expression was significantly increased in hypoxia compared to normoxia A549 cells (Fig 6.8 B). In H1299 cells, IGFBP-3 expression increased at 4 h and 9 h hypoxia compared to

normoxic cells but this was not significant. In HBEC 4 cells, IGFBP-3 expression gradually increased over 24 h hypoxia compared to normoxic cells. This increase in IGFBP-3 expression reached significance at 9 h ($P=0.0387$) and 24 h ($P=0.0077$) hypoxia (Fig 6.8 C). There was no change in IGFBP-4 expression under hypoxia compared to normoxia in A549 cells (Fig 6.7 D). IGFBP-5 expression gradually significantly decreased in A549 cells at 2 h ($P=0.0051$), 4 h ($P=0.0199$), 9 h ($P=0.0055$) and 24 h ($P=0.0020$) hypoxia compared to normoxic cells over 24 h (Fig 6.8 D). There was no expression of IGFBP-4 or IGFBP-5 detected in either H1299 or HBEC 4 cell lines.

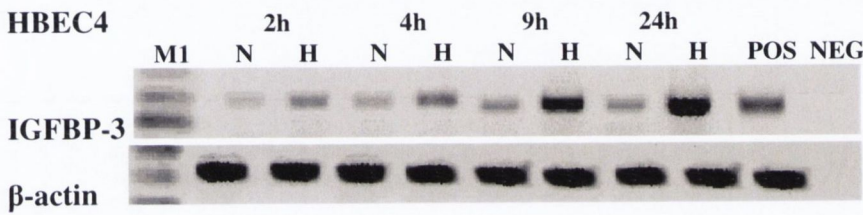
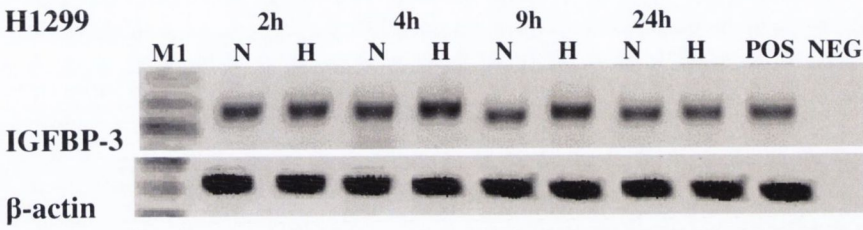
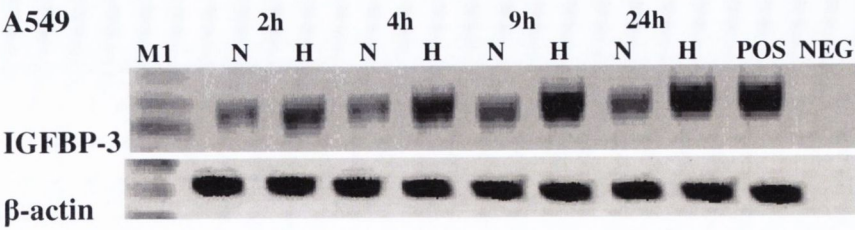
A.



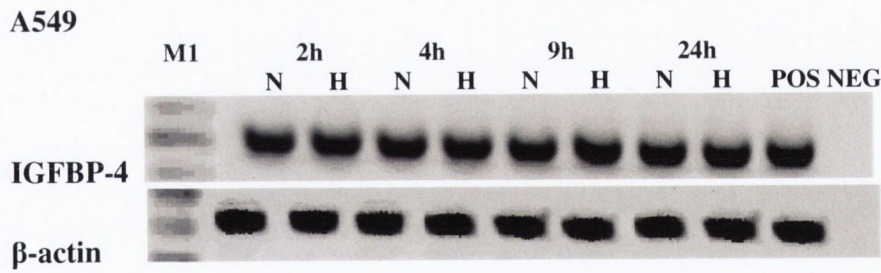
B.



C.



D.



E.

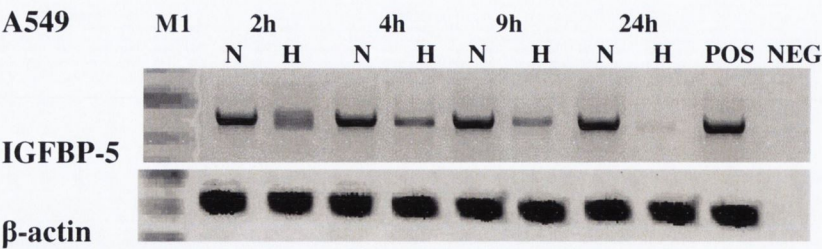


Fig 6.7: IGFBP1-5 mRNA expression under normoxia and hypoxia at different time intervals in A549, H1299 and HBEC 4 cell lines. (A) IGFBP-1, (B) IGFBP-2, (C) IGFBP-3, (D) IGFBP-4, (E) IGFBP-5. These images are representative of three independent experiments. cDNA was generated from the isolated RNA, and a PCR carried out to examine the expression of IGFBP-1-5 at the level of the mRNA transcript. A PCR was also carried out for β -actin in all samples to determine loading efficiency. A 1Kb-plus DNA ladder was loaded in lane 1 (M). A negative sample was set-up with sterile water to control for contamination (NEG). A positive sample was also set-up using Hep3B cDNA to control for positive expression (POS). PCR reactions were loaded in the remaining lanes. (N – Normoxia, H – Hypoxia)

(IGFBP-1 -272bp, IGFBP-2 - 518bp, IGFBP-3 - 434bp, IGFBP-4 – 239bp, IGFBP-5 - 403bp, IGFBP-6 - 345bp, β -actin 294bp)

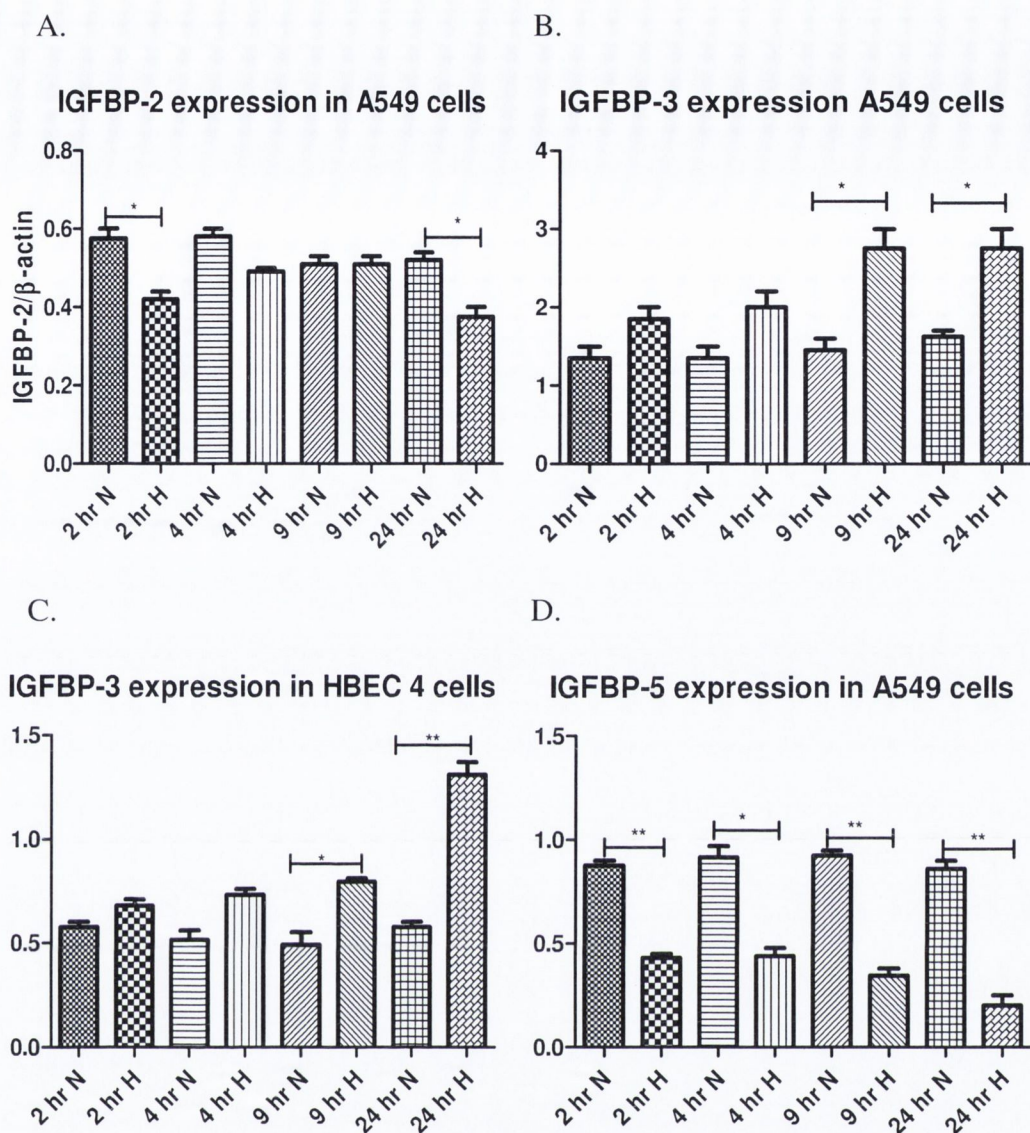


Fig 6.8 Densitometry results of IGFBP-2, IGFBP-3 and IGFBP-5 mRNA in A549 and HBEC 4 cell lines under normoxia and hypoxia. (A) IGFBP-2, (B) IGFBP-3, (C) IGFBP-3, (D) IGFBP-5. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test. [Only significantly different results shown]

6.1.4 Effect of hypoxia on IGFBP-3 protein expression at different time intervals

The effect of hypoxia (0.5% O₂) on IGFBP-3 protein expression in HBEC 4, H1299 and A549 cells was examined and compared to the changes observed at the RNA level. Cells were seeded at 1×10^6 /T75 cm³ flask and left to adhere overnight. The subsequent morning, cells were incubated in normoxia or hypoxia for periods between 2 and 24 h. The lung cancer cells were serum starved overnight in media containing 0.5% FBS prior to insertion into the hypoxia chamber.

Cell culture media was removed from the flask and protein lysis buffer (20 mM Tris-HCl pH 7.5, 1.5M NaCl, 2mM EDTA, 10% glycerol, NP40, Na₃SO₄ and Protease Inhibitor Cocktail) was added. The lysate was used for the isolation of protein. Total protein was loaded on a gel and separated by SDS-page gel electrophoresis followed by transfer to PVDF membrane. Expression of IGFBP-3 was determined by antibody probing of membranes. A representative gel is shown in Fig 6.9. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using a two tailed student t-test.

Hypoxic values were compared to normoxic densitometry values. IGFBP-3 protein expression in A549 and H1299 cells was decreased compared to normoxic cells at 4 h and 9 h hypoxia. In normal lung cells, IGFBP-3 protein expression remained stable under hypoxia and no change in expression was found up to 24 h hypoxia compared to normoxic cells. Densitometry results were not statistically significant (not shown).

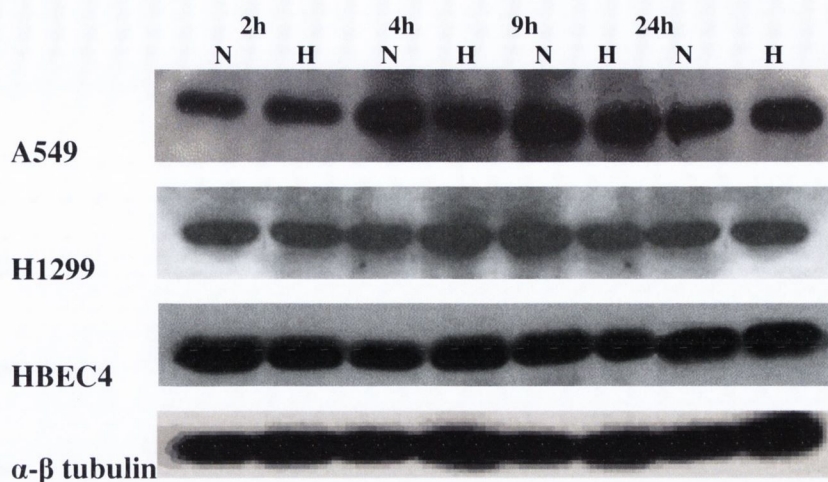


Fig 6.9: The protein expression of IGFBP-3 under normoxia and hypoxia at different time intervals. This image is representative of three independent experiments. Total protein was loaded on a gel and separated by electrophoresis followed by transfer to PVDF membrane. Expression of IGFBP-3 was determined by antibody probing of membranes. Membranes were probed with α - β -tubulin to determine loading efficiency. Statistical analysis was carried out using a two tailed student t-test.

(N – Normoxia, H – Hypoxia) (IGFBP-3 -31.7 kDa)

6.1.5 Effect of hypoxia on IGF-1R mRNA and protein expression at different time intervals in normal and lung cancer cells

To determine the effect of hypoxic (0.5% O₂) conditions on the expression of IGF-1R at the mRNA and protein level in normal and lung cancer cells, cells were seeded at 1×10^6 /T75 cm³ flask and left to adhere overnight. The subsequent morning, cells were incubated in normoxia or hypoxia for periods between 2 and 24 h. The lung cancer cells were serum starved (media containing 0.5% FBS) prior to insertion into the hypoxia chamber.

RNA and protein were isolated from the cell pellet. Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGF-1R mRNA was examined by PCR analysis. Total protein was loaded on an 8% gel and separated by SDS-PAGE gel electrophoresis followed by transfer to PVDF membrane. Expression of IGF-1R was determined by antibody probing of membranes. A representative gel is shown in Fig 6.10 B. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. Statistical analysis was carried out using a two tailed student t-test.

Hypoxic values were compared to normoxic densitometry values. In A549 cells, IGF-1R mRNA expression was significantly decreased at 24 h hypoxia compared to normoxic cells ($P=0.0389$) (Fig 6.10 A). At the protein level however, IGF-1R expression was significantly decreased at 4 h ($P=0.0077$) and 9 h ($P=0.0022$) hypoxia compared to normoxic cells (Fig 6.10 B). In H1299 cells, IGF-1R mRNA expression was decreased at 2 h and 4 h hypoxia compared to normoxic cells. This was significant at 4 h hypoxia ($P=0.0283$) (Fig 6.10 A). IGF-1R mRNA expression increased between 4 h and 24 h under hypoxia in normoxic cells but this was not significant. There was no change in IGF-1R protein expression at 4 and 9 h between normoxic and hypoxic cells but at 24 h IGF-1R protein expression decreased in normoxic cells compared to hypoxic cells ($P=0.0011$) (Fig 6.10 B). In HBEC4 cells, IGF-1R protein expression was significantly higher in hypoxic cells compared to normoxic cells at 9 h ($P=0.0050$) and 24 h ($P=0.0044$) (Fig 6.10 B). At the mRNA level there was no change in IGF-1R expression between normoxic and hypoxic cells (Fig 6.10 A).

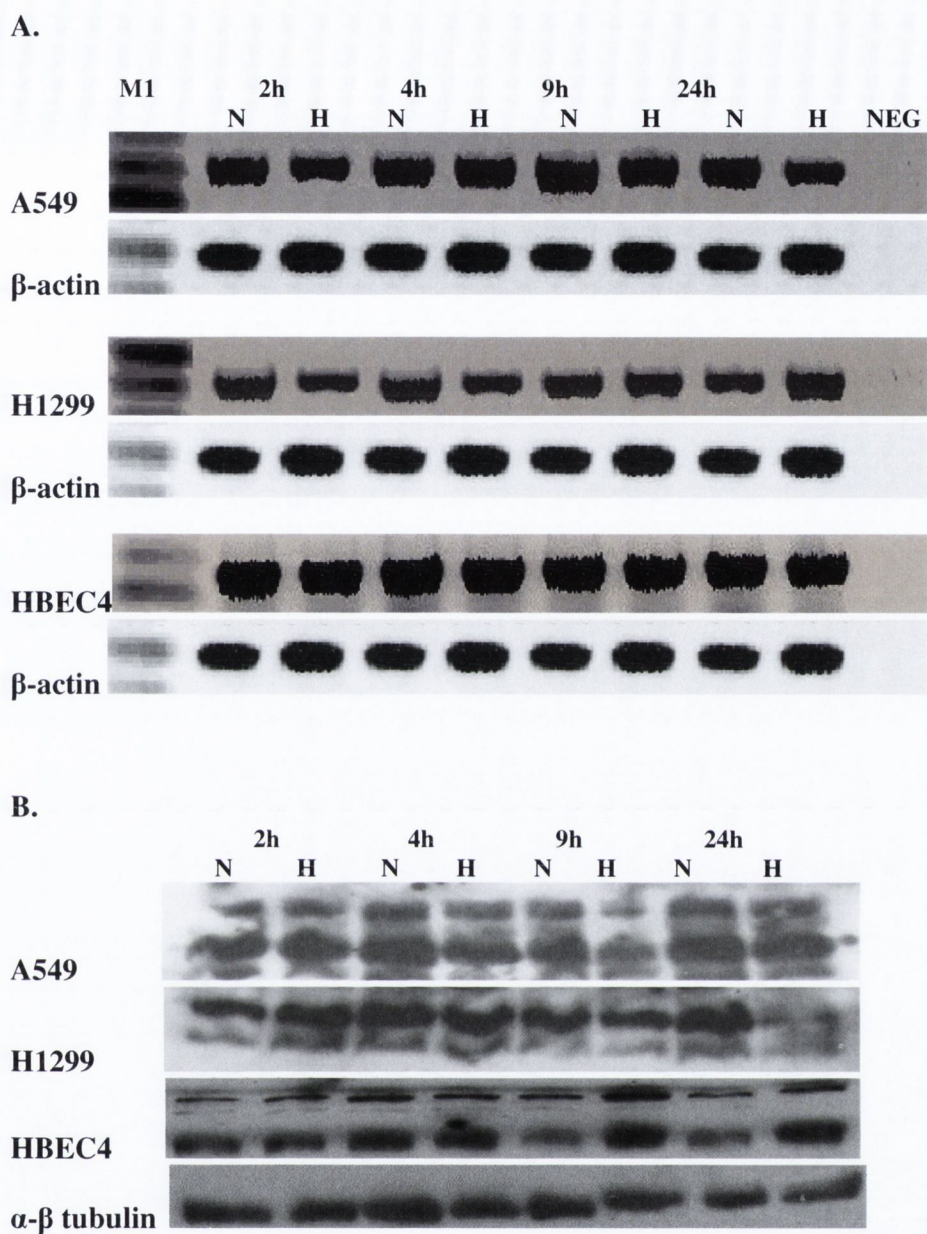


Fig 6.10: The mRNA and protein expression of IGF-1R under normoxia and hypoxia at different time intervals in normal and lung cancer cell lines. These images are representative of three independent experiments. (A) IGF-1R mRNA expression. A PCR was carried out for β -actin in all samples to determine loading efficiency. A 1Kb-plus DNA ladder was loaded in lane 1 (M). A negative sample was set-up with sterile water to control for contamination (NEG). PCR reactions were loaded in the remaining lanes. (B) IGF-1R protein expression. Expression of IGF-1R was determined by antibody probing of membranes. Membranes were probed with α - β -tubulin to determine loading efficiency.

(N – Normoxia, H – Hypoxia) (IGF-1R 265bp, 130 kDa (α) and 90kDa (β))

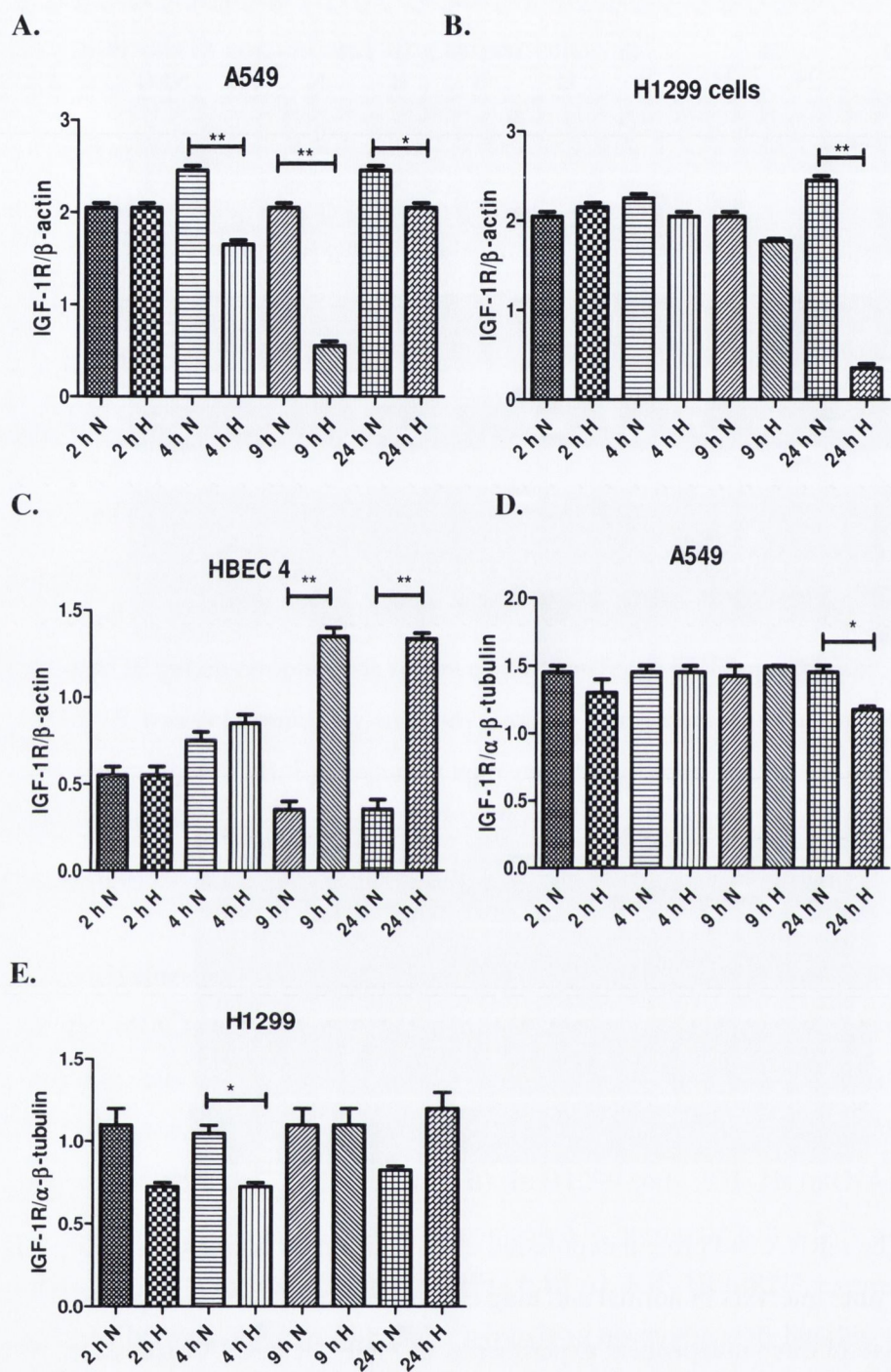


Fig 6.11 Densitometry results of IGF-1R mRNA and protein expression under normoxia and hypoxia at different time intervals in normal and lung cancer cell lines. These images are representative of three independent experiments. (A-C) IGF-1R protein expression, (D and E) IGF-1R mRNA expression. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test. [Only significantly different results shown]

6.1.6 Effect of hypoxia on IGF-1 mRNA expression at different time intervals in normal and lung cancer cell lines

To examine whether IGF-1 mRNA expression was changed under hypoxic (0.5% O₂) conditions over a time course, cells were seeded at 1 x 10⁶/T75 cm³ flask and left to adhere overnight. The subsequent morning, cells were incubated in normoxia or hypoxia for periods between 2 and 24 h. The lung cancer cells were serum starved overnight in media containing 0.5% FBS prior to insertion into the hypoxia chamber. RNA was isolated from the cell pellet. Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGF-1 mRNA was examined by PCR analysis. A representative gel is shown in Fig 6.12. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software.

Hypoxic values were compared to normoxic densitometry values (Fig 6.12). IGF-1 mRNA expression in A549 cells increased significantly under hypoxia at 2 h (P=0.0222), 4 h (P=0.0422) and 24h (P=0.0233) compared to normoxic cells (Fig 6.13). IGF-1 mRNA expression in H1299 cells also increased significantly under hypoxia at 2 h (P=0.0385), 4 h (P=0.0381) and 24h (P=0.0313) compared to normoxic cells (Fig 6.13). In normal lung cells, IGF-1 mRNA expression significantly decreased under hypoxia up to 24h compared to normoxic cells. No IGF-1 mRNA expression was present at 2 to 9 h hypoxia. At 24 h IGF-1 mRNA expression was significantly increased in normoxic and hypoxic HBEC 4 cells however IGF-1 mRNA was still significantly reduced in HBEC 4 cells under hypoxia at 24 h (P=0.0282) (Fig 6.13).

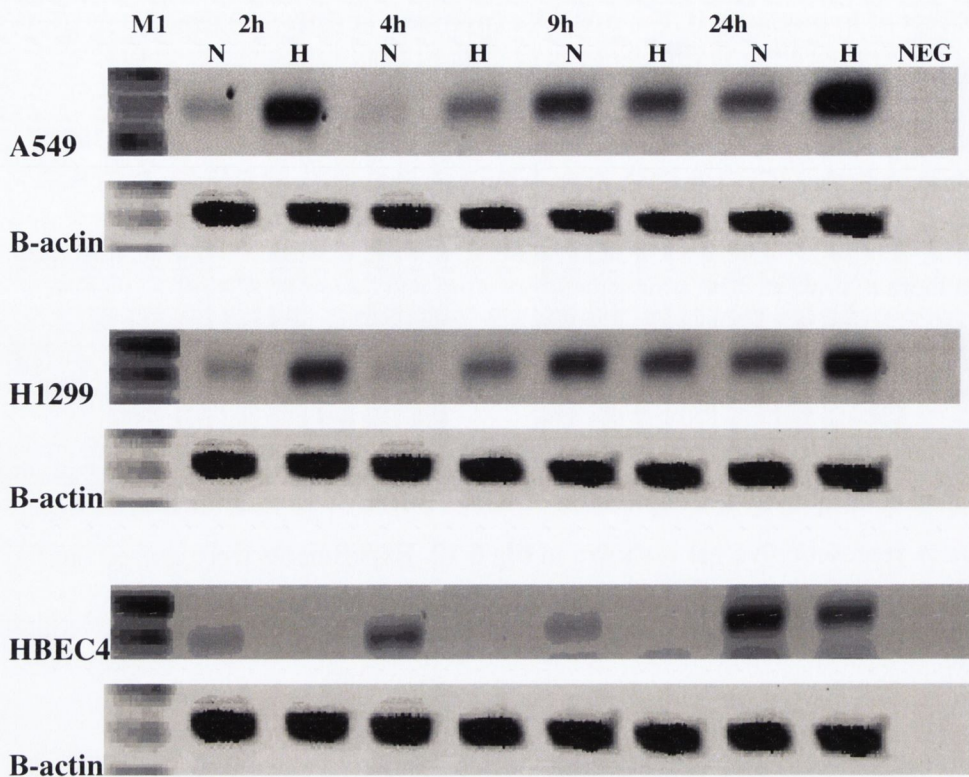
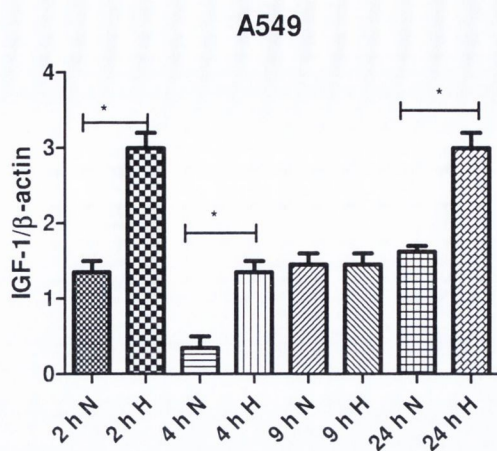


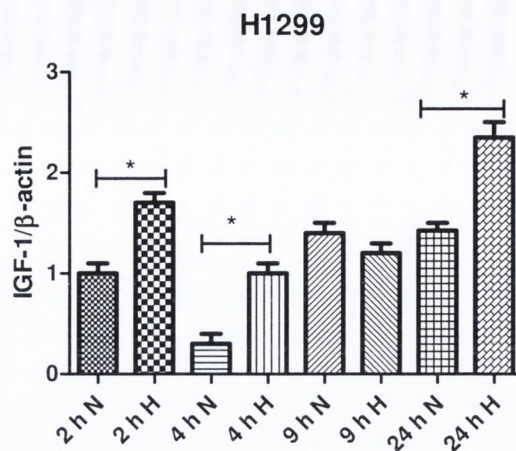
Fig 6.12 The mRNA expression of IGF-1 under normoxia and hypoxia at different time intervals in normal and lung cancer cells. This image is representative of three independent experiments. cDNA was generated from the isolated RNA, and a PCR carried out to examine the expression of IGF-1 at the level of the mRNA transcript. A PCR was also carried out for β -actin in all samples to determine loading efficiency. A 1Kb-plus DNA ladder was loaded in lane 1 (M). A negative sample was set-up with sterile water to control for contamination (NEG). PCR reactions were loaded in the remaining lanes.

(N – Normoxia, H – Hypoxia) (IGF-1 428bp)

A.



B.



C.

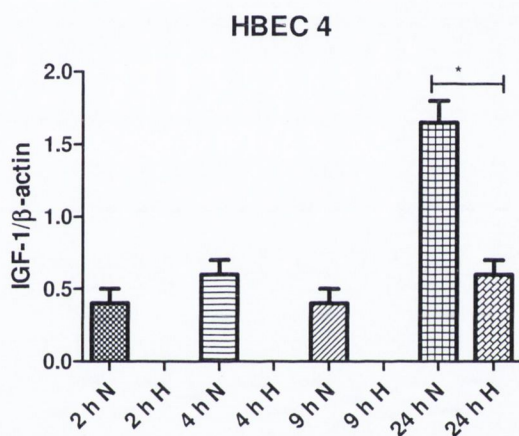


Fig 6.13 Densitometry results of IGF-1 mRNA expression under normoxia and hypoxia at different time intervals in normal and lung cancer cell lines. These images are representative of three independent experiments. (A) A549, (B) H1299 and (C) HBEC 4. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test.

6.1.7 IGFBP-3, IGFBP-5, IGF-1R and IGF-1 mRNA expression in matched normal and tumour tissue from NSCLC patients

IGFBP-3, IGF-1R and IGF-1 mRNA expression was examined in 10 matched tumour and normal tissue from NSCLC patients who had undergone a lung tumour resection. RNA was isolated from the fresh frozen tissue samples through homogenization of the tissue sample in TRI Reagent.

Total RNA was reverse transcribed into cDNA, and subsequently the expression of IGFBP-3, IGF-1R and IGF-1 mRNA was examined by PCR analysis. A representative gel is shown in Fig 6.14. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software. IGFBP-3 expression was significantly higher in tumour compared to matched normal tissue ($P=0.0009$) (Fig 6.15 A). IGFBP-5 expression was also significantly higher in tumour compared to matched normal tissue ($P=0.0189$) (Fig 6.15 B). IGF-1R expression was also significantly higher in tumour compared to matched normal tissue ($P=0.0001$) (Fig 6.15 C). IGF-1 expression was significantly reduced in tumour compared to matched normal tissue ($P=0.0001$) (Fig 6.15 D).

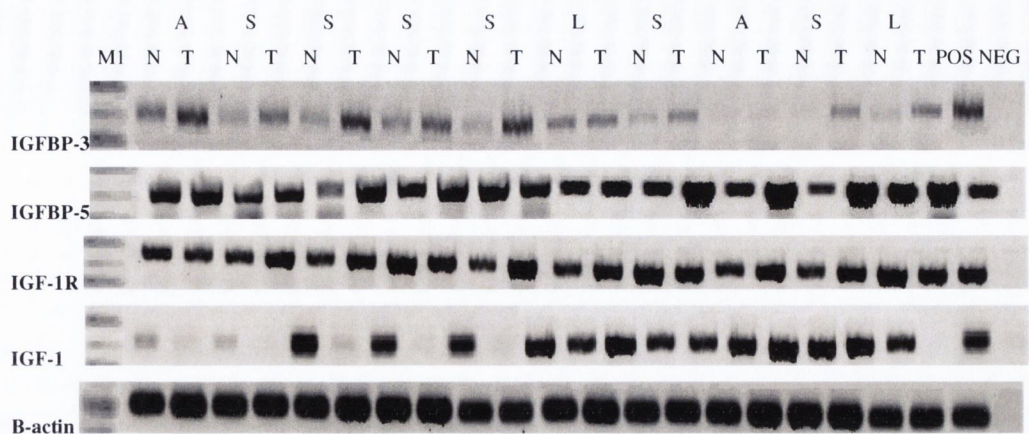


Fig 6.14: The mRNA expression of IGFBP-3, IGFBP-5, IGF-1R and IGF-1 in matched tumour and normal patient samples. This image is representative of three independent experiments. cDNA was generated from the isolated RNA, and a PCR carried out to examine the expression of IGFBP1-6 at the level of the mRNA transcript. A PCR was also carried out for β -actin in all samples to determine loading efficiency. A 1Kb-plus DNA ladder was loaded in lane 1 (M). A positive sample was also set-up using Hep3B cDNA to control for positive expression (POS). A negative sample was set-up with sterile water to control for contamination (NEG). A positive sample was also set-up using Hep3B cDNA to control for positive expression (POS). PCR reactions were loaded in the remaining lanes.

(N – Normal, T – Tumour, A- Adenocarcinoma, S- Squamous cell carcinoma, L- NSCLC) (IGFBP-3 - 434bp, IGF-1R 265bp, IGF-1 428bp)

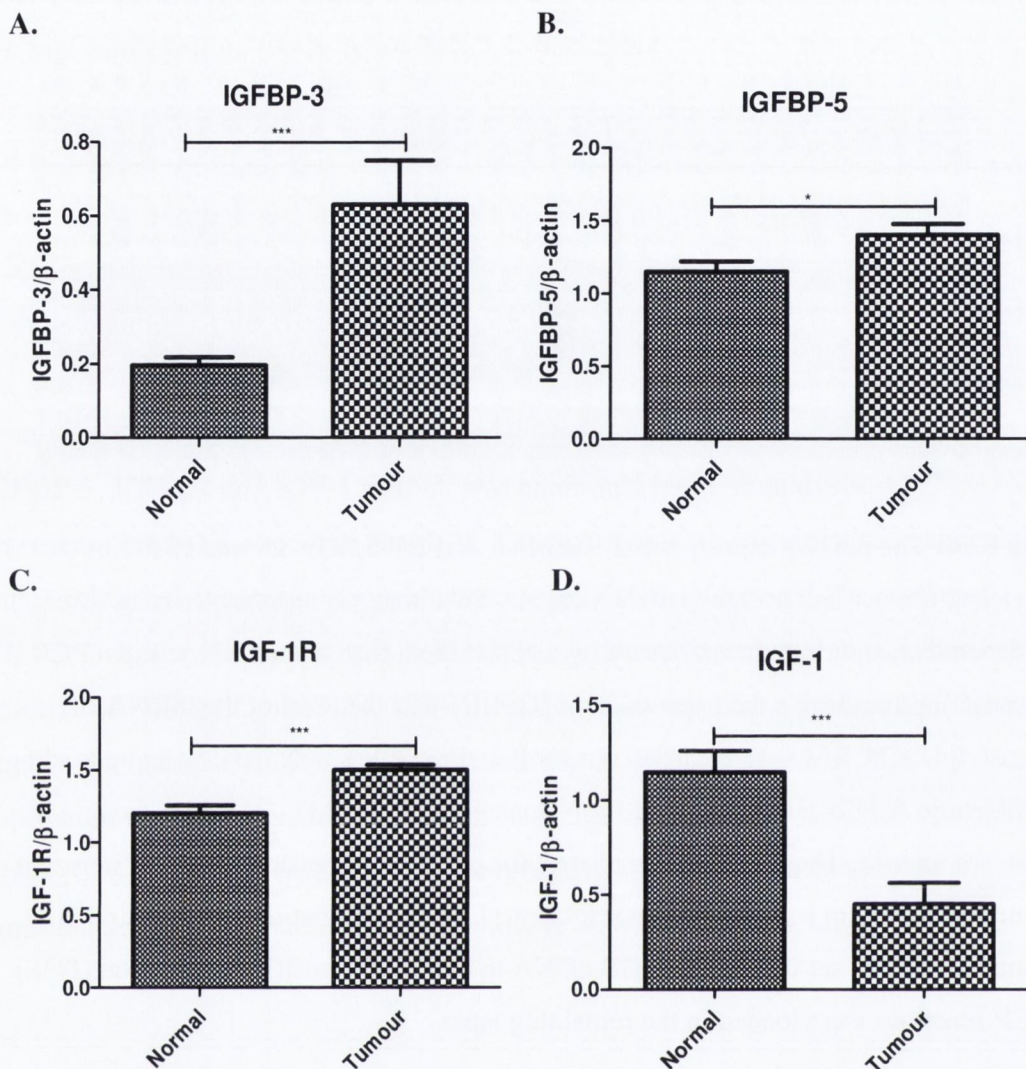


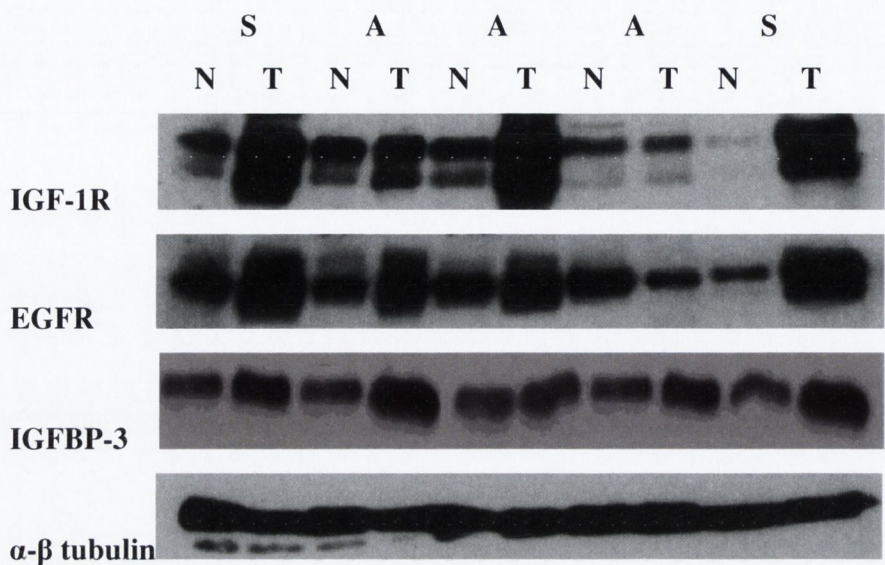
Fig 6.15 Densitometry results of IGFBP-3, IGFBP-5, IGF-1R and IGF-1 mRNA in matched normal and tumour tissue. These images are representative of three independent experiments. (A) IGFBP-3, (B) IGFBP-5, (C) IGF-1R and (D) IGF-1. Data is expressed as mean of triplicate experiments $\pm$ SEM. Statistical analysis was carried out using a two tailed student t-test.

6.1.8 IGFBP-3, IGF-1R and EGFR protein expression in matched tumour and normal tissue from NSCLC patients

IGFBP-3, IGF-1R and EGFR protein expression was examined in 10 matched tumour and normal tissue from NSCLC patients which had undergone lung tumour resection surgery. The NSCLC matched normal and tumour samples are the same samples which were used in chapter 4 and section 6.1.7. Protein was isolated from the tumour and normal tissue samples. Total protein was loaded on a gel and separated by electrophoresis followed by transfer to PVDF membrane. Protein expression was determined by antibody probing of membranes. A representative gel is shown in Fig 6.16. Experiments were carried out in triplicate and densitometry analysis was performed using TINA (Version 2.09c) software.

In the fresh frozen resected tumours IGFBP-3 protein expression was significantly increased in tumour tissue compared to matched normal tissue in NSCLC patients ($P=0.0001$) (Fig 6.16). IGF-1R and EGFR were significantly overexpressed relative to matched normal tissues in NSCLC patients, $P=0.004$ and $P=0.0015$ respectively (Fig 4.8).

A.



B.

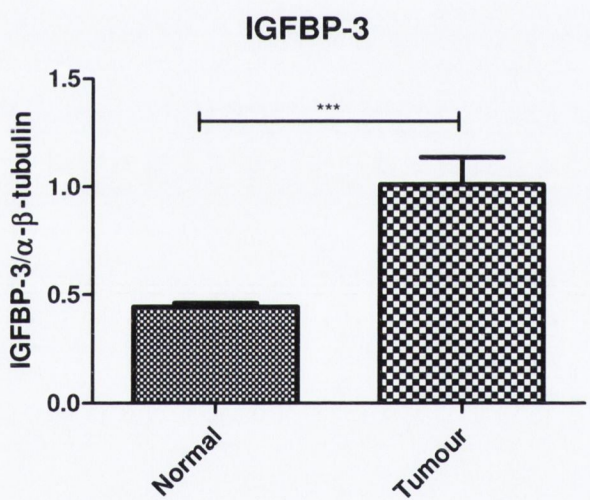


Fig 6.16: The protein expression of IGFBP-3, IGF-1R and EGFR in matched tumour and normal patient samples. (A) Protein expression of IGFBP-3, IGF-1R and EGFR and (B) densitometry results of IGFBP-3 protein expression. This image is representative of three independent experiments. Total protein was loaded on a gel and separated by electrophoresis followed by transfer to PVDF membrane. Expression of IGF-1R/EGFR/IGFBP-3 was determined by antibody probing of membranes. Membranes were probed with α - β -tubulin to determine loading efficiency. (B). Densitometry analysis for IGFBP-3 expression. Densitometry analysis for IGF-1R and EGFR is shown in Fig 4.8. (N – Normal, T – Tumour, A- Adenocarcinoma, S- Squamous cell carcinoma)

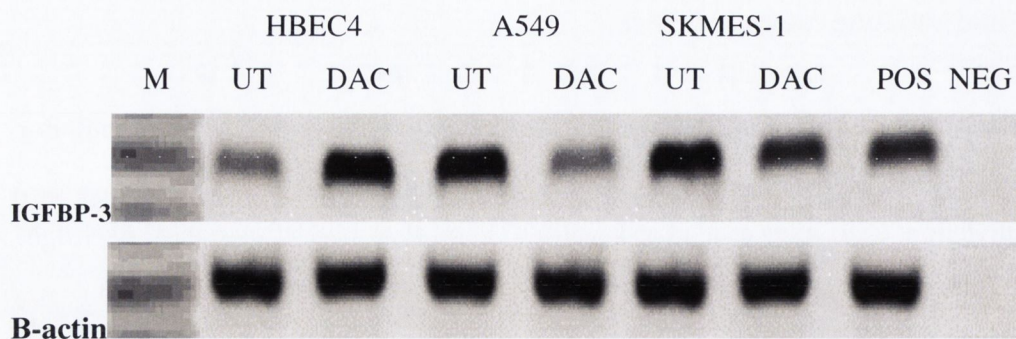
6.1.9 Effects of 5-aza-2'-deoxycytidine (DAC) on IGFBP-3 mRNA expression in normal and lung cancer cell lines

HBEC-4, A549 and SKMES-1 were treated with a DNA methyltransferase inhibitor (DNMTi) to examine IGFBP-3 mRNA expression upon inhibition of DNA methylation. Cells were seeded at 1×10^6 /T75 cm³ flask and left to adhere overnight. The following day the cells were serum starved (lung cancer cell lines- media containing 0.5% FBS) for 24 h before drug treatment. To determine if methylation was involved in the regulation of IGFBP-3 mRNA expression, cells were treated with 1 μ M DAC. Cells were treated for 48 h with DAC and replaced every 24 h in fresh media.

RNA was isolated from the cell pellet. The RNA was reverse transcribed into cDNA, and subsequently, the mRNA expression of IGFBP-3 and IGF-1 were examined by PCR analysis. Representative gels are shown in Fig 6.17. Experiments were carried out in triplicate. The densitometric values of DAC treatment was compared to untreated cells (UT).

Treatment with DAC decreased IGFBP-3 mRNA expression in A549 cells ($P=0.0036$) and SKMES-1 cells ($P=0.0154$) (Fig 6.17). The opposite was found in HBEC 4 treated cells as treatment with DAC increased IGFBP-3 RNA expression ($P=0.0122$) (Fig 6.17).

A.



B.

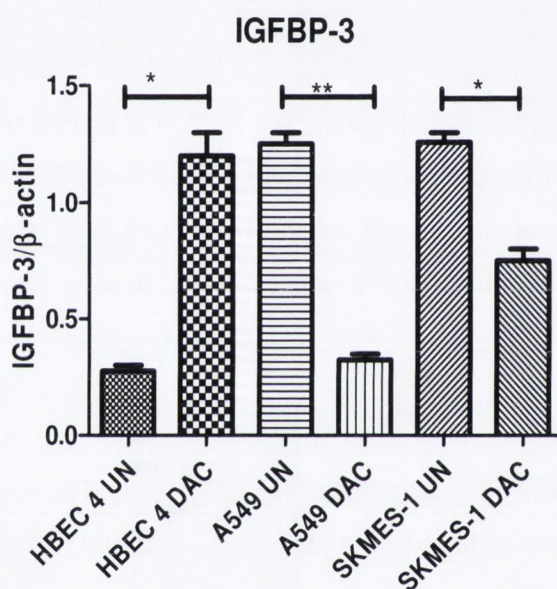


Fig 6.17 Effects of a DNMTi on IGFBP-3 mRNA expression. The mRNA expression of IGFBP-3 in HBEC-4, A549 and SKMES-1 after treatment with DAC. The image is representative of three independent experiments. Cells were treated with DAC (1 μ M) for 48 h, with DAC replaced every 24 h. cDNA was generated from the isolated RNA, and RT-PCR carried out to examine the expression of IGFBP-3 at the level of the mRNA transcript. RT-PCR was also carried out for β -actin in all samples to determine loading efficiency. A 1Kb-Plus ladder was loaded in lane 1 (M). A negative sample was set-up with sterile water to control for contamination (NEG). PCR reactions were loaded in the remaining lanes. (IGFBP-3 - 434bp)

6.2 Discussion

A typical microenvironment for most solid tumours is hypoxia, low-oxygen conditions that are under the normal physiological level. Tumour hypoxia has been shown to increase the metastatic and angiogenic potential of cancer cells making them resistant to radiation and chemotherapy [331]. A complex cross-talk system exists between the transcription factor hypoxia-inducible factor (HIF-1) and IGF signalling pathways that has important implications on tumour cell metabolism, growth and survival it is important to understand the role IGFBPs play in these hypoxic environments. Cells adapt to lower oxygen levels through a mechanism known as metabolic suppression where the cellular ATP-utilizing processes is decreased. Suppression of the metabolic rate involves the activation of HIF-1, which regulates ATP generation (metabolic supply). Tumour hypoxia has been shown to increase IGF-1 expression which in turn induces expression of IGF-1R and promotes increased cell survival under these conditions preventing apoptosis [332].

The aim of this chapter was to examine the expression levels of IGFBP-1 to -6 in a panel of NSCLC cell lines and normal bronchial epithelial cells (HBECs) grown under normoxic and hypoxic conditions. IGFBPs which both enhance and inhibit the physiological and biological actions of the IGFs, have been shown to be secreted *in vitro* in lung tumours [333]. Little is known about the regulation of IGFBPs by hypoxia and as tumour hypoxia is a common occurrence in NSCLC, there is a need to learn more about the importance of these binding proteins in the context of tumour aggressiveness and drug resistance.

The mRNA expression levels of IGFBPs were firstly examined under normoxia in a panel of normal and lung cancer cell lines. The pattern of IGFBP expression differed significantly between HBEC and NSCLC cell lines. In order to identify the IGFBPs expression from NSCLC cell lines the RNA from 5 different NSCLC cell lines and 3 HBEC cell lines was analyzed by RT-PCR. A549 cells displayed the highest level of expression of IGFBPs out of the NSCLC cell lines (Fig 6.1 A). A549 cells had significantly higher expression of IGFBP-1 compared to SKMES-1 ($P=0.0010$) and HCC827 ($P=0.0068$) (Fig 6.2 A). The results for IGFBP-1 mRNA expression were similar to what was found in a study by Reeve *et al.* by RT-PCR but not by northern blot analysis [334]. IGFBP-2 and IGFBP-3 were expressed in all NSCLC and HBEC

cell lines (Fig 6.1 A and Fig 6.1 B). This is not surprising as IGFBP-2 and IGFBP-3 influence the actions of IGFs and as IGFBP-3 is the most abundant binding protein in the circulation. A study by Jaques *et al.* also found IGFBP-2 and IGFBP-3 were predominantly expressed when they examined the expression of IGFBPs in primary tumours [335] [333]. The results of section 6.1.1 demonstrate that IGFBP-2 and IGFBP-3 are widely expressed and play important roles in both NSCLC and normal lung cell lines.

When IGFBP mRNA expression was then examined in normal and lung cancer cell lines under normoxia and hypoxia (2 h) it was found that different cell lines had increased or decreased expression of IGFBPs under hypoxic conditions (6.1.2). Hypoxic values were compared to normoxic densitometry values. Relative to normoxic controls, hypoxia had various effects between both the normal and lung cancer cell lines at the mRNA level. IGFBP-1 expression in A549 cells decreased under hypoxia whereas in HBEC5 cells hypoxia upregulated expression of IGFBP-1 (Fig 6.4 A and Fig 6.4 B). Other studies have found that IGFBP-1 increases under hypoxia in cancers such as pancreatic [236] and liver [336]. Hypoxia increased IGFBP-2 expression in H1819 or H1299 cells while it decreased expression in SKMES-1 cells and HCC827 cells (Fig 6.4 A). There was no significant difference of IGFBP-2 expression between normoxic and hypoxic HBEC 3 and HBEC 4 cell lines but expression was significantly decreased in HBEC 5 cells under hypoxia ($P=0.0034$) (Fig 6.6 A). An increase in IGFBP-2 expression has been demonstrated in other cancer cells lines under hypoxia through stimulation by IGF and induced HIF-1 expression [232].

IGFBP-3 expression increased in H1299, A549, SKMES-1 and HCC827 cells as well as in all normal cell lines under hypoxia. There was a significant increase in IGFBP-3 expression in HBEC 4 ($P=0.0072$) and HBEC 5 ($P=0.0006$) cell lines under hypoxia however this was not significant in HBEC 3 ($P=0.0808$) cells (Fig 6.6 B). The effect of hypoxia on IGFBP-3 expression has been well documented in the literature and has been shown to induce IGFBP-3 mRNA through p53-independent and -dependent mechanisms [232] [234]. Many cancer cell lines have increased IGFBP-3 mRNA expression by hypoxia regardless of their p53 load/status [234].

There was no expression of IGFBP-4 in any of the HBEC cell lines under normoxia or hypoxia. This was also observed in studies by Feldser *et al.* and Tucci *et al.* [327]

[232]. There are no reports of IGFBP-4 mRNA expression under hypoxia in NSCLC. IGFBP-5 was expressed in HBEC 3 under normoxia and hypoxia with no significant difference in expression between them. IGFBP-6 expression significantly decreased under hypoxia in HBEC 5 ($P=0.0115$) (Fig 6.6 D).

As there is not much in the literature about the effect of hypoxia on IGFBP expression in NSCLC further investigation to examine the reasons for these increases and decreases in IGFBP expression is needed. As IGFBPs have been shown to have both inhibitory and stimulatory effects on cell growth, their expression levels under hypoxia could either initiate apoptosis or survival as has been shown by Koga *et al.* that IGFBPs contribute to survival of pancreatic cancer cells under severely hypoxic conditions [236].

After the initial screen of IGFBP1-6 mRNA expression under normoxia and hypoxia in normal and lung cancer cell lines, the changes in IGFBP expression under hypoxia were then examined over different time points in a 24 h period. As HBEC4 cells are derived from an individual who eventually went on to develop cancer, this cell line was the only normal cell used for the remaining experiments.

To examine whether IGFBP mRNA and protein expression changed under hypoxic (0.5% O₂) conditions over 24 h a time course was used to compare A549, H1299 and HBEC 4 cell lines (6.1.3). In A549 cells, while IGFBP-1 expression was increased under hypoxia after 2 h no difference in expression was seen when cells were subjected to increased hypoxia exposure upto 24h. IGFBP-2 mRNA expression was differentially expressed over 24 h hypoxia in normal and lung cancer cells. IGFBP-2 expression increased at 2 h and 9 h hypoxia but this was not significant whereas IGFBP-2 expression significantly decreased at 2 h hypoxia and 24 h hypoxia in A549 cells. There was no change in IGFBP-2 expression in HBEC 4 cells over 24 h hypoxia compared to normoxic cells.

IGFBP-3 expression was increased at 2 h and 4 h in hypoxic compared to normoxic A549 cells and was significantly increased at 9 h ($P=0.0468$) and 24 h ($P=0.0498$) compared to normoxic cells. In H1299 cells, IGFBP-3 expression increased at 4 h and 9 h hypoxia compared to normoxic cells but this was not significant. In HBEC 4 cells, IGFBP-3 expression gradually increased over 24 h hypoxia compared to normoxic cells. This increase in IGFBP-3 expression reached significance at 9 h

($P=0.0387$) and 24 h ($P=0.0077$) hypoxia. In A549 and HBEC4 cells, IGFBP-3 mRNA expression under hypoxia continued to be higher than normoxic cells at 24 h.

There was no change in IGFBP-4 expression under hypoxia compared to normoxia in A549 cells. IGFBP-5 expression gradually significantly decreased in A549 cells at 2 h ($P=0.0051$), 4 h ($P=0.0199$), 9 h ($P=0.0055$) and 24 h ($P=0.0020$) hypoxia compared to normoxic cells over 24 h. This demonstrates the effect of hypoxia on the regulation of IGFBP-5 expression and that hypoxia downregulates IGFBP-5 expression at the RNA level in A549 cells. The importance of IGFBP-5 has been reported in the literature. It has been shown that breast cancer patients with positive lymph nodes and increased IGFBP-5 mRNA expression have a poor outcome [187]. Studies in ovarian cancer, showed that IGFBP-5 acts as a tumour suppressor by inhibiting angiogenesis [184]. Endogenous IGFBP-5 expression in two ovarian cancer cells was found to be lower than those of normal cells such as HEK293 cells (human normal kidney epithelial cell line) and MRC-5 cells (human normal lung fibroblast line). IGFBP-5 was found to be upregulated at the protein level from SCLC cell lines [337]. Expression levels of IGFBP-5 seem to vary depending on the type of cancer.

The IGFBP mRNA expression changes observed in the normal and lung cancer cell lines under normoxia and hypoxia are a possible example of what may occur in hypoxic tumours which are known to be a more aggressive phenotype and develop resistance to chemotherapy drugs. IGFBP-2, IGFBP-3 and IGFBP-5 expression changes were evidently seen under hypoxic exposure which seemed to be cell-type specific and also varied under length of hypoxia exposure. The decrease in IGFBP-5 mRNA expression seen in A549 cells may be a contributing factor to an aggressive and drug resistance phenotype in adenocarcinoma patients due to its important role as a tumour suppressor [184]. IGFBP-3 mRNA expression was clearly increased in all normal and most lung cancer cell lines which may enhance or suppress cell growth under hypoxic conditions.

A study by Grimberg *et al.*, found that IGFBP-3 induction under hypoxic conditions is independent of p53 status in tumour cell lines as IGFBP-3 induction by hypoxia also induces p53, however p53 is not always needed [234]. When p53^{+/+} lung carcinoma cells were exposed to increasing hypoxic time points IGFBP-3 mRNA expression increased. When this experiment was repeated using p53^{-/-} PC-3 cells,

this was also observed, suggesting that hypoxia can modulate IGFBP-3 mRNA expression independent of p53. As IGFBP-3 is responsible for inhibiting cell proliferation and inducing apoptosis in IGF-dependent and IGF-independent manner [338], its induction by hypoxia implies IGFBP-3 plays a role in protecting cells against abnormal cell growth.

IGFBP-3 protein expression was then examined in the normal and lung cancer cell lines to see if there was also increased IGFBP-3 expression under hypoxia at the protein level (6.1.4). However there was no difference in expression between normoxic and hypoxic cells over 24 h in any of the normal and lung cancer cell lines. Although hypoxia altered mRNA expression of IGFBP-3, this was not translated to the protein level.

It is not well understood what the relationship is between gene expression at the mRNA level and the corresponding protein level in cancer cells. Expression at the mRNA level is not a predictor of expression at the protein level in lung cancer samples. A study which compared mRNA and protein expression of a group of genes in lung adenocarcinomas found that there was no significant correlation between mRNA and protein expression and that only 17% of proteins correlated with corresponding mRNA expression [339]. Other studies found in other cells that there was also no correlation between mRNA and protein expression [340] [341]. There may be some transcriptional mechanism underling the quantity of these proteins in lung adenocarcinomas.

The expression levels of IGF-1R were then examined at the mRNA and protein level in normal and lung cancer cells (6.1.5). A recent study showed that IGFBP-3 in association with IGF-1R, can predict prognosis in squamous cell carcinoma of the head and neck (SCCHN) [342]. The results of intratumoural expression of IGF-1R and IGFBP-3 by immunohistochemistry showed that positive IGF-1R and IGFBP-3 expression was observed in 96 (73.3%) and 70 cases (53.4%), respectively and that when IGF-1R and IGFBP-3 were co-expressed patients had a poorer prognosis. The prognostic role of IGFBP-3 expression was found to be dependent on IGF-1R expression in the study of four subgroups classified according to IGF-1R and IGFBP-3 expression. Co-expression of IGFBP-3 with IGF-1R may also be used as a predictor for poor prognosis in other cancers and should be further investigated.

In comparing IGF-1R expression under hypoxia it was also found that mRNA and protein expression results were different. In A549 cells, IGF-1R mRNA expression was significantly decreased at 24 h hypoxia compared to normoxic cells ($P=0.0389$). At the protein level however, IGF-1R expression was decreased at 4 h ($P=0.0077$), 9 h ($P=0.0022$) and 24 h ($P=0.0299$) hypoxia compared to corresponding normoxic cells. In H1299 cells, IGF-1R mRNA expression was decreased at 2 h and 4 h hypoxia compared to normoxic cells. This was significant at 4 h hypoxia ($P=0.0283$). IGF-1R mRNA expression then increased at 24 h under hypoxia in normoxic cells but this was not significant. There was no change in IGF-1R protein expression at 4 and 9 h between normoxic and hypoxic cells but at 24 h IGF-1R protein expression decreased in normoxic cells compared to hypoxic cells ($P=0.0011$). These changes in IGF-1R expression between the mRNA and protein at different time intervals need to be investigated further as other factors may be involved.

IGF-1R activation protects cells from a variety of apoptosis-inducing agents, including hypoxia. It has been shown that overexpression of IGF-1R promotes transformation of normal cell lines and enhances cell survival in response to hypoxia [332]. In HBEC4 cells, IGF-1R protein expression was significantly higher in hypoxic cells compared to normoxic cells at 9 h ($P=0.0050$) and 24 h ($P=0.0044$). At the mRNA level there was no change in IGF-1R expression between normoxic and hypoxic cells (Fig 6.11). The increase in IGF-1R protein expression in HBEC4 cells under prolonged hypoxia exposure may prevent apoptosis in these cells and enable the survival of these normal cells under hypoxia which may lead to the transformation of these cells to cancerous cells.

IGF-1 mRNA expression was examined in response to hypoxic stress in normal and lung cancer cells up to 24 h and to see if changes in IGF-1 expression may coincide with changes in IGFBP-3 and IGF-1R expression. IGF-1 mRNA expression in A549 and H1299 cells increased significantly under hypoxia at 2 h, 4 h and 24h compared to normoxic cells (Fig 6.12). In HBEC 4 cells, IGF-1 mRNA expression significantly decreased under hypoxia up to 24h compared to normoxic cells. The results in the NSCLC cell lines were as expected as it has been reported that high IGF-1 levels are associated with an increased risk of lung cancer [343]. The reason for the decrease in IGF-1 suggests that it may be bound to the IGF-1R which explains the high IGF-1R protein expression in HBEC4 cells.

Expression levels of IGF-1, IGF-1R, IGFBP-3 and IGFBP-5 mRNA were then examined in 10 matched normal and tumour samples from NSCLC patients (6.1.7). The expression of IGFBP-3 and its significance in non-small cell lung cancer (NSCLC) tissue samples are unknown. These samples were a mixture of adenocarcinoma and squamous cell carcinoma tumours. There was a significant increase in expression of IGFBP-3 ($P=0.0009$), IGFBP-5 ($P=0.0189$) and IGF-1R ($P=0.0001$) in tumour compared to matched normal tissue (Fig 6.15). IGF-1 expression was significantly reduced in tumour compared to matched normal tissue ($P=0.0001$). Increase in IGFBP-3 expression was also found in breast [344] and esophageal tumour samples [161].

Expression of IGFBP-3, IGF-1R and EGFR were examined at the protein level in 10 matched normal and tumour tissue in NSCLC patients. IGFBP-3 protein expression was significantly increased in tumour tissue compared to matched normal tissue in NSCLC patients ($P=0.0001$). This result was compared with results for IGF-1R and EGFR protein expression found in the same group of NSCLC patients as shown in Fig 4.8. High IGFBP-3 expression was observed in samples also expressing high IGF-1R and EGFR.

It has been reported that upregulation of both EGFR and IGFBP-3 was observed in around 60% of primary esophageal squamous cell carcinomas [178]. IGFBP-3 mRNA and protein expression was increased in EGFR overexpressing tumours suggesting IGFBP-3 may function through the EGFR signalling pathway to modulate EGFR activity during carcinogenesis. It was found that when EGFR kinase activity was blocked using an inhibitor, IGFBP-3 overexpressing cells were growth inhibited compared with controls treated with inhibitor. The interaction between IGFBP-3 and the EGFR may influence whether IGFBP-3 acts as a growth stimulator or inhibitor in cancer cells. Therefore, EGFR targeted therapies may have increased effect on tumours expressing high levels of IGFBP-3.

It has been documented that loss of IGFBP-3 expression occurs in the early stages of NSCLC due to methylation of the IGFBP-3 promoter [180][345]. A DNA methyltransferase inhibitor was used to examine IGFBP-3 expression between normal and lung cancer cells. A549 and SKMES-1 cells were chosen to compare expression between an adenocarcinoma and a squamous cell carcinoma cell line.

Treatment with DAC decreased IGFBP-3 mRNA expression in A549 cells ($P=0.0036$) and SKMES-1 cells ($P=0.0154$). The opposite was found in HBEC 4 treated cells as treatment with DAC increased IGFBP-3 RNA expression ($P=0.0122$) due to promoter demethylation. Further investigation is needed to explain the difference in IGFBP-3 mRNA expression between normal and lung cancer cells. Translational or post-translational mechanisms may be involved in regulating its expression following treatment with DAC. The concentration of DAC used in this experiment was $1\mu\text{M}$. Different results may be found using higher or lower concentrations and may be investigated further.

Further studies investigating the role of the IGFBPs in NSCLC are needed to determine if they are involved in mediating drug resistance in hypoxic environments. The changes in IGFBP expression in NSCLC and HBEC cell lines under hypoxia in this chapter suggest that they are regulated by hypoxia. These changes need to be examined further at longer time points under hypoxia and also at the protein level. The expression of these binding proteins also needs to be examined in additional matched normal and tumour NSCLC patients. Due to the time constraints of this project IGFBP-3 and IGFBP-5 expression were only examined in 10 patients. It would also be interesting to examine changes in IGFBP-3 and IGFBP-5 expression in tissue from healthy disease free individuals and compare these results to NSCLC patients.

Concluding Discussion

Lung cancer is the leading cause of cancer related death in Ireland and worldwide. Due to difficulties in early detection, lung cancer has a high morbidity/mortality, with five year survival rates continuing to remain poor at approximately 15% [3]. New therapeutics and/or biomarkers are required to aid in the treatment of the disease and to prolong survival.

The EGFR and IGF-1R are two powerful pathways in NSCLC. EGFR targeted therapies are an established first and second line treatment for NSCLC [251] [252] [253]. In recent years [258] the IGF-1R has emerged as an attractive target for anti-cancer drug development [259] [260]. Like the EGFR it is over-expressed in several tumour types where it plays a role in promoting tumour growth by inhibition of apoptosis, transformation, metastasis, and angiogenesis [261] [262] [263]. IGF-1R and EGFR share common downstream signalling molecules. Crosstalk occurs between these receptors as they share common downstream signalling molecules [267] [193]. This complex cross-talk between EGFR and IGF-1R activates the downstream signalling cascades in both pathways and this may have an adverse effect on current anti-EGF receptor treatments in lung-cancer patients [268] [269].

Due to the success of EGFR inhibitors in the treatment of patients with EGFR amplification and mutations identifying functional polymorphisms in the IGF-1R pathway may also be a way to select patients that would benefit from combined EGFR and IGF-1R inhibitors resulting in improved treatment for individuals with reduced toxicities. Given the important role of the IGF-1R signalling pathway in tumour cell survival and growth, it is possible that genetic variation may play a role in the activation of this pathway in cancer [138] [139].

In this thesis 198 NSCLC patients and 866 control patients were screened for the presence of SNPs in exons 16-20 of the IGF-1R TK domain. The only SNP found in the coding region was in exon 16 (rs2229765) (Table 3.1). No significance was found in the genotype ($p=0.5487$) frequency or in the allelic ($p=0.9082$) frequency. When patients were age and sex matched there was also no significance found in the genotype ($p=0.4351$) frequency or in the allelic ($p=0.2636$) frequency. The Kaplan-Meier survival analysis showed no significant difference in genotype distribution (Figure 3.2) or IGF-1R expression (Figure 3.3) in NSCLC patients. Even though no significant association of SNP rs2229765 was found with NSCLC it may play a

possible role in drug resistance, the function of this SNP should be investigated further. In the case of IGF-1R polymorphisms in the tyrosine kinase domain, their use as biomarkers in patient drug targeted therapy seems unlikely.

In immunohistochemistry analysis it was found that IGF-1R and EGFR expression when examined in isolation does not have prognostic relevance in NSCLC. It was revealed though that high coexpression of EGFR and IGF-1R were identified in 35% of our cohort of NSCLC patients and was associated with a poorer overall survival on univariate and multivariate analysis ($P=0.048$; $P=0.05$) indicating that these two biomarkers taken together correlate to a poor prognosis in resected NSCLC. A study by Ludovini et al [273] also showed that high coexpression of both IGF-1R and EGFR is associated with shorter disease-free survival and a trend toward a poorer OS ($p=0.09$) in patients with resected NSCLC. The abundance of both receptors in a subset of NSCLC patients, in particular SCC, indicates that both pathways could potentially be activated to cause an aggressive tumour phenotype. Treating this subset of patients with a combination of IGF-1R and EGFR targeted therapies may prove to be an effective strategy for the future [285].

IGF-1R and EGFR were also significantly overexpressed relative to matched normal tissues in NSCLC patients ($P=0.004$ and $P=0.0015$ respectively) in fresh frozen resected tumours when analysed by Western blot. When receptor expression was examined in terms of histology in NSCLC patients, SCC patients had significantly higher expression of IGF-1R ($P=0.0021$) and EGFR ($P=0.0039$). However in ADC patients IGF-1R ($P=0.1994$) and EGFR ($P=0.0913$) expression were not found to be significantly expressed. In the immunohistochemistry analysis SCC patients were also found to have higher co-expression of IGF-1R and EGFR than non-SCC (χ^2 13.59, $p=0.0002$). These findings are in contrast to the initial report by Merrick et al which demonstrated that high IGF-1R expression was associated with ADC histology and a poorer survival in 191 resected NSCLC patients [276].

A panel of NSCLC cell lines were then screened for EGFR and IGF-1R protein expression by Western blot analysis. HCC827 cells had the highest level of EGFR expression out of the 5 NSCLC cell lines. HCC827 cells had significantly higher EGFR expression than A549 cells ($P<0.05$) and SKMES-1 ($P<0.05$) (Fig 4.7). HCC827 cells did not have significantly higher expression than H1819 and H1299

cell lines. A549 cells had the highest level of IGF-1R expression. A549 cells had significantly higher IGF-1R expression than H1819 ($P<0.001$), H1299 ($P<0.001$) and HCC827 ($P<0.001$) cells.

This chapter highlights the importance of the interaction between the IGF-1R and EGFR in NSCLC, particularly SCC. Targeting both receptors simultaneously may prove a more effective anti-tumour strategy than using EGFR targeted therapies alone in the management of NSCLC. This is supported by pre-clinical studies and a number of ongoing clinical trials are addressing this issue, particularly in the second-line treatment of NSCLC [346] [94] [311].

A549 and HCC827 cells were chosen to examine the effects of the monoclonal antibody R1507 and also in combination with the EGFR TKI erlotinib. HCC827 cells have mutated as well as amplified EGFR which are highly sensitive to erlotinib at nanomolar concentrations [315] and A549 cells containing the KRAS mutation and high IGF-1R expression are known to be resistant to erlotinib [316]. Sensitivity of NSCLC cell lines to R1507 was examined in order to identify what subset of NSCLC patients would benefit from treatment with this antibody.

Both NSCLC cell lines were sensitive to the R1507 though neither cell line displayed more than 50% growth inhibition by R1507 even at high concentrations of the antibody. This was unexpected as HCC827 cells express low levels of IGF-1R and it has been previously shown that this cell line was not sensitive to R1507. The concentration of 25 $\mu\text{g/ml}$ was chosen for further experiments as both HCC827 ($P<0.05$) and A549 ($P<0.05$) cell lines had significantly lower cell proliferation at this concentration. Results to these experiments suggest that IGF-1R expression levels are not a predictor of response to treatment with R1507 considering A549 and HCC827 cells which express high and low levels of IGF-1R respectively were both moderately sensitive to the antibody. This will need to be investigated further in a panel of NSCLC cell lines expressing various levels of IGF-1R.

Results of the treatment of cells with erlotinib were as expected as HCC827 cells contain an EGFR mutation sensitive to treatment with EGFR TKIs they were sensitive to erlotinib at low concentrations. A549 cells were resistant to treatment with erlotinib at lower concentrations. There was no effect on cell proliferation and

cell growth in A549 cells treated with erlotinib at concentrations less than 1000nM. The concentration of erlotinib chosen for the remaining experiments in the chapter was 100nM as this ($P=0.0204$) resulted in a significant decrease in cell proliferation in HCC827 cells.

HCC827 and A549 cell lines were then treated with combined 25 μ g/ml R1507 and 100 nM erlotinib to examine the effect on cell proliferation and apoptosis. Compared to untreated A549 cells, those treated with R1507 and erlotinib had significant reduction in cell growth than R1507 treated cells alone ($P<0.05$). In HCC827 cells there was no further reduction to cell growth when R1507 was added in combination with erlotinib compared to R1507 treated cells alone. The effect of combined R1507 and erlotinib treatment on apoptosis in A549 and HCC827 cells was then examined by measuring caspase 3/7 activation. This showed that levels of caspase 3/7 activation were increased in A549 cells treated with combined 25 μ g/ml R1507 and 100nM erlotinib treatment. In HCC827 cells, increased concentrations of 100 μ g/ml R1507 and 1000nM erlotinib significantly increased caspase 3/7 expression ($P<0.01$). This may mean that concentrations of 25 μ g/ml R1507 and 100nM erlotinib retard cell growth in HCC827 cells whereas higher concentrations induce activation of caspase 3/7 and lead to cell apoptosis. The ability of combined R1507 and erlotinib to induce apoptosis needs to be confirmed by flow cytometry.

The aim of the final chapter was to examine the expression levels of IGFBP-1 to -6 in NSCLC under hypoxic conditions. There is not a lot known about the role IGFBPs play in hypoxic conditions and as tumour hypoxia is a common occurrence in NSCLC, there is a need to learn more about the importance of these ligands of the IGF-1R signalling pathway in the context of tumour aggressiveness and drug resistance. IGFBPs expression levels were examined using a panel of normal and lung cancer cell lines as well as matched tumour and normal tissue from NSCLC patients. A549 cells had significantly higher expression of IGFBP1-6 than HCC827, H1819, H1299 and SKMES-1 cells. A change in IGFBP-3 expression under hypoxia was observed across lung cancer and normal cell lines. IGFBP-3 expression increased (not significant) in H1299, A549, SKMES-1 and HCC827 cells under hypoxia. IGFBP-3 expression increased in all normal cell lines under hypoxia. There was a significant increase in IGFBP-3 expression in HBEC 4 ($P=0.0072$) and HBEC 5 ($P=0.0006$) cell lines under hypoxia (Fig 6.6). This increase in expression of

IGFBP-3 under hypoxia in HBEC 3 ($P=0.0808$) cells was not quite significant. When IGFBP-3 expression was examined over 24 h hypoxia, IGFBP-3 expression increased significantly in A549 and HBEC4 cell lines under hypoxia ($P<0.05$). A decrease in IGFBP-5 expression was observed in A549 cells under hypoxia over 24 h.

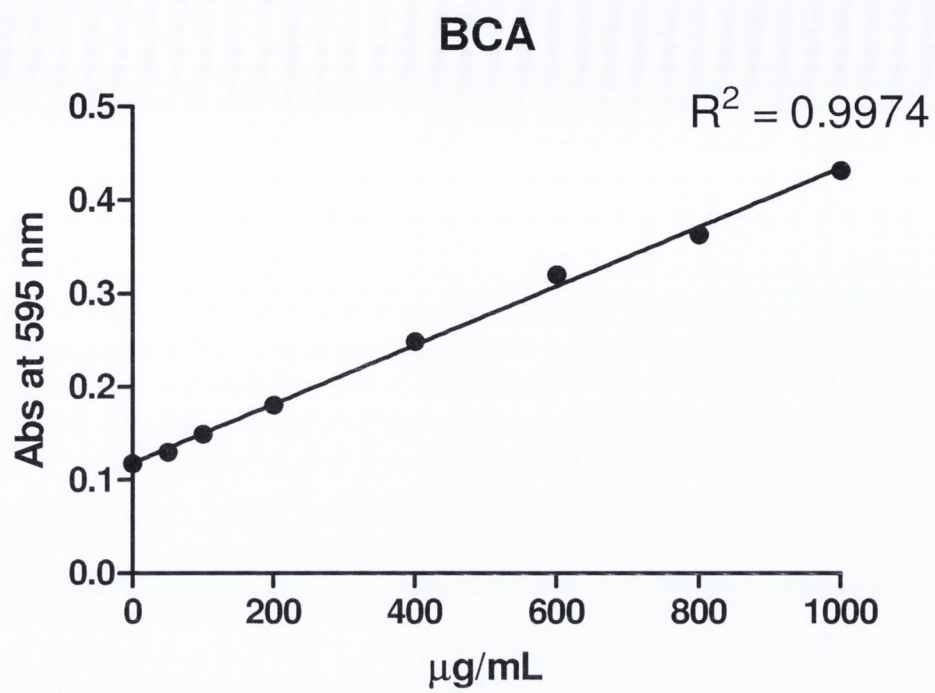
There was a significant increase in mRNA expression of IGFBP-3 ($P=0.0009$), IGFBP-5 ($P=0.0189$) and IGF-1R ($P=0.0001$) in tumour compared to matched normal tissue. IGF-1 expression was significantly reduced in tumour compared to matched normal tissue ($P=0.0001$). IGFBP-3 protein expression was significantly increased in tumour tissue compared to matched normal tissue in NSCLC patients ($P=0.0001$). High IGFBP-3 expression was observed in samples also expressing high IGF-1R and EGFR. The interaction between IGFBP-3 and the EGFR may influence whether IGFBP-3 acts as a growth stimulator or inhibitor in cancer cells. Therefore, EGFR targeted therapies may have increased effect on tumours expressing high levels of IGFBP-3. This may play a positive or negative role in NSCLC.

HBEC-4, A549 and SKMES-1 were also treated with a DNA methyltransferase inhibitor (DNMTi) to examine IGFBP-3 mRNA expression upon inhibition of DNA methylation. Treatment with DAC decreased IGFBP-3 mRNA expression in A549 cells ($P=0.0036$) and SKMES-1 cells ($P=0.0154$) indicating that translational or post-translational mechanisms may be involved in regulating its expression. The opposite was found in HBEC 4 treated cells as treatment with DAC increased IGFBP-3 RNA expression ($P=0.0122$) due to promoter demethylation.

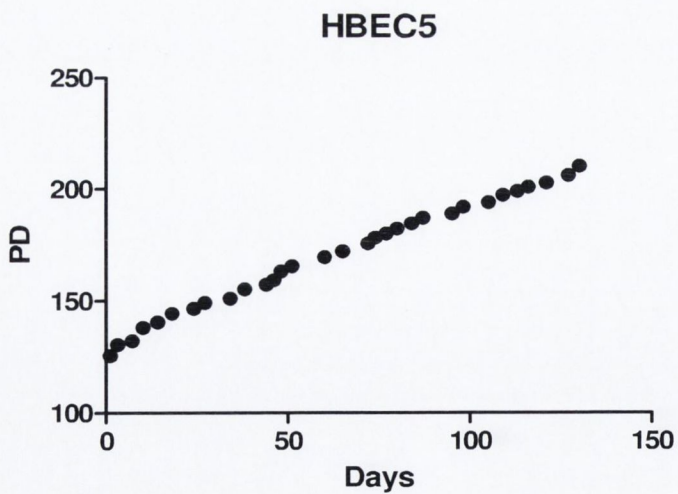
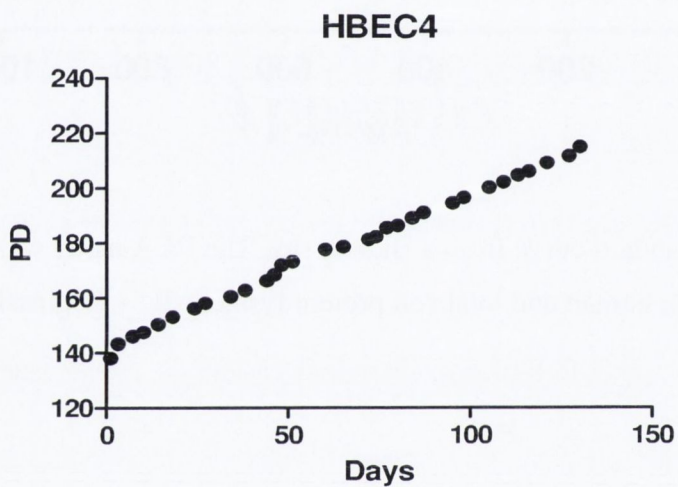
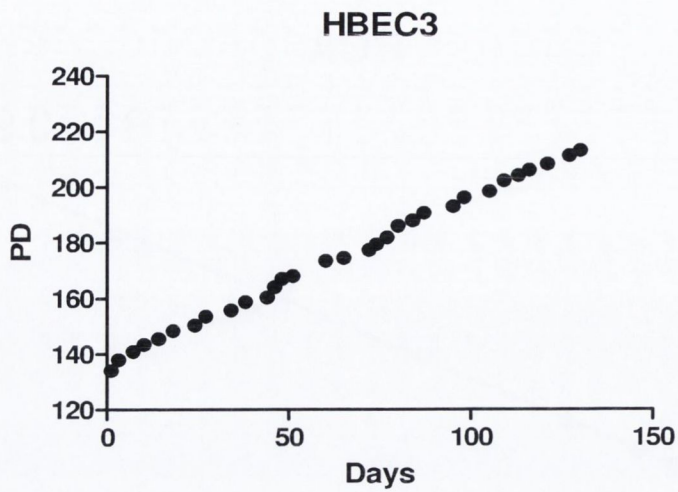
This thesis highlights the importance of IGF-1R and EGFR in NSCLC. High coexpression of EGFR and IGF-1R were identified in 35% of our cohort of NSCLC patients and was associated with a poorer overall survival on univariate and multivariate analysis ($P=0.048$; $P=0.05$) indicating that these two biomarkers taken together correlate to a poor prognosis in resected NSCLC patients. SCC patients had significantly higher expression of IGF-1R ($P=0.0021$) and EGFR ($P=0.0039$). Targeting both receptors simultaneously may prove a more effective anti-tumour strategy than using EGFR targeted therapies alone in the management of NSCLC. When this was examined in NSCLC cell lines, combined treatment of 25 μ g/mL R1507 and 100nM erlotinib in A549 cells significantly decreased cell growth

($P < 0.01$) and increased caspase 3/7 ($P < 0.01$) more than treatment with either inhibitor alone. IGF-1R protein expression was also significantly reduced ($P < 0.001$) at concentrations of combined $25\mu\text{g/mL}$ R1507 and 100nM erlotinib in HCC827 cells. The apoptosis assay and the BrdU assay confirms that HCC827 cells are sensitive to treatment with R1507 which has not been shown before in the literature. This suggests that patients with low IGF-1R expression should not be excluded from treatment with the IGF-1R targeting antibody R1507. Results in this thesis confirm that patients with high expression of IGF-1R and EGFR may benefit from combined R1507 and erlotinib treatment. This treatment strategy may increase survival in this subset of patients who have the poorest overall survival of NSCLC patients. Dual targeting of the IGF-1R and EGFR with R1507 and erlotinib treatment may also prevent resistance to erlotinib in NSCLC patients.

Appendix



Representative standard curve from a BCA assay. The BCA assay was used to quantify protein in human and total cell protein lysates. (R^2 – Regression coefficient)



Representative growth curves for HBEC3-5. (PD – Population doubling)

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