The interaction between the PI3K/Akt cascade and the Androgen Receptor in the development and progression of castrate resistant prostate cancer

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The work presented in this thesis was performed entirely by the author except as acknowledged. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Pamela McCall
June 2010

Dedication

This thesis is dedicated to my husband Ross for all his love, patience and support.

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TABLE OF CONTENTS

List of Figures

List of Tables

List of Publications

Published Papers

Edwards J, **Traynor P**, Munro AF, Pirret CF, Dunne B, and Bartlett JM
The role of HER1-HER4 and EGFRvIII in hormone-refractory prostate cancer.
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McCall P, Orange C, Seywright M and Edwards J
Nuclear Factor κB/p65 signallingis constitutively activated in castrate resistant prostate cancer and correlates with disease progression and metastases.

List of Abbreviations

ACTH	Adrenocorticotrpoic hormone
ADT	Androgen depravation therapy
AF-1	Transactivation Function 1
AF-2	Transactivation Function 2
AR	Androgen receptor
ASAP	Atypical Small Acinar Proliferation
BPH	Benign Prostatic Hyperplasia
Cap	Cancer of the prostate
CRPC	Castrate resistant prostate cancer
DAB	Diaminobenzidine
DAPI	4'-6-Diamidino-2-phenylindole
DBD	DNA binding domain
DHT	Dihydrotestosterone
DMSO	Dimethyl sulphoxide
ECL	Enhanced chemiluminesence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EtOH	Ethanol
FISH	Fluorescent in situ hybridisation
FITC	Fluorescent Isothiocyanate
FOXO	Forkhead transcription factors
GnRH	Gonadotrophin hormone releasing hormone
HAT	Histone acetyltranserases
HDAC	Histone deacetalase
HDR	High dose rate
HER 2-4	Human epidermal growth factor receptor
Hrg	Heregulin
HREs	Hormone response elements
HRP	Horse radish peroxidase
HSP	Heat shock protein
IF	Immunofluorescence
IGF-1	Insulin growth factor 1
IHC	Immunohistochemistry
Iκβα	Inhibitory Kappa Beta alpha
IKK	Inhibitory Kappa Beta Kinase (IKK)
IL-1-6	Interleukin 1-6
LBD	Ligand binding domain
LDR	Low dose rate
LH	Leutinising hormone
LHRH	Leutinising hormone releasing hormone
LUTS	Lower urinary tract symptoms
MAB	Maximum androgen blockade
MAPK	Mitogen activated protein kinase
MR	Mineralcorticoid Receptor

MMP-9	Matrix metalloprotease 9
mTOR	Mamalian target of rapamycin
PBS	Phosphobuffered saline
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3 Kinase
PIP2	Phosphatidyl inositol (4,5) bisphosphate
PIP3	Phosphatidyl inositol (3,4,5) trisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PIN	Prostatic intra neoplasia
PR	Progesterone receptor
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PVDF	Polyvinlylidene Difluoride
RHD	Rel homology domain
RTK	Receptor tyrosine kinases
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis
SHBG	Sex hormone binding globulin
SMRT	Silencing mediator for retinoid and thyroid
TBS	Tris buffered saline
TNFα	Tumour necrosis factor alpha
TNM	Tumour node metastasis
TTBS	Tris buffered saline-tween
PSMA	Prostate specific membrane antigen
UPS	Ubiquitin proteasome system
WST	Water soluble tetrazolium salts

Summary

Prostate cancer has high cancer incidence in males and is the second highest cause of male cancer related mortality. Currently the main stay therapy for localised and metastatic disease is maximum androgen blockade (MAB). This aims to inhibit androgen production or action, thereby reducing stimulation of the androgen receptor (AR). This in turn prevents the activation of androgen-regulated genes, which normally result in on-going growth and survival. Inhibition of testicular androgen production may be achieved surgically (bilateral orchidectomy) or chemically, using gonadotropin-releasing hormone (GnRH) agonists. The latter induces castrate levels of testosterone by down-regulating pituitary GnRH receptors (and therefore gonadotropin hormone production) through constant stimulation. The action of androgen may be blocked at a peripheral level using anti androgens, which inhibit ligand binding to AR and subsequent activation. Although this approach has initial response rates of over 80% the majority of men relapse with castrate resistant prostate cancer (CRPC) and this is the cause of significant morbidity and mortality. To overcome this and to improve patients treatment options the mechanisms of castrate resistance need to be addressed.

The PI3K/Akt cascade regulates several cellular processes such as proliferation and apoptosis. Akt activation results in phosphorylation of multiple substrates and has been implicated in prostate carcinogenesis and castration resistance. Research has suggested that Akt interacts with signallingcascades implemented in carcinogenesis, in particular the NFkB cascade and AR signalling. The current study investigated the hypothesis that the expression and activation of PI3K/Akt cascade influences the

progression to castrate resistant disease using clinical prostate cancer tumours. Fluorescent *insitu* hybridisation and Immunohistochemistry revealed that *PTEN* deletion was a common event in castrate resistant prostate cancer and low PTEN protein expression was significantly associated with a poor outcome. PTEN negatively regulates PI3K signalling. Consequently increased levels of PI3K and activated Akt (pAkt ser ^{308} and pAkt ser ^{473}) were significantly associated with a shorter time to biochemical relapse and shorter disease specific survival. Inhibition of PI3K resulted in a significant reduction in cellular proliferation and Akt phosphorylation.

The downstream affects of Akt activation were also investigated. Akt has been reported to directly activate the NFkB signallingcascade both directly and indirectly but no correlations between Akt and NFkB were observed in the current study. Using an immunohistochemical approach NFkB, IκBα and MMP-9 expression were observed to be significantly associated with shorter time to death from relapse and disease specific death. MMP-9 and IκBα expression were also significantly associated with metastases at relapse. Using paired hormone naive and castrate resistant LNCaP cells lines allowed the functional consequences of NFkB inhibition to be investigated. Reduced NFkB activation significantly inhibited cellular proliferation and induced apoptosis in both cell lines.

Having shown a significant link between expression and activation of the PI3K cascade and progression to castrate resistant disease, the interaction between Akt and the AR was investigated in both clinical prostate tumours and cell lines. The phosphorylation of AR at the Akt consensus site serine 213 (pARser^{213}) was

significantly associated with disease progression. Patients with high expression pARser^{213} had a significantly shorter time to death from relapse and disease specific survival. Additionally 42% of patients displayed an increase in pARser^{213} expression, these patients also had a significantly shorter time to death from relapse and disease specific survival. Inhibition of PI3K resulted in a reduction of pARser^{213} expression in both cell lines and using siRNA knockdown to target PI3K p85 regulatory subunit reduced pARser^{213} expression. This research highlights the impact of both the PI3K/Akt and NFkB signallingcascades on prostate cancer progression and development of castrate resistant disease. In particular this study highlights the impact of Akt phosphorylation in castrate resistant prostate cancer patients. Therefore phosphorylation of AR at serine 213 may serve as a diagnostic tool to predict patient outcome in response to maximum androgen blockade and inhibition of AR 213 phosphorylation via the Akt cascade may be an effective therapeutic avenue to investigate for treatment of prostate cancer.

1. Introduction

1.1 Prostate cancer incidence and prevalence

Cancer of the prostate is recognised as a significant problem facing the male population world wide. It is the most common cancer in men in the UK accounting for nearly a quarter of all new male cancer diagnoses (1). In 2006, there were 35,515 new cases of prostate cancer diagnosed in the UK, which amounts to an incidence of 97 men each day or one man every 15 minutes being diagnosed with prostate cancer. For men in the UK the lifetime risk of being diagnosed with prostate cancer is 1 in 10 (1).

As men live longer an increase in both incidence and mortality of prostate cancer is observed. Prostate cancer incidence rates increase rapidly with age and the highest rates occur in the oldest age groups. The incidence of prostate cancer per 100,000 men aged 55-59 is 144; ten years later, the rate more than triples to 500 and by 85+ the rate is more than five times higher at 789 (1). Results from post-mortem data, indicate that roughly half of all men in their fifties have histological evidence of cancer in the prostate, which rises to 80% by age 80, but only 1 in 26 men (3.8%) will die from this disease (2;3). Thus, men are more likely to die with prostate cancer than from it.

At present little is known about what causes prostate cancer but there are some factors that are thought to increase a man's chance of developing the disease. Apart from age, the primary risk factor is hereditary. Men who have close male relatives who have had prostate cancer are slightly more likely to develop the disease

themselves. It is belived that a man's risk of developing prostate cancer is significantly increased if their father or brother developed prostate cancer at or under the age of 60, or if more than one man on the same side of the family has had prostate cancer. Only a small number of prostate cancers (5–10% or less than one in ten cases) are thought to be due to an inherited altered gene running in the family. However, a major susceptibility locus for prostate cancer, the "Human Prostate Cancer Gene 1" has been identified on chromosome 1. This gene locus has been implicated in around 30% of hereditary prostate cancers in one study (4). A strong family history of breast cancer may also increase the risk of prostate cancer, particularly if a close relative has been diagnosed with breast cancer under the age of 40. The BRCA1 and BRCA2 genes associated with a higher risk of breast cancers, are also linked to a higher risk of prostate cancer. Research has shown that carrying the BRCA2 gene, increases the risk of getting prostate cancer before the age of 65 by seven times, when compared with the average man under 65 (5). Once over 65, the risk is reduced to 4.5 times, when compared to the average man.

Other risk factors include diet, lifestyle and race. In the USA there are significant differences in prostate cancer incidence between racial groups. Prostate cancer incidence rates for black Americans are more than 50% higher than for white Americans while rates for Asian Americans are 40% lower than for white Americans. In 2001-2005 the age-standardised (to the 2000 US population) incidence rate was 249 per 100,000 for black men, 157 per 100,000 for white men and 93.8 per 100,000 for Asian men (6). Prostate cancer occurs less frequently in

Asia. However, when Asians leave their homelands to live in the west, the incidence rate multiplies by 5 times suggesting the role of diet plays a part.

1.2 Prostate cancer pathology

1.2.1 Normal Prostate pathology

The prostate is a small gland about the size of a walnut that sits under the bladder and in front of the rectum (Figure 1). The function of the prostate is to store and secrete a slightly alkaline (pH 7.29) fluid, which usually constitutes 25-30% of the volume of the semen along with spermatozoa and seminal vesicle fluid. The prostate gland anatomy is divided into glandular tissue or non-glandular tissue. The glandular tissue is epithelial and comprises of ducts and glands that secrete fluid and non-glandular tissue is fibromuscular stroma. The prostate glandular epithelium has three types of cells: basal, luminal secretory and neuroendocrine. Basal cells are fewer in number and their function is not wholly understood although they secrete components of the basement membrane. It is suggested that a subset of basal cells may be epithelial stem cells for the luminal epithelial cells (7). The luminal cells secrete components of the Prostatic fluid, express the Androgen receptor (AR) and also secrete prostate specific antigen (PSA) in an androgen dependent manner. The stroma is composed of fibroblasts, smooth muscles, endothelial cells, dentric cells, nerve cells, some inflammatory infiltrates such as mast cells and lymphocytes. Some stromal cells are androgen responsive and produce growth factors which act in a

paracrine fashion on the epithelial cells. Homeostasis is therefore modulated in part by the stromal-epithelial cross talk (8).

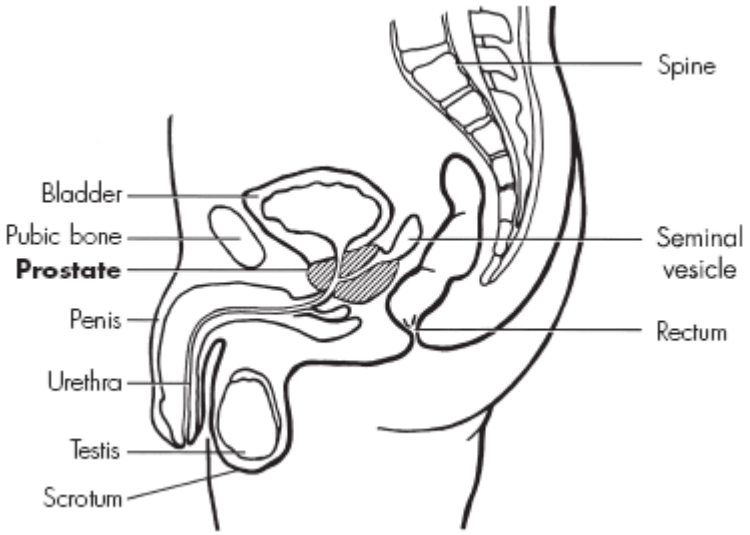

Figure 1.1: Prostate location

The position of the prostate gland and surrounding organs

The prostate gland is classified according to the Prostatic capsule and three zones: the transitional, the central, and the peripheral zones (Figure 1.2). The clinical significance of the zonal anatomy of the prostate is in understanding which areas of the gland are susceptible to benign and malignant disease.

- **Prostatic capsule**

The Prostatic capsule is a fibromuscular layer which encases the prostate. This layer is most prominent along the base of the prostate gland.

- **Transitional Zone of the Prostate Gland**

The transitional zone is the innermost part of the prostate gland and surrounds the urethra where it passes through the prostate. The transition zone, along with the central zone, begins to enlarge as men pass the age of 40. Due to the immediate proximity to the urethra, the enlargement of this part of the gland can cause bladder outflow obstruction. The transition zone makes up about 5% of the glandular volume and is the site of about 10% of prostate cancers.

- **The Central Zone of the Prostate Gland**

The central zone also begins to enlarge after men pass the age of 40. The central zone surrounds the transitional zone and constitutes about 25% of the non-glandular volume. About 5% of prostate cancer cases originate in the central zone.

- **The Peripheral Zone of the Prostate Gland**

The peripheral zone of the prostate gland is located in the back of the prostate gland closest to the rectum. The peripheral zone constitutes about 80% of the prostatic volume and is the site of about 80% of prostate cancers.

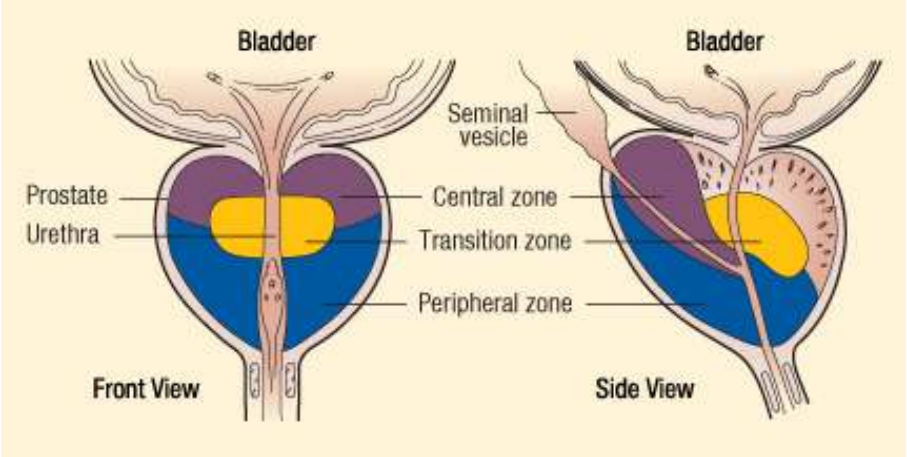

Figure 1.2: Zones of the prostate gland

The position of the prostate and the three zones

1.2.2 Prostate Histology

1.2.2.1 Benign Prostatic hyperplasia (BPH)

In this condition the epithelium and the fibrous tissue of the prostate undergo proliferative growth, causing enlargement of the prostate gland. It is extremely common, being found to some degree in most men beyond middle age. Histological BPH is characterized by the benign overgrowth of the transitional zone of the prostate which results in the part of the urethra that is surrounded by the prostate becoming constricted. This can cause various urinary symptoms, known collectively as lower urinary tract symptoms (LUTS) and obstruction to the neck of the bladder usually results in;

- difficulty in starting
- slowing of the urinary stream
- dribbling at the end of the stream

- bladder irritability

Currently BPH is treated with medical management or surgery.

1.2.2.2 Prostatic intraepithelial neoplasia (PIN)

Premalignant change in the prostate gland was identified in 1986 (9); McNeal and Bostwick identified the existence of dysplastic prostatic lesions which they named intraductal dysplasi. These lesions exhibited many features, including cytological atypia and nuclear polymorphism, which are associated with other premalignant conditions. In 1989, intraductal dysplasia was renamed prostatic intraepithelial neoplasia or PIN which brought the nomenclature into line with other pre malignant conditions such as CIN (cervical intraepithelial neoplasia). The clinical implications of PIN are controversial. PIN has been shown to occur approximately a decade before the onset of prostate cancer (2). Areas of PIN have been shown to be closely associated with foci of prostate cancer and the distribution of clinically relevant prostate cancer and PIN is similar (10). Although high grade PIN is not a cancerous condition it is a recognised risk factor of prostate cancer. There is a high percentage (40 to 50%) chance that a person with PIN will go on to develop prostate cancer at some stage of his life. Therefore people diagnosed with PIN are usually carefully monitored by techniques such as watchful waiting and repeated biopsy tests may be taken to monitor any development of the condition.

1.2.2.3 Atypical small acinar proliferation (ASAP)

ASAP is another premalignant change that denotes a focus of atypical glands that are suspicious for cancer. The histological findings of ASAP are characterized by the presence of abnormal or atypical glands that have insufficient cytological or architectural atypia for a definitive diagnosis of cancer and follow-up of the patient is warranted. ASAP has been observed in 2% of biopsy specimens, whereas PIN is present in 4% to 16% of contemporary needle biopsy specimens, (11-13). These findings can occur together in specimens from the same set of biopsies without cancer being present.

1.2.2.4 Cancer of the prostate (CaP)

The majority of prostate cancers are adenocarcinoma in origin. Adenocarcinoma is the term used to describe a cancer that has originated in epithelial cells that line certain internal organs and have gland-like properties (Figure 1.3). Prostate cancer may also have the form of small cell carcinoma and squamous cell carcinoma. Small cell carcinoma is made up of small round cells, and typically forms at nerve cells. Small cell carcinoma is very aggressive in nature and as it does not lead to an increase in PSA, it can be difficult to detect in comparison to adenocarcinoma and generally has reached an advanced form upon detection. Squamous cell carcinoma is very aggressive in nature and like small cell carcinoma there is no increase in PSA when it progresses. There are other, more rare forms of prostate cancer, these include sarcomas and transitional cell carcinoma; the latter seldom develops in the prostate but derives from primary tumours present in the bladder or urethra. Prostate

cancer is divided into two subgroups: locally confined or latent prostate cancer and locally advanced and metastatic prostate cancer.

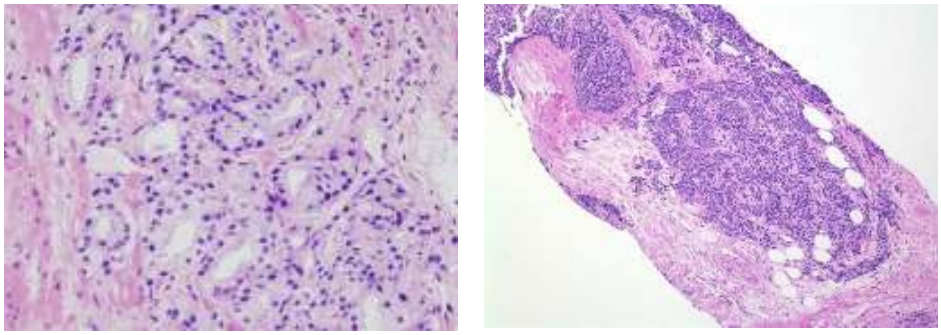

Figure 1.3: Prostate cancer histology

Adenocarcinoma (a) and (b) Small cell carcinoma of the prostate gland

- **Locally confined cancer of the prostate**

The development of clinically progressive disease does not always occur within the lifetime of every patient with histological evidence of cancer. As a result, while some patients have a high risk of dying of prostate cancer, the remainder lives with localised disease, eventually succumbing to death from other causes. Prostate cancer is unique among potentially fatal human cancers in that a wide difference exists between the incidence of histologically confirmed cancer and clinical disease. Scardino (14) attempted to estimate the extent of the discrepancy between the high prevalence of the disease at autopsy and the low incidence of clinical disease and estimated that only 1 in 95 men with cancer were diagnosed with the disease, and only 1 in 323 men with prostate cancer died of the disease. This indicates that the

majority of small prostatic tumours which are detected at autopsy do not progress to clinical prostatic disease. It is thought that many of these small, well differentiated prostate cancers lack the ability to grow, differentiate and metastasize within a patient's lifetime. At diagnosis, localised or organ-confined disease may be treated and potentially cured with treatments such as radical surgery or radiotherapy.

However many patients present with extracapsular or advanced disease; this is termed locally or regionally advanced, or metastatic disease. It is this subset of prostate cancer patients who make up the majority of the 12,000 deaths per year in the UK alone, which contributes in making prostate cancer the second most common cause of cancer death in men in Europe (15)

- **Locally advanced and metastatic cancer of the prostate**

At diagnosis, approximately ¼ of men present with locally advanced or metastatic prostate cancer. This indicates that the cancer has spread beyond the capsule to local tissues or to distant lymph nodes and other sites, such as the bones. For patients presenting with advanced stage prostate cancer, the prognosis is significantly worse, with survival time limited.

1.3 Predictive and prognostic markers of prostate cancer

1.3.1 Prostate specific Antigen (PSA)

Prostate specific antigen (PSA) is a 32-kilodalton (kD) glycoprotein protease. It is synthesised by the ductal and acinar epithelium of the prostate gland and is secreted into the seminal fluid after ejaculation. The basement membrane of the prostatic

epithelium normally creates a barrier preventing the escape of PSA into the circulation. Disruption of the basement membranes by disease allows PSA to enter the systemic circulation. Serum PSA is measured in nanograms per millilitre (ng/ml) quantities and is a sensitive marker for detecting prostate cancer. After radical surgery, serum measurements should nadir to undetectable levels. The recurrence of measurable PSA is the initial sign of therapeutic failure. However many disease processes within the prostate gland including BPH and Prostitis are associated with elevated circulating PSA concentrations. Thus PSA evaluation is not a diagnostic test for prostate cancer but is useful in helping to identify men in whom further investigations such as a prostate biopsy would be appropriate. The test has dramatically changed the way that men are evaluated and treated for prostate cancer (16). Prior to the PSA evaluation era nearly 70% of men diagnosed with prostate cancer had progressed to the locally advanced or metastatic stage. Since the test was introduced into clinical practice in 1986, the early diagnosis and management of prostate cancer has been revolutionized and much has been learned about the strengths and weaknesses of this assay. PSA testing not only helps in the early diagnosis but also assists in assessing the response to therapy, determining tumor progression, and, in its most controversial role, screening for prostate cancer.

1.3.2 Prostate specific membrane Antigen (PSMA)

Prostate specific membrane antigen (PSMA) is an antigenic marker to prostate epithelial cells which can be detected in the serum. PSMA is a 100-kd type II membrane protein that is expressed in normal Prostatic epithelial cells, BPH, PIN,

and cancer. PSMA represents an attractive antigen for antibody-based diagnostic and therapeutic intervention in prostate cancer, as it is highly restricted to the prostate and over expressed in all tumour stages. Immunohistochemical studies indicte that PSMA expression is increased in castrate resistant disease and also increased concentrations of PSMA are present in the serum of prostate carcinoma patients compared with healthy individuals (17).Additionally, PSMA primers used in a reverse transcriptase–polymerase chain reaction (RT-PCR) study have been used to detect micro metastases in lymph nodes removed during radical prostatectomy (18). At present PSMA is being evaluated as a means for therapy. Vaccines including dendritic cell (DC)-based vaccines hold promise as a safe therapy for prostate cancer, here dendritic cells are primed with PSMA and infused into the patient. This is intended to produce a specific immune response to prostate cells. Using PSMA as a guide to identify and target prostate cells, radioactive isotopes and cytotoxic agents can be delivered to these cells. These findings have spurred development of PSMA-targeted therapies for cancer, and first-generation products have entered clinical testing.

1.3.3 Prostate tumour stage

Tumour stage is a measure of the tumour size and the extent of disease spread. The TNM (Tumour Node Metastasis) classification breaks tumour stage into three components; the T component which reflects the extent of disease at the primary site, the N component which reflects the presence or absence of nodal metastases and the M component which indicates the presence or absence of distant tumour metastases.

The UICC (International union against cancer) 2002 TNM classification is used for staging cancer of the prostate (Table 1).

1.3.4 Grading of prostate cancer

The grade of a tumour depends on its histological appearance and reflects differentiation associated neoplasia. The most commonly used system for grading cancer of the prostate is the Gleason grading system (19). This classification describes tumour growth according to the degree of cellular differentiation, which is graded 1-5. Tumours can be graded as well differentiated, moderately differentiated or poorly differentiated based on their microscopic appearance. A Gleason score is calculated using combined scores of the two most prominent tumour areas which can range from 2-10. Gleason grade 2-4 (well differentiated), 5-7 (moderately differentiated) and 8-10 (poorly differentiated). This score has been shown to provide significant prognostic information and also to be readily reproducible (20).

T - Primary tumour		
	TX	Primary tumour cannot be assessed
	T0	No evidence of primary tumour
T1 Clinically unapparent tumour not palpable or visible by imaging		
	T1a	Tumour incidental histological finding in 5% or less of tissue resected
	T1b	Tumour incidental histological finding in more than 5% of tissue resected
	T1c	Tumour identified by needle biopsy (e.g., because of elevated prostate-specific antigen (PSA) level)
T2 Tumour confined within the prostate[1]		
	T2a	Tumour involves one half of one lobe or less
	T2b	Tumour involves more than half of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3 Tumour extends through the prostatic capsule[2]		
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumour invades seminal vesicle(s)
T4 Tumour is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, levator ani and/or pelvic wall		
N - Regional lymph nodes[3]		
	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph node metastasis
	N1	Regional lymph node metastasis
M - Distant metastasis[4]		
	MX	Distant metastasis cannot be assessed
	M0	No distant metastasis
	M1	Distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s)
1	*Tumour found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c*	
2	*Invasion into the prostatic apex, or into (but not beyond) the prostate capsule, is not classified as T3, but as T2.*	
3	*The regional lymph nodes are the nodes of the true pelvis, which are essentially the pelvic nodes below the bifurication of the common iliac arteries. Laterality does not affect the N classification.*	
4	*When more than one site of metastasis is present, the most advanced category should be used*	

Table 1.1 : TNM classification used for prostate cancer staging

1.4 Treatment of prostate cancer

There are many different ways of treating prostate cancer. The optimal management of prostate cancer is determined primarily by tumour stage and grade, patient's age and general health and individual patient preference.

1.4.1 Watchful Waiting

The watchful waiting approach a standard treatment option applied in the management of localised prostate cancer. This is based on the knowledge that a large proportion of patients with prostate cancer will not die from the disease. Patients who are placed on Watchful waiting have well or moderately differentiated tumours and a <10 year life expectancy. Watchful waiting allows clinicians to monitor a patient's progress and intervene if the patient develops symptoms or evidence of disease progression. Watchful waiting tends to be offered to elderly men, especially those who have other illnesses, such as coronary heart disease or diabetes.

1.4.2 Radiotherapy

- **External beam**

Radiotherapy kills cancer cells in the treated area. This treatment uses a high dose of radiation just to the area of the prostate gland. Radiotherapy is a potentially curative treatment in patients with localised prostate cancer. Radiotherapy is generally offered to patients whose prostate cancer is between stage T1 and T3 whereby the cancer may have spread through the prostate capsule but is still localised. If the cancer has spread any further, radiotherapy is not likely to be used as a curative option. It is known that both increasing T-stage and a high Gleason score

compromise the chance of achieving a cure with radiotherapy (21;22). However radiotherapy is also given to locally advanced prostate cancer that has started to spread from the prostate into nearby tissues, with the aim of shrinking the tumour or, when the cancer has relapsed after treatment but has not spread to other parts of the body. This therapy is also used to treat bone pain due to metastatic disease.

- **Conformal Beam radiotherapy& Intensity Modulated Radiotherapy (IMRT)**

In conformal radiotherapy a computer is used to shape the radiotherapy beams to a more exact shape of the patients prostate. This technique aims to cut down the amount of healthy body tissue that receives radiation and reduce side effects to the bladder and the bowel. However, this is still being investigated in clinical trials.

Intensity Modulated Radiotherapy (IMRT) is a newer type of conformal radiotherapy where the radiotherapy beams are shaped with more precision to the cancer and vary the dose across the area of the tumour.

- **Brachytherapy**

Brachytherapy is another method of delivering radiation doses directly to the prostate whilst minimising exposure to the surrounding non-target tissues. The dose rate of brachytherapy refers to the intensity with which the radiation is delivered to the surrounding medium and is expressed in Grays per hour (Gy/h). Brachytherapy can be given as permanent Lowdose rate (LDR) by implanting radiation sources that emit radiation at a rate of up to 2 Gy.hr-1(23). This involves placing small radioactive pellets (about the size of a grain of rice) in the prostate tumour area and

leaving them there permanently to gradually decay. Over a period of weeks or months, the level of radiation emitted by the sources will decline to almost zero. The inactive seeds then remain in the treatment site with no lasting effect (22). Temporary high dose rate (HDR) brachytherapy is a newer approach to treating prostate cancer and is currently less common than seed implantation. It is used to boost therapy, as an extra dose in addition to external beam radiotherapy. This method offers an alternative way to deliver a high dose of radiation therapy that conforms to the shape of the tumour within the prostate, while sparing radiation exposure to surrounding tissues (24-28). HDR brachytherapy as a boost for prostate cancer allows the external beam radiotherapy course to be shorter than when using alone (24;25;28;29).

1.4.3 Radical prostatectomy

The purpose of surgery is to remove disease and classify the extent of disease staging. A radical prostatectomy is a surgical procedure in which the prostate, seminal vesicles and a sample of some nearby lymph nodes are removed. Radical prostatectomy offers the advantage of a single potentially curative intervention in patients with localised prostate cancer. This surgery is offered to patients who have localised but clinically significant disease in whom life expectancy is estimated to be at least 10 years. In these patients the expected benefits of surgery out-weigh the risk of morbidity.

1.4.4 Chemotherapy

Chemotherapy is used in castrate resistant prostate cancer patients to provide effective palliation of symptoms. Several chemotherapeutic agents are available to treat prostate cancer. The most common ones are docetaxel (Taxotere) and mitoxantrone (Novantrone). Chemotherapy is often given alongside other treatments such as palliative radiotherapy, bisphosphonates or pain-relieving drugs. Steroids such as prednisolone may also be given along side chemotherapy to make the treatment more effective. Research has shown that using a combination of docetaxel (Taxotere) and prednisolone can help to reduce symptoms such as pain, improve quality of life and increase survival (30). The molecular basis for taxol-based chemotherapy is well characterised. Taxanes, like other chemotherapeutic agents such as estramustine, inhibit microtubule disassembly by binding to tubulin and arresting cells at the G2/M phase.

1.4.5 Bisphosphonates

Bisphosphonates are drugs which are used to slow down or prevent bone damage. These drugs target areas of increased bone turnover. The osteoclast cells which breakdown old bones absorb the bisphosphonate, which subsequently slows down their activity and reduces bone destruction. Cancer cells that have spread to the bone release cytokines and growth factors that stimulate the osteoclasts making them overactive and bone is destroyed faster than it is rebuilt. Subsequently, the bones can become thinner and weaker, causing bone pain, hypercalcaemia and increased risk of fractures. Several types of bisphosphonates are used in cancer treatment, Zoledronic acid is used in treating castrate resistant prostate cancer with bone

metastases. Bisphosphonates may benefit men with castrate resistant disease with evidence of bone metastases (31;32). They do not actually alter the prognosis but they bind to areas of bone metastases where there is a lot of damage and can help to relieve bone pain. They may also help to prevent and slow down the bone breakdown (33).

1.4.6 Cryotherapy

Cryotherapy is a new method of killing cancer cells by freezing them. However it is still undergoing clinical trials due to uncertainties surrounding it. It can be used to treat men with localised prostate cancer that has not spread beyond their prostate gland. Tiny probes (cryoneedles) are inserted into the prostate gland through the wall of the rectum. This freezes the prostate gland killing the cancer cells, but some normal cells also die. The aim is to kill cancer cells while causing as little damage as possible to healthy cells. The main side effects of cryotherapy include impotence, urinary symptoms and rectal problems.

1.4.7 Hormonal therapy/androgen depravation therapy

Hormone therapy controls prostate cancer by stopping production of the male hormone testosterone. The key treatment for patients with advanced disease is based on androgen-deprivation therapy, and this approach has remained unchanged ever since Huggins and Hodges first demonstrated the hormonal dependence of prostate cancer in 1941 (34). Their work showed the growth suppressive effect of reducing endogenous androgen levels, by surgical castration in patients with prostate cancer. Androgen deprivation therapy aims to inhibit androgen production or action, thereby

reducing stimulation of the Androgen receptor (AR). This in turn prevents the activation of androgen-regulated genes, which normally result in on-going growth, survival and inhibition of apoptosis.

Front line therapy for locally advanced prostate cancer includes therapies that reduce the levels of androgen. The European Association of Urology Guidelines currently recommends androgen depravation therapy (ADT) as first-line management in these patients. Response rates are initially high (70-80%) (35) but after 18-24 months (36), almost all patients relapse and develop castrate resistant prostate cancer (CRPC).

- **Luteinizing hormone releasing hormone agonists (LHRH)**

Production of testicular androgen may be inhibited surgically by Orchidectomy, which is the removal of both testicles. Most men (more than 80%) respond positively to this treatment, with the progression of their cancer slowing markedly for around 18 months or more. Testosterone production may also be inhibited chemically, using Gonadotrophin realising hormone super agonists (GnRH) which are generally referred to as Luteinizing hormone releasing hormone agonists. LHRH agonists induce castrate levels of testosterone by down-regulating pituitary gonadotropin-releasing hormone GnRH receptors (and therefore gonadotropin hormone production) through constant stimulation. Goserelin (Zoladex) is a GnRH receptor agonists used in the treatment of prostate cancer. This drug mimics GnRH, a hormone produced by the hypothalamus. This hormone stimulates the release of Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. LH in turn stimulates the production of androgens in the testes.

Administration of GnRH agonists initially causes a surge in circulating concentrations of testosterone and dihydrotestosterone which can lead to tumour flare. Eventually these agonists downregulate the hypothalamic-pituitary axis and consequently inhibit the production of LH and therefore androgen production (37). Hormonal therapy is also used in patients who present with metastatic disease. It also reduces bone pain, improves obstructive urinary symptoms, reduces prostatic bleeding, and decreases circulating PSA concentrations.

- **Anti androgens**

The action of androgen may be blocked at a peripheral level using anti androgens, which prevent testosterone and DHT from binding to the AR. An antiandrogen is often used in combination with LHRH therapy to prevent tumour flare seen at the beginning of therapy. Antiandrogens can be used along with Orchidectomy and may be used 2 months before radiation or from 4 months to 2 years after radiation for men with intermediate-risk prostate cancer. Bicalutamide (Casodex) was launched in 1995 as a combination treatment (with surgical or medical castration) for advanced prostate cancer and subsequently launched as monotherapy for the treatment of earlier stages of the disease. Other anti-androgens include flutamide (Drogenil or Chimax) and cyproterone acetate (Cyprostat). Oestrogens that reduce production of testosterone include diethylstilboestrol.

- **Combination treatment**

Different methods of deceasing androgens are often used in the same patient: using LHRH agonists with anti-androgens can achieve what is known as a maximum

androgen blockade (MAB). Hormone therapy can also be used in conjunction with other treatments, especially in the case of advanced stage prostate cancer being treated with radiation therapy. In that case, hormonal therapy is often given before the radiation and this is known as neoadjuvant hormonal therapy and also before surgery.

After a while, all prostate cancers will become resistant to hormonal therapy. For some this often takes many years and hormonal therapy can buy a lot of time in patients with extensive disease or patients who choose not to undergo surgery or radiation. However CRPC is a lethal and heterogeneous disease. Despite secondary treatment, median survival of CRPC patients ranges from only 9 - 27 months (38;39). The development of castrate resistance remains a significant clinical problem, as it leads to disease progression and metastasis.

1.5 Androgens and the Androgen receptor (AR)

1.5.1 Androgen

Androgens control the development, differentiation, and function of male reproductive and accessory sex tissues, such as the seminal vesicle, epididymis, and prostate. Other organs and tissues, such as skin, skeletal muscle, bone marrow, hair follicles, and brain, are also under the influence of androgens. The principal action of androgens is to regulate gene expression through the androgen receptor (AR). Androgens are the major regulators of prostate growth (40). Their known effects are mediated by a cytoplasmic AR that is translocated into the nucleus upon hormone binding to positively or negatively modulate the expression of specific genes (41).

Testosterone is the main circulating androgen produced primarily by the Testes. However, a smaller source is also produced from the adrenal cortex, by adrenocorticotropoic hormone (ACTH), which releases androstene and androsteriedione (42). Testosterone circulates in the blood bound to albumin and sex hormone binding globulin (SHBG). When free testosterone enters prostate cells it is converted, by the enzyme 5α reductase, to the more active metabolite dihydrotestosterone (DHT) in the cell cytoplasm. Testosterone and DHT wield their biological effects through binding the AR and inducing transcriptional activity.

1.5.2. The Androgen Receptor (AR)

The gene for the androgen receptor is located on the X chromosome at Xq11-12 and encodes a protein with a molecular mass of approximately 110 kDa. AR is a member of the nuclear receptor super-family. Nuclear receptors are ligand-inducible transcription factors that mediate the signals of a broad variety of fat-soluble hormones, including steroid and vitamin D3 hormones, thyroid hormones and retinoids (41;43;44). AR can alter gene expression directly by interacting with specific elements in the regulatory regions of target genes (45) or indirectly by activating various growth factor signallingpathways (46).

1.5.3 AR Structure

The androgen receptor gene was cloned in 1988 by Chang and co-workers (47) and Lubahn *et al*. (48;49) followed by several others Tilley *et al* (50) Trapman *et al* (51), Brinkmann *et al* (52). AR has four major functional regions (Figure. 1.4): the N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD), a C-

terminal ligand-binding domain (LBD), and a hinge region connecting the DBD and LBD (53). Two autonomous transactivation functions, a constitutively active activation function (AF-1) originating in the N-terminal and a ligand-dependent activation function (AF-2) arising in the LBD, are responsible for the transcriptional activity of nuclear receptors (54).

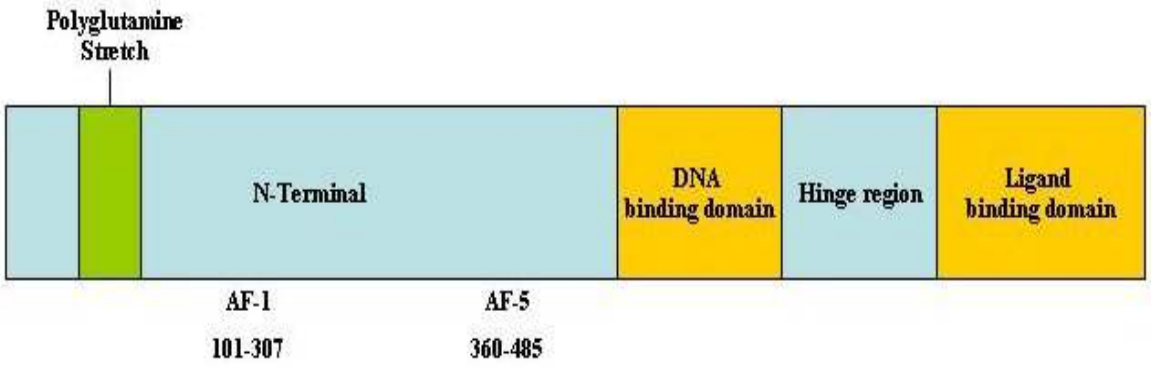

Figure 1.4 Functional domains of Androgen receptor

The Androgen receptor comprises of four functional domains, the diagram shows the function of each domain.

- **N-terminal transactivation domain (N-terminal)**

The N-terminal domain harbors the major transcription activation functions and several structural subdomains. Within its 538 amino acids, two independent activation domains have been identified, these are activation function 1 (AF-1) and activation function 5 (AF-5) (Figure 1.4). AF-1 is located between residues 101 and 370 and is essential for transactivity of full length AR. AF-5 is located between residues 360-485 and is required for transactivity of a constitutively active androgen receptor, which lacks its LBD (55). In addition, the N-terminal contains a variable

polyglutamine tract usually located at positions 57-78, which affects AR stability and transcriptional activity (56)

- **DNA binding domain**

The DNA-binding domain (DBD) is an independently folded protein domain which contains at least one motif that recognizes double- or single-stranded DNA. The DBD of AR exhibits a high degree of amino acid sequence identity to other members of the glucocorticoid receptor (GR) subfamily, the progesterone receptor (PR), and mineralocorticoid receptor (MR). Consequently, the four receptors recognize very similar, if not identical, hormone response elements (HREs). Steroid receptors have long been known to bind DNA elements that are organized as inverted repeats of hexameric binding sites separated by three nucleotide spacers. For AR, GR, PR and MR, the consensus reads 5'-TGTTCT-3' (57;58). The DNA-binding domain has a compact, globular structure with two zinc finger motifs with four conserved cysteines. The first zinc finger contains a P-box (sequence critical for DNA-DNA recognition and specificity) and is responsible for binding to HRE of target genes (59-61). The second zinc finger contains a five amino acid-residue long D-box and controls dimerisation of the receptor on the HREs.

- **Hinge Domain**

The hinge region connects the DBD with the LBD. It is defined by residues 628-669 and is a multifunctional domain involved in DNA binding (62-65) and nuclear translocation (66). The AR hinge contains a serine at position 650 that can be

phosphorylated by MEKK-kinases and which seems to be involved in the regulation of receptor translocation (67;68). A mutation of Serine 650 to Alanine reduced the nuclear export of the AR (67). The hormone-dependent nuclear translocation of AR is mainly mediated through a bipartite nuclear localization signal (NLS) consisting of two clusters of basic residues that are located in the DBD and the hinge region (66). The AR can also be acetylated in its hinge region at residues K630, K632, and K633 (69). Acetylation of these residues has been reported to regulate transcriptional activity (69), subcellular distribution and folding of the AR (70), and coactivator and corepressor binding (69-71).

- **Ligand binding domain**

The ligand binding domain (LBD) contains the ligand dependent transactivation function 2 (AF-2). This is a highly conserved hydrophobic cleft flanked by opposing charge residues (72). This area contains a fold comprised of up to 12 α helices (H12) forming a ligand binding pocket (LBP). Ligand binding changes the conformation and the H12 serves as a 'lid' to close the ligand-binding pocket (LBP), whereas in the antagonist-bound conformation, H12 is positioned in a different orientation, thus opening the entrance to the LBP.

1.5.4 Androgen Receptor Isoforms

Two isoforms of the androgen receptor (A and B) have been identified (73). Androgen receptor A (AR-A) is approximately 87 kDa. The N-terminus is truncated

and lacks the first 187 amino acids. Androgen receptor B (AR-B) is the full length AR and is approximately 110kDa.

- **Splice variants**

Novel splice variants have been identified in prostate cancer cell lines designated AR-v3, ARv-4, ARv-5 and AR-v7; these variants lack the LBD (74). AR-v3 has been shown to be expressed in human prostate cancer tissues and to be constitutively active and its transcriptional activity does not appear to be regulated by androgen or anti androgens. AR-v3 was also significantly up-regulated during prostate cancer progression and expression levels correlate with the risk of tumour recurrence after radical prostatectomy (74). Hu et al also reported that levels of AR-v7 are elevated by approximately 20 fold in castrate resistant prostate cancer cell lines derived from metastatic prostate cancer specimens failing hormone ablation therapy. In hormone naïve prostate cancers which had not undergone hormone therapy a low ARv-7 expression was observed. Interestingly higher ARv-7 expression was observed in these patients flowing therapy and this was also predictive of PSA recurrence (75).

1.5.5 Androgen Receptor Cofactors

The AR interacts with nuclear proteins, which modify its effects on transcription. AR co-activators participate in DNA modification of target genes, either directly through modification of histones or indirectly by the recruitment of chromatin-modifying complexes, as well as functioning in the recruitment of the basal transcriptional machinery (76).

- **Co activators**

To facilitate transcription, nuclear receptor proteins recruit coactivator proteins, which increase the rate of transcription by promoting histone acetylation and interactions with the basal transcriptional machinery (77). The best-characterised coactivators include Steroid Coactivator 1 (SRC1) and Creb-Binding Protein (CBP). The p160/SRC family consists of three members: SRC-1 (p160-1, N-CoA1), SRC-2 (TIF-2, GRIP1, NCoA2), and SRC-3 (RAC3, TRAM1, ACTR, ABI1, P/CIP). Most nuclear receptors recruit SRCs to their LBD using a leucine-rich motif (L*XX*LL). However, the LBD of the AR preferentially recruits its own AF-1 domain via an N-terminal F*XX*LF motif, which interacts strongly with the hydrophobic cleft of the LBD created by ligand binding (78-81). The subsequent recruitment of coactivators, in particular the SRC family, is then mediated primarily by the AF-1 domain rather than by the LBD (77;81-84).

- **Co-Repressors**

Co repressors interact with nuclear receptors in the absence of ligand or the presence of antagonists, and repress transcription. Nuclear receptor co-repressor 1 (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) are key co-repressors. Both prevent AR mediated transcription by recruiting proteins that have histone deacetylases (HDAC) to the promoter region of the genes, which maintains chromatin in a condensed state thus inhibiting access of the transcription machinery. Prohibitin and Hey 1 have recently been identified as AR corepressors (85-87).

1.5.6 Ligand dependent activation of androgen receptor

Ligand free AR is maintained in a multiprotein inhibitory complex with heat shock proteins (HSPs 70 & 90). A vital component of this complex is the co-chaperone p23. HSPs bind to regions of the receptor required for homodimerisation, nuclear localisation and DNA binding. Androgen binding induces a conformational change in the AR that leads to dissociation from the HSPs, homodimerisation, receptor phosphorylation and nuclear translocation (Figure 1.5). Nuclear AR binds directly to HRE sequences in the promoter region of androgen regulated genes. This leads to the recruitment of co-activators, co repressors and transcription machinery to the promoter (88). Androgen/AR interaction functions in promoting the survival of the secretory epithelia (89), controlling expression of genes that drive cell growth by promoting G1-S phase progression and inhibition of apoptosis. (90).

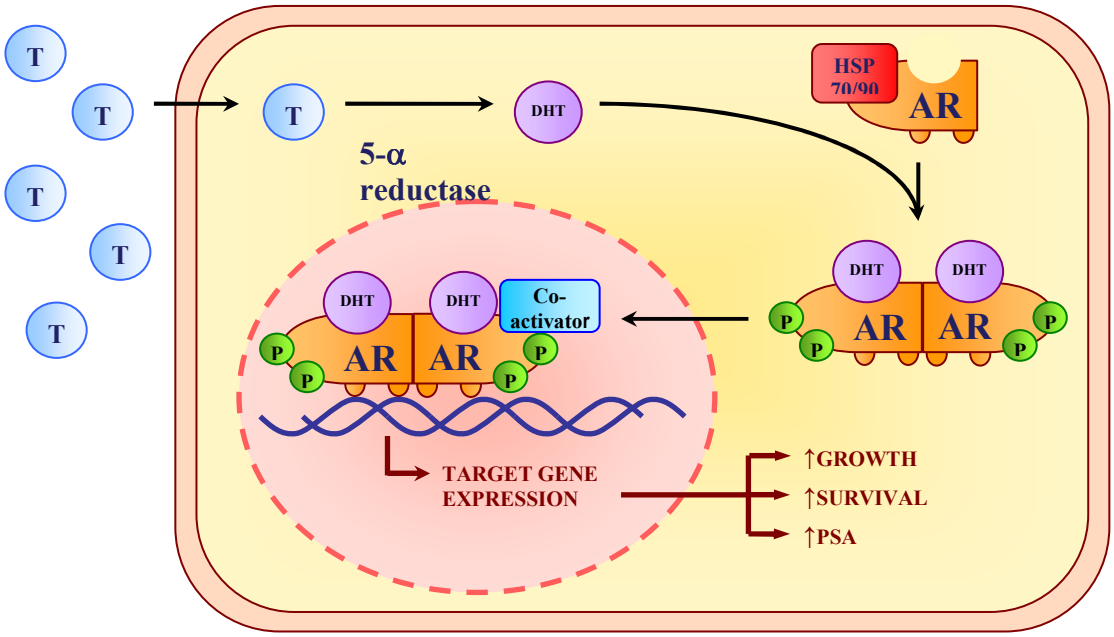

Figure 1.5 Activation of the AR by androgens

Testosterone enters prostatic epithelial cell and is converted to dihydrotestosterone (DHT) by 5α-reductase. DHT binds to AR, resulting in dissociation of inhibitory heat-shock proteins (HSPs) and transactivation of the AR involving AR phosphorylation and dimerisation. The AR homo-dimer transolcates to the nucleus and binds to androgen response elements in the promoter regions of target genes, resulting in biological responses such as growth, survival and PSA expression.

1.5.7 Androgen receptor phosphorylation

Phosphorylation of AR is stimulated in a ligand dependent manner via androgen binding and also in a ligand independent manner through signal transduction cascades. Phosphorylation of Serine and Tyrosine residues on the AR has been identified. Phosphorylation of Serine residues stabilizes the AR and protects it from proteolytic degradation (91). Phosphorylated amino acids within the AR include

serines (Ser) 16, 81, 94, 256, 308, 424,578 and 650 (Figure 1.6) (92-94) . All of these sites show increased phosphorylation in the presence of androgen, with the exception of Ser-94, which is constitutively phosphorylated. In response to androgen-binding, Ser-81 is the most frequently phosphorylated site on the AR, giving the highest stoichiometric phosphorylation yield in LNCaP cells treated with androgens (94). Phosphorylation of Ser-81 is associated with AR stability and transcriptional activity (95). It is also associated with prostate cancer cell growth in cell line studies, suggesting some significance of AR Ser-81 phosphorylation in AR function (96). AR transcriptional activity can be induced by EGF, this is dependent on AR phosphorylation at serines 515 (MAPK consensus site) and 578 (PKC consensus site), which regulates nuclear cytoplasmic shuffling of the AR, through interactions with the Ku-70/80 regulatory subunits of DNA-dependent protein kinase (97). The AR can be phosphorylated in response to a variety of signals, which facilitates the recruitment of coactivators or components of the transcription machinery and therefore acts in conjunction with the ligand to enhance transcription activation. Phosphorylation of the AR at serine residues 213 and 791 is mediated by Akt which results in increased AR transactivation (98). Tyrosine kinases, Ack1 (activated cdc42-associated kinase) and Src, phosphorylate and enhance AR activity and promote prostate xenograft tumour growth in castrated animals. Ack1 mediates heregulin and Gas6-induced AR Tyrosine 267 phosphorylation, and Src mediates Tyrosine 534 phosphorylation induced by EGF, IL-6 and bombesin. Dasatinib, a Src inhibitor, inhibits EGF-induced Tyrosine 534 phosphorylation and also inhibits heregulin induced Ack1 phosphorylation of the AR at Tyrosine 267 (99).

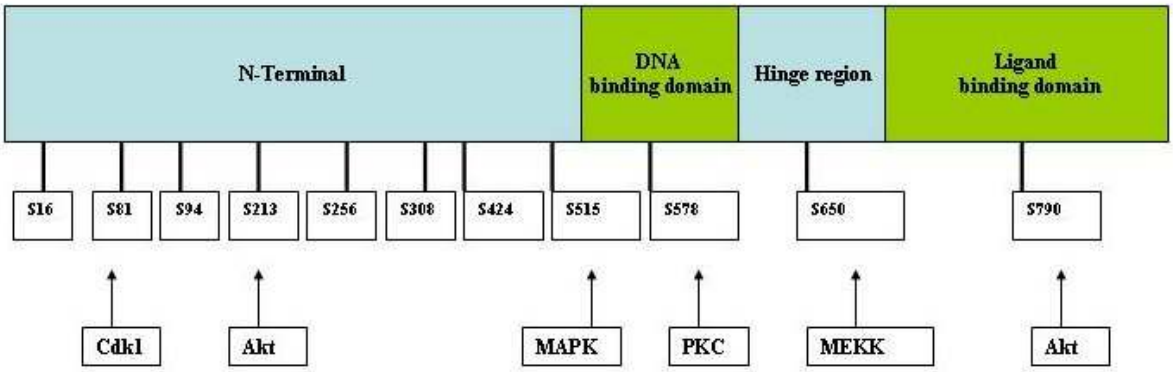

Figure 1.6 AR serine phosphorylation sites

Diagram of the AR displaying the serine residues that are known to undergo phosphorylation and the kinases reported to mediate this.

1.5.8 Mechanisms of castrate resistance

Depriving prostate cancers of androgen inhibits cell proliferation and induces apoptosis resulting in tumour regression however disease regression is temporary for most and the castrate resistant phenotype proceeds. The mechanisms underlying both the development of hormone naïve and castrate resistant disease are poorly understood however several possible mechanisms are currently being investigated.

1.5.8.1 Genetic modification of the AR

- **AR gene amplification**

AR expression is observed in hormone naïve and castrate resistant prostate cancers despite low levels of circulating androgens during treatment. Increased AR abundance is one mechanism that can aid tumour cell proliferation despite the low androgen levels. AR gene amplification has been associated with castrate resistance

(100). Approximately 30% of tumours that progress to castrate resistance have AR gene amplification, resulting in increased AR protein expression. However studies have reported that AR gene amplification was not present in the hormone naïve tumours before the commencement of hormone therapy (100;101). This suggests that AR amplification was possibly due to clonal selection of cells that could proliferate in the reduced androgen environment. AR amplification rates have also been shown to significantly increase in the transition from hormone naïve to castrate resistant disease (102;103). Interestingly, 80% of these patients displayed a corresponding increase in AR protein expression, indicating that AR gene amplification does not always lead to increased AR protein expression. This study also observed that 22% of patients exhibited an increase in AR protein expression in the absence of AR amplification but it was the patients who acquired AR gene amplification that had a significantly shorter survival time, suggesting that in a sub set of prostate cancer patients AR gene amplification is involved in the progression to castrate resistant disease. However, this observation does not account for the 70% of patients who do not acquire AR gene amplification but progress to a castrate resistant disease thus suggesting another method to disease progression.

Animal models of prostate cancer have suggested that increased AR levels can initiate prostate cancer development (104) and castrate resistant xenografts have shown that the AR exhibits an increased sensitivity to reduced androgen levels (105). Kim et al observed that castrate resistant tumour cells were hypersensitive to the growth promoting effects of DHT. These androgen independent cells were

stimulated by DHT concentration four times lower than that required for androgen dependent LNCaP cells. These results therefore, have shown that in some models of prostate cancer the AR is transcriptionally active at the low levels of androgens found to be circulating in castrated patients.

- **AR Mutations**

In untreated prostate cancer the incidence of AR mutations is found to increase with cancer stage. AR mutations are found in 0-4% of latent and stage B prostate tumours (106-108). However, they are more commonly observed in distant metastasis, AR mutations were found in 21-44% of metastatic tumours sampled before therapy (107;109). Therefore the acquisition of mutations within the AR is likely to be a mechanism for castrate resistance in some prostate cancer patients. The majority of AR mutations identified are point mutations resulting from a single amino acid substitution. The replacement of threonine 877 with alanine in the AR LBD is a frequent mutation in prostate cancer patients and corresponds to the mutation found in LNCaP cells (110-113). In cells with AR mutations, androgen signalling is maintained by the broader number of ligands that can bind to and activate the receptor. The T877A substitution allows the AR to be activated by binding to cortisol and progesterone and other ligands that the wild type receptor cannot accommodate. This mutation allows antiandrogens such as flutamide to bind, changing its function from an antagonist to agonist, thereby promoting prostate cancer cell growth (114;115). Additionally several point mutations in and around the hinge region have been identified (116-118). Some of these mutations have been

shown to increase the transcriptional response which indicates an inhibitory function of the hinge domain. Various other AR mutations have been identified which are catalogued in the Androgen Receptor Gene Mutation Database (119). At present it is uncertain how many of these other mutations use the same promiscuous receptor mechanism that allow prostate cancer cells to become castrate resistant.

- **CoRegulator alterations**

Modulation of coregulatory proteins and their function is most likely to contribute to the progression to castrate resistant disease. This may be by increasing the AR transcriptional activity at low androgen concentrations or by altering the ligand specificity. In prostate cancer several coactivators have been shown to alter the ligand specificity of the AR, including CBP, β catenin, AR-associated protein 55 (ARA55) and ARA70 (120-122). In addition, the expression of three members of the SRC family, SRC-1, TIF-2 and SRC-3 have been shown to be high in prostate cancer (76;88). SRC-1 has been shown to be overexpressed in 50% of hormone naïve prostate cancers compared to normal and BPH samples. In castrate resistant tumours 63% displayed both SRC-1 and TIF-2 overexpression (123). Moreover an increase in SRC-3 expression has been shown to significantly correlate with increased tumour stage and Gleason grade and decreased disease specific survival (124). Another study also highlighted that higher expression of RAC-3 resulted in higher PSA levels in the presence and absence of androgen. Here it was observed that RAC-3 facilitates RNA polymerase II recruitment to a distant enhancer element of the PSA gene, resulting in a increase in PSA expression (125). The AR coactivator cdc25B (cdk-activating phosphatase) has also been associated with

prostate cancer progression. High expression of cdc25B was observed in late stage prostate tumours with a high Gleason grade (126). In the CWR22 prostate xenograft and LNCaP cell line the expression and nuclear localisation of AR coactivator Tat interactive protein 60 kDa (Tip 60) was shown to increase upon androgen withdrawal (127). AR co-activator overexpression appears to play a role in prostate cancer progression, which coactivators most significantly contribute to prostate cancer progression remains to be determined.

Several mechanisms have been implicated with the development of castrate resistant disease. AR mutations have been identified in a small number of prostate cancers and mutated AR may cause anti-androgens to activate the AR rather than inhibiting it. AR gene amplifications are present in 20-30% of castrate resistant prostate cancers. Amplification of the AR gene may result in increase protein which is hypothesised to enable low levels of circulating androgens to bind and activate the AR even in the presence of anti-androgens. However, there remain a large number of cases that cannot be explained by AR mutations or amplifications. Cell line studies suggest that castrate resistance may be a result of AR activation by phosphorylation or may be due to a mechanism completely independent of the AR.

1.5.8.2 Post-translational modifications of the AR

Upon AR expression, post-translational modification takes place. These modifications, which are generated by signal transduction pathways, include acetylation, ubiquitination, sumoylation and phosphorylation (128). The progression

of prostate cancer has been associated with alteration of growth factor or growth factor receptor expression by the tumour (129;130). Growth factors and cytokines regulate cellular responses through binding to membrane receptors. This binding initiates a phosphorylation cascade that ultimately results in phosphorylation of transcription factors or transcription factor interacting proteins.

1.6 Cross talk between AR and growth factor receptor pathways

In prostate cancer a number of signal transduction cascades are known to influence AR transcriptional activity by direct phosphorylation of the AR or by phosphorylation of AR coregulators. *In vivo,* this may allow the AR to be activated in the absence of androgens, by other steroids or be sensitised to the low levels of circulating adrenal androgens that remain present during androgen-deprivation therapy. Several growth factors such as Insulin like growth factor -1 (IGF-1) and EGF can activate the AR and induce AR regulated genes in the absence of androgen (131). These growth factors are ligands for receptor tyrosine kinases and initiate intracellular signallingcascades. A full description of the signallingcascades implicated in castrate resistant disease progression is beyond the scope of this thesis however current advances in signal transduction association will briefly be discussed.

The Human Epidermal Growth Factor Receptor (HER) family has been implicated in prostate cancer. Increased expressions of the EGFR, HER2, HER3, HER4, and EGFRvIII have all been associated in prostate cancer progression (132-136). AR activation has been reported through HER2 induced Mitogen activated protein

kinase (MAPK) and Phosphatidylinositol 3 Kinase (PI3K) signalling(137). In LNCaP cells HER2 mediated AR transactivation is reduced by transfection of a dominant negative mutant of Akt suggesting Akt phosphorylation of AR can enhance AR transcription at a low level of androgen (98).

The IL-6/JAK/STAT cascade is thought to play a key role in the progression of castrate resistant disease. IL-6 cytokine was originally described as a regulator of the immune and inflammatory responses, but is now known to influence tumour cell growth in an autocrine/paracrine manner (138). As prostate cancer progresses from hormone naïve to castrate resistant disease, the circulating concentrations of IL-6 in the serum of patients increase (139). Functional cell line studies demonstrate that the AR/STAT-3 complex can promote androgen-regulated gene transcription even in the absence of androgens (140). This mechanism is supported by data that demonstrates IL-6 can activate the AR in a ligand-independent manner (141;142). Evidence in clinical tissue to support these in vitro observations are sparse, although it is reported that IL-6 receptor (IL-6 R) expression is eightfold higher in prostate cancer tissue compared to normal tissue (143). Additionally, IL-6R and pSTAT3^{Tyr705} expression are associated with reduced time to biochemical relapse and survival in matched hormone naïve and castrate resistant tumour samples (144)

Work carried out in our lab previously using CGH gene array identified that several members of key signallingcascades were upregulated in clinical castrate resistant prostate cancer samples (145). This study observed that key members of the PI3K and MAPK cascade were more commonly amplified in castrate resistant tumours. Both of these signallingcascades can be activated by growth factors via tyrosine

kinase receptors. The Ras/Raf/MAP Kinase pathway may increase prostate cancer cell growth both via and independently of the AR, further implicating it in the development of castrate resistant disease. Multiple members of this pathway have been implicated in prostate cancer progression. In studies using human prostate tumours, phosphorylated MAPK expression increases with tumour stage and grade and is over-expressed in castrate resistant disease (146). *H, K* and *N RAS* genes are amplified in approximately 50% of hormone naïve and castrate resistant prostate tumours (145) and N-Ras expression is associated with the development of castrate resistant disease and correlates with MAPK activation (147). Additionally both an increase in Raf-1 and MAPKexpression are significantly associated with prostate cancer survival (148). Recent evidence suggests that MAPK activation induces phosphorylation of AR at Serine 81 to stimulate prostate cancer cell growth and induce PSA expression (96). MAPK has been shown to increase transcription of androgen dependent genes independently of androgens via phosphorylation of the AR itself at serine 515 or via phosphorylation of AR co factors such as SRC-1 (141;149).

1.7 The PI3K/Akt cascade

The PI3K cascade has been shown to regulate multiple cellular events in prostate cancer. Activation of the PI3K cascade is initiated by receptor tyrosine kinases (RTKs) and non RTKs. PI3K activation results in the catalytic conversion a phosphate from ATP to the D-3 position of the inositol ring of phospatidylinositol (4, 5) bisphosphate (PIP2) to phospatidylinositol (3,4,5) triphosphate (PIP3) (150).

These lipids act as secondary messengers and recruit pleckstrin homology (PH) domain containing proteins such as Akt to the inner surface of the plasma membrane, driving their conformational change resulting in their activation. Activated Akt translocates to the cytoplasm and nucleus and activates down stream targets such as mammalian target of Rapamycin complex 1 (mTOR 1). The PI3K cascade is negatively regulated by the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10).Activation of this pathway is believed to be a key mechanism driving castrate resistant disease progression (Figure 1.7)

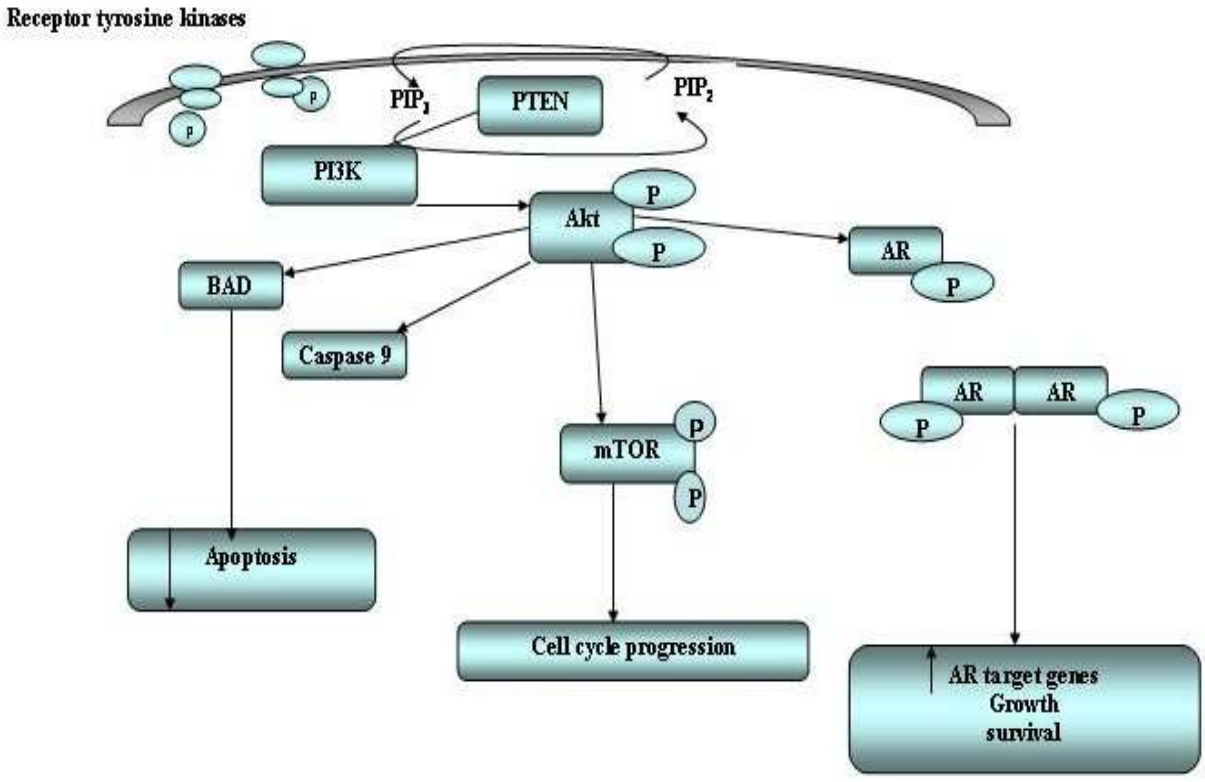

Figure 1.7 PI3K cascade

Schematic representation of the PI3K cascade

1.7.1 PI3K

The oncogenic form of PI3K, *v-p3k*, was first discovered in an avian sarcoma virus (151), here the expression of *v-p3k* was shown to induce tumours when the transformed cells were injected into chicken. The PI3K family is composed of three Classes; I, II, and III. Class I PI3K is further divided into two groups: PI3K-IA and PI3K-IB and activation is dependent upon the specific regulatory and catalytic isoforms that associate into the heterodimers. Class IA PI3K heterodimers contain specific isoforms of the 85 kDa adaptor subunit (p85α, p55α, p50α, p85β, or p55γ) that facilitates interaction with receptor tyrosine kinases (RTK) and either an alpha, beta or delta p110 catalytic subunit (p110α, p110β, or p110γ). The preferred substrates of class I, PI3K are phosphoinositides (4, 5) bisphosphate (PIP2), Phosphorylation of PIP2 by PI3K generates PtdIns (3, 4, 5) PIP3. The class IA PI3Kp110α is mutated in many cancers causing the kinase to be more active (152;153). Functional analyses of PIK3CA mutations revealed that they increase its enzymatic activity, stimulate Akt signalling, and allow growth factor independent growth as well as increasing cell invasion and metastasis (153).

1.7.2 Akt

Three Akt family members Akt 1, 2 and 3 have become known as critical mediators of signal transduction cascades downstream of activated tyrosine kinases and PI3K. An ever increasing list of Akt substrates has specifically defined the multiple functions of this kinase family in normal physiology and disease states. Cellular processes regulated by Akt include cell proliferation and survival, cell size and

response to nutrient availability, intermediary metabolism, angiogenesis, and tissue invasion. Akt proteins contain a central kinase domain with specificity for serine or threonine residues in substrate proteins. They also contain a pleckstrin homology (PH) domain, which mediates lipid-protein and/or protein-protein interactions. Akt1 has been implicated as a major factor in many types of cancer. Akt2 has shown to be an important signallingmolecule in the Insulin signallingpathway and is required to induce glucose transport.These separate roles for Akt1 and Akt2 were demonstrated in study using mice which had either Akt1 or Akt2 gene deletion. In Akt 1 null mice with normal Akt2, glucose homeostasis is undisrupted however the animals were smaller, consistent with a role for Akt1 in growth. In contrast, Akt2 null mice with normal Akt1, have mild growth deficiency and display a diabetic phenotype again consistent with the idea that Akt2 is more specific for the insulin receptor signallingpathway (154). The role of Akt3 is less clear, though it appears to be predominantly expressed in brain tissue (155). In unstimulated cells Akt is constituatively phosphorylated at serine 124 and threonine 450. Once correctly positioned in the cell membrane via binding of PIP3 Akt can then be phosphorylated by its activating kinases at two regulatory residues. Phosphoinositide dependent kinase 1 (PDK-1) phosphorylates Akt at threonine 308, this is essential for Akt activation as it causes a charge induced conformational change that allows substrate binding and an increased rate of catalysis. Without this threonine phosphorylation the hydrophobic motif of Akt is more susceptible to the action of phospatases. Mammalian target of rapamycin complex 2 (mTORC2) phosphorylates Akt at serine 473 (156;157). Phosphorylation of Akt at serine 473 increases Akt activity 10-fold

(156;158). Once activated Akt translocates from the inner surface of the cell membrane to the cytoplasm and nucleus where it phosphorylates proteins in several pathways regulating aspects of metabolism, proliferation and apoptosis. Akt also plays significant roles in protein translation, particularly by regulating those proteins involved in growth and survival. Akt regulates the apoptotic response to a variety of stimuli via its ability to interact with a number of key players in the apoptotic process. BAD is a pro-apoptotic protein of the Bcl-2 family. Akt phosphorylates BAD on Serine 136 which makes BAD dissociate from the Bcl-2/Bcl-X complex and lose the pro-apoptotic function (159). Akt has also been shown to regulate cell cycle progression via a pathway that ultimately down regulates the cell cycle inhibitor p27 (160).

1.7.3 mTOR

Mammalian target of rapamycin (mTOR) is a large protein kinase that exists in two distinc complexes within cells, one that contains mTOR, GβL, and raptor (mTOR C1) and another containing mTOR, GβL and rictor (mTOR C2). Akt can directly phosphorylate and activate mTOR, as well as cause indirect activation of mTOR by phosphorylating and inactivating TSC2 (tuberous sclerosis complex 2), which normally inhibits mTOR through the GTP-binding protein Rheb (Ras homolog enriched in brain). When TSC2 is inactivated by phosphorylation, the GTPase Rheb is maintained in its GTP-bound state, allowing for increased activation of mTOR. In the mTORC1 complex, mTOR signals to its downstream effectors S6 kinase/ribosomal protein S6 and Eukaryotic translation initiation factor 4E (4EBP-

1/eIF-4E) to control protein translation. Although mTOR is generally considered a downstream substrate of Akt, mTOR can also phosphorylate Akt when bound to Rictor in TORC2 complexes, this is thought to provide a level of positive feedback on the pathway (156). The downstream mTOR effector S6 kinase-1 (S6K1) can also regulate the pathway by catalyzing an inhibitory phosphorylation on insulin receptor substrate (IRS) proteins. This prevents IRS proteins from activating PI3K, thereby inhibiting activation of Akt. Due to its central position in the PI3K cascade i.e downstream of Akt and upstream of S6K the activation of mTOR is part of a fundemental step in controlling tumour cell growth.

1.7.4 PTEN

PI3K dependent Akt activation can be regulated through the tumor suppressor PTEN which works essentially as the opposite of PI3K. The *PTEN* tumour suppressor gene is located on chromosome 10q23, an area known to undergo loss of heterozygosity (LOH) in many human cancers. *PTEN* Mutations and somatic deletions have been identified in an array of tumours placing *PTEN* as one of the most commonly mutated tumour suppressor genes in human cancer (161). PTEN mutants that retain protein tyrosine phosphatase activity but loose the ability to dephosphorylate PIP3 are found in many tumours, indicating that the lipid phosphatase activity is needed for tumour suppression (162). PTEN acts as a phosphatase to dephosphorylate PIP3 back to PIP2. This removes the membrane-localization factor from the Akt signallingpathway. Without this localization, the rate of Akt activation decreases significantly, as do the all the downstream pathways that depend on Akt for

activation. The ability of PTEN to inhibit PI3K/Akt signallingallows it to block nuclear entry of Mdm2. Phosphorylation of Mdm2 at serines 166 and 188 by Akt induces the translocation of Mdm2 to the nucleus. In the nucleus Mdm2 ligates ubiquiton to the p53 tumour suppressor thus targeting it for degradation. PTEN also negatively regulates cell migration and invasion by dephosphorylation of focal adhesion kinase (FAK) and also negatively regulates growth factor receptor signallingthrough inhibition of the adaptor protein Shc, (163;164).

1.7.5 The PI3K/Akt cascade and prostate cancer

Several key components of the PI3K/Akt cascade have been implemented in prostate carcinogenesis and castration resistance. PI3K inhibition has been studied *in vitro* for some time and evidence of it key role in carcinogenesis continue to emerge. Genetic analysis of high Gleason grade prostate cancers revealed 3% of patients had PIK3CA mutation and 13% had PIK3CA amplification (165). Up regulation of PI3K signallingmay also be due to overexpression of RTKs which have been previously reported to be overexpressed in prostate tumours and cell lines (133;134;137). PI3K has shown to be an important signallingmolecule and key survival factor involved in prostate cancer proliferation and invasion. Previous studies have reported that treatment LNCaP, PC-3 and DU145 with PI3K pharmacological inhibitor, LY294002, potentially suppressed the invasive properties in each of these cell lines and restoration of the *PTEN* gene to highly invasive prostate cancer PC-3 cells or expression of a dominant negative version of Akt also significantly inhibited invasion and down regulated protein expression of urokinase-type plasminogen

activator (uPA) and matrix metalloproteinase (MMP)-9, markers for cell invasion, indicating a central role of the PI3K/Akt cascade in this process. Increased levels of PI3K (p110α/β) and regulatory (p85) and Akt were also observed in these cell lines (166).

A somatic mutation in AKT1 (E17K) has been detected in breast, colorectal, lung, ovarian and prostate cancers (167;168). In AKT1, the E17K substitution leads to a PI3K-independent activation of AKT1. In prostate cancer, AKT1 mutation was reported to have a prevalence of just 1.4% and the mutation seemed to be associated with favourable clinical outcome and was not associated with a specific tumour growth pattern (168). Overexpression of Akt in prostate cancer is hypothesised to be due to defective *PTEN* gene as discussed below. Prostate tumours are reported to have significantly higher Akt expression than BPH (169), and only 10% of well-differentiated prostate tumours strongly express pAkt compared to 92% of poorly differentiated tumours (161;170-172). Additionally, in hormone-naive tumours Akt1 and Akt2 expression has been associated with shorter time to biochemical relapse; however, no association was reported with the activated forms or with survival (173).

Loss of *PTEN* has been associated with advanced prostate cancer (174) and loss of PTEN expression is associated with increased risk of recurrence in human tumours (175-178). Prostate cancer cell lines that have been cultured from metastatic sites such as the lymph nodes (LNCaP) or brain metastasis (PC3) have highly active PI3K/Akt signallingand *PTEN* deletion (179;180) The magnitude of loss of function of PTEN is best described in both localised and metastatic prostate cancers and

includes homozygous deletions, loss of heterozygosity (LOH) and inactivating mutations (181). The reported frequency and mode of inactivation at different stages of prostate cancer vary. Homozygous deletions of PTEN have been detected in up to 15% of locally confined prostate cancers and up to 30% in metastatic cases (182-186). Heterozygous loss has been reported in 13% of locally confined prostate cancers and up to 39% in metastatic cases (183-185;187;188). *PTEN* mutation has been associated with 5-27% of localised and 30-60% of metastatic prostate tumours (182;189;190) In addition, loss of PTEN expression is associated with disease progression and increased risk of recurrence (175;176;178) although substantial heterogeneity has been observed between different metastatic sites within the same patients (182).

Many oncoproteins and tumor suppressors intersect in the PI3K cascade, regulating cellular functions at the interface of signal transduction and classical metabolic regulation. This careful balance is altered in human cancer by a variety of activating and inactivating mechanisms that target both Akt and interrelated proteins. Numerous studies have suggested that PI3K signallingenhances its oncogenic signal through interaction with other signallingnetworks such as the transcription factor Nuclear factor Kappa B (NFκB) (191;192). Bai et al have recently reported that suppression of NFκB activity by IκB superepressor induces a strong and selective resistance to PI3K or Akt induced oncogenic transformation which suggests an essential role for NFκB in the transforming mechanisms induced by this signallingcascade (193). NFκB signallinghas been reported to regulate various genes involved in the invasion, angiogenesis and metastasis of cancer cell (194).

1.8 NFκB cascade

Aberrant NFκB nuclear activation has been implicated in the pathogenesis of several human malignancies including prostate cancer. NFκB has been shown to play important roles in the control of growth, differentiation, and apoptosis (195;196). NFκB has been shown to be related to the initiation and progression of many types of cancer through its target genes. These target genes include c-myc, cyclin D and IL-6 which promote cell growth, Bcl-2 which inhibits apoptosis, IL-8 and VEGF which promote angiogenesis, and MMP9 which promotes invasion and metastases (197;198). Many different stimuli have been reported to cause nuclear localisation and transcriptional activation of NFκB via activation of the IKK complex (IKK α, IKK β and IKKγ/NEMO), resulting in phosphorylation of IκBα at serine 32 and serine 36. NFκB normally exists in a dormant state bound to IκBα, when IκBα is phosphorylated NFκB is released from the complex and IκBα is marked for degradation. Following release NFκB translocates from the cytoplasm to the nucleus where it binds to the promoter region of multiple genes (198).

1.8.1 NFκB

Transcription factor NFκB was first identified by Sen and Baltimore over twenty years ago as a B cell- specific nuclear factor that bound to an enhancer element in the immunoglobulin kappa (κ) light chain gene. NFκB proteins are found in essentially all cell types and are involved in the activation of a remarkably large number of genes in response to infections, inflammation, and other stressful conditions which call for prompt reprogramming of gene expression. NFκB exists in

the cytoplasm in the majority of cell types as a variety of homo or heterodimeric isoforms. Five family members; c-Rel, Rel A (p65), Rel B, NF- κB1 (p50/p105), and NF- κB2 (p52/p100) have been identified. These proteins share a high sequence homology within a conserved Rel homology domain (RHD) near the N-terminal. This area comprises of DNA binding domains, a dimerisation domain, and interacts with the inhibitory IκB proteins. Additionally the RHD contains a nuclear localisation signal (NLS). In the majority of cell types the Rel A (p65)/ p50 heterodimer is most commonly expressed. The c-Rel, Rel A and Rel B proteins have c-terminal transactivation domains responsible for their function as a transcriptional activator. Alternately NF- κB1 and NF- κB2 are created as precursor forms p105 and p100 respectively where a series of ankaryin repeats are present in the c-terminal domain in place of a transactivation domain. These proteins undergo proteolytic processing removing the c-terminal sequences generating the products p50 NF- κB1 and p52 NF- κB2 respectively. Due to the lack of the transactivation domain, p50 and p52 homodimers are thought to function as transcriptional repressors while heterodimers of p50 or p52 with transactivation domain- containing Rel proteins lead to activation of target genes (194).

1.8.2 NFκB activation

Activation of NFκB is stimulated through several mechanisms including signal transduction pathways involving tyrosine kinases, NFκB inducing kinase (NIK), IKK and PI3K. At present it appears that all NFκB complexes are regulated in the same manner which is through interaction with IκBs. Seven IκB proteins have been

identified IκBα, IκBB, IκBε, p105, p100, BCL-3 and IκBζ. Based on their domain organization, selectivity toward specific NFκB dimmers and subcellular location in resting cells this family of proteins has three groupings; canonical, NFκB precursor and nuclear IκB. IκBs contain five to seven ankaryin repeats of 30-33 amino acids. These stacked helical domains bind to the NLS of NFκB proteins, blocking nuclear localisation. Only Iκβα, Iκββ, and Iκβ ε contain N-terminal regulatory regions which are necessary for stimulus induced degradation, the crucial step in NFκB activation. IκBα plays a central role in termination of NFκB activation. Newly synthesized IκBα enters the nucleus and binds to NFκB, thus enhancing the dissociation from the DNA and causing its re-exportation to the cytoplasm by means of a nuclear export sequence (NES) present on IκBα . Following cellular stimulation, IκBs become phosphorylated by the multisubunit IκB kinase (IKK) complex at serine residues 32 and 36. The phosphorylated IκB is then ubiquitinylated leading to their proteolysis by the 26s proteosome in an ATP-dependent manner (199). A key step in NFκB activation is phosphorylation of the p65 and p50 subunits. A number of positions of phosphorylation in the p65 subunit have been acknowledged and are associated with the action of multiple stimulus coupled kinases that act in both the cytoplasm and the nucleus. A key phosphorylation event involves serine 536 of the p65 subunit. The phosphorylation of this site is catalysed by multiple kinases that are activated by diverse stimuli (200-202). The involvement of PI3K and Akt in phosphorylation of p65 is controversial. Some studies suggest that PI3K/Akt directly phosphyorylates p65 at serine 536 in response to IL-1 whereas, other suggest it serves as an intermediate kinase that

activates IKKα by phosphorylation at threonine 23 which in turn phosphorylates serine 536 (193;203). Other p65 phosphorylation sites have been identified. Casein kinase II and protein kinase A have been shown to phosphorylate p65 at serine 529 and 276 (204;205). GSK-3 β phosphorylates p65 at ser 468; this reduces the basal activity of NFκB to basal activity in resting cells (206).

1.8.3 NFκB and prostate cancer

NFκB has been shown to be constitutively activated in prostate cancer cells, and elevated NFκB activity is also sustained in androgen-responsive human prostate cancer cells by androgen treatment (207). Huang *et al* have demonstrated that suppression of NFκB activity in human prostate cancer cells by IκBα mutation transfection inhibits their tumorigenic and metastatic properties in nude mice by suppressing angiogenesis and invasion. IκBαM transfection-blocked NFκB activity was associated with down regulation of VEGF, IL-8 and MMP-9 promoter activities and decreased expression of these genes in cultured cells and in cells implanted into the prostate gland of nude mice. The decreased expression of VEGF, IL-8 and MMP-9 *in vivo* directly correlated with decreased neovascularization and production of lymph node metastasis. This provides direct involvement of NFκB in the regulation of angiogenesis and metastasis of prostate cancer cells. Numerous reports have demonstrated that in prostate cancer as well as other tumour types that the metastatic potential of tumour cells directly correlates with the expression level of several angiogenic genes, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (Bfgf), interleukin 8 (IL-8) and matrix

metalloproteases MMP-2 and MMP-9 (198;208). Expression of MMPs is associated with poor prognosis in a variety of cancers including prostate. MMPs are enzymes that are capable of degrading the extracellular matrix and basement membrane. An increase in MMP-2 and MMP-9 is associated with tumour progression but how the constitutive expression of these genes is regulated in prostate cancer is at present unclear. In addition NFκB has been associated with the development of castrate resistant prostate cancer. The association between steroid hormone receptor expression and NFκB activation has been of substantial interest in both prostate and breast cancers. Primary investigations of breast cancer cell lines and also of solid tumours suggest an inverse correlation between NFκB activation and oestrogen receptor (ER) expression. However this correlation has not been found in all studies. Prominent constitutive NFκB has been observed in the prostate cancer cell lines PC-3 and DU-145 which lack AR expression however, only very low levels of NFκB were seen in the AR positive cell line LNCaP (197). This data suggests that either the presence of AR actually inhibits NFκB activity in prostate cancer or alternatively that constitutive activation of NFκB may correlate with AR loss, which in turn may contribute to compensatory cellular changes, allowing cell survival and growth in the absence of AR activation. Chen *et al*, have observed markedly higher NFκB activity in an androgen independent prostate cancer xenograft model than in its androgen dependent counterpart (209). Here NFκB activated expression of AR regulated gene PSA. This suggests that NFκB is contributing to androgen independent prostate cancer cell growth in the absence of the AR signallingpathway. It was suggested that the absence of PTEN might contribute to constitutive

activation of NFκB induced by PI3K/Akt pathway. However no direct correlation has been observed in prostate cancer cell lines. NFκB has been implicated with prostate cancer progression via two mechanisms, promotion of metastases via MMP-9 expression or promotion of androgen independence via an as yet unknown mechanism.

The signallingcascades described in brief highlight some of the many possibilities of the complex intermolecular signallingmechanisms that are believed to contribute to the progression of the castrate resistant disease. As previously mentioned ligand independent phosphorylation of the AR has been reported to enhance AR transcription in cell lines and there is now unquestionable evidence that AR signallingcontinues to play a critical role in many patients with castrate resistant disease. The PI3K and NFκB cascades have both been implicated in castrate resistant disease and PI3K activity has been reported to influence NFκB signalling. However the precise role of the interaction of signallingcascades and the AR in the development of clinical castrate resistant disease is still poorly understood.

1.9 Statement of research aims

The research presented in this thesis investigated the role of the PI3K/Akt and NFκB cascades in the development and progression of castrate resistant prostate cancer. Using prostate tumours consisting of matched histological tissue specimens obtained before commencement of androgen deprivation therapy and after the development of castrate resistant disease, the members of the PI3K signallingcascade were investigated at a genetic and protein expression level. This tested the hypothesis that

genetic alterations lead to functional consequences in the terms of protein expression. It was speculated that increased protein expression of the components of this pathway are linked to clinical parameters such as time to relapse, time to death from relapse and overall survival. As Akt is believed to be a key mediator in the development of castrate resistance, via its interaction with multiple signallingcascades, the association between the PI3K/Akt and NFκB signallingcascades were investigated. The expression and activation levels of NFκB were determined in the clinical samples and *in vitro* studies investigated the effects of NFκB inhibition on cell proliferation and apoptosis.

The protein expression of AR and phosphorylated AR at the Akt consensus site was also investigated in the clinical samples and correlated with clinical parameters. *In vitro* studies investigated the effect of PI3K inhibition and siRNA silencing on Akt and ARexpression in castrate resistant LNCaP cell lines.

Ultimately the goal of this research was to further our understanding of the mechanisms that drive the development of resistance to anti androgen therapy in prostate cancer patients.

Research Aims

- To determine if members of the PI3K cascade have gene amplifications or deletions in prostate cancer patients
- To determine the protein expression levels of members of the PI3K cascade in prostate cancer patients and to investigate if protein expression is related to patient outcome.

- To determine the protein expression levels of components of NFκB cascade in prostate cancer patients and investigate if protein expression is associated with patient outcome.
- To investigate the effect of NFκB inhibition on prostate cancer proliferation, apoptosis using matched hormone naïve and castrate resistant LNCaP cell lines.
- To determine the expression levels of AR and AR phosphorylated at the Akt consensus site in prostate cancer patients and investigate if protein expression is associated with patient outcome.
- To determine the levels of activated Akt and AR in matched hormone naïve and castrate resistant LNCaP cell lines and determine if these mirror the tissue observations.
- To investigate the effect of PI3K siRNA silencing and inhibition on Akt and AR expression in matched hormone naïve and castrate resistant LNCaP cell lines.

2. Materials and Methods

2.1. *In- vitro* studies

2.1.1 Culturing of Prostate cancer cell lines

Matched hormone sensitive LNCaP (lymph node metastasis) and castrate resistant LNCaP-CR prostate cancer cell lines were a kind gift from Professor C Robson (Northern Institute for Cancer Research, Newcastle). LNCaP cells were routinely maintained in RPMI 1640 (Invitrogen, UK) containing phenol red and supplemented with 10% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK), and penicillin/streptomycin (50 units/ml, 50μg/ml (Invitrogen, UK)). These cell lines were selected because they are androgen responsive and is an androgen receptor expressing prostate cancer cell line. LNCaP-CR cells have been developed using parental LNCaP cells as a model of hormone resistant prostate cancer by gradual withdrawal of androgens from the medium. Castrate resistant cells are defined by their ability to survive testosterone depravation by castration and to sustain androgen receptor activation through ligand dependent and ligand independent mechanisms. These cells were routinely cultured in RPMI 1640 supplemented with 10% charcoal-stripped foetal calf serum (Invitrogen, UK) known to contain negligible amount of androgens, 2mM L-glutamine (Invitrogen, UK), and penicillin/streptomycin (50 units/ml, 50μg/ml (Invitrogen, UK).

Cells were grown in T-75 flasks (Gibco**)** and maintained in 5% CO2 at 37°C, with the medium changed twice weekly, as it is rapidly acidified.

2.1.2 Trypsinisation of cells

Sub-confluent cultures (70-80%) were routinely passaged 1:6 using trypsin (Invitrogen, UK) to prevent the cells becoming confluent and forming clumps. Used medium was removed from the flasks and the cells washed twice with warmed Phospho-Buffered Saline (PBS) (Invitrogen) to eliminate traces of serum, which includes trypsin inhibitors. Cells were then incubated in 3 mls of trypsin for 5 minutes in 5% CO_2, 37°C, in order to detach the cells from the flask. Once cells were no longer adherent, 3mls of RPMI was added to inactivate the trypsin. The cells were disaggregated from their clusters by gentle pipetting and seeded into new T -75 flasks containing 10mls of fresh RPMI. LNCaP cells grow slowly in clusters and were left for up to 48 hours to reattach.

2.1.3 Freezing Cells

Once cells are trypsinised, aliquots of the cells can be stored for future use. The cell suspension was transferred from the flask to a 15ml centrifuge tube and cell pellets were collected by centrifugation at 1200rpm for 5 minutes. The medium was removed and the pellet resuspended in 1ml of RPMI (supplemented 10% foetal calf serum (Invitrogen, UK), 2mM L-glutamine (Invitrogen, UK), and penicillin/streptomycin (50 units/ml, 50μg/ml (Invitrogen, UK)) and 10%DMSO which serves as a cyroprotectant. The cells were immediately transferred in an alcohol bath (Mr Frosty, Sigma) to -80°C for 24 hours before being transferred to liquid nitrogen (-180°C) for long-term storage.

When cell aliquots were required, they were removed from liquid nitrogen and warmed for 1 minute in a 37°C water bath before being promptly transferred to a flask containing 10mls of pre-warmed RPMI. It was essential not to leave the cells defrosting longer than necessary, as DMSO is toxic.

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2.1.4 Drug Treatments

Extracellular stimulus was used to measure the phosphorylation status of several proteins investigated throughout this study in both hormone naive and castrate resistant LNCaP cell lines. All drugs were prepared in large enough volumes to provide enough for all time points and to ensure experimental equality. Details of all the drugs used are listed in Table 2.1

- **Heregulin α (Sigma)**

Heregulin α (HRG) is the ligand for HER3/4. Ligand binding to HER3 results in phosphorylation and dimerisation with HER2 and activation of the growth factor receptor cascades. Cell lines were treated with 10nM HRG. A stock solution of 1.42μM was made and stored at –20°C. Treatment time with HRG ranged from five minutes to three hours.

- **Epidermal Growth Factor (Sigma)**

Binding of Epidermal Growth Factor (EGF) to its receptor (EGFR) results in phosphorylation and, either homo- or hetero-dimerisation, leading to the activation of growth factor receptor signallingcascades. Cell lines were treated with 10nM

EGF. A stock solution of 10μg/ml was prepared in PBS. Treatment time with EGF ranged from five to thirty minutes.

- **Insulin like Growth factor 1 (Calbiochem)**

Binding of Insulin like growth factor-1 (IGF-1) to its receptor (IGFR-1), activates growth factor receptor signallingcascades. Both cell lines were treated with 10ng/ml. A stock solution of 10μg/ml was prepared in PBS, 1%BSA. Treatment time with IGF-1 ranged from five minutes to three hours.

- **LY 294002**

LY294002 is a cell permeable compound that acts as a potent and selective inhibitor of PI3K. It blocks the catalytic activity of PI3K without affecting other kinases including PKC, PKA, MAPK, EGFR and Src. A stock solution of 3.25mM LY294002 was prepared in ethanol and treatment concentrations ranged from 10 to 1000uM for 48 hours.

- **Tumour necrosis factor Alpha (TNFα) (Sigma)**

In response to inflammatory stimulation, macrophages or monocytes secrete TNFα that can induce apoptotic or necrotic cell death of certain tumour cell lines. TNFα is also capable of inducing cell proliferation and differentiation in many types of cells under certain circumstances. Functional characteristics of TNFα are executed through specific members of the TNF receptor (TNFR) superfamily. These receptors trigger several intracellular signallingpathways, most importantly, the IKK and

MAPK cascades, which govern gene expression through NFkB and AP-1 transcription factors, respectively. A stock solution of 90ng/ml was prepared in distilled water and cell lines were treated with 30ng/ml.

- **NFκB inhibitor (2607 and 2070)**

Two NFκB inhibitors were used in this study: 2070 and 2607, and were supplied by Caledonian Biotech. These inhibitors were designed to directly block phosphorylation hence activation of NFκB. A stock solution of 2mg/ml of each was prepared in ethanol and treatment concentrations ranged from 0.10 to 100uM.

- **Dihydrotestosterone (DHT)**

DHT binding to the AR results in AR phosphorylation, translocation to the nucleus and transcription of AR genes. Cell lines were treated with 10nM DHT. A stock solution of 10μg/ml was prepared in DMSO. Treatment time with DHT ranged from five minutes to three hours.

Drug	Molecular Weight (Daltons)	Drug prepared in	Stock Concentration	Final Concentration	Pre-treatment
Heregulin (HRG)	7000	PBS	1.42μM	10nM	NO
Epidermal Growth Factor (EGF)	6200	PBS	1.61mM	10nM	NO
Insulin growth factor (IGF)	7500	PBS	50μM	10ng/ml	NO
LY 294002	307.4	Ethanol	3.25mM	20μM	YES
2607	500	Ethanol	2mg/ml	100μM	NO
2070	400	Ethanol	2mg/ml	100μM	NO
TNFα	25,600	Water	90ng/ml	30ng/ml	NO
DHT	290.44	DMSO	10nm	10nm	NO

Table 2.1: Drug informations

Details of all the drugs used throughout the course of this study; pre-treatment involved exposure to drugs for 30 minutes prior to other treatments.

2.1.5 Time course treatments of LNCaP and LNCaP-CR

Cell lines were grown in T-75 flasks until 80% confluent, the medium was removed and the cells washed in warmed PBS. Then, cells were incubated in serum free RPMI overnight. The following day, medium was removed and the cells washed in warmed PBS in preparation for the appropriate drug treatment. The first time course treatment carried out was with 10nM HRG at five different times, 5, 15, 30, 60 and 180 minutes; all treatments were performed identically and in duplicate.

Additionally an untreated control (cells treated with serum free RPMI and PBS) was included as was a completely untreated control, LNCaP cells, that received nothing but serum free RPMI (without vehicle). Each flask was treated with 3mls of drug or control, ensuring that all cells were completely covered, and incubated at 37°C in 5% CO_2 for the required time. The drug was removed from the flask and cells carefully rinsed twice, with ice-cold PBS. It was important to remove as much of the PBS, using a pipette, as possible, as it can dilute the lysis buffer. Cells were then lysed in 500µl Phosphosafe buffer (Calbiochem) and 1:100 Protease inhibitor cocktail set 1 (Calbiochem) for 5 minutes on ice, and collected using a cell scraper (Gibco). The cell lysates were then transferred to an appropriately labelled 1.5ml eppindorff tube (Gibco) and stored on ice until all treatments were completed. All samples were centrifuged at 14 000 rpm at 4°c for 15 minutes, the supernatant removed and subsequently stored at -70°C. This experiment was performed twice; therefore, in total there were four protein samples for each treatment condition available for analysis. Time course treatments using EGF, IGF-1 and DHT were performed identical to that described above. TNFα time course treatments were carried out as above excluding the 180 minute time point.

2.1.6 Inhibition of PI3K using LY294002

Cell lines were grown in T-75 flasks until 80% confluent, the medium was removed and the cells washed in warmed PBS. Subsequently, cells were incubated in serum free RPMI overnight. The following day, medium was removed and the cells washed in warmed PBS. This was followed by incubation in the presence or absence of 20 uM LY294002 for 30 minutes, followed by stimulating with IGF-1 for 1 hour.

Additionally cells were treated in the presence or absence of IGF-1 for an hour without any inhibitor. Protein was extracted as previously described in section 2.1.5.

2.1.7 Inhibition of NFKB using 2607 and 2070

Cell lines were grown in T-75 flasks until 80% confluent, the medium was removed and the cells washed in warmed PBS. Subsequently, cells were incubated in serum free RPMI overnight. The following day, medium was removed and the cells washed in warmed PBS. This was followed by incubation in the presence or absence of 2607 or 2070 diluted in culture media at concentrations ranging from 0.1 uM to 100uM for 24 hours. Protein was extracted as previously described in section 2.1.5.

2.1.8 Determination of protein samples

The method used to determine protein concentration of the cell lysates was Bio-Rad's protein assay, which was based on the Bradford dye-binding procedure (Bradford 1976), and involves a colorimetric assay for measuring total protein concentration. Protein standards were prepared using Bovine Serum Albumin (BSA) (Sigma). The BSA was supplied at a concentration of 2mg/ml, but for the purpose of this study it was diluted to 1mg/ml with dH_2O. One reference and seven protein standards were prepared in disposable cuvettes (Gibco) in triplicate as shown in Table 2.2.

Volume of 1mg/ml BSA required (µl)	Volume of dH_2O required (µl)
0 (REFERENCE)	800
1	799
5	795
10	790
15	785
20	780
25	775
50	750

Table 2.2: Protein standards

Samples were prepared in triplicate for a low-concentration assay in disposable cuvettes (Gibco). 200µl of Bio-Rad Protein Assay Reagent (Bio-Rad) was added to the cuvette, followed by 795µl of dH_2O. Then 5µl of protein sample was included to the mix. The solution was thoroughly mixed with a pipette to ensure even distribution of the protein for an accurate concentration reading. Once prepared the reference and standards were used to calibrate the spectrophotometer (Bio-Rad) Protein 595 assay programme.

The optical density at 595nm (O.D. 595nm) was measured for the reference and the seven protein standards. The O.D 595 was then read for all the samples and the concentration of protein present generated from the standards concentration. The spectrophotometer calculated the amount of protein (µg/ml) present, but the theory behind it involves plotting a graph of absorbance at 595nm against protein concentration of standards. This standard curve is then used to determine the concentration of the protein present in the samples from its O.D. 595 value. The concentration (µg/ml) was calculated from a diluted protein sample (1:200). From this the final concentration in mg/ml was determined as follows:

Protein reading (µg/ml) x 0.2 = Final protein concentration (mg/ml)

Proteins were aliquotted into (100µl) samples and stored at -80°c until required. For western blotting 50µg of protein was used and the volume of sample required was calculated from the final concentration.

2.2 Western blotting of protein samples

Western blotting (immunoblotting) was used to verify the specificity of all antibodies and also to measure the levels of protein in samples obtained from the time course and inhibitor treatments.

- **Preparation of SDS-PAGE (Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis) gels**

Western blots were carried out using the Bio-Rad Mini-PROTEAN 3 Electrophoresis System. For all the proteins analysed it was suitable to use 10% resolving gels, which were prepared as follows**:**

Reagents	10% Gel
40% Acrylamide/Bis-Acrylamide (Sigma)	12.49ml
0.5M EDTA	330µl
2M Tris, pH 8.9	8.35ml
10% SDS	500µl
dH_2O	28.33ml
10% APS	300 µl
TEMED	30µl

Gels are formed from the polymerisation of the acrylamide and N-N-methylene-bis-acrylamide (bis). Bis acts as the cross linking agent for the gel, and the TEMED and APS are the catalysts for gel polymerisation. The separation of proteins within the

gel is directed by the size of the pores within the gel, this is determined by the amount of acrylamide-bis present. As the amount of acrylamide present increases, the pore size decreases. In general a higher percentage of resolving gels are used for smaller proteins, and lower percentage gels are more effective for separating larger proteins.

A gels thickness of 1.5mm was used throughout the various studies; these were produced using 1.5mm spacer plate. Once the gel solution was prepared, excluding the TEMED and APS, the gel plates and gel casting apparatus were assembled. Having correctly assembled the plates, the TEMED and APS were added to the resolving gel solution, using a plastic pastette the gel mix was poured in between the two glass plates, to a level that allowed for the stacking gel and comb to be added. A layer of isopropanol was applied to the top of the gel to remove any air bubbles and to flatten it out at the top. The resolving gel was then left to polymerise for approximately one hour.

Once set, the isopropanol was removed from the top of the resolving gel. At this stage the gel (wrapped in damp tissue and saran wrap) could be stored at 4°C until required.

When required 4.5% stacking gel was prepared as follows:

Reagents	**4.5 % Gel**
40% Acrylamide/Bis-Acrylamide	5.63ml
0.5M EDTA	400µl
1M Tris, pH 6.8	6.25ml
10% SDS	500µl
dH_2O	37.22ml
10% APS	30µl
TEMED	10µl

Again, the TEMED and APS were the last two reagents to be added to the gel solution. The stacking gel was then poured onto the resolving gel to fill the plate, the gel comb (15 well combs) was then inserted and the gel left to polymerise for approximately 30 minutes.

- **Protein denaturation**

To enable the primary antibody to recognise and bind to its epitope, it was necessary to denature the proteins. Denaturing the protein the gives the antibody easier access to the epitope and enables them to run more efficiently through the gels. Having previously obtained the concentration of protein within each sample, the 50μg removed from each sample and transferred to a new Eppendorf tube. This was stored on ice, and 2X Laemmli's sample reducing buffer was added to each sample. This buffer consisted of:

1.0ml 0.5M Tris-HCl

0.8ml Glycerol

1.6ml 10% sodium dodecyl sulphate (SDS)

0.4ml 2-Mercaptoethanol

0.2ml Bromophenol Blue (0.05% w/v)

4.0ml dH_2O

The samples were thoroughly mixed and then boiled at 100°C for four minutes. The molecular weight marker (Biotinylated Protein Ladder –Cell SignallingTechnology) that was used to determine the size of the detected protein was also boiled at 100°C for four minutes.

The SDS within the sample buffer is a detergent, with a highly negative charge. It has a hydrophobic tail that binds to the proteins, causing them to become negatively charged. SDS also disrupts the tertiary structure of the protein, resulting in their unfolding. The 2-Mercaptoethanol prevents the reformation of disulphide bonds and helps maintain the protein in its denatured state. Boiling the samples also contributed to the denaturing of the proteins by unfolding them completely. Once boiled samples were immediately stored back on ice.

- **Gel Electrophoresis**

The principle behind electrophoresis is that an electrical charge moves the proteins down the polyacrylamide gel. The proteins are negatively charged, due to the SDS and they are attracted towards the positive anode. The pores produced by the acrylamide result in smaller protein molecules travelling through the gel at a faster rate than the larger molecules; thus, they progress further down the gel. Therefore, proteins are separated by gel electrophoresis according to their molecular weight.

Gels were placed into the electrode assembly in the mini buffer tank and combs were removed from gels. The wells were then rinsed and the tank filled with 1X running buffer (details in table 2.2). Denatured protein samples (50μg) and ladder (10μl) were then carefully loaded into the wells using a fine tipped pipette. It was important to prevent overspill between the wells. Once all samples were loaded the gel was run at 100V for approximately one hour.

- **Protein Transfer**

To allow protein detection by a primary antibody, the protein had to be transferred from the polyacrylamide gel to PVDF (polyvinylidene difluoride) membrane. Once the proteins had run sufficiently through the gel, proteins were transferred to the PVDF membrane (Biorad). For optimal transfer, PVDF membrane was pre-treated in 100% methanol for five minutes before being soaked in 1X Transfer Buffer (Details of which can be found in table 2.2.). Fibre pads and 3M Whatman paper were also soaked in transfer buffer. The gels were then carefully removed from the glass plates and the stacking gel was discarded, the remaining resolving gel equilibrated, for approximately five minutes in transfer buffer. This step prevented the gel from shrinking during the transfer process.

The transfer sandwich was then assembled. This comprised of a fibre pad, 3 squares of 3M Whatmann paper, gel, and PVDF membrane, 3 squares of 3M Whatmann paper and finally a fibre pad. This is shown below in Figure 2.1.

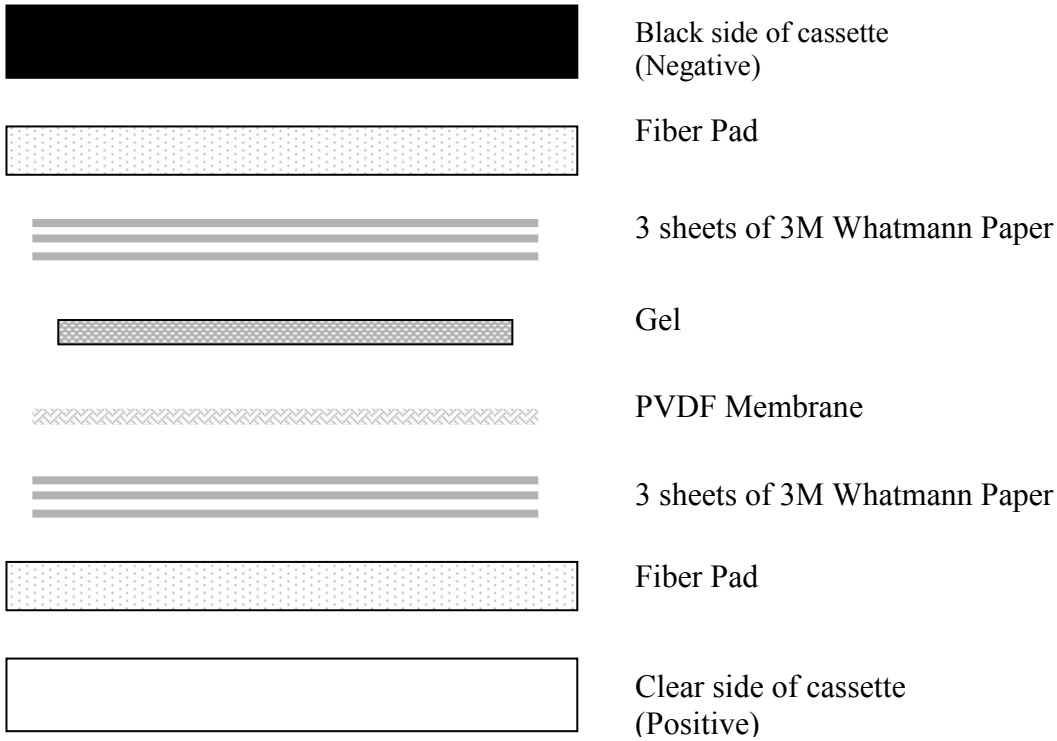


Figure 2.1: Schematic Representation of Western Blot Transfer Sandwich

Schematic representation of the assembly of the transfer sandwich on the cassettes, the sandwich was comprised of fiber pads, 3M Whatmann paper, gel and PVDF membrane, all of which had been equilibrated in transfer buffer.

While building the sandwich, it was important to make sure that there were no air bubbles present; this would prevent efficient transfer. After each new layer was applied, air bubbles were removed by gently rolling a glass rod over the top surface. The assembled sandwich was then slotted into the electrode assembly and placed in the mini-tank, which was filled with transfer buffer. To reduce the temperature of the buffer during the transfer process, the Bio-Ice cooling unit was used. A magnetic stirrer was also added to maintain even buffer temperature and ion distribution. Proteins were transferred from the gel (negative/cathode) to the membrane (positive/anode) at 100 volts for one hour.

- **Blocking of Membrane**

To prevent the primary antibodies from binding non-specifically to the membrane and also to reduce background staining, it was necessary to block the membrane. This was achieved by incubating the membrane in 5% Non-Fat Dry Milk (Marvel) blocking solution, prepared in TBS-Tween (TTBS) for one hour at room temperature. This step and all future steps were performed on an orbital shaker.

Buffer	Reagents
10X Running Buffer	200mM Tris, 2M Glycine, 1% SDS (For 1X dilute in dH_2O)
10X Transfer Buffer	248mM Tris, 1.3M Glycine, 20% Methanol (For 1X dilute in dH_2O)
10X TBS	0.1M Tris/HCl, 1.5M NaCl, pH = 7.4 (For 1X dilute in dH_2O)
0.001% TTBS	1000µl of Tween 20 per litre of 1X TBS

Table 2.3: Buffers used in western blotting

Details of the buffers and solutions used in western blotting

- **Incubation of membrane with primary antibody**

Having blocked the membrane, the following step was incubation in primary antibody. All antibodies were prepared in 10mls of 5% Non-Fat Dry Milk/TTBS solution, to further reduce non-specific binding. Membranes were incubated with primary antibody overnight (approximately 18 hours) at 4°C.

- **Incubation of membrane with secondary antibody**

Following incubation with primary antibody, the membrane was washed in TBST three times for 20 minutes, to remove any excess antibody. Detection of the protein of interest required a secondary antibody bound to either biotin or an enzyme conjugate, such as horseradish peroxidase (HRP), which was species-specific to the

primary antibody. The secondary antibodies used were HRP-linked anti-mouse IgG (Cell SignallingTechnology) anti-rabbit IgG (Cell SignallingTechnology) and anti-goat IgG (Dako). The anti-mouse and anti-goat antibodies were used at dilution of 1:10,000 and the anti-rabbit was used at a dilution of 1:2000. In addition a secondary antibody for the detection of the biotinylated ladder was required. For this the anti-biotin HRP linked antibody (Cell SignallingTechnology) was used at a dilution of 1:1000. The anti-biotin and anti-mouse/rabbit /goat secondaries were prepared together in 10mls of 5% Non-Fat Dry Milk/TTBS solution and were incubated with the membranes for one hour at 37°C.

- **Protein visualisation**

The final steps in the western blotting protocol involved detection of the protein of interest using a chemiluminescent method. Luminescence is the emission of light due to the dissipation of energy from a substance in an excited state. Horse-radish peroxidase catalyzes oxidation of luminol, a chemiluminescent substrate, in alkaline conditions. Oxidation results in the luminol being in an excited state which then decays to ground state via a light emitting pathway. For this method ECL plus (Amersham) was used. The principle behind this is that horse-radish peroxidise, conjugated to the secondary antibody, oxidises the ECL Plus chemiluminescent substrate Lumigen PS-3 Acridan, which produces thousands of acridinium ester intermediates per minute. These intermediates then react with the peroxide to produce a sustained, high intensity chemiluminescence with a maximum emission at 430nm. This light is then detected on autoradiography film. Following incubation with the secondary antibody, membranes were washed three times in TTBS for 20

minutes. While the membranes were washing, the ECL plus reagents were decanted into a universal and warmed to room temperature. Once the reagents had warmed, solution A and solution B were mixed in a ratio of 40:1; 3mls of solution was an adequate amount per membrane. When using the ECL reagents all steps were performed in semi-darkness. Membranes were placed protein side up on a sheet of saran wrap. The ECL solution was pipetted onto the membrane ensuring complete coverage. The membrane was incubated with the reagents for five minutes, and excess solution was then removed and the membrane transferred to a fresh piece of saran wrap, which it was then enveloped in. Lastly the membrane was transferred to a film cassette where it was exposed to autoradiography film for various times. Generally the incubation times were 30 seconds and 1, 5, 15 minutes. The film was then developed using a Kodack X-OMAT x-ray processor and both the marker and protein bands visualised.

- **Stripping Membranes**

To confirm equal sample loading, primary antibody was removed from probed membranes using 10X Stripping buffer (Chemicon). The membrane was washed in TBST 3 times for 10 minutes, to remove any excess antibody. Membranes were incubated in 20mls of stripping buffer (diluted 1:10 in dH_2o) at 37°C for 20 minutes, subsequently, membranes were blocked in 5% Non-Fat Dry Milk/TTBS and then probed with anti-αTubulin HRP linked antibody (1:1 000 AbCam) to confirm equal protein loading.

2. 3 ELISA

2.3.1 pAKT ser 473 ELISA

The phosphoELISA™ kit (Biosource, UK) was used to assess Akt activity in both cell lines. This assay is a solid phase Sandwich Enzyme linked-Immuno-Sorbent Assay (ELISA) and begins with a monoclonal antibody specific for Akt (regardless of phosphorylation state) coated onto the wells of microtitre strips. Samples, including a standard containing Akt, control specimens, and unknowns, are added to these wells. During the first incubation, the protein antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody specific for total protein or protein phosphorylated at a specific residue is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase–labelled anti–rabbit IgG antibody (anti-rabbit IgG-HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess anti-rabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of total or phosphorylated protein present in the original specimen.

ELISA was performed according to the manufacturer's instructions. In brief; the Protein standards were prepared by reconstituting the standard 1 with Standard

diluent buffer and left at room temperature for ten minutes, this was to ensure complete reconstitution. The standards were then set up as follows.

Standard:	Add	Into
100 Units/ ml	Prepare as described	
50 Units/ ml	250 µl of 100 Units/ml std	250 µl of Diluent buffer
25 Units/ ml	250 µl of 50 Units/ml std	250 µl of Diluent buffer
12.5 Units/ ml	250 µl of 25 Units/ml std	250 µl of Diluent buffer
6.25 Units/ ml	250 µl of 12.5 Units/ml std	250 µl of Diluent buffer
3.12 Units/ ml	250 µl of 6.25 Units/ml std	250 µl of Diluent buffer
1.6 Units/ ml	250 µl of 3.12 Units/ml std	250 µl of Diluent buffer
0	250 µl of Diluent buffer	Empty tube

Firstly, 100 µl of the standard diluent buffer was added to the blank wells to zero the wells. EGF treated LNCaP cell lysates as described in section 2.1.5 were then diluted 1:10 in standard diluent buffer.100 µl of standards and the prepared samples were placed in the appropriate wells, this was then incubated for two hours at room temperature. The solution was then carefully removed from the wells and washed four times with washing buffer. 100 µl of anti-Akt (detection antibody) solution was added into each well (excluding the chromagen blanks). The plate was then tapped gently on the side to carefully mix the sample and antibody and incubated for one hour at room temperature. The solution was carefully removed and wells washed four times with washing buffer. Following this, 100 µl anti-rabbit IgG-HRP working solution was added to each well (except the chromagen blanks) and incubated for thirty minutes at room temperature. The antibody was then removed and wells washed four times with washing buffer. Subsequently 100 µl of Stabilised Chromagen was added to each well and the plate incubated for thirty minutes in the dark, at room temperature. Then 100 µl of Stop Solution was added to each well and

the side of the plate tapped gently to ensure thorough mixing and even distribution of the protein for an accurate concentration reading. This solution changes the colour from blue to yellow. The absorbance was measured using a 96 well microplate reader at 450 nm having blanked the reader against a chromagen blank composed of Stabilised Chromagen and Stop Solution. Using Excel the absorbance of the standard against the standard concentration was plotted. The values obtained for the samples were multiplied by the dilution factor (10) to correct for the dilution.

2.3.2 Histone/DNA ELISA for detection of apoptosis

The Cell Death Detection ELISA Kit (Roche, USA) was used to detect apoptosis in both prostate cancer cell lines treated with increasing concentrations of 2607 and 2070, diluted in culture media for 24 and 72 hours. This is a one step sandwich ELISA for relative quantification of histone-complexed DNA fragments (mono- and oligonucleosomes) from the cytoplasm of cells after the induction of apoptosis. LNCaP and LNCaP CR cells were seeded $5x10^3$ cells (100 µl) per well and cultured for 24 hours followed by incubation for either 24 or 72 hours with 1, 10 and 50 µM of 2607 or 2070, diluted in culture media. After the incubation, the cells were pelleted by centrifugation at 200xg for 10 minutes at room temperature and the supernatant was discarded. The cells were then resuspended with 100 µl of lysis buffer and incubated for thirty minutes at room temperature. After lysis, the cells were collected by centrifugation at 200x g and 20 µl of the supernatant was transferred to a streptavadin coated microtiter plate. 100 µl of Immunoreagent (two monoclonal antibodies, antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated) was added to the wells and incubated at room temperature for two

hours. The immunoreagent was carefully removed and wells washed three times with washing buffer to remove cell components that were not immunoreactive. Following this samples were incubated with peroxidase substrate for fifteen minutes at room temperature and absorbance of the samples was measured using a 96 well microplate reader at 405 nm.

2.4 Cell proliferation assay

Proliferation was assessed using the WST-1 (Water Soluble Tetrazolium Salts) assay (Millipore, UK). Proliferation is based on the cleavage of the tetrazolium salt WST-1 (water soluble tetrazolium salt, in the presence of 1-methoxy PMS) to formazan by cellular mitochondrial dehydrogenases. Expansion of viable cell numbers results in an increase in activity of the mitochondrial dehydrogenases in the sample, corresponding to an increase in formazan dye metabolism. The formazan dye produced by the viable cells is measured at an absorbance of 440 nm using a standard multiwell spectrophotometer.

Cells were seeded in 96 well plates at a density 5x103 cells (100 µL) per well in standard culture media. The assay was performed at 48, 72 and 96 hours by adding 10 µL of WST-1 reagent prior diluted in Electro Coupling Solution (ECS) to each well. The optical absorbance level was measured after 2 hours incubation at 370C, (this time point was determined in previous studies performed within our group) using a 96 well microplate reader at 450 nm with reference wavelength 600 nm. Each experiment was repeated three times and each condition was done in triplicate.

2.4.1 The effect of PI3K/Akt on proliferation

To elucidate the role of PI3K/Akt cascade in regulating proliferation, LNCaP cells were seeded in 96-well plates at $5x10^3$ cells (100 µL) per well and cultured for 24 hours followed by incubation with 30 uM, 100 uM, 300 uM and 1000 uM of LY 294002, diluted in culture media for 72 hours. WST assay was performed at 48 and 72 hours as described in section 2.1.8.

2.4.2 The effect of NFKB on proliferation

To elucidate the role of NFKB in regulating proliferation, LNCaP cells were seeded in a 96 well plate, at $5x10^3$ cells (100 µL) per well and cultured for 24 hours followed by incubation with 1 uM , 10 uM and 50 uM of 2607 and 2070, diluted in culture media for 48 hours. WST assay was performed at 48 hours as described in section 2.1.8.

2.4.3 Statistical analysis for cell line studies

All statistical analysis was performed using the SPSS version 15 for Windows. To assess if the novel NFκB inhibitors were sufficient to inhibit proliferation (WST) and stimulate apoptosis (cell death ELISA) the Dunnets and LSD tests were performed. This compares values of untreated and treated samples. A value of $p < 0.05$ was considered statistically significant.

2.5 siRNA Knock down of PI3K

LNCaP CR cells were transfected with the Cell Line Nucleofector® Kit R, Program T-009 as described by manufacturer (Lonza, Cologne, AG). Briefly, 8-well chamber

slides were filled with 0.5mls of RPMI supplemented with heat-inactivated foetal calf serum (10%) and 2mM L-glutamine, (penicillin/streptomycin was omitted to allow the cell membrane to recover after transfection). Chamber slides were then placed in a humidified 37°C/5% CO_2 incubator to equilibrate. 70% confluent cells were harvested by trypsinization (section 2.1.2) and cells were counted using a haemocytometer. $1x10^6$ cells per sample were centrifuged at 1000xg for 10 minutes at room temperature and supernatant completely removed. The cell pellets were then resuspended in 100 µl room-temperature Nucleofector® Solution and combined with 300 nM PI3K siRNA SMARTpool or nonspecific siRNA pool (Dharmacon, Lafayette, CO, USA). Samples were then transferred to cuvettes and transfected using Nucleofector® Program T-009 (Lonza). 400 µl of warmed culture medium was added to each cuvette and 50 µl of cells were gently transferred into the prepared 8-well chamber slide to give a final cell number of $5 x10^4$ cells per well . The cells were allowed to settle for 24 hours before the medium was replaced with RPMI supplemented with heat-inactivated foetal calf serum (10%), 2mM L-glutamine and penicillin/streptomycin (50 units/ml, 50µg/ml) for another 48 hours. The cells were analysed 72 hours post siRNA transfection by Immunofluorescence (IF).

2.6 Immunofluoresence (IF)

Immunofluorescence (IF) is a method that allows the detection of a specific protein or antigen in cells or tissue sections by fluorescent visualisation. There are two types of IF direct and indirect. Direct IF requires using a primary antibody labelled with

fluorescent dye, whereas the indirect approach involves the use of a secondary antibody labelled with a fluorescent marker capable of binding to the primary antibody which is bound to the antigen. The protein of interest is then visualised using a fluorescent microscope. The indirect method was used to investigate the expression of proteins in prostate cancer cells following PI3K siRNA knockdown as described in section 2.5. Following knockdown cells were washed twice with ice-cold PBS, before being fixed to the culture slides by incubation in ice-cold methanol (500µl of methanol per well) for three minutes. Excess methanol was removed by washing the cells three times in dH_20 for five minutes. To reduce non-specific binding of the antibody, cells were incubated in 500µl of 1.5% normal horse serum (Vector Laboratories) in antibody diluent (DAKO) for twenty minutes. As of this point all steps were performed on an orbital shaker and at room temperature. Cells were incubated with the appropriate primary antibody for 1 hour (100µl of antibody per well) before being washed three times with TBS for ten minutes. Following this, cells were incubated with either 100µl biotinylated mouse or rabbit secondary antibody (3µg/ml in antibody diluent) (Vector Laboratories) for thirty minutes. Cells were again washed three times with TBS for ten minutes, before being treated with 100µl of Fluorescein Avidin D (diluted 1:100 in TBS) (Vector Laboratories) for thirty minutes. Once the cells were treated with the fluorescent protein, slides were covered in foil and all future steps performed in semi-darkness, to prevent the fluorescence from fading. Finally cells were washed with TBS three times for ten minutes. At this stage culture slides were mounted onto coverslips, using Vectashield mounting media with 4'-6-Diamidino-2-phenylindole (DAPI) (635µl

mountant media : 65µl mountant media with DAPI)(Vector Laboratories). DAPI stains nuclei specifically, with little or no cytoplasmic labelling. The protein of interest was then visualised using a fluorescent microscope with either a DAPI or FITC filter.

2.7 Patients

Sixty-eight patients with matched hormone-naive and castrate resistant tumour pairs were retrospectively selected for analysis. All tumours had patient identification removed, including block number and hospital number and were coded to make the database anonymous. Ethical approval was obtained from the Multicentre Research Ethics Committee for Scotland (MREC/01/0/36) and Local Research and Ethical Committees. Patients were only selected for analysis if they initially responded to hormone treatment (in the form of subcapsular bilateral orchidectomy or maximum androgen blockade), but subsequently relapsed (two consecutive rises in PSA greater than 10%) and had a pre- and post castrate resistant tissue sample available for analysis. The database used for the study consisted of matched pairs of tissue obtained sequentially from patients before androgen deprivation therapy and following the development of castrate resistant prostate cancer. These sections originated from either a transurethral resection of the prostate (TURP) or from diagnostic transrectal ultrasound (TRUS) biopsies. Following the development of biochemical relapse, castrate resistant tissue was obtained from palliative TURP, carried out to relieve clinical outflow symptoms.

2.8 Fluorescence in situ hybridisation (FISH)

Fluorescence *In Situ* Hybridisation is a method that uses fluorescently labelled DNA probes to visualise chromosomal or genetic abnormalities. FISH probes are single stranded DNA that are able to bind to the complimentary DNA strand on the chromosome/gene of interest and consequently allow the fluorescence detection of the chromosome/gene.

Probes were provided by Dako A/S to investigate genetic changes (gene amplifications and deletions) of *PIK3CA* (p110 catalytic subunit of PI3K), *PTEN*, *Akt 1-3* and *mTOR*. FISH was performed on 5 μm, archival formalin fixed; paraffin embedded prostate tumour tissue arrays (TMA's; 4 cores from each patient for prostate carcinomas). The probe mix consisted of a mixture of Texas Red-labelled DNA cosmid clones covering the sequence of *PIK3CA* (p110 catalytic subunit of PI3K), *PTEN*, *AKT1*, *AKT2*, *AKT3* or *mTOR* genes and a fluorescein isothiocyanate (FITC)-labelled chromosome specific reference for determining numerical gene aberration. Specificity of both genetic and reference probes was tested by restriction analysis. Localization of the probe mix was validated by hybridisation to metaphase spreads of normal cells which showed hybridisation to all associated chromosomes (all performed by Dako A/S before sending the probes). FISH was performed using a Histology FISH accessory kit (Dako Denmark A/S, Glostrup, Denmark) according to the manufacturer's instructions but with minor modifications. In brief slides were incubated for 1 hour at 56^{o}C, dewaxed and re-hydrated through graded alcohols. Slides were rinsed twice in wash buffer and then incubated for 10 min in pre-treatment buffer at 96^{o}C, followed by 3 min incubation at room temperature in wash

buffer. Slides were then incubated in Pepsin for 26 mins at 25^{o}C, followed by 3 min incubation at room temperature in wash buffer and then incubated for 10 minutes in 10% formalin. The slides were then washed, dehydrated and air dried. Ten µl probe mix was applied, the slide mounted and sealed with rubber cement. The probe and target DNA were denatured at 82°C for 22 minutes and the tissue incubated overnight at 45°C in a humidified hybridizer (Dako Denmark A/S, Glostrup, Denmark). After washing in stringent wash buffer for 10 minutes at 65°C, the tissue was dehydrated, air-dried and the slide mounted using vectasheild mounting media with 0.5 µg/ml 4.6-diamindino-2 phenylindole (DAPI) (135µl Vectasheild with DAPI:565µl Vectasheild) (Vector Laboratories). Signals corresponding to both gene and chromosome were visualized using a fluorescent microscope.

2.8.1 FISH Scoring

Gene amplification status was determined in 20 nuclei for each TMA core as the ratio of total number of red signals for the gene of interest probes over the total number of green signals for the chromosome reference probes (chromosome 3 (*PI3KCA*), 10 *(PTEN)*, 14 *(AKT1)*, 19 *(AKT2)*, 1 *(AKT3)* and 1 (*mTOR*)). A total of 4 cores from each patient was analysed and the gene/chromosome ratio was calculated separately for each core. A tumour was considered amplified if the gene/chromosome ratio in at least one core was equal to or more than 1.5 and deleted if the gene/chromosome ratio in at least one core was below 0.8(210).

Borderline cases (cases with gene/chromosome ratio between 0.70 and 0.90 and between 1.8 and 2.2) and 10% of all cases were scored independently by two observers. Gene and chromosome copy number was addressed for each tumour. A tumour was defined as disomic when the mean chromosome copy number (MCCN) (mean number of chromosome signals per counted cancer cell) was between 1.35 – 1.85 (211). When the MCCN was below 1.35 or above 1.85 the tumour was regarded as aneusomic as previously defined (211). I am indebted to Dr Tove Kirkegaard who carried out the *PI3KCa* and *Akt 1-3* FISH.

2.9 Immunohistochemistry IHC)

Immunohistochemistry is a method that allows for the detection of a cellular protein or other antigen within cells and tissues using an antibody specific for the desired antigen. The simplest immunohistochemical methods attach the marker directly to the primary antibody. In general, this direct immunohistochemical method does not have very high sensitivity. An alternative more sensitive method is indirect immunohistochemistry. This involves using a second or "secondary" antibody, labelled with either a visible marker (fluorochrome) or an enzyme that binds to the primary antibody bound to the antigen. This indirect approach generates an amplified signal. The method for this study was the Envision system (DAKO) that involves dextran polymer technology. Dako Envision detection reagent consists of a dextran backbone to which a large number of peroxidase (HRP) molecules and secondary antibody molecules have been coupled. A unique chemistry is used for the coupling reaction, which permits the binding of up to 100 HRP molecules and up

to 20 antibody molecules per backbone. The secondary antibody coupled to the dextran backbone has been raised in goats. It reacts equally well with rabbit and mouse immunoglobulins. Following incubation with the Envision, the tissue is incubated with a substrate solution that consists of diaminobenzidine (DAB) chromagen and hydrogen peroxide. The HRP molecules on the Envision interact with the substrate solution to produce a crisp brown end product at the site of the target antigen/protein, which can be viewed using a light microscope. Immunohistochemistry involves the following steps

- **Tissue preparation**

All IHC was performed on 5μm, archival formalin-fixed, paraffin-embedded prostate tumour sections. Sections were dewaxed in xylene (2x4 minutes) and rehydrated through graded alcohol (100 %(2x2minutes), 90 %(1x2minutes), 70 %(1x2minutes)) washes.

- **Antigen Retrieval**

After formalin fixation and paraffin embedding of tissues, many antibodies react only weakly or not at all with their antigen. This is due to the fact that solvents, heat and fixatives can mask the antigen site. Formation of methylene bridges during fixation, which cross link proteins, mask antigenic sites therefore, it was necessary to include an antigen retrieval step, to break the protein cross-links and expose the antigenic binding site, in order to optimise immunohistochemical staining. Two different heat mediated methods of antigen retrieval were used for the antigens studied. The first involved incubating the tissue sections under pressure in 1L of TE

buffer (1mM EDTA (Sigma), 5mM Tris (VWR), pH 8.0) or 1L Antigen Unmasking Solution (pH 6, 1:100 (Vector)) for five minutes. The alternative method incubated tissue sections for twenty minutes at 96°C in a water bath in 100ml Antigen Unmasking Solution (pH 6, 1:100 (Vector)), or Tris EDTA buffer (10mM EDTA (Sigma), 0.25mM Tris (VWR)) pH 9. All antigen retrieval steps were followed by a twenty minute cool down period.

- **Reduction of background staining: - Blocking steps**

Peroxidase reacts with diaminobenzidine, therefore the presence of endogenous peroxidase activity in tissues is a common problem in IHC as it is a cause of background staining. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide (H_2O_2) (VWR) for ten minutes, followed by a wash in water. A further cause of background staining is the formation of hydrophobic bonds between immunoglobulins and tissue proteins that results in the primary and secondary antibodies binding non-specifically to the tissue section rather than just the target antigen. To reduce this non-specific binding, tissue sections were incubated in 5% normal horse serum (Vector Laboratories) in TBS buffer (0.1M Tris/HCl, 1.5M NaCl, pH 7.4) for twenty minutes.

- **Incubation with Primary Antibody**

Antibody dilutions, incubation times and temperature were established for each protein investigated. All antibodies were diluted to the desired concentration in antibody diluent (DAKO). For each, a dilution series was performed, investigating

various antibody titrations, incubation times and temperatures, to establish the optimal conditions in which to achieve the highest quality of staining, i.e. strongest specific antigen staining with the lowest non-specific background. It was crucial to have both a positive and negative control each time IHC was performed. The positive control confirmed that the chosen IHC method was working while the negative control checked the specificity of the antibody involved. Tonsil tissue, placenta tissue or prostate tissues previously shown to have strong expression of the desired antigen were incubated with the appropriate antibody and used as positive controls. The same tissues were used for negative controls by treating them with a negative isotype matched control reagent (DAKO).

- **Incubation with Secondary Antibody**

Following incubation with antibody or negative control, the slides were thoroughly washed in TBS buffer twice for five minutes. The Envision detection method was used for all antigens. The slides were incubated with Envision for thirty minutes then washed twice for five minutes in TBS

- **Detection & Visualisation**

The chromagen used for staining the tissue sections was 3,3'-diaminobenzidine (DAB) –(5ml distilled water (dH_2O), 2 drops of buffer solution, 4 drops of DAB stock solution, and 2 drops of Hydrogen Peroxidase solution -Vector Laboratories). Slides were incubated with DAB for five to ten minutes to allow brown staining to develop and were then washed in running water for ten minutes.

- **Counterstaining**

Tissue sections were counterstained with haematoxylin and Scots Tap Water Substitute (S.T.W.S). Slides were immersed in the haematoxylin for approximately thirty seconds, until a red colour was produced in the tissue section. Following this slides were then submerged in S.T.W.S for another thirty seconds, to produce a blue colour, in contrast to the brown positive staining of the antigen. The last steps involved dehydrating the tissues through a series of alcohol washes: (70% (1x1min), 90% (1x1min), 100 %(2x1min)) and xylene (2x1min), and then mounting the slides onto coverslips using DPX mountant (VWR) (Dibutyl Phtalate containing Xylene).

2.9.1 Histoscore

Staining was scored blind by two independent observers (Myself and Dr Joanne Edwards) using a semi-quantitative weighted histoscore method (212) also known as the Hscore system (213). Histoscores were calculated using the following formula: 0 x % negative tumour cells + 1 x the % of cells staining weakly positive + 2x the % of cells staining moderately positive + 3 x the % of cells staining strongly positive. The histoscore ranged from a minimum of zero to a maximum of 300. Results were considered discordant if the histoscores differed by more than 50. These cases were re evaluated by both observers. Also, both intra-(variation in individual scoring) and inter-(variation between two observers) class correlation coefficients were calculated Agreement was considered excellent if the ICCC value was > 0.7, an ICCC of 1 indicates identical score (212). The mean of the two observer's histoscores was used for analysis. Changes in protein expression staining between

hormone naive and castrate resistant cases were defined as an increase or decrease out with the 95% confidence interval for the difference in inter-observer variation (i.e. the mean difference between the histoscore that each observer assigns for protein expression plus or minus 2 standard deviations) (102).

2.9.2 Statistical Analysis for Immunohistochemistry Studies

All statistical analysis was performed using the SPSS version 15 for Windows. Basic descriptive statistics were performed to calculate the frequencies, mean, median and inter-quartile ranges for the histoscore for each protein investigated. These values were then used to establish appropriate cut-off points to define tumours as either low or high expressers of the desired protein.

Wilcoxon signed Rank tests were used to compare protein expression between hormone naive prostate cancer and castrate resistant prostate cancer tumours. Correlations between protein expression levels in various sub cellular compartments and associations between the expressions of different proteins were calculated using the Spearman's Rank Correlation Test.

Kaplan-Meier life table analysis and Cox's multiple regression (multivariate survival analysis) were performed to estimate differences in prostate cancer related survival, in terms of time to biochemical relapse, time to death from relapse, disease specific death and overall survival. Multivariate analysis combined the biological marker of interest with tumour grade at diagnosis, tumour grade at relapse (Gleason score), PSA at diagnosis, PSA at relapse, presence of metastasis at diagnosis, presence of metastasis at relapse and age to establish if it was independent of these known prognostic markers in influencing patient outcome.

For survival analysis patients were split into groups, those whose tumours expressed high levels of protein (above the median) and those who expressed low levels of protein (below the median). To establish the relative risk of a patient relapsing or dying as a result of either high or low levels of a particular protein in their tumour hazard ratios (HR) were calculated using Cox regression analysis. A value of $p < 0.05$ was considered statistically significant.

3. The role of the PI3K/Akt cascade in the progression to CRPC

3.1 Introduction

Activation of the PI3K/Akt cascade is reported to play a role in the development and progression of prostate cancer. In order to investigate the clinical significance of this cascade in the progression to castrate resistant disease paired hormone naïve and castrate resistant tumours and cell lines were utilized. As deregulation of this cascade can occur through an array of processes, members of this cascade were investigated at the genetic level using FISH and protein expression levels were determined using IHC.

3.2 Expression and activation of Akt in LNCaP cell lines

To establish the phosphorylation hence activation levels of Akt in the matched hormone sensitive and castrate resistant prostate cancer cell lines ELISA and western blotting were carried out. Both cell lines were incubated with or without EGF for 15 minutes (Figures 3.1 and Figure 3.2). It is evident that Phosphorylation of Akt at serine 473 in LNCaP-CR is notably higher in untreated cells as compared to LNCaP cells and is also significantly higher when stimulated with EGF for fifteen minutes. Phosphorylation of Akt in LNCaP cells increases to a similar level to the basal levels in LNCaP-CR after fifteen minutes of EGF stimulation (Figure 3.1). LNCaP cells that are hormone sensitive still depend on androgens for growth and survival, these results are indicative that in the presence of androgen the activity of

the PI3K/Akt is lower than that of castrate resistant LNCaP suggesting androgens become less important factors in tumour cell growth in late stage prostate cancer.

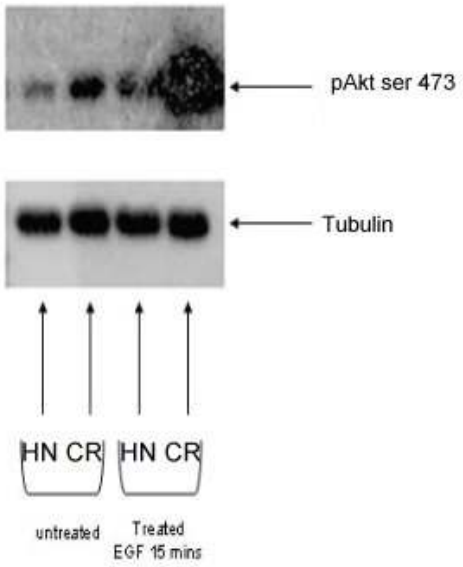


Figure 3.1: Akt expression LNCaP cell lines.

Western blot analysis of LNCaP and LNCaP-CR cells both untreated and treated with10nM EGF for fifteen minutes. Tubulin confirms equal loading of samples.

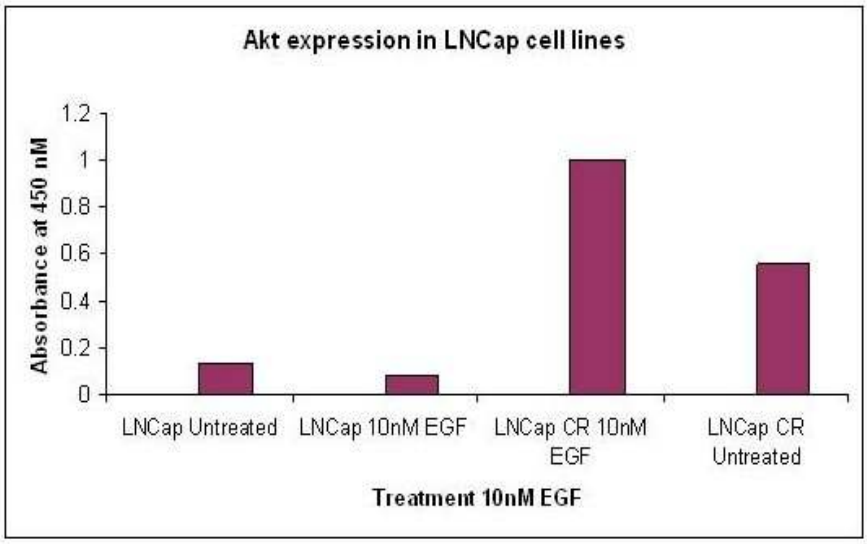


Figure 3.2 EGF induced Akt expression in LNCaP cell lines

ELISA demonstrating Akt phosphorylated at serine 473 is up regulated in castrate resistant LNCaP prostate cells compared to the hormone naïve LNCaP prostate cells when untreated or treated with 10nm EGF.

3.2.1 Stimulation of Akt phosphorylation

Current models suggest that phosphatydilinositol-3, 4, 5 triphopshates produced upon growth factor stimulation recruit Akt to the plasma membrane by binding to its N-terminal pleckstrin homology (PH) domain. To reflect the vigorous recruitment and activation of PI3K by growth factor pathways Hrg and IGF-1 time course treatments were carried out. Treatment with 10nM Hrg appears to induce Akt phosphorylation in a time dependent manner (Figure 3.3). In the hormone naïve LNCaP the basal levels of Phosphorylated Akt are notably higher in the untreated sample (grown in serum free medium) and expression of phosphorylated Akt serine 473 appears to be expressed at a lower level up until the three hour time point. This

data suggests that stimulation with Hrg alone for a shorter time period is not sufficient in enhancing PI3K activity in comparison to overnight serum withdrawal in hormone sensitive prostate cancer cells. In the castrate resistant cells however the expression of phosphorylated Akt is reduced for a brief period between the five and thirty minute time points which further increases as the time progresses to the three hour time point.

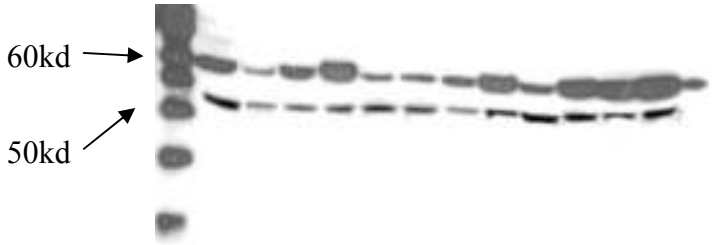


Figure 3.3 Hrg stimulation of Akt phosphorylation

Western blot was performed on 50ug of extracts from LNCaP and LNCaP-CR cells treated or untreated with 10nM HRG and probed for pAkt (ser 473) (60kd) expression. Lane 1 LNCaP unstimulated, 2 LNCaP-CRunstimulated.Lanes3-12 loaded LNCaP, LNCaP-CRfor5,15,30 minutes,1, and 3 hours respectively.Tubulin(50kd) was used as a loading control.

IGF-1 stimulation of LNCaP cells appears to have an inhibitory effect on the phosphorylation of Akt at serine 473 (Figure 3.4.) The basal levels of phosphorylated Akt in the serum starved extracts are notably higher and the stimulation with IGF-1 appears to reduce the expression of phosphorylated Akt at serine 473 with time. The reasons for these inhibitory effects of growth factors on the phosphorylation of Akt are unclear at present. Elevated phosphorylation of Akt in LNCaP cells was inhibited by treating cells with the PI3K inhibitor LY294002 for thirty minutes (Figure 3.5), indicating that constitutive phosphorylation of Akt at

serine 473 is dependent on constitutive production of 3'-phosphoinositides Also, inhibition of PI3K activity was sufficient to inhibit phosphorylation of Akt in LNCaP cells stimulated with IGF-I (Figure 3.5).

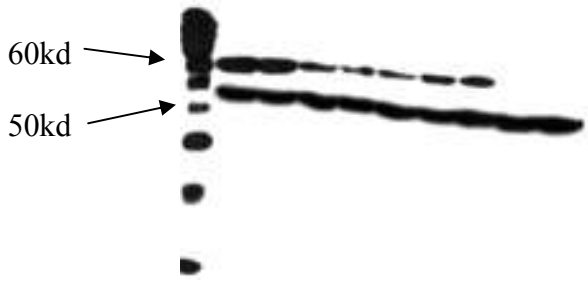


Figure 3.4 IGF stimulation of Akt phosphorylation

Western blot was performed on 50ug of extracts from LNCaP cells treated or untreated (serum free medium) with 10ng IGF-1 and probed for pAkt (ser 473) (60kd) expression. Lane 1 LNCaP unstimulated, 2 LNCaP unstimulated (vehicles, acetic acid and ethanol).Lanes3-7 LNCaP cell extracts stimulated for 5, 15, 30 minutes, 1 and 3 hours. Lane 8 extracts treated with LY294002 20uM for 30 minutes, lane 9 extracts treated with LY294002 20uM 30 minutes then stimulated for one hour with 10ng IGF-1.Tubulin confirms equal loading of samples

Figure 3.5 Phosphorylation of Akt was regulated by PI3K in LNCaP cells.

Lane 1 LNCaP unstimulated 2, LNCaP cell extracts treated with 20uM LY294002,3 LNCaP cell extracts stimulated for1 hour with10ng IGF-1,4 extracts treated with LY294002 20uM 30 minutes then stimulated for one hour with 10ng, 5 LNCaP unstimulated(vehicles, acetic acid and ethanol . Tubulin confirms equal protein volumes.

3.2. Prostate cancer cell proliferation.

To determine the proliferation rate of both cell lines, proliferation was assessed using the WST assay (Figure 3.6). The data shown indicates that both cell lines proliferate at an almost equal rate and have a doubling time of approximately forty hours.

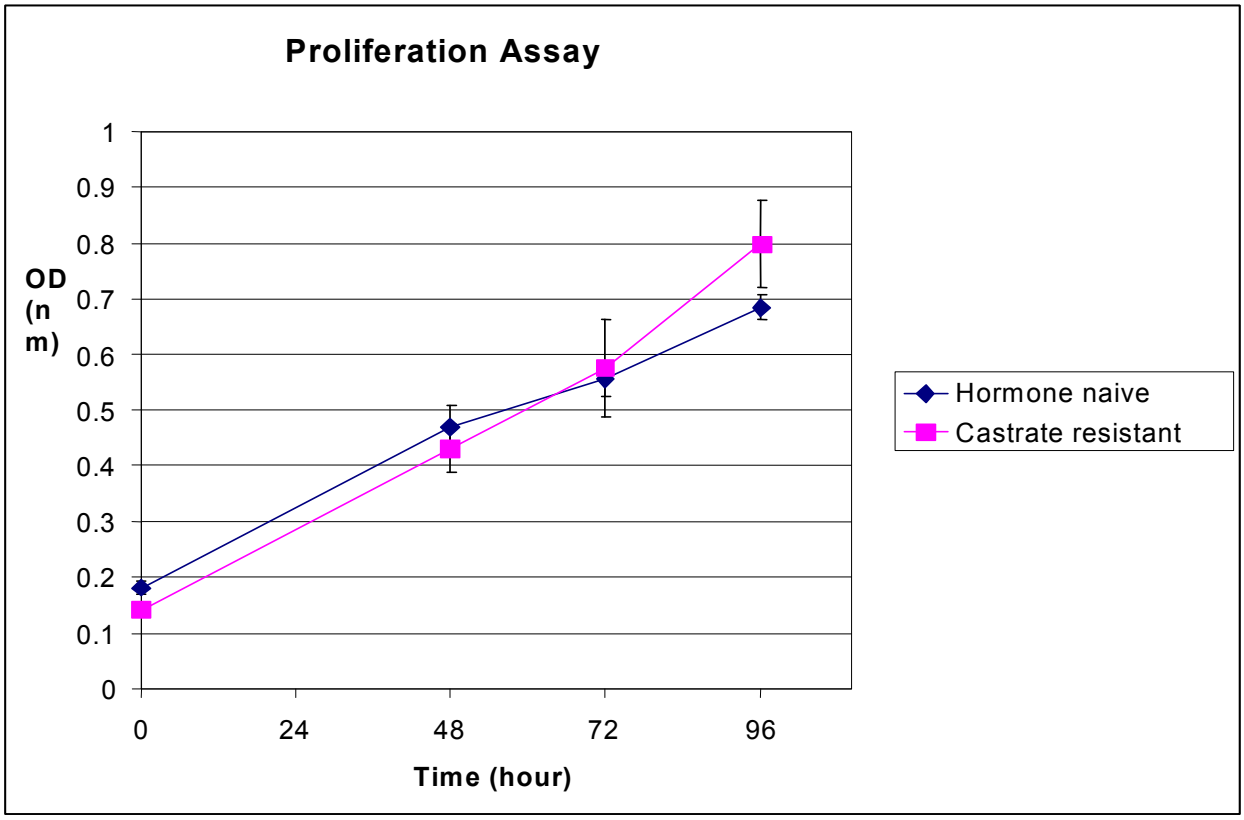


Figure 3.6 LNCaP and LNCaP CR cell proliferation

Proliferation rates in hours of both cell lines studied

3.2.2 PI3K regulates prostate cancer cell proliferation

Reports suggest that PI3K signallingmay play a critical role, allowing prostate cancers cells to maintain continued proliferation in low androgen environments. To clarify the role of PI3K/Akt cascade in regulating proliferation, LNCaP cells were incubated with increasing concentrations of LY 294002 for 48 hours. This time was deemed to be the optimum from the previous proliferation experiment. Treatment of LNCaP cells with LY 294002 resulted in a dose-dependent reduction in proliferation, the IC_{50} was calculated as 15uM (Figure 3.7). This data further supports the reports that PI3K plays a role in the proliferation of prostate cancer cells in the absence of PTEN.

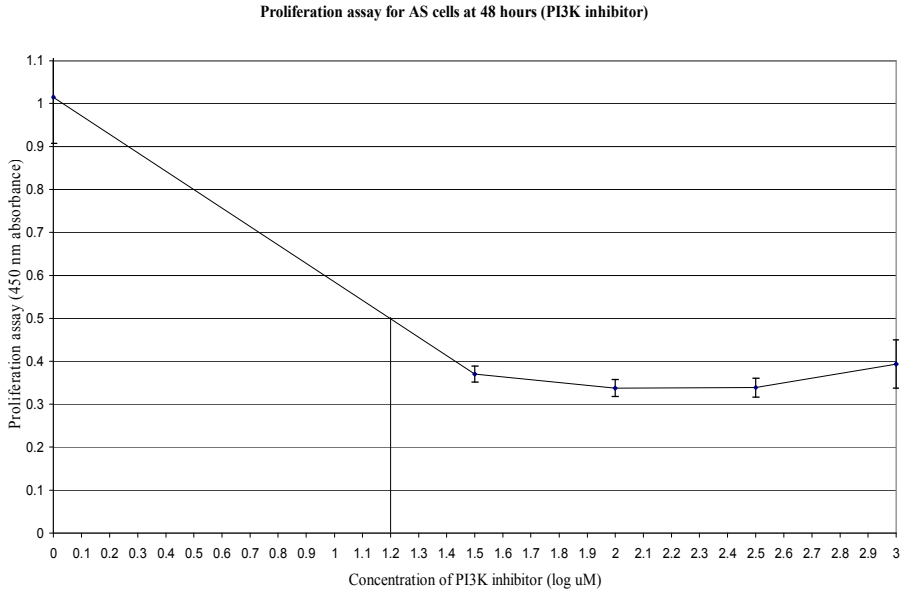


Figure 3.7 LY294002 inhibits prostate cancer cell proliferation.

LNCaP cells were treated with different doses of LY294002 over a 48 hour period and the IC_{50} calculated was 15uM.

Using pAkt ser ^{473} as a marker for PI3K activation has provided additional evidence that the PI3K signallingcascade is upregulated in the transition to castrate resistant disease as expression of pAkt ser ^{473} is markedly higher in castrate resistant LNCap cells in comparison to hormone naïve LNCaP cells. The expression of pAkt ser ^{473} can be reduced by treatment with LY204002 as can cellular proliferation. Therefore having observed that the PI3K cascade is upregulated in prostate cancer cell lines it was deemed appropriate to investigate this cascade in the clinical samples.

3.3 Patients

A total of 68 prostate cancer patients were included in this study, all were available for IHC analysis (136 tumours in total) and approximately 25 matched pairs were available for FISH analysis (31 hormone naive and 25 castrate resistant). Patients in this cohort were diagnosed with locally advanced (50) or metastatic prostate cancer (18) and subsequently received surgery and androgen deprivation therapy (26 sub capsular bilateral orchidectomy, 44 GnRH analogue, 2 had both). Forty-five of the 68 patients also received anti androgen therapy and this included all those who received GnRH analogues. At initial diagnosis the median age was 70 years (66-74) and 26% of patients had metastatic disease. The median time to biochemical relapse was 2.32 (1.48-4.00) years and the percentage of patients with metastatic disease had increased to 57%. Sixty-one patients (89.7%) died during follow-up and median survival for these patients was 4.34(2.94-6.63). Seven patients were alive at last follow-up; the median time of follow-up for all 68 patients was 4.34(2.86-6.74) years.

When serum PSA level, age, metastasis and Gleason grade at diagnosis were analysed by univariate analysis for this patient cohort, PSA at diagnosis ($p=0.036$) and Gleason score at diagnosis ($p=0.010$) were associated with shorter time to biochemical relapse. Death from time of biochemical relapse was associated with PSA level at relapse ($p=0.016$). Overall survival was associated with presence of metastases at relapse ($p=0.0019$) and Gleason score at diagnosis ($p=0.049$).

3.3.1 Genetic changes for members of the PI3K cascade.

FISH was performed for *PIK3CA* (p110 catalytic subunit of PI3K), *PTEN, AKT1-3* and *mTOR*.

PI3KCA//chromosome 3 ratio was successfully evaluated in 57 cases (22 hormone naive and 35 castrate resistant tumours). The remaining cases were excluded from the study because of insufficient tumour material in the cores. Gene amplification was observed in only 1 (5%) hormone naive tumour, and 1 (3%) in castrate resistant tumour, therefore no significant alteration was observed in the transition form hormone naive to castrate resistant disease. No *PI3KCA* gene deletions were observed in this cohort. Due to the low level of genetic changes observed correlations with clinical parameters were not performed.

PTEN/chromosome 10 was successfully evaluated in 31 cases (17 hormone naive and 14 castrate resistant tumours), *PTEN* FISH is shown in Figure 3.8. The remaining cases were excluded from the study because of insufficient tumour material in the cores. Gene deletion was observed in 23% of hormone naive tumours, this increased significantly to 52% in castrate resistant tumours (p=0.044). Loss of one copy of *PTEN* was commonly observed, and this was heterogeneous in nature, being frequently observed in only one area of tumour. Loss of *PTEN* was correlated with prostate cancer progression; however, no correlation was observed between loss of *PTEN* and Gleason score at diagnosis, loss of *PTEN* and presence of metastasis at diagnosis or loss of *PTEN* and PSA at diagnosis. When loss of *PTEN* was correlated with survival, a trend between loss of *PTEN* and poorer disease

specific survival was noted (p=0.086), this was not independently significant by Cox regression analysis.

AKT1 FISH was successfully evaluated in 38 prostate carcinomas (20 hormone naive and 18 castrate resistant tumours). One castrate resistant tumour was identified with *AKT1* whereas no *AKT1* deletions were found. Due to the low level of genetic changes observed correlations with clinical parameters were not performed.

AKT2/chromosome 19 ratio was successfully evaluated in 35/38 cases (92.1%). The remaining cases were excluded because of insufficient tumour material in the cores. Neither *AKT2* gene amplifications nor deletions were identified in any of the evaluated tumours. Due to the low level of genetic changes observed correlations with clinical parameters were not performed.

AKT3/chromosome 1 ratio was successfully evaluated in 34/38 (89.5%) cases. The remaining cases were excluded from the study because of lack of tumour material in the cores. *AKT3* was neither amplified nor deleted in any of the analysed carcinomas. Due to the low level of genetic changes observed correlations with clinical parameters were not performed.

*mTOR /*chromosome 1 ratio was successfully evaluated in 50 cases (25 hormone naive and 25 castrate resistant tumours). The remaining cases were excluded from the study because of insufficient tumour material in the cores. Gene amplification or deletion as identified, therefore correlations with clinical parameters were not performed.

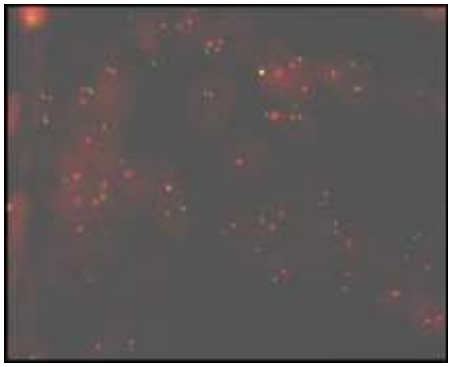
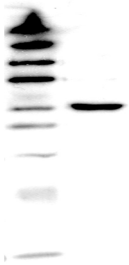


Figure 3.8 PTEN FISH

Fluorescent in situ hybridisation for chromosome 10 (red signal) and PTEN (green signal).

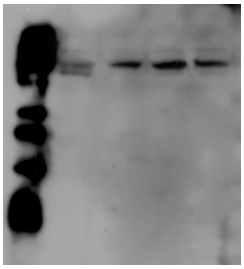
3.3.2 Protein expression of the PI3K/Akt signallingcascade

Expression of all proteins was monitored using an immunohistochemical approach. All IHC was performed as descrided in materials and methods (Chapter 2.9). Before IHC commenced antibody specificity was confirmed by western blotting (Figure 3.9). Information on all antibodies employed are displayed in Table 3.1 and examples of immunohistochemical staining for each are displayed in Figure 3.10. I am grateful to Dr Lisa Gemmel for carrying out both the mTOR and phosphorylated mTOR IHC.



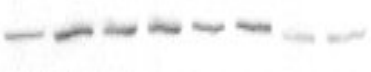
A. Phosphorylated Akt at serine 308.*Western blot analysis of extracts from Jurkat cells treated (lane1)or untreated (lane2)with calyculin A(Cell signallingtechnologies)*

B. Phosphorylated Akt at serine 473. *Western blot analysis of extracts from Jurkat cells treated or untreated with calyculin A (lanes 1&2),LNCaP(3&4) and LNCaP-CR(5&6)cell extracts treated with charcol stripped and full medium.*



C. PI3K. *Western blot analysis of extracts from Jurkat cells untreated or treated with calyculin A (lanes 1&2.) LNCaP (3) and LNCaP-CR (4) cell extracts grown in standard culture media.*

D.PTEN. *LNCaP cells do not express PTEN protein, where as DU145 cells do express PTEN protein. LNCaP&LNCaP-CRcell lysates (lanes1& 2) lane 3 DU145 cell lysates.*



E.mTOR. *Western blot analysis of extracts from MCF7 cells untreated and treated with 10nm EGF(lanes 1&2)BT474 (lanes 3&4)* ,MDAMB 231*(lanes 5&6),MDAMB 361(lanes 7&8).*

F. Phosphorylated mTOR at serine 2448. *Western blot analysis of extracts from MCF7 cells untreated and treated with 10nm EGF(lanes 1&2)BT474 (lanes 3&4)* ,MDAMB 231*(lanes 5&6),MDAMB 361(lanes 7&8).*

Figure 3.9: Specificity of Antibodies

Western blotting was performed to confirm the specificity of all antibodies used in this study.

Protein	Antibody	Antigen Retrieval	Antibody concentration	Incubation temperature and time
PTEN	PTEN Mouse Monoclonal Cell Signalling	Citrate Buffer	1µg/ml	Overnight at 4ºC
PI3K	PI3K(p110 catalytic subunit) Rabbit Polyclonal Cell Signalling	Citrate Buffer	1µg/ml	Overnight at 4ºC
pAkt (ser473)	pAkt serine 473 Rabbit Polyclonal Biosource	TE Buffer	4µg/ml	Overnight at 4ºC
pAkt (ser308)	pAkt (ser308) Rabbit polyclonal Cell Signalling	Citrate Buffer	5µg/ml	Overnight at 4ºC
*mTOR	mTOR Goat Polyclonal Santa Cruz Biotechnology	Citrate Buffer	5µg/ml	Overnight at 4ºC
pmTOR(ser2448)	pmTOR(ser2448) Rabbit polyclonal Cell Signalling	Citrate Buffer	2µg/ml	Overnight at 4ºC
Akt 1	Akt 1 Mouse monoclonal Santa Cruz Biotechnology	TE Buffer	1µg/ml	Overnight at 4ºC
Akt 2	Akt 1 Mouse monoclonal Santa Cruz Biotechnology	TE Buffer	2µg/ml	Overnight at 4ºC
Akt 3	Akt 1 Mouse monoclonal Santa Cruz Biotechnology	TE Buffer	2µg/ml	Overnight at 4ºC

Table 3.1: Antibody Information

*Details of the antibodies used to detect members of the PI3K/Akt cascade.Information regarding the source, antigen retrieval method, concentation and incubation are all recorded. *For the mTOR antibody only, incubation with*

rabbit anti-goat antibody (Dako A/S) (1 : 4000) for 1 h at room temperature was also required.

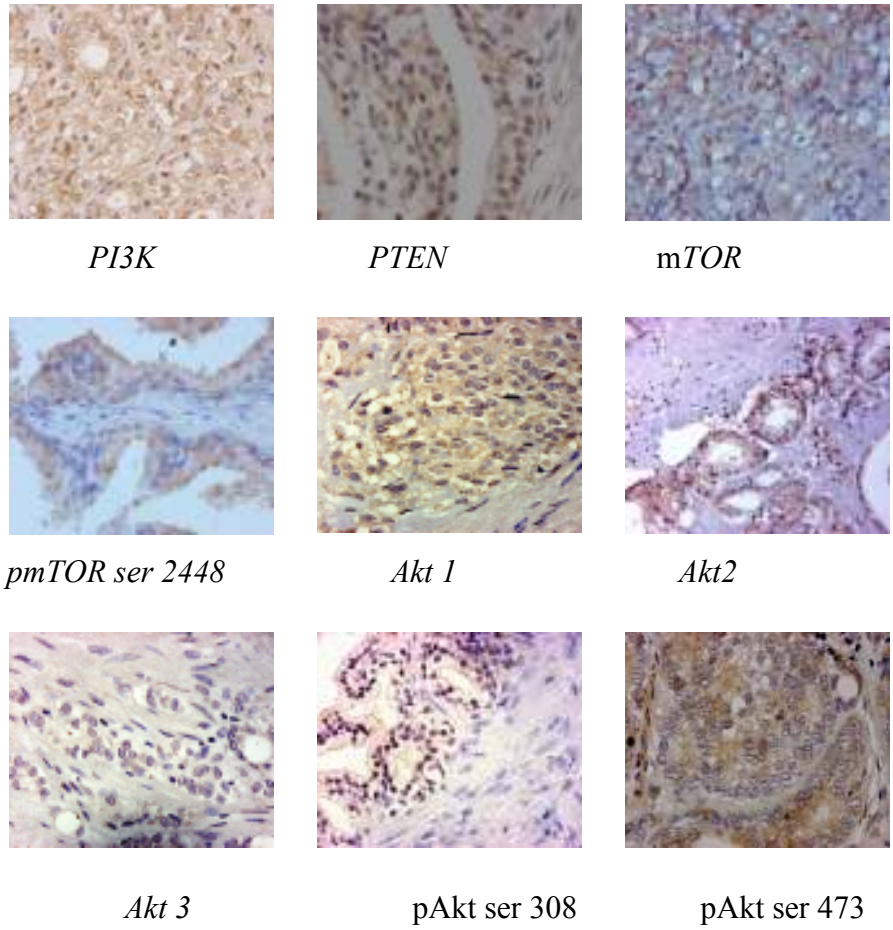


Figure 3.10: PI3K /Akt cascade Immunohistochemistry

Prostate tumours displaying immunohistochemical staining magnification x400

Akt1, pAkt^{473,} PTEN and pmTOR^{2448} protein expression was observed in the cell membrane, cytoplasm or nucleus. mTOR expression was observed at the membrane and cytoplasm, and PI3K, Akt2 and Akt3 expression was observed only in the cytoplasm. All IHC statistical analysis was carried out as previously described in (Chapter 2.9.1&2.9.2).

Expression of all proteins investigated was non parametric, median values and inter quartile ranges for each protein at each location are provided in Table 3.2 for both hormone naive and castrate resistant tumours.

Protein	HSPC (IQR)	CRPC (IQR)	P value	ICCC	Histoscore units	Fallers	Risers
PI3Kc	100(58-140)	100(79-134)	0.875	0.85	60	23	23
Akt1m	0(0-11)	0(0-90)	0.798	0.88	30	14	13
Akt1c	75(20-100)	70(0-90)	0.488	0.82	55	20	13
Akt1n	0(0-18)	0(0-0)	0.110	0.95	21	19	9
Akt2	125(100-185)	120(98-165)	0.551	0.81	61	22	13
Akt3	50(0-100)	60(0-95)	0.619	0.84	48	20	30
pAkt^{473}m	40(0-90)	33(0-90)	0.988	0.90	58	22	23
pAkt^{473}c	88(54-110)	80(40-105)	0.671	0.89	49	25	23
pAkt^{473}n	0(0-25)	0(0-35)	0.465	0.93	40	15	18
mTORm	0(0-20)	0(0-10)	0.134	0.92	47	10	3
mTORc	43(15-87)	40(10-62)	0.123	0.83	31	35	23
pmTOR^{2448}m	0(0-22)	0(0-15)	0.330	0.95	33	14	8
pmTOR^{2448}c	61(20-100)	40(8-70)	0.044	0.91	45	33	23
pmTOR^{2448}n	0(0-10)	0(0-0)	0.575	0.90	19	3	6
PTEN m	0(0-65)	0(0-40)	0.086	0.84	27	33	15
PTEN c	100(80-150)	100(80-105)	0.104	0.90	37	33	23
PTEN n	0(0-50)	50(0-80)	0.588	0.82	30	28	25

Table 3.2: Protein expression patterns

For each protein, descriptive statistics were performed on the generated histoscores. Median histoscore and interquartile range (IQR) for hormone naive tumours (HSPC) and castrate resistant tumours (CRPC) and the p value of these values compared using a Wilcoxon sign rank test. ICCC= interclass correlation coefficient. The mean difference in observer scores plus 2 standard deviations is also shown as the number of histoscore units that is defined as a change in protein expression (change). The percentage of tumours that were defined as having a fall or rise in protein expression (calculated using the number of histoscore units that is defined as a change in expression) are also shown. m, c and n relates to protein

cellular location, m = membrane, c= cytoplasm and n = nucleus. P before a protein indicates that the antibody detects phosphorylated protein and the number following the protein represents the site of phosphorylation.

The Wilcoxon signed-rank test was used to compare expression levels in the hormone naive tumours compared to castrate resistant tumours. None of the proteins studied were shown to have a significant increase with the development of castrate resistant disease.

3.3.3 Factors associated with time to biochemical relapse.

When presence of metastases at diagnosis, age at diagnosis, Gleason score at diagnosis and PSA at diagnosis were analysed for this patient cohort only PSA at diagnosis (p=0.036) and Gleason score at diagnosis (p=0.038) were associated with shorter time to biochemical relapse.

3.3.4 Protein expression in the hormone naïve cohort

To determine if protein expression was linked to time to biochemical relapse, Kaplan-Meier graphs were plotted for the hormone naive tumours expressing low levels of protein (< median) versus high levels of protein (> median) and compared using the log rank test. Only PTEN was shown to have significant results. The patients whose tumours expressed low levels of PTEN in the cytoplasm were shown to have relapsed significantly earlier than those patients whose tumours expressed high levels of PTEN in the cytoplasm (Figure 3.11(a), p=0.027). Cox regression analysis indicates that cytoplasmic PTEN expression is independent of known clinical prognostic factors (p=0.028, hazard ratio 0.51 (95%CI 0.27-0.93). It was noted, however, that the Kaplan Meier curves did not separate until approximately

2.5 years after diagnosis. Therefore PTEN loss appeared to be influencing relapse in those patients who took more than 30 months to relapse. If patients that relapsed within 30 months were excluded from analysis the median time to relapse for those with low PTEN expression was 3.9 (IQR 2.98-4.92) years compared to 5.6 (4.36-6.84) years for those with high PTEN expression (p=0.0035)(Figure 3.11(b)).

In addition, those patients with high levels of cytoplasmic PTEN expression in their hormone naive tumours were observed to have longer median overall survival (6.1 years (IQR 2.8-9.4)) compared to those with low PTEN expression (4.4 years (IQR 3.3-5.4)), although this did not reach significance (p=0.072)(Figure 3.12). Again the curves first separate approximately 30 months after diagnosis. Levels of $pAkt^{473}$ expression in tumours that expressed low levels of PTEN were higher compared to tumours that expressed high levels of PTEN (p=0.047).

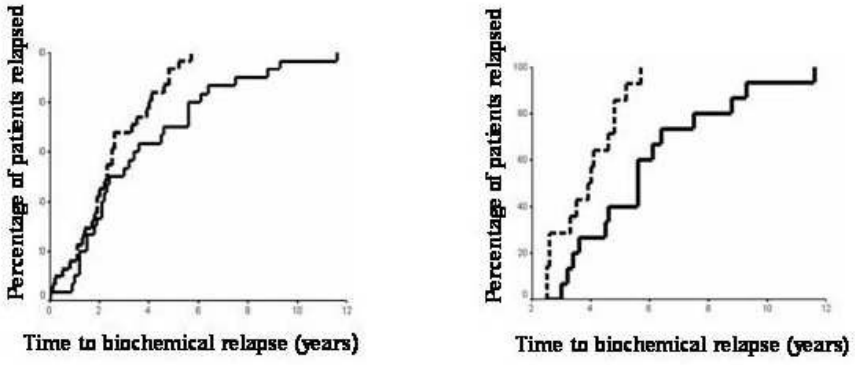


Figure 3.11(a & b) : PTEN cytoplasmic expression

Kaplan Meier Plot for high (above the median, solid line) and low (below the median, dotted line). The figure on the left (a) shows the time to biochemical relapse with with low cytoplamic PTENexpression(p=0.027), figure 3.11(b) shows the time to biochemical relapse of patients with low cytoplamic PTENexpression that took more than 30 months to relapse (p=0.0035)

The median for both PTEN membrane and nuclear expression in the hormone naive tumours was 0; therefore patients were divided into those patients whose tumours did not express PTEN at these locations and those that did. Patients with PTEN membrane expression in their hormone naive tumour had significantly longer overall survival than those patients without (Figure 3.13, p=0.002). The median time to relapse for patients whose tumours did not express PTEN in the membrane was 3.8 years (IQR 2.7-4.85) compared to 6.5 (IQR 5.8-7.2) years for patients with membrane expression.

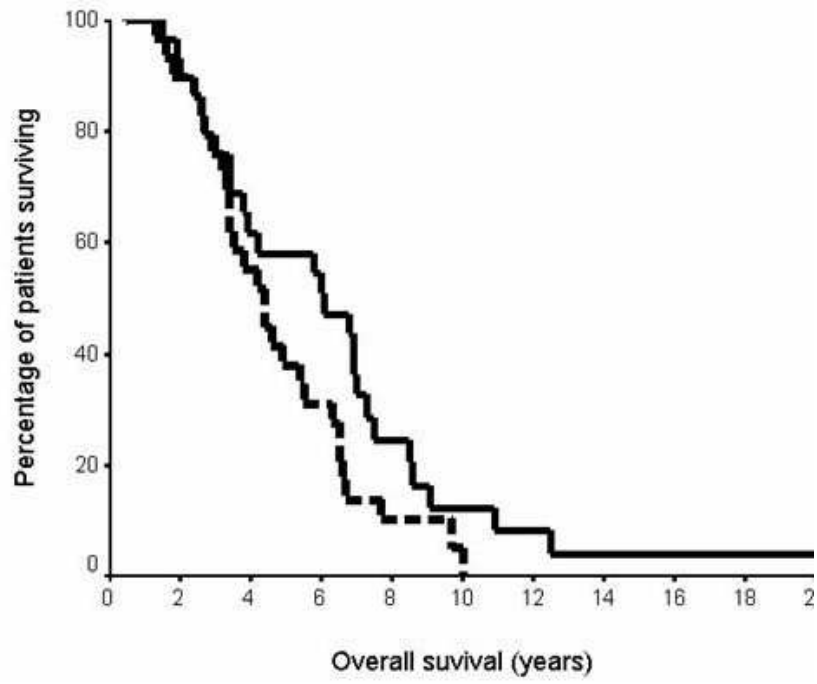


Figure 3.12: PTEN cytoplasmic expression and disease specific survival

Kaplan Meier Plot for high (above the median, solid line) and low (below the median, dotted line) PTEN cytoplasmic expression and disease specific survival (labeled overall survival) (p=0.072).

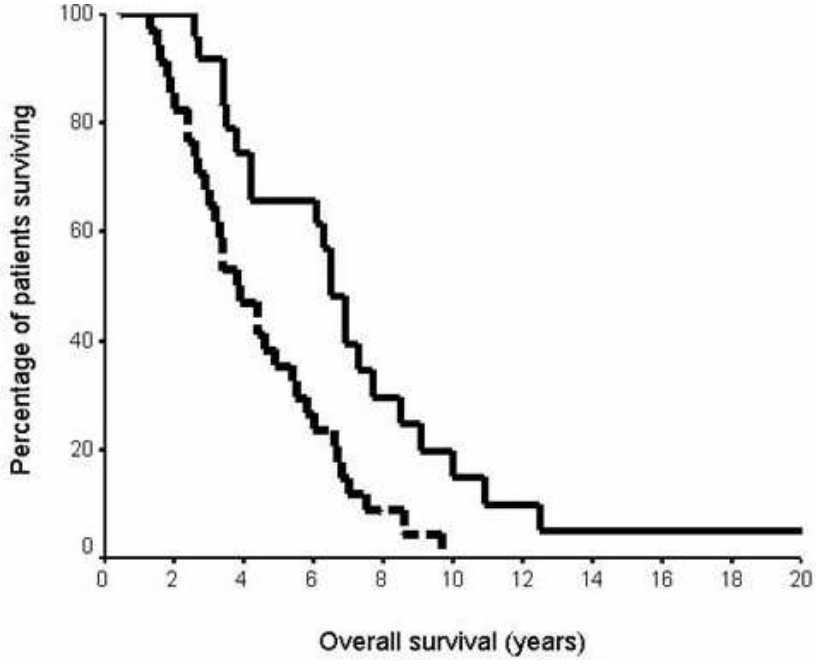


Figure 3.13: PTEN membrane expression

Kaplan Meier Plot for patients with membrane expression, (solid line) and no membrane expression (dotted line) and disease specific survival (labeled overall survival) (p=0.002).

PTEN nuclear expression was associated with overall survival, those patients whose tumours had no nuclear PTEN expression had a significantly shorter overall survival compared to those patients with PTEN nuclear expression (Figure 3.14, p=0.003). Median overall survival was 3.4 years (IQR2.6-4.2) compared to 6.5 years (IQR 5.1-7.8), which confers a survival advantage of 3 years for those patients whose tumours express nuclear PTEN. Nuclear PTEN expression was demonstrated to be an independent prognostic marker by Cox regression analysis when compared with known clinical prognostic parameters (p=0.031, hazard ratio 0.52 (95%CI 0.29-0.95).

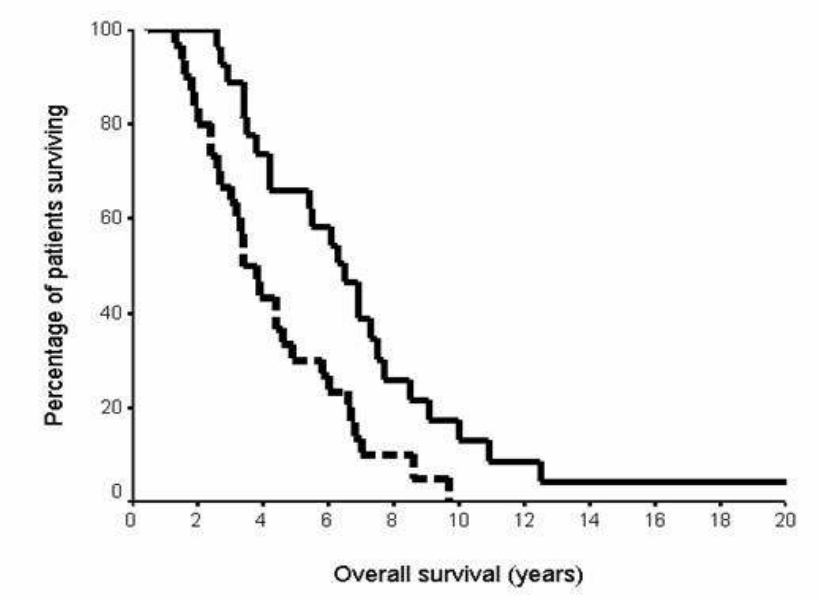


Figure 3.14: Nuclear PTEN expression

Kaplan Meier plot for patients that have nuclear PTEN express (solid line) and those who do not (dotted line) and disease specific survival. (p=0.003)

As observed with membrane expression, $pAkt^{473}$ expression was lower in the nucleus of tumours with high levels of nuclear PTEN than those with low levels but this did not reach significance (p=0.132). In contrast, PTEN membrane expression correlated strongly with nuclear PTEN expression ($p<0.001$, R_S 0.66)

3.3.5 Protein expression in the castrate resistant cohort

When expression levels of each protein investigated in the castrate resistant tumours were divided into to high or low expression (levels above or below the median) none of the proteins investigated were associated with time to death from relapse or disease specific survival.

When presence of age at diagnosis, Gleason score at diagnosis, metastases at relapse and PSA at relapse were analysed for this patient cohort only PSA at relapse (p=0.016) was associated with disease specific death from time of biochemical relapse.

3.3.6 Changes in protein expression

Due to the nature of the cohort it is possible to investigate if those patients whose tumours exhibit an increase or decrease in expression of members of the PI3K cascade in the transition from hormone naive to castrate resistant disease were more likely to relapse or die quicker. The cutoff histoscore selected to separate subgroups of patients is displayed in Table 3.2. Using this method, an increase in PI3K (Figure 3.15; *P*=0.014, HR=2.11 (95% CI: 1.14–3.91)) and pAkt^{308} (Figure 3.16, p=0.038, HR 2.4 (1.01-5.67)) were associated with quicker time to relapse. The median time to relapse for those patients whose tumours have a decrease or no change in PI3K expression was 2.57 (1.74-3.40) years compared to 1.36 (1.20-2.72) years for those patients whose tumours had an increase in PI3K expression. The median time to relapse for those patients whose tumours had no change or decrease in pAkt^{308} was 2.36 (1.61-3.11) years compare to 1.14 (0.17-2.63) years for those patients whose tumours had an increase in pAkt^{308} expression.

Additionally, an increase in pAkt^{473} (Figure 3.17, p=0.0019, HR 2.89(95% C.I., 1.43-5.8)) was associated with shorter disease specific survival. The median survival from diagnosis for those patients whose tumours had no change or decrease in pAkt^{473} was 6.68 (IQR, 6.22-7.14) years compared to 4.15 (IQR, 2.65-6.5) years for those patients whose tumours had an increase in pAkt^{473}expression.

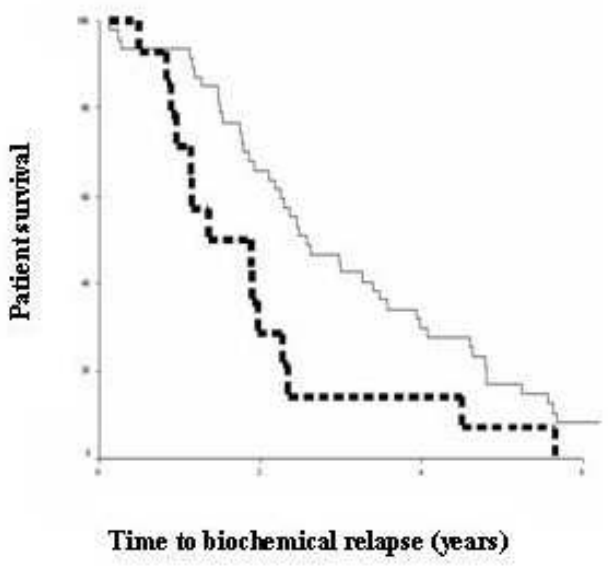


Figure 3.15: Increased PI3K expression

Kaplan Meier plot for patients that have increased PI3K expression (dotted line) and those who do not (solid line) and time to relapse (p=0.014)

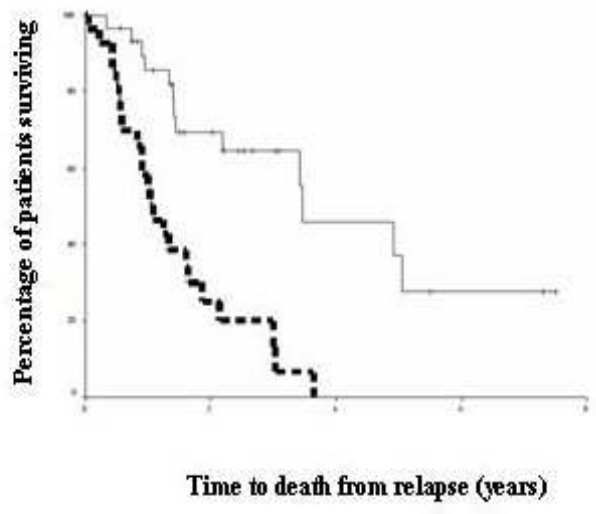


Figure 3.16 Increased pAkt (ser 308)

Kaplan Meier plot for patients that have increased pAkt (ser 308) expression (dotted line) and those who do not (solid line) and time to relapse (p=0.038).

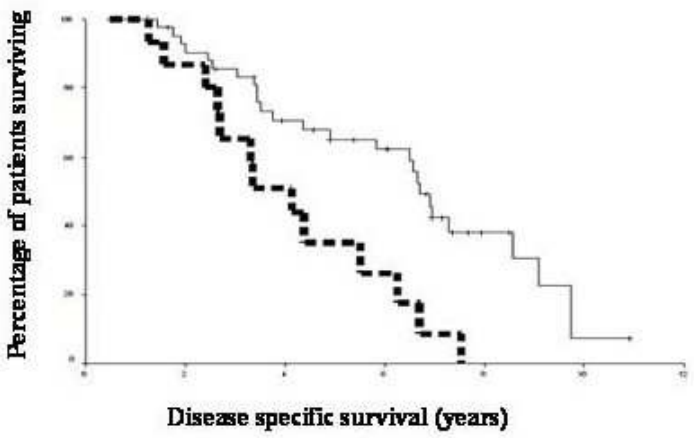


Figure 3.17: Increased pAkt (ser 473) expression

Kaplan Meier plot demonstrates that those patients whose tumours exhibit an increase in pAkt^{473} cytoplasmic expression (broken line) have shorter time to disease specific death than those patients whose tumours exhibit no change or a decrease in pAkt^{473} expression (solid line).

When all significant factors associated with disease specific survival were combined in multivariate analysis, presence of metastases at relapse, PTEN nuclear expression in hormone naive tumours and an increase in pAkt^{473} expression were independently significant (Table 3.4).

Factor	P value (uni)	P value (multi)	Hazard ratio	95% C.I.
Metastases at relapse	0.0019	0.043	3.54	1.03-12.07
PTEN nuclear expression	0.011	0.0054	0.27	0.11-0.68
Change $pAkt^{473}$ expression	0.0019	0.0314	2.74	(1.09-6.88)

Table 3.4: Multivariate analysis for factors effecting disease specific survival

Factors significantly associated with disease specific survival using Kaplan Meier analysis and those independently associated with disease specific survival using Cox Regression analysis. PTEN nuclear expression represents those patients whose hormone naive tumours expressed PTEN in the nucleus compared to those patients whose hormone naive tumours did not express PTEN in the nucleus. Change in $pAkt^{473}$expression represents those patients whose tumours exhibited an increase in expression in the transition from hormone naive disease to castrate resistant disease compared to those patients whose tumours had no change or a decrease in expression

3.4 Discussion

Evidence from several groups indicates that the PI3K cascade may influence the progression to castrate resistant prostate cancer despite only castrate levels of circulating androgens. Indicating that this pathway could serve as a novel target for therapeutic intervention in the treatment of prostate cancer. Due to the significance of PI3K signallingin prosate cancer cell lines this study further examined if the protein expression levels of several key components of this signallingcascade played a role in prostate cancer relapse to castrate resistant disease aiming to identify which proteins would make the optimum targets for treatment and most effectively, identify patients suitable for therapy.

Functional loss of PTEN has been associated with increased Akt phosphorylation, higher gleason grade, advanced stage and poor prognosis, predicting disease recurrence after primary treatment (214). The generation of transgenic mouse models that recaptilate

features of the disease has enhanced understanding of the pathway (215) and studies of knockout mice with targeted deletion of prostate specific PTEN (PTEN-/-) have revealed prostate intraepithelial neoplasia formation, invasive adenocarcinoma progressing to metastatic disease, an initial response to androgen ablation therapy, and eventual tumour growth despite castration (216). This study presents data in keeping with PI3K signallinginducing continued prostate cancer growth.

Akt activity has been shown to increase during androgen ablation to stimulate cell growth and survival when androgen reliance is weaker, and therefore promote development of CRPC (150;172). LNCaP cells are PTEN deficient, therefore PI3K pathway signallingis evident by high levels of phosphorylated Akt..In this study it was established that both hormone naïve and castrate resistant LNCaP express phosphorylated Akt during serum starvation which indicates PI3K signallingis active at both stages of the disease regardless of the growth conditions. Phosphorylated Akt expression was markedly higher in the castrate resistant cells than hormone naïve cells (Figure 3.1&3.2) and treatment with EGF induces the phosphorylation of Akt in hormone naïve cells to a similar level of that observed in the untreated castrate resistant cells. Heregulin was shown to induce Akt phosphorylation in both cell lines but at a quicker rate in the castrate resistant cells (Figure 3.3). IGF-1 stimulation did not appear to enhance the phosphorylation of Akt to a higher than basal level in the hormone naïve LNCaP cells (Figure 3.4). This observation that Akt phosphorylation increases with prostate cancer progression is supported by several reports in the literature. Androgen ablation in LNCaP cells stimulated the activation of Akt, which eventually resulted in androgen-independence of the cell line in culture (180). The PI3K inhibitor LY294002 was sufficient in blocking Akt

phosphorylation in LNCaP cells with and without IGF-1 stimulation (Figure 3.5). LY294002 was also observed to inhibit the proliferation of LNCaP cells. In keeping with previous reports this study established that the PI3K cascade is upregulated both hormone naïve and castrate resistant prostate cancer cell lines (217). It was therefore fitting to investigate the members of the PI3K cascade at the genetic level in matched hormone naive and castrate resistant clinical samples.

One mechanism by which protein expression is altered is by gene amplification or deletion, however this study failed to show any link between gene amplification and increased protein expression. Loss of *PTEN* was noted in 23% hormone sensitive tumours compared to 52% castrate resistant tumours, these rates of loss are similar to those previously reported by FISH analysis (185) suggesting that *PTEN* loss is associated with tumour progression. Although this was not significantly associated with clinical outcome measures a trend was observed that demonstrated that those patients with *PTEN* loss had shorter overall survival. If the FISH studies were expanded to a larger dataset these results may have reached significance. FISH depending on the region that the probe binds to does not always detect small deletions and in the case of this study the probe covers the whole of the *PTEN* gene, hence loss of the whole gene is being measured. It was observed that very few tumours had homogeneous *PTEN* deletion and complete loss of PTEN expression (2%), but almost all have heterogeneous loss of expression, this is consistent with previous reports (185). In the current study, PTEN loss does not correlate with *PTEN* gene deletion, although all tumours with PTEN deletion have low PTEN expression. An explanation for low PTEN expression in tumours that appear not to have *PTEN* deletion is hypermethylation of the *PTEN* promoter region.

Evidence for promoter hypermethylation has been reported in prostate cancer xenografts (190) and the promoter region of PTEN in the LNCaP cell lines used in this study were observed to be methylated (data not shown) additionally western blot analysis confirmed that these LNCaP cell lines did not express PTEN (Figure 3.1). Although the mechanism of PTEN inactivation is currently controversial and possibly due to different mechanisms in different tumours, it is widely accepted that PTEN loss is one of the most common events associated with prostate cancer (218).

Work using human prostate tissue confirms that $pAkt^{473}$ is expressed in PIN and invasive prostate cancer, and staining intensity positively correlated with PSA levels and Gleason grades (172;217). Additionally, a large study of 640 radical prostatectomy specimens demonstrated that high levels of pAkt were predictive of biochemical recurrence (171). However, to date this had not been investigated in castrate resistant tumour samples. The current study demonstrates for the first time that the PI3K/Akt pathway is up regulated in clinical castrate resistant disease and that it is independently associated with reduced disease specific patient survival. An increase in PI3K expression and low PTEN cytoplasmic expression were both associated with shorter time to biochemical relapse. The consequence of low PTEN expression displays an increase in Akt activation, in a sub cohort of the patients. It is observed that those patients with an increase in $pAkt^{308}$ (partially activated) and $pAkt^{473}$ expression (fully activated Akt) have shorter time to relapse and disease specific survival respectively (Figures 3.16 & 3.17). As expected the relationship between disease specific survival and fully activated Akt ($pAkt^{473}$) appears to be stronger than that with partially activated Akt ($pAkt^{308}$). The increase in $pAkt^{473}$ expression predicts disease specific survival independent of Gleason score and presence

of metastasis. By investigating PTEN protein expression as predicted, loss or low cytoplasmic PTEN expression was independently associated with time to relapse and linked with increased Akt activation. However this was observed to be a late event with curves separating 30 months following diagnosis, suggesting that other factors such as PI3K expression may also contribute to Akt activation and disease progression. In addition to cytoplasmic PTEN expression, nuclear PTEN expression was also observed. Unlike cytoplasmic PTEN expression, loss of nuclear PTEN expression was weakly associated with time to relapse and this did not reach significance. Nuclear PTEN expression was independently associated with overall survival where the curves on the Kaplan Meier plot begun to separate approximately after diagnosis. These results in combination with the lack of correlation with Akt activation suggest that the role of PTEN in the nucleus is independent of cytoplasmic PTEN.

It is possible that nuclear PTEN is simply a surrogate marker of PTEN activation, as *in vitro* studies demonstrate that following phosphorylation, PTEN is released from the membrane bound scaffolding proteins and enters the nucleus. In support of this a correlation between membrane and nuclear PTEN expression was observed ($p<0.001$, R_S 0.66) and PTEN membrane expression is also linked to survival. However, in contrast to cytoplasmic PTEN expression, no correlations were observed between nuclear PTEN expression and Akt activation, therefore the evidence to support nuclear PTEN as a surrogate marker of PTEN cytoplasmic activation is not convincing in the current study. In addition, both cytoplasmic and nuclear PTEN are independently associated with good outcome measures in hormone sensitive prostate cancer, yet appear to have independent

roles. Results gained for this study suggest that the increase in Akt activity we observe may be due to PTEN loss and/or an increase in PI3K activity.

The protein mTOR is also downstream of Akt and was also investigated in this study as it has previously been linked to CRPC (219). In the current cohort, $pAkt^{473}$ expression correlates with $pmTOR^{2448}$ expression, however, expression levels or changes in expression levels of $pmTOR^{2448}$ do not correlate with any clinical parameters in the cohort suggesting that mTOR may not be involved in the development of CRPC. This is surprising as stimulation of mTOR ultimately results in increase protein synthesis and enhances translation of proteins involved in growth control via turning off 4EBP and activating S6Kinase. Although Akt has been demonstrated to phosphorylate mTOR directly, the role of these phosphorylation sites remains unclear. A more appropriate marker of mTOR activation could be S6Kinase (220). Apart from activating its substrate S6 it is also involved in a negative feedback loop to inactivate Akt (221). Interestingly Munders et al have recently demonstrated that VEGF-C increased the survival of prostate cancer cells during hydrogen peroxide stress by the activation of Akt. This activation was mediated by mTORC2 and was not observed in the absence of oxidative stress. It is therefore possible that during stress cells prefer to shut off protein synthesis which is energetically expensive and growth and enhance cellular processes that mediate survival. Thus the absence of mTOR activity facilitates prolonged activation of Akt during severe stress providing suvival advantage. Agents including radiotherapy, chemotherapeutic drugs such as pacitaxel and histone deacetalase inhibitors increase oxidative stress (222;223). Thus a reccurrence of the tumour after therapy likely results from a subset of cells that have developed the ability to overcome oxidative damage (223).

It has long been hypothesised that interaction with growth factor receptors and the Androgen receptor drive the development of CRPC. This study presents clinical evidence that expression and activation of the components of the PI3K cascade is associated with poor clinical outcome. Loss of PTEN expression as prostate cancer progresses results in constitutive activation of Akt. These prelimanary results demonstrate an increase in phosphorylated Akt (serine 473) expression in the transition from hormone naïve to castrate resistant disease, suggesting that the PI3K cascade is up regulated in a sub group of patients, this was also associated with a reduced survival period from relaspe. Cell line arm of this study also indicated tha PI3K cascade was upregulated at castrate levels of androgen and that this pathway is involved in prostate cancer cell proliferation. As PI3K signallingat castrate levels of circulating androgen appears to play a role in prostate cancer progression it was deemed appropriate to investigate the downstream effects of Akt activity. As mentioned earlier Akt interacts with NFκB a transcription factor involved in a vast many cellular processes and also with the Androgen receptor phosphorylating it at serine residue 213 and 791. Accordingly the next two chapters shall investigate the effects of Akt on NFκB signallingand interaction with the androgen receptor.

4. The role of NFκB signalling in clinical prostate cancer progression.

4.1. Introduction

In the previous chapter the significance of the PI3K/Akt cascade in the transition for hormone naive to castrate resistant disease was highlighted using in vivo and in vitro models. Based on these results it was decided to further investigate the activity and expression levels of proteins down stream of Akt. Akt has been shown to stimulate signallingcascades that regulate the activity of the transcription factor NFκB which has been proven to be directly involved in a number of human disorders including a variety of cancers.

4.2 Patients

A total of 61 prostate cancer patients were included in this study, all were available for IHC analysis (132 tumours in total). Clinical data, recorded for each patient included age (median 70, inter quartile range 67-74), PSA at diagnosis (median 31 ng/ml, inter quartile range 7.8-109), PSA at relapse (median 10 ng/ml, inter quartile range 4-11) and Gleason grade at diagnosis (median 8, range 6-9). All patients under went biochemical relapse (median time to relapse 2.48 years, inter quartile range 1.76-4.43 years). At last follow-up, 40 patients had died of their disease and 15 patients had died of other causes and 6 were still living. The median follow up for those patients still alive was 6.4 years and inter quartile range was 3.7-9.2 years. Following diagnosis 10 % patients received surgical orchidectomy and 90% patients received LHRH analogue in combination with

anti androgen therapy. Following biochemical relapse 64% patients received radiotherapy, no patients received taxane therapy. Clinical parameters associated with time to biochemical relapse, time to death from biochemical relapse and disease specific survival are shown in table 4.1.

	Time to relapse	Time to death from relapse	Disease specific survival
Age (<70/>70/unknown)	0.809 29/29/3	0.137 28/29/4	0.434 30/29/2
Gleason (**<7/=7/>7**/unknown)	0.473 14/12/28/7	**0.026** 14/12/27/8	**0.013** 14/12/29
Metastasis at diagnosis (No/Yes/Unknown)	0.182 40/14/7	**0.013** 40/14/7	**0.002** 40/16/unknown
PSA at diagnosis (<4/4-10/>10) unknown	0.063 9/7/38/7	0.568 9/7/37/7	0.664 9/7/37/8
Metastasis at relapse (No/Yes/Unknown)		**0.001** 10/33/unknown	**0.012** 10/35/unknown
PSA at relapse		0.933 39/18/unknown	**0.045** 39/18/unknown

Table 4.1: Patient characteristics

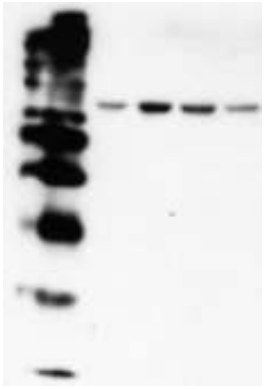
An overview of the cohort's characteristics where each clinical parameter, were appropriate has been correlated with time to relapse, time to death from relapse and disease specific survival (p-values).

4.3 Immunohistochemisrty

Before IHC commenced antibody specificity was confirmed by western blotting (Figure 4.1). Information on all antibodies employed are displayed in table 3.2 and examples of immunohistochemical staining for each are displayed in figure 3.2. This study was carried out to investigate the significance of NFκB activation in clinical prostate cancer specimens using an immunohistochemical approach therefore by investigating the

expression of pIκBα serine $^{32/36}$ gives an indication of NFκB activation as phosphorylation of IκBα at this serine residue leads to the activation of NFκB.

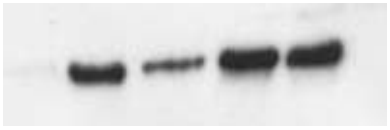
A. pIκBα serine $^{32/36}$ *Western blot of Hela cells extracts treated (lane1)or untreated (lane2)with TNFα (Cell signallingtechnologies)*



B. NFκB p65 *Western blot of LNCaP, LNCaP-CR, PC3 and DU145 cell extracts grown in full medium.*

C. pNFκB p65 serine ^{536} *Western blot of LNCaP, LNCaP-CR, PC3 and DU145 cell extracts grown in full medium.*

D. NFκB p65 serine^{276} *Western blot of LNCaP, LNCaP-CR, and PC3 cell extracts grown in full medium.*

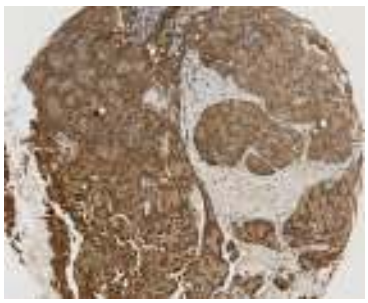


E.MMP-9 *Western blot of PC3, LNCaP, LNCaP-CR, and DU145 cell extracts grown in full medium.*

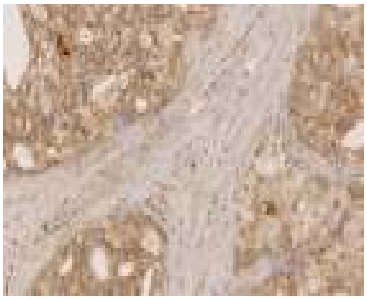
Figure 4.1: Antibody specificity *Western blotting was performed to confirm the specificity of all antibodies used in this study.*

Protein	Antibody	Antigen Retrieval	Antibody concentration	Incubation temperature and time
NFκB	NFκBp65 Mouse Monoclonal IgG1 Ab F Santa Cruz	Citrate Buffer	8ug/ml	Two hours at 25ºC
NFκB	NFκB p serine 536 Rabbit Polyclonal Abcam	Citrate Buffer	2ug/ml	Overnight at 4ºC
NFκB	NFκB p serine 276 Rabbit Polyclonal Cell Signalling	Citrate Buffer	2ug/ml	Overnight at 4ºC
IκBα	IκBα p serine 32/36 Mouse Monoclonal Cell Signalling	Citrate Buffer	2ug/ml	Overnight at 4ºC
MMP-9	MMP-9 Rabbit Polyclonal Millipore	N/A	0.5ug/ml	Thirty Minutes at 25ºC

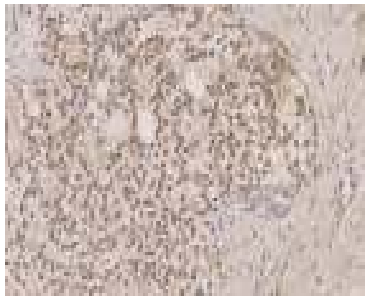
Table 4.2: Antibody Information. *Details of the antibodies used to detect members of the* NFκB *cascade.Information regarding the source; antigen retrieval method, concentation and incubation are all recorded.*



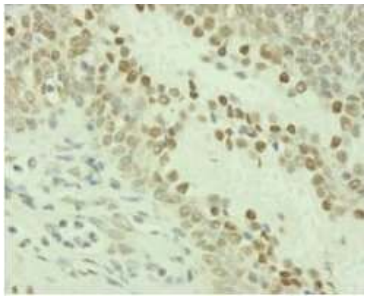
IκBα p ser $^{32/36}$



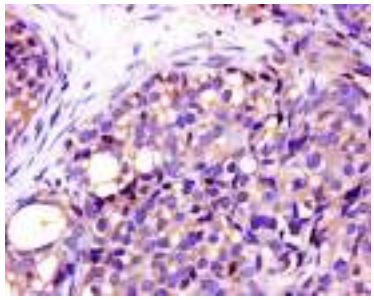
NFκBp65



NFκB p ser ^{536}



NFκB p ser ^{276}



MMP-9

Figure 4.2: Immunohistochemistry

Prostate tumours displaying immunohistochemical staining, magnification x400

4.3.1Protein expression patterns

NFκB p65, pNFκB p65 serine ^{276}, pNFκB p65 serine ^{536,} MMP-9 and pIκBα serine $^{32/36}$ protein expression was observed in the cell cytoplasm, pNFκB p65 serine ^{276}, pNFκB p65 serine ^{536} and pIκBα serine $^{32/36}$ expression was also observed in the nucleus. All IHC statistical analysis was carried out as previously described in (Chapter 2.9.1&2.9.2). Expression of all proteins investigated was non parametric, median values and inter quartile ranges for each protein at each location are provided in Table 3.2 for both hormone naive and castrate resistant tumours.

	HSPC (IQR)	*CRPC (IQR)*	*P value*	*ICCC*	*Histoscore units*
NFκB p65, c	100 (140-180)	100 (150-180)	P=0.755	0.86	40
NFκB $p65^{276}$,c	0 (0-41)	0(0-30)	P=0.010		
NFκB $p65^{276}$,n	7(50-80)	50(70-92)	P=0.544	0.77	64
NFκB $p65^{536}$,c	70(100-120)	42(90-123)	P=0.276	0.83	80
NFκB $p65^{536}$,n	0(20-73)	0(40-80)	P=0.159	0.85	55
pIκBα$^{32/36}$, c	110 (116-196)	133(160-186)	P=0.392	0.80	80
pIκBα$^{32/36}$, n			P=0.963	0.93	55
MMP-9	80(120-170)	90(120-160)	P=0.922	0.83	80

Table 4.3: Protein expression pattern

The median histoscore and interquartile range (IQR) for hormone naive tumours (HNPC) and castrate resistant tumours (CRPC) and the p value of these values compared using a Wilcoxon sign rank test. ICCC= interclass correlation coefficient. The mean difference in observer scores plus 2 standard deviations is also shown as the number of histoscore units that is defined as a change in protein expression (change). c and n relates to protein cellular location, c= cytoplasm and n = nucleus. P before a protein indicates that the antibody detects phosphorylated protein and the number following the protein represents the site of phosphorylation.

4.3.2 Protein expression in the Hormone Naïve cohort

To determine if protein expression was linked to time to biochemical relapse, Kaplan-Meier graphs were plotted for the hormone naïve tumours expressing low levels of protein (< median) versus high levels of protein (> median) and compared using the log rank test. Only cytoplasmic p65 ser ^{536} was shown to have significant results. The patients whose tumours expressed low levels of p65 ser ^{536}in the cytoplasm were shown to have relapsed significantly earlier than those patients whose tumours expressed high

levels of p65 ser ^{536} in the cytoplasm p= 0.024 (Figure 4.3), the median time to relapse for those with low expression was 2.3 (2.1-2.6) years compared to 3.5 years (1.6-5.4 years) for those with high p65 ser ^{536} expression indicating that cytoplasmic p65 ser ^{536} has a protective role in hormone naïve prostate cancer..

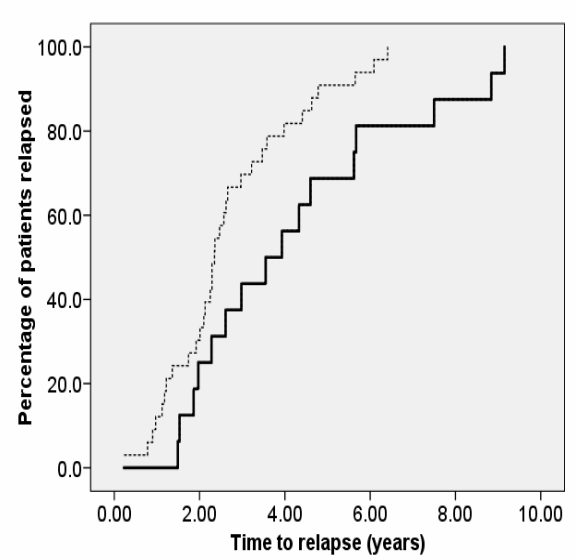


Figure 4.3 High cytoplasmic p65 ser ^{536} expression

Kaplan Meier plot demonstrates that those patients whose tumours express high p65 ser ^{536} in the cytoplasm (black line) relapse later than those patients whose tumours exhibit low p65 ser ^{536} expression (broken line).

When protein expression patterns were correlated with clinical parameters cytoplasmic NFκB p65 expression was significantly associated with PSA at relapse (p=0.02) (Figure 4.4). The median p65 histoscore value for patients with PSA below 4(ng/ml) was 135 (80-160) vs. 170(120-200) for patients with a PSA greater than 4 (ng/ml). In addition, MMP9 expression was significantly associated with the presence of metastasis at relapse (p=0.026), the median histoscore value for patients with no metastasis was 85 (72.5-115) vs. 125 (100-200) for those patients with metastasis at relapse (Figure 4.5).

Figure 4.4: p65 expression & PSA at relapse
p65 expression is significantly associated with higher PSA at relapse (p=0.02).

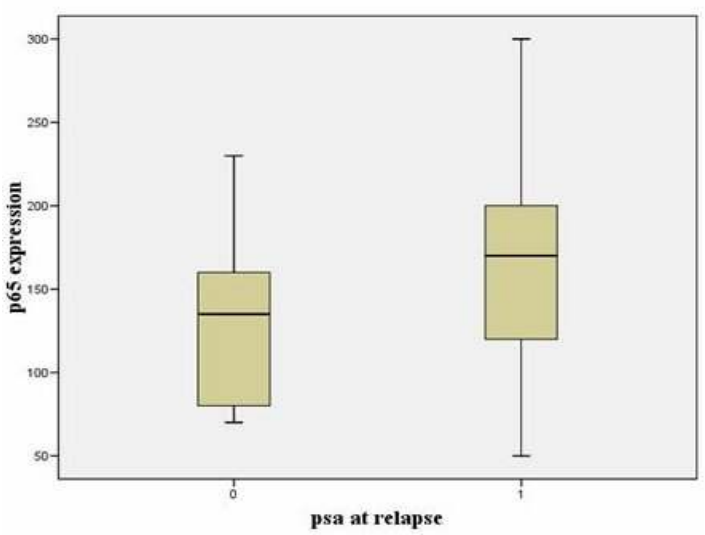
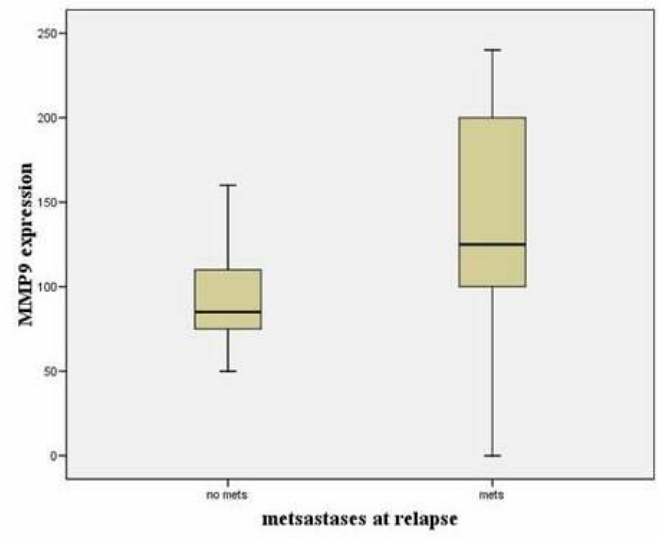


Figure 4.5: MMP-9 expression & metastases at relapse *MMP-9 expression is significantly higher in patients with metastases at relapse (p=0.026)*



To determine whether NFκB ser ^{536} phosphorylation was influenced by the Akt a correlation between phosphorylated Akt ser ^{473} and phosphorylated NFκB ser ^{536} was carried out. A negative correlation was observed between cytoplasmic Akt ser 473 and cytoplasmic p65 ser 536, r2=0.433 p=0.008 (Figure 4.6)

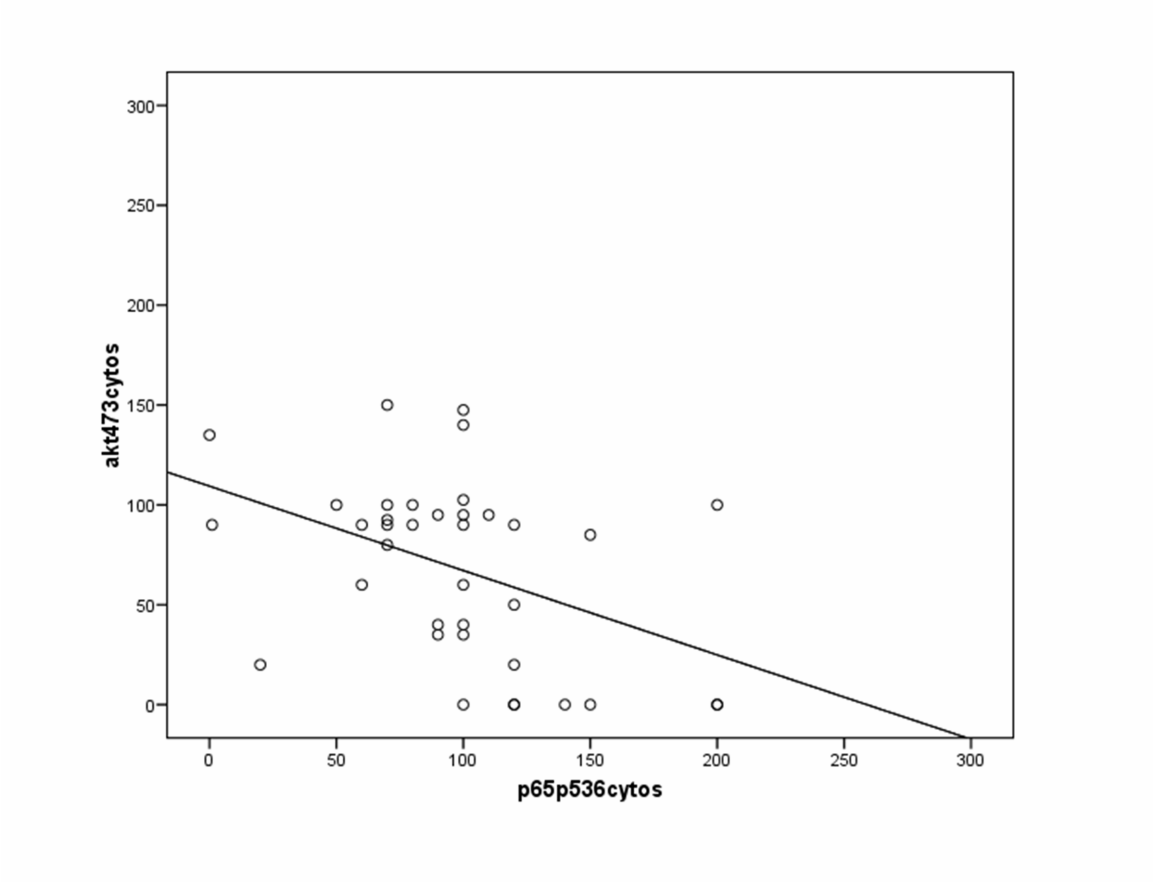


Figure 4.6: Scatter Graph

Scatter Graph comparing p65 ser536 cytoplasmic expression and cytoplasmic pAkt ser 473 expression, P=0.008

4.3.3 Protein expression in the Castrate resistant cohort

To establish if protein expression was linked to time to death from biochemical relapse, Kaplan-Meier graphs were plotted for the castrate resistant tumours expressing low levels

of protein (< median) versus high levels of protein (> median) and compared using the log rank test.

The patients whose tumours expressed low levels of nuclear p65ser ^{536} were shown to have a significantly shorter time to death from relapse compared to those patients whose tumours expressed high levels of nuclear p65 ser ^{536} p= 0.028 (Figure 4.7). These patients had a shorter time to death from relapse, 1.1 years (0.2-2.1 years) compared to 3years (1.2-4.8). Patients expressing a high level of nuclear p IκBα ser $^{32/36}$ had a significantly shorter time to death from relapse p=0.029 (Figure 4.8). Here it was observed that the patients who expressed a higher level of nuclear p IκBα ser $^{32/36\ had}$ a median time to death from relapse of 1.9 years (0.9-2.8) compared to those patients who expressed levels below the median, 4.1 years (0.5-7.9).

Additionally high MMP-9 expression resulted in a significantly shorter time to death from relapse p=0.015, (Figure 4.9), these patients survived for 1.3 years (0.5-2.2) compared to 3.6 years (0.6-6.6).

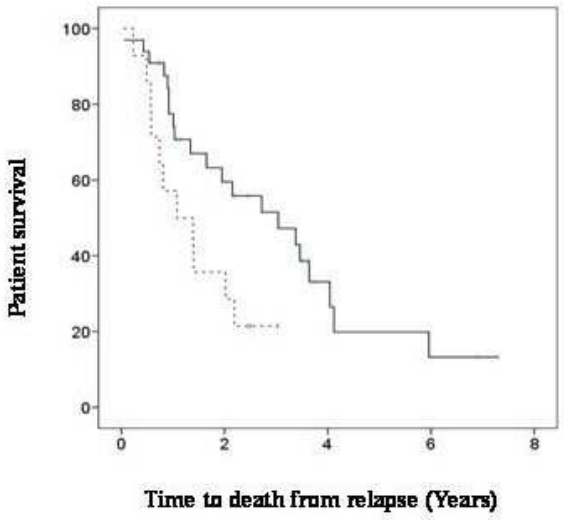


Figure 4.7: Nuclear p65ser ^{536} expression

Kaplan Meier plot demonstrates that those patients whose tumours express high p65 ser ^{536} in the nucleus (black line) have a longer time to death from relapse than those patients whose tumours exhibit low p65 ser ^{536} expression (broken line).

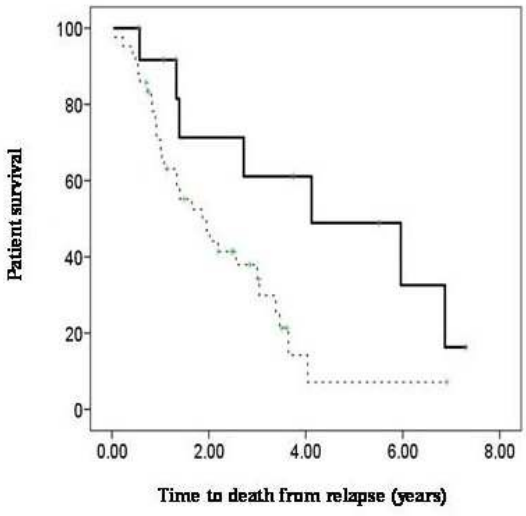


Figure 4.8: Nuclear IκBα ser $^{32/36}$ expression

Kaplan Meier plot demonstrates that those patients whose tumours express high p IκBαser $^{32/36}$ in the nucleus (broken line) have a longer time to death from relapse than those patients whose tumours exhibit low p65 ser ^{536} expression (black line).

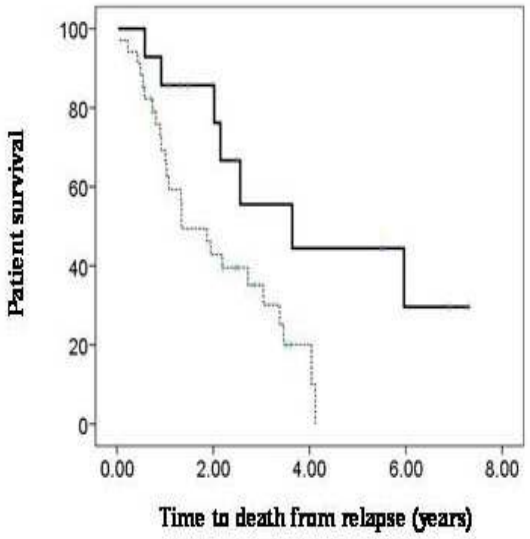


Figure 4.9: MMP-9 expression

Kaplan Meier plot demonstrates that those patients whose tumours express high MMP-9 (broken line) have a longer time to death from relapse than those patients whose tumours exhibit low MMP-9 expression (black line).

Correlations between clinical parameters and protein expression revealed Gleason grade at relapse was significantly associated with nuclear p65 ser ^{536} and cytoplasmic p65 ser^{276} expression p=0.001 and p=0.020 respectively and metastasis at relapse was significantly associated with nuclear p IκBα ser $^{32/36}$ p=0.003 (Figure 4.10). The median histoscore value for patients with no metastasis was 130 (40-161) vs. 195 (171-213) for those patients with metastasis at relapse.

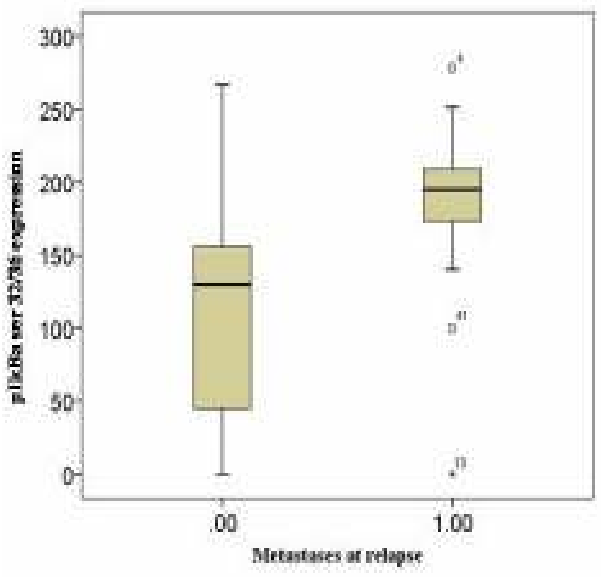


Figure 4.10: IκBα ser 32/36 expression and metastases at relapse

IκBα ser $^{32/36}$ expression is significantly higher in patients with metastases at relapse (p=0.003)

4.3.4 Changes in protein expression

As mentioned in the previous chapter the strength of this patient cohorts is the ability to investigate if those patients whose tumours exhibit an increase or decrease in protein expression, in the transition from hormone naïve to castrate resistant disease are more likely to relapse or die quicker. Table 4.3 provides the cutoff histoscore selected to separate subgroups of patients and the associations observed between time to biochemical relapse and disease specific survival for each protein investigated are shown in table 4.5.

Protein	Time to death from relapse	Overall survival
NFκ-B p65	**P=0.044**	P=0.081
pNFκ-B p65ser ^{276}, c	P=0.983	P=0.901
pNFκ-B p65^{276,}n	P=0.428	P=0.839
pNFκ-B p65^{536,}c	P=0.259	**P=0.009**
pNFκ-B p65^{536},n	P=0.137	P=0.200
pIκBα$^{32/36}$, c	P=0.399	P=0.172
pIκBα$^{32/36}$, n	P=0.114	P=0.260
MMP-9	P=0.129	**P=0.022**

Table 4.4: Changes in protein expression and survival

Kaplan meier survival analysis was performed to investigate if changes in protein expression were linked to time to death from biochemical relapse, and overall survival.

Nuclear p65 ser ^{536} and nuclear p65 ser ^{276} significantly increased with the progression to castrate resistant disease P=0.021 and P=0.001 respectively. To determine if an increase in protein expression was linked to time to death from biochemical relapse, and overall survival Kaplan-Meier graphs were plotted for the castrate resistant tumours expressing increased levels of protein versus decreased/no change of protein and compared using the log rank test, (Table 4.). An increase in cytoplasmic p65 expression was significantly associated with time to death from relapse p=0.004 (Figure 4.11). Those patients who had an increase in p65 expression had a significantly shorter time to death from relapse, 1.9 years (0.1-3.8) compared to those who displayed a decrease or no change 3.4 years (2.4-4.3).

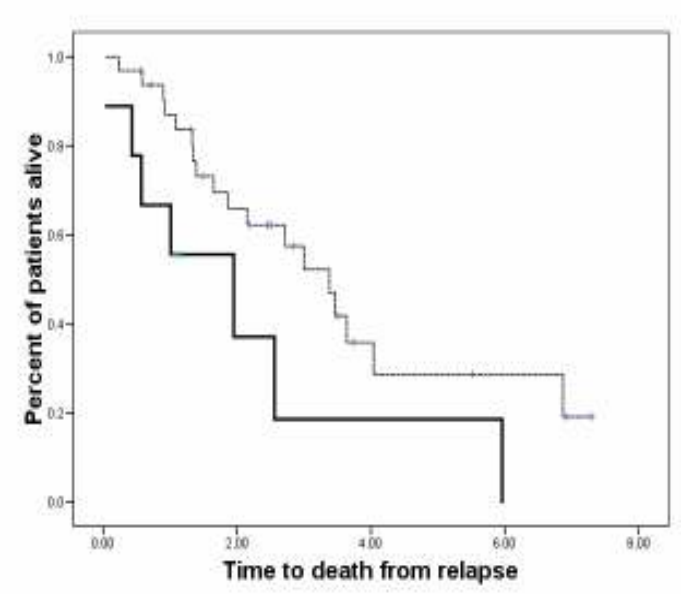


Figure 4.11 Increased p65 expression *Kaplan Meier plot demonstrates that those patients whose tumours express an increase in P65 expression (black line) have shorter time to death from relapse than those patients whose tumours exhibit decrease/no change in expression (broken line).*

4.4 Cell line

4.4.1 Activation of p65

To establish the activation levels of p65 in the matched hormone naive and castrate resistant prostate cancer cell lines a TNF α time course treatment was carried out (Figure 4.12). Phosphorylation of p65 at serine 536 was used as a measure of p65 activation and is seen to be rapidly achieved in the presence of TNF α. A higher phosphorylation level is evident the untreated LNCaP-CR suggesting p65 phosphorylation is a more common event in castrate resistant prostate cancer. By stimulating with TNF α both cell lines have undergone phosphorylation of the p65 subunit at serine 536 after 15 minutes, and both are observed to be markedly higher than in the untreated samples. Consequently it was

fitting to examine the effect of p65 inhibitors on NFκB activation, downstream protein expression, cell proliferation and apoptosis.

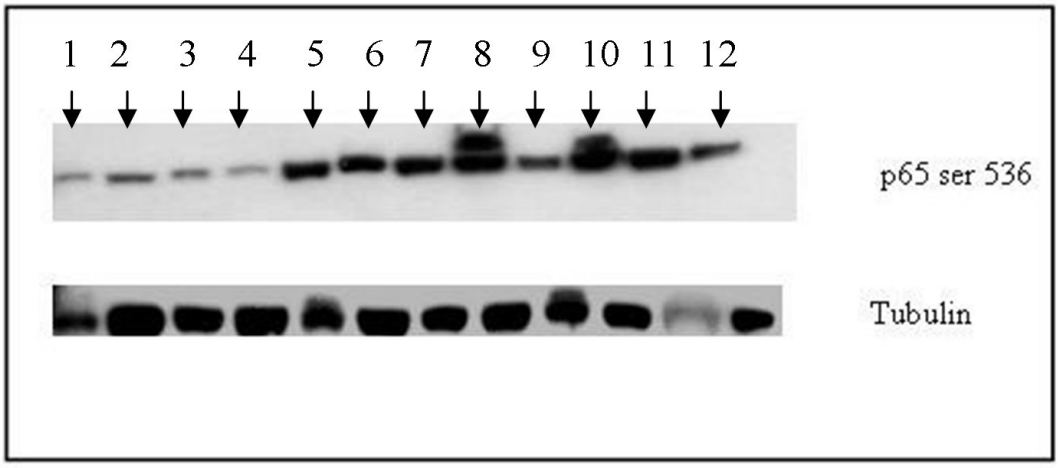


Figure 4.12 TNF stimulation of p65

Western blot was performed on 50ug of extracts from LNCaP& LNCaP-CR cells treated or untreated(serum free medium) with 30ng TNFα and probed for p65 (ser 536) expression.Lane 1LNCaP, 2 LNCaP-CR (unstimulated), 3&4 LNCaP& LNCaP-CR unstimulated(vehicle,dh_2o).Lanes5-12LNCaP& LNCaP-CR cell extracts stimulated for 5,15,30 minutes, and 1 hour. Double bands in lanes 8 and 10 are due to the volume of protein required to amount to 50ug. The loading wells in the gels only hold 30ul and for these samples extra had to be added as the proteins began to run through the stacking gel. Tubulin confirms loading of samples.

4.4.2 NFκB Inhibition

It was observed that treatment of LNCaP cells with NFκB inhibitors 2607 and 2070 resulted in a dose dependent decrease in constitutive expression of p65 ser ^{536} (Figure 4.13a & 4.13 b) while total p65 levels remained unchanged. To assess the downstream effect of p65 inhibition MMP-9 expression was examined. A reduction in MMP-9 expression is also shown (Figures 4.13 a & 4.13 b), here it is observed that 2607 and to a

lesser extent 2070 both inhibit the down stream activity of p65. The above results indicate that both inhibitors attenuate constitutive phosphorylation of p65 at ser ^{536} which in turn inhibit its down stream actions.

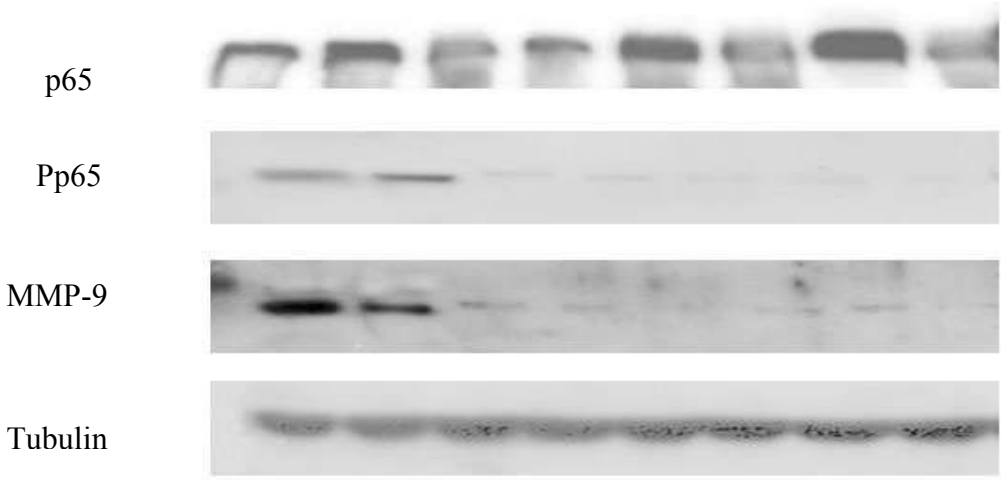


Figure 4.13(a) Effect of 2607 on p65 Inhibition

Western blot was performed on 50ug of extracts from LNCaP cells and probed for p65, p65 (ser 536) and MMP-9 expression. Lane 1&2 LNCaP unstimulated & unstimulated (vehicle, ethanol).Lanes 3-8 LNCaP cell extracts incubated for 24 hours in 0.1,1,3,10,30,100 uM of drug 2607respectively. Tubulin (50kd) was used as a loading control.

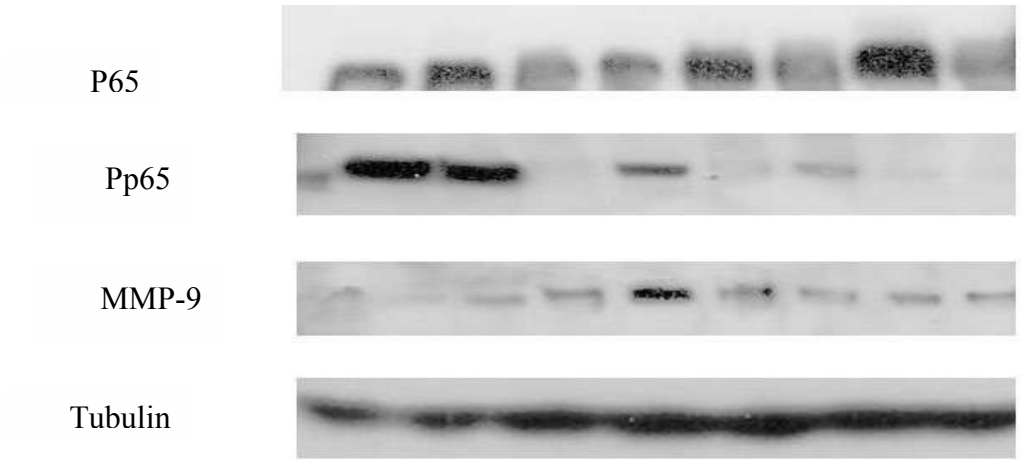


Figure 4.13 (b) Effect of 2070 on p65 Inhibition

Western blot was performed on 50ug of extracts from LNCaP cells and probed for p65, p65 (ser 536) and MMP-9 expression. Lane 1&2 LNCaP unstimulated & unstimulated (vehicle, ethanol).Lanes 3-8 LNCaP cell extracts incubated for 24 hours in 0.1,1,3,10,30,100 uM of drug 2070 respectively. Tubulin (50kd) was used as a loading control.

To further examine the affect of NFκB inhibition both cell lines were incubated with 1uM, 10 uM and 50 uM of inhibitors 2607 and 2070 and the proliferation measured at 24 and 72 hours by WST assay. Both cell lines showed a dose dependent inhibition in proliferation after 72 hours, (Figures 4.14(a), 4.14(b) and 4.15). Additonally the effect of these inhibitors on apoptosis was also examined. It was observed that 2070 stimulated apoptosis during 24 hours in response to all concentrations tested and 2607 stimulated apoptosis during 24 hours in response to 50uM only (Figure 4.16 & 4.17).

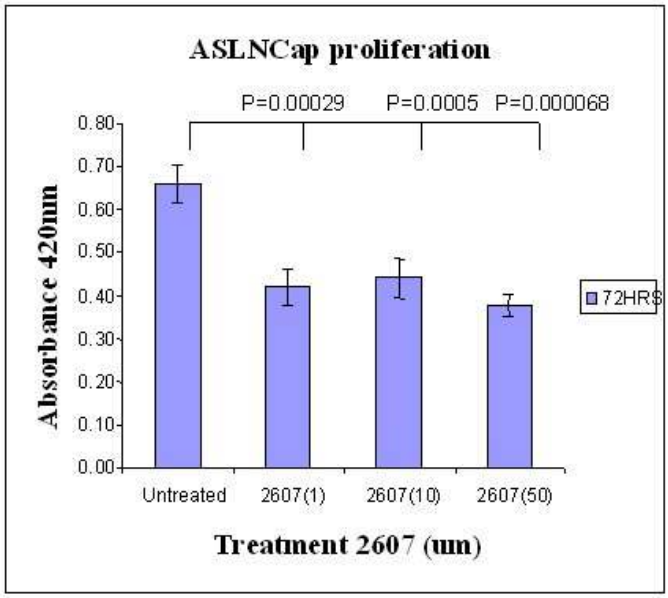


Figure 4.14(a): Drug 2607 inhibits cellular proliferation

LNCaP cells were treated with 1, 10 &50 uM of drug 2607 and proliferation was significantly reduced at 72 hours

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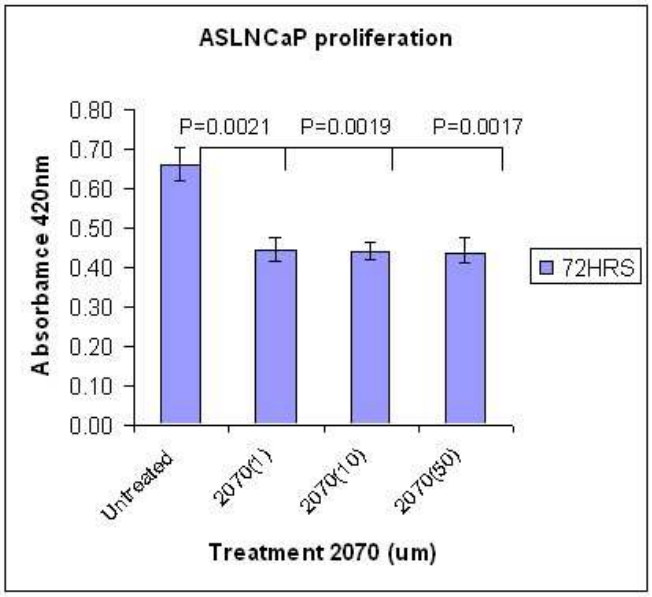


Figure 4.14(b): Drug 2070 inhibits cellular proliferation

LNCaP cells were treated with 1, 10 &50 uM of drug 2070 and proliferation was significantly reduced at 72 hours.

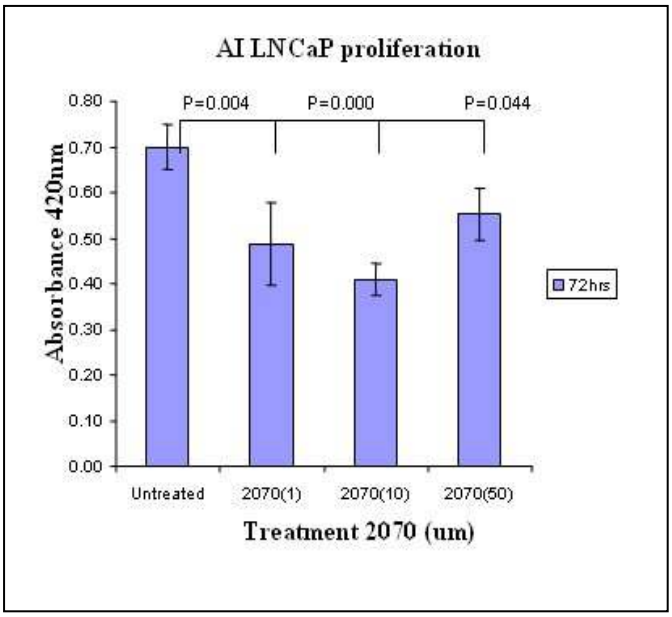


Figure 4.15: Drug 2070 inhibits proliferation in LNCaP-CR cells

LNCaP cells were treated with 1, 10 &50 uM of drug 2070 and proliferation was significantly reduced at 72 hours.

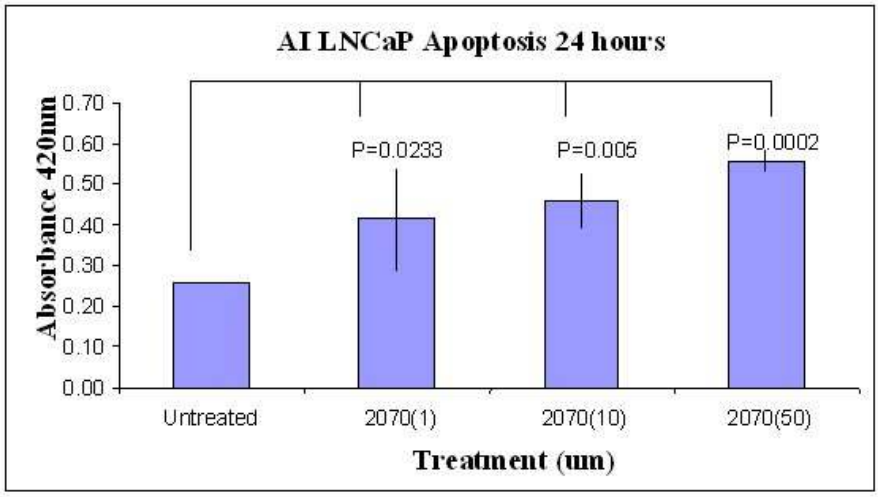


Figure 4.16: Drug 2070 stimulates apoptosis in LNCaP-CR cells

LNCaP-CR cells were treated with 1, 10 &50 uM of drug 2070 and apoptosis was significantly induced at 24 hours.

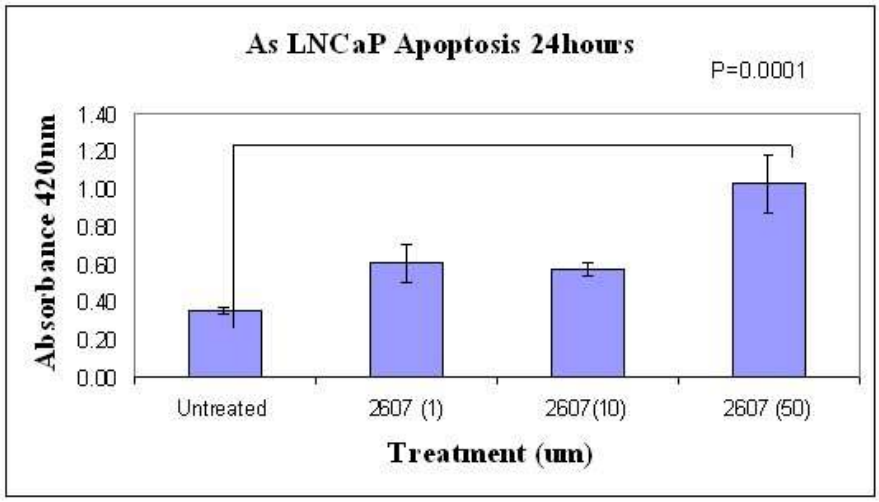


Figure 4.17: Drug 2607 stimulates apoptosis in LNCaP cells

LNCaP cells were treated with 1, 10 &50 uM of drug 2607 and apoptosis was significantly induced at 24 hours

4.5 Discussion

The NFκB system is viewed as a hopeful anticancer target due to its role in oncogenesis and chemotherapy resistance in many tumour types. As the biological significance of constitutive NFκB activation in prostate cancer is unclear at present, this study was undertaken to provide further evidence on the expression and role of NFκB in prostate cancer progression and aimed to characterise the expression and subcellular location of p65 protein and explore the value of p65 in predicting patient outcome. Phosphorylated and total NFκB p65 protein expression and localisation were determined in prostate cancer tissue and cell lines.

In this study it was observed that phosphorylation of p65 at serine residues 276 and 536 was a common event in prostate cancer with all samples showing some degree of expression of both phosphorylation sites in the nucleus. Both activation sites were found to significantly increase with the progression to castrate resistant disease however neither were associated with survival. In the hormone naïve tumours a high expression level (above the median) of cytoplasmic p65p^{536} resulted in a longer time to biochemical relapse than those patients who expressed low levels, (Figure 4.3). This result is not unforeseen as it is frequently stated in the literature that p65 subunit of NFκB is further activated in the nucleus where it then binds to and activates a wide number of genes. Surprisingly a high level of nuclear p65 ^{536} in castrate resistant tumours was also shown to have protective role. Patients whose tumours expressed low levels of nuclear p65 ^{536} were observed to have a significantly shorter time to death from relapse (Figure 4.7). However when a change in p65 ^{536} expression from hormone naïve to castrate resistant disease was examined it was observed that increased levels of cytoplasmic p65 ser^{536}

resulted in a shorter overall survival (Table 4.4) however this result was only observed in four patients and data was therefore not shown, if the cohort were expanded this may have been evident in a larger population of the cohort. Thus these results indicate that the activation of p65 ser ^{536} is not playing a direct role in the development of castrate resistant prostate cancer however, in a sub group of patients increased levels p65 ser^{536} is seen to have a negative effect on patient survival. We hypothesis that another phosphorylation site of p65 is driving this observation or it could simply be that nuclear localisation is a more appropriate marker of NFkB and would serve as a more robust biomarker/prognostic marker. The phosphorylation sites chosen in the current study had been extensively reported in the literature as being associated with p65 transactivation in many cancer types (224-226).

A review of the literature, demonstrated that Akt can activate NFκB via serval stimuli and this in turn functions to prevent apoptosis. In particular, two studies have indicated that Akt, via TNFα signallingor in response to growth factor stimulation, stimulated NFκB nuclear translocation via the IKK complex (192;203). Another study indicated that Akt alone could not induce nuclear translocation of NFκB but synergized with PMA to induce this response (191). It has been shown that Akt signallinginvolves the stimulation of the transcription function of NFκB and that the ability of oncogenic Ras to activate NFκB transcriptional activity is dependent on Akt activity (227;228). Madrid et al have also observed that the ability of Akt to stimulate the transactivation potential of the p65 subunit of NFκB actually requires IKK and p38 (229). In this study a negative correlation between activated Akt and p65 ser^{536} was observed which corroborates with others suggesting that Akt does not directly phosphorylate p65 at ser ^{536}.

Studies showing that NFκB is active (i.e. nuclear) in a number of tumours are consistent with a role for NFκB in cancer, although some tumour cell lines exhibit NFκB activity without significant nuclear accumulation hence suggesting a cytoplasmic role for p65 other than a nuclear transcription factor. In this study it was observed that an increase in cytoplasmic p65 expression in the transition from hormone naïve to castrate resistant disease was significantly associated with a shorter time to death from relapse (Figure 4.11) and cytoplasmic p65 expression also correlated with PSA at relapse (Figure 4.5). The PSA gene promoter has κB site (230). PSA is involved in prostate epithelial growth therefore suggesting that p65 is involved in prostate cancer progression. When expression levels of p65 inhibitor pIκBα$^{32/36}$ were examined it was noted that high nuclear levels were significantly associated with a shorter time to death from relapse this result indicates that p65 is active in the cell. Moreover metastasis at relapse was significantly associated with nuclear p IκBα ser $^{32/36}$ (Figure 4.10), further suggesting a role for p65 in prostate cancer progression. As previously mentioned IκBα is phosphorylated at serines 32/36 in by IKKα/β in response to multiple stimuli and marked for degradation by the UPS, this is known to occur in the cytoplasm and leads to NFκB activation and nuclear translocation. Newly synthesized IκBα shuttles in and out the nucleus controlling NFκB activity however evidence now indicates that proteasomes occur in the cytoplasm and the cell nucleus (231-233) (234;235). Additionally, IKKα has recently been reported to have a nuclear role (236). In prostate cancers infiltrating immune cells which express RANKL (Receptor activator of NFκB) have been shown to bind to its receptor, RANK which induces activation and nuclear translocation of IKKα.

IKKα then interacts with the Maspin gene promoter silencing its expression, in turn promoting prostate cancer metastases (237).

NFκB has been shown to be constitutively activated in prostate cancer cells, and elevated NFκB activity is also sustained in androgen-responsive human prostate cancer cells by androgen treatment. Huang *et al* have demonstrated that suppression of NFκB activity in human prostate cancer cells by IκBα mutation transfection inhibits their metastatic properties in nude mice by suppressing angiogenesis and invasion providing direct involvement of NFκB in the regulation of angiogenesis and metastasis of prostate cancer cells (198).

Numerous reports have demonstrated that in prostate cancer as well as other tumour types that the metastatic potential of tumour cells directly correlates with the expression level of several angiogenic genes, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (Bfgf), interleukin 8 (IL-8) and matrix metalloproteases MMP-2 and MMP-9 (16). Expression of MMPs is associated with poor prognosis in a variety of cancers including prostate. An increase in MMP-2 and MMP-9 is associated with tumour progression but how the constitutive expression of these genes is regulated in prostate cancer is at present unclear. It is known however that NFκB binds the MMP-9 promoter (13). In this study MMP-9 expression was significantly associated with the presence of metastasis at relapse and was also significantly associated with shorter time to death from relapse (Figure 4.5 & Figure 4.9). An increase in MMP-9 expression was also significantly associated with a shorter overall survival (Table 4.5), again this was only observed in a few patients and the data is not shown. This study provides evidence that

NFκB signallingis an important event in the progression of castrate resistant prostate cancer and metastasis in a subset of patients.

In prostate cancer cells androgen depravation induces cell cycle arrest and apoptotic cell death. NFκB mediates cell cycle progression through direct binding of the Cyclin D1 promoter at multiple sites and regulates progression through G_1-S cell cycle check point (238). The expression of Cyclin D1 was investigated in another study done in our Laboratory using the current patient cohort. Therefore the association between NFκB pathway members and Cyclin D1 expression was calculated using the Spearman's Rank Correlation Test. In the hormone naïve tumours a positive correlation between nuclear p65 ser ^{276} and nuclear Cyclin D1 was observed p=0.040 r^2 =4.32. Moreover a positive correlation between nuclear pIκBα$^{32/36}$ and Cyclin D1 expression p=0.020 r^2 =.516 was observed in the castrate resistant cohort. NFκB has also been shown to inhibit apoptosis by directly binding the promoter and inducing genes encoding BCL-2 homologue BCL-XL and survivin (239). Therefore inhibition of p65 is an important strategy to inhibit prostate cancer tumourgenesis. Due to the diverge ranges of upstream activators of NFκB, the effect of inhibiting NFκB activity directly was investigated. In the current study it was demonstrated using two novel NFκB inhibitors that these drugs can significantly inhibit proliferation and stimulate apoptosis in both LNCaP and LNCaP CR cells. These observations support the hypothesis that NFκB could offer a therapeutic target for treatment of castrate resistant prostate cancer. Additionally, the suggested biomarker for prediction of patient response for these novel therapies would be nuclear localization of p65 and not phosphorylation of the sites investigated in the current study.

In summary, it was hypothesised that NFκB expression could be increased via activation of the Akt cascade however no positive correlation was observed between activated Akt and activated NFκB. However these findings suggest that activation of the NFκB pathway is sufficient to maintain androgen independent growth of prostate cancer. Inhibition of NFκB activity leads to a decrease in cellular proliferation and induced apoptosis. Thus the NFκB pathway may be a potential target for therapy in a subset of patients with castrate resistant disease.

5. The relationship between Akt and the Androgen receptor

5.1 Introduction

The mechanisms driving the development of castrate resistance are not fully understood and there are several hypotheses. As discussed in chapter 1, the AR can be activated in a ligand dependent manner, via androgen or in a ligand independent manner via signal transduction cascades. During the progression of prostate cancer from hormone naïve to castrate resistant disease prostate cancer cells retain AR expression which suggests that AR plays a critical role in the development and progression of prostate cancer. Research suggest that activation of the PI3K/Akt cascade contributes to the development of castrate resistant disease through phosphorylating and activating the AR. Results from chapter 3 suggested that low levels of PTEN, increased levels of PI3K, and activated Akt are associated with reduced time to relapse and patient survival. Inhibition of PI3K also resulted in decreased Akt activation and decreased cellular proliferation. As mentioned in chapter 1, Akt specifically binds to AR and phosphorylates serines 213 and 791, thereby activating AR. Blocking the Akt cascade by a dominant – negative Akt or an inhibitor of Akt inhibits HER2 induced AR signalling. Thus, these results suggest that Akt is an activator of AR signalling. The interaction between Akt and the AR is well documented however the consequence of AR phosphorylation by Akt on patient survival has not been investigated in clinical samples.

This study investigates whether phosphorylation of AR by Akt influences clinical outcome and the progression to castrate resistant disease. Matched patients samples (hormone naïve and castrate resistant) were analysed for AR, AR phosphorylated at serine 213 (Akt consensus site) and AR phosphorylated at serine 81 expression (which

has previously been shown to be phosphorylated in response to androgens). In vitro models were employed to investigate the effect of PI3K inhibition on AR phosphorylation and PI3K expression was knocked down using small interfering RNA specific to the p85 regulatory subunit of PI3K, and the consequences of this on AR phosphorylation were investigated by Immunofluoresence.

5.2 Patients

Sixty two patients with matched hormone naïve and castrate resistant tumour pairs were retrospectively selected for analysis (124 tumours in total) Clinical data, recorded for each patient included age (median 70, inter quartile range 66-74), PSA at diagnosis (median 31 ng/ml, inter quartile range 7.8-109), PSA at relapse (median 13.4 ng/ml, inter quartile range 4.5-31) and Gleason grade at diagnosis (median 8, range 6-9). All patients under went biochemical relapse (median time to relapse 2.49 years, inter quartile range 1.63-4.27 years) and median follow-up was 6.7 (2.8-8.4) years. Patients in this cohort were diagnosed with locally advanced (39) or metastatic prostate cancer (23) and subsequently received surgery and androgen deprivation therapy (26 sub capsular bilateral orchidectomy, 43 GnRH analogue, 3 had both). Forty four of the 62 patients also received anti androgen therapy and this included all those who received GnRH analogues. Forty patients (64%) had metastases at relapse. Fifty nine patients (95%) died during follow-up and median survival for these patients was 4.73 (3.75-6.74). Clinical parameters associated with time to biochemical relapse, time to death from biochemical relapse and disease specific survival are shown in table 5.1.

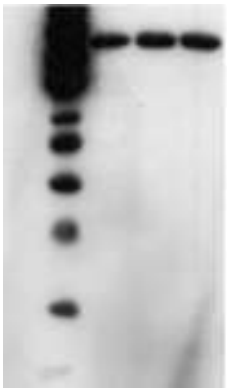
	Time to relapse	Time to death from relapse	Disease specific survival
Age	0.210	0.247	0.246
Gleason	0.031	0.002	0.001
Metastasis at diagnosis	0.007	0.016	<0.001
PSA at diagnosis	0.025	0.471	0.141
Metastasis at relapse	NA	0.001	0.006
PSA at relapse	NA	0.146	<0.001

Table 5.1: Patient Characteristics

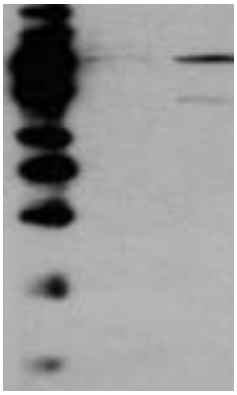
An overview of the cohort's characteristics where each clinical parameter, were appropriate has been correlated with time to relapse, time to death from relapse and disease specific survival (p-values).

5.3 Immunohistochemistry

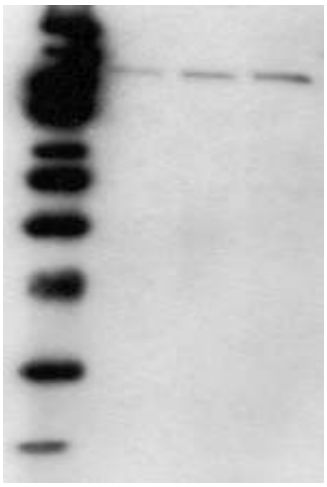
Before IHC commenced antibody specificity was confirmed by western blotting (Figure 5.1). Information on all antibodies employed are displayed in table 5.2 and examples of immunohistochemical staining for each are displayed in figure 5.2 . I am grateful to Dr Yin Rue Lim for carrying out the AR IHC.



A.Androgen receptor *(110kDa). Western blot of LNCaP, LNCaP-CR and casodex resistant LNCaP cell extracts grown in full medium.*



B. Androgen receptor p serine [81] *(110kDa). Western blot of LNCaP cell extracts grown in full medium untreated and treated with 10nm DHT.*



C. Androgen receptor p serine [213] *(110kDa). Western blot of LNCaP, LNCaP-CR, and casodex resistant LNCaP cell extracts grown in full medium.*

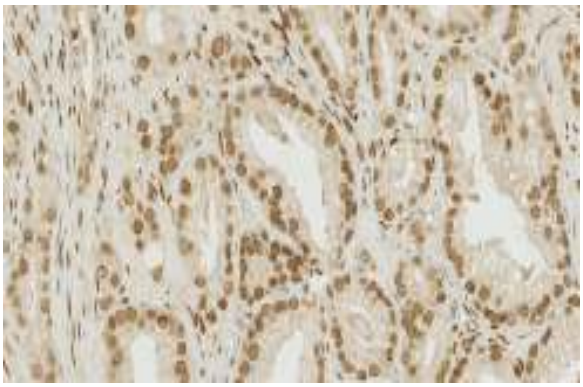
Figure 5.1: Antibody specificity

Western blotting was performed to confirm the specificity of all antibodies used in this study

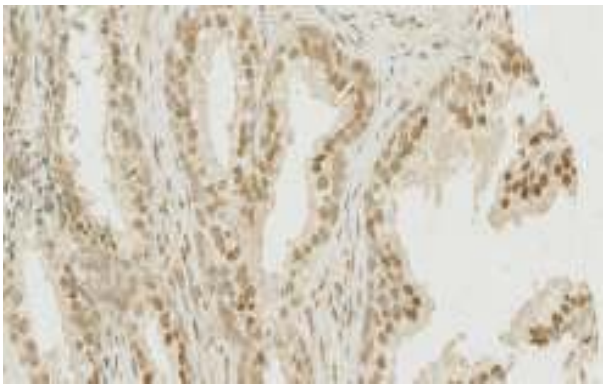
Protein	Antibody	Antigen Retrieval	Antibody concentration	Incubation temperature and time
AR	AR Mouse Monoclonal IgG1 Ab Dako (A/S)	TE Buffer	1ug/ml	Overnight at 4ºC
AR	AR p serine 81 Rabbit Polyclonal IgG1 Ab Upstate	TE Buffer	10ug/ml	Overnight at 4ºC
AR	AR p serine 213 Rabbit Polyclonal IgG1 Ab Imgenex	TE Buffer	50ug/ml	One hour at 25ºC

Table 5.2: Antibody Information

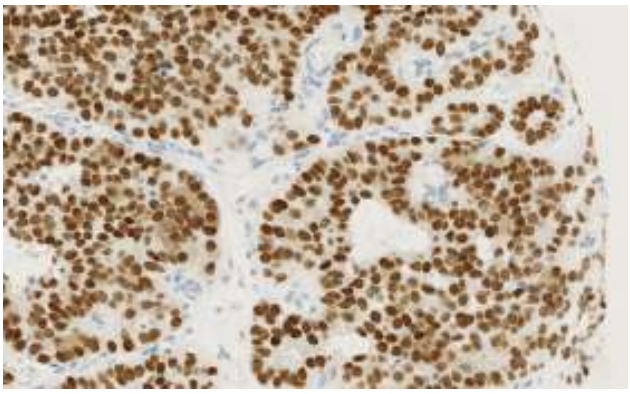
Details of the antibodies used to detect AR, pAR serine 81 and pAR serine 213.Information regarding the source; antigen retrieval method, concentation and incubation are all recorded.



Androgen Receptor



Phosphorylated AR serine 81



Phosphorylated AR serine 213

Figure 5.2: Immunohistochemistry

Prostate tumours displaying immunohistochemical staining magnification x400

5.3.1 Protein expression patterns

Protein expression of AR, pAR serine ^{213} and pAR serine ^{81} was observed in the cell cytoplasm and nucleus. Expression of all proteins investigated was non parametric, median values and inter quartile ranges for each protein at each cellular location are provided in table 5.3 for both hormone naïve and castrate resistant tumours. All IHC

statistical analysis was carried out as previously described in Materials and methods (Chapter 2.9.1&2.9.2).

Both cytoplasmic and nuclear AR expression increased significantly with the development of castrate resistant disease. Nuclear pAR ser ^{213} increased significantly with the development of castrate resistant disease and cytoplasmic pAR ser ^{81} significantly decreased with the development of castrate resistant disease (Table 5.3).

	HNPC (IQR)	***CRPC (IQR)***	***P value***	***ICCC***	***Histoscore units***
AR, c	0	5 (0-30)	**P=<0.0001**	0.89	50
AR, n	65.0 (36.7-90.7)	120.0 (78.8-147.5)	**P=<0.0001**	0.81	48
AR p ser ^{81} c	90(50-115)	60(30-95)	**P=0.001**	0.75	73
AR p ser ^{81}n	100(50-135)	90(50-120)	P=0.816	0.77	108
AR p ser ^{213}n	35(0-85)	103(50-169)	**P=<0.0001**	0.93	52

Table 5.3. Protein expression patterns

The median histoscore and interquartile range (IQR) for hormone naive tumours (HNPC) and castrate resistant tumours (CRPC) and the p value of these values compared using a Wilcoxon sign rank test. ICCC= interclass correlation coefficient. The mean difference in observer scores plus 2 standard deviations is also shown as the number of histoscore units that is defined as a change in protein expression. c and n relates to protein cellular location, c= cytoplasm and n = nucleus. P before a protein indicates that the antibody detects phosphorylated protein and the number following the protein represents the site of phosphorylation.

5.3.2 Protein expression in the Hormone Naïve cohort

As done in the previous chapters, protein expression levels were analysed for association with clinical outcome by dividing into high (above median values) and low expression (below median values) and Kaplan-Meier graphs were plotted to determine if protein expression was linked to patient outcome. None of the protein investigated were associated time to biochemical relapse or patient survival.

5.3.3 Protein expression in Castrate resistant tumours

When expression levels of each protein investigated in the castrate resistant tumours were divided into to high or low expression, nuclear AR expression was associated with a quicker time to death from relapse (Figure 5.3). Patients with high AR expression had a median survival from time from relapse of 1.34 yrs (0.80-1.88) compared to 3.0 years (1.68-4.32) for those with low AR expression (p=0.038 HR 1.98 (1.03-3.82)) however, this was not significant on Cox Regression multivariate analysis. High expression of pAR ser ^{213} was associated with quicker time to death from relapse (figure 5.4(a), p=0.003 HR 2.85 (1.38-5.87)) and shorter disease specific survival (figure5.4 (b), p=0.0136, HR 2.33 (1.16-4.66). Median survival from time from relapse for those patients with tumours that expressed low levels of pAR ser ^{213} was 3.42 (2.82-4.02) years compared to 1.40 (0.85-1.95) for those who had tumours that expressed high levels of pAR ser ^{213} . The median disease specific survival for those patients with tumours that expressed low levels of pAR ser ^{213} was 8.57 (5.41-11.73) years compared to 5.82 (3.18-8.46) for those who had tumours that expressed high levels of pAR ser ^{213}. This represents a survival difference of almost 3 years for patients expressing high levels of pAR ser ^{213} in their castrate resistant tumour. Additionally, correlations between clinical parameters and protein expression

revealed high expression of AR and pAR ser [213] was associated with presence of metastases at relapse figure 5.5 (p=0.018 and p=0.046, respectively). The phosphorylation of AR ser [81] was not associated with patient survival.

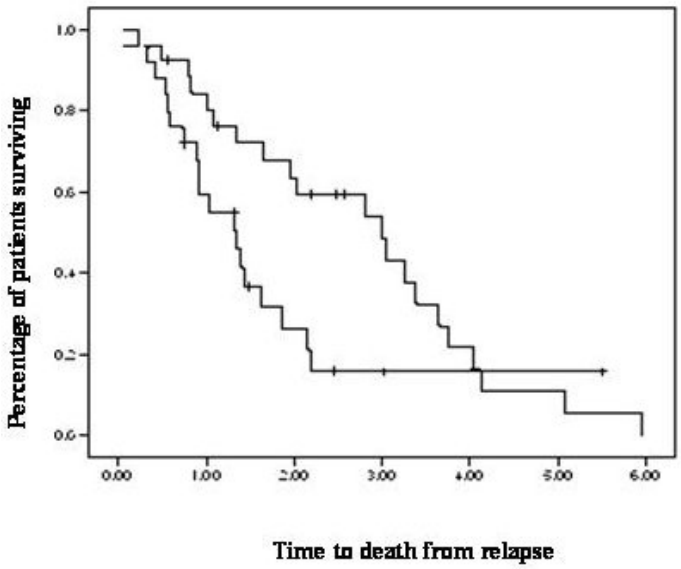


Figure 5.3: Nuclear AR expression

Kaplan Meier plot demonstrates that those patients whose castrate resistant tumour has high AR nuclear expression (bottom line) have shorter time to disease specific death from time of biochemical relapse than those patients whose castrate resistant tumour have low AR nuclear expression (top line)(p=0.038).

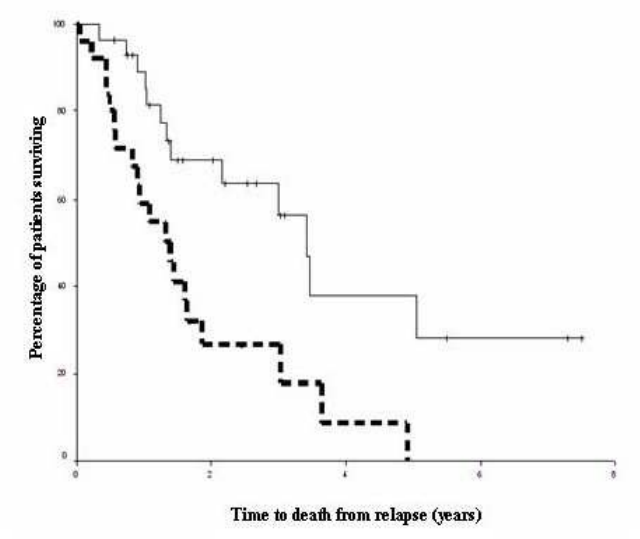


Figure 5.4(a): Nuclear pAR ser ^{213} expression

Kaplan Meier plot demonstrates that those patients whose castrate resistant tumour has high pAR nuclear ser ^{213} expression (broken line) have shorter time to disease specific death from time of biochemical relapse than those patients whose castrate resistant tumour have low pAR nuclear ser ^{213} expression (solid line)(p=0.003).

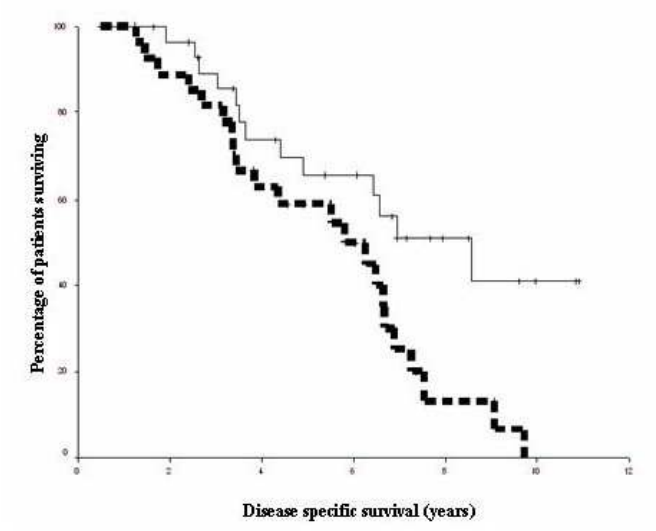


Figure 5.4(b): Nuclear pAR ser ^{213} expression and disease specific survival

Kaplan Meier plot demonstrates that those patients whose castrate resistant tumour has high pAR nuclear ser ^{213} expression (broken line) have shorter time to disease specific

death than those patients whose castrate resistant tumour have low pAR nuclear ser [213] expression (solid line)(p=0.0136).

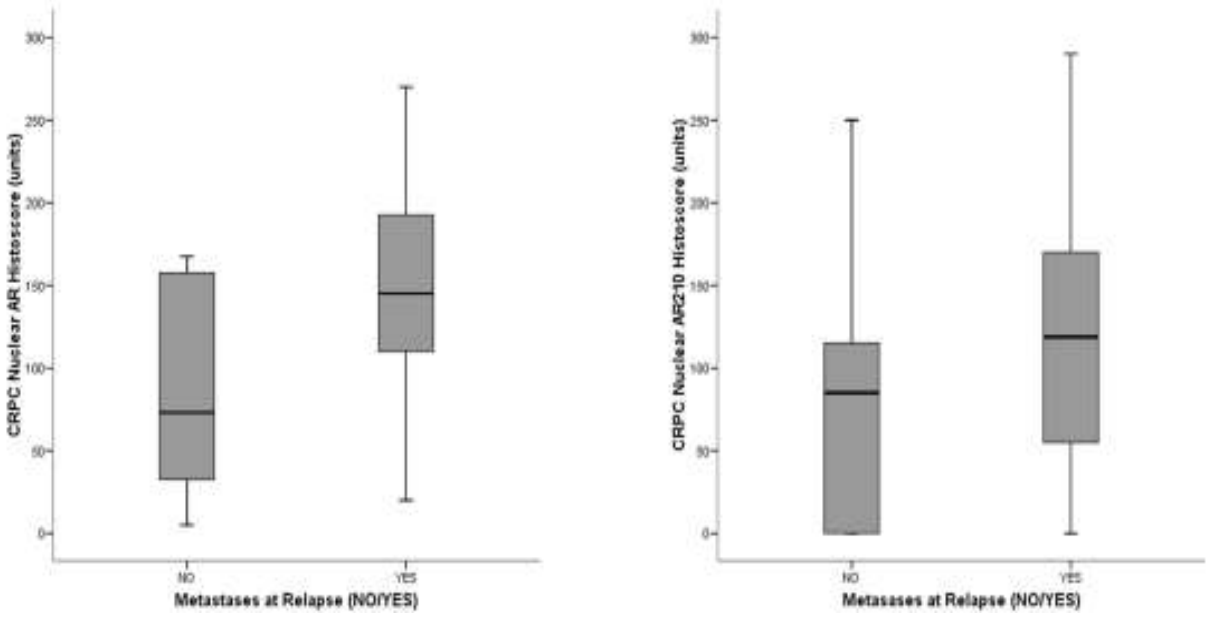


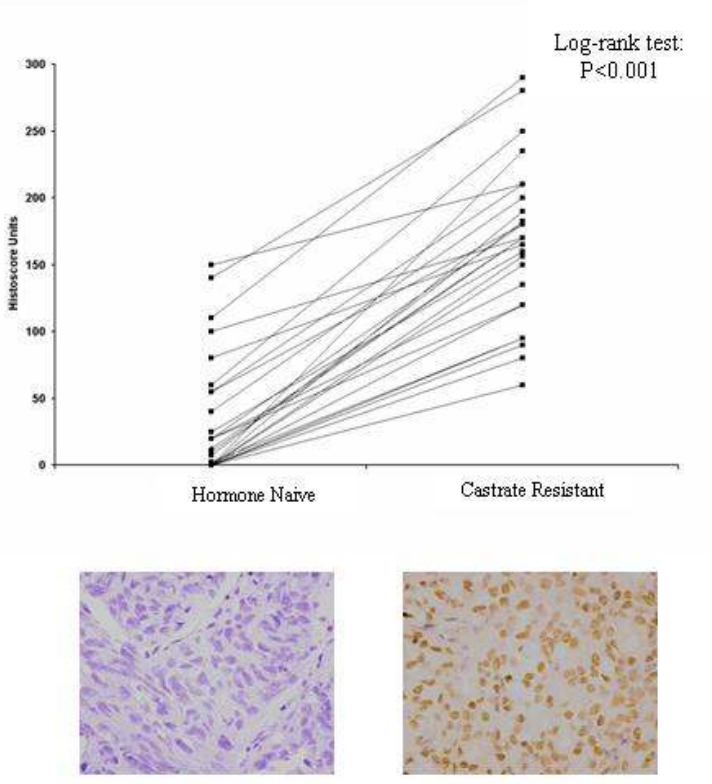
Figure 5.5: AR & pAR ser [213] expression and metastasis at relapse

Box plot demonstrating difference in expression of total AR expression (left), and pAR ser 213 expression (right) between metastatic and non metastatic patients at relapse.

5.3.4 Changes in Protein Expression

As with the previous two chapters changes in protein expression levels in the transition from hormone naive to castrate resistant disease were used to investigate a link with clinical outcome. In the current cohort, castrate resistant tumours express significantly higher levels of pAR ser [213] compared to hormone naïve tumours, approximately 42% of patients have an increase in pAR ser [213] expression, (Figure 5.6). An increase in pAR ser [213] expression (figure 5.7(a), p<0.0001, HR 4.18 (1.99-8.74)) was significantly associated

with quicker time to death from relapse, the median survival from biochemical relapse for those patients whose tumours had a decrease or no change in pAR ser ^{213} expression was 3.46 (1.39-5.53) years compared to 1.25 (0.83-1.67) years for those patients whose tumours had an increase in pAR ser ^{213} expression. Additionally an increase in pAR ser ^{213} (figure 5.7 (b) p=0.0015, HR 2.86 (1.45-5.67)) was associated with a shorter disease specific survival. The median survival from diagnosis for those patients whose tumours had a decrease or no change in pAR ser ^{213} expression was 6.95 (4.07-9.83) years compared to 4.36 (1.67-7.10) years for those patients whose tumours had an increase in



pAR ser ^{213} expression.

Figure 5.6: Change in pAR ser 213 expression

Displays the number of patients who had an increase in pAR ser ^{213} protein expression. IHC staining of a patient who had an actual increase of 150 histoscore units in the

transition tcastrate resistant disease. Brown nuclear staining denotes pAR ser ^{213}expression. Magnification x400.

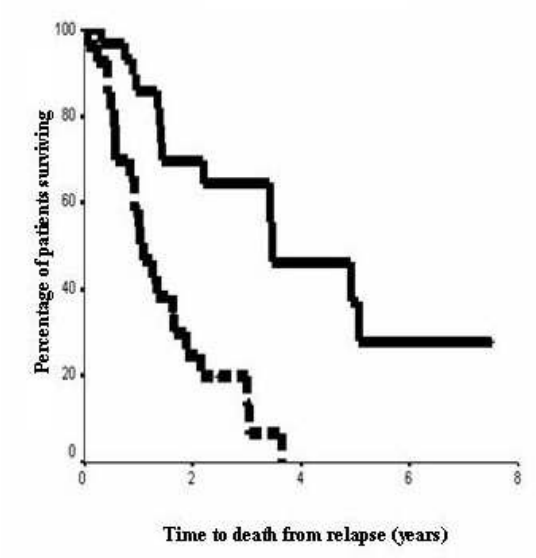


Figure 5.7(a): Increased pAR ser 213 and time to death from relapse

Kaplan Meier plot demonstrates that those patients whose tumours exhibit a increase in pAR^{213} expression (broken line) have shorter time to disease specific death from time of biochemical relapse than those patients whose tumours exhibit no change or a fall in pAR^{213} expression (solid line).

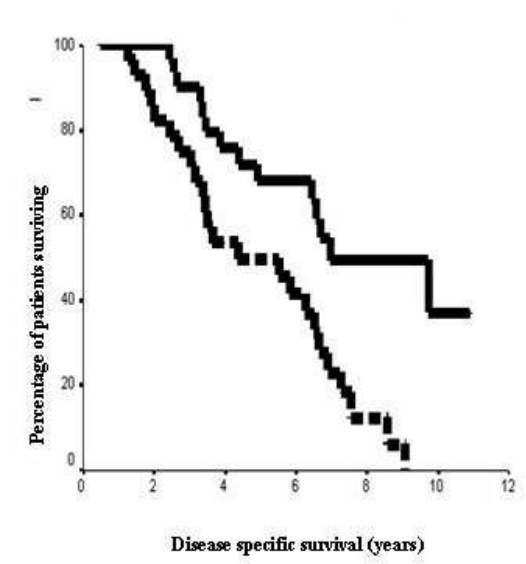


Figure 5.7(b): Increased pAR ser 213 and disease specific death

Kaplan Meier plot demonstrates that those patients whose tumours exhibit a increase in pAR^{213} expression (broken line) have shorter time to disease specific death than those patients whose tumours exhibit no change or a fall in pAR^{213} expression (solid line).

5.3.5 Correlations between active members of the PI3K/Akt cascade

Protein expression was also correlated with members of the PI3K cascade. In hormone naïve tumours expression levels of the phosphorylated proteins did not correlate, however in the castrate resistant tumours pAkt ser ^{473} correlated with pAR ser ^{213} (r_s=0.711, p<0.001) and pmTOR2^{448} (r_s.=0.489, p=0.003).

5.3.6 Association between AR gene amplification and protein expression

Data for AR gene amplification from the same cohort of patients was available for analysis to determine if AR amplification was associated with an increase in expression of AR. Those patients who exhibited AR amplification, (Figure 5.8) (AR: X ratio >1.5) in

castrate resistant tumours had a significantly shorter time to death from relapse compared to patients who had no AR amplification (p=0.0002) (102).

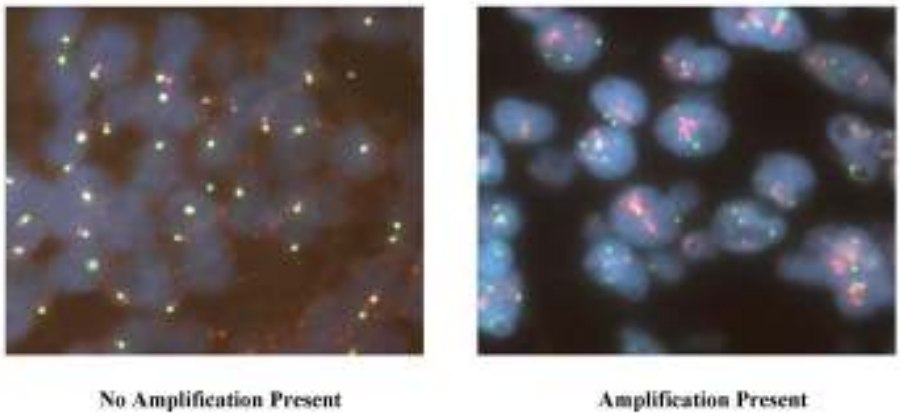


Figure 5.8: AR gene amplification
Hormone sensitive tumour with normal AR:X chromosome ratio (left) and castrate resistant tumour with AR amplification; ratio of orange to green signal of > 1.5

AR amplification was correlated with AR expression level, and a significant correlation was observed (p=0.033, correlation coefficient=0.25). By dividing AR expression into tumours with or without AR amplification, tumours with amplification had significantly higher AR expression compared to those without amplification (p=0.005) (Figure 5.9(a)). Median AR expression for castrate resistant tumours without amplification is 125 (IQR 73-156) histoscore units compared to 181 (IQR, 126-219) histoscore units for tumours with amplification. In addition, AR amplification significantly correlated with pAR ser ^{213} expression (p=0.007, CC=0.315). Castrate resistant tumours with gene amplification have significantly higher pAR ser^{213} expression levels (p=0.004). Median pAR^{213} expression for castrate resistant tumours without amplification is 90 (IQR, 23-130)

histoscore units compared to 165 (IQR, 83-193) histoscore units, for tumours that do have amplification (Figure 5.9(b). Expression of pAR ser [81] did not correlate significantly with AR amplification p=0.705.

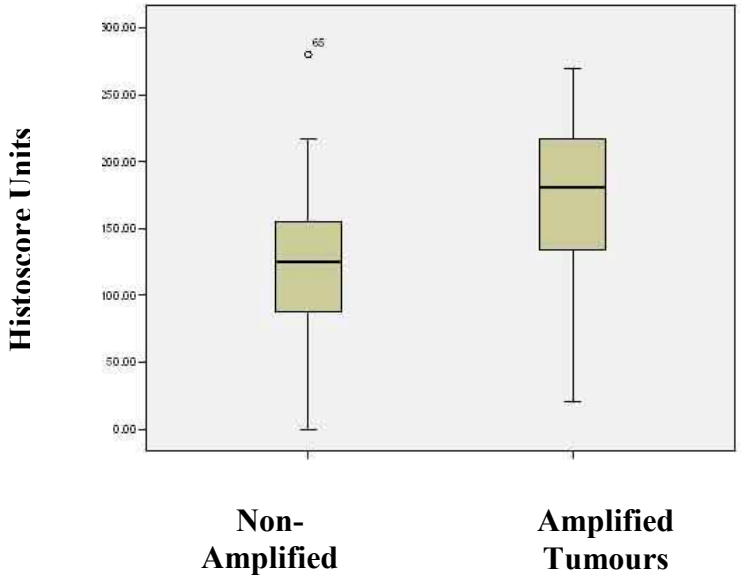


Figure 5.9(a): AR amplification and AR protein expression

Box plot demonstrating difference in expression of total AR expression between AR amplified and Non-amplified tumours.

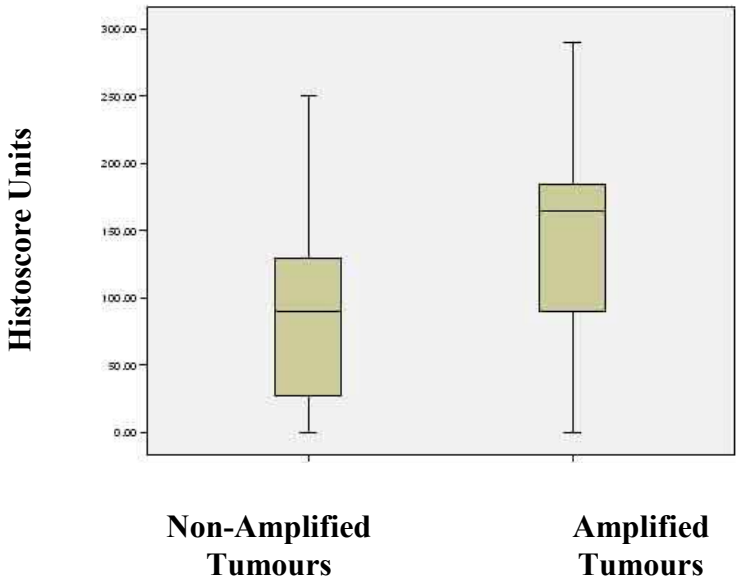


Figure 5.9 (b): AR amplification and pAR ser ^{213} protein expression

Boxplot demonstrating differences in expression of pAR ser ^{213} between AR amplified and non-AR amplified tumours.

5.4 Cell line

5.4.1 The phosphorylation of AR

Work carried out previously in our laboratory confirmed the LNCaP cell lines used in this study to be a good in-vitro model of hormone naïve and castrate resistant prostate cancers (2505). Hormone naïve LNCaP cells response to DHT was measured by phosphorylation of the AR at serine ^{81} (Figure 5.10). Here it was observed that DHT stimulates AR ser ^{81} phosphorylation within five minutes while levels of AR remain constant.

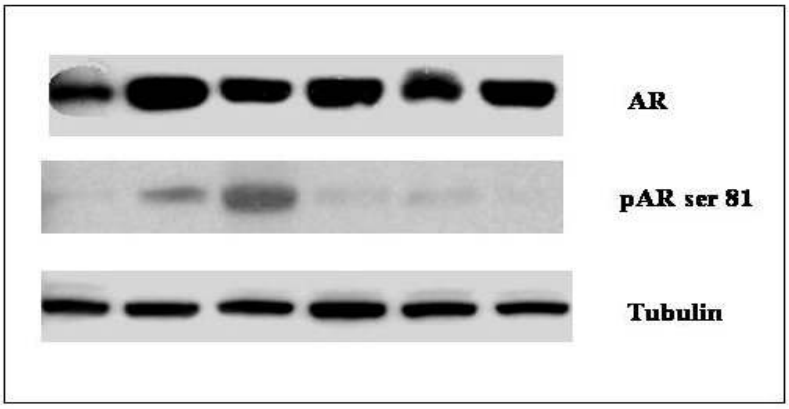


Figure 5.10: DHT stimulation of LNCaP cells

Western blot was performed on 50ug of extracts from LNCaP cells and probed for AR & pAR ser 81 (110kDa). Lane 1 unstimulated LNCaP. Lanes 2-6 treated with 10nM DHT for 5,15,30,60,180 minutes (respectively) Tubulin (50kd) was used as a loading control.

5.4.2 Effect of PI3K inhibition on AR phosphorylation.

As shown in chapter 3 the PI3K inhibitor LY294002 inhibits pAkt ser ^{473} in LNCaP cells, (Figure 3.5). It was therefore deemed appropriate to determine whether this inhibition of Akt also results in down regulation of pAR at the Akt consensus site serine 213. The tissue results above indicate that phosphorylation of the AR at serine 213 is associated with the progression of castrate resistant disease it was therefore necessary to investigate the consequences of PI3K inhibition in LNCaP CR to mirror the tissue expression study. Therefore to determine whether PI3K/Akt cascade plays a role in the phosphorylation of AR serine 213 LNCaP CR cells were treated with LY294002 and with or without the stimulation with IGF-1 (chapter 2.1.6). Expression of pAR ser^{213} was determined by western blotting. Here it is observed that the expression of pAR ser^{213} is markedly reduced in the castrate resistant cell lines (Figure 5.11). This indicates that a kinase

activated by the PI3K cascade, likely Akt is involved in phosphorylation of AR at serine 213.

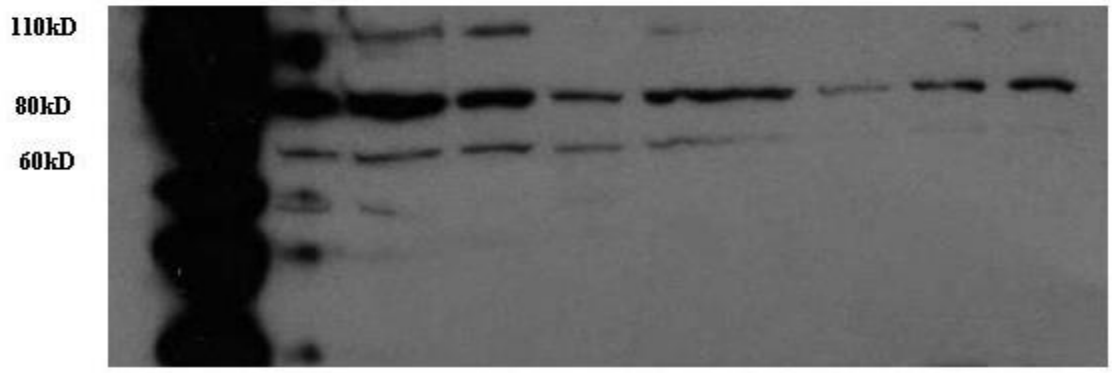


Figure 5.11: LY294002 inhibition of pAR ser ^{213}

Western blot was performed on 50ug of extracts from LNCaP &LNCaP CR cells and probed for pAR ser^{213} (110kDa). Lane 1&2 unstimulated LNCaP &LNCaP CR , Lane 3&4 LNCaP &LNCaP CR treated with LY294002 20uM for 30 minutes Lanes 5&6 LNCaP &LNCaP CR with 10ng IGF-1 for 1 hour, lanes 7&8 LNCaP &LNCaP CR treated with LY294002 20uM for 30 minutes then with 10ng IGF-1 for 1 hour. Bands at 110kD respresent full length AR, 80kD represent AR V-7 and 60kD represent AR V-3.Extra bands in lane 1 are shadow from the protein ladder.

5.4.3 Effect of siRNA knockdown of PI3K on AR phosphorylation

PI3K was silenced in LNCaP CR cells and to visualize the effects of this silencing on down stream targets of PI3K an Immunofluorescent (IF) study was designed. As shown in Figure 5.12 successful knockdown of PI3K was achieved. PI3K expression is observed in the non silenced control cells and barely expressed in the PI3K siRNA cells. The expression of downstream targets of PI3K was also observed to be markedly lower. Phosphorylation of both Akt ser ^{473} and AR ser ^{213} are also reduced in the PI3K siRNA

cells compared with the controls. Additionally as expected, silencing PI3K has no apparent effect on AR or phosphorylated AR at ser ^{81} expression.

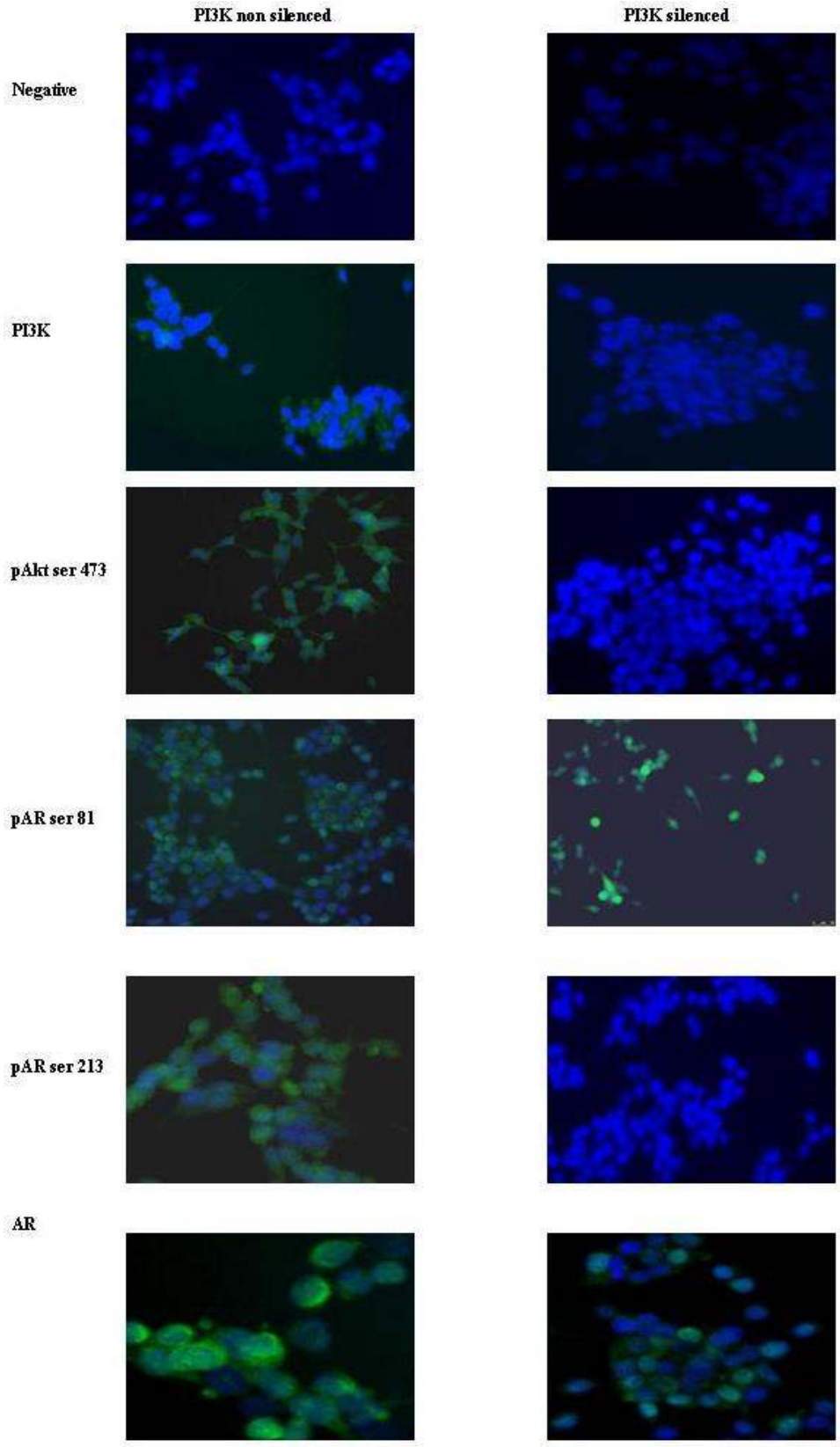


Figure 5.12: PI3Kp85 silencing in LNCaP cells

5.5 Discussion

It has been well documented that in addition to protecting and stabilizing AR, phosphorylation of specific serine residues can influence a ligand-independent induction of transcription by the AR (92;240). The AR exists as a phosphorylated protein in cells even in the absence of ligand-binding (94;241). Ligand-free AR is unstable and undergoes degradation. Upon binding of androgens, the AR undergoes additional phosphorylation predominantly at serine residues (94;242). Translating the *in vitro* evidence into the clinical scenario, this study established that phosphorylation at specific serine residues on the AR are involved in the development of castrate resistant disease. The current study investigated phosphorylation of the AR at serine residues 81 and 213 with the aim of identifying if phosphorylation of AR is of clinical importance and to establish if phosphorylation at a specific site played a key role in castrate resistant disease. The phosphorylation at serine 81 is located in the N-terminus between the polyglutamine stretch and AF-1(243). In response to androgen binding, Serine 81 is the most frequently phosphorylated site on the AR, giving the highest stoichometric phosphorylation yield in LNCaP cells treated with androgens (94).Phosphorylation of Serine 81 is associated with AR stability and transcriptional activity (95) and prostate cancer cell growth in cell line studies (96).Cell lines studies investigating mutagenesis at serine 81 signify that phosphorylation at this site alone does not drive AR transcriptional activity or stabilization, which suggests multiple sites may be involved.

In the current study expression of pAR ser ^{81} significantly decreased with the progression to castrate resistant disease (Table 5.3). These results indicate that AR phosphorylation at serine 81 decreases with the development of castrate resistant disease. This is in

agreement with reports in the literature stating that serine 81 occurs in response to androgen binding. As androgen levels are higher in hormone naïve prostate cancers, a decrease in pAR ser ^{81} expression would be expected in castrate resistant prostate cancer due to hormone depravation therapy which all patients in the cohort received. In LNCaP cells pAR ser ^{81} expression was induced by DHT stimulation (Figure 5.9) and was barely detectable without the addition of ligand. Interestingly, the expression of pAR ser ^{81} returns to barely detectable levels between 15 and 30 minutes whereas the expression of total AR remains unchanged. This result is similar to that published by Chen et al, however there are slight experimental difference with the conditions of Cell culturing and DHT exposure (244). Therefore the data gained from this study is in agreement with previous reports suggesting that multiple site of AR phosphorylation are involved in the transcriptional activity of the AR and thus the progression to castrate resistant disease.

AR expression (above the median) was associated with a quicker time to death from relapse (Figure 5.3) and was also observed to be significantly higher in patients who acquired metastatic disease at relapse (Figure 5.5), this result suggests AR involvement in the progression of castrate resistant disease and metastases despite patients undergoing hormone depravation therapy which has been well documented in the literature. Interestingly phosphorylation of the AR at serine 213 was shown to be significantly associated with castrate resistant disease. A high protein expression (above the median) of pAR ser ^{213} in castrate resistant tumours was observed to be significantly associated with a shorter time to disease specific death from biochemical relapse and a shorter overall survival, (Figures 5.4 a&b), in addition expression of pAR ser ^{213} was also observed to be significantly higher in patients who acquired metastatic disease at relapse

(Figure 5.5). Furthermore, castrate resistant tumours express significantly higher levels of pAR ser ^{213} compared to hormone naïve tumours, (Table 5.3). It was observed that approximately 42% of patients have an increase in pAR ser ^{213} expression and these patients have a significantly shorter survival period from time of relapse and a shorter disease specific death than those with no increase in expression (Figures 5.7 a & b).The expression levels of pAR ser ^{213} were correlated with members of the PI3K cascade and it was established that pAR ser ^{213} and pAkt ser ^{473} expression levels correlated significantly in the castrate resistant tumours only, suggesting that it is only when androgen levels are low that this signallingcascade is activated.

Moreover, these results confirmed previous findings in the literature that AR amplification and protein over-expression may be involved in castrate resistant disease. Using the AR gene amplification data from this cohort it was possible to determine whether AR amplification resulted in increased protein expression and it was observed that both AR and pAR ser ^{213} expression was significantly higher in castrate resistant patients with AR gene amplification, (Figure 5.9 a& b). These results confirmed previous findings in the literature that AR amplification and protein over-expression may be involved in CRPC development (101-103). However, AR amplification and/or increase in AR expression are too low to wholly explain castrate resistant disease development.

This study was also investigated the hypothesis that targeted depletion of PI3K will inhibit AR activation in prostate cancer cells. The AR remains the most important nuclear receptor in prostate cancer therefore this study used two prostate cancer cell lines which represent prostate cancers cells *in vivo* which express the AR. The PI3K inhibitor LY294002 targets the p110 catalytic site of PI3K. The use of this inhibitor in LNCaP cell

lines has indicated a role for PI3K in the phosphorylation of AR at serine 213.Both LNCaP and LNCaP CR cells were observed to have a markedly reduced expression of pAR ser ^{213} when treated with LY294002 (Figure 5.11). Interestingly the castrate resistant LNCaP cells appear to be more sensitive. This may be due to the fact that in hormone naïve cells phosphorylation of serine 213 occurs in the presence of androgens suggesting that ligand binding induces a conformational change in the N-terminus that reveals the serine 213 site to cellular kinases (245) whereas in castrate resistant cell lines AR phosphorylation at serine 213 is ligand independent. However as the PI3K catalytic subunit is highly conserved amoung the PI3K family members LY294002 does not discriminate amoung the various isoforms of PI3K and therefore affects many cellular processes (246). Therefore an alternative and more selective approach is to block the phosphotyrosine binding of the p85 SH2 domains thus averting the recruitment and activation of class 1a PI3K by growth factor tyrosine kinases (247). As shown in Figure 5.12 siRNA silencing of PI3K results in reduced expression of PI3K, pAkt ser ^{473} and also pAR ser ^{213} without altering AR or pAR ser ^{81} expression levels. The results show that it is possible to inhibit the phosphorylation of AR through targeted silencing of the p85 subunit of PI3K in prostate cancer cells and may therefore be therapeutic approach to the treatment of prostate cancer patients who display higher levels of p AR at serine 213. The functional consequences of siRNA silencing of PI3K remains to be investigated however it is expected that depletion of PI3K should result in a significant decrease in cell proliferation and induction of apoptosis as this cascade serves to inhibit many tumour suppressor like proteins that negatively regulate cell survival, proliferation and growth. Thus blocking this pathway could therefore inhibit the proliferation of tumour cells, (as

discussed in chapter 3.2.1) and sensitize them to apoptosis. A similar study in breast cancer cell lines, reported that targeted depletion of PI3K resulted in a significant decrease in cell viability and induction of apoptosis irrespective of their ER or HER 2 status and resulted in a significant G_1 phase cell cycle arrest in ER positive breast cancer cell lines (247).

These results provide additional evidence that the PI3K/Akt cascade is up-regulated during development of castrate resistant disease, resulting in phosphorylation of the AR and sensitisation to circulating adrenal androgens. Cell line studies demonstrate that this occurs *in vitro*, however the current data demonstrates for the first time that this may be one possible mechanism allowing development of castrate resistant disease in the clinical setting and targeting PI3K cascade is a possible means of therapeutic intervention.

6. General Discussion

One of the many challenges in the effective management of prostate cancer is the identification of molecular markers capable of predicting disease progression. Castrate resistant disease represents the lethal phenotype of prostate cancer. The main aim of this study was to investigate the role of the PI3K/Akt cascade in the development and progression of prostate cancer and to determine if it influenced patient outcome. Key functions of the PI3K/Akt cascade are mediating cell survival, as well as cell cycle progression and neoplastic transformation (159). The outcome of this study suggested that the PI3K/Akt cascade is in part accountable for mediating patient outcome.

Aberrant signallingof the PI3K/Akt cascade can occur through a variety of processes including gain of function oncogenic mutations of PI3KCA, which has been reported in Breast, Ovarian and Colorectal cancer (187), amplification or mutation of Akt isoforms and loss of function PTEN, (248;249). Loss of function of PTEN can occur through gene deletion, mutation, microRNA expression or epigenetic silencing (182;183;249;250). Patients in the current study were analysed for modifications of *PIK3CA*, *PTEN, AKT1-3* and *mTOR* at the genetic level using FISH. As outlined in chapter 3, very few gene amplifications were detected for *PI3K* and *AKT1* to warrant analysis and no gene amplifications were detected for *mTOR*, *AKT2* and *AKT 3*. However, *PTEN* deletions were detected in 23% of hormone naive tumours, which increased significantly to 52% in castrate resistant tumours (p=0.044). Loss of one copy of *PTEN* was commonly observed, and this was heterogeneous in nature, being frequently observed in only one area of tumour. Immunohistochemistry indicated that low levels of both cytoplasmic and

nuclear PTEN protein expression in hormone naïve tumours have a significantly quicker time to relapse and this also translates into shorter overall survival. Negative regulation of the PI3K pathway is primarily accomplished through the action of PTEN. This mechanism may therefore provide prostate cancer with the ability to evade apoptosis induced by hormone depravation therapy and hence the progression to castrate resistant disease. Loss of PTEN function in prostate cancer is quite common and results in an abundance of lipid second messengers (PtdIns (3, 4, 5) P3), which can cause constitutive activation of PH domain containing proteins including Akt. This is a likely reason why Akt is found to be highly activated in advanced prostate cancers. In the current study low levels of PTEN were associated with high levels of activated Akt. However, additional factors such as PI3K overexpression may also contribute to Akt activation and disease progression.

Consequently, it would stand to reason that loss of PTEN would initiate heightened activation of this cascade, which could then enable the cell to adopt a number of cancer like properties. A recent study by Zhu et al reported that gene expression analysis of clinical specimens showed that both PI3Kp85α and PI3Kp110β were highly expressed in malignant prostate tissues compared to the nonmalignant compartments, and their expression levels correlated significantly with disease progression (251). However, in this study expression of PI3Kp110 α was investigated and it was observed that in a sub cohort of patients increased levels of PI3Kp110α, pAkt thr ^{308} and pAkt ser ^{473} expression were observed to be significantly associated with shorter time to biochemical relapse and shorter disease specific survival moreover, the increase in pAkt ser ^{473} expression predicts disease specific survival independent of Gleason grade and presence of metastases. Taken

together these results suggest that the PI3K cascade is functionally active in castrate resistant disease.

The most widely used *in vitro* model of prostate cancer is the LNCaP cell line (252) LNCaP contains a frameshift mutation in the *PTEN* gene (253). Consistent with LNCaP having defective PTEN, this study observed that Akt is constitutively phosphorylated in LNCaP cells after 24 hours of culture in serum-free media and treatment of LNCaP with PI3K inhibitor LY294002 diminishes the phosphorylation of Akt and also results in inhibition of proliferation. Abrogation of PI3K/Akt activity by PI3K inhibitors has also been shown to stimulate apoptosis in LNCaP cells (254;255). These findings are in agreement with the concept that it is the constitutive activation of the PI3K/Akt pathway that renders these cells capable of surviving without exogenous growth and survival factors.

Akt is able to target a number of proteins known to affect cell survival including caspase-9, BAD, and glycogen-synthase kinase-3β (GSK-3β) (256-258). Phosphorylation of these proteins by Akt may result in either their activation or inactivation but the end result is to promote survival of the cell. Once activated, Akt is able to translocate to the nucleus where it affects the activity of a number of transcriptional regulators (259). Cyclic-AMP response element binding protein (CREB), E2F, NFκB, Forkhead transcription factors (FOXO) and the AR are all either direct or indirect substrates of Akt and each can promote either cellular proliferation or survival (98;191;260-262). Aberrant Akt activation is able to elicit the pro-survival properties observed in prostate cancer cells

through a number of mechanisms, hence inhibiting Akt or restoring PTEN activities are potential therapeutic targets in prostate cancer.

This study is unique as it examines multiple components of the PI3K/Akt cascade, and also because it investigates PI3K/Akt interaction with down stream signallingcascades and links these with progression to castrate resistant prostate cancer. Given that NFκB signalling has been reported to play an important role in castrate resistant disease this study investigated if Akt signalling in prostate cancer involves the NFκB cascade. There is accumulating evidence associating NFκB in prostate cancer progression and metastasis (263). The NFκB pathway has been implicated as a downstream target of PI3K signalling(227). Akt is believed not to directly phosphorylate NFκB, but to activate it indirectly through IKKα phosphorylation. Although this study did not observe any correlations between Akt and NFκB it provided further evidence that NFκB signallingplays a role in progression of castrate resistant disease and metastases. As discussed in chapter 4, increased levels of NFκBp65, pIκβα ser $^{32/36}$ and MMP-9 were significantly associated with disease progression and patient survival. MMP-9 and pIκβα ser $^{32/36}$ expression were also significantly associated with metastases. NFκB is an important regulator of cell proliferation through its direct role in cell cycle progression (238) Studies have shown prognostic significance of Cyclin D1 in prostate cancer and in the development of castrate resistant disease (264;265). Interestingly this study has shown a positive correlation between Cyclin D1 and NFκBp65 expression in hormone naïve prostate cancer and also a positive correlation between cyclin D1 and pIκβα ser $^{32/36}$ in castrate resistant disease suggesting NFκB signallingto be involved in cell cycle progression in prostate cancer. A significant reduction in cellular proliferation and

stimulation of apoptosis was observed in both hormone naïve and castrate resistant LNCaP cells using two novel NFκB inhibitors. Interestingly, reports in the literature suggest that NFκB can directly interact with the AR. Like Akt and AR the relationship between NFκB and AR remains controversial. However it is reported that there are NFκB binding sites on the AR promoter (2309). Elevated expression of NFκBp65 has been shown to repress AR mediated transactivation in a dose dependent manner (266). Yet, it has been reported by Lee et al that IL-4 induced NFκB is required for AR transactivation (267). The transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis and suppression of apoptosis appears to lie at the heart of the ability of NFκB to promote prostate cancer and castrate resistant disease progression. Supporting these findings this study, performed in vivo and *in vitro* investigations, which implicate NFκB inhibition as an important approach for the treatment of castrate resistant prostate cancer. A study using the anticarcinogen 3,3′-Diindolylmethane (DIM) which induces cell cycle arrest and apoptosis, through unknown mechanisms has reported that B-DIM (a formulated derivative with greater bioavailibility) significantly inhibited Akt activation, NFκB DNA binding activity, AR phosphorylation and the expression of both AR and PSA suggesting cross talk between Akt, AR and NFκB. Induction of apoptosis was also observed in both hormone naïve and castrate resistant prostate cancer cells (268). Interestingly, five cancer treatment trials of DIM have been registered in the USA. Four of these trials have been completed or are not currently recruiting and formal results are awaited. The fifth trial in prostate cancer is ongoing and recruiting subjects. This trial is stated “To measure the level of diindolylmethane in prostate tissue after treatment with

diindolylmethane (DIM) in patients with stage I or II adenocarcinoma of the prostate undergoing radical prostatectomy" (269).

Evidence strongly suggests that interactions of PI3K/Akt and AR signallingpathways provide prostatic epithelium with the necessary signallingevents to escape the apoptotic response associated with androgen withdrawal. Lin et al have previously shown that in low passage number LNCaP cells the PI3K/Akt signallingcascade suppresses AR activity and induces AR ubiquitylation and degradation by the 26S proteasome when PTEN is active (270). Additionally they observed that PTEN also suppresses AR activity in low passage LNCaP cells independently of PI3K/Akt signalling. PTEN was observed to directly interact with AR and induce caspase-3 activation. It is hypothesized that the interaction between PTEN and AR may lead to AR to expose the active site for caspase-3 recognition, resulting in AR degradation (271). However, in high passage number LNCaP cells PI3K/Akt signallingwas reported to enhance AR activity via an unknown mechanism and PTEN was shown to suppress AR activity via PI3K/Akt signalling. The relationship between PI3K/Akt cascade and AR has been well documented as discussed in chapter 5. In the current study increased levels of AR and phosphorylated AR at the Akt consensus site serine 213 was significantly higher in castrate resistant disease, with 42% of patients showing an increase in AR ser ^{213} expression. This also leads to a shorter time to relapse and a shorter disease specific survival time in castrate resistant prostate cancer tumours. In addition, AR ser ^{213} expression was further increased in patients who acquired AR amplification in the transition to castrate resistant disease. Also patients who presented with metastases at relapse had significantly higher AR and AR ser ^{213} expression. This data is in keeping with PI3K signallinginducing continued AR gain of

function despite reduced androgen levels (272). *In vitro* studies were also carried out to investigate the effect of PI3K inhibition and knockdown on AR phosphorylation. There were several key findings from these studies. The first confirmed that AR phosphorylation by Akt could be reduced in LNCaP CR cells by treating cells with the PI3K inhibitor LY294002 and that inhibition of PI3K activity was sufficient to reduce phosphorylation of AR in LNCaP CR cells stimulated with IGF-I. PI3Kp85 knockdown resulted in reduced levels of p85, pAkt ser ^{473} and pAR ser ^{213} expression while levels of AR and pAR ser ^{81} remained unchanged in LNCaP CR cells, supporting the evaluation of PI3K inhibitors in prostate cancer treatment. Reports suggest an important role for PI3Kp110β in prostate cancer. Both PI3Kp85α and PI3Kp110β appear to be essential for androgen induced AR transactivation because they are required for cell proliferation and tumour growth (2569). It has been demonstrated that PI3K inhibitors are unable to block AR nuclear translocation but can suppress AR-mediated gene expression indicating that a PI3K dependent mechanism is involved in AR–DNA binding, or the assembly of AR-mediated transcription complex (273). Zhu et al reported that that PI3K inhibitor abolished androgen-induced AR–DNA binding and knocking down p110β expression also resulted in loss of AR–DNA binding (251). Conditional knock out mice studies have evaluated the impact of deletion of p110β in the presence of PTEN loss in Prostatic epithelium. Here it was reported that the epithelium had normal appearance in the absence of p110β only. However, in the absence of PTEN alone high grade PIN was detected in the anterior lobe by 12 weeks therefore it seems ablation of p110β prevented tumourigenesis caused by PTEN loss (2571). PTEN loss led to increased Akt phosphorylation in the prostate and ablation of p110β diminished Akt phosphorylation.

These changes were shown to be p110β specific as p110α knockout did not abrogate tumour formation of Akt phosphorylation (274).

The PI3K/Akt and NFκB signallingcascades has been considered promising targets for anticancer therapies. The research presented here further highlights the importance of these proteins as possible therapeutic targets. Potent and isoform selective PI3K inhibitors are now entering clinical trials (157). PI 103 inhibits the PI3K cascade at multiple sites including all class 1A isoforms as well as mTOR C1 and mTOR C2. This dual PI3K/mTOR inhibitor has been shown to have significant anti tumour activity in xenografts tumour models (275). Phase 1 studies of the oral PI3K pathway inhibitors XL147 (Exelixis), BEZ235 (Novartis) and GDC-0941 (Genetech) are currently in progress (276;277). Formal published results of the clinical trial are awaited. Furthermore there are several classes of Akt inhibitors currently in development. These include isoform selective Akt catalytic-domain inhibitors and inhibitors of the PH domain. Alkylphospholipid, perifosine an inhibitor of the PH domain has undergone phase II clinical trials in patients with castrate resistant disease however it has shown no evidence of significant activity (181). Targeting both Akt-1 and Akt-2 has been reported to be superior to the inhibition of a single isozyme for induction of caspase- 3 activity in tumour cells. This suggests that pan-Akt inhibitors such as the ATP-competitive inhibitor GSK690639 (GlaxoSmithKline) are likely to be more effective although toxicity may be a potential issue (278). A number of other small molecule Akt inhibitors are in early clinical trials including MK2206 (Merck, Inc) (279) and formal published results are awaited. Additionally mTOR inhibitors have also been developed. mTOR is the target of the antibiotic Rapamycin, an immunosuppressive macrolide which inhibits metastatic

prostate tumor growth and angiogenesis in in vivo mouse models (280). At present there are several rampamycin analogues in development including: Temsirolimus (CCI-779), Everolimus (RAD001) and Deforolimus (AP23573) however prelimary results for studies using these analogues as single agents in the treatment of castrate resistant disease have been disappointing (281). A phase II trial using CC1-779 has recently finished however no results are available at present (282).

It is evident that multiple signal transduction cascades are critical to prostate cancer progression and resistance to therapy. The data accumulated in this study provides the clinical evidence to support the hypothesis that the PI3K/Akt and NFκB cascades are functional in castrate resistant disease. Both play a role in promoting disease development and progression as their expression significantly impacts on time to biochemical relapse and overall survival in a sub cohort of patients where *in vitro* studies showed that targeting both cascades can result in reduction of cellular proliferation and induction of apoptosis. Increasing our understanding of the biology of this disease has lead to the hope that novel inhibitors of these cascades will result in therapeutic benefit.

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Appendix : Patient Information

Study No	Date of Birth	Age at Diagnosis	Date Diagnosis	Diagnosis-BX / Turp	Gleeson sum	Date of bone scan	Result of bone scan	Relapse op type	Op date 2(relapse)
AR1&2	24/02/1925	72	01/05/1997	TURP	8		No metastases	TURP	01/12/1999
AR3&4	12/06/1920	78	01/05/1999	BIOPSY	7		No metastases	TURP	01/11/1999
AR5,6&7	06/06/1922	69	01/01/1992	BIOPSY	5		No metastases	TURP	01/01/1997
AR8&9	04/08/1927	70	01/04/1998	TURP	7			TURP	01/05/1998
AR14&15	01/03/1923	71	14/05/1994	TURP	5		No metastases	TURP	14/08/1998
AR16&17	02/12/1920	72	26/03/1993	TURP	6		No metastases	TURP	22/12/1998
AR18&19	14/07/1917	74	16/04/1992	TURP	4		No metastases	TURP	00/00/98
AR23-26	04/05/1937	54	03/04/1992	TURP	7		No metastases	TURP	20/12/1997
AR29&30	06/08/1923	71	08/02/1995	TURP	9	10/02/1997	No Metastases	TURP	01/01/1997
AR31 &32	06/04/1929	66	04/09/1995	TURP	10			TURP	01/01/1998
AR33&34	15/07/1934	62	19/02/1997	Tru-cut Bx	9	24/07/1997	N0 Metastases	TURP	01/01/2000
AR35&36	07/03/1915	83	12/03/1998	Tru-cut Bx	10	23/11/1998	No Metastases	TURP	01/06/1995
AR37&38	01/04/1920	67	20/07/1987	Tru-cut Bx;TURP	8			TURP-	06/11/1995
AR39&40	13/09/1925	71	16/09/1996	Trucut Bx	8	17/12/1998	No metastases	TURP-	31/08/1998
AR41&42	14/01/1922	76	07/08/1998	Tru-cut Biopsy	8	04/08/1998	multiple metastases	TURP-	23/06/2000
AR43&44	05/04/1928	68	10/12/1996	TURP	9			TURP--	03/08/1999
AR45&46	24/07/1919	76	07/05/1996	TURP	7			TURP--	17/03/2000
AR47&48	17/08/1928	69	09/02/1998	Tru-cut Bx	6	05/07/1999	No metastases	TURP-	18/10/1999
AR49&50	06/07/1929	64	22/09/1993	TURP	8	11/04/1994	Metastases	TURP	00/00/96
AR51&52	22/02/1929	70	16/04/1999	TURP	6			TURP	00/00/2000
AR53&54	06/12/1914	71	30/10/1986	TURP	7	40/4/87	No metastases	TURP-	12/11/1990
AR55&56	13/04/1916	73	08/08/1989	TURP	9		No metastases	TURP-	14/12/1992
AR57&58	06/11/1906	79	09/05/1986	TURP	7	09/05/1986	No metastases	TURP-	09/05/1986
AR59&60	08/09/1922	63	19/08/1986	TURP	7		No metastases	TURP-	29/12/1990
AR61&62	03/03/1905	81	11/02/1987	TURP	7		No metastases	TURP-	06/02/1992
AR63&64	21/11/1910	77	01/03/1988	TURP	7	01/03/1988	no metastases	TURP-	08/02/1991
AR65&66	03/09/1912	75	29/04/1988	TURP	9		No metastases	TURP-	14/03/1994
AR67&68	14/08/1916	73	21/08/1989	TURP	9	21/08/1989	Metastases	TURP-	18/02/1991
AR69,70&71	14/01/1922	67	09/05/1989	Trucut Bx	8		No metastases	TURP-	08/03/1993
AR72&73	01/05/1927	63	02/05/1990	Trucut Bx	7		No metastases	TURP-	02/12/1997
AR74&75	15/11/1940	49	11/07/1990	TURP	6		No metastases	TURP-	09/01/1996
AR76&77	05/12/1920	68	11/09/1989	Trucut Bx	6		Metastases	TURP-	14/02/1994
AR78&79	19/08/1922	68	08/01/1991	Trucut Bx	6		No metastases	TURP-	17/05/2000
AR80&81	05/03/1915	75	28/02/1991	TURP	4	28/02/1991	No metastases	TURP-	17/07/1992
AR82&83	12/07/1921	70	29/04/1992	TURP	8	29/04/1992	Metastases	TURP-	28/03/1994

Study No	time to 2nd op(days)	Gleason sum(relapsed)	PSA at Relapse	Relapse PSA(date)	time to PSA relapse(days)	time to PSA relapse(yrs)	Date of bone scan 2
AR1&2	944	8	15	15/08/1999	836	2.29	
AR3&4	184	8	470	01/11/1999	184	0.50	
AR5,6&7	1827	9	6.4	29/03/1997	1914	5.24	
AR8&9	30	8	64	25/04/1998	24	0.07	
AR14&15	1553	6	7.6	04/05/1998	1451	3.98	
AR16&17	2097	9	39.3	17/11/1998	2062	5.65	11/8//98
AR18&19		10,9	29	27/11/1997	2051	5.62	19/06/1998
AR23-26	2087	8,8,8,not given	0.1	02/12/1997	2069	5.67	
AR29&30	693	9	116.1	13/09/1999	1678	4.60	01/02/1999
AR31 &32	850	10	43.7	12/01/1998	861	2.36	09/06/1998
AR33&34	1046	8	30.6	10/07/2000	1237	3.39	04/08/1998
AR35&36		8	6.5	31/08/1999	537	1.47	08/03/2000
AR37&38	3031	9	7.2	02/11/1995	3027	8.29	03/05/1996
AR39&40	714	8	15.8	30/06/1998	652	1.79	17/12/1998
AR41&42	686	8	796.4	15/06/2000	678	1.86	
AR43&44	966	9	2.5	24/03/1999	834	2.28	26/01/2000
AR45&46	1410	9	140	05/08/1998	820	2.25	19/01/1999
AR47&48	616	10	?				21/03/2000
AR49&50		7	?				03/10/1996
AR51&52		9	16.6	10/07/1999	85	0.23	17/05/2000
AR53&54	1474	9	2.3	03/09/1990	1404	3.85	27/05/2000
AR55&56	1224	7	52	01/07/1991	692	1.90	30/09/1991
AR57&58		9	2	05/11/1990	1641	4.50	01/07/1991
AR59&60	1593	9	32.8	20/11/1989	1189	3.26	
AR61&62	1821	8	2.7	25/09/1989	957	2.62	23/11/1992
AR63&64	1074	7	10.9	10/07/1989	496	1.36	08/08/1988
AR65&66	2145	9	1.3	25/11/1991	1305	3.58	
AR67&68	546	9	4.1	16/07/1990	329	0.90	
AR69,70&71	1399	9	3.1	04/05/1992	1091	2.99	
AR72&73	2771	9	1	24/11/1995	2032	5.57	11/07/1997
AR74&75	2008	8	4.5	10/09/1992	792	2.17	25/04/1997
AR76&77	1617	9	1.7	11/03/1991	546	1.50	04/10/1993
AR78&79	3417	7	1.9	15/10/1999	3202	8.77	
AR80&81	505		6.1	04/05/1992	431	1.18	31/01/1995
AR82&83	698	8	4.6	09/08/1993	467	1.28	

Study No	Result of bone scan 2	Orchidectomy	anti-androgen	GnRH analoggue	Stilboestrol-Date	Radiotherapy-date
AR1&2		01/06/1997	flutamide & casodex		nil	No
AR3&4			nil	zoladex 4/5/99	nil	No
AR5,6&7			nil	zoladex 11/1/98	nil	No
AR8&9			nil	zoladex 24/4/98	nil	No
AR14&15			nil	finesteride 14/8/98	nil	No
AR16&17	No metastases	25/03/1993	nil		nil	Yes
AR18&19	Metastases	10/05/1992	Casodex-Feb 99	Zoladex-May 98	nil	Yes
AR23-26		31/08/1992	Flutamide-25/9/96		yes-12/12/97	yes
AR29&30	Metastases		Casodex-Oct 1998	Zoladex-March 95		yes
AR31 &32	No metastases	31/08/1995	Flutamide-July 1998		Yes--Nov 98	
AR33&34	No metastases		Casodex- July 2000	Zoladex-01/04/1997		
AR35&36	Metastases		Flutamide-January 2000	Zoladex-April 1998		yes
AR37&38	No metastases			Zoladex-August 1987	Aug-87	yes
AR39&40	No metastases		Casodex-March 1999	Zoladex Sept 1996		yes
AR41&42				Zoladex-August 98		
AR43&44	No metastases			Zoladex-Jan 97	Yes--Jan 2ooo	yes
AR45&46	Metastases		? Flutamide-14/12/99	Zoladex-May 96		
AR47&48	Metastases		Casodex-Jan 2000	Zoladex -March 98		
AR49&50	Metastases			Zoladex-March 96		
AR51&52	Metastases			Zoladex-OCT99		Yes-
AR53&54	Metastases	30/10/1986				
AR55&56	No metastases	16/08/1989				
AR57&58	Metastases	09/05/1986				
AR59&60		19/08/1986		20/02/1989		
AR61&62	Metastases	23/10/1987				
AR63&64	Metastases	24/08/1988				
AR65&66		04/05/1988				
AR67&68		16/08/1989				
AR69,70&71		02/11/1990				yes-palliative
AR72&73	Metastases	07/05/1990				Yes-palliative
AR74&75	No metastases	06/03/1991	Casodex-?11/7/90			
AR76&77	Metastases	27/09/1989				Yes-palliative
AR78&79		27/03/1991	Flutamide			
AR80&81	No metastases	03/05/1991	casodex			
AR82&83				Zoladex-April 92		Yes-palliative

Study No	Last Visit-Date	Alive/ Dead	code for alive/dead	follow-up(years)	followup from relapse(years)	deadthcode alive=0, cd=1, ncd=2
AR1&2	10/09/2000	Alive	0	3.36	1.07	0
AR3&4	01/11/1999	Alive	0	0.50	0.00	0
AR5,6&7	29/11/1999	Alive	0	7.92	2.67	0
AR8&9	25/08/1999	Alive	0	1.40	1.33	0
AR14&15	23/11/2000	DEAD	1	6.53	2.56	1
AR16&17	29/09/2005	DEAD	1	12.52	6.87	1
AR18&19	22/07/1999	DEAD	1	7.27	1.65	1
AR23-26	27/10/1998	DEAD	1	6.57	0.90	1
AR29&30	12/08/2000	DEAD	1	5.51	0.92	1
AR31 &32	11/02/1999	DEAD	1	3.44	1.08	1
AR33&34	21/01/2001	DEAD	1	3.92	0.53	1
AR35&36	10/08/2000	DEAD	1	2.42	0.95	1
AR37&38	15/05/1998	DEAD	1	10.83	2.53	2
AR39&40	01/10/1999	DEAD	1	3.04	1.25	1
AR41&42	06/01/2001	DEAD	1	2.42	0.56	2
AR43&44	01/04/2001	DEAD	!	4.31	2.02	.
AR45&46	18/07/2000	DEAD	1	4.20	1.95	1
AR47&48	05/05/2000	DEAD	1	2.24		1
AR49&50	29/01/1997	DEAD	1	3.36		1
AR51&52	09/11/2000	DEAD	1	1.57	1.34	1
AR53&54		DEAD	1			1
AR55&56	18/07/1996	DEAD	1	6.95	5.05	1
AR57&58	04/03/1992	DEAD	1	5.82	1.33	1
AR59&60	15/03/1991	DEAD	1	4.57	1.32	2
AR61&62	16/05/1993	DEAD	1	6.26	3.64	1
AR63&64	02/09/1991	DEAD	1	3.51	2.15	1
AR65&66	13/05/1994	DEAD	1	6.04	2.47	2
AR67&68	18/07/1991	DEAD	1	1.91	1.01	1
AR69,70&71	02/06/1995	DEAD	1	6.07	3.08	2
AR72&73	23/11/1998	DEAD	1	8.57	3.00	1
AR74&75	14/03/1998	DEAD	1	7.68	5.51	2
AR76&77	12/08/1994	DEAD	1	4.92	3.42	1
AR78&79	12/08/2000	DEAD	1	9.60	0.83	2
AR80&81	19/08/1999	DEAD	1	8.48	7.30	2
AR82&83	23/06/1996	DEAD	1	4.15	2.87	1

Study No	Date of Birth	Age at Diagnosis	Date Diagnosis	Diagnosis-BX / Turp	Gleeson sum	Date of bone scan	Result of bone scan	Relapse op type	Op date 2(relapse)
AR84&85	26/09/1922	70	08/12/1992	TURP	9		No metastases	TURP-	09/12/1996
AR86&87	19/12/1925	68	06/07/1994	TURP	6		No metastases	TURP-	10/11/1995
AR-88&89	16/11/1916	67	16/11/1984	Trucut Bx	7			TURP-	28/11/1995
AR90&91	11/09/1930	63	08/08/1994	TURP	6		No metastases	TURP-	27/10/1998
AR92&&93	01/08/1919	74	31/05/1994	TURP	8			TURP-	13/08/1996
AR94&95	?		20/02/1984	TURP				TURP--	22/07/1994
AR96&97	16/07/1916	74	07/11/1990	Trucut Bx	7		No metastases	TURP--	04/03/1998
AR98&99	04/05/1928	59	07/09/1987	Trucut Bx		07/09/1987	metastases	TURP--	19/03/1993
AR100&101	11/10/1916	80	07/05/1997	Trucut Bx	9		No metastases	TURP-	21/04/1998
AR102&103		98	08/01/1998	TURP	10			TURP--	15/03/1999
AR104,105&106	23/07/1913	70	16/05/1984	TURP	6		No metastases	TURP-	23/04/1993
AR107&108	05/11/1926	68	10/05/1995	TURP	9		No metastases	TURP-	10/02/1998
AR109&110	21/06/1922	73	08/02/1996	TURP	9	08/02/1996	No metastases	TURP-	11/08/1998
AR111,112&113	07/12/1911	67	26/02/1979	TURP	6		No metastases	TURP-	26/09/1990
AR114,115	29/01/1930	63	25/08/1993	TURP	8	26/09/1990	Metastases	Trucut biopsy	11/10/1993
AR116,117	25/10/1953	41	17/11/1994	Trucut Bx	7	02/12/1994	no metastases	TURP	25/03/1998
AR118,119	21/11/1937	59	11/06/1997	Trucut Bx	9	25/07/1997	No metastases	TURP	24/09/1998
AR120,121	25/01/1936	59	21/12/1995	Turp	10	03/03/1996	No metastases	TURP	26/11/1996
AR122,123	26/10/1921	68	13/02/1990	Trucut Bx	8	23/05/1991	No metastases	TURP	29/07/1993
AR124,125	07/02/1929	62	12/01/1992	Trucut Bx	7	25/01/1992	Metastases	TURP	11/06/1993
AR126,127	27/10/1920	72	12/07/1993	TURP	7	31/4/00	Metastases	TURP	11/05/1999
AR128,129	25/02/1929	70	14/02/2000	BIOPSY	8	28/02/2002	Metastases	TURP	28/02/2002
AR130,131	15/04/1935	60	05/09/1995	TURP	6	31/11/02	No Metastases	TURP	28/02/2002
AR132,133	20/04/1936	58	07/10/1994	BIOPSY	7	Nov-94	No metastases	TURP	01/12/1998
AR134,135	19/02/1928	67	08/01/1996	TURP	8	29/05/1998	Metastases	TURP	27/01/1998
AR136,137	14/03/1920	72	03/07/1992	TURP	8	Jul-92	Metastases	TURP	01/01/2000
AR138,139	15/10/1919	71	16/07/1991	BIOPSY	8	Jul-91	Metastases	TURP	01/07/1997
AR140,141	22/12/1912	80	13/12/1993	TURP	9	31/12/1993	Metastases	TURP	01/01/1995
AR142,143	07/03/1929	66	12/04/1995	BIOPSY	10	May-95	Metastases	TURP	01/01/1999
AR144,145	28/09/1922	67	15/03/1990	TURP	7	Feb-99	No metastases	TURP	01/10/1996
AR146,147	05/11/1925	71	27/08/1997	TURP	8	Jul-00	Metastases	TURP	01/06/2000
AR150, 151	19/06/1919	77	28/05/1997	BIOPSY	6	Jul-97	No metastases	TURP	01/06/2001
AR152,153	12/06/1923	76	13/01/2000	TURP	9	Jan-00	Metastases	TURP	01/12/2001
AR154,155	17/03/1929	70	07/05/1999	TURP	9	May-99	No metastases	TURP	01/02/2002
AR156,157	22/12/1928	67	01/06/1996	BIOPSY	10	01/08/1996	No metastases	TURP	01/05/1999

Study No	time to 2nd op(days)	Gleason sum(relapsed)	PSA at Relapse	Relapse PSA(date)	time to PSA relapse(days)	time to PSA relapse(yrs)	Date of bone scan 2
AR84&85	1462	9	25.5	19/09/1997	1746	4.78	09/01/1998
AR86&87	492	5	31.8	25/08/1995	415	1.14	
AR-88&89	4029	7	10.8				11/07/1997
AR90&91	1541	8	15.3	24/03/1999	1689	4.63	
AR92&&93	805		4.5	15/11/1996	899	2.46	
AR94&95	3805		12.7	06/11/1992	3182	8.72	
AR96&97	2674	8	1.5	16/12/1994	1500	4.11	
AR98&99	2020	7	258	12/06/1992	1740	4.77	07/04/1995
AR100&101	349	8	10.7	15/08/1997	100	0.27	
AR102&103	431		261	05/03/1999	421	1.15	
AR104,105&106	3264	10	1.2	06/09/1993	3400	9.32	
AR107&108	1007	9	33.6	14/02/1997	646	1.77	14/02/1997
AR109&110	915	10	27.1	14/06/1998	857	2.35	
AR111,112&113	4230	4,5	34.3				
AR114,115	47	8	29	11/10/1993	47	0.13	23/03/1995
AR116,117	1224	10	22.4	07/05/1997	902	2.47	
AR118,119	470	9	33.4	10/08/1998	425	1.16	17/05/1999
AR120,121	341	10	41.4	22/10/1996	306	0.84	09/12/1996
AR122,123	1262	9	24	04/02/1993	1087	2.98	06/02/1993
AR124,125	516	10	5.6	10/08/1993	576	1.58	21/07/1994
AR126,127	2129	9	95.8	01/08/1999	2222	6.09	
AR128,129	745	9	100	02/02/2002	719	1.97	
AR130,131	2368	7	8	01/02/2002	2341	6.41	
AR132,133	1516	10	37	Nov-98	1486	4.07	Apr-98
AR134,135	750	9	131.4	08/12/1997	700	1.92	
AR136,137	2738	8	702	01/01/2000	2738	7.50	
AR138,139	2177	9	10.3	Jan-95	1265	3.47	
AR140,141	384	10	510	Dec-94	353	0.97	
AR142,143	1360	8	55	Jul-98	1176	3.22	
AR144,145	2392	8	15.5	Jan-95	1753	4.80	
AR146,147	1009	10	7.2	Oct-99	765	2.10	
AR150, 151	1465	7	64.8	May-01	1434	3.93	Nov-99
AR152,153	688	9	153.1	Mar-02	778	2.13	
AR154,155	1001	9	40.5	Dec-01	939	2.57	Apr-02
AR156,157	1064	10	33.5	Dec-98	913	2.50	Feb-99

Study No	Result of bone scan 2	Orchidectomy	anti-androgen	GnRH analoggue	Stilboestrol-Date	Radiotherapy-date
AR84&85	Metastases	14/11/1994				Yes-palliative
AR86&87			Flutamide-6/7/94			
AR-88&89	Metastases		Casodex-11/9/97			
AR90&91			Casodex-date	Prostap-date	Yes--date	
AR92&&93				Zoladex--31/5/94		
AR94&95				Zoladex--29/1/88		
AR96&97		18/06/1991		Zoladex-7/11/90		Yes-palliative
AR98&99	Metastases		Flutamide-11/9/92	Zoladex-7/9/87		Yes-palliative
AR100&101			Casodex-7/5/97	Zoladex-7/5/97		
AR102&103				Zoladex-8/1/98		
AR104,105&106		19/02/1990				
AR107&108	No metastases		Casodex-10/5/95	Zoladex-10/5/95		
AR109&110				Zoladex-8/2/96		Yes-palliative
AR111,112&113		26/02/1979			YES	
AR114,115	Metastases	26/10/1993	Flutamide-21/11/94			yes-palliative
AR116,117		23/01/1995	Casodex-24/4/97	21/06/1995		yes-Radical radiotherapy-7/6/97
AR118,119	Metastases		Casodex-Feb 99	Zoladex-July 97	yes-april 99	
AR120,121	Metastases		Flutamide-March 97	Zoladex-18/3/96		
AR122,123	Metastases		Flutamide-6/8/93	Zoladex- Feb 1990		yes-palliative to pelvis & orbit
AR124,125	Multiple metastases		Flutamide-15/1/93			
AR126,127				Jul-93		yes 1/00
AR128,129				01-Jan		
AR130,131				01-Dec		
AR132,133	No metastases			May-98		May-95
AR134,135				Apr-96		
AR136,137		Aug-92				
AR138,139		Jul-91	Sep-96			
AR140,141			Apr-95	Feb-94		
AR142,143			Sep-98	May-95		
AR144,145		JUNE-96 FLUTAMIDE APRIL-98 C		May-93		JUN-90, MAY-98
AR146,147				Apr-98		01/09/2000
AR150, 151	Metastases		Sep-98	Jan-98		
AR152,153				Feb-00		
AR154,155	Metastases		Apr-02	Jun-99		
AR156,157	Metastases		Jan-99	Jul-96		

Study No	Last Visit-Date	Alive/ Dead	code for alive/dead	follow-up(years)	followup from relapse(years)	deadthcode alive=0, cd=1, ncd=2
AR84&85	02/08/1999	DEAD	1	6.65	1.87	1
AR86&87	20/02/1997	DEAD	1	2.63	1.49	2
AR-88&89	10/11/2000	Alive	0			0
AR90&91	04/06/2001	DEAD	1	6.83	2.20	2
AR92&&93	16/05/2004	DEAD	1	9.97	7.50	2
AR94&95	01/12/1995	DEAD	1	11.79	3.07	2
AR96&97	17/12/1999	DEAD	1	9.12	5.01	1
AR98&99	07/06/1996	DEAD	1	8.76	3.99	2
AR100&101	28/12/1998	DEAD	1	1.64	1.37	2
AR102&103	15/03/1999	Alive	0	1.18	0.03	0
AR104,105&106	05/04/1995	DEAD	1	10.89	1.58	2
AR107&108	06/04/1998	DEAD	1	2.91	1.14	2
AR109&110	24/06/2001	DEAD	1	5.38	3.03	2
AR111,112&113	10/06/2000	DEAD	1			2
AR114,115	27/05/1995	DEAD	1	1.75	1.62	1
AR116,117	18/10/1999	DEAD	1	4.92	2.45	2
AR118,119	01/01/2000	DEAD	1	2.56	1.39	1
AR120,121	27/03/1997	DEAD	1	1.27	0.43	1
AR122,123	20/07/1996	DEAD	1	6.44	3.46	1
AR124,125	18/03/1995	DEAD	1	3.18	1.60	1
AR126,127	30/05/2000	DEAD	1	6.89	0.83	1
AR128,129	29/06/2003	DEAD	1	3.37	1.40	1
AR130,131	31/10/2002	Alive	0	7.16	0.75	0
AR132,133	27/02/1999	DEAD	1	4.39	0.32	1
AR134,135	03/09/1998	DEAD	1	2.65	0.74	1
AR136,137	18/01/2000	DEAD	1	7.55	0.05	1
AR138,139	13/01/1998	DEAD	1	6.50	3.04	1
AR140,141	30/05/1995	DEAD	1	1.46	0.49	1
AR142,143	03/04/2002	DEAD	1	6.98	3.76	1
AR144,145	Dec-99	DEAD	1	9.72	4.92	1
AR146,147	01/02/2001	DEAD	1	3.44	1.34	1
AR150, 151	06/10/2001	DEAD	1	4.36	0.43	1
AR152,153	25/09/2002	DEAD	1	2.70	0.57	1
AR154,155	02/07/2002	DEAD	1	3.16	0.58	1
AR156,157	21/09/1999	DEAD	1	3.31	0.81	1

Study No	Date of Birth	Age at Diagnosis	Date Diagnosis	Diagnosis-BX / Turp	Gleeson sum	Date of bone scan	Result of bone scan	Relapse op type	Op date 2(relapse)
AR158,159	13/01/1915	83	18/02/1998	BIOPSY	5	Feb-98	No metastases	TURP	01/09/1999
AR160,161	06/11/1922	76	18/10/1999	TURP	10	Nov-99	No metastases	TURP	01/03/2001
AR164,165	30/05/1924	73	03/10/1997	TURP	9	Apr-01	Metastases	TURP	01/06/1999
AR166,167	19/10/1926	67	01/12/1993	TURP	8	Dec-93	No metastases	TURP	11/01/2002
AR168,169	23/01/1926	74	07/07/2000	BIOPSY	8	Aug-00	No metastases	TURP	12/04/2002
AR170,171	13/08/1928	62	01/01/1991	TURP	9	Jan-91	mets	TURP	1993
AR172,173	17/04/1933	62	18/12/1995	BIOPSY	8	Mar-02	Metastases	TURP	01/03/2000
AR174, 175	07/10/1930	71	22/07/2002	BIOPSY	9		No metastases	TURP	20/03/2004
AR176,177	07/07/1927	69	07/11/1996	BIOPSY	7		No metastases	TURP	19/09/2002
AR178,179	22/09/1917	79	22/04/1997	BIOPSY	7		No metastases	TURP	29/01/2003
AR180,181	07/09/1939	62	30/07/2002	BIOPSY	7		No metastases	TURP	18/12/2002
AR182,183	05/08/1938	58	30/06/1997	BIOPSY	7		No metastases	Biopsy	03/09/2002
AR184,185	28/03/1934	57	06/11/1991	BIOPSY	7	07/11/1991	No metastases	turp	28/02/2003
AR186,187	25/10/1921	77	11/06/1999	TURP	8	NO SCAN	No metastases	TURP	06/06/2005
AR 188,189,190	24/07/1936	63	03/04/2000	BIOPSY	5	Mar-00	Metastases	TURP	08/01/2002
AR191,192	19/08/1938	59	23/01/1998	TURP	9	Feb-01	Metastases	Trucut biopsy	31/05/2001
AR193,194	01/03/1934	67	12/06/2001	BIOPSY	8	Jul-01	Metastases	TURP	06/08/2003
AR195,196	04/01/1941	54	13/09/1995	TURP	8	Sep-95	No metastases	TURP	22/07/2001
AR197,198	25/12/1925	69	02/02/1995	TURP	4	Feb-95	No metastases	TURP	17/11/1998
AR199,200	06/08/1923	69	05/05/1993	TURP		Mar-93	No metastases	TURP	15/04/1999
AR201,202	24/04/1927	72	03/04/2000	TURP	5	Apr-00	Metastases	TURP	27/05/2005
AR203,204	21/08/1924	68	26/03/1993	BIOPSY		Mar-93	No metastases	TURP	30/04/1998
AR205,206	18/11/1920	80	13/03/2001	BIOPSY	6	Mar-01	Metastases	TURP	27/05/2004
AR207,208	27/04/1926	75	21/12/2001	BIOPSY	8	Dec-01	Metastases	TURP	18/08/2004
AR209,210	25/03/1931	68	26/03/1999	BIOPSY	8	Feb-99	Metastases	TURP	18/10/2001
AR211,212	12/04/1927	71	30/10/1998	BIOPSY		Oct-98	Metastases	TURP	23/05/2002
AR213,214	30/05/1913	84	02/02/1998	BIOPSY	3	Feb-98	Metastases	TURP	25/06/2001
AR215,216	27/11/1926	69	22/12/1995	TURP	9	Nov-95	No metastases	TURP	28/06/2001
AR217,218	06/04/1924	70	18/11/1994	TURP	6	Nov-94	No metastases	TURP	30/03/1999
AR219,220	11/08/1922	72	12/10/1994	BIOPSY		Oct-94	No metastases	TURP	28/06/2004
AR221,222	03/10/1931	63	02/06/1995	BIOPSY		Jun-95	No metastases	TURP	05/10/2005
AR223,224	14/05/1923	72	15/01/1996	BIOPSY		Feb-96	No metastases	TURP	12/02/2004
AR225,226	16/05/1923	75	23/04/1999	BIOPSY	5	Jun-99	No metastases	TURP	23/11/2004
AR227,228	02/01/1926			BIOPSY				TURP	17/08/2004
AR229,230,231,232	03/12/1920	67	19/09/1988	TURP	5	Oct-88	No metastases	TURP	14/06/1995

Study No	time to 2nd op(days)	Gleason sum(relapsed)	PSA at Relapse	Relapse PSA(date)	time to PSA relapse(days)	time to PSA relapse(yrs)	Date of bone scan 2
AR158,159	560	8	108.7	Sep-99	560	1.53	5/00
AR160,161	500	10	8	Dec-00	410	1.12	01-Mar
AR164,165	606	8	3.4	Jul-99	636	1.74	
AR166,167	2963	8	26.9	Oct-02	3226	8.84	Oct-02
AR168,169	644	9	10	Jan-02	543	1.49	Mar-02
AR170,171		9				0.00	
AR172,173	1535	8	10.9	Aug-97	592	1.62	
AR174, 175	607	10	16	14/12/2003	510	1.40	Jan-05
AR176,177	2142	9	5.7	04/04/2001	1609	4.41	
AR178,179	2108	7	2	07/03/2000	1050	2.88	
AR180,181	141	9	56.6	28/11/2002	121	0.33	
AR182,183	1891	6	6.7	03/06/1999	703	1.93	
AR184,185	4132	7	9.9	19/02/2003	4123	11.30	25/03/2004
AR186,187	2187	9	17.7	23/05/2005	2173	5.95	NO SCAN
AR 188,189,190	645	9	6.2	Nov-01	602	1.65	
AR191,192	1224		9.7	Jan-01	1074	2.94	Jun-01
AR193,194	785		1.2	Mar-02	286	0.78	Aug-03
AR195,196	2139	10	2.1	Oct-98	1126	3.08	
AR197,198	1384	8	3.7	Feb-97	733	2.01	Oct-97
AR199,200	2171	7	2.3	Apr-96	1083	2.97	Mar-00
AR201,202	1880		4.7	Oct-03	1295	3.55	
AR203,204	1861		6.5	Jul-95	837	2.29	Apr-98
AR205,206	1171	7	6	Jan-03	686	1.88	
AR207,208	971	9	7.6	Mar-03	445	1.22	Jul-04
AR209,210	937	10	5.8	Apr-00	389	1.07	Oct-01
AR211,212	1301	6	11.4	01/02/2000	459	1.26	May-02
AR213,214	1239	10	8.1	Jun-00	878	2.41	
AR215,216	2015	9	1.2	Mar-99	1175	3.22	
AR217,218	1593	9	1.5	Jul-97	971	2.66	Mar-99
AR219,220	3547	9	3.5	Dec-03	3338	9.15	May-04
AR221,222	3778	9	1.6	Sep-99	1579	4.33	Aug-05
AR223,224	2950	10	8.6	Oct-01	2111	5.78	Dec-03
AR225,226	2041	9	6.8	Sep-02	1227	3.36	Jul-02
AR227,228		9	4.3	Nov-03			Aug-04
AR229,230,231,232	2459	9	14.5	Aug-94	2142	5.87	Aug-01

Study No	Result of bone scan 2	Orchidectomy	anti-androgen	GnRH analoggue	Stilboestrol-Date	Radiotherapy-date
AR158,159	Metastases		Jun-00	Mar-98		
AR160,161	Metastases		Mar-01	Sep-00		
AR164,165				Nov-97		Apr-01
AR166,167	Metastases			Mar-01		Mar-94
AR168,169	Metastases			Oct-00		
AR170,171		1991				1993
AR172,173			Jul-98	Jan-96		
AR174, 175	Metastases		Casodex-Aug-02-Sept-02, Feb	Aug-02		
AR176,177	No metastases		Flutamide-Dec-96-Jan-97	Zoladex-Dec-96-Jun-98		Yes-Jan-98
AR178,179			Flutamide-May-97-Jun-97	Zoladex-Jun-1997		
AR180,181	No metastases		Flutamide-Jul-02-Aug-02	Zoladex-Aug-02		
AR182,183	No metastases	de-Jul-97-Aug-97, Nov-97-Feb-98. Casodex		Zoladex-Aug-97-May-98, Oct-99		Yes-Nov-Dec-02
AR184,185	mets		flut feb 1992, flut nov1999	zoladex nov 1999		
AR186,187	No metastases			23.7.99		
AR 188,189,190	Metastases		Dec-01	Mar-00		
AR191,192	Metastases		Aug-98	Mar-98		11/09/2000
AR193,194	Metastases		Jul-03	Apr-01		01/09/2003
AR195,196	Metastases			Jul-97		15/02/1996
AR197,198	No metastases		18/10/95-10/11/95	Nov-95		
AR199,200	Metastases		20/06/1993-30/07/1994	Aug-95		
AR201,202						
AR203,204	No metastases			Jul-93		
AR205,206		7/04/2001-28/04/2001, 14/12/2		Apr-01		
AR207,208	Metastases	30/03/2002-21/04/2002, 10/02/2004-2		Apr-02		
AR209,210	Metastases			Apr-99		
AR211,212	Metastases		Nov-00	Nov-98		
AR213,214			May-01	Feb-98		
AR215,216				1/4/1996-5/10/1999,		
AR217,218	No metastases			Jul-95		04/09/2000
AR219,220	Metastases		4/2/2004-24/5/2004	Jan-03		01/03/1995
AR221,222	Metastases		4/8/1995-18/10/1999	8/8/1995-17/2/203, 1/4/2005		
AR223,224	No metastases		24/01/1996, 9/12/2003	Apr-96		01/05/2004
AR225,226	Metastases		Sep-02	Mar-99		
AR227,228	Metastases		09/03/2005-28/03/2005, 01/05/	Apr-02		31/08/2005
AR229,230,231,232	Metastases	05/06/1989	Apr-02			01/05/2002

Study No	Last Visit-Date	Alive/ Dead	code for alive/dead	follow-up(years)	followup from relapse(years)	deadthcode alive=0, cd=1, ncd=2
AR158,159	31/07/2000	DEAD	1	2.45	0.92	1
AR160,161	07/02/2003	DEAD	1	3.31	2.19	1
AR164,165	Jul-01	DEAD	1	3.77	2.02	1
AR166,167	Dec-02	DEAD	1	9.07	0.23	1
AR168,169	16/07/2002	DEAD	1	2.02	0.54	1
AR170,171	01/11/1994	dead	1	3.84		1
AR172,173	31/08/2002	DEAD	1	6.71	5.08	1
AR174, 175	20/03/2005	DEAD	1	2.66	1.27	1
AR176,177	22/12/2003	DEAD	1	7.13	2.72	1
AR178,179	21/08/2003	DEAD	1	6.33	3.46	2
AR180,181	25/08/2006	ALIVE	0	4.07	3.74	0
AR182,183	06/07/2005	DEAD	1	8.02	6.10	1
AR184,185	27/03/2005	DEAD	1	13.40	2.10	1
AR186,187	25/08/2006	ALIVE	0	7.21	1.26	0
AR 188,189,190	23/02/2005	DEAD	1	4.90	3.25	1
AR191,192	20/07/2002	DEAD	1	4.49	1.55	1
AR193,194	10/08/2005	DEAD	1	4.16	3.38	1
AR195,196	24/02/2002	DEAD	1	6.45	3.37	1
AR197,198	18/03/2001	DEAD	1	6.13	4.12	1
AR199,200	07/05/2000	DEAD	1	7.01	4.04	1
AR201,202	25/08/2006	Alive	0	6.40	2.85	0
AR203,204	13/01/1999	DEAD	1	5.81	3.51	2
AR205,206	25/08/2006	Alive	0	5.45	3.58	0
AR207,208	12/09/2005	DEAD	1	3.73	2.51	2
AR209,210	08/06/2004	DEAD	1	5.21	4.14	1
AR211,212	19/11/2002	DEAD	1	4.06	2.80	1
AR213,214	28/02/2003	DEAD	1	5.07	2.67	1
AR215,216	17/10/2002	DEAD	1	6.82	3.61	2
AR217,218	29/06/2003	DEAD	1	8.62	5.96	1
AR219,220	17/08/2004	DEAD	1	9.85	0.71	2
AR221,222	25/08/2006	Alive	0	11.24	6.91	0
AR223,224	31/01/2006	DEAD	1	10.05	4.27	1
AR225,226	25/08/2006	Alive	0	7.35	3.98	0
AR227,228	25/08/2006	Alive	0		2.81	0
AR229,230,231,232	15/11/2002	DEAD	1	14.16	8.30	1

Appendix : Publications