THE ROLE OF AP-1 TRANSCRIPTION FACTOR EXPRESSION IN ANDROGEN SENSITIVE AND ANDROGEN INDEPENDENT PROSTATE CANCER

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Doctor of Medicine

By

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Declaration

i) I, Sarath Krishna Nalagatla, hereby certify that this thesis, which is approximately 42,326 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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ii) I was admitted as a candidate for the degree of MD in April 2001. The higher study for the degree of MD of which this is a record was carried out at Glasgow Royal Infirmary.

Signed Date

iii) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of MD and that the candidate is qualified to submit this thesis in application for that degree.

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Ethical Approval

Ethical approval for the use of tumour material and associated anonymised case details was granted by Local Research Ethics Committees and Multi Research Ethics Committee for Scotland.

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I dedicate this thesis to Rohini for her patience and support, and to Sarika and Niharika for their cooperation.

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Abstract

Development of androgen independent prostate cancer (AIPC) carries a poor prognosis. The underlying molecular mechanisms are complex and not completely understood. Androgen receptor dependent and independent mechanisms have been postulated. AP-1 is a transcription factor whose components are nuclear proteins encoded by c-Jun and c-Fos proto-oncogenes. The possible role of c-Jun and c-Fos in contributing to the development of androgen independent prostate cancer in-vivo using paired human prostate cancer tissue samples has not been studied before.

Study hypothesis- AP-1 transcription factor related proteins are dysregulated in the emergence of androgen independent prostate cancer.

Aims of the study-

1. To establish a paired clinical cohort of patients in which prostate cancer tissue is available at diagnosis of prostate cancer and also following the development of androgen independence in order to test the hypothesis by immunohistochemistry.

2. To evaluate the expression of AP-1 related proteins in androgen sensitive and androgen independent prostate cancer, by immunohistochemistry.

We demonstrated that both androgen sensitive and androgen independent prostate cancers express c-Jun, c-Fos, phosphorylated c-Jun, PKC, COX-2 and AR proteins.

A significant proportion of tumours expressed high levels of AP-1 at relapse (c-Jun 68.6%, 35/51; P-Jun, 47%, 24/51; c-Fos 40%, 20/51). An increase or no change in AP-1 (c-Jun, P-Jun & c-Fos) was observed in >80% of AIPCs. Also An increase or no change in PKC (55%), COX-2(70%) and AR (90%) protein expression in AIPC was observed.

Differences in the levels of protein expression were observed as androgen sensitive prostate tumours progressed to androgen independent state.

In androgen sensitive prostate tumours, we demonstrated a statistically significant correlation in expression of c-Jun, c-Fos and p-Jun proteins (table 14).

In androgen independent prostate tumours c-Jun expression correlated with phosphorylated c-Jun with statistical significance ($p=<0.0001$).

We also demonstrated that, high levels of phosphorylated c-Jun and an increase in Protein Kinase C expression at relapse were associated with a decrease in the duration of survival from relapse ($p=0.003$).

Protein Kinase C expression correlated with COX-2 expression in both androgen sensitive as well as in androgen independent prostate tumours ($p=0.014$ & 0.002 respectively).

Also AR protein expression correlated with phosphorylated c-Jun expression in both ASPC and AIPC ($p=0.003$ & 0.07 respectively). This confirms that both the proteins

are involved in the progression of ASPC to AIPC as demonstrated in cell line studies [110, 204].

The study has demonstrated that AP-1 related proteins are dysregulated with the emergence of androgen independent prostate cancer in a subset of patients thus proving the hypothesis.

Abbreviations

ABC	Avidin biotin complex
ARE	Androgen response element
AIPC	Androgen independent prostate cancer
ASPC	Androgen sensitive prostate cancer
AP-1	Activator protein 1
AR	Androgen receptor
cAmp	Cyclic Adenosine monophosphate
CBP	CREB binding protein
COX	Cyclooxygenase
DAB	Diaminodenzidine tetrachloride
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
FSH	Follicular stimulating hormone
GnRH	Gonadotropin releasing hormone
GTP	Guanosine triphosphate
HETE	Hydroxyeicosatetraenoic acid
HRPC	Hormone refractory prostate cancer
HSP	Heat shock protein
IGF	Insulin like growth factor
IGFBP	Insulin like growth factor binding protein
IHC	Immunohistochemistry
IL-6	Interleukin 6
JAK	Janus activation kinase
JNK	Jun N- terminal kinase
LBD	Ligand binding domain
LH	Luteinising hormone

MAB	Maximum androgen blockade
LHRH	Luteinising hormone releasing hormone
MAPK	Mitogen activated protein kinase
NE cells	Neuroendocrine cells
NTD	N- terminal domain
PI3K	Phosphatidyl Inositol 3 Kinase
PIN	Prostatic intra epithelial neoplasia
PKC	Protein kinase c
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
SEER	Surveillance Epidemiology & End Result
SELECT trial	Selenium, Vitamin E chemo-prevention trial
SHBG	Sex hormone binding globulin
SRC-1	Steroid receptor coactivator-1
STAT3	Signal transducer and transactivation of transcription-3
TNM	Tumour, node, metastases
TPA	12-0-tetradeconoyl-phorbol-13 acetate
TRE	TPA response element
TRUS	Trans rectal ultrasound
TURP	Transurethral resection of prostate
XRT	External beam radiotherapy

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CHAPTER 1

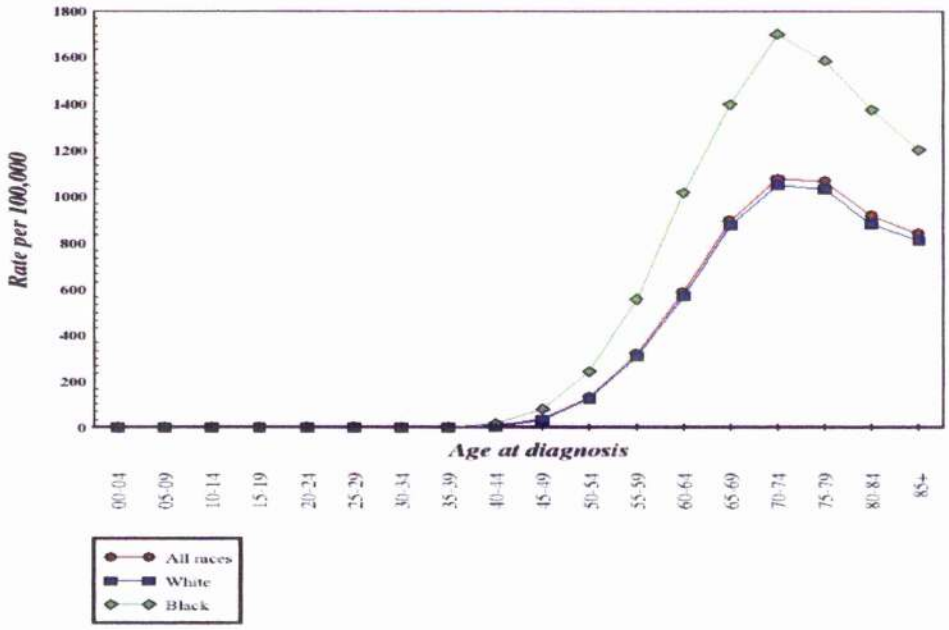
INTRODUCTION

1.1 Incidence and Epidemiology

Prostate cancer is the most common cancer of the male genitourinary tract. In the UK, by incidence prostate cancer is the fourth commonest cancer in men, after skin, lung and bowel cancer. The incidence of prostate cancer increases with age such that the probability of prostate cancer developing in a man under the age of 40 years is approximately 0.01%; increasing to 1% for men aged 40-59 and to >12% in men aged 60-79 [1]. Even if the age standardised incidence rates were to remain at their present level, an ageing population means that the number of prostate cancers requiring treatment and care is bound to increase over the next 10-15 years. Every year around 19,000 men are diagnosed with prostate cancer in England and Wales. In England and Wales the incidence of prostate cancer tripled between 1971 and 1993 (the number of registered cases increasing from 6,174 to 17,210 [2] In the USA, information on cancer epidemiology is provided by the Surveillance, Epidemiology, and End results (SEER) program of the National Cancer Institute. At present the number of new cases of prostate cancer diagnosed in the USA is estimated at over 220,000 per annum and incidence is expected to increase to more than 380,000 cases per annum by 2025 [3] The contributing factors to the continuing increase in prostate cancer incidence include screening, greater awareness of the disease and improved diagnostic techniques (e.g. PSA, transrectal ultrasound etc).

These tests have led to the diagnosis of many cancers, some of which might not have presented within the life-times of the men concerned.

Figure 1: Incidence rates of prostate cancer in different age groups in USA, 1996-2000, from the SEER data base.



1.1.1 Geographical and Ethnic variation

There are well recognised geographic and racial differences in prostate cancer incidence (Figure 2). The incidence is highest in the American population and lowest in the Far East (Figure 3). Japan and China have the lowest prostate cancer incidence rates in the world (3.4 per 100,000 and 2.5 per 100,000 respectively) [4,5]. African-American men have a particularly high incidence of prostate cancer, greater than that of US Whites (170 per 100,000 vs 110 per 100,000) [6]. Whilst in Asian-

American men the annual incidence was lowest (82 per 100,000). Hispanic-American men had an intermediate incidence in this period of 104 per 100,000 [6].

Figure 2:Incidence rates of prostate cancer in different races (55-64 yrs) in USA, 1992-2001, from the SEER data base.

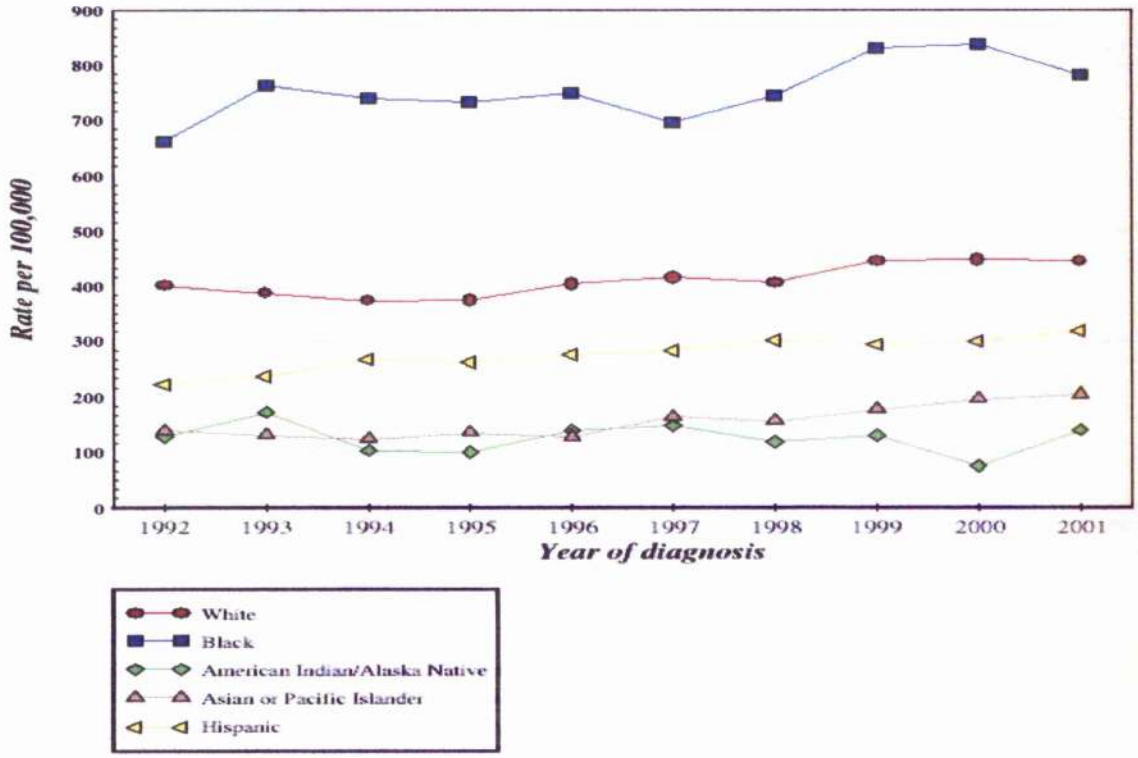
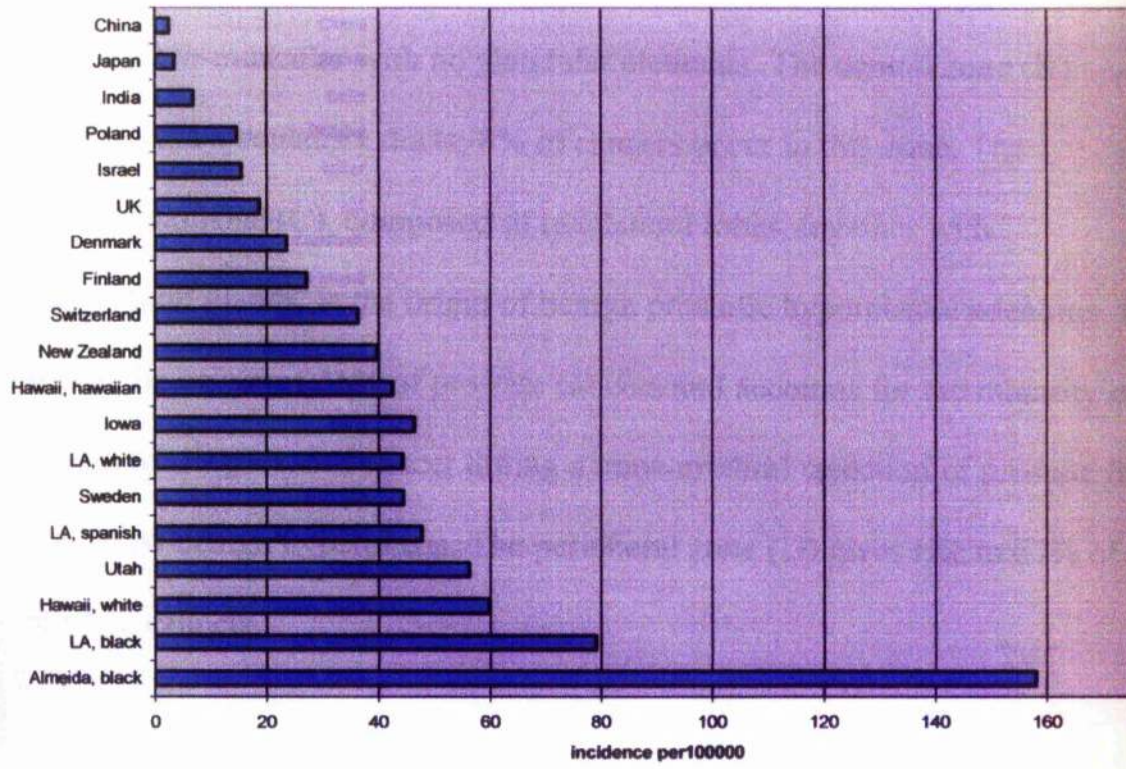


Figure 3: International variation in prostate cancer incidence

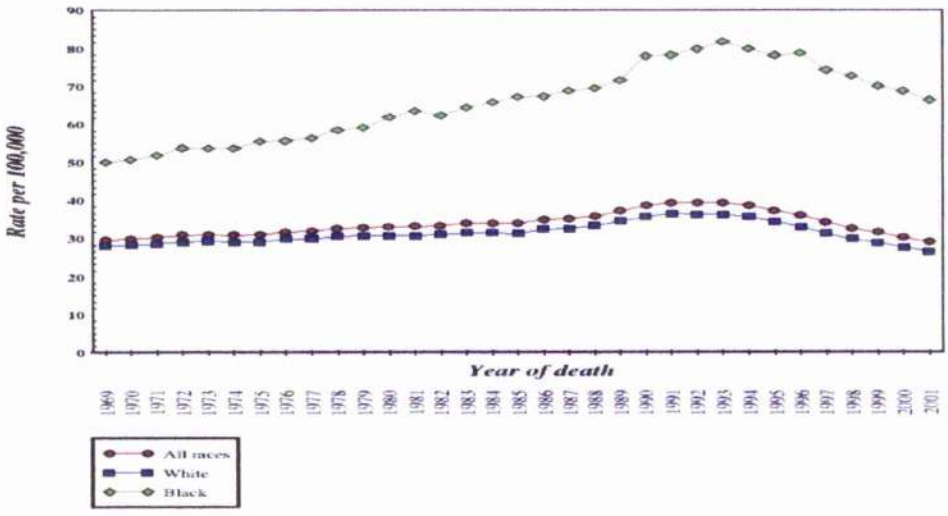


1.1.2 Mortality from prostate cancer

In the western world prostate cancer is the second leading cause of male cancer related death [7]. By 80 years of age about 50% of all men have a focus of cancer in their prostate. But only 1 in 25 i.e. 4% will actually die from this disease. Prostate cancer mortality rates have geographical and ethnic variations (Figure 4a-b). In the European Union more than 100,000 men are diagnosed with prostate cancer and 35,000 die from it each year [8]. Scandinavian countries have a particularly high rate of prostate cancer diagnosis and death compared with southern European countries. For

example prostate cancer mortality is twice as high in Norway as in Spain (24 per 100,000 vs 13 per 100,000). Asian countries such as Japan and China have the lowest mortality rates in the world [4,5]. These differences could be secondary to low incidence of prostate cancer in general as well as differences in the early diagnosis and treatment of prostate cancer. In USA mortality from prostate cancer increased slowly during the 1970s and 1980s, with an annual increase of 0.7% & 1.6% in whites and African-Americans respectively. Between 1987 and 1991 an annual increase of 3.1% was observed, which then decreased at rate of 1.9% annually up to 1995 [9,10]. It is possible that aggressive screening and treatment are responsible for the decrease in prostate cancer mortality.

Figure 4a: Prostate cancer mortality rates from the SEER data base



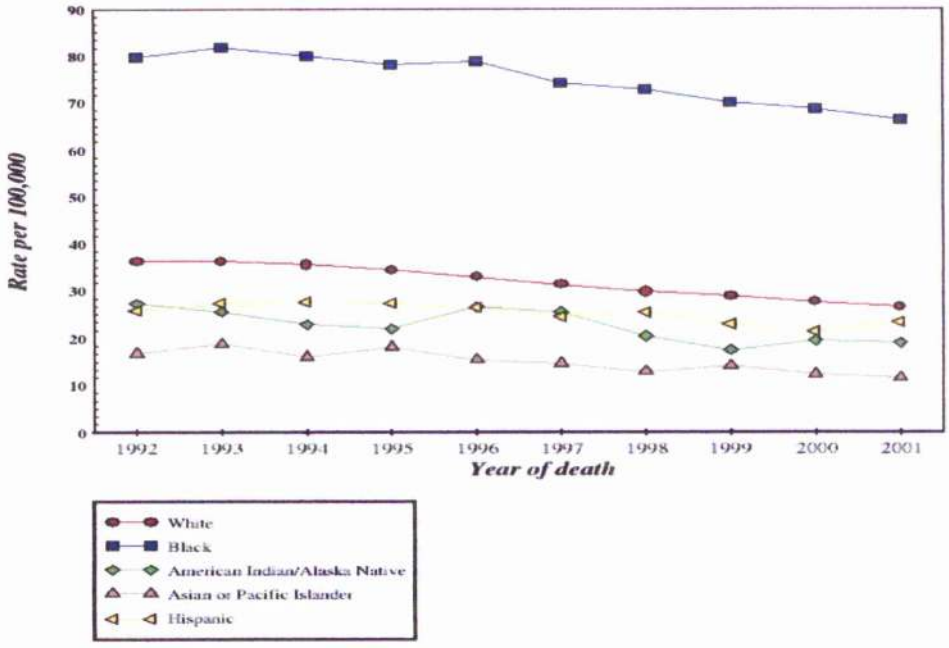


Figure 4b: Prostate cancer mortality rates from the SEER data

1.2 Risk Factors Associated With The Development Of Prostate Cancer

The precise factors responsible for prostate cancer initiation and progression are not yet known. But considerable evidence suggests that a number of factors play a role in the evolution of this disease.

1.2.1 Hereditary prostate cancer

Members of the same family may be affected by prostate cancer suggesting that some prostate cancers may be inherited [11]. Men with one first-degree relative with prostate cancer have a 2-fold increased risk

of developing prostate cancer, while men with two or three affected first degree relatives have a 5 to 10-fold increased risk [12,13,14] The risk increases if the prostate cancer, in the affected relative, is diagnosed at an early age (< 55 years). Although hereditary prostate cancer accounts for 10% of all prostate cancers, it is implicated in 43% of prostate cancers diagnosed before 55 years of age [13] Hereditary prostate cancer is a complex problem and the presence of genetic locus heterogeneity makes discovery of prostate cancer susceptibility genes a challenging process.

1.2.2 Genetic Polymorphisms

Gene polymorphisms are inherited variations in gene sequences which may result in altered function. Molecular epidemiological studies have discovered a number of associations between specific gene polymorphisms and risk of developing prostate cancer.

Androgen Receptor (AR) Polymorphisms

Two polymorphisms of the AR gene appear to influence the risk of prostate cancer. Exon 1 contains a trinucleotide $(CAG)_n$ repeat that encodes polyglutamine [15] The length of the polymorphic repeat is inversely related to the transcriptional activity of the AR gene [16,17] Long CAG repeats are associated with androgen insensitivity in patients with spinobulbar muscular atrophy [Kennedy's disease]. Short CAG repeat lengths are hypothesized to result in enhanced AR mediated activity and

increased susceptibility to benign prostatic hyperplasia and prostate cancer. CAG repeat lengths less than 18 are linked to a 1.5- fold relative risk of prostate cancer compared with those with repeat lengths >26 [18] Shorter CAG lengths were associated with a higher risk of advanced stage (relative risk-2.2) and high grade (relative risk-1.9) disease. In studies of men without prostate cancer, the prevalence of short CAG repeat lengths was highest in African Americans, intermediate in non-Hispanic whites, and lowest in Asians [19]. These data could account in part for the observed ethnic differences in prostate cancer incidence. However Mir et al [20] found no correlation between the AR CAG repeat length and the age of onset, stage and grade of prostate cancer. Similarly Correa-Cerro et al (1999) did not find an association between CAG and GGN repeats in the human androgen receptor gene and susceptibility for prostate cancer In a French-German population. It is possible that AR polymorphisms have very little clinical significance in prostate cancer.

5α-reductase Type 2 Gene (SRD5A2) Polymorphisms

SRD5A2 polymorphisms appear to result in increased enzymatic activity and risk of prostate cancer, possibly via increased conversion of testosterone to DHT [21,22] The 3' untranslated region (UTR) of SRD5A2 contains the polymorphic dinucleotide repeat $(TA)_n$. African-American men tend to have longer TA alleles (17 or more repeats) than do other ethnic groups [21] However this observation has not been found in all

studies [22,23]. Two other SRD5A2 polymorphisms have been described [24,25] In one variant, alanine at codon 49 is replaced with threonine (A49T). This is most common in African-Americans. This allele has 5-fold higher enzymatic activity than the wild type enzyme. V89L is another SRD5A2 polymorphism in which valine is substituted by leucine. This allele is more common in Asian-Americans than in African-Americans [26] This allele is less efficient in converting testosterone to DHT and these genetic variations could partly explain the lower risk of prostate cancer in Asians vs African Americans.

Cytochrome P459C17α (CYP17) Polymorphism

The 17 α- hydroxylase and 17, 20 lyase enzymes are involved in the biosynthesis of testosterone. The 5'UTR of CYP17 contains a single base polymorphism which differentiates two allelic variants- A1 and A2. Men homozygous for the A1 allele were found more commonly in a Swedish prostate cancer patient population than in a control population, suggesting that this allele may increase prostate cancer risk [27] The A1 allele is believed to encode a more active enzyme than A2. Women carrying two A2 alleles were reported to be at higher risk of developing breast cancer [22] Each of the described polymorphic variations supports the general hypothesis that increased androgen production may correlate with prostate cancer risk.

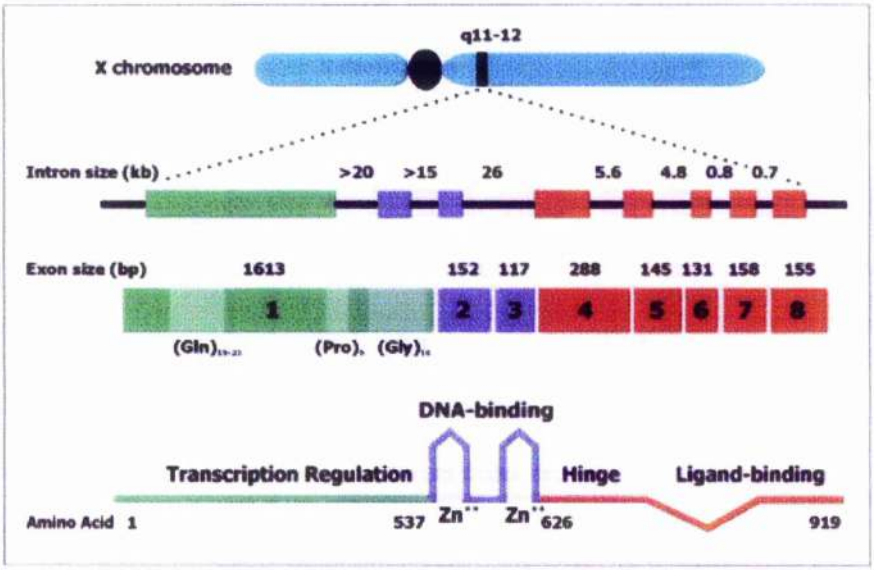
1.2.3 Androgens

Circulating androgens are produced primarily in the form of testosterone by Leydig cells in the testes. The adrenal cortex also secretes androgens predominantly androstenedione and dehydroepiandrosterone. These adrenal androgens are not as potent as testosterone in activating androgen receptors nor are they present in such high concentrations. The production of androgens by Leydig cells is regulated primarily via the hypothalamic-pituitary-gonadal axis.The hypothalamus secretes gonadotropin-releasing hormone (GnRH) in pulses every 90-120 minutes. GnRH stimulates the anterior pituitary to release Luteinizing hormone (LH) and follicular stimulating hormone (FSH). LH stimulates the production of androgens by the Leydig cells in the testes. Androgens in turn have a negative feed back effect and inhibit the secretion of GnRH and LH.

The androgen receptor is a phosphoprotein that mediates the actions of testosterone and dihydrotestosterone (DHT) by acting as a transcription factor [28] The androgen receptor is a member of the steroid nuclear receptor superfamily [29] and maps to band q11-12 of the X chromosome [30,31]. The androgen receptor (figure 5) has three major domains namely

- An N-terminal transcription activation domain
- A central DNA binding domain
- A C-terminal ligand binding domain.

Figure 5: Androgen receptor



In the prostate the AR is expressed in both epithelial and stromal cells. Once testosterone enters the cell, it is converted to dihydrotestosterone by the 5α-reductase enzymes. Whilst the AR is capable of binding both testosterone and DHT, DHT has a 2-10 fold higher affinity than testosterone and is the primary androgen bound to the AR under normal physiological conditions. Prior to ligand binding the AR appears to be in an inactive state stabilized by the heat shock proteins bound to it. Following ligand binding a conformational change takes place and the heat shock proteins (hsp90, hsp70 and hsp56) [32] dissociate from the AR. Subsequently the AR interacts with co-activators, such as AR associated

160-kd proteins, ARA70, ARA55, ARA54, and cyclic adenosine monophosphate (cAMP) response element-binding protein-binding protein (CBP). The AR then forms a homodimer and is further phosphorylated. Reversible phosphorylation appears to play a role in both ligand-dependent and ligand-independent AR activation [28] Finally the AR binds to a specific DNA binding site (androgen response element) in the promoter of androgen-responsive genes, to activate transcription of these genes.

Prostate cancer initiation and progression appears to be influenced by androgen concentrations. Prostate tumours are extremely sensitive to male sex hormones and they regress after hormone deprivation by medical or surgical castration. Studies carried out to investigate the relationship between circulating androgen levels and risk of prostate cancer have reported conflicting results.

African Americans have a higher incidence of prostate cancer and also elevated levels of circulating androgen [33]. A study carried out by Ross et al [21] reported that young African American men had 15% higher total circulating testosterone levels than white men. On the other hand Guess and colleagues [34] in their prospective study found no relationship between testosterone, sex hormone binding globulin [SHBG, which binds to and inactivates testosterone] or 5α-reductase activity and risk of prostate cancer.

A recent meta-analysis of previously published studies on hormonal predictors of prostate cancer risk [35] suggests that men whose total testosterone level is in the highest quartile are 2.34 times more likely to develop prostate cancer.These studies indicate that people with higher levels of serum testosterone are at risk of developing prostate cancer. However this is a contentious issue as there are a number of technical and theoretical issues in these studies. For example circulating testosterone levels have a significant diurnal variation and this affects measurement of overall androgen levels. It is difficult to measure the degree of exposure of the prostate to endogenous testosterone. It is not known whether early versus late exposure to androgens during a mans life or a change in the concentration over time is important [36].

1.2.4 Insulin-Like Growth Factor I [IGF-I]

Insulin like growth factor-1 has both mitogenic and antiapoptotic effects on normal and transformed prostate epithelial cells [37,38,39]. IGF-1 is implicated in both initiation and progression of prostate cancer. Chan and colleagues [40] studied IGF and IGFBP (IGF-binding protein) levels in prospectively collected sera and found a strong association between raised serum IGF levels and development of prostate cancer. Men with IGF levels in the highest quartile had a 4.3 fold higher risk of prostate cancer compared with men in the lowest quartile. Similarly Wolk et al [41] measured serum IGF-1 and IGFBP-3 levels in 210 Swedish patients with

newly diagnosed prostate cancer and in 224 controls. Increased serum IGF levels were significantly associated with prostate cancer, especially in men younger than 70 years (relative risk 2.93). These studies provide considerable evidence to suggest that the IGF axis could play an important role in the development of prostate cancer. Latif et al [42] studied circulating IGF, IGFBP-3, PSA and C-reactive protein concentrations in patients with BPH as well as prostate cancer. They found that IGF and IGFBP-3 concentrations were similar in both BPH and prostate cancer patients. There was no correlation between their concentrations and stage of prostate cancer. These findings are contradictory to those reported in previous studies.

1.2.5 Dietary Factors

People who migrate from one area to another will eventually assume the cancer rates of their host population. This suggests that dietary habits could be playing an important role in modulating the risk of prostate cancer development. However Asian-American men have a lower prostate cancer incidence than white or African-American men indicating that genetic factors still play an important role in determining prostate cancer predisposition. Migration studies have shown an increased incidence of prostate cancer in first generation immigrants from Japan and China to America [43,44] There has been considerable interest in evaluating the link between diet and prostate cancer.

Fat

Fat is the dietary component most frequently associated with prostate cancer. Several epidemiological studies have shown a correlation between dietary fat and prostate cancer risk. Giovannucci et al [45] prospectively studied the relationship between diet and prostate cancer in men. Men who consumed a high fat diet had an increased risk of developing advanced prostate cancer (Relative risk 1.6). Countries such as America where dietary fat consumption is high, have a high incidence of prostate cancer, whereas Japan, with one of the lowest rates of fat consumption has a low incidence rate. Fat may increase the risk of prostate cancer by altering androgen levels. Men who consume less fat have lower plasma testosterone levels [46].

Essential fatty acids are derived from the diet and are all unsaturated (Linoleic, Linolenic & arachidonic acids). They affect prostaglandin synthesis. Linoleic acid stimulates the growth of an androgen-unresponsive prostate cell line whereas the derivatives of linolenic acid have an inhibitory effect [47,48,49]. Arachidonic acid is a polyunsaturated fatty acid that can stimulate growth of prostate cancer cells in vitro. It is converted into 5-hydroxyeicosatetraenoic acid (5-HETE). Inhibition of 5-HETE specifically inhibited growth of prostate cancer cells. This suggests that 5-HETE may be involved in prostate cancer formation or growth [50,51,52] In the future, dietary manipulation may be a valid component in the prevention of prostate cancer.

Lycopene

Lycopene is a carotenoid antioxidant present at high concentrations in tomatoes. [53,54] reported that high lycopene intake was associated with a 21% lower risk of prostate cancer. The mechanism by which lycopene may decrease prostate cancer risk is not known. It may block IGF-mediated cellular proliferation by blocking Tyrosine phosphorylation of IGF receptor [55], however at present this remains unsubstantiated.

Selenium

Selenium is a trace mineral and a component of the antioxidant glutathione peroxidase enzyme that protects the cell from oxidative damage. A prospective randomized study that was carried out to determine if selenium supplementation could reduce the risk of skin cancer [56] found no effect of Selenium on skin cancer, but incidentally found a dramatic reduction in the incidence of prostate cancer. [57] found a similar reduction of prostate cancer incidence in men with a high levels of selenium in their toe nails. Further studies are underway to clarify the protective role of selenium in the development of prostate cancer.

Vitamin E (α- tocopherol)

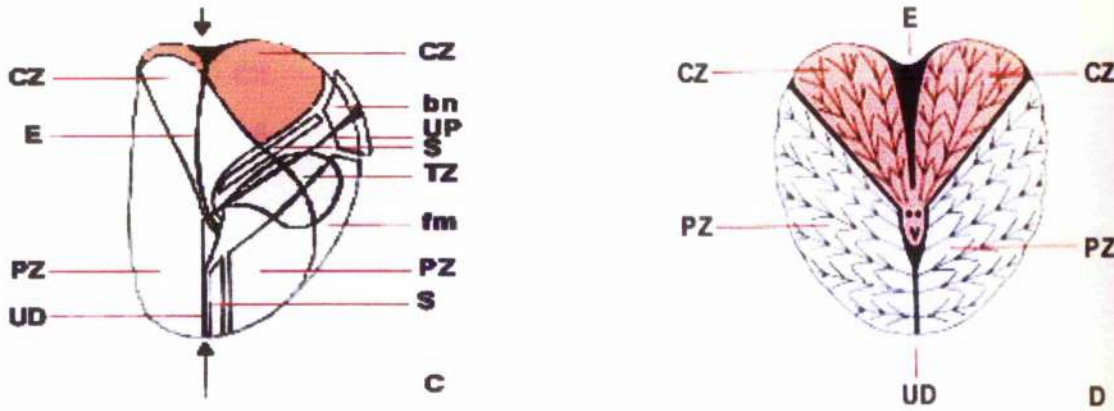
Vitamin E is a major intracellular antioxidant with anticancer properties. Vitamin E protects cell membranes from free-radical damage. In vitro studies have shown a pro-apoptotic and antiproliferative effect of vitamin E

on prostate cancer cells [58] A Finnish study [59] carried out to assess the effect of Vitamin E supplements on the incidence of lung cancer in smokers showed a 32% decrease in prostate cancer incidence and a 41% decrease in prostate cancer mortality compared with placebo. No reduction in the incidence of lung cancer was found. An American SELECT trial (Selenium Vitamin E chemo-prevention trial) is being conducted, which is a double-blinded randomized study to assess the efficacy of Selenium and Vitamin E in preventing prostate cancer, initial results are expected in 2012.

1.3 Surgical Anatomy Of The Prostate

The prostate is a fibromuscular and glandular organ lying just below the urinary bladder. There are three zones in the prostate [60] namely peripheral, central and transitional zones. Seventy percent of the prostate volume is formed by the peripheral zone, 25% by the central zone and the remaining 5% by the transitional zone (Figure 6). The majority of prostate cancers (60-70%) arise in the peripheral zone, 10-20% in the transitional zone, and 5-10% in the central zone [61]. Conversely benign prostatic hyperplasia (BPH) almost always develops in the transitional zone.

Figure 6- zonal anatomy of the prostate



Diagrammatic representation of the zones in the prostate gland.
Sagittal) and frontal view of the histological zones. Peripheral PZ = zone, CZ = central zone, TZ = zone of transition, E = ejaculatory channels, UD = distal urethra, UP = urethra proximal, BN = vesical collar, V = véru-montanum, FM = stroma fibro-muscular, S = sphincter

1.4 Pathology Of Prostate Cancer

1.4.1 Prostatic intraepithelial neoplasia (PIN)

Prostatic intraepithelial neoplasia consists of architecturally benign prostatic acini or ducts lined by cytologically atypical cells with prominent nucleoli. Pathologists cannot reproducibly differentiate low-grade PIN and benign prostate tissue [62] Hence low-grade PIN should not be commented on in diagnostic reports. Several studies have noted an increase in high grade PIN in the peripheral zone of the prostate, where 70% of prostatic adenocarcinomas arise. High grade PIN is most critical when found on needle biopsy of the prostate. A 23% to 35% risk of prostate cancer is reported after repeat biopsy in patients with High grade PIN [63,64] High grade PIN is associated with invasive carcinoma of prostate in approximately 80% of cases. High grade PIN appears to be a precursor lesion to many peripheral adenocarcinomas of the prostate. However PIN need not be present for carcinoma to arise.

1.4.2 Pathological typing of prostate cancers

More than 95% of prostate cancers are adenocarcinomas and arise from the epithelium lining the prostatic acini. Of the remaining 5%, 90% are transitional cell carcinomas [65] and the rest are neuroendocrine carcinomas [66], small cell carcinomas [67], prostatic duct adenocarcinoma [68], squamous carcinoma [69] and sarcomas [70] .

1.4.3 Gleason Grading Of Prostate Cancer

The Gleason grading system [71,72] is the most widely used numeric grading system for evaluating prostatic adenocarcinoma and is based on the low-power microscopic appearance of the prostatic glandular architecture. Grades from 1 to 5 are assigned, with increasing numeric values denoting increasing dedifferentiation of the carcinoma. Due to the high degree of variation in grade observed within prostatic carcinomas, pathologists commonly assign a primary grade to the pattern of cancer that is most commonly observed and a secondary grade to the second most commonly observed pattern in the tissue specimen. The numbers of the two most widely represented grades are added together to produce the Gleason score or sum ranging in value from 2 to 10. Well differentiated tumours have a Gleason sum of 2-4; moderately differentiated tumours have a Gleason sum of 5-7; and poorly differentiated tumours have a Gleason sum of 8-10. The Gleason score or sum provides useful prognostic information.. Gleason scores above 4 are associated with an increased risk of more rapid disease progression, increased metastatic potential and decreased survival.

1.5 Patterns of Progression of Prostate Cancer

The pattern of progression of prostate cancer is well defined. Small and well differentiated cancers are usually confined to the prostate, whereas large volume (>4cm^3) or poorly differentiated cancers are more often

locally advanced or metastatic to regional lymph nodes or bones [73]. Penetration of the prostatic capsule by prostate cancer often occurs along perineural spaces. Seminal vesicle involvement by prostate cancer can occur by either direct extension or haematogenous spread [74] and is associated with a high likelihood of regional or distant disease. Locally advanced carcinoma of prostate can cause ureteral obstruction by invading the trigone of the bladder. Involvement of the rectum is rare as Denonvillier's fascia acts as a strong barrier. Lymph node metastases are most often identified in the obturator lymph node chain. Other groups of lymph nodes that are frequently involved are the common iliac, presacral and periaortic lymph nodes. The axial skeleton is the most usual site of distant metastases. The lumbar vertebra is the most common site followed by, in decreasing order, the proximal femur, pelvis, thoracic spine, ribs, sternum, skull, and humerus [75] Bone metastases are typically osteoblastic. Lung, liver, and adrenal gland are the most frequent sites of visceral metastases.

1.6 Clinical Presentation of Prostate Cancer

There has been a significant shift in presentation of prostate cancer in most countries in the last decade. Traditionally, most men presented with a combination of weight loss, bone pain, lethargy and bladder outflow obstruction, as a result of locally advanced or metastatic disease. Increasingly the disease is being diagnosed in younger, asymptomatic patients, with much less advanced disease [9,76] This earlier

presentation of prostate cancer has posed the medical profession with difficult dilemmas concerning management. Prostate cancer patients may present with a variety of symptoms. They could present with symptoms of bladder outflow obstruction such as frequency, hesitancy and poor flow or symptoms resulting from local extension of the tumour, such as haematuria, haemospermia, erectile dysfunction, bilateral ureteric obstruction etc. Worldwide, many men still present with metastatic disease. The most common presenting symptoms are low back pain, anaemia and weight loss. Pathological fractures may also occur, particularly affecting the neck of the femur. Metastases within the vertebrae can lead to spinal cord compression and paraplegia [77,78]. Spread of prostate cancer to the lymph nodes could cause bilateral ureteric obstruction and renal failure secondary to lymph node enlargement.

1.7 Early Detection of Prostate Cancer

The majority of the patients in whom prostate cancer is suspected are identified on the basis of abnormal findings on DRE (Digital rectal examination) or, more commonly, by raised serum PSA levels.

1.7.1 Digital Rectal Examination

Not all tumours are palpable on DRE, either due to an anterior location of the cancer or due to the fact that not all are large or hard enough to be

palpated. [79] reported that approximately 50% of palpable lesions on DRE were cancerous on histological examination after prostate biopsy. Most other series report an overall detection rate of less than 40% for an abnormal DRE [80,81].

1.7.2 Prostate Specific Antigen

PSA is a glycoprotein (34kDa) protease secreted exclusively from the epithelial cells that line the acini of the prostate. PSA is recognized as the most useful tumour marker available in clinical practice for diagnosis, staging and monitoring of prostate cancer [82] The approximate chance of cancer on prostate biopsy is 1 in 50 for men with PSA levels below 4ng/ml; 1 in 4 for PSA of 4-10 ng/ml; and 1 in 2 for PSA greater than 10ng/ml [83,84,85] The early detection of prostate cancer is feasible using PSA screening which advances the diagnosis of the disease by 5-10 years [86]. Whether prostate cancer screening will reduce prostate cancer mortality currently remains unknown and the application of screening remains controversial

1.7.3 Transrectal Ultrasound (TRUS) And Prostate Biopsies

Patients with an DRE, elevated PSA, or both are offered prostate biopsies. [87] first reported that TRUS guided sextant prostate biopsy was better than digital guidance, since then this technique has become a standard

procedure for diagnosing prostate cancer. As most cancers are impalpable or invisible on TRUS, systematic biopsies must be taken.

1.8 Clinical Staging of Prostate Cancer

The first clinical staging system for prostate cancer was introduced by [88] and modified by [89]. The American Joint Committee for Cancer Staging and End Results Reporting (AJCC) introduced the tumour, node, metastases (TNM) staging system. In 1992, the AJCC and the UICC (International Union Against Cancer) adopted a new TNM classification system for prostate cancer which was further modified in 1997 as follows:

T1-Nonpalpable tumour (Incidental Prostate Cancer)

T1a-≤5% of tissue resected (TURP) for benign disease has cancer

T1b->5% of tissue resected (TURP) for benign disease has cancer

T1c-Detected from elevated PSA alone (normal DRE and TRUS)

T2-Palpable or visible tumour confined to the prostate

T2a-Tumour palpable or visible by TRUS and it involves one lobe or less

T2b-Tumour palpable or visible by TRUS and it involves both lobes

T3- Palpable tumour beyond the prostate

T3a- Extracapsular extension of the tumour on one or both sides

T3b-Tumour involves the seminal vesicles

T4-Tumour is fixed or invades adjacent structures

T4a-Tumour invades bladder neck and/or external sphincter and/or rectum

T4b-Tumour invades levator muscles and/or is fixed to pelvic wall

N-Regional Lymph nodes [obturator, internal & external iliac,presacral]

N0-No regional lymph node metastases

N1-Metastases in single regional lymph node, ≤2cm in dimension

N2- Metastases in single[>2 but < 5cm] or multiple nodes with none >5cm

N3- Metastases in regional lymph node >5cm in dimension

M-Distant Metastases

M0-No evidence of distant metastases

M1a-Metastases in non-regional lymph nodes

M1b-Metastases to bone

M1c-Metastases to other sites

1.9 Role of Axial Imaging (CT, MRI) & Bone Scan In Prostate cancer

Once the diagnosis of prostatic adenocarcinoma is histologically confirmed an accurate assessment of stage is essential to predict prognosis and select appropriate therapy. The extent of the disease correlates directly with prognosis in men with newly diagnosed prostate cancer. Cross-sectional imaging of the pelvis in patients with carcinoma of the prostate is selectively carried out to exclude lymph node metastases and seminal vesicle involvement in high risk patients (PSA >20 ng/ml; Gleason score 8-10) who are believed to be candidates for curative local

therapy (Radical prostatectomy or radical radiotherapy). The sensitivity of CT and MRI for detecting nodal metastases is approximately 36% and the specificity is 97% [90]. Radionuclide bone scanning is the most sensitive imaging technique to detect skeletal metastases. The isotope most commonly used is 99mTc-methylenediphosphonate. Overall, the sensitivity of bone scans in skeletal metastases from prostate cancer exceeds 95%.

1.10 Management of Prostate Cancer

1.10.1 Localized Prostate Cancer

The aim of treating a patient with localized prostate cancer is to achieve cure. To achieve this, the selection of the patient is critical. Ideally, patients should have life expectancy of at least 10 years. Men with localized disease do not experience significant disease related morbidity for several years after diagnosis, and curative treatment itself could result in some morbidity. Those men with a shorter life expectancy would benefit least from such treatment. The treatment options for localized prostate cancer are watchful waiting, radical prostatectomy (retropubic, perineal and laparoscopic) and radical radiotherapy (external beam or conformal) or brachytherapy.

Watchful Waiting

Watchful waiting may be recommended for older patients (>70 yrs) with low volume, well differentiated tumours, particularly when other significant illnesses are also present. Patients should be counselled, reviewed and PSA levels measured at regular intervals The development of metastatic disease during watchful waiting is believed to be 2.1% per year in patients with well differentiated tumours (Gleason scores 2-4), compared to 13.5% per year in patients with aggressive tumours (Gleason scores 7-10). Patients with low-grade tumours appear to have a disease specific survival of 87% after 10 years compared with only 26% in patients with poorly differentiated tumours [91]. However, if PSA levels start to rise or the cancer begins to cause symptoms, then initiation of therapy should be considered.

Radiotherapy

External beam radiotherapy (XRT) is widely used in the treatment of localised prostate cancer. Survival rates are often comparable to those achieved by radical prostatectomy with reported 15 year survival rates of 40-60%. One of the disadvantages with XRT technique is, it fails to provide adequate coverage of the target volume in 20-40% of patients with prostate cancer. Improved imaging and use of 3-dimentional treatment planning software can now guarantee accurate coverage. Retrospective

studies from several centres suggest that acute toxicity is reduced with the use of conformal radiotherapy compared with standard XRT [92,93].

Brachytherapy

This technique involves placing Iodine-125 seeds into the prostate via the transperineal route under TRUS (Transrectal Ultrasound) guidance. The indications for brachytherapy are T1a - T2a cancer with a gleason score 2-6, PSA <10 ng/ml, prostate volume <60ml and urinary flow rate >15ml/s. The initial 10 year results showed a 66% biochemical disease-free survival, which was maintained in the 12-year follow-up results [94].

Radical Prostatectomy

In USA, radical prostatectomy is the first line treatment option for men aged 70 years or less with clinically localised prostate cancer [95]. When carried out on men with localised prostate cancer and a life expectancy of 10 years or more, radical prostatectomy is considered an acceptable and effective treatment. Patients with organ confined prostate cancer have 10 year disease free survival ranging from 70% to 85% in several series [96] Principal adverse effects associated with radical prostatectomy are urinary incontinence (2-18%) and erectile dysfunction (30-100%).

1.10.2 Locally Advanced Prostate Cancer

A diagnosis of locally advanced prostate cancer implies that there is extra-capsular spread of prostate cancer but no evidence of metastases to

regional lymph nodes or distant sites. External beam radiation therapy has been the mainstay of treatment for clinical stage T3 disease. The 10 year survival rate is approximately 15-30% [97,98]. Neoadjuvant hormone deprivation therapy before radiotherapy has been studied in an attempt to improve local control and survival [99]. Local control and disease free survival rates at 5 years are superior in the neoadjuvant hormone deprivation group in comparison to the radiotherapy alone [100,101]. Monotherapy with the antiandrogen Bicalutamide is currently licenced to treat locally advanced prostate cancer with out metastases.

1.10.3 Metastatic Prostate Cancer

In UK, around 30% of prostate cancer patients currently present with metastatic disease. Metastatic prostate cancer is associated with high mortality approximately 70% within 5 years [102]. Hormone deprivation therapy in the form of surgical castration has been the gold standard since Huggins and Hodges demonstrated that the prostate cancer is dependent on androgens [103] A favourable response rate of 70-80% is expected with hormone deprivation treatment. Hormone deprivation can be achieved by surgical castration (bilateral orchidectomy), medical castration (estrogens, LHRH agonists), androgen receptor blocking agents (pure antiandrogens & steroidal antiandrogens) and maximal androgen blockade.

Orchidectomy

In 1941 Huggins and Hodges demonstrated that castration slowed progression of prostate cancer. Bilateral orchidectomy or bilateral subcapsular orchidectomy [104] are simple surgical procedures and carry little morbidity. Clinical responses (decreased bone pain and reduced PSA concentrations) are achieved in more than 75% of patients. Because of the psychological and cosmetic effects of orchidectomy, many patients prefer treatment with LHRH analogues.

LHRH Analogues (Goserelin acetate, Leuprolide acetate)

Schally et al [105] isolated and described the structure of gonadotropin-releasing hormone which is active in stimulating the release of FSH and LH by the pituitary. This discovery was honoured by the Nobel Prize.

LHRH agonists cause an initial stimulation of luteinizing hormone production with a rise in serum testosterone (flare response) [106,107] Within 2 weeks, they cause an inhibition of luteinizing hormone secretion resulting in castrate levels of serum testosterone. The principal side effect is tumour flare as a result of the initial increase in serum testosterone. This could result in increased bone pain or worsening of symptoms of bladder outflow obstruction [107]. There is also a potential risk of spinal cord compression.

Antiandrogens

Megestrol and cyproterone acetate are steroidal antiandrogens. Where as Flutamide, Bicalutamide and Nilutamide are nonsteroidal antiandrogens. Steroidal antiandrogens have properties similar to progesterone and they cause suppression of gonadotrophins resulting in decreased production of testosterone by Leydig cells. Nonsteroidal antiandrogens competitively bind to the androgen receptor in the target tissue and prevent the action of androgens. Bicalutamide or Cyproterone acetate are used to prevent the flare response that occurs following initiation of treatment with LHRH agonists. Seidenfeld et al [108] carried out a literature based metaanalysis of endocrine monotherapy of prostate cancer. Twenty-four trials involving 6600 patients were reanalysed. There was a trend towards shorter overall survival with nonsteroidal antiandrogens compared with castration or LH-RH agonists.

Maximum Androgen Blockade [MAB]

Suppressing both testicular and adrenal androgens simultaneously is known as maximal androgen blockage (MAB). This form of treatment has been tried as an initial treatment for metastatic prostate cancer. However metaanalysis of 22 randomised trials where MAB was used did not find a significant improvement in 5 year survival (22.8% for castration versus 26.2% for MAB) [109] over castration.

1.11 Androgen Independent Prostate Cancer [AIPC]

In 1941, Charles Huggins and Hodges [103] reported that castration or oestrogen administration in patients with metastatic prostate cancer induced regression of the cancer. This was associated with a decrease in serum acid phosphatase and alkaline phosphatate. Testosterone administration in orchiectomised patients resulted in stimulation of cancer growth and an increase in acid phosphatate levels. Huggins noted that orchiectomy was often more effective than oestrogen administration and favourable responses occurred more frequently in patients having normal sized testes and those having well differentiated tumours. Since then hormone deprivation therapy has been the main therapeutic intervention for the treatment of metastatic prostate cancer.

Seventy percent of patients with advanced prostate cancer respond to androgen deprivation therapy as measured by a decrease in serum PSA to normal levels during the first 8 months of therapy and most will relapse within 2-3 years from the initiation of treatment [110]. In contrast, in the remaining 30% of the patients, serum PSA will fail to decline to a plateau in the normal range, after an initial response to therapy, higher readings are observed, indicating early progression to androgen independent prostate cancer.

AIPC is incurable and systemic therapy for patients with hormone refractory prostate cancer has been disappointing. Chemotherapy has only a palliative role to play in the management of some patients with

AIPC [111] The median time to progression after endocrine ablation therapy is around 18-24 months, with a median survival of 6-18 months from the time of development of AIPC [112,113].

Understanding the molecular mechanisms that lead to HRPC is the most important step towards developing therapies capable of treating this disease.

1.11.1 Prostate Cancer And The Androgen Receptor

Prostate cancer is a hormone dependant tumour, androgens promote cellular proliferation and down regulate apoptosis [114]. The majority of prostate cancers initially regress after androgen deprivation therapy, however most tumours eventually begin to re-grow despite continuing androgen deprivation. Studies on prostate cancer specimens show that AR continues to be expressed in nearly all tumours of the prostate before and after hormone deprivation therapy [115-118]. PSA, which is encoded by an androgen responsive gene, is also detected in almost all hormone refractory prostates cancers. Prostate cancer cells appear to have several possible mechanisms by which they could become hormone refractory [119]

1. Mutations in the androgen receptor (AR) ligand binding domain or amplification of the AR gene.
2. Alterations of interactions between the AR and its co-activators.
3. Activation of AR by phosphorylation.

4. Activation of regulatory molecules down stream of the AR (bypassing AR).

It is possible that more than one mechanism will be utilized in each case of hormone refractory prostate cancer. Certainly there is growing evidence that each mechanism may promote hormone relapse in different patient sub-groups.

1.11.2 Androgen Receptor Mutations

Normally, the AR is specifically activated by testosterone and dihydrotestosterone, but androgen receptor mutations involving the ligand binding domain may allow the receptor to bind and be activated by hormones that are normally present in the body but do not normally cause substantial activation of the androgen receptor (e.g: adrenal androgens such as dehydroepiandrosterone (DHEA) and androstenedione) [120].

Two androgen receptor mutants T877A and H874Y were identified *in vitro* [121]. T877A was initially described in the LNCaP cell line, and H874Y is found in the hormone refractory prostate cancer xenograft CWR22. These mutations affect the ligand-binding domain of the AR and enable the AR to be activated by DHEA (several fold higher than that of wild-type AR).

Mutant ARs that respond to adrenal androgens have also been identified in tumours of patients who have failed to respond to hormone deprivation. Also other steroid hormones (progestins, estrogens) and anti-androgens (Flutamide) illicitly bind to the mutant AR and act as agonists [122]

Prostate cancer cells continue to proliferate and avoid apoptosis by utilizing DHEA when the level of androgens is low.

Flutamide is an AR antagonist. Hence it is used in the treatment of metastatic prostate cancer. 'Flutamide withdrawal syndrome' is a condition where patients show clinical worsening with Flutamide [123]. In such patients Flutamide acts as an agonist and the AR's with T877A mutations are activated by Flutamide. Discontinuing Flutamide results in initial tumour regression before the tumour starts to regrow.

MDA PCa 2a and 2b cell lines, derived from bone metastases that developed after orchidectomy [124] also express ARs. Androgens stimulate their growth and PSA expression. However the AR in these cell lines has reduced affinity for androgens and the MDA cells are less sensitive to androgens than LNCaP cells [125]. Two mutations (T877A & L701H) in the AR ligand binding domain were identified in these cell lines [125]. In the presence of L701H mutation, the affinity of the AR to bind and respond to DHT decreases [126]. This mutation enhances the binding of glucocorticoids (cortisol and cortisone) to the AR. The T877A mutation has a synergistic effect by increasing the affinity of the AR for glucocorticoids by 300% more than the L701H alone. In this doubly mutated AR, cortisol and cortisone function as AR agonists. In tumour cells with this mutation glucocorticoids can substitute for androgens and promote hormone independent growth [126] Although there is a large body of in vitro evidence supporting a role for AR mutations in the development of

hormone refractory prostate cancer, the overall frequency of AR mutations in the clinical setting cannot account for the majority of hormone refractory cancers. Gaddipati et al [127] reported T877A mutation in 6 out of 24 tumour samples (25%) from patients with metastatic prostate cancer indicating that the mutation is not uncommon in patients with HRPC.

1.11.3 Androgen Receptor Gene Amplification

AR gene amplification occurs in 20-30% of hormone refractory prostate cancers [128-130]. The resulting increase in AR protein expression is thought to sensitise the cell to the residual amounts of androgens remaining after androgen deprivation therapy, and in the presence of anti androgens [129]. Xenograft studies have demonstrated that an increase in AR expression not only allows AR to be activated in the presence of anti-androgens, but converts the anti-androgens to a weak agonist [131]

Edwards et al [118] studied AR gene amplification status and AR protein expression in a unique cohort of matched hormone sensitive and hormone refractory tumours from 51 patients. AR gene amplification was demonstrated in 20% of the patients. The AR protein expression increased as the disease progressed to hormone refractory state. Eighty percent of cases in which AR amplification was observed, also exhibited an increase in AR protein expression.

AR gene amplification and increased AR protein expression are associated with the development of hormone refractory prostate cancer in

a subgroup of prostate cancer patients only. Therefore there must be alternative mechanisms causing hormone refractory disease in the remaining prostate cancers.

1.11.4 Activation of the Androgen Receptor by AR co-activators

AR co-activators generally do not bind DNA, but are recruited to the promoter region through protein protein interactions with AR, usually in a ligand dependent manner. These co-activator proteins interact with the androgen receptor and enhance androgen receptor dependent gene transcription

The function of a co-activator is to act as a bridge and facilitate assembly of transcription factors into a stable pre-initiation complex. Included in this group are the glucocorticoid receptor-interacting protein 1, steroid receptor coactivator-1 (SRC-1) and the receptor-associated co-activator-3. These co-activator proteins interact with the N-terminal activation domain of the androgen receptor [132] or bind to the c-terminal ligand binding domain of the androgen receptor and thereby enhance AR-mediated transcription of target genes [133]. It is also possible that an increase in the protein levels of the p160 co-activators may allow adrenal androgens to function as AR ligands [119]

In addition to their bridging function, some coactivators including steroid receptor coactivator-1 (SRC-1), cAMP response element binding protein binding protein (CBP), and p300 can also remodel chromatin by

acetylating histones and recruiting the p300/CBP associated factor which harbours intrinsic histone acetyltransferase activity [134]. Therefore, when the ligand bound AR binds to the ARE, co-activators and p300/CBP associated factor are recruited. This loosens the nucleosome structure of the gene, by targeting histone acetylation, and initiates the stable assembly of the pre initiation complex via their bridging function. The end result is an enhanced rate of transcription initiation by RNA polymerase II.

In addition AR co-activators may act to alter the specificity of the ligand activation of the androgen receptor. These co-activators include the AR-associated proteins ARA54, ARA55 and ARA70 [135-137]. ARA55 and ARA70 allow activation of the AR by 17β-estradiol [136-137] and the antiandrogen hydroxyflutamide [136,138]

Thus an increase in these co-activator levels or mutations in co-activators could provide a way for hormone refractory prostate cancer to develop. AR mutations in combination with co-activators that alter the steroid specificity can result in activation of the androgen receptor and the development of hormone independence. However there is currently no direct evidence of altered expression levels of the co-activators or of altered interactions between the co-activators and the androgen receptor in hormone refractory prostate cancer.

1.11.5 Activation of the Androgen receptor by protein kinases

Dihydrotestosterone binds to AR and it protects it from proteolytic degradation by recruiting protein kinases resulting in hyperphosphorylation of the AR [139,140]. Following phosphorylation both the stability and transcriptional activity of the AR protein increase. The transcriptional activity of the AR has also been shown to correlate strongly with phosphorylation of specific serine residues [140-144]. Factors that induce androgen receptor transcriptional activity by mediating phosphorylation may therefore provide a mechanism for hormone dependent prostate cancer to develop into hormone refractory disease.

Several proteins, e.g. Mitogen activated protein kinase [140,142,145,146] and AKT or protein kinase B (PKB) [140,142,145-147], phosphorylate AR *in vitro* in the absence of androgens. This phosphorylation may facilitate the development of hormone refractory disease.

In the absence of dihydrotestosterone, AR activity increases in response to phosphorylation suggesting that the AR is activated in the absence of the natural ligand [145]. It is more likely that, in vivo, phosphorylation of AR by kinases, sensitizes the AR to low circulating levels of adrenal androgens during hormone deprivation therapy. It has been demonstrated that following DHT binding, phosphorylation in the N-terminal domain of the AR by MAP kinase results in enhanced transcription. MAP kinase and Akt (protein kinase B), regulate AR activity via phosphorylation at serine 515 and serine 213 respectively, and modulate the function of the AR.

There is no evidence that AR phosphorylation can induce dimerization of the AR, resulting in AR translocation to the nucleus to induce transcription [148]. Current evidence therefore suggests that phosphorylation of AR may influence the development of hormone escape by sensitisation to circulating androgens.

1.11.5.A Ras / Raf / MAP kinase Pathway

Cell growth, differentiation and programmed cell death are mediated by the activation of the Ras/ Raf / MAP kinase cascade. N-Ras, H-Ras and K-Ras are three different genes, which share homology and encode Ras GTPase proteins [148]. Raf is a serine / threonine protein kinase. Ras is known to interact and activate Raf in a GTP dependent manner [149]. Raf is recruited to the plasma membrane where it binds to Ras, causing RAF phosphorylation conformational changes and activation [150]

The immediate downstream target of Raf is MAP kinase kinase (MEK) which is implicated in invasion, metastasis and angiogenesis [150]. Both metastatic and non metastatic prostate cancer cell lines express Raf [151]. MEK activates MAP kinase by phosphorylation at specific tyrosine and threonine sites [150]. Activated MAP kinase stimulates various transcription factors including AR, Activator protein-1 and c-myc [118].

The MAP kinase pathway is implicated in the development of hormone escape

[147]. AR is phosphorylated by MAP kinase, which results in an increase in transcription of target genes, helps in recruitment of co-factors and increases growth stimulation [145]. MAP kinase has the ability to activate other transcription factors, which promote proliferation, independent of AR activation.

Activation of AR via phosphorylation by MAP kinase has been demonstrated in prostate cancer cell line studies. MAP kinase activity is elevated in androgen independent prostate cancer tissue [152]. Activated MAP kinase also correlates with advanced stage and grade in prostate cancer [153], and cell line studies have demonstrated that MEK and MAP kinase are expressed and activated during prostate cancer progression [154].

Cell line studies also demonstrated that transfection with Ras results in increased expression and activation of MAP kinase resulting in the development of hormone escape [118,153], also demonstrated amplification of members of the Ras/MAP kinase pathway with the development of clinical hormone escape.

It appears that activation of MAP kinase may stimulate prostate cancer cell growth in the absence of androgens via phosporylation of AR. However in-vitro studies cannot rule out increased proliferation due to activation of transcription factors that bypass the AR e.g. AP-1 and c-myc [152]. In a clinical setting, where low levels of circulating androgens are present, phosphorylation of the AR by MAP kinase could induce androgen

hypersensitivity, and this has been demonstrated in vivo using LNCaP cells [153] Whatever the route that the MAP kinase pathway takes to promote prostate cancer growth, it is evident that it is involved in the development of hormone escape.

It has also been reported that the MAP kinase pathway may increase AR activity by phosphorylating AR co-factors (SRC-1) independent of AR phosphorylation [155,156] This offers another route for the MAP kinase signal transduction to influence the development of hormone escape [156].

1.11.5.B PI3 Kinase/AKT pathway

The AKT family (AKT1-3 or Protein kinase B) is a group of serine / threonine protein kinases that inhibit apoptosis by phosphorylating targets such as BAD, procaspase-9 and the forkhead family of transcription factors [157,158]. AKTs are also involved in regulation of cell differentiation and/or proliferation via c-Myb, a sequence specific DNA-transcriptional activator.

AKT-1 and AKT-2 directly phosphorylate AR at Ser 210/213 and Ser 790/791 [147]. PI3K (phosphotidylinositol-3-kinase) generates PIP3 (phosphotidylinositol-3,4,5-triphosphate) which activates AKTs.

In vitro studies suggest that the PI3K/AKT pathway can promote prostate cancer cell survival via phosphorylation of the AR or by bypassing the AR and activating BAD, procaspase-9 [159]. AKT is constitutively activated in

both LNCaP and PC-3 cells [160] and over expression of activated AKT-1 in LNCaP cells dramatically accelerates xenograft tumour growth [161]. Prostate cancer cell line studies have also demonstrated that the activity of AKT increases as cells progress to androgen independence [162].

In contrast to MAP kinase, phosphorylation of AR by AKT decreases AR trans-activation in LNCaP, PC3 and DUI45 cells. Phosphorylation of AR by AKT prevents binding of co-activators and hence decreases AR trans-activation. So if PI3K/AKT pathway is not regulated, trans-activation of AR by DHT is greatly diminished. This reduction in AR trans-activation results in a decrease in AR regulated genes activity that normally induce apoptosis e.g. p21. This results in an increase in prostate cancer cell survival [163]

It has also been demonstrated that the PI3K/AKT pathway is required for basal and DHT induced AR expression [164] Therefore activation of the PI3K/AKT pathway could result in up-regulation of AR protein and increased sensitivity to low circulating levels of DHT, thus providing a pathway to hormone relapse. Similar to MAP kinase, phosphorylation of AR by AKT, increases AR transcriptional activity with subsequent increase in PSA, cell proliferation in androgen independent cell lines and xenografts has been reported [118,165] identified amplification of several members of the AKT pathway in prostate cancer tissue using gene array technology. These data suggest that alterations of AKT function could be a key to the development of hormone relapse prostate cancer.

1.11.5.C JAK/STAT3 pathway

Interleukin-6 can activate signal transducer and activators of transcription (STATs) such as STAT3 (signal transducer and transactivator of transcription-3), via the Janus kinase pathway (JAK). Activation of the JAK/STAT3 pathway via the IL-6 receptor has also been reported as one promoting possible mechanism to the development of hormone escape [156,163,166] Clinical data has shown that serum IL-6 levels are elevated in men with hormone refractory prostate cancer and that these high levels were accompanied by high levels of serum PSA [167,168]. Activation of the IL-6 receptor results in activation of both the MAP kinase pathway and / or the JAK/STAT3 pathway. IL-6 receptor activation results in activation of JAK, and which inturn activates STAT3. Activated STAT3 forms a homodimer prior to entering the nucleus [156]. In LNCaP cells the STAT3 homodimer binds to the AR and then enters the nucleus and thus facilitating AR translocation to the nucleus in the absence of androgens [166]. The AR/STAT3 complex can activate transcription and elevate PSA expression in the absence of androgens, and can therefore possibly offer a route to hormone escape [156,166].

1.11.6 AR activation by c-Jun

Transcription factors are constitutive proteins that are directly responsible for the 'switching on' of genes. They are usually the end targets of several signaling pathways. AP-1 is composed of c-Jun and c-Fos heterodimers or

c-Jun homodimers [169]. c-Fos is known to inhibit AR transcriptional activity [170]. Where as c-Jun can either stimulate or inhibit AR activity.

The c-Jun monomer is reported to function as an AR co-activator by binding to the N-terminal domain at amino acids 503-555 [171]. This domain is involved in the dimerization of the AR and it contains the AF-1 region [145] . The AF-1 region contains many consensus phosphorylation sites and is critical for ligand independent transactivation of the AR [145]. Binding of c-Jun to the NTD allows the AR to form a homodimer (an AR N-C domain interaction) which allows the AR to translocate into the nucleus and bind to the DNA in a sequence specific manner and hence act as a transcription factor [172]. This action of c-Jun is independent of c-Jun phosphorylation, c-Fos and AP-1 DNA binding and ligand binding [172] However the AR c-activator action of c-Jun is inhibited by phosphorylation of c-Jun and the presence of c-Fos [172]. In prostate cancer cell lines, c-Jun alone can increase AR activation [172].

In androgen independent cell lines and in the absence of androgens, transcriptional activaton of AR is increased by over expression of c-Jun [173]. AF-2 region of LBD is involved in ligand dependent transactivation of the AR [145]. It is thought that c-Jun can act in conjunction with the co-activator TIF2 which acts at the AF-2 region to potentiate AR transactivation. The effects of c-Jun and TIF2 binding to the AR on AR transactivation is additive [172]. Therefore c-Jun and TIF2 function together by targeting different regions of the AR.

Overexpression of c-Jun in hormone refractory prostate cancer offers a route by which the AR may be activated in the absence of androgens, or may potentiate AR activity when circulating levels of androgens are low. In vivo studies are needed to investigate the involvement of c-Jun in the development of clinical hormone refractory prostate cancer.

1.11.7 Progression to AIPC Via Bypassing the Androgen Receptor

Although the AR plays a major role in the progression to hormone-refractory prostate cancer, prostate cancer cells may use other pathways for proliferation independent of the AR in the absence of androgens.

1.11.7.A Neuroendocrine differentiation

The prevalence of neuroendocrine (NE) cells in prostate adenocarcinoma varies from 30-100% depending on the sources of tumour samples and the methods used to detect NE cells. NE cells become more prevalent after long-term anti-androgen therapy both in vitro and in vivo [174]. These NE cells are non-mitotic and do not express the AR. As the NE cells do not proliferate, they are relatively resistant to radiation therapy and endocrine therapy [175]. The prevalence of proliferating carcinoma in the vicinity of the NE cells [176,177] suggests that these NE cells may play a role in the growth of hormone refractory prostate cancer.

NE cells produce a number of products such as parathyroid hormone-related protein, Serotonin, bombesin, calcitonin, chromagranin A,

neurotensin and thyroid stimulatory hormone which may play a role in the progression to hormone refractory prostate cancer [178,179]. Cell line studies have demonstrated that IL-6 treatment induces LNCaP and PC-3 cells to undergo NE differentiation [180,181]. IL-6 induced NE differentiation in LNCaP cells via activation of PI3K (Phosphatidylinositol 3 Kinase). This can be blocked by a dominant-negative mutant form of Etk. STAT3, one of the downstream targets of Etk [182] mediates IL-6 induced NE differentiation in LNCaP and PC-3 cells [183]. Thus IL-6 induces NE differentiaton in prostate cancer cell lines by activating PI3K [184], Etk and STAT3. There is no in vivo data to account for the increase in NE cells after androgen ablation therapy. It is possible that elevated serum levels of IL-6 in patients with hormone refractory disease [167] activate the PI3K-Ekt-STAT3 signalling pathway to cause NE differentiation in prostate tumours.

1.11.7.B Anti-apoptotic signaling Pathways

Withdrawal of androgen triggers apoptosis in both normal and malignant androgen-dependent prostate epithelial cells. However, androgen-refractory prostate cancer cells do not undergo apoptosis [185]. PI3 Kinase and related pathways that activate anti-apoptotic signaling pathways should be critical for the survival of hormone refractory prostate cancer cells.

PI3 Kinase is activated by a number of survival factors, such as IL-6 etc. PI3 Kinase phosphorylates phosphatidylinositol to generate D-3 phosphatidylinositol, including phosphatidylinositol-3,4,5- triphosphate and phosphatidylinositol-3,4- biphosphate, both of which activate protein kinase B (AKT). Protein kinase B is one of the key regulatory molecules involved in the protection of cells against apoptosis [186,187]. The tumour suppressor gene, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a lipid phosphatase whose primary function is to negatively regulate PI3-Kinase / AKT signaling. PTEN is inactivated in many cancers, including prostate, brain, breast, endometrium, and kidney [188]. In prostate cancer loss of PTEN function can result from a number of mechanisms such as deletion, mutation and in a xenograft model, methylation [189-192]. The frequency of PTEN mutation in prostate cancer is relatively low overall, but inactivation of PTEN is more frequent in advanced stages of the tumour [190,193,194]). Thus, loss of PTEN function may favour tumour cells survival in the environment resulting from androgen ablation therapy. Loss of PTEN and activation of P13K / AKT could favour Bcl-2 and Bcl-XL proteins which are inhibitors of apoptosis. In the normal prostate, Bcl-2 is expressed in the basal epithelial cells but not in the luminal epithelial cells [195,196] Bcl-2 is over-expressed in early stage disease, but most studies have shown higher frequencies of over-expression in advanced prostate cancer [197] . Apoptosis in LNCaP cells as a result of androgen withdrawal is prevented

by forced expression of Bcl-2, which enables cells to be less dependent on androgens [198]. Over-expression of Bcl-2 is implicated in the conversion of hormone-dependent to hormone refractory prostate cancer [197,198].

1.11.7.C.c-Jun and c-Fos expression in prostate cancer

The Activator Protein 1 (AP-1) is a transcription factor involved in control of cell growth and differentiation. Its activity is modulated by growth factors, cytokines and Protein kinase c (PKC) [199]. c-Jun and c-Fos are encoded by c-Jun and c-Fos proto oncogenes. Jun/Fos heterodimers have higher stability than Jun homodimers which accounts for their increased DNA binding activities [200]. Formation of either dimer requires c-Jun phosphorylation at serine residues 63 and 73 by c-Jun N-terminus kinase (JNK), which is activated by PKC or MAP Kinase cascades. Dimerisation is mediated via the leucine zipper domain of both proteins [201]. The basic region adjacent to the leucine zipper on Jun and Fos proteins mediates AP-1 DNA binding activity [202,203] AP-1 induces transcriptional activation by binding to the TPA (12-o-tetradecanoyl-phorbol-13 acetate) response element (TRE) [201]. TREs are recognized by both the AP-1, c-Jun homodimers nd c-Jun / c-Fos heterodimers [110,204] Activation of AP-1 via PKC or the Raf/MAP kinase pathway has been reported to be involved with the development of androgen escape [172,204,205]. AP-1 influences the development of hormone

escape by competing with the AR to alter expression of AR regulated genes [172,204].

AR and AP-1 are capable of binding to each other, this protein-protein interaction prevents either from being able to bind to DNA and hence decreases gene transcription [110, 204]. However evidence also suggests that AP-1 can increase expression of androgen regulated genes by binding to a TRE within the promoter region [204]. Therefore the effect of AP-1 on androgen regulated gene expression depends on the ratio of AR to AP-1 and the ability of AP-1 to bind to specific promoter regions within the androgen regulated genes [110,204]. Such competition could influence the ability of AP-1 to increase androgen regulated genes expression in the absence of androgens and hence might influence the development of androgen escape [110]. This is especially important in androgen regulated genes which contain multiple TREs in the promoter region e.g. PSA and PSMA [110]. In a situation where the ratio of AP-1 to AR is high (e.g. in the absence of androgens), there would be less AR available to initiate transcription by binding to the ARE [204]. There would be excess AP-1 available for binding to an alternative TRE, resulting in an increase in androgen regulated gene expression. Therefore it is conceivable that such a situation could influence the development of androgen escape via increases in androgen regulated gene expression in the absence of androgens. In vitro studies demonstrate that in PC3 cells, the intracellular concentration of c-Jun and c-Fos is 7 fold greater than in

LNCaP [204]. This suggests that AP-1 influences androgen escape in PC-3 cell line [206]. Although cell line work suggests that AP-1 could be involved in the development of androgen escape, little work has been conducted to investigate its action in vivo.

The role of AP-1 is further complicated by the fact that c-Jun can act as an AR co-factor. In addition to interactions between AP-1 and AR, there is evidence that the c-Jun monomer may also influence the development of AIPC by functioning independently of AP-1. c-Jun acts as an AR co-activator binding to the N-terminal domain at amino acids 503-555 [171], this promotes AR dimerisation and gene transcription [193] . This action of c-Jun is independent of c-Jun phosphorylation, c-Fos, AP-1 DNA binding and AR ligand binding [193,207].

The potential role of c-Jun and AP-1 in AIPC is therefore highly complex, with c-Jun being an AR co-activator, whilst phosphorylated c-Jun may both inhibit AR activation via AP-1 binding to AR and promote AIPC via AP-1 mediated gene transcription.

1.11.7.D Protein kinase C (PKC)

The protein kinase c family of enzymes promote lipid hydrolysis. Diacylglycerol and phorbol esters are potent PKC activators [208]. Receptor tyrosine kinases or non-receptor tyrosine kinases can cause diacylglycerol production by either activating specific phospholipase C or

phospholipase D [209-211]. Protein kinase C (PKC) is an isozyme family with at least eleven mammalian members that play important roles in cell growth regulation and differentiation [212]. Diacylglycerol and phorbol esters cause PKC to translocate to membranes. All isozymes of PKC require phosphatidylserine located on the cytoplasmic face of cell membranes and some isozymes require Ca^{2+} for its optimal activity [] [213,214].

Phorbol-ester is a tumour promoter, which indicates PKC involvement in tumorigenesis [212,215]. In vitro studies have demonstrated involvement of PKC in androgen regulated transcription. Transfection studies have shown enhancement of AR-induced transactivation by PKC activator phorbol 12-myristate 13-acetae (PMA) [216]. No changes in AR expression levels, DNA binding, or phosphorylation were observed. The effect of PMA was attributed to the activation by PKC of proteins downstream. Two well studied proteins activated by PKC are c-Jun and c-Fos [169,172] demonstrated that c-Jun stimulates hAR activity without altering hAR expression or DNA binding and they suggested that PKC activation of c-Jun may be responsible for the PMA effect on AR transactivation. They also demonstrated c-Fos as a negative regulator of c-Jun action on the AR.

Activation of AP-1 via PKC or the Raf/MAP kinase pathway has been linked to the development of androgen escape [172,204,205]. AP-1

influences the development of hormone escape by competing with the AR to alter expression of AR regulated genes [172,204].

PKC-α is selectively over-expressed in androgen independent human prostate cancer cell line (PC3- 8 fold & DU 145- 40 fold) in comparison to LNCaP [207]. Activation of the isozyme PKC-α results in apoptosis of LNCaP cells [218,219]. Androgen- independent human prostate cancer cell lines DU 145 and PC3 do not suffer apoptosis in response to phorbol esters [219,220]. Chelerythrine is a highly selective PKC inhibitor which elicits apoptoic response in androgen-independent human prostate cancer cell lines that express constitutively active PKC but not in LNCaP, which do not express constitutive PKC activity [211]. It appears that constitutive PKC-α activity is required for survival and growth of androgen independent human prostate cancer cells [215].

1.11.7.E Cyclooxygenase 2 (COX-2)

Cyclooxygenase (COX), is also known as prostaglandin (PG) endoperoxide synthase. It is a key enzyme in the conversion of arachidonic acid to PGs. There are two isoforms of COX. COX-1 is constitutively expressed in most tissues and mediates the synthesis of PG required for normal physiological functions. COX-2 is not detectable in most normal tissues, but is inducible by trauma, growth factors, tumour promoters and cytokines, and is involved in inflammation and proliferation. Increased COX-2 expression has been described in a

number of human cancers including colonic, breast and prostate cancers [222-226]. Overexpression and persistence of COX-2 has been linked to promotion of tumorigenesis, resistance to apoptosis and defective regulation of cell-cycle [228]. COX-2 is also implicated in tumour progression and there is a strong link between COX-2 expression and hypoxia induced tumour angiogenesis [228]. COX-2 induced PGE2 has been reported to stimulate angiogenesis [229]. Both androgen sensitive LNCaP and androgen independent PC-3 cell lines express COX-2 protein . Treated both cell lines with a selective COX-2 inhibitor NS-398, resulted in apoptosis [230-231].

Ceramide is a novel lipid second messenger. It is formed when the membrane phospholipids sphingomyelin undergoes hydrolysis by sphingomyelinase (SMase). Subbaramaiah et al [223] demonstrated enhanced prostaglandin E2 synthesis and increased COX-2 protein expression in human mammary epithelial cells following treatment of these cells with neutral sphingomyelinase. The induction of ceramide was inhibited by calphostin C, an inhibitor of protein kinase C. Stimulation of ceramide pathway also resulted in increased expression of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and MAPK activities. Inhibition of MAPK kinase blocked the induction of COX-2 by SMase. Overexpressing ERK1, JNK or MAPK led to several fold increases in COX-2 promoter activity. A dominant negative for c-Jun inhibited the activation of COX-2 promoter activity by ceramide. Hence in

response to ceramide, increased MAPK signalling activates c-Jun, which in turn induces COX-2 gene expression via the cAMP response element in the COX-2 promoter. Therefore it appears that both PKC and MAPK can induce COX-2 expression by activating c-Jun.

1.12 HYPOTHESIS & AIMS / OBJECTIVES OF THE STUDY

Study hypothesis- AP-1 transcription factor related proteins are dysregulated in the emergence of androgen independent prostate cancer.

Aims of the study-

1. To establish a paired clinical cohort of patients in which prostate cancer tissue is available at diagnosis of prostate cancer and also following the development of androgen independence in order to test the hypothesis by immunohistochemistry.

2. To evaluate the expression of AP-1 related proteins in androgen sensitive and androgen independent prostate cancer, by immunohistochemistry.

To test the hypothesis, expression of AP-1 transcription factor related proteins- c-Jun, phosphorylated c-Jun, c-Fos, Protein kinase c, COX-2 and AR in paired prostate cancer tissue specimens will be evaluated using immunohistochemistry. Immunohistoscoring will be carried out by two independent observers. An increase or decrease in expression of AP-1

related proteins with the development of androgen independence will be determined.

An increase or decrease in expression of AP-1 transcription factor related proteins will be correlated with time to development of hormone relapse and also duration of survival from relapse.

Also a change in the expression of one protein will be correlated with other AP-1 related proteins to determine if AP-1 pathway is involved in the emergence of androgen independent prostate cancer.

CHAPTER 2

MATERIALS AND METHODS

2.1 Patient Selection

2.1.1 Ethical Approval

Ethical approval was obtained from the Multi-centre Research Ethics committee [MREC] for Scotland in Edinburgh. MREC approval has to be obtained if the study would be undertaken in five or more LREC sitesAs the study was retrospective and majority of the patients were not alive at the time of the study, it was not possible to obtain written consent from patients whose prostate cancer tissue was studied. This aspect was made clear to the MREC, which gave approval for the study to be carried out. All together It took 5 months for getting MREC approval.

Subsequently Local Research Ethics Committee [LREC] approval was sought from each hospital involved. It was time consuming and It took several months before approval could be obtained from all the LRECs. One LREC did not give approval for the study as it was not possible to obtain written consent from patients and they suggested a prospective study to be carried out with written consent taken from the patients.

2.1.2 Recruitment of Patients

After having obtained ethical approval to carry out the research project, pathology departments of each hospital involved in the study were contacted. The project was discussed with them in detail. From the pathology data base, a list of prostate cancer patients who had two

procedures carried out on their prostate [either TURP or Prostate biopsies] was obtained first. From this list of prostate cancer patients, their medical records were accessed.

If all of the following criteria were satisfied then the patients were included in the study.

1. Patients diagnosed to have carcinoma of the prostate.
2. Prostate cancer tissue available at the time of diagnosis.
3. Patients should have received hormonal treatment and have their PSA values monitored.
4. Evidence of response to hormonal treatment as indicated by decreasing levels of serum PSA.
5. Subsequently developed hormone relapse disease as indicated by rising PSA levels in spite of continuing hormonal treatment.
6. Had TURP for symptoms of bladder outflow obstruction after developing relapse and their prostate cancer tissue available.

Prostate cancer patients who did not satisfy all of the above criteria were excluded from the study.

A total of 51 patients out of 1021, who satisfied all the above criteria were selected for the study. Full clinical follow-up was retrieved from patient medical records. All the relevant details were entered into an anonymised database protected with a password and kept within the University Department of Surgery. The details that were recorded include date of birth, date of diagnosis, serial PSA values through out follow-up, results of

staging investigations such as CT scan, Bone scan, hormonal treatment received, date of relapse, date of last visit and date of death where applicable.

In our study Androgen independent prostate cancer was defined as rising levels of PSA inspite of hormonal treatment after having a good initial PSA response to hormonal treatment.

2.2 Preparation of silanised slides

To study protein expression by IHC, tissue sections were mounted on Silanised slides. Plain glass slides were coated with silane using the following procedure.

Slides were loaded into racks and placed in acetone for 5 minutes and then in 2% v/v Aminopropyltriethoxysilane in acetone for another 5 minutes in a fume hood. Slides were washed in running tap water for 25 minutes and then dried overnight in a fume hood. They were then labeled and stored in dated boxes for no more than 6 months.

2.3 Tissue sections

Archival formalin fixed, paraffin-embedded tissue blocks were cooled by placing them on a cooled stage (Tissue TEX II) prior to sectioning. 4 μm sections were cut using a microtome (Leica RM 2135). The cut sections were transferred into a warm water bath at 50^0C and then mounted on to

Aminopropyltriethoxysilane-treated glass slides or non-silanised slides. The slides with tissue sections were then loaded into slide holding racks and placed in an oven at 56^0C overnight to allow the tissue to fix. Silanised slides were used for IHC and non- silanised slides were used for haematoxylin and eosin staining.

2.4 Haematoxylin and Eosin staining

For histological examination and identification of tumour areas Haematoxylin and Eosin staining was performed using the following protocol.

1. Removal of wax from the sections Before any staining procedure is carried out it is necessary to remove the wax from the sections. This was done by placing the slides in Xylene for 6 minutes twice.
2. Rehydration of tissue sections- The sections were gradually rehydrated through graded alcohols (100% alcohol for 4 minutes, repeat; 90% alcohol for 2 minutes & 70% alcohol for 2 minutes. Then the sections were washed in running tap water for 2 minutes.
3. Staining of sections: sections were placed in Haematoxylin stain for 3 minutes, washed in running tap water. They were then rinsed in Scott's tap water untill blue and cleared in acid alcohol. Sections were then

rinsed in running tap water. They were then placed in eosin for 3 minutes and again rinsed in running tap water.

4. Dehydrating and mounting the sections- The sections were then dehydrated through a series of alcohols (70% alcohol for 30 seconds, 90% alcohol for 30 seconds & 100% alcohol for 30 seconds) and cleared in Xylene. The sections were then covered with glass cover slips using DPX mountant (contains dibutyl phthalate, xylene).

   The slides were viewed using a light microscope.

2.5 Immunohistochemistry

2.5.1 General Principles

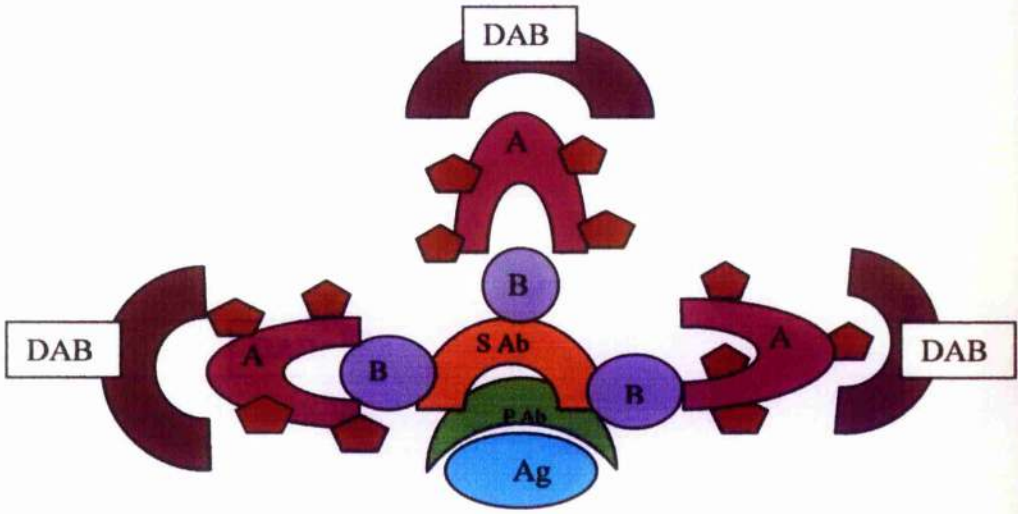
Immunohistochemistry (IHC) is a technique for localising and visualising an antigen in a tissue section by using an antibody specific for the tissue antigen. The procedure consists of tissue preparation, antibody incubation, and a series of detection reactions. There are a number of different IHC techniques. All aim to attach a visible marker to the primary antibody that will specifically recognise the antigen of interest.

The visualisation method we used is known as indirect IHC, which is more sensitive than the direct method that employs labelling the antibody with a substance that can be visualised. In indirect IHC another antibody (secondary antibody) is used to amplify the signal and enhance the detection of the antigen. The secondary antibody would be raised in a species different to that of the primary antibody. If the primary antibody is

made in the mouse then the secondary antibody would be made in a horse or a rabbit. The secondary antibody is labelled with the marker (e.g. peroxidase). The peroxidase label would then be visualised with DAB (diaminobenzidine). The advantage of using this procedure is greater sensitivity. Each primary antibody will end up with several secondary antibodies attached. Therefore we get more peroxidase molecules attached to each antigen.

The procedure we used was slightly more complicated and is known as Avidin Biotin Peroxidase Complex procedure (ABC). This is a 3-step procedure, the first two are similar to the indirect method, but the secondary antibody is labelled not with the peroxidase but with biotin. Biotin has a very high affinity for avidin (present in high amounts in egg white and acts as an antibiotic by removing biotin from any bacteria that could infect the egg), which is a large molecule and can be labeled with many peroxidase molecules. So an avidin-peroxidase conjugate forms the third layer.

Figure 7: Avidin biotin peroxidase complex



Ag- Antigen; P Ab- Primary antibody; S Ab- Secondary antibody; B- Biotin; A- Avidin labelled with peroxidase; DAB- Diamino benzidine.

2.5.2 Quality control for Immunohistochemistry

When demonstrating the antigen of interest in tissue using IHC techniques, it is essential to include appropriate control sections (positive and negative) for quality control. Negative controls serve as "blanks" and any staining on this specimen is nonspecific. The primary antibody is added to the positive control section and an isotope matched IgG is added to the negative control section. A positive reaction with the positive control

section indicates that the antibodies are working properly and that the procedure has been carried out correctly. A negative reaction with the positive control indicates that the fixatives may have reduced access of antibody to antigen, the antibody may be too dilute, the secondary antibody may not be recognising the primary antibody or the enzyme/substrate system is defective or incompatible. A positive reaction with the negative control indicates either a technical mistake or non-specific staining as a result of secondary antibody binding non specifically to the tissue. Therefore a negative control slide must always be included.

2.5.3 Primary Antibodies

Primary antibodies may be either polyclonal or monoclonal. Polyclonal antibodies are produced by in vivo immunisation usually of mammals such as a goat, sheep, mouse or a rabbit. Polyclonal antibodies are derived from a variety of B-cells that differ in the genetic material that encodes for antibody production. In a polyclonal sample some of the antibodies will be specific for the antigen with which the animal was immunised. The remaining antibodies are produced from interaction with other antigens that the animal was exposed to throughout its lifetime.

Monoclonal antibodies are derived using a more complex process. Here, a mammal, an inbred mouse, is immunised with an antigen. After repeated immunisations, the spleen of the animal is removed. The spleen is responsible for B-cell production and so the spleen cells contain the

genetic information that gives rise to antibody production. But these spleen cells cannot be cultured. So they are fused with "immortal" myeloma cells, so-called because of their ability to proliferate *in vitro*. The resulting fused cells, called hybridoma cells, are screened by enzyme-linked immunoabsorbant assay (ELISA) using the antigen in question. This assay allows selection of hybridoma cells that produce antigen-specific antibodies. Because a given hybridoma cell is derived from a single B-cell, it produces a "monoclonal" cell population producing the required antibody. Once a single hybridoma line is selected, it is injected into a healthy mouse. Hybridoma cells, like myeloma cells, have the ability to produce tumors. After injection with a hybridoma line, a tumor grows inside the host mouse. When this tumor grows, it produces ascites, a fluid that is rich in monoclonal antibodies. These days this is being carried out by culturing hybridomas in-vitro

2.5.4 Immunohistochemistry procedure

The steps described below are only general principles in carrying out IHC. After optimising the IHC technique for each antibody the following steps were tailored accordingly. The steps taken to optimise the IHC technique for each antibody is described in detail separately.

Step I Removal of wax and rehydration of tissue sections

The wax was dissolved from paraffin embedded tissue by placing the slides in Xylene (Fishers chemicals) for 6 minutes and this was repeated. Xylene is miscible with alcohol. The sections were gradually rehydrated through graded alcohols (100% alcohol for 4 minutes,repeat; 90% alcohol for 2 minutes & 70% alcohol for 2 minutes. Then the sections were washed in running tap water for 2 minutes.

Step II Quenching of endogenous peroxidase activity

Some cells, especially red blood cells, muscle cells, granulocytes, monocytes, kidney and liver contain high concentrations of peroxidase enzymes. If endogenous peroxidase is not destroyed, it could result in staining of the negative control and tissue sections due to nonspecific oxidation of DAB. Endogenous peroxidase is generally inactivated by incubating tissues with H_2O_2. Sections are treated with 0.3% H_2O_2 (VWR International limited; supplied at concentration of 30%) for 10 minutes in a staining dish using a magnetic stirrer and then rinsed in distilled water to remove excess H_2O_2.

Step III Retrieval of Antigen

After formalin fixation and paraffin embedding of tissues, many antibodies react only weakly or not at all with their antigen(s). This is because solvents, heat and fixatives denature proteins, and promote protein protein and protein nucleic acid cross linking, these alterations can mask antigens by obscuring the epitope recognised by the antibody.

Formaldehyde fixes tissue by reacting with the basic amino acid lysine to form cross-linking methylene bridges. This may result in the partial loss of the characteristic 3D protein structure so that the change in physical shape of the antigen means that it will not be recognised by its corresponding antibody: therefore antigen retrieval. If the formaldehyde cross-links directly with the epitope in question it means that its chemical characteristics are altered and again it will not be recognised by its antibody until unmasked. Trypsin exposes the antigenic determinant by cleaving arginine and lysine residues. Micro waving or pressure cooking in EDTA at pH 8 is the latest antigen retrieval method and although the exact mechanism is unknown, it is thought that the fixative protein cross-linking is broken thus restoring the proteins physical and chemical characteristics and therefore antigenic properties.

Antigen retrieval was carried out by one of the following techniques that were under use in our laboratory, depending upon the antibody used.

1. Microwave/ pressure cooking- Tris EDTA buffer solution (0.37g sodium EDTA-BDH Laboratory supplies and 0.55g Tris base-Sigma, made up to 1 litre with distilled water, pH 7.5) was warmed for 13.5 minutes on full power and the sections were microwaved in a pressure cooker for 2 minutes to bring it to pressure and for another 5 minutes under pressure. Then the weight on the pressure cooker was lifted to allow the steam to escape and the lid was finally removed. The sections were allowed to cool

for 20 minutes, washed in water and then transferred to a staining dish with water.

2. Trypsin antigen retrieval technique-

Sections were incubated in 80 mls of 0.1% Trypsin calcium chloride solution (0.08 gm of Trypsin (Sigma) in 80 mls of 0.1% $CaCl_2$ (Sigma) solution) for 25 minutes in a water bath at 37^0C. Subsequently the slides were washed in water and transferred to a staining dish with water.

Step IV-Blocking non-specific back ground staining

Non specific background staining was blocked by incubating the sections in normal horse serum(Vector) at a concentration of 15μl/ml for 15 minutes. After incubation time the serum was tapped off the sections.

Step V- Blocking of endogenous Biotin

Some tissues may bind avidin, biotinylated peroxidase or other Biotin/Avidin system components without prior addition of biotinylated antibody because Biotin is a recognised enzyme co-factor in normal physiology. This binding may be due to endogenous biotin or biotin binding proteins, lectins, or non-specific binding substances present in the section.

If a high background was present using the ABC reagents (Vector) in the absence of biotinylated secondary antibody, pre-treatment of the tissue with avidin, followed by biotin (to block the remaining binding sites on the

avidin), was required. The sections were incubated with Avidin for 15 minutes, rinsed in Tris Base solution (pH 7.5 and 100 mls of 10 times TBS in 900 mls of distilled water), then incubated with Biotin for 15 minutes and finally rinsed in Tris Base solution.

Step VI- Incubation with Primary antibody

Excess serum was blotted from the sections and then incubated with the primary antibody in a humidified chamber or in a cold room (4^0C) depending upon the antibody used .The concentration of the primary antibody and Incubation period varied, depending on the antibody. After incubation the sections were washed in Tris Base saline solution for 2 x 5 minutes.

Step VII- Binding with the secondary antibody

The sections were incubated with the biotinylated secondary antibody solution (15µl of normal serum + 5µl of secondary antibody per ml of Tris Base saline solution) for 30 minutes and washed in Tris Base saline solution for 2 x 5 minutes.

Step VIII- Avidin / biotin labelled peroxidase binding

The avidin and peroxidase labeled biotin must be allowed to interact for at least 30 minutes before being applied (to the sections), resulting in the formation of a large and highly labelled complex, the labeled molecules (peroxidase) being shared by several biotin molecules.

The proportion of avidin to labelled biotin must be such that some binding sites on the avidin are left free to attach to the biotin of the secondary antibody. Avidin has an extraordinarily high affinity for biotin making binding of avidin to biotin essentially irreversible. In addition, avidin has 4 binding sites for biotin and peroxidase can be conjugated with several molecules of biotin resulting in a very sensitive detection system.

The sections were incubated with ABC solution (Vectastain Elite ABC reagent- 20μl of Reagent A added to 1 ml of Tris Base saline solution, mixed, then 20μl of Reagent B added and mixed again) for 30 minutes and washed in Tris Base saline solution for 2 x 5 minutes.

Step IX- Localizing peroxidase using Diaminobenzidine

Diaminobenzidine tetrahydrochloride (DAB) is a chromogen used to localise peroxidase in the tissue sections. DAB in the presence of peroxidase will polymerize to form a visible insoluble brown precipitate which is insoluble in alcohol and clearing agents, allowing sections to be permanently mounted. DAB is poisonous and a known carcinogen therefore precautions must be taken. The sections were incubated with DAB (Vector Laboratories) solution (2 drops of buffer stock added to 5 ml of saline, mixed, then 4 drops of DAB added, mixed and finally 2 drops of hydrogen peroxide is added and mixed) for 2-10 minutes_until brown colour developed and washed in running water for 10 minutes.

Step X- Counterstaining

It is helpful to use a nuclear counter stain, which will produce a colour contrast with the labelled antigen. With the deep brown colour produced by the DAB and peroxidase, haematoxylin produces a satisfactory blue counter stain.

The sections were counter stained with heamatoxylin for 60 seconds and then washed in water. The sections are then immersed in Scotts tap water till they appear blue and then immersed in acid alcohol and then washed in running tap water.

Step XI- Dehydrating and mounting

The sections were then dehydrated through a series of alcohols (70% alcohol for 1 minute; 90% alcohol for 1 minute and 100% alcohol for 2 minutes), cleared in xylene (2 minutes) and mounted in a synthetic resin (DPX- xylene, dibutylphthalate). The protein in tissue sections, the synthetic resin and the glass slides all have similar refractive index thus producing the maximum resolution when viewed down the microscope.

2.5.5 Choice of Antibodies

In the present study c-Jun, Phosphorylated c-Jun, c-Fos, Protein Kinase C, AR, PSA and COX-2 protein expressions were studied immunohistochemically.

An antibody is defined as an immunoglobulin capable of specific combination with the antigen that caused its production in a susceptible

animal. The classical Y shape of IgG is composed of the two variable, antigen specific F(ab) arms, which are critical for actual antigen binding, and the constant Fc "tail" that binds immune cell Fc receptors and also serves as a useful "handle" for manipulating the antibody during most immunochemical procedures.

Immunochemical techniques capitalize upon the extreme specificity, at the molecular level, of each immunoglobulin for its antigen, even in the presence of high levels of contaminating molecules. The multivalancy of most antigens and antibodies enables them to interact to form a precipitate.

In our study all the antibodies used were monoclonal. In general monoclonal antibodies are reported to provide more specific immunostaining results than those obtained with polyclonal antibodies. Also they give much lower background staining than polyclonal antibodies. They also provide highly reproducible results. In view of these advantages monoclonal antibodies were used in our study. These antibodies were shown to be effective in staining tissue embedded in paraffin wax. Immunohistochemical protein expressions of c-Jun, phosphorylated c-Jun, c-Fos, Protein Kinase C, AR, PSA and COX-2 using these monoclonal antibodies have been reported in various studies and the results were encouraging.

2.6 c-Jun Immunohistochemistry

c-Jun IHC was performed using the DK4 mouse monoclonal c-Jun antibody (IgG1) specific to the human c-Jun oncoprotein (Novocastra laboratories). DK4-c-Jun has been reported to be effective on frozen tissue as well as paraffin wax embedded tissue. The data sheet for this antibody recommended a typical working dilution between 1:40 to 1:80, Trypsin digestion of paraffin sections and over night primary antibody incubation at 4^0C for IHC. Reconstituted antibody was frozen at -20^0C and stored in a freezer. Working dilutions of the antibody was prepared on the day of use.

As a large proportion of breast cancers express c-Jun protein, breast cancer sections were used in the work up of this antibody in the initial stages. Subsequently prostate cancer tissue was used for the study. Antibody concentrations of 1:40, 1:50, 1:60, 1:70, 1:80, 1:90, 1:100 & 1:120 were incubated for 30 or 60 minutes at room temperature and at 4^0C in a cold room overnight and the optimum antibody concentration was found to be at a dilution of 1:70 (40 μl of reconstituted primary antibody in 2800 μl of DAKO antibody diluent). The optimum duration of incubation was overnight at 4^0C in a cold room. Negative control sections did not demonstrate any background staining. However to reduce the background staining in the positive slides DAKO antibody diluent with background

reducing components was used. The following protocol was used for c-Jun IHC.

2.6.1 IHC protocol for c-Jun

Clone DK4, NCLc-JUN

Mouse monoclonal antibody IgG_1

NovoCastra Laboratories

A) Dewax & rehydrate

1. Dewax the slides-2 x 4 minutes in Xylene
2. Rehydrate-2 x 2 minutes in 100% alcohol, 2 minutes in 90% alcohol and 2 mins in 70% alcohol
3. Rinse in water
4. Treat with 0.3% Hydrogen peroxide for 10 minutes on a magnetic stirrer (30% Hydrogen peroxide- 5 mls in 500 mls of distilled water)
5. Rinse in water

B) Antigen Retrieval

1. Make up 0.1% Ca Cl_2 (0.08g in 80ml of distilled water)
2. Incubate sections in 0.1% Trypsin in 80 mls of CaCl2 solution for 25 mins at 37^0C
3. Rinse in water
4. Transfer to a staining dish with water (slides can be stored like this)
5. Keep TBS ready. (1 in 10 TBS – 200 mls in 2 litres of distilled Water)

C) Staining

1. Ring the sections with DAKO pen to create a barrier.
2. Horse serum- incubate the sections for 15 minutes

(Vector ABC Kit- add 15 μl of horse serum per 1 ml of TBS- buffer; i.e. 0.5ml per slide)

3. Blot serum from sections
4. Primary antibody- Incubate the sections with c-Jun monoclonal antibody (1 in 70 dilution) over night in a cold room at 4^0C. Use DAKO solution for dilution

   (Use isotope matched IgG antibody for negative control ie 5 μl of serum in 500 μl of DAKO solution)
5. Wash in TBS- 2 X 5 minutes. (use magnetic stirrer)
6. Secondary antibody- Incubate the sections with secondary antibody for 30 minutes. (15 μl of serum + 5 μl of secondary antibody per 1 ml TBS)
7. Wash in TBS- 2 X 5 minutes. (use stirrer)
8. ABC reagent- Incubate for 30 minutes.(20 μl of A, 20 μl of B per 1 ml of TBS)
9. Wash in TBS- 2 X 5 minutes.(use magnetic stirrer)
10. DAB- Incubate the sections with DAB until colour develops ie for 2-10 minutes.(5 mls of distilled water + 2 drops of buffer stock & mix, 4 drops of DAB stock & mix, 2 drops of hydrogen peroxide & mix)
11. Wash in water for 10 minutes

D) Counter Staining

1. Stain in haematoxylin for 60 seconds
2. Rinse in running tap water
3. Place the slides in Scots tap water substitute till they appear blue
4. Rinse in running tap water

E) Dehydrating & mounting

1. 1 minute 70% alcohol
2. 1 minute 90% alcohol
3. 2 x 1 minutes 100% alcohol
4. 2 x 1 minutes xylene & mount in DPX

2.7 Phosphorylated c-Jun Immunohistochemistry

A mouse monoclonal IgG_1 antibody raised against a peptide corresponding to amino acids 56-69 of c-Jun of human origin was used for IHC. Phosphorylated c-Jun (KM-1) antibody reacts with c-Jun p39 phosphorylated on Ser-63 of mouse, rat and human origin. No cross reactivity with Jun B or Jun D phosphorylated on the analogous serine residues or with c-Jun non-phosphorylated at Ser-63. It is a product of Santa Cruz Biotechnology, Inc (p-c-Jun (KM-1): sc-822). The data sheet for this antibody recommended a dilution range of 1:100-1:1000 for western blot analysis. Recommended starting dilution was 1:100. Each vial contained 200μg IgG_1 in 1.0 ml of PBS containing 0.1% sodium azide and 0.2% gelatin. The antibody was stored at 4^0C in a cold room. Prostate cancer tissue was used to work out the antibody. 1:100 dilution was tried to start with. Negative control sections did not demonstrate any background staining. Satisfactory staining was observed with this dilution. The following protocol was used for p-c-Jun IHC.

2.7.1 IHC protocol for p-c-Jun

p-c-Jun (KM-1): sc-822
Mouse monoclonal antibody IgG_1
Santa Cruz Biotechnology,Inc.

A) Dewax & rehydrate

1. Dewax the slides-2 x 4 minutes in Xylene

2. Rehydrate-2 x 2 minutes in 100% alcohol, 2 minutes in 90% alcohol and 2 mins in 70% alcohol
3. Rinse in water
4. Treat with 0.3% Hydrogen peroxide for 10 minutes on a magnetic stirrer (30% Hydrogen peroxide- 5 mls in 500 mls of distilled water)
5. Rinse in water

B) Antigen Retrieval

1. Make up 0.1% $CaCl_2$ (0.08g in 80ml of distilled water)
2. Incubate sections in 0.1% Trypsin in 80 mls of CaCl2 solution for 25 mins at 37^0C
3. Wash in water
4. Transfer to a staining dish with water (Slides can be stored like this)
5. Keep TBS ready. (1 in 10 TBS – 200 mls in 2 litres of distilled Water)

C) Staining

1. Ring the sections with DAKO pen to create a barrier.
2. Horse serum- Incubate the sections for 15 minutes

(Vector ABC Kit- add 15 µl of horse serum per 1 ml of TBS- buffer; i.e. half ml per slide)

3. Blot serum from sections

4. Primary antibody- Incubate the sections with phosphorylated c-Jun monoclonal antibody (1 in 100 dilution) over night at 4^0C. Use DAKO solution for dilution (Use isotope matched IgG antibody for negative control i.e. 5µl of serum in 500 µl of DAKO solution)

5. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)

6. Secondary antibody- Incubate the sections with secondary antibody for 30 minutes (15 μl of serum + 5 μl of secondary antibody per 1 ml TBS)

7. Wash in TBS- 2 X 5 minutes (use stirrer)

8. ABC reagent- Incubate with it for 30 minutes (20 μl of A, 20 μl of B per 1 ml of TBS)

9. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)

10. DAB- Incubate the sections with the DAB until colour develops i.e. for 2-10 minutes (5 mls of distilled water + 2 drops of buffer stock & mix, 4 drops of DAB stock & mix, 2 drops of hydrogen peroxide & mix)

11. Wash in water for 10 minutes.

D) Counter Staining

1. Stain in haematoxylin for 60 seconds
2. Rinse in running tap water
3. Place the slides in Scots tap water substitute till they appear blue
4. Rinse in running tap water

E) Dehydrating & mounting

1. 1 minute 70% alcohol
2. 1 minute 90% alcohol
3. 2 x 1 minutes 100% alcohol
4. 2 x 1 minutes xylene & mount in DPX

2.8 c- Fos Immunohistochemistry

A mouse monoclonal antibody IgG_1 was used for c-Fos IHC. The antibody is manufactured by BioGenex using c-Fos 88 clone. This antibody specifically localizes c-Fos protein in formalin-fixed, paraffin-embedded tissue sections. It is sourced from ascites and diluted in phosphate buffered saline, pH 7.6, containing 1% BSA and 0.09% sodium azide. The data sheet recommends to storing the antibody at 2-8^{0}C. Antigen retrieval was tried by microwave pressure cooking as recommended in the data sheet. Due to inconsistency in staining, Trypsin digestion technique was used for antigen retrieval. Breast cancer tissue as well as prostate cancer tissue were used for working up the antibody. The data sheet for the antibody recommended a dilution of 50-100, incubation time of 30 minutes at room temperature. Satisfactory staining was achieved with 1:50 dilution. The following protocol was used for c-Fos IHC.

2.8.1 IHC protocol for c-Fos

c-Fos Mouse monoclonal antibody IgG_1
Ab No.348M
Biogenex

A) Dewax & rehydrate

1. Dewax the slides-2 x 4 minutes in Xylene
2. Rehydrate-2 x 2 minutes in 100% alcohol, 2 minutes in 90% alcohol and 2 mins in 70% alcohol

3. Rinse in water
4. Treat with 0.3% Hydrogen peroxide for 10 minutes on a magnetic stirrer (0.3% Hydrogen peroxide- 5 mls in 500 mls of distilled water)
5. Rinse in water

B) Antigen Retrieval

1. Make up 0.1% $CaCl_2$ (0.08g in 80ml of distilled water)
2. Incubate in 0.1% Trypsin in 80 mls of CaCl2 solution for 25 mins at 37^0C
3. Wash in water
4. Transfer to a staining dish with water (Slides can be stored like this)
5. Keep TBS ready (1 in 10 TBS – 200 mls in 2 litres of distilled Water)

C) Staining

1. Ring the sections with DAKO pen to create a barrier
2. Horse serum- Incubate the sections for 15 minutes

(Vector ABC Kit- add 15 µl of horse serum per 1 ml of TBS- buffer; i.e. half ml per slide)

3. Blot serum from sections
4. Primary antibody- Incubate the sections with c-Fos monoclonal antibody (1 in 50 dilution) over night in a cold room at 4^0C. Use DAKO solution for dilution. (Use isotope matched IgG antibody for negative control i.e. 5 µl of serum in 500 µl of DAKO solution)
5. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)

6. Secondary antibody- Incubate the sections with secondary antibody for 30 minutes (15 μl of serum + 5 μl of secondary antibody per 1 ml TBS)
7. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
8. ABC reagent- Incubate with it for 30 minutes (20 μl of A, 20 μl of B per 1 ml of TBS)
9. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)

10. DAB- Incubate the sections with the DAB until colour develops i.e. for 2-10 minutes (5 mls of distilled water + 2 drops of buffer stock & mix, 4 drops of DAB stock & mix, 2 drops of hydrogen peroxide & mix)

10. Wash in water for 10 minutes.

D) Counter Staining

1. Stain in haematoxylin for 60 seconds
2. Rinse in running tap water
3. Place the slides in Scots tap water substitute till they appear blue
4. Rinse in running tap water.

E) Dehydrating & mounting

1. 1 minute 70% alcohol
2. 1 minute 90% alcohol
3. 2 x 1 minutes 100% alcohol
4. 2 x 1 minutes xylene & mount in DPX.

2.9 Protein Kinase C Immunohistochemistry

PKC IHC was carried out using a mouse monoclonal IgG2a antibody against purified bovine brain protein kinase C. The antibody (anti-protein kinase C ab31) is a product of abcam limited. It has cross reactivity with mouse, rat and human PKC. It was synthesized from MC5 clone and P3x63. This antibody recognizes alpha, beta and gamma PKC isoforms. The data sheet recommended storage of the antibody at 4^0C. For long term (>6 months) storage the recommended temperature is -20^0C.

Trypsin digestion technique was initially tried for antigen retrieval. This did not work. Hence pressure cooking technique was tried subsequently. This worked well. Different dilutions of the primary antibody were tried (1:100, 1:200, 1:300 & 1:400). Optimum concentration of the primary antibody was found to be at 1:200.

The following protocol was used for PKC IHC.

2.9.1 IHC protocol for PKC

PKC Mouse monoclonal antibody IgG2a
Anti Protein Kinase C ab31
Abcam Limited

A) Dewax & rehydrate

1. Dewax the slides-2 x 4 minutes in xylene
2. Rehydrate-2 x 2 minutes in 100% alcohol, 2 minutes in 90% alcohol and 2 mins in 70% alcohol

3. Rinse in water
4. Treat with 0.3% Hydrogen peroxide for 10 minutes on a magnetic stirrer (0.3% Hydrogen peroxide- 5 mls in 500 mls of distilled water)
5. Rinse in water

B) Antigen Retrieval

1. Make up Tris EDTA buffer, pH 8 (0.37g EDTA+0.55G Tris Base + 1 Litre distilled water)
2. Microwave on full power for 13.5 minutes to warm the solution
3. Add the slides and close the lid and microwave on full power for 2 minutes to bring to pressure
4. Microwave for 5 minutes under pressure
5. Carefully remove the weight to allow the steam to escape, and remove the lid. Cool it for 20 minutes
6. Wash the slides in water
7. Transfer to a staining dish with water (Slides can be stored like this)
8. Keep TBS ready (1 in 10 TBS – 200 mls in 2 litres of distilled Water)

C) Staining

1. Ring the sections with DAKO pen to create a barrier & Keep incubator ready
2. Horse serum- Incubate the sections with it for 15 minutes

(Vector ABC Kit- add 15 µl of horse serum per 1 ml of TBS- buffer [i.e. half ml per slide)

3. Blot serum from sections

4. Blocking step- Avidin- for 15 minutes, quick rinse in TBS; Biotin-for 15 minutes, quick rinse in TBS (No dilution of Avidin/Biotin)
5. Primary antibody- Incubate the sections with PKC monoclonal antibody (1 in 200 dilution) over night in a cold room at 4^0C. Use DAKO solution for dilution.

   (Use isotope matched IgG antibody for negative control i.e. 5 μl of serum in 500 μl of DAKO solution)
6. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
7. Secondary antibody- Incubate the sections with secondary antibody for 30 minutes (15 μl of serum + 5 μl of secondary antibody per 1 ml TBS)
8. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
9. ABC reagent- Incubate with it for 30 minutes (20 μl of A, 20 μl of B per 1 ml of TBS)
10. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
11. DAB- Incubate the sections with the DAB until colour develops i.e. for 2-10 minutes (5 mls of distilled water + 2 drops of buffer stock & mix, 4 drops of DAB stock & mix, 2 drops of hydrogen peroxide & mix)
12. Wash in water for 10 minutes.

D) Counter Staining

1. Stain in haematoxylin for 60 seconds.
2. Rinse in running tap water.
3. Place the slides in Scots tap water substitute till they appear blue.

4. Rinse in running tap water.

E) Dehydrating & mounting

1. 1 minute 70% alcohol
2. 1 minute 90% alcohol
3. 2 x 1 minutes 100% alcohol
4. 2 x 1 minutes xylene & mount in DPX.

2.10 COX-2 Immunohistochemistry

I used a mouse monoclonal (CX229) antibody a product of Cayman chemical USA. Our laboratory has previous experience in using this antibody in breast cancer and prostate cancer samples. I followed this protocol without any modifications as described below.

2.10.1 IHC protocol for COX 2

COX 2 Mouse monoclonal antibody IgG1
CX229 clone
Cayman Chemical, USA.

A) Dewax & rehydrate

1. Dewax the slides-2 x 4 minutes in Xylene
2. Rehydrate-2 x 2 minutes in 100% alcohol, 2 minutes in 90% alcohol and 2 mins in 70% alcohol
3. Rinse in water
4. Treat with 0.3% Hydrogen peroxide for 10 minutes on a magnetic stirrer (0.3% Hydrogen peroxide- 5 mls in 500 mls of distilled water)
5. Rinse in water

B) Antigen Retrieval

1. Make up Tris EDTA buffer, pH 8 (0.37g EDTA+0.55G Tris Base + 1 Litre distilled water)
2. Microwave on full power for 13.5 minutes to warm the solution

3. Add the slides and close the lid and microwave on full power for 2 minutes to bring to pressure
4. Microwave for 5 minutes under pressure
5. Carefully remove the weight to allow the steam to escape, and remove the lid. cool it for 20 minutes
6. Wash the slides in water
7. Transfer to a staining dish with water (slides can be stored like this)
8. Keep TBS ready (1 in 10 TBS – 200 mls in 2 litres of distilled Water)

C) Staining

1. Ring the sections with DAKO pen to create a barrier.
2. Horse serum- Incubate the sections for 15 minutes

   (Vector ABC Kit- add 15 μl of horse serum per 1 ml of TBS- buffer; i.e. half ml per slide)
3. Blot serum from sections and incubate with primary antibody at room temperature for 30 minutes

   (Use isotope matched IgG antibody for negative control i.e. 5 μl of serum in 500 μl of DAKO solution)
4. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
5. Add yellow link solution and incubate for 30 minutes
6. Wash in TBS- 2 X 5 minutes (use stirrer)
7. Add red streptavidin solution and incubate for 1 hour
8. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)

9. DAB- Incubate the sections with the DAB until colour develops i.e. for 2-10 minutes (5 mls of distilled water + 2 drops of buffer stock & mix, 4 drops of DAB stock & mix, 2 drops of hydrogen peroxide & mix)
10. Wash in water for 10 minutes

D) Counter Staining

5. Stain in haematoxylin for 60 seconds.
6. Rinse in running tap water.
7. Place the slides in Scots tap water substitute till they appear blue.
8. Rinse in running tap water.

E) Dehydrating & mounting

5. 1 minute 70% alcohol
6. 1 minute 90% alcohol
7. 2 x 1 minutes 100% alcohol
8. 2 x 1 minutes xylene & mount in DPX.

2.11 Androgen Receptor Immunohistochemistry

A mouse monoclonal (clone 2F12) IgG1 antibody was used for AR IHC. It is available from Novocastra Laboratories Ltd (NCL-AR-2F12). The data sheet recommends high temperature antigen unmasking technique and 60 minutes primary antibody incubation at 25^0C. Work up of the antibody was carried out using prostate cancer sections. The reconstituted antibody is stable for at least two months when stored at 4^0C and for long-term storage, aliquots of the antibody are frozen at -20^0C. A primary antibody dilution of 1:100 gave optimal staining. The following protocol was used for AR IHC.

2.11.1 IHC protocol for AR

AR Mouse monoclonal (clone 2F12) antibody IgG1
NCL-AR-2F12
Novocastra Laboratories Ltd

A) Dewax & rehydrate

1. Dewax the slides-2 x 4 minutes in Xylene
2. Rehydrate-2 x 2 minutes in 100% alcohol, 2 minutes in 90% alcohol and 2 mins in 70% alcohol
3. Rinse in water
4. Treat with 0.3% Hydrogen peroxide for 10 minutes on a magnetic stirrer (0.3% Hydrogen peroxide- 5 mls in 500 mls of distilled water)
5. Rinse in water

B) Antigen Retrieval

1. Make up Tris EDTA buffer ,pH 8 (0.37g EDTA+0.55G Tris Base + 1 Litre distilled water)
2. Microwave on full power for 13.5 minutes to warm the solution
3. Add the slides and close the lid and microwave on full power for 2 minutes to bring to pressure
4. Microwave for 5 minutes under pressure
5. Carefully remove the weight to allow the steam to escape, and remove the lid Cool it for 20 minutes
6. Wash the slides in water
7. Transfer to a staining dish with water (slides can be stored like this)
8. Keep TBS ready (1 in 10 TBS – 200 mls in 2 litres of distilled Water)

C) Staining

1. Ring the sections with DAKO pen to create a barrier.
2. Horse serum- Incubate the sections for 15 minutes

(15 μl of horse serum per 1 ml of TBS- buffer; i.e. 0.5 per slide)

3. Blot serum from sections
4. Blocking step- Avidin- for 15 minutes, quick rinse in TBS; Biotin-for 15 minutes, quick rinse in TBS (No dilution of Avidin/Biotin)
5. Primary antibody- Incubate the sections with primary antibody (1 in 100 dilution) for 30 minutes. Use DAKO solution for dilution

   (Use isotope matched IgG antibody for negative control i.e. 5 μl of serum in 500 μl of DAKO solution)

6. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
7. Secondary antibody- Incubate the sections with secondary antibody for 30 minutes (15 μl of serum + 5 μl of secondary antibody per 1 ml TBS)
8. Wash in TBS- 2 X 5 minutes (use stirrer)
9. ABC reagent- Incubate with it for 30 minutes (20 μl of A, 20 μl of B per 1 ml of TBS)
10. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
11. DAB- Incubate the sections with the DAB until colour develops i.e. for 2-10 minutes (5 mls of distilled water + 2 drops of buffer stock & mix, 4 drops of DAB stock & mix, 2 drops of hydrogen peroxide & mix)
11. Wash in water for 10 minutes.

D) Counter Staining

1. Stain in haematoxylin for 60 seconds.
2. Rinse in running tap water.
3. Place the slides in Scots tap water substitute till they appear blue.
4. Rinse in running tap water.

E) Dehydrating & mounting

1. 1 minute 70% alcohol
2. 1 minute 90% alcohol
3. 2 x 1 minutes 100% alcohol
4. 2 x 1 minutes xylene & mount in DPX.

2.12 PSA Immunohistochemistry

A monoclonal antibody (A 0562) to human PSA was used. It is available from DAKO laboratory, Denmark. A 1:200 primary antibody dilution was used for IHC. Satisfactory staining was observed with this dilution. The following protocol was used for PSA IHC.

2.12.1 IHC protocol for PSA

PSA human monoclonal
A 0562
Dako Laboratories Ltd

A) Dewax & rehydrate

1. Dewax the slides-2 x 4 minutes in Xylene
2. Rehydrate-2 x 2 minutes in 100% alcohol, 2 minutes in 90% alcohol and 2 mins in 70% alcohol
3. Rinse in water
4. Treat with 0.3% Hydrogen peroxide for 10 minutes on a magnetic stirrer (0.3% Hydrogen peroxide- 5 mls in 500 mls of distilled water)
5. Rinse in water

A) Antigen Retrieval

1. Make up Tris EDTA buffer, pH 8 (0.37g EDTA+0.55G Tris Base + 1 Litre distilled water)
2. Microwave on full power for 13.5 minutes to warm the solution
3. Add the slides and close the lid and microwave on full power for 2 minutes to bring to pressure

4. Microwave for 5 minutes under pressure
5. Carefully remove the weight to allow the steam to escape, and remove the lid. Cool it for 20 minutes
6. Wash the slides in water
7. Transfer to a staining dish with water (Slides can be stored like this)
8. Keep TBS ready (1 in 10 TBS – 200 mls in 2 litres of distilled Water)

B) Staining

1. Ring the sections with DAKO pen to create a barrier.
2. Horse serum- Incubate the sections for 15 minutes

(Vector ABC Kit- add 15 µl of horse serum per 1 ml of TBS- buffer i.e. half ml per slide)

3. Blot serum from sections
4. Blocking step- Avidin- for 15 minutes, quick rinse in TBS; Biotin-for 15 minutes, quick rinse in TBS (No dilution of Avidin/Biotin)
5. Primary antibody- Incubate the sections with primary antibody (1 in 200 dilution) 30 minutes. Use DAKO solution for dilution

   (Use isotope matched IgG antibody for negative control i.e. 5 µl of serum in 500 µl of DAKO solution)
6. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
7. Secondary antibody- Incubate the sections with secondary antibody for 30 minutes (15 µl of serum + 5 µl of secondary antibody per 1 ml TBS)
8. Wash in TBS- 2 X 5 minutes (use stirrer)

9. ABC reagent- Incubate with it for 30 minutes (20 μl of A, 20 μl of B per 1 ml of TBS)
10. Wash in TBS- 2 X 5 minutes (use magnetic stirrer)
11. DAB- Incubate the sections with the DAB until colour develops i.e. for 2-10 minutes (5 mls of distilled water + 2 drops of buffer stock & mix, 4 drops of DAB stock & mix, 2 drops of hydrogen peroxide & mix)
11. Wash in water for 10 minutes.

C) Counter Staining

1. Stain in haematoxylin for 60 seconds
2. Rinse in running tap water
3. Place the slides in Scots tap water substitute till they appear blue
4. Rinse in running tap water

D) Dehydrating & mounting

1. 1 minute 70% alcohol
2. 1 minute 90% alcohol
3. 2 x 1 minutes 100% alcohol
4. 2 x 1 minutes xylene & mount in DPX.

2.13 Scoring Principles

Scoring was performed independently by two observers (NSK- N Sarath Krishna and JE- Dr Joanne Edwards). The percentage of cells labelled and the labelling intensity were estimated by scoring the percentage of tumour cells falling into negative, weakly positive, moderately positive and strongly positive. intensity, distribution and pattern of immunostaining were recorded. If there was a discrepancy between the observer scores, a consensus was reached after further evaluation. For example in a tumour section, 50% of the cells are negative (no brown staining i.e. 0), 20% weakly positive (light brown staining i.e. 1+), another 20% are moderately positive (brown staining i.e. 2+) and the remaining 10% are strongly positive (dark brown staining i.e. 3+). To calculate the histoscore, each percentage is multiplied by staining intensity (50 X 0=0; 20 X 1=20; 20 X 2=40; 10 X 3=30). The sum of these gives the histoscore (20+40+30=90). So the histoscore in this case is 90. Maximum histoscore that could be obtained is 300. The means of the two observer's scores were used for analysis. Protein expression was defined as increased or decreased in the tumours sections only if there was a difference between paired pre and post hormone refractory prostate cancer samples of greater than 2 X the mean observer variation in scores.

2.14 Statistics

2.14.1 Inter-observer variation

Mean histoscores for each protein assigned by the two observers (NSK & JE) were compared. Intra-class correlation coefficient (ICCC) and Pearson correlation coefficient were calculated. The Pearson correlation coefficient is a measure of linear association, rather than of agreement. A correlation coefficient of +1.00 signifies a perfect positive relationship. While –1.00 signifies a negative relationship. The ICCC is a true index of agreement between measurements. The criteria for assessing the degree of agreement were: ICCC<4- poor; ICCC ≥ 0.4 but <0.59-fair; ICCC ≥ 0.6 but < 0.74- good; ICCC of ≥ 0.75 is regarded as excellent.

Analysis of agreement for each set of measurements was conducted using the methods of Bland and Altman.This plot provides a direct impression of the agreement, distribution of the differences and detection of possible outliers. The mean difference between the two sets of measurements was obtained. This mean difference would be zero for optimal agreement between the measurements. 95% confidence intervals for the mean were calculated to assess the precision of the estimate of mean difference.

The differences between the two sets of measurements were assessed to determine that they followed a normal distribution. On establishing

normality , the 95% limits of agreement (mean difference ± 2 SD) the limits within which 95% of the differences would be expected to lie in the population were calculated.

This information was displayed graphically by plotting the differences against the means of the two sets of measurements. The mean difference and 95% limits of agreement were displayed on the graphs. Random scattering of the points above and below the mean line reflects no systematic bias between observers.

Scatter plots of one set of measurements against the other further illustrated the relationship between the two sets of measurements.

2.14.2 Correlation of Protein Expression

Spearman's rank test was used to correlate protein expression within the two groups (hormone sensitive and hormone refractory tumours). Spearman's rho is a measure of the linear relationship between two variables. Wilcoxon signed rank tests were used to compare expression between pre and post AIPCs.

The primary end points of the study time to relapse (measured from the time of response to treatment) and the time to death from relapse. The principal methods of analysis were of time to events. Univariate analyses used the log rank test with life table analysis of overall survival and time to death from relapse performed using Kaplan-Meier estimates.

2.14.3 Power Calculation

Statistical power is the probability of getting a statistically significant result given that there is a biologically real effect in the population being studied. If a particular test is not statistically significant, is it because there is no effect or because the study design makes it unlikely that a biologically real effect would be detected? Power analysis can distinguish between these alternatives, and is therefore a critical component of designing experiments and testing results.

Power analysis is most useful when planning a study. Such "prospective" power analyses investigate the relationship between the range of sample sizes that are thought to be feasible, effect sizes thought to be biologically important, levels of variance that could exist in the population (usually taken from the literature or from pilot data), and desired levels of a and statistical power. The result is a decision about the sample size and a level that will be used in the study, and the target effect size that will be "detectable" with the given level of statistical power. After the study is completed and the results analyzed, a "retrospective" power analysis can also be useful if a statistically non-significant result was obtained.

Protein	SD of difference in histoscore [Between Pre and Post AIPC]	Power to detect specified difference at 5% significance level for n=51 paired sets of measurements			
		Difference = 10	Difference = 20	Difference = 30	Difference = 40
c-Jun	64.9	19.0 %	57.9 %	89.9 %	99.1 %
P c-Jun	47.4	31.5 %	84.0 %	99.3 %	100.0 %
c-Fos	62.5	20.2 %	61.1 %	91.9 %	99.4 %
PKC	39.7	42.3 %	94.2 %	100.0 %	100.0 %
Cox-2	45.9	33.2 %	86.3 %	99.6 %	100.0 %
AR	61.8	20.5 %	62.0 %	92.5 %	99.5 %

With 51 paired sets of measurements (51 patients), a difference of 40 in the histoscores for each protein tested would be statistically significant with a power of 99%.

With a similar paired number of measurements, a difference of 30 in the histoscores for each protein tested would also be statistically significant with a power of 90%.

A generic power calculation was performed to estimate the size of the difference in protein expression between pre and post AIPCs that the study could reasonably be expected to detect. To perform this calculation it was assumed that the standard deviation of the difference in histoscores was 50 units for all proteins (based on pilot data) and that statistical significance would be assessed at the 5% level using Student's paired t-test.

No. of paired samples	**Power to detect specified difference at 5% significance level assuming a standard deviation of 50 histoscore units**			
	Difference = 10	**Difference = 20**	**Difference = 30**	**Difference = 40**
30	18.5	56.3	88.8	98.9 %
40	23.5	69.4	95.9	99.9%
50	28.4	79.2	98.6	100.0%
60	33.2	86.2	99.6	100.0%
70	37.9	91.0	99.9	100.0%
80	42.4	94.2	100.0	100.0%
90	46.7	96.4	100.0	100.0%
100	50.8	97.7	100.0	%

If the paired sample size is 40 or more and the difference in histoscore for each paired sample is over 30, then the difference would be statistically significant with a power of 95.9%.

CHAPTER 3

RESULTS

3.1 Patient information

3.1.1 Patient Inclusion Criteria

Based on the inclusion and exclusion criteria only 51 out of 1021 patients were suitable for the study. If all of the following criteria were satisfied then the patients were included in the study.

1. Patients diagnosed to have carcinoma of the prostate.
2. Prostate cancer tissue available at the time of diagnosis.
3. Patients should have received hormonal treatment and have their PSA values monitored.
4. Evidence of response to hormonal treatment as indicated by decreasing levels of serum PSA.
5. Subsequently developed hormone relapse disease as indicated by rising PSA levels in spite of continuing hormonal treatment.
6. Had TURP for symptoms of bladder outflow obstruction after developing relapse and their prostate cancer tissue available.

A total of 960 patients were excluded as they did not satisfy all of the above criteria. In total 102 (51 pairs) prostate cancer tissue samples were analysed.

The median age of the patients at diagnosis was 69 yrs (41-83 years). Median follow-up of the patients was 4.57 years (2.9-6.65) and median time to hormone relapse was 2.46 years (1.47-4.63).

3.1.2 Descriptive patient data

Study number, age of the patients, gleason sum, type of biopsy at diagnosis and at relapse, PSA nadir, PSA at relapse, PSA velocity, time to relapse, time from relapse to second biopsy, presence of bone metastases at diagnosis and development of bone metastases are illustrated below (Table 1-4).

Table1 Descriptive Patient Data

Study no	Age (yrs)	Gleason sum at diagnosis	Type of biopsy at	
			Diagnosis	Relapse
AR1&2	72	8	TURP	TURP
AR3&4	78	7	TRUCUT	TURP
AR5,6	69	5	TRUCUT	TURP
AR8&9	70	7	TURP	TURP
AR14&15	71	5	TURP	TURP
AR16&17	72	6	TURP	TURP
AR18&19	74	4	TURP	TURP
AR23-24	54	7	TURP	TURP
AR29&30	71	9	TURP	TURP
AR31 &32	66	10	TURP	TURP
AR33&34	62	9	TRUCUT	TURP
AR35&36	83	10	TRUCUT	TURP
AR37&38	67	8	TRUCUT	TURP
AR41&42	76	8	TRUCUT	TURP
AR43&44	68	9	TURP	TURP
AR45&46	76	7	TURP	TURP
AR47&48	69	6	TURP	TURP
AR49&50	64	8	TURP	TURP
AR51&52	70	6	TURP	TURP
AR55&56	73	9	TURP	TURP
AR57&58	79	7	TURP	TURP
AR59&60	63	7	TURP	TURP
AR61&62	81	7	TURP	TURP
AR63&64	77	7	TURP	TURP
AR65&66	75	9	TURP	TURP

Table 2 Descriptive Patient Data

Study no	Age (yrs)	Gleason sum at diagnosis	Type of biopsy at Diagnosis	Relapse
AR67&68	73	9	TURP	TURP
AR69,70	67	8	TRUCUT	TURP
AR72&73	63	7	TRUCUT	TURP
AR74&75	49	6	TURP	TURP
AR76&77	68	6	TRUCUT	TURP
AR78&79	68	6	TRUCUT	TURP
AR80&81	75	4	TURP	TURP
AR82&83	70	8	TURP	TURP
AR84&85	70	9	TURP	TURP
AR86&87	68	6	TURP	TURP
AR-88&89	67	7	TRUCUT	TURP
AR90&91	63	6	TURP	TURP
AR92&&93	74	8	TURP	TURP
AR96&97	74	7	TRUCUT	TURP
AR100&101	80	9	TRUCUT	TURP
AR102&103	98	10	TURP	TURP
AR104,105	70	6	TURP	TURP
AR107&108	68	9	TURP	TURP
AR109&110	73	9	TURP	TURP
AR111,112	67	6	TURP	TURP
AR114,115	63	8	TURP	TRUCUT
AR116,117	41	7	TRUCUT	TURP
AR118,119	59	9	TRUCUT	TURP
AR120,121	59	10	TURP	TURP
AR122,123	68	8	TURP	TURP
AR124,125	62	7	TRUCUT	TURP

Table 3 Descriptive Patient Data

Study no	PSA Nadir ng/ml	PSA at Relapse ng/ml	PSA Velocity ng/ml/yr	Time to Relapse (days)	Time from PSA relapse to 2nd biopsy in days	Bone Metastases at Diagnosis/Relapse
AR1&2			4.97	836	108.00	
AR3&4	37	470	216	184	0.00	
AR5,6	1.2	6.4	1.16	1914	9.00	
AR8&9	1.3	64	62.7	24	6.00	
AR14&15	7.6	19.4	11.8	1451	102.00	
AR16&17	6.4	39.3	14.65	2062	35.00	
AR18&19	0.9	29	29.2	2051	156.00	+
AR23-24	1	6	1.66	2069	18.00	+
AR29&30	13.4	38	45.25	1678	22.00	
AR31 32	15.6	43.7	21	861	323.00	
AR33&34	7.9	30.6	9.24	1237	22.00	
AR35&36	0.8	2.6	3.9	537	28.00	
AR37&38	5.5	7.2	1.3	3027	4.00	
AR41&42	85.2	796.4	683.6	678	8.00	+ +
AR43&44	0.5	6.4	7.75	834	20.00	
AR45&46	126	243	43.6	820	94.00	+
AR47&48	1.9	7.7	1.15	605	109.00	+
AR49&50	35	384	107.2	365	62.00	+ +
AR51&52	7.5	16.6	11.7	85	268.00	+
AR55&56	26	101	39	692	252.00	
AR57&58	0.1	19.8	4.9	1641	14.00	+
AR59&60	14.5	106	238.1	1189	313.00	
AR61&62	2.0	58	11	957	129.00	+
AR63&64	10.7	29	20.8	496	389.00	+
AR65&66	0.1	1.3	29.9	1305	147.00	

Table 4 Descriptive Patient Data

Study no	PSA Nadir ng/ml	PSA at Relapse ng/ml	PSA Velocity ng/ml/yr	Time to Relapse In days	Time from PSA relapse to 2nd biopsy in days	BoneMetastases at Diagnosis/Relapse
AR67&68	0.6	20.0	36.2	329	119.00	+ +
AR69,70	0.2	3.1	28.8	1091	308.00	
AR72&73	0.1	2.2	8.6	2032	543.00	+
AR74&75	1.6	4.5	12.5	792	1216.00	
AR76&77	0.9	2.6	32.2	546	602.00	+ +
AR78&79	0.1	1.9	2.9	3202	215.00	
AR80&81	4.1	6.1	8.4	431	74.00	
AR82&83	1.3	8.7	11.8	467	140.00	+ +
AR84&85	6.1	25.5	8.6	1746	81.00	+
AR86&87	5.0	31.8	24.3	415	77.00	
AR-88&89	2.0	13.1	8.6	5306	81.00	+
AR90&91	1.0	7.0	15.9	1689	118.00	
AR92&&93	0.8	4.5	1.9	899	172.00	
AR96&97	0.4	1.5	1.4	1500	1174.00	
AR100&101	4.9	22.7	50.2	100	172.00	
AR102&103	7.6	261	252.4	421	10.00	
AR104,105	0.1	1.2	0.67	3400	1.00	
AR107&108	3.3	33.6	34.5	646	361.00	
AR109&110	1.7	27.1	24.2	857	58.00	
AR111,112	18.2	34.3	11.2	4230	0.00	
AR114,115	29	114	247.2	47	183.00	+ +
AR116,117	4.8	22.4	12.7	902	322.00	
AR118,119	3.9	33.4	61.7	425	45.00	+
AR120,121	11.1	41.4	30.3	306	35.00	+
AR122,123	8.1	24	125.3	1087	175.00	+
AR124,125	2.5	5.6	489.8	576	32	+ +

3.1.3 Statistical Association Between PSA and Time to Relapse & Survival

There was a statistically significant difference in time to relapse in prostate cancer patients whose PSA level at diagnosis was >10ng/ml compared to those with PSA <10ng/ml (p= 0.037) (Figure 8a).

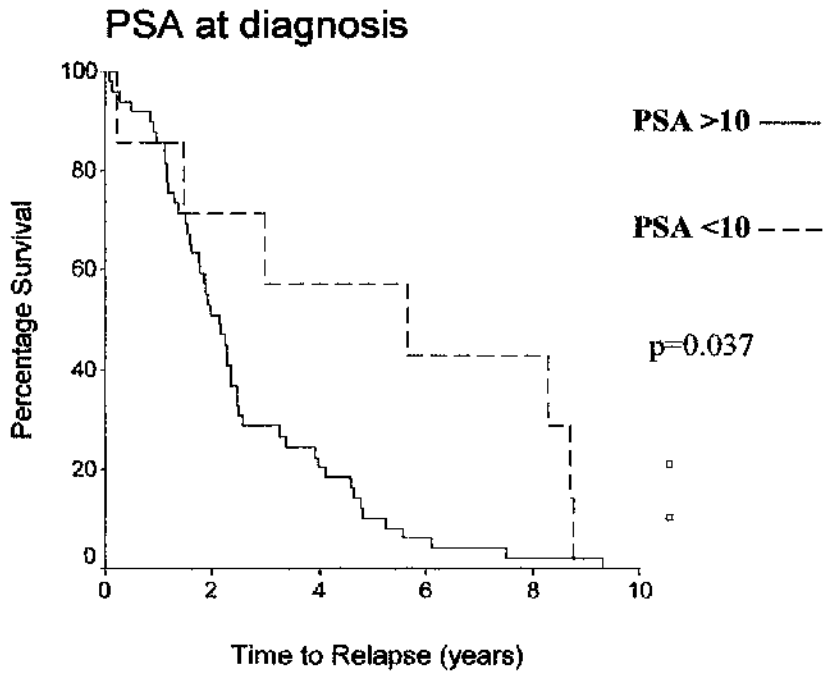


Figure 8a- Kaplan-Meier Plot showing time to relapse in Prostate cancer patients with PSA levels <10ng/ml and >10ng/ml at diagnosis. Patients with PSA >10ng/ml at diagnosis relapsed earlier compared to those with PSA <10ng/ml (P= 0.037).

PSA levels at diagnosis did not have a significant effect on overall survival (p=0.076) (figure 8b).

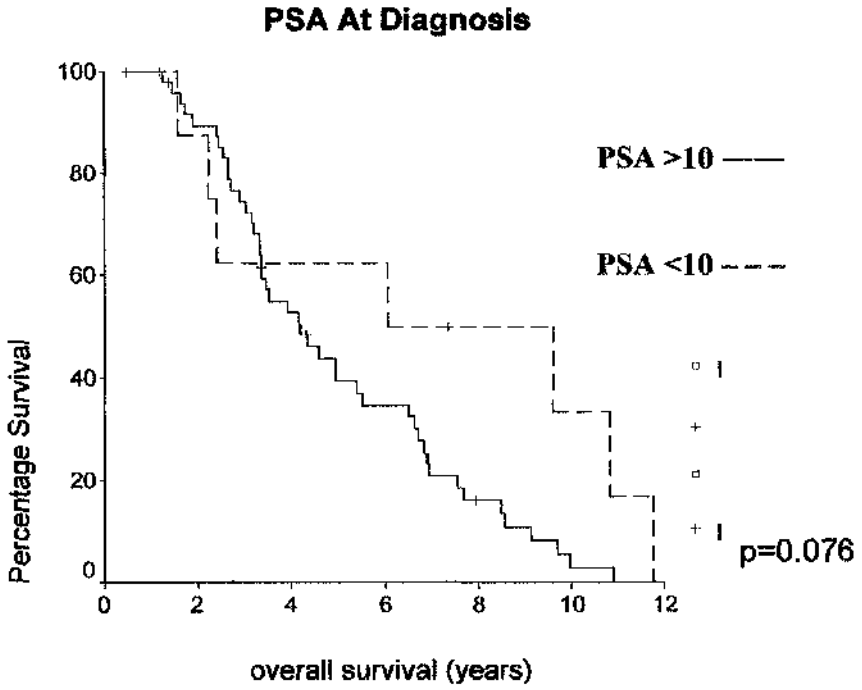


Figure 8b- Kaplan-Meier Plot showing over all survival in Prostate cancer patients with PSA levels <10ng/ml and >10 ng/ml at diagnosis. No significant difference was observed (p=0.076).

3.1.4 Statistical Association Between Gleason sum and Time to Relapse & Survival

Mean Gleason sum at diagnosis was 7.63 (STDEV=1.40) and after the development of androgen independent disease it was 8.44 (STDEV=1.16). Gleason sum in androgen sensitive tumours was significantly lower than in androgen independent tumours ($p<0.001$) (Table 5).

Table 5 Gleason sum in androgen sensitive and androgen independent prostate tumours

Gleason sum	Number of androgen sensitive prostate tumours	Number of androgen independent prostate tumours	p-value
≤6	24% (12/51)	4% (5/51)	
=7	27% (14/51)	12% (7/51)	
≥8	49% (25/51)	78% (40/51)	<0.001

We also correlated Gleason sum at diagnosis with time to relapse as well as overall survival. There was no significant difference in time to relapse i.e. development of androgen independent prostate cancer (figure 8c).

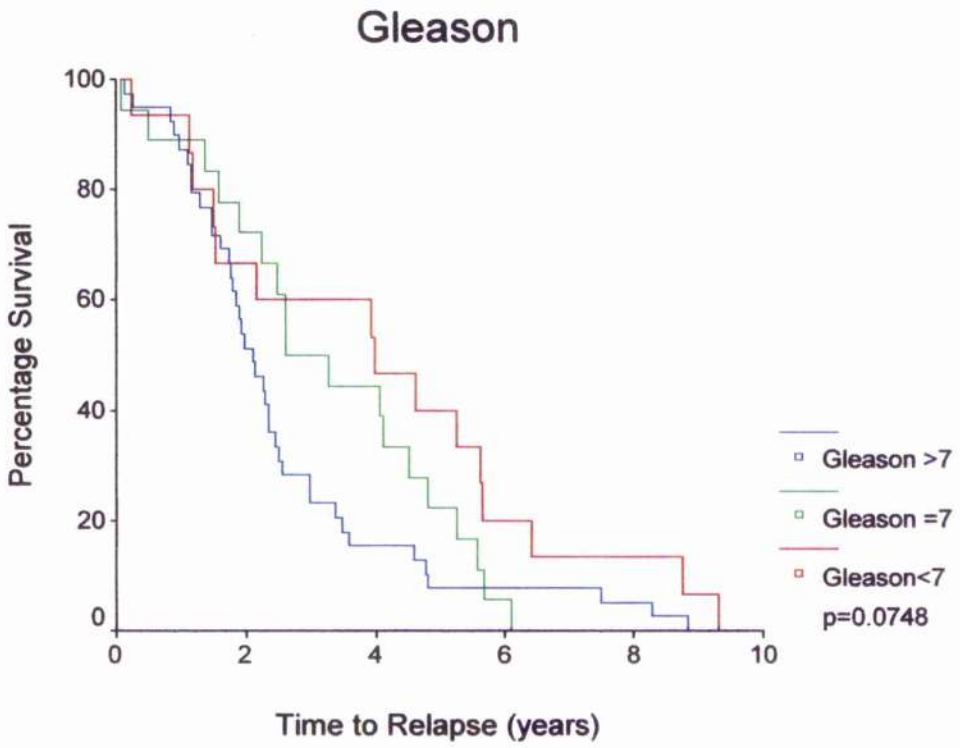


Figure 8c- Kaplan-Meier Plot showing no difference in time to relapse, in patients with Gleason sum <7, =7 or >7 at the time of diagnosis of prostate cancer (p=0.075).

Gleason sum however had a significant effect on overall survival. Those patients with gleason sum >7 had a shorter overall survival that was statistically significant (p=0.05).

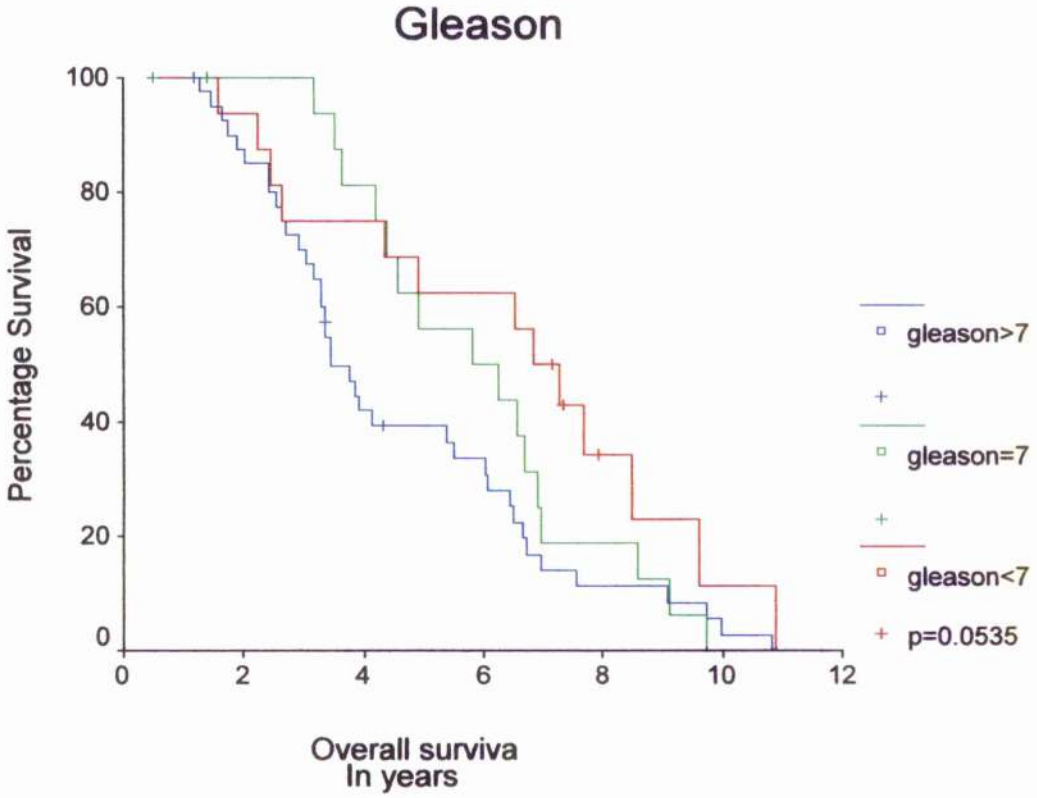


Figure 8d- Kaplan-Meier Plot showing overall survival in prostate cancer patients who had different Gleason sum at diagnosis (Gleason <7 ,=7 & >7). The difference in survival in the three groups was significant (p=0.05).

3.2 Protein location and expression in prostate tumours.

3.2.A Site of protein expression in prostate tumours

c-Jun and c-Fos proteins expression was observed in the cytoplasm and nucleus of the tumour cells. However phosphorylated c-Jun and AR expression was observed only in the nucleus. PKC and COX-2 protein expression was observed only in the cell cytoplasm (Figures 11a-f).

3.2.B Inter-observer variation

3.2.B.1 Correlation of histoscores assigned by two independent observers

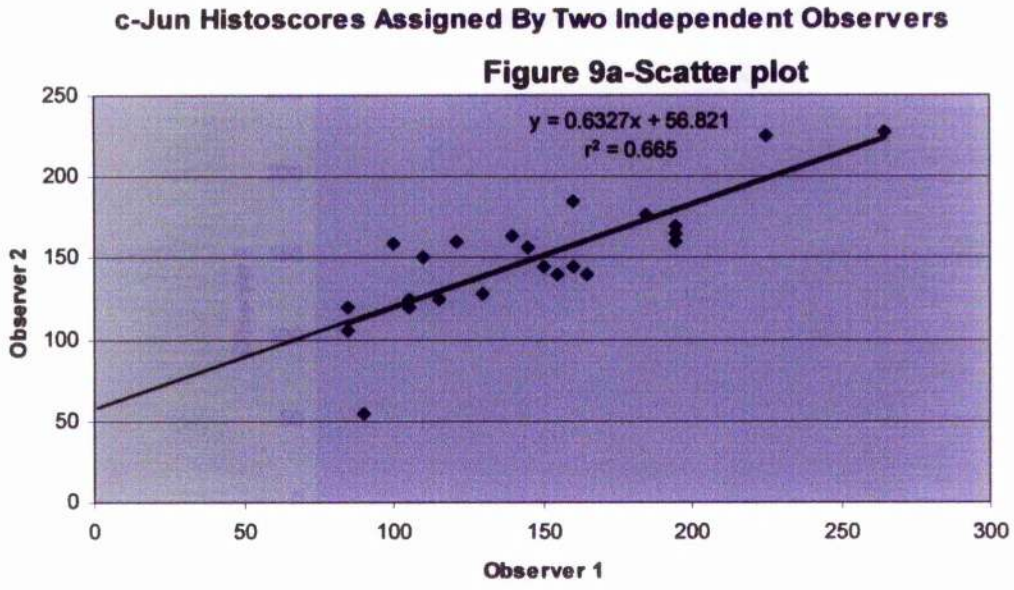
Evaluating inter-observer variation was a specific objective of the study. Intensity of protein expression was scored by two independent observers (NSK and JE). The scores assigned by the observers were then compared. The mean difference plus 2 standard deviations, the inter-class correlation coefficient (ICCC) and Pearson correlation coefficient (PCC) for each protein investigated are shown in Table 6.

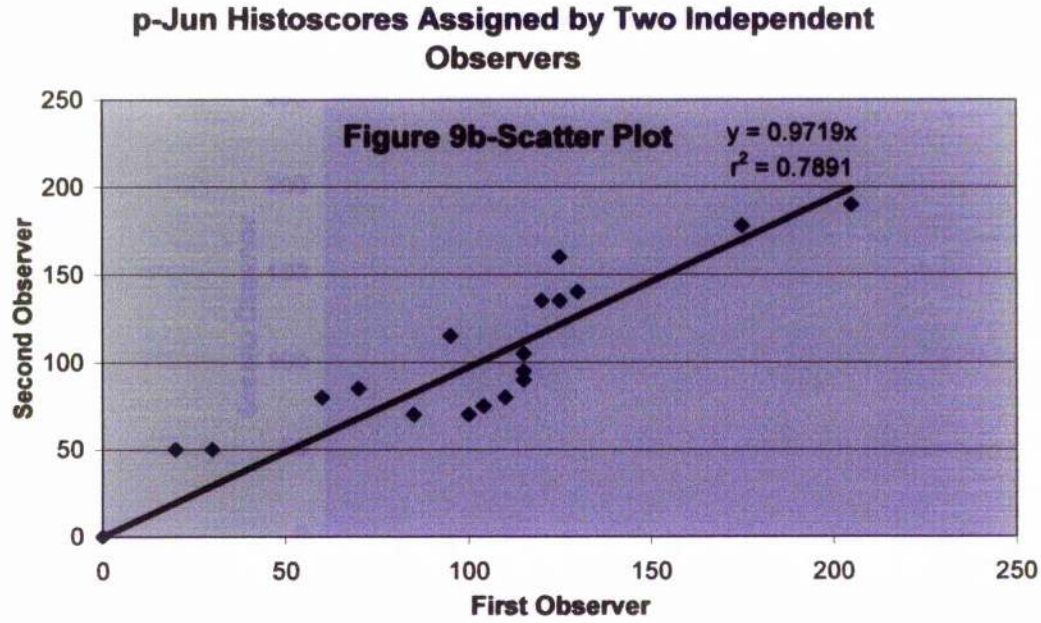
The ICCC is a true index of agreement between the scores assigned by both observers. All the proteins that were investigated had ICCC's greater than 0.75, which is regarded as an excellent correlation (Table 6). However for AR the ICCC was 0.68 which is regarded as a good correlation.

The PCC was gained from scatter diagram which illustrates the relationship between protein histoscores assigned by the two observers. The square root of r^2 indicates correlation between the histoscores assigned by the two observers (figures 9a-f).

Table 6- Mean difference plus 2SD of histoscores assigned by two independent observers, ICCC & PCC are shown

Protein	Mean difference plus 2 standard deviations	ICCC	PCC
c-Jun	57.7	0.79	0.82
P c-Jun	64.78	0.90	0.90
c-Fos	58.1	0.90	0.91
PKC	16.6	0.87	0.86
COX-2	33.4	0.89	0.90
AR	29.98	0.68	0.87

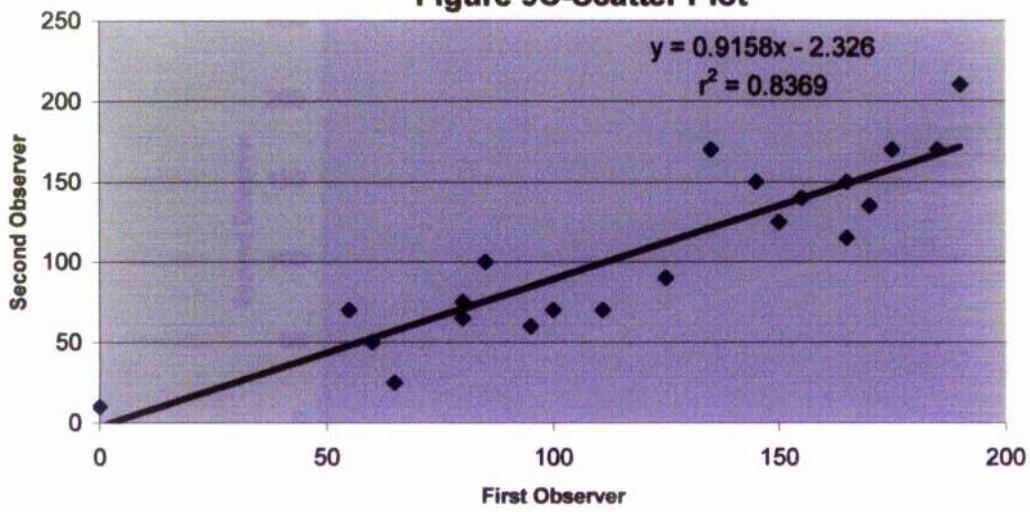




The above scatter diagrams illustrate the relationship between protein histoscores assigned by the two observers. PCC is the square root of r^2 and it indicates correlation between the histoscores assigned by the two observers. It is a measure of linear association.

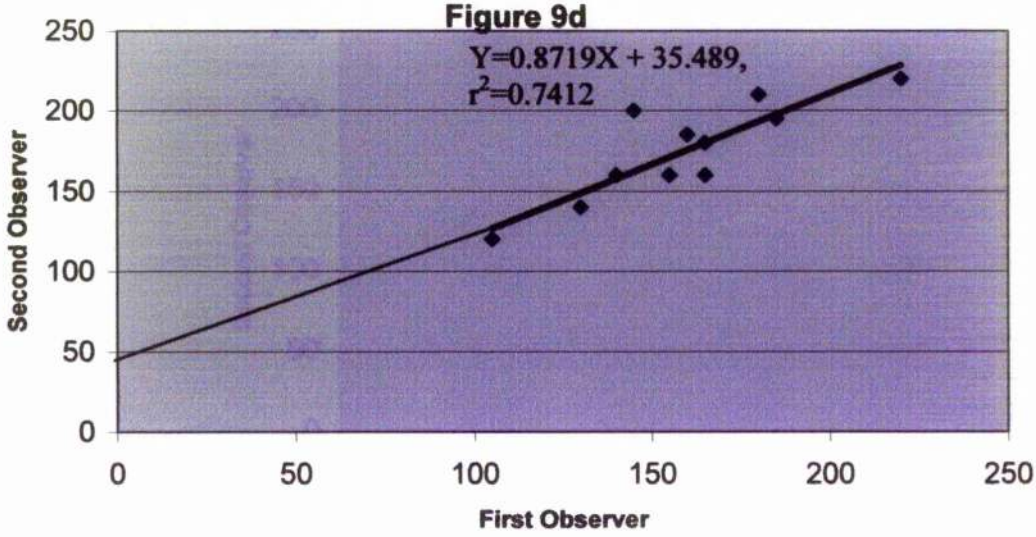
c-Fos Histoscores Assigned By Two Independent Observers

Figure 9C-Scatter Plot



Scatter Diagram Of PKC Histoscores Assigned By Two Independent Observers

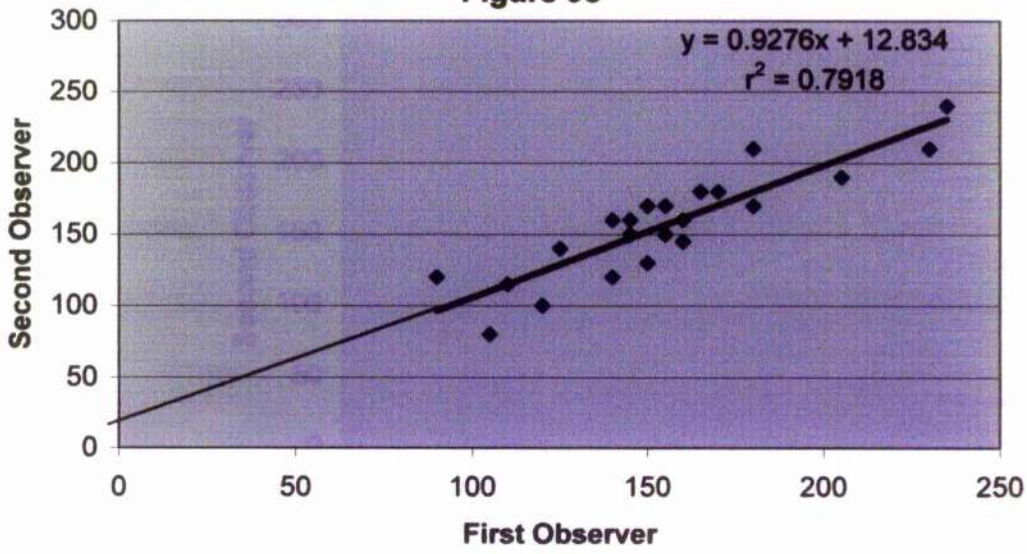
Figure 9d



The above scatter diagrams illustrate the relationship between protein histoscores assigned by the two observers. PCC is the square root of r^2 and it indicates correlation between the histoscores assigned by the two observers. It is a measure of linear association.

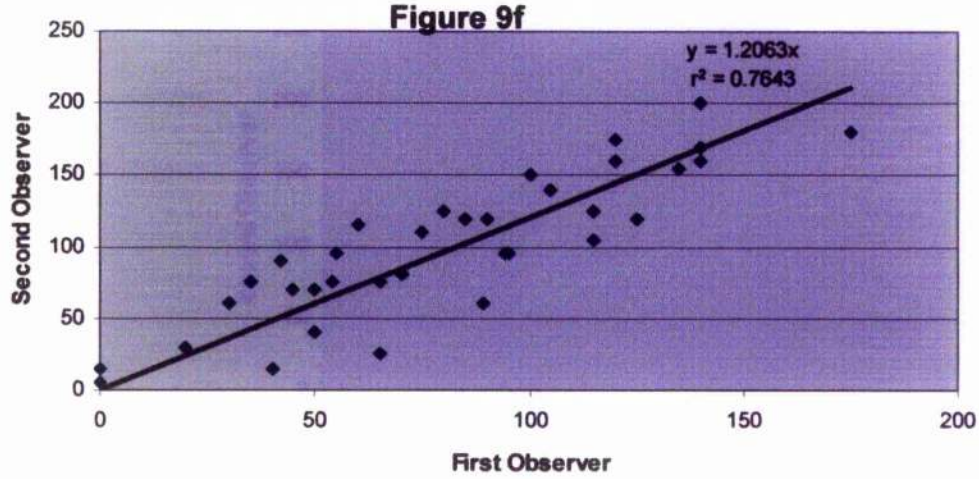
Scatter Diagram Of COX-2 Histoscores Assigned By Two Independent Observers

Figure 9e



Scatter Diagram of AR Histoscores Assigned By Two Independent Observers

Figure 9f

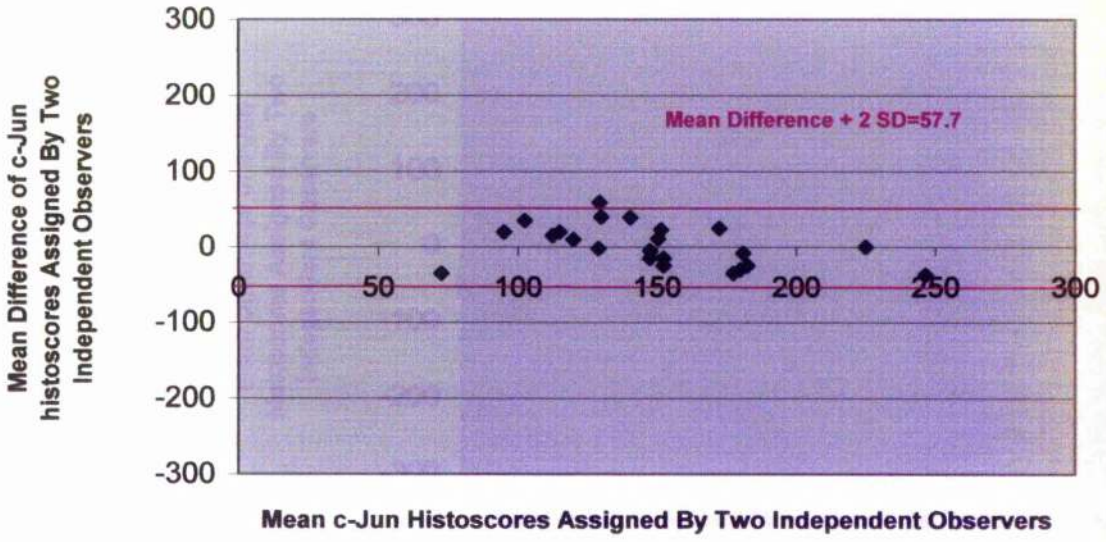


The above scatter diagrams illustrate the relationship between protein histoscores assigned by the two observers. PCC is the square root of r^2 and it indicates correlation between the histoscores assigned by the two observers. It is a measure of linear association.

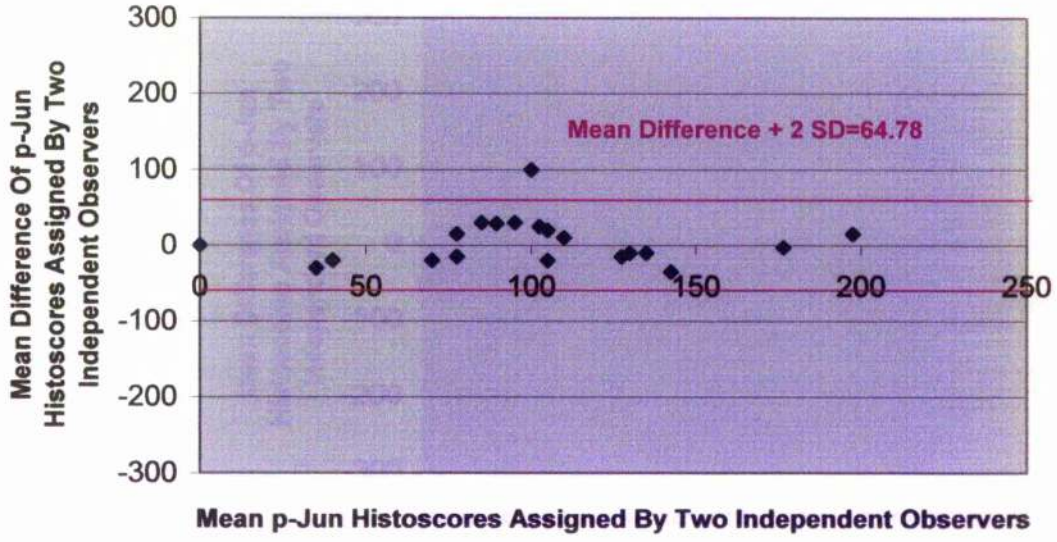
3.2.B.2 Analysis of agreement for each set of histoscores assigned by the two independent observers

This was conducted using the methods of Bland and Altman. The information is displayed graphically. The differences between histoscores assigned by observers are plotted against the means of the observer's histoscores. If the points in the graph are randomly scattered above and below the zero line, then this reflects no systematic bias in the method. The graph provides a direct visual impression of the agreement between the observer's scores, distribution of the differences in scores and detection of possible outliers. Bland and Altman plots for each protein investigated are shown in figures 10a-f.

Bland And Altman Plot For c-Jun Histoscores
Figure 10a

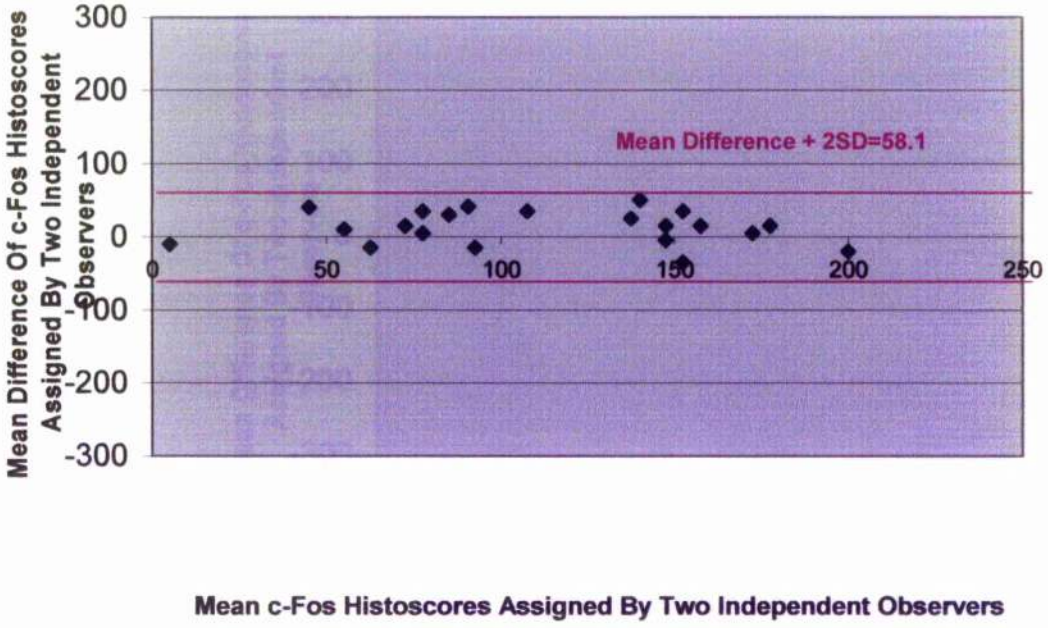


Bland And Plot For p-Jun Histoscores
Figure 10b

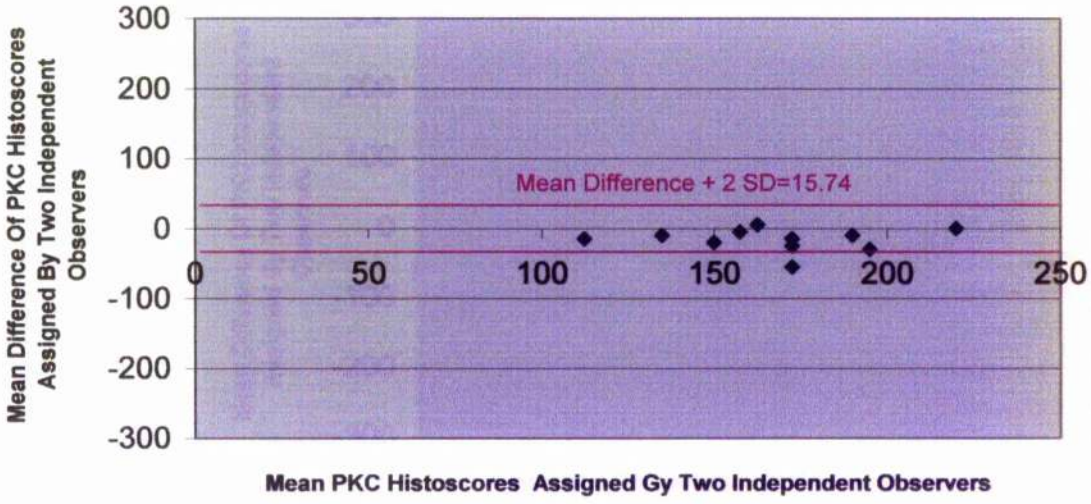


In the above Bland & Altman plots- mean histoscores of two independent observers are randomly scattered above & below the zero line reflecting no systemic bias. The mean difference + 2SD line is marked.

Bland And Altman Plot For c-Fos Histoscores
Figure 10c

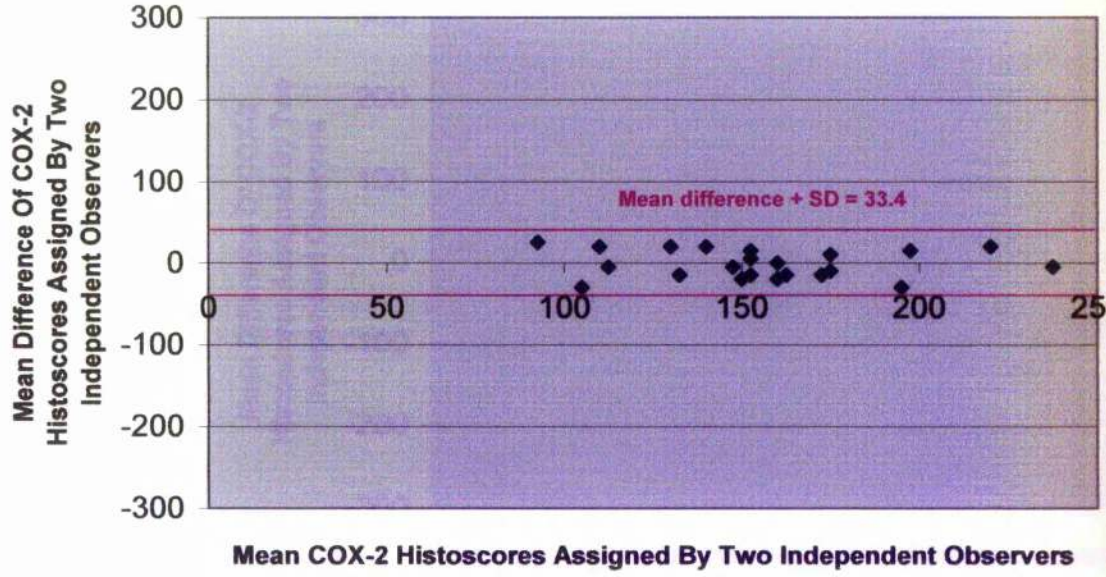


Bland And Altmann Plot For PKC Histoscores
Figure 10d

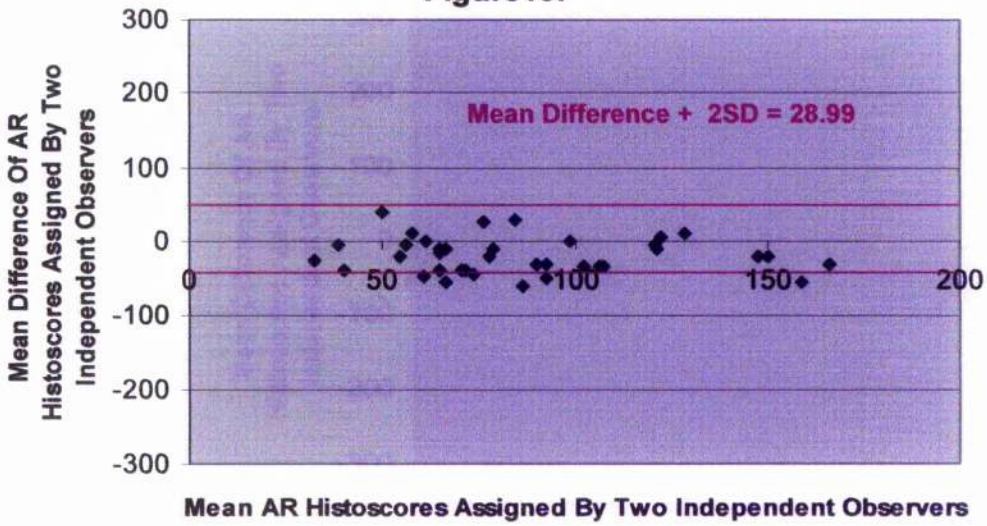


In the above Bland & Altman plots- mean histoscores of two independent observers are randomly scattered above & below the zero line reflecting no systemic bias. The mean difference + 2SD line is marked.

Bland And Altman Plot For COX-2 Histoscores
Figure 10e



Bland And Altman Plot For AR Histoscores
Figure10f



In the above Bland & Altman plots- mean histoscores of two independent observers are randomly scattered above & below the zero line reflecting no systemic bias. The mean difference + 2SD line is marked.

Figure 11 a c-Jun protein expression (brown staining in the cytoplasm and nucleus)

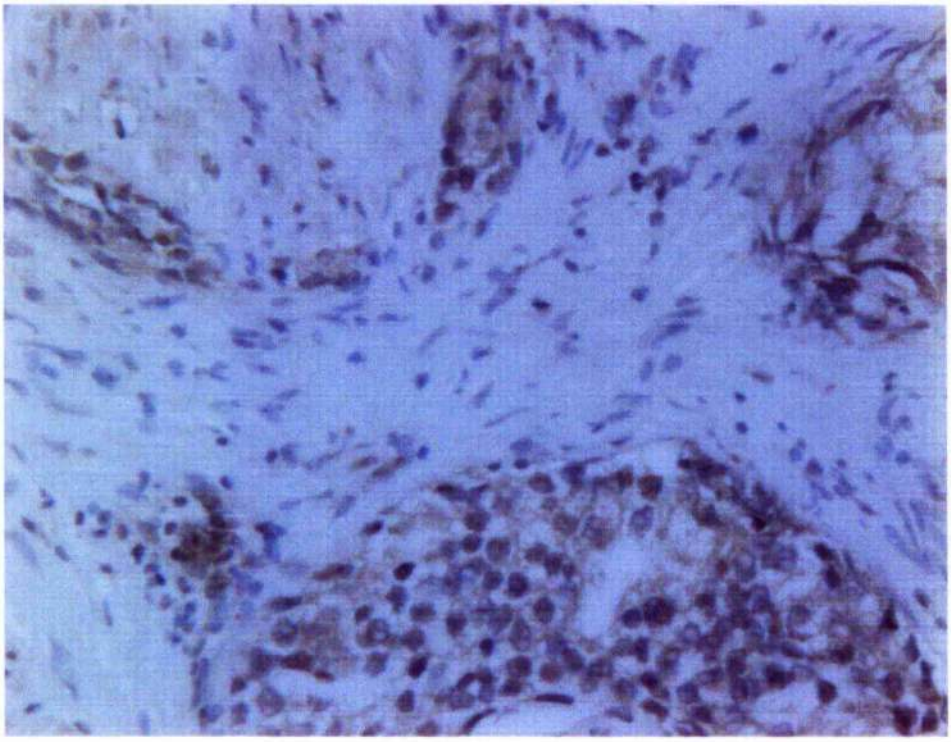


Figure 11 b Phosphorylated c-Jun protein expression (brown staining in the nucleus)

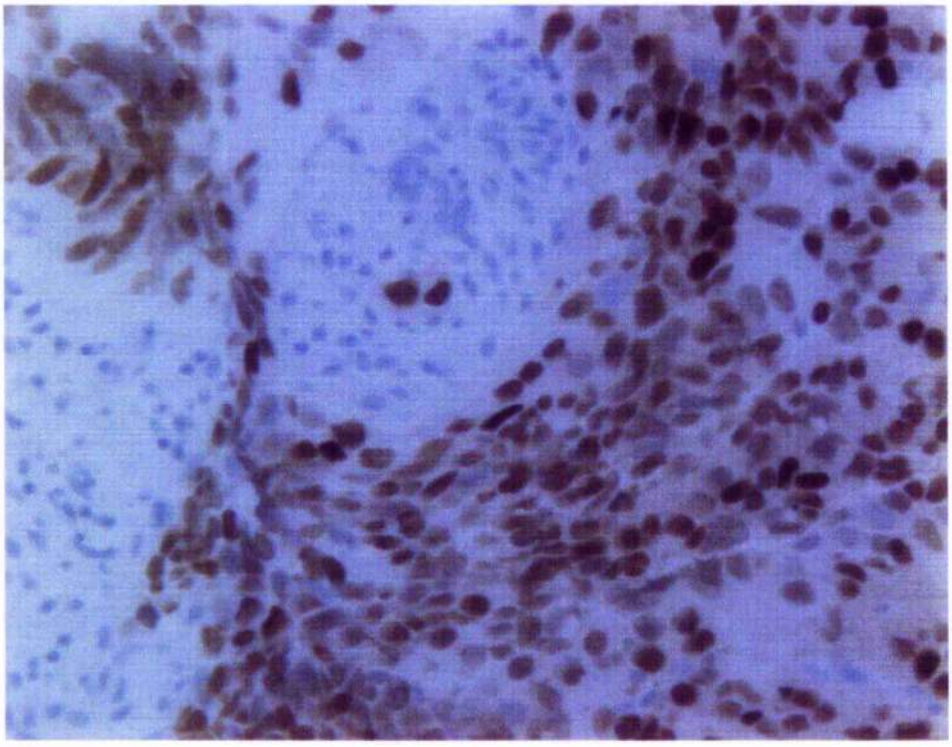


Figure 11 c c-fos protein expression (brown staining in the cytoplasm and nucleus)

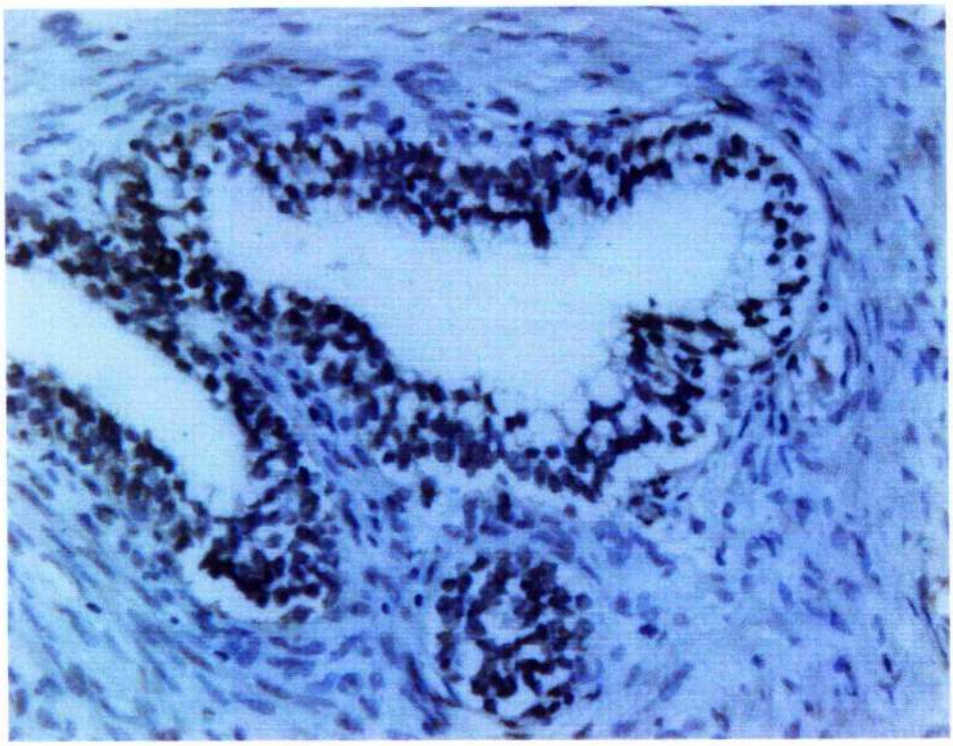


Figure 11 d PKC protein expression (brown staining in the cytoplasm

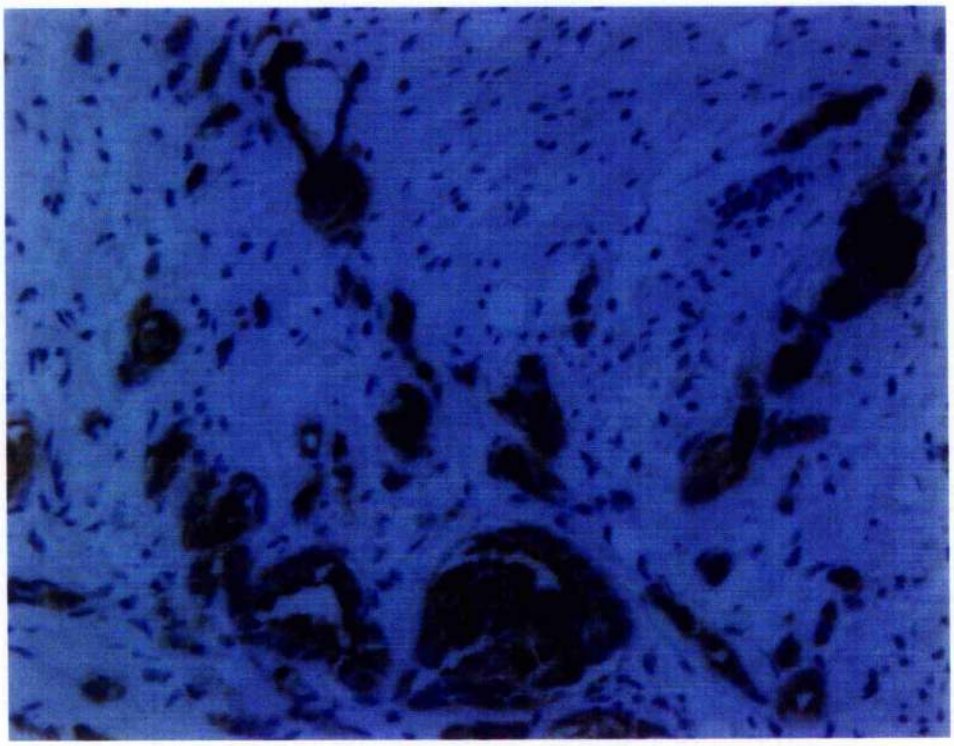


Figure 11 e Cox-2 protein expression (brown staining in the cytoplasm)

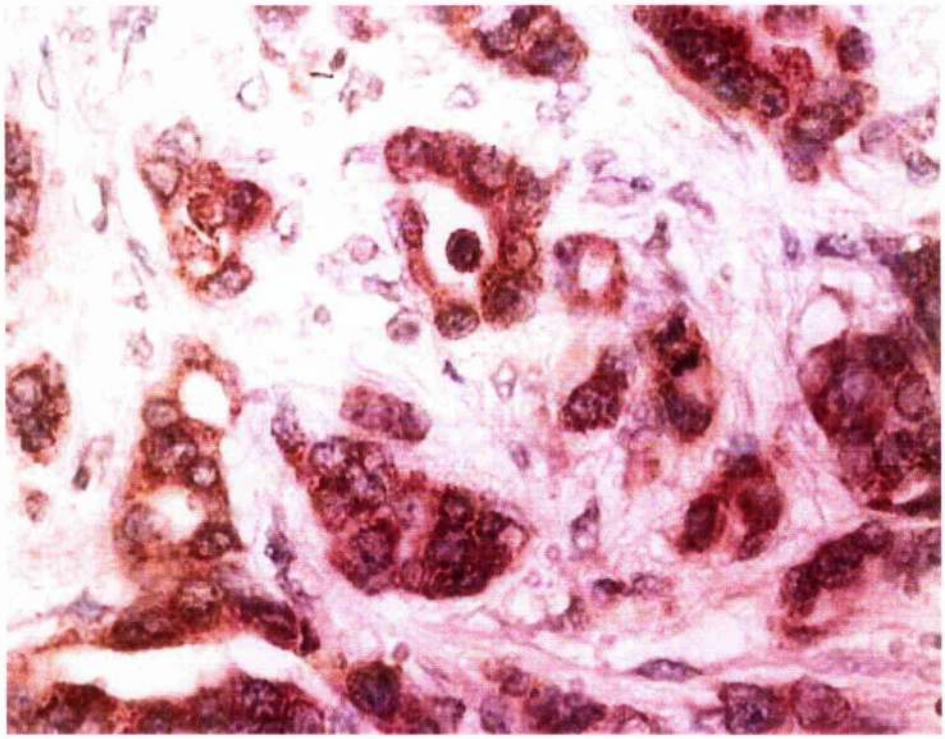
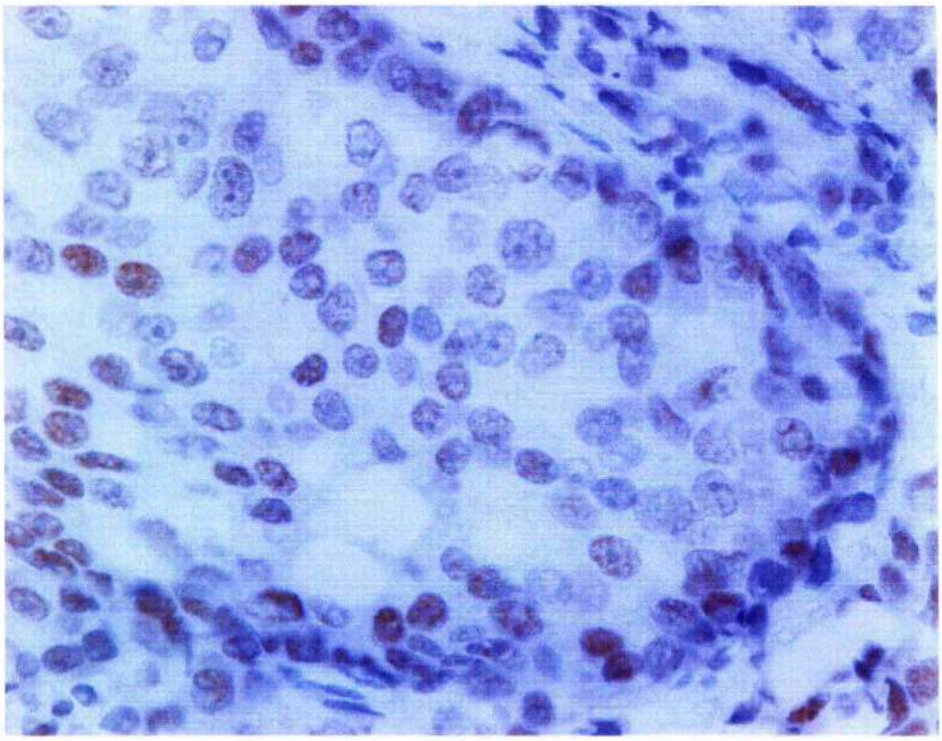


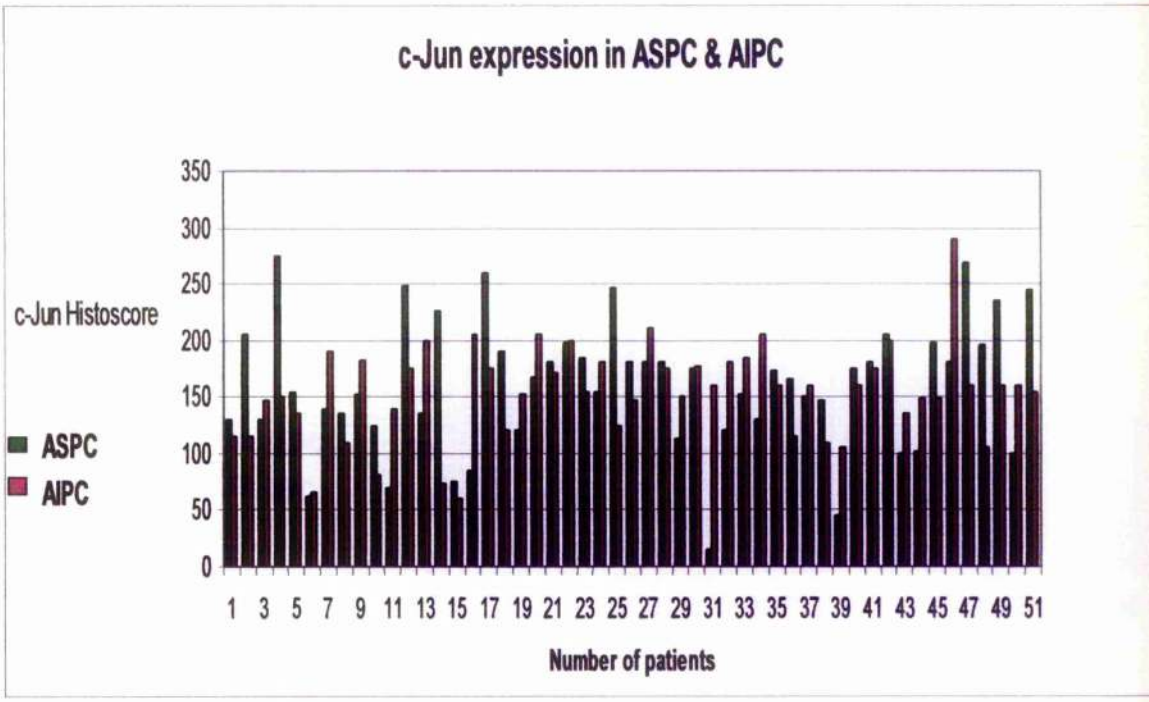
Figure 11 f AR protein expression (brown staining in the nucleus)



3.3.A Distribution of the staining intensity (histoscores) for androgen sensitive and androgen independent prostate tumours

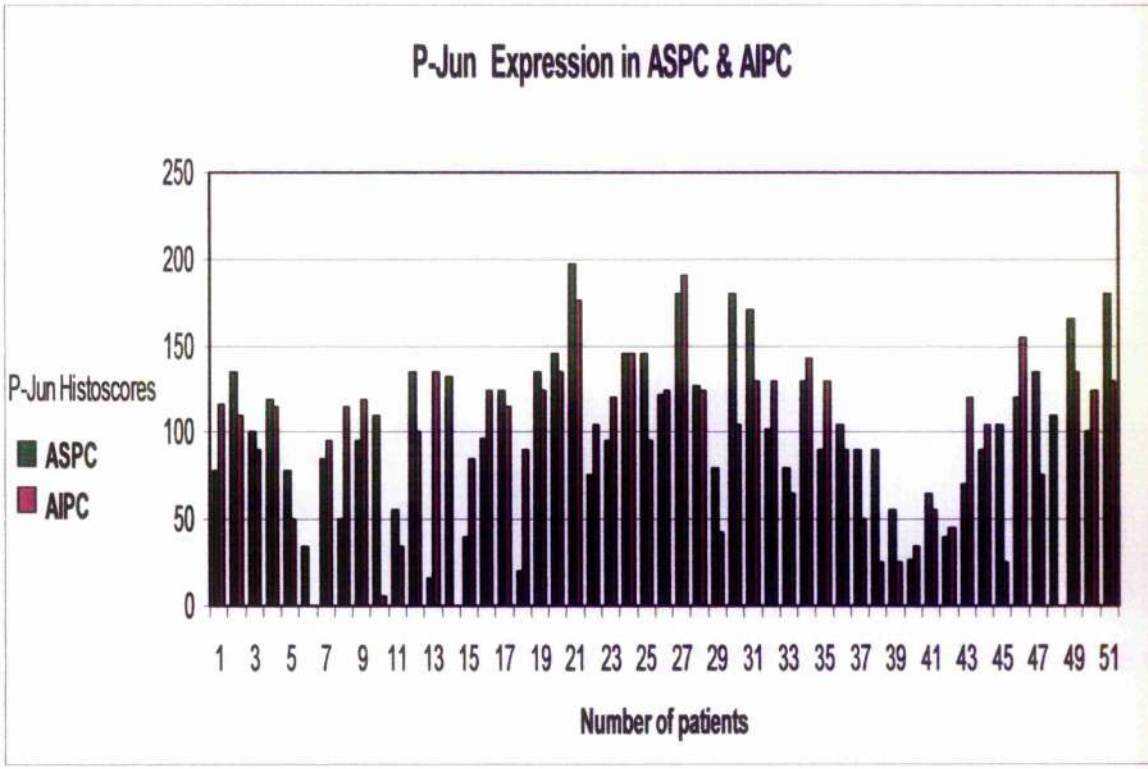
The histograms depicted below, demonstrate the distribution of staining intensity (histoscores) for androgen sensitive and androgen independent prostate tumours for each protein investigated (Figures 12A-F). None of the protein expressions shown in the histograms had a normal distribution in AIPC, therefore all statistical tests used in this study were non parametric. The median histoscore and interquartile ranges (Summary statistics) for each protein investigated are shown in table 7. No significant difference in c-Jun, p-Jun, c-Fos, PKC and COX-2 protein expression was observed for any of the proteins investigated in the transition from ASPC to AIPC. There was a significant increase in AR protein expression in the transition from ASPC to AIPC.

Figure 12 A- c-Jun Histoscores in Androgen Sensitive And Androgen Independent Prostate Cancers.



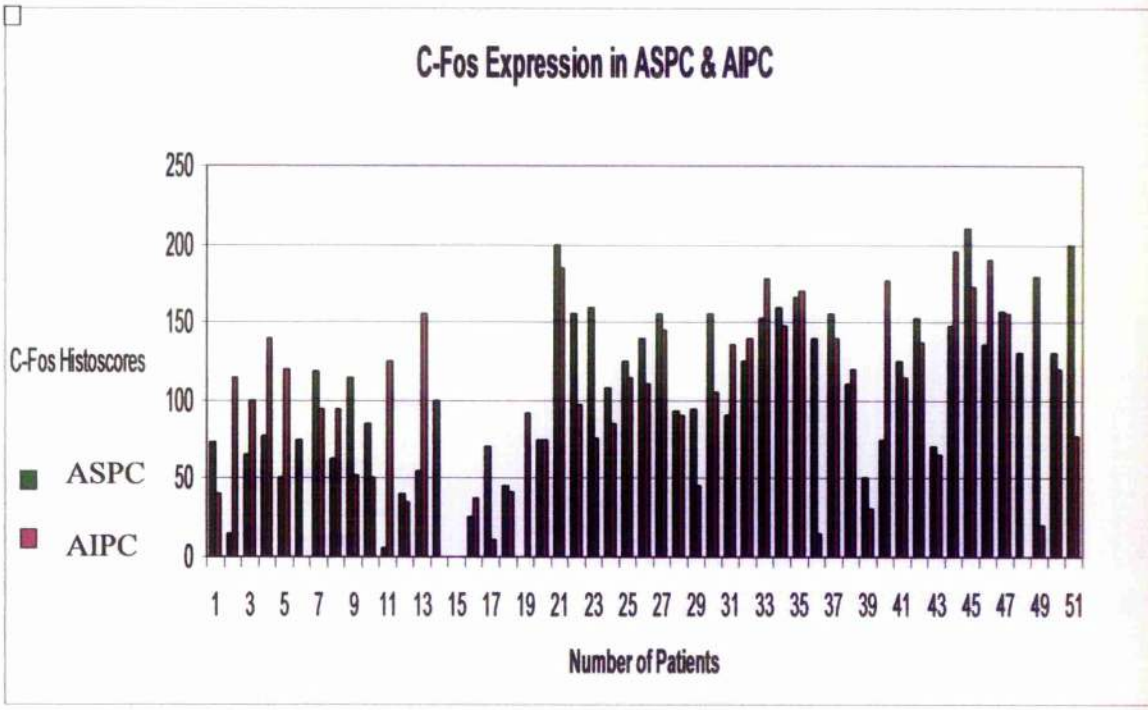
The above histogram illustrates c-Jun histoscores in ASPC (Green bars) and AIPC (Pink). The distribution of histoscores was not a normal distribution.Increase in c-Jun expression was observed in 15.7%, no change in 64.7% and decrease in 19.6% at relapse (development of AIPC.) c-Jun histoscores scores are given in Appendix III.

Figure 12 B p-Jun Histoscores in Androgen Sensitive And Androgen Independent Prostate Cancers.



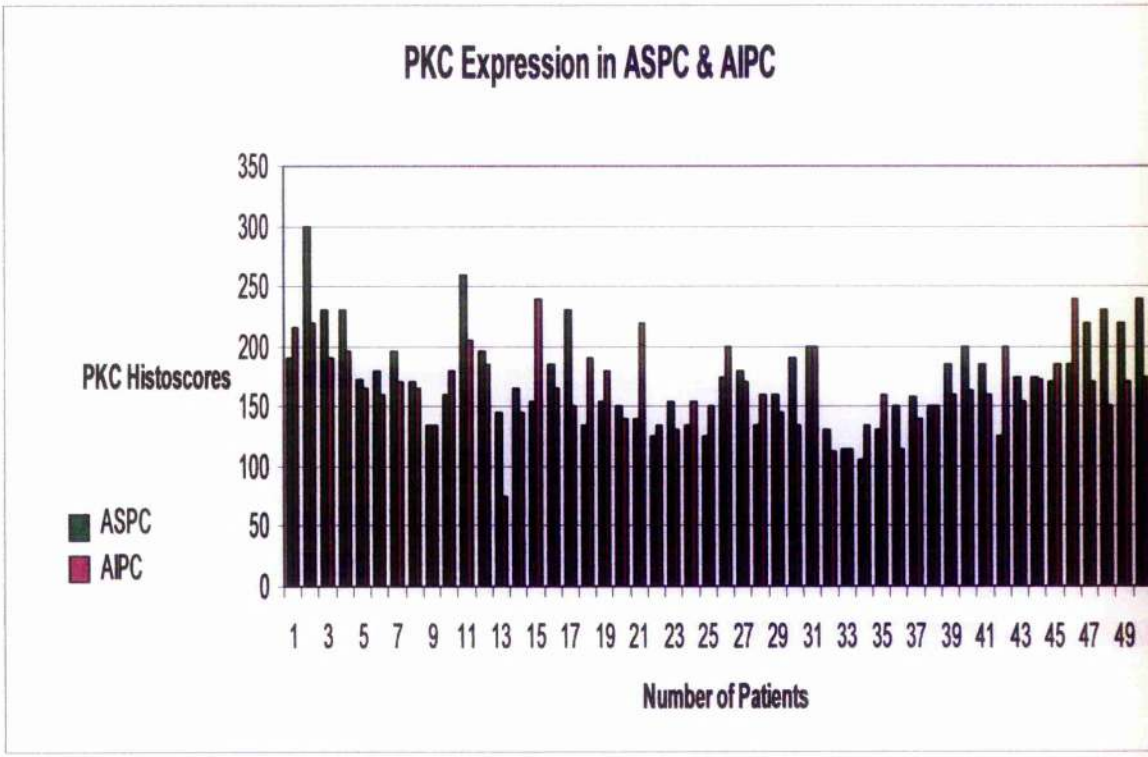
The above histogram illustrates p-Jun histoscores in ASPC (Green bars) and AIPC (Pink). The distribution of histoscores was not a normal distribution.Increase in p-Jun expression was observed in 7.8%, no change in 82.3% and decrease in 9.8% at relapse (development of AIPC.) p-Jun histoscores scores are given in Appendix IV.

Figure 12 C c-Fos Histoscores in Androgen Sensitive And Androgen Independent Prostate Cancers.



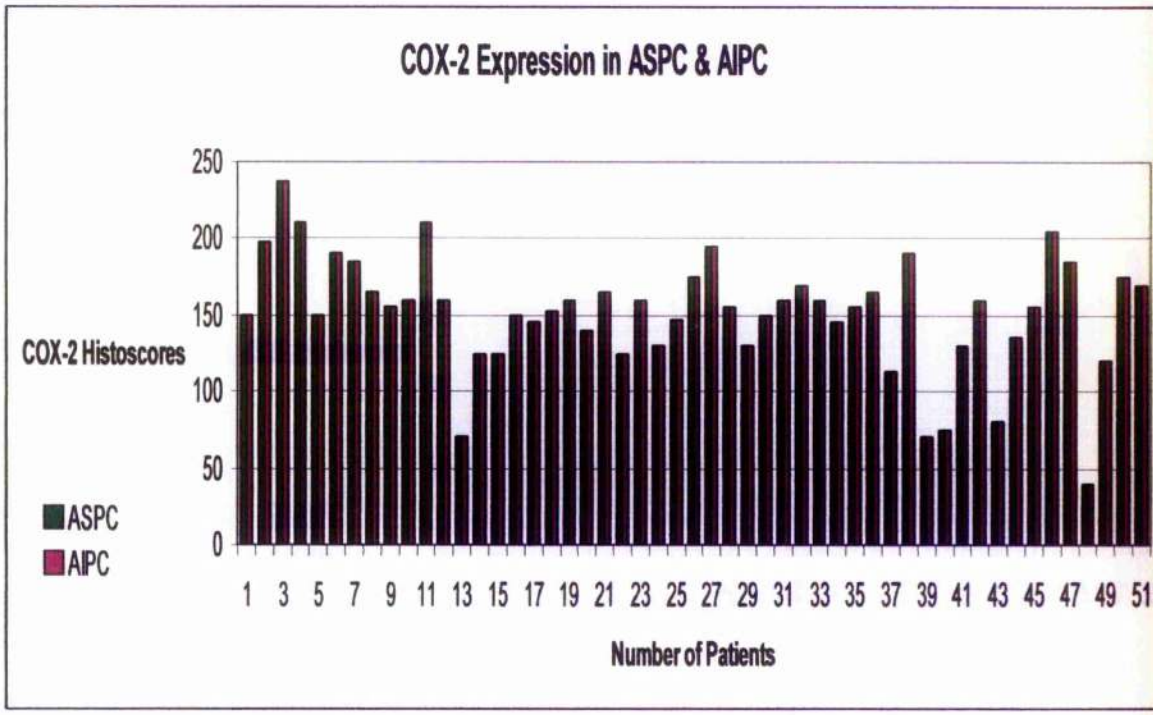
The above histogram illustrates c-Fos histoscores in ASPC (Green bars) and AIPC (Pink). The distribution of histoscores was not a normal distribution.Increase in c-Fos expression was observed in 13.7%, no change in 68.6% and decrease in 17.6% at relapse (development of AIPC.) c-Fos histoscores scores are given in Appendix V.

Figure 12 D PKC-Jun Histoscores in Androgen Sensitive And Androgen Independent Prostate Cancers.



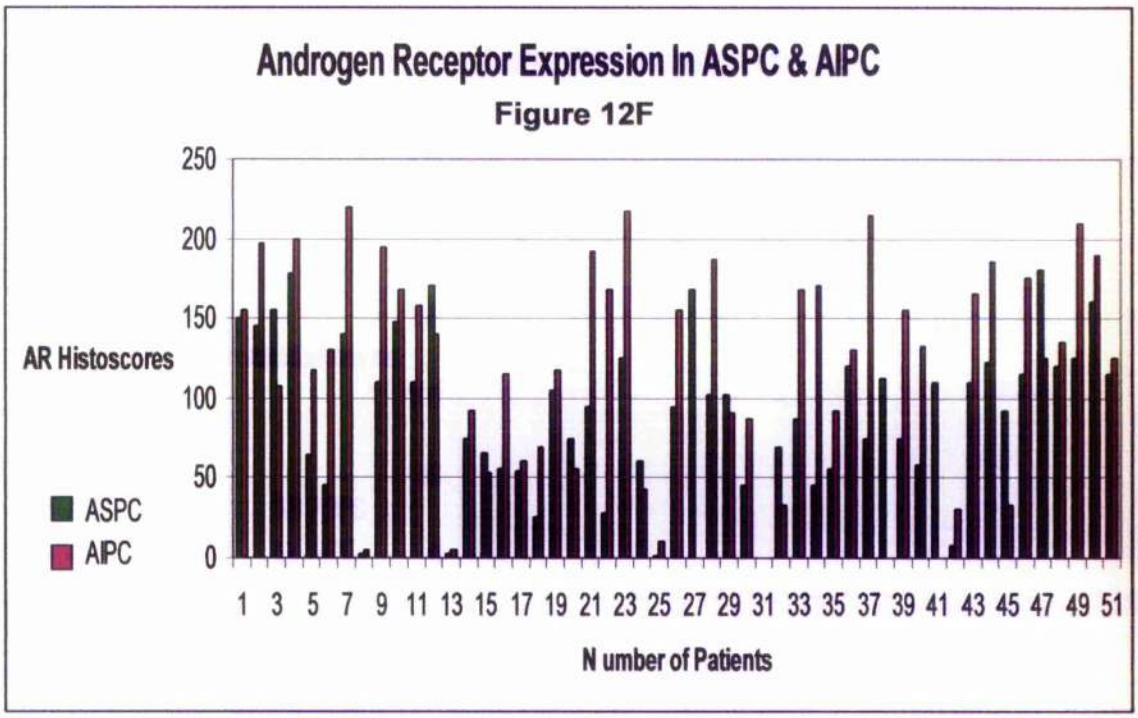
The above histogram illustrates PKC histoscores in ASPC (Green bars) and AIPC (Pink). The distribution of histoscores was not a normal distribution.Increase in PKC expression was observed in 27.5%, no change in 27% and decrease in 45% at relapse (development of AIPC.) PKC histoscores scores are given in Appendix VI.

Figure 12 E COX-2 Histoscores in Androgen Sensitive And Androgen Independent Prostate Cancers.



The above histogram illustrates COX-2 histoscores in ASPC (Green bars) and AIPC (Pink). The distribution of histoscores was not a normal distribution.Increase in COX-2 expression was observed in 33.3%, no change in 39.2% and decrease in 27.5% at relapse (development of AIPC.) COX-2 histoscores scores are given in Appendix VII.

Figure 12 F Androgen Receptor Histoscores in Androgen Sensitive And Androgen Independent Prostate Cancers.



The above histogram illustrates AR histoscores in ASPC (Green bars) and AIPC (Pink). The distribution of histoscores was not a normal distribution.Increase in AR expression was observed in 50.98%, no change in 39.2% and decrease in 9.8% at relapse (development of AIPC.) AR histoscores scores are given in Appendix VIII.

Table 7 Protein expressions in androgen sensitive and independent prostate cancer

Protein	ASPC median histoscore (1^{st} - 3^{rd} quartile)	AIPC median histoscore (1^{st} - 3^{rd} quartile)	p value
c-Jun	155 (129-185)	160 (143.75-181.75)	0.882
P c-Jun	101 (77.5-132.8)	105 (57.5-125)	0.388
c-Fos	111 (66.88-154.4)	100 (52-140)	0.642
PKC	172 (147.5-195)	164 (146.25-185)	0.125
COX-2	145 (122.5-175)	155 (132.5-170)	0.957
AR	94.5 (55-120)	132.5 (89.25-172.5)	0.000

3.3.B Expression of c-Jun, c-Fos, Cox-2 & AR in BPH tissue

c-Jun, c-Fos, AR and COX-2 Proteins were expressed in the BPH tissue on immunohistochemical examination. Median histoscores of protein expressions are given in table 8.

Table 8 Expression of c-Jun, c-Fos, AR and COX-2 Proteins in BPH Tissue

NO	C JUN HISTOSCORE	C FOS HISTOSCORE	AR HISTOSCORE	COX 2 HISTOSCORE
BPH 1	140	170	105	105
BPH 2	60	185	65	115
BPH 3	110	195	135	195
BPH 4	60	180	60	60
BPH 5	110	190	120	190
BPH 6	100	170	130	135
BPH 7	150	195	170	170
BPH 8	130	205	130	170
BPH 9	130	170	100	155
BPH 10	130	160	120	65
Mean Histoscore	112	182	113.5	136
SD	31.19	14.37	32.91	48.64
Skewness	-0.863	0.059	-0.274	-0.468
Median	120	182.5	120	145
Inter Quartile range	90-132.5	170-195	91.25-131.25	95-175

c-Jun, c-Fos, AR and COX-2 Protein expressions In BPH tissue and ASPC tissue were compared (Median histoscores- c-Jun in BPH- 112, AIPC- 160; c-Fos in BPH-182, AIPC-100; AR- in BPH-120, AIPC-132; COX-2 in BPH-145, AIPC-155). There was no significant correlation for c-Jun, c-Fos and COX-2 expression in BPH and AIPC tissue. However Androgen receptor protein expression in BPH tissue and ASPC tissue correlated well and it was statistically significant (p=0.04).

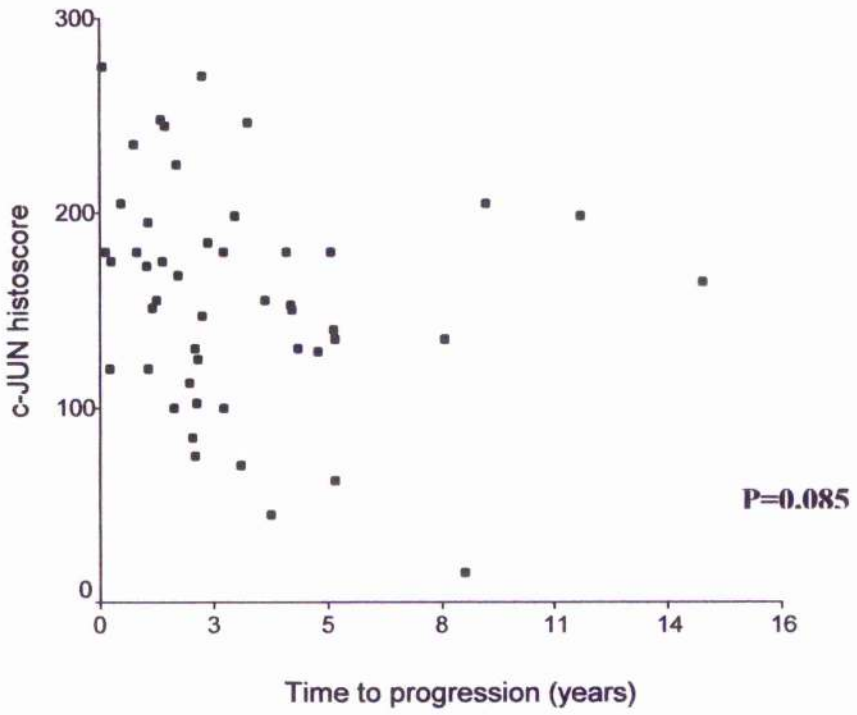
3.3.C Relation of protein expression in androgen sensitive tumours to time to relapse.

When the protein expression for c-Jun, p-Jun, c-Fos, PKC and COX-2 in the primary androgen sensitive tumours was investigated, we were unable to demonstrate a significant correlation between any of the proteins investigated and time to relapse (table 9). Time to progression (relapse) against c-Jun, p-Jun and PKC expression in ASPC are given as scatter plots (Figure-13 A-C).

Table 9 Protein Expressions In ASPC and Time To Relapse

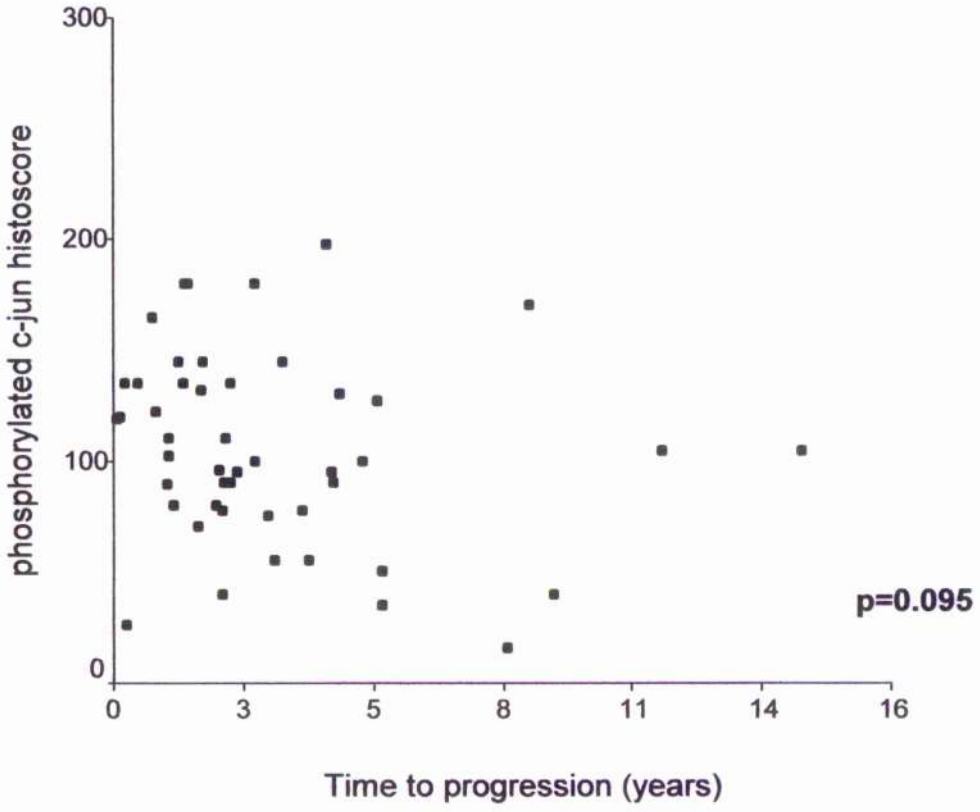
Protein	Spearman's rank order Correlation Coefficient	P value
c-Jun	-0.252	0.085
P c-Jun	-0.242	0.095
c-Fos	0.062	0.678
PKC	-0.248	0.086
COX-2	-0.099	0.500

Figure 13-A Scatter Plot of Time to progression (Relapse) against c-Jun protein Expression (Histoscores) in ASPC



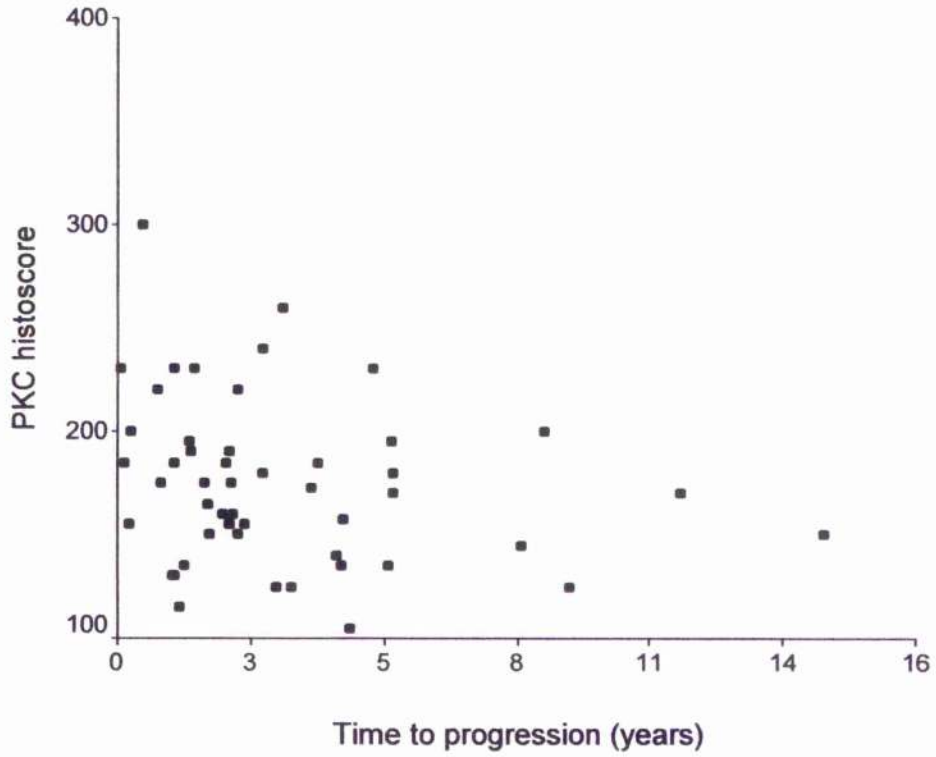
The above scatter plot clearly illustrates no correlation between c-Jun expression in androgen sensitive prostate cancers and time to relapse.

Figure 13-B Scatter Plot of Time to progression (Relapse) against p-Jun protein Expression (Histoscores) in ASPC



In the scatter plot there is no statistically significant correlation between p-Jun histoscores and time to relapse.

Figure 13-C Scatter Plot of Time to progression (Relapse) against PKC protein Expression (Histoscores) in ASPC



Similar to c-Jun and p-Jun protein expression, PKC expression had no effect on time to progression.

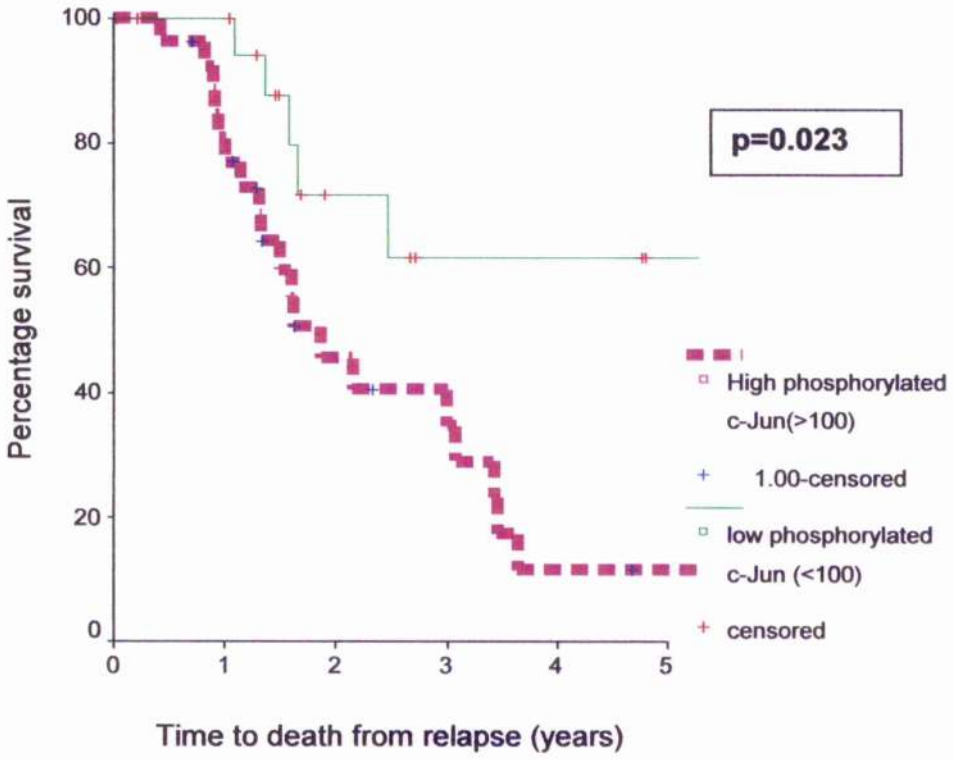
3.3.D Relation of high protein expression in androgen independent tumours to time to death from relapse

Protein expression was considered to be high in AIPC tumours if the histoscore was > 100. However for PKC and COX-2, so few cases had histoscores of less than 100 (1 for PKC and 5 for COX-2), it was not an appropriate cut off, therefore for these two proteins high expression was defined as a histoscore > 150. Using these definitions it was noted that approximately 60% of patients had a high c-Jun, PKC, COX-2 and AR expression with the development of AIPC and (table10). Approximately half of patients had high phosphorylated c-Jun expression and these patients were noted to have a significantly shorter period of survival following relapse in comparison to patients with low expression (figure 14A). However high expression levels of other proteins at relapse had no effect on duration of survival from relapse (figures 14 B-F) & (Table 11).

Table 10 High Protein expression in AIPC

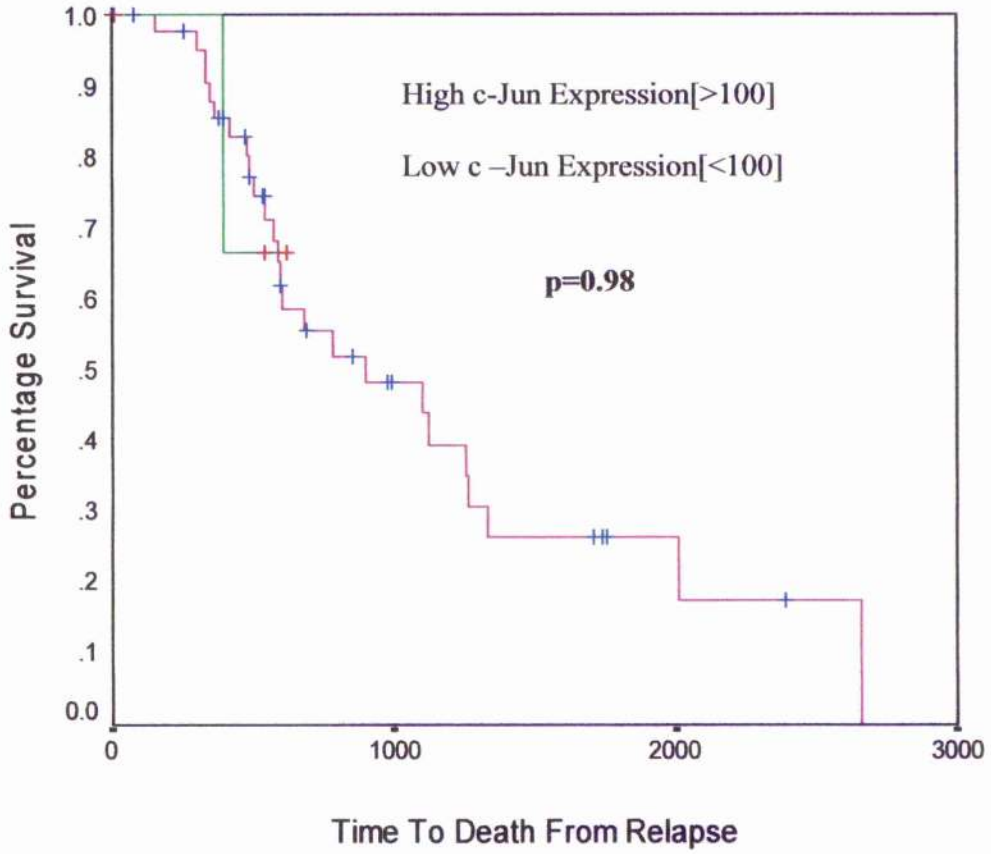
Protein	High Protein Expression No of tumours (%)	Low protein expression No of tumours (%)
c-Jun	35 (68.6%)	16 (31.3%)
P c-Jun	24(47%)	27(52.9%)
c-Fos	20(39.2%)	31(60.8%)
PKC	30(58.8%)	21(41.2%)
COX-2	29(56.8%)	22(43.2%)
AR	32(62.7%)	19(37.3%)

Figure 14-A Kaplan-Meier survival plot of patients with High (——) and low (——) phosphorylated c-Jun expression in Androgen Independent prostate cancers



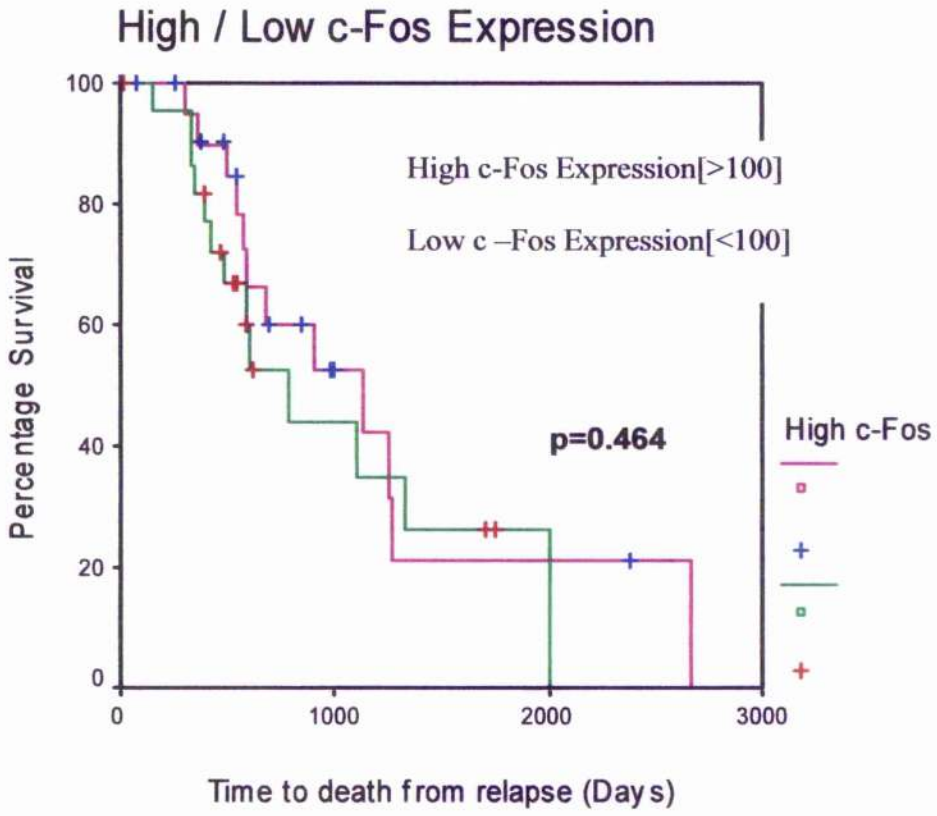
Patients with high phosphorylated c-Jun expression survived for a significantly shorter period of time than those with low phosphorylated c-Jun expression (p=0.023, log-rank test)

Figure 14-B Kaplan-Meier survival plot of patients with High (——) and low (——) c-Jun expression in AIPC.



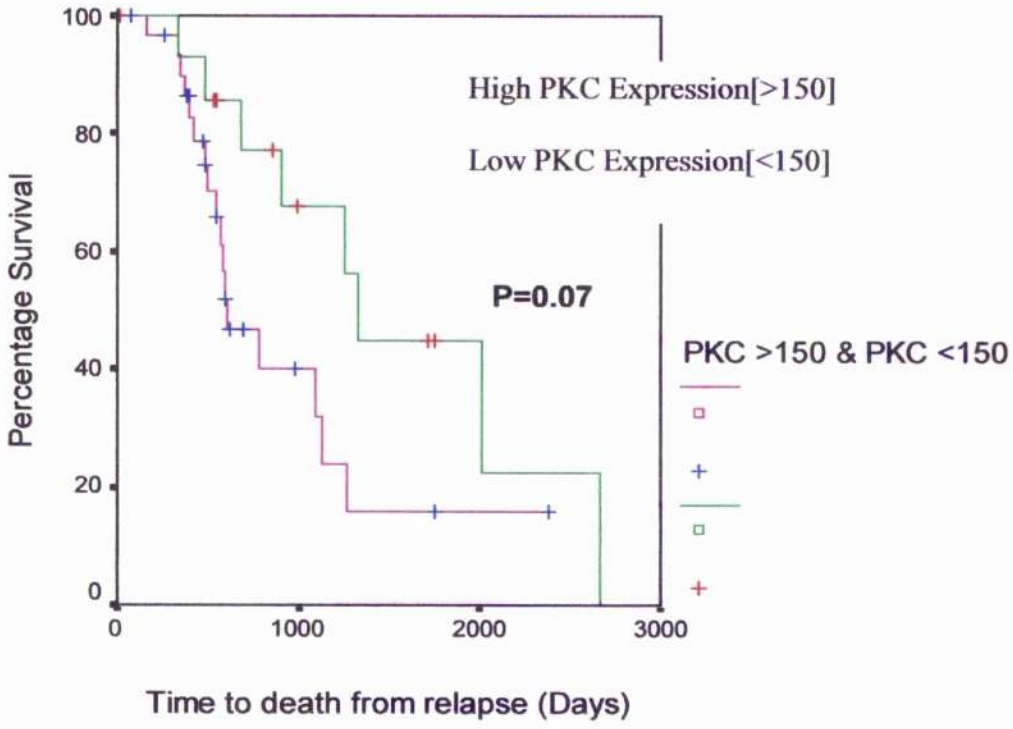
Patients with high and low c-Jun expression in AIPC had no difference in survival from relapse (p=0.98, log rank test).

Figure 14-C Kaplan-Meier survival plot of patients with High (——) and low (——) c-Fos expression in AIPC.



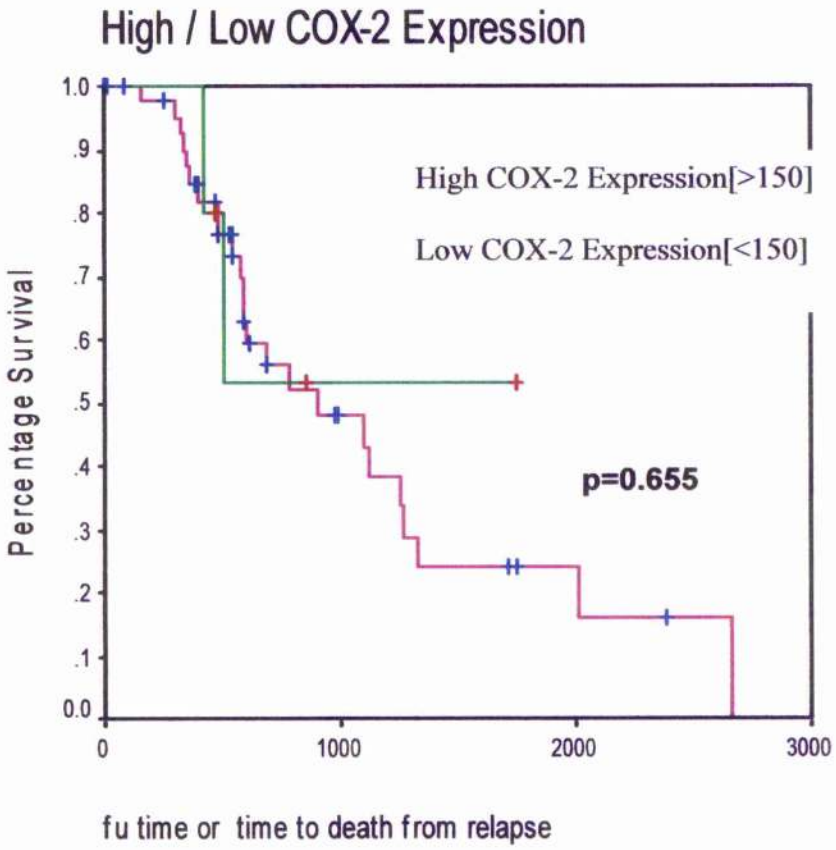
Patients with high and low c-Fos expression in AIPC did not have significant difference in survival (p=0.464, log rank).

Figure 14-D Kaplan-Meier survival plot of patients with High (——) and low (——) PKC expression in AIPC.



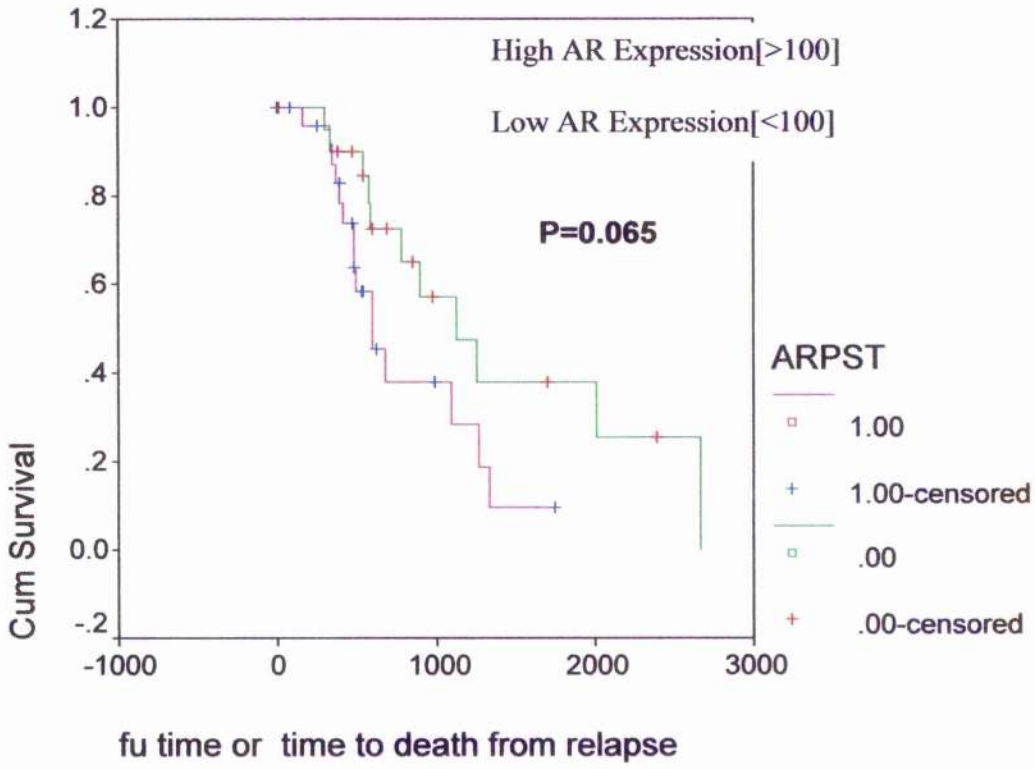
Duration of survival following relapse, in patients (AIPC) with high and low PKC expression did not have a significant difference in survival (p=0.07, log rank).

Figure 14-E Kaplan-Meier survival plot of patients with High (——) and low (——) COX-2 expression in AIPC.



COX-2 expression in AIPC patients, either high or low did not effect survival from relapse (p=0.655, log rank test).

Figure 14-F Kaplan-Meier survival plot of patients with High (——) and low (——) AR expression in AIPC.



No correlation between androgen receptor protein expression (low or high) in AIPC patients and time to death from relapse was demonstrated.

Table 11 Duration of survival after relapse in relation to high and low protein expression

Protein	High protein expression Median survival from relapse in yrs (Interquartile range)	Low protein expression Median survival from relapse in yrs (Interquartile range)	p value
c-Jun	2.5(0.78-4.1)	2.56(0.82-4.32)	0.978
P c-Jun	1.87(1.1-2.6)	5.5(1.07-9.9)	0.023
c-Fos	3.07(1.28-4.9)	2.1(1.30-2.99)	0.464
PKC	1.65(0.97-2.32)	3.6(3.0-4.2)	0.070
COX-2	3.0(1.1-4.9)	2.5(1.6-3.3)	0.655

3.3.E Changes in protein expression with the development of AIPC

When the changes in protein expression were investigated for each individual patient it was noted that sub groups of patients had increases, no changes or decreases in protein expression. A real change in protein expression is defined as mean difference of inter-observer variation plus 2 standard deviations, table 2 gives the values in histoscores for each

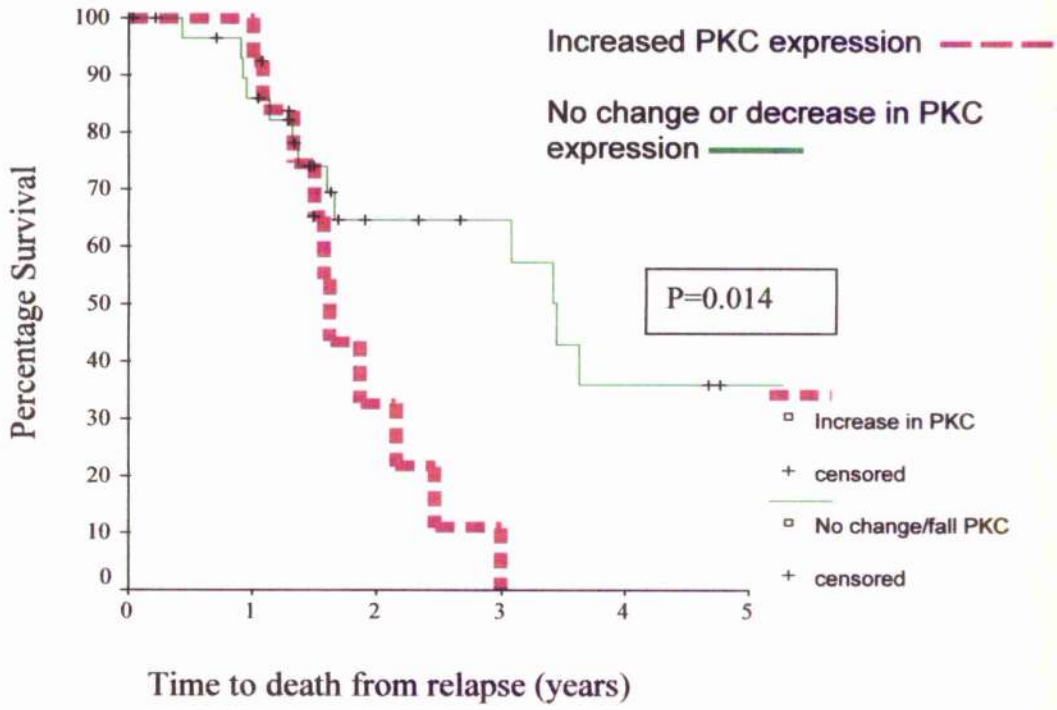
protein investigated. PCK and COX-2 were the 2 proteins with the highest rate of increase in expression. Approximately 1/3 of patients had an increase in PKC or COX-2 expression with the development of AIPC, while increases in expression of c-Jun and c-Fos were observed in less than 20% of patients (table 12).

Table 12 Changes in protein expression with development of relapse

Protein	Increase in expression (%)	No change in expression (%)	Decrease in expression (%)
c-Jun	8/51 (15.7%)	33/51 (64.7%)	10/51 (19.6%)
P c-Jun	4/51 (7.8%)	42/51 (82.3%)	5/51 (9.8%)
c-Fos	7/51 (13.7%)	35/51 (68.6%)	9/51 (17.6%)
PKC	14/51 (27.5%)	14/51 (27%)	23/51 (45%)
COX-2	17/51 (33.3%)	20/51 (39.2%)	14/51 (27.5%)
AR	26/51(50.98%)	20/51(39.2%)	5/51(9.8%)

An increase in PKC protein expression in the transition from androgen sensitive to androgen independent prostate cancer resulted in a significant reduction in patient survival from time to relapse (figure 15-A). For all other proteins investigated no significant difference in patient survival from time to relapse was noted with an increase in protein expression (table 13) (figures 15C-F). When changes in protein expression with the development of AIPC were compared to overall survival no significant observations were made.

Figure 15-A Kaplan Meier Survival Plot of AIPC Patients with increase and decrease in PKC Expression



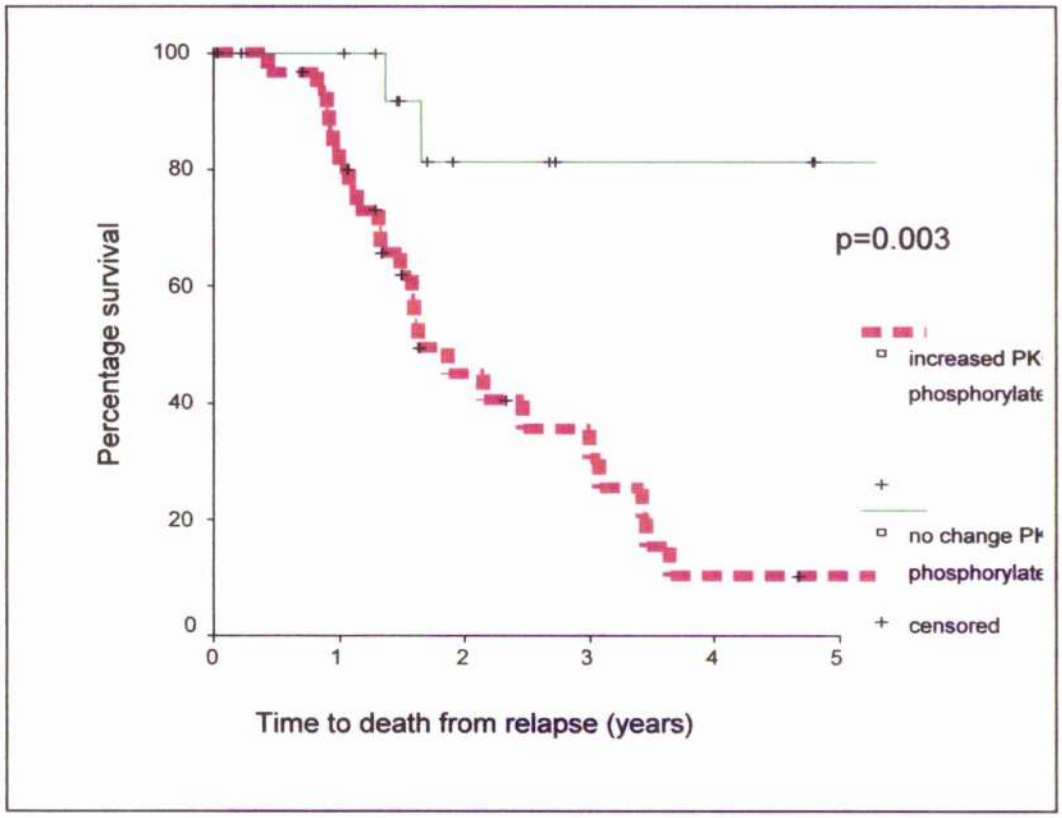
An increase in PKC expression was associated with shorter patient survival from time to relapse when compared to those with no change or a decrease in PKC expression in AIPC patients (p=0.014, log rank).

Table 13 Changes in protein expression and duration of survival from time to relapse

Protein	Increase in expression Median survival from relapse in yrs (Inter quartile range)	No change or fall in expression. Median survival from relapse in yrs (Inter quartile range)	p value
c-Jun	3.45(0.29-6.62)	2.47(1.05-6.62)	0.301
P c-Jun	1.65(0.45-2.85)	3.0(1.7-4.3)	0.466
c-Fos	1.31(1.10-1.36)	2.47(0.79-4.14)	0.645
PKC	1.61(1.4-1.8)	3.5(28.4.1)	0.014
COX-2	1.33 (0.48-2.17)	3.08 (2.12-4.04)	0.112

It was noted that high levels of phosphorylated c-Jun and an increase in PKC expression were both associated with shorter patient survival and that these represented to individual patient cohorts. We therefore combined these patient and we observed that those patients with high phosphorylated c-Jun expression or an increase in PKC expression with development of AIPC survived for a significantly shorter period of time compared to those with low phosphorylated c-Jun or no increase in PKC expression (figure 15-B, p=0.0036). Median survival changed from 5.5 years (3.99-6.38) to 1.62 years (0.99-2.24 years), depending on changes in PKC expression and levels of phosphorylated c-Jun.

Figure 15-B Kaplan Meier Survival Plot of AIPC Patients with High phosphorylated c-Jun and an increase in PKC expression

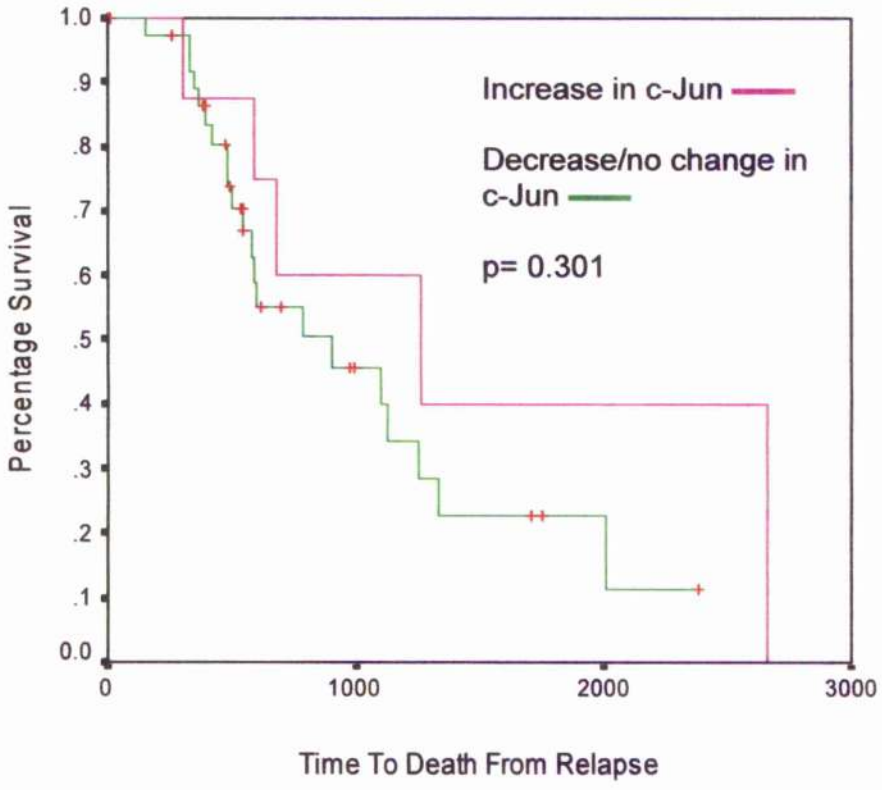


Increased PKC & high p-Jun expression

No change/ Low / decrease In p-Jun & PKC expression

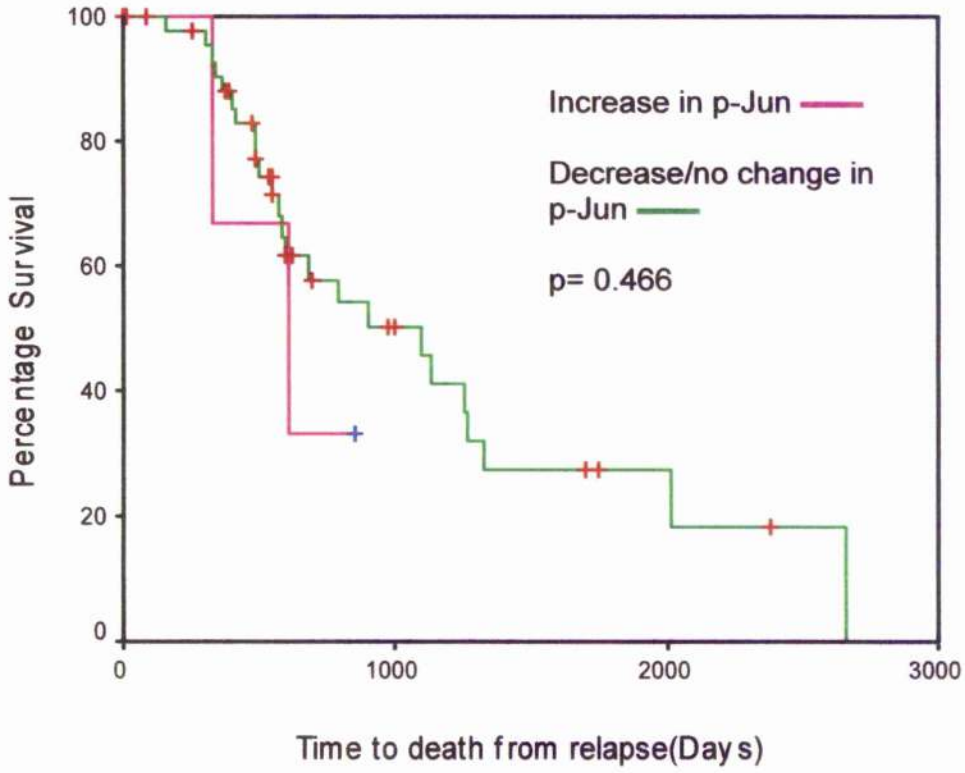
The above graph shows that AIPC patients with high p-Jun and increased PKC expression survived for a significantly shorter period of time than those with low or no change or a decrease in p-Jun / PKC expression (p=0.003, log rank).

Figure 15-C Kaplan Meier Survival Plot of AIPC Patients with Increase and a decrease or no Change in c-Jun Expression



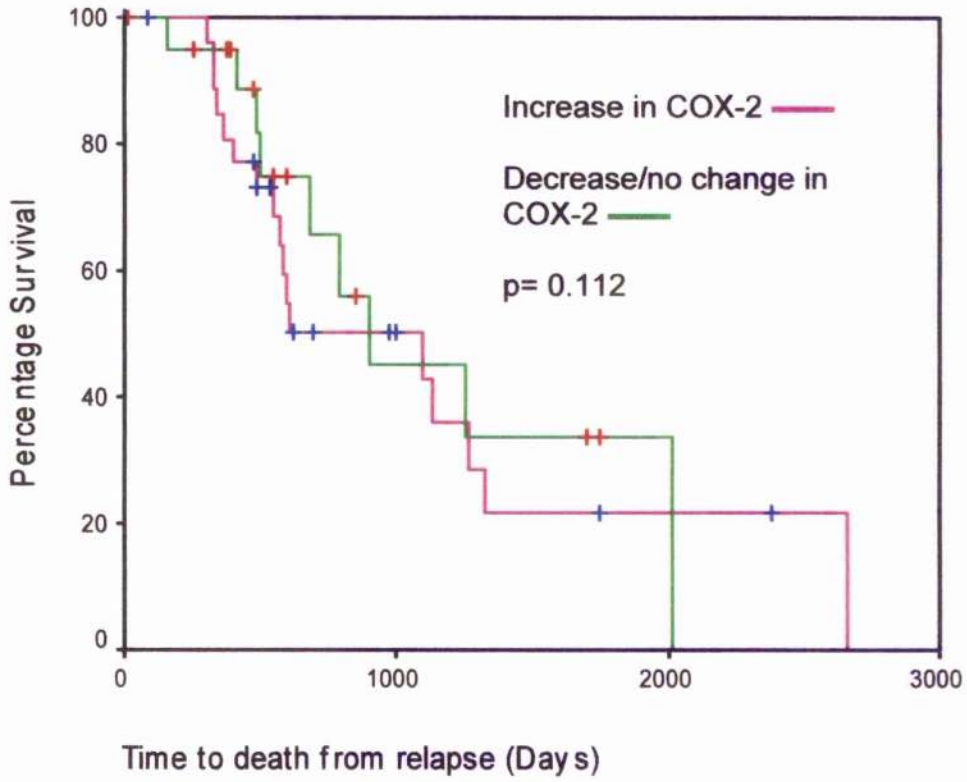
AIPC patients with an Increase, decrease or no change in c-Jun expression had no significant difference in survival from the time of development of hormone relapse (p=0.301, log rank).

Figure 15-D Kaplan Meier Survival Plot of AIPC Patients with Increase and a decrease or no Change in p-Jun Expression



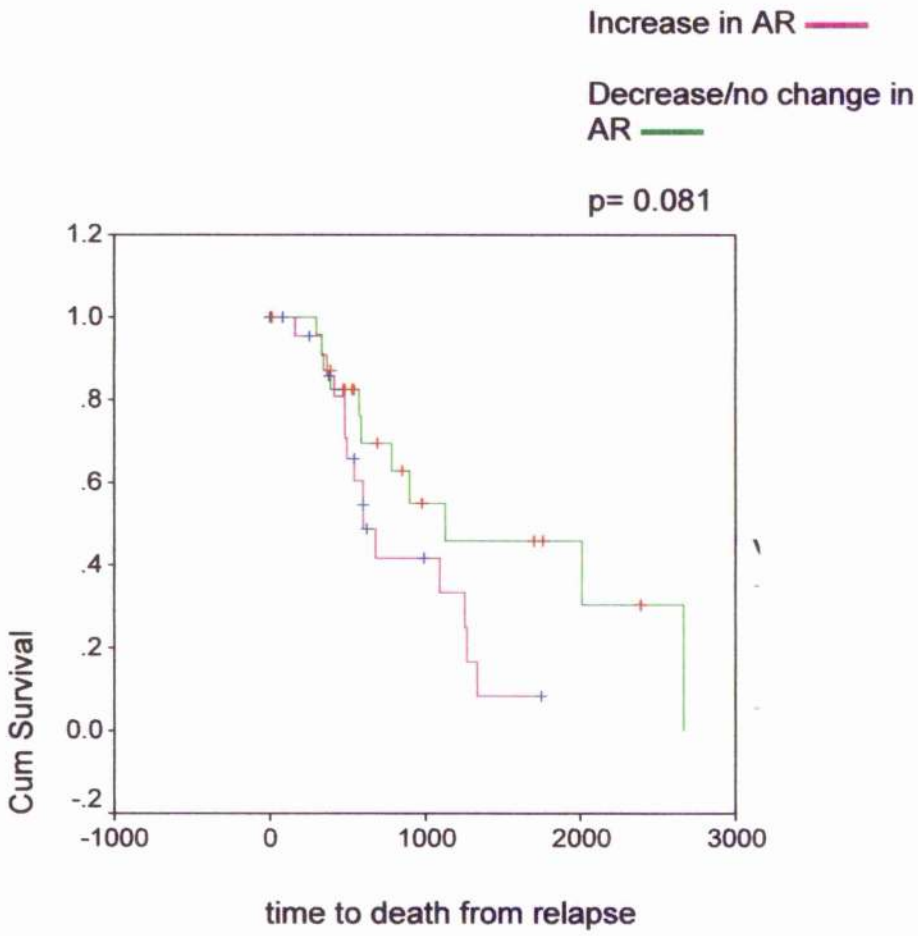
The above survival plot demonstrates no significant difference in survival in patients with increased p-Jun expression in comparison to those with a decrease or no change (p=0.466, log rank).

Figure 15-E Kaplan Meier Survival Plot of AIPC Patients with Increase and a decrease or no Change in COX-2 Expression



The Kaplan Meier Survival Plot of AIPC patients with Increase and a decrease or no change in COX-2 Expression shows no significant difference in survival from time to relapse (p=0.112, log rank).

Figure 15-F Kaplan Meier Survival Plot of AIPC Patients with Increase and a decrease or no Change in AR Expression



AIPC patients with an Increase, decrease or no change in AR expression had no significant difference in survival from the time of development of hormone relapse (p=0.081, log rank).

3.3.F Protein Correlation in Androgen Sensitive and Independent prostate cancer

c-Jun homodimers or heterodimers of phosphorylated c-Jun and c-Fos form the transcription factor AP-1. In our patient cohort protein expression of c-Jun, phosphorylated c-Jun and c-Fos all correlate in ASPC, however with the development of AIPC c-Fos expression no longer correlates with c-Jun or phosphorylated c-Jun (table 14)

Table 14 Correlation between components of AP-1

	Phosphorylated c-Jun	c-Fos
ASPC c-Jun	0.008 (c.c 0.396)	0.02 (c.c 0.343)
AIPC c-Jun	<0.0001 (c.c 0.608)	0.083 (c.c 0.274)
ASPC c-Fos	0.027 (c.c 0.332)	
AIPC c-Fos	0.480 (c.c 0.113)	

It was previously reported that PKC may phosphorylate and activate c-Jun and also increase expression of c-Fos. We therefore correlated PKC expression in ASPC and AIPC tumours to c-Jun, phosphorylated c-Jun and c-Fos expression. In ASPC no correlations were seen with c-Jun and

c-Fos and PKC, however following the development of AIPC c-Jun protein expression negatively correlates with PKC protein expression (table 15).

Table 15, Protein correlations with c-Jun, c-Fos and PKC

Protein	c-Jun	Phosphorylate c-Jun	c-Fos
ASPC PKC	0.662 (c.c 0.067)	0.433 (c.c. 0.123)	0.280 (c.c.-0.165)
AIPC PKC	0.012 (c.c.- 0.387)	0.877 (c.c - 0.025)	0.825 (cc - 0.037)

COX-2 has a consensus phosphorylation site for both PKC and AP-1, in our patient cohort COX-2 protein expression correlated with PKC expression in both ASPC and AIPC tumours however no correlations were observed for phosphorylated c-Jun (AP-1) in either ASPC or AIPC tumours (table 16)

Table 16 Protein correlations with COX-2, PKC and phosphorylated c-Jun.

	PKC	Phosphorylated c-Jun
ASPC COX-2	0.014 (c.c. 0.387)	0.684 (c.c. -0.59)
AIPC COX-2	0.002 (c.c. 0.451)	0.262 (c.c.0.162)

c-Jun may act as an AR co-activator or compete with AR to regulated gene transcription, however in this patient cohort AR does not correlate with protein expression of c-Jun in ASPC or AIPC tumours. However AR correlated with phosphorylated c-Jun in both androgen sensitive as well as androgen independent psostate tumours (table 17).

Table 17 Protein correlations with AR, c-Jun, c-Fos and phosphorylated c-Jun

	c-Jun	Phosphorylated c-Jun	c-Fos
ASPC AR	0.176 (c.c. 0.197)	0.003 (c.c. 0.460)	0.925 (c.c. –0.016)
AIPC AR	0.799 (c.c. –0.037)	0.07 (c.c. 0.659)	0.408 (c.c. 0.142)

CHAPTER 4

DISCUSSION

4.1 Back Ground To The Study

Prostate cancer is the second most common cancer in men in the UK, with more than 21 000 new cases diagnosed each year in the UK [232]. Most men at diagnosis have early localised disease, but almost 30-35% are diagnosed with locally advanced or metastatic disease [232].

Since the landmark experiments by Huggins and coworkers (103,233] it has been known that prostate cancer can be treated by endocrine therapy. Initially endocrine therapy was accomplished by surgical orchidectomy and later by the use of estrogens. However the main stay of endocrine therapy in the modern era has been through the use of long acting Luteinising hormone releasing hormone (LH-RH) analogues [234,235] they act to down-regulate the production of luteinizing hormone (LH) by the pituitary gland, resulting in castrate levels of serum testosterone [236]. Serum PSA levels fall (PSA $\leq$ 4.0 ng/ml) in around 70-80% of men after endocrine therapy, and that is maintained for a variable period of time [237,238]. The earliest manifestation of relapse after endocrine deprivation therapy is a rise in serum PSA level, which usually precedes the development of clinically detectable disease progression [239,240]. At this stage, such disease is often labelled as androgen independent prostate cancer (AIPC) or hormone refractory prostate cancer (HRPC). The median time to progression after endocrine ablation therapy is around 18-24

months, with a median survival of 6-18 months from the time of development of AIPC [78,112].

Currently, there is no standard approach to the treatment of AIPC. Treatment options include second-line endocrine therapy, chemotherapy with or without endocrine agents and radiotherapy, depending on the site of relapse, symptoms at relapse, performance status of the patient, and presence or absence of co-morbid medical conditions [232]. They do not provide significant control of the disease but improve quality of life [234].

Two phase III trials demonstrated the efficacy of Mitoxantrone plus corticosteroid in AIPC [241,242]. In both trials, a benefit in palliation was observed with out any survival benefit. How-ever two further randomised, phase 3 multi-centre studies (TAX 327 and SWOG 99-16) used taxane based chemotherapeutic agent, docetaxel [236] which binds to tubulin and causes disruption of the microtubular network essential for mitotic and interphase cellular functions. In the TAX 327 study [243] an actual median survival improvement of 2.4 months by comparison was observed (18.9 months in docetaxel vs 16.5 months in mitoxantrone groups). In the SWOG 99-16 study [244] a similar result to that observed with TAX 327 was observed. In both the studies a 24% improvement in overall survival was demonstrated. The efficacy of docetaxel is not universally effective in all patients. The median survival benefit of 2.4 months is small. This suggests that AIPC is a difficult disease to treat and none of the currently available chemotherapeutic agents are able to improve survival

significantly. Due to lack of proper understanding of the underlying molecular mechanisms involved in the pathogenesis of AIPC, there are only few treatment options available.

Studies on human prostate cancer specimens show that the androgen receptor (AR) is expressed in nearly all cancers of the prostate before and after hormonal therapy [115-117]. Prostate specific antigen (PSA) production by prostate tumours is regulated by androgens initially, but with endocrine treatment the levels decline sharply. An early sign of AIPC is a rising PSA level that may eventually surpass the pre-treatment value (Miller et al, 1992; Gleave et al, 1996). These findings does suggest that the AR is still functional in an androgen independent manner [245].To explain the progression of androgen sensitive prostate cancer (ASPC) to AIPC, androgen receptor dependent and independent molecular mechanisms have been postulated [119,246,247].

Androgen receptor dependent mechanisms include mutations in the androgen receptor (AR) ligand binding domain or amplification of the AR gene, alterations of the interactions between the AR and its co-activators and ligand independent activation of AR via AR phosphorylation. Androgen receptor independent mechanisms completely bypass the androgen receptor and they activate the regulatory molecules down stream of the AR. It is possible that more than one mechanism will be utilised in each case of hormone refractory prostate cancer.

Androgen receptor gene mutations are reported in both *in-vitro* as well as *in-vivo* studies. AR mutations were reported in 10-25% of prostate cancer tissue specimens [127,155] and they were more common in AIPC in

AR gene amplifications [118,128,129,130,250,251] are more common in AIPC (0-5%) in comparison to ASPC (20-30%). An increase in AR protein expression as a result of AR amplification is postulated to enables low circulating androgen levels after endocrine manipulation, to activate AR promoting prostate cancer progression [249] However both AR mutations and amplification can explain development of AIPC only in a small subgroup of prostate tumours.

AR transcriptional activity depends on its phosphorylation status [252,253]. DHT binding to AR protects it from proteolytic degradation by protein kinases resulting in hyperphosphorylation of the AR at multiple serine residues [139,140]. Several proteins phosphorylate AR *in-vitro* in the absence of androgens. These include MAP kinase [140,142,145,146] and Akt [140,142,145-147]. Hence AR phosphorylation may offer a possible route for progression to AIPC.

Even though AR plays a major role in the progression to AIPC, androgen independent prostate cancer cells may use other pathways for proliferation and survival via bypassing the AR entirely. Bcl-2 is a member of a family consisting of interacting pro-survival proteins e.g. Bcl-2 and pro-apoptotic proteins e.g. Bax [254,255]. Bcl-2 protects cells against

apoptosis, however when it forms a dimer with pro-apoptotic protein BAX, the ability of Bcl-2 to block apoptosis is inhibited [256]. Bcl-2 has been shown *in-vitro* and *in-vivo* model systems to suppress apoptosis following androgen ablation [257]. Animal studies have shown increased expression of Bcl-2 protein with the development of androgen escape after surgical castration [257].

PI3K (phosphatidylinositol 3-kinase) also plays a critical role in cell survival by inhibiting several downstream pro-apoptotic proteins Bad, caspase-9 via a pathway that includes PIP (3) (3-phosphoinositide protein kinase-1 and 2) and Akt [119]. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a lipid phosphatate whose primary function is to negatively regulate PI3K/Akt signalling. So loss of PTEN and activation of PI3K /Akt in prostate cancer cells might provide a favourable environment for Bcl-2 protein to function as inhibitor of apoptosis.

The role of activator protein 1 (AP-1) in the development of AIPC is more complicated by the fact that it acts by two different mechanisms. In the first, c-Jun forms AP-1 transcription factor and actually competes with the AR to bind to AR regulated genes [173,204]. In the other, instead of forming AP-1 transcription factor, c-Jun monomer acts as an AR co-activator by binding to the N-terminal domain at amino acids 503-555 [171,172].

AP-1 is a transcription factor whose components are nuclear proteins encoded by c-Jun and c-Fos proto-oncogenes [169,204,258]. AP-1 is

implicated in cell growth, differentiation and development, its activity modulated by growth factors, cytokines, oncogenes and protein kinase c (PKC) [204]. AP-1 can either be a c-Jun/ c-Jun homodimer or a c-Jun/c-Fos heterodimer, the c-Jun/c-Fos heterodimer is more stable than the c-Jun/c-Jun homodimer [204]. Formation of either dimer requires phosphorylation of c-Jun by c-Jun N-terminal kinase (JNK), which is activated by either PKC / MAP kinase cascade [259] c-Jun homodimers and c-Jun/Fos heterodimers are formed through the leucine Zipper domain of both proteins [204,171]. AP-1 induces transcriptional activation by binding to the TPA-responsive element (TRE or AP-1 DNA binding site [204].

Activation of c-Jun via PKC / MAP kinase pathway is reported to be involved in the development of AIPC [172,204,205]. AP-1 influences the development of AIPC by competing with the AR to alter androgen regulated gene expression [172-204].

Cross talk between the androgen receptor and PKC signal transduction has been reported in prostate cells [170,204,260]. TPA increased AP-1 activity inhibited androgen induced PSA promoter activity in LNCaP cells. This was due to protein protein interaction between AR and AP-1 [204. Mutual inhibition of DNA binding by AR and AP-1 resulted from formation of complexes between the two proteins [204]. Hence the effect of AP-1 on androgen regulated gene expression depends on the ratio of AR to AP-1 and the ability of AP-1 to bind to specific promoter regions in the androgen

regulated gene [110,206]. Such AP-1 and AR protein protein interaction could influence the ability of AP-1 to enhance androgen regulated gene transcription in the absence of androgens and hence might influence the development of AIPC [110,204]. *In-vitro* work on PC3 cells (poorly differentiated prostate cancer cells and are androgen independent) demonstrated a 7 fold greater intracellular c-Jun concentration than in LNCaP cells that are androgen sensitive [204]. This suggests that AP-1 influences androgen escape in PC-3 cells. Matrix metalloproteinases (MMPs) degrade all of the protein components of the extracellular matrix such as collagens, proteoglycans, laminin, elastin, and fibronectin [261,262]). This is considered to be a prerequisite for tumour invasion and metastases. Also MMPs contribute to angiogenesis, cellular differentiation, proliferation and apoptosis [261-263]. MMPs gene expression is primarily regulated through AP-1 via mitogen activated protein kinase (MAPK) pathways [264-266]. demonstrated inhibition of MMP-2 and MMP-9 in DU145 cell by EGCG (epigallocatechin-3-gallate) via inhibition of activation of c-Jun and NF-kB.

The possible role of c-Jun and c-Fos in contributing to the development of androgen independent prostate cancer *in-vivo* using paired human prostate cancer tissue samples has not been studied before. We had opportunity to study this in a unique cohort of prostate cancer patients who were hormone sensitive to start with and subsequently progressed to hormone relapse. Prostate cancer tissue at diagnosis and at relapse was

available for the study. The present study was carried out to test the following hypothesis

"AP-1 transcription factor related proteins are dysregulated in the emergence of androgen independent prostate cancer".

The aims of the study were

1. To establish a paired clinical cohort of patients in which prostate cancer tissue is available at diagnosis of prostate cancer and also following the development of androgen independence in order to test the hypothesis by immunohistochemistry.

2. To evaluate the expression of AP-1 related proteins in androgen sensitive and androgen independent prostate cancer, by immunohistochemistry.

4.2 Study Results And Issues Raised

4.2.1 Scoring Criteria For Immunohistochemical Staining Of Protein Expression

We investigated immunohistochemical expression of c-Jun, phosphorylated c-Jun, c-Fos, PKC, COX-2 and AR in 51 paired prostate tumour samples, the first specimen in a pair was obtained when the tumour was androgen sensitive and the other at relapse. Immunohistochemical staining of protein expressed was quantified using intensity weighted histoscore method. This is a semi-quantitative method of scoring based on intensity of staining and the proportion of cells stained [118,267,268].

Pertschuk et al [269] reported use of similar Intensity weighted histoscore technique in scoring immunohistochemical AR expression in paraffin embedded prostate cancer tissue specimens.

Regitnig et al [270] studied estrogen and progesterone receptor expression in breast cancer specimens by immunohistochemical technique. The expression of these receptors was evaluated semiquantitatively by estimating the percentage and intensity of stained nuclei.

Kerfoot et al [268] in their study to evaluate PKC α immunohistochemical expression in advanced human breast carcinoma also reported the use of

similar intensity weighted histoscore method to evaluate PKC immunostaining.

In our study intensity of protein expression was scored as negative (0), weak (1), moderate (2), or strong (3). The proportion of cells stained also noted. The histoscore was then calculated from the following equation. Histoscore = (1 x % weak staining cells) + (2 x % moderate staining cells) + (3 x strong staining cells), with a maximum score of 300.

Two independent observer's scores of the samples and the mean of the scores was used. The inter-class correlation coefficients (ICCC) between the two independent observers were calculated as 0.79 for c-Jun, 0.90 for phosphorylated c-Jun and c-Fos, 0.87 for PKC, 0.89 for COX-2 and 0.68 for AR. By definition an ICCC of 0.7 is regarded as an excellent correlation and an ICCC of >0.6 as a good correlation.

Changes in protein expression between ASPCs and AIPCs were defined based on the variation in scores between the two independent observers (NSK and JE), as the mean plus 2 standard deviations of the inter-observer variation of all sections scored. Therefore, an increase in histoscore of more than 57.7 for c-Jun, more than 64.7 for phosphorylated c-Jun, more than 58.1 for c-Fos, more than 16.6 for PKC, more than 33.4 for COX-2 and more than 29.98 for AR would represent a significant increase in protein expression in paired ASPC and AIPC samples. Those cases in which an increase was demonstrated were classed as having significant rise in protein expression for each protein investigated.

4.2.2 Protein Expressions In Androgen Sensitive And Androgen Independent Prostate Cancer

c-Jun, phosphorylated c-Jun, phosphorylarted c-Jun, c-Fos, PKC, COX-2 and AR protein expressions in ASPC and AIPC paired samples were studied by immunohistochemical method.

Protein expressions in both groups of tumours were not normally distributed (ASPC- Skewness- c-Jun -134, phosphorylated c-Jun -.076, c-Fos -.185, PKC .741,COX-2 .442 and AR -.150; AIPC-Skewness-c-Jun -.942 phosphorylated c-Jun -.656, c-Fos -.184 , PKC -.031, COX-2 -.703 and AR -.435)

Median expression of c-Jun, phosphorylated c-Jun, c-Fos, PKC, COX-2 and AR proteins in androgen sensitive samples and at relapse were compared (table 7). There was no significant difference in their median protein expression between the two groups except for AR expression (ASPC vs AIPC median histoscores and p value- c-Jun 155 vs 160, p=0.88; phosphorylated c-Jun 101 vs 105, p=0.39; c-Fos 111 vs 100, p=0.64; PKC 172 vs 164, p=0.13; COX-2 145 vs 155, p=0.96; and AR 145 vs 155, p=0.000).

c-Jun and c-Fos expression was observed in the nucleus and cytoplasm of the tumour cells (figure 11a and c). However staining for phosphorylated c-Jun and AR was found in the nucleus (figure 11b and

11f). PKC and COX-2 proteins were expressed only in the cytoplasm of the tumour cells (figures 11d & e).

Changes in protein expression were investigated in our paired cohort of tumours. A change in protein expression at relapse (between ASPCs and AIPCs) was defined, as the mean plus 2 standard deviations of the inter-observer (NSK and JE) variation of all sections scored. Therefore, an increase in histoscore of more than 57.7 for c-Jun, more than 64.78 phosphorylated c-Jun, more than 58.1 for c-Fos, more than 16.6 for PKC, more than 33.4 for COX-2 and more than 29.98 for AR would represent a significant increase in protein expression in paired samples (table 6). Those cases in which an increase was demonstrated were classed as having significant rise in protein expression for each protein investigated (table 12).

At relapse c-Jun protein expression was increased in 15.7%(8/51), Phosphorylated c-Jun in 7.8%(4/51), c-Fos in 13.7%(7/51), PKC in 27.5%(14/15), COX-2 in 33.3%(17/51) and AR 50.98%(26/51). A decrease in protein expression was also noted at relapse (c-Jun in 19.6% [10/51]; phosphorylated c-Jun in 9.8% [5/51]; c-Fos in 17.6%[9/51]; PKC in 45%[23/51; COX-2 in 27.5% [14/51] and AR in only 9.8% [5/51].

We also defined high and low protein expression by a tumour at relapse depending on the histoscore for that protein assigned by the observers. If the histoscore was over 100, then it was considered that the protein expression by that tumour was high. Similarly a low expression at relapse,

if the histoscore was less than 100. But for PKC and COX-2, so few tumours had a histoscores less than 100 (1/51 for PKC and 5/51 for COX-2), we felt that it was not an appropriate cut off, therefore for these proteins, a histoscore of 150 was used to define high and low protein expression. Using these definitions we noted that approximately 60% of tumours had a high c-Jun, PKC, COX-2 and AR protein expressions (c-Jun 68.6%[35/51]; PKC58.8% [30/51]; COX-2 56.8%[29/51]; AR [32/51]) with progression to AIPC (table 10). Also approximately 45% had high phosphorylated c-Jun and c-Fos expression at development of relapse (phosphorylated c-Jun 47%[24/51]; c-Fos 39.2%[20/51]).

4.2.2.A Role of Threshold Setting In Survival Analysis

Determination of factors that predict outcome measures such as time to relapse, survival time and probability of death from disease are referred to as prognostic variables. Several methods have been used to assess whether these variables are genuinely associated with the outcome. Such prognostic variables can be entered directly into a proportional-hazards regression (e.g. Cox regression) or logistic regression. However many practitioners choose a threshold value so that patients with values on one side of the threshold are designated as high risk and the remaining patients as low risk. The final assessment and use of the marker is then based on the low risk / high risk dichotomy and not on the actual value of

the marker. The method of choosing the threshold requires care. Not all studies report how the threshold has been determined. Keegan et al [298] reported that optimal threshold setting using several methods have serious statistical flaws that could undermine conclusions of the study. Some of the flawed methods widely used are, chosing the threshold value that yields the smallest p-value when the groups above and below this value are compared, using a hypothesis test such as a chi-squared or log-rank test which uses just a single marker. All the above methods choose the threshold value on the basis of the data; which is not legitimate and can give p-values that have a large downward bias which can lead to misrepresentation of the data. Hence methods such as proportional-hazards regression (e.g. Cox regression) or logistic regression should be regarded as the method of choice.

In our study we defined high and low protein expression by a tumour at relapse depending on the histoscore for that protein assigned by the observers. If the histoscore was over 100, then it was considered that the protein expression by that tumour was high. Similarly a low expression at relapse, if the histoscore was less than 100. But for PKC and COX-2, so few tumours had a histoscores less than 100 (1/51 for PKC and 5/51 for COX-2), we felt that it was not an appropriate cut off, therefore for these proteins, a histoscore of 150 was used to define high and low protein expression. Using these definitions we noted that approximately 60% of tumours had a high c-Jun, PKC, COX-2 and AR protein expressions (c-

Jun 68.6%[35/51]; PKC58.8% [30/51]; COX-2 56.8%[29/51]; AR 62.7% [32/51]) with progression to AIPC (table 10). Also approximately 45% had high phosphorylated c-Jun and c-Fos expression at development of relapse (phosphorylated c-Jun 47% [24/51]; c-Fos 39.2%[20/51]). We also demonstrated that high phosphorylated c-Jun expression was associated with shorter patient survival from time to relapse.

4.2.2.B c-Jun, phosphorylated c-Jun and c-Fos Proteins Expression

Our study was carried out in prostate cancer tissue clinical specimens. The findings presented here are based on immunohistochemistry. Studies looking into immunohistochemical protein expressions of c-Jun, phosphorylated c-Jun, c-Fos in prostate cancer as well as other cancers like breast, bladder, lung, have been carried before [271-274]. The present study is unique as paired prostate cancer tissue samples were investigated.

Tiniakos et al [272] showed c-Jun-specific immunostaining in normal and benign breast tissues, which was weaker and in fewer cells compared to that observed in breast carcinomas. Ninety per cent of all breast carcinomas studied showed c-Jun-specific nuclear staining. There were no statistically significant differences in the intensity of c-Jun immunoreactivity among different grades of infiltrating ductal carcinomas.

In ASPCs and AIPCc, we observed c-Jun staining in the nucleus and cytoplasm of the tumour cells. c-Jun reactivity increased in 15.7%(8/51) of tumours and was high in 68% tumours (38/51) at relapse. No relation to Gleason sum was seen (p=0.098) in ASPCs.

c-Jun oncoprotein expression in transitional cell carcinomas (TCCs) of the urinary bladder using immunohistochemistry and to determine its relationship to tumour grade and stage was investigated by Tiniakos et al in 1994 (273) They found a positive association between intense c-Jun immunoreactivity and muscle invasive growth, and EGFR positivity in TCCs. Ninety two (44/48) tumours showed c-Jun specific nuclear immunoreactivity of variable intensity. The intensity of c-Jun immunostaining was significantly related to tumour stage and EGFR status. c-Jun expression was not related to clinical outcome in terms of patient survival or rate of tumour recurrence.

Walker et al [274] investigated c-Fos protein expression in both benign and malignant human breast tissue using immunohistochemistry. There was a greater degree of staining in the carcinomas than in the normal and benign tissue. In all the instances, staining was nuclear. In our study, we also demonstrated similar nuclear and also cytoplasmic staining for c-Fos in prostate cancer tissue specimens examined. Poorly differentiated breast carcinomas had a greater degree of reactivity, but this did not apply to well differentiated tumours. They could not find any clear relationship between c-Fos detection and proliferation or differentiation. However in

the prostate tumours we studied, c-Fos expression varied in both well and poorly differentiated androgen sensitive prostate tumours. No statistical significance was reached in relation to Gleason sum (p=0.161). In both groups ASPC and AIPC, median c-Fos expression was not different (p=0.642).

Gavrilov et al [271] examined c-Jun and c-Fos expression in high-grade prostate cancers. Strong staining for c-Jun and c-Fos occurred in approximately 35% of cancers. No staining was detected in BPH tissue. Remarkably, c-Jun and c-Fos staining was frequently localised to the cytoplasm of tumour cells. Also PC3 cells were reported to exhibit cytoplasmic staining. Where as we observed both nuclear and cytoplasmic staining in our cohort of tumours.

4.2.2 C COX-2 Protein Expression

COX-2 is a key enzyme in the conversion of arachidonic acid to prostaglandins and it is highly expressed in a number of human cancers and cancer cell lines, including prostate cancer [275]. *In-vivo* studies reported COX-2 expression in prostate tumours by immunohistochemical analysis. In one study 87% of the prostate tumour samples demonstrated immunoreactivity to COX-2 [275]. The enzyme was strongly expressed in smooth muscle cells of both the normal and cancerous prostate. Its expression in benign epithelial cells was limited to the basal cell layer. But it was expressed in epithelial cells of high grade PIN and cancer.

Madan et al [226] assessed COX-1 and COX-2 expression in human prostates. COX-2 expression was significantly greater in cancer compared to BPH Its expression was significantly higher in poorly differentiated tumours (P=<0.001) The results were supported by immunoblotting. In BPH there was membranous expression of COX-2 in luminal glandular cells and no stromal staining. In cancer the stromal expression was similar, but the staining pattern changed from membranous to cytoplasmic (p=<0.001). Our study findings were consistent with some of the findings in this study. COX-2 staining was cytoplasmic in prostate cancer cells. However COX-2 expression was unrelated to tumour grade (Gleason sum) in ASPCs (p=0.278). High COX-2 expression was observed in 57% of tumours at relapse. Median COX-2 expression in sensitive and independent prostate tumour groups was no different (p=0.957).

In-vitro cell line studies [230,231,276,277] have demonstrated COX-2 expression in both LNCaP and PC3 cells. Hsu et al,(2000) compared COX-2 expression in LNCaP and PC3 cells vs normal human prostate epithelial cells by western blot analysis. COX-2 expression was lower in normal prostate epithelial cells than LNCaP and PC3 cells. Between the two cancer cell lines, LNCaP cells displayed a higher COX-2 level than PC3 cells in a ratio of approximately 2:1.

4.2.2.D PKC Protein Expression

Protein kinase c (PKC) is a family of serine/threonine protein kinases, with at least twelve mammalian members that play an important role in cell growth and differentiation [278-280]. Different isoforms of PKC are categorized under 3 main categories classic'novel and atypical according to cofactor requirements [281]. Classic PKCs (α, β_1, β_2, and γ) and novel PKCs (δ, ε, $\eta$and θ) can be activated *in-vivo.* by second messenger diacylglycerol and *in-vitro* by phorbol esters. Classic PKC isoenzymes are also dependent on calcium. In contrast atypical PKCs (ζ, λ, μ, and ι) are calcium independent and are not activated by phorbol esters.

PKC α is present in both androgen sensitive LNCaP cells as well as androgen independent cells such as PC-3, PC-3M and DU 145 [217,282]. Androgen independent cells express 6-38 fold more PKCα mRNA and 4-16 fold less PKC μ mRNA than LNCaP cells.(Powell et al, 1996; O'Brian yet al, 1998). PKC α protein expression was respectively 5, 8, and 40 fold higher in PC3M, PC3, and DU145 cells compared with LNCaP [215,217]. They demonstrated a selective over expression of PKC α protein in androgen independent human prostate cancer cells.

Lahn et al [282] obtained 44 prostate cancer biopsy samples to compare over all PKC α with phosphorylated PKC α expression using western immunoblotting. PKC α was present in 100% of the samples where as

phosphorylated PKC α in 98%. They did not have information whether the tissue samples were from ASPCs or AIPCs.

Jaggi et al [279] investigated PKCμ activity in androgen independent C4-2 cells and compared it with LNCaP cells by immunoblot analysis. Also Immunohistochemical analysis of prostate cancer tissue from patients progressing to AIPC was carried. PKCμ expression was seen as diffuse cytoplasmic staining in the tumour tissue. PKCμ was decreased in 100% of androgen independent prostate cancers. This consistent down regulation in both cell line models and human prostate cancer tissue suggests a possible functionally significant role for PKCμ in progression to AIPC.

In our cohort of tumours examined pan PKC immunoreactivity was seen in both tumour groups. All the tumours (100%) were positive for PKC staining. The staining was identified in the cytoplasm of the tumour cells. These findings were consistent with the findings in other studies. Approximately 60% of tumours in the androgen independent group had a high PKC expression (Histoscore >100). Comparison of median pan PKC expression in ASPCs and AIPCs did not have any statistical significance ($p=0.125$).

4.2.2.E AR Protein Expression

In our study AR protein expression was demonstrated in the nucleus of prostate cancer cells. Both androgen sensitive and androgen independent tumours expressed AR protein. AR protein expression was significantly higher in AIPC when compared to ASPC (p=0.000). Median histoscores for AR protein expression in ASPC and AIPC were 94.5 and 132.5 respectively.

We used AR expression data to correlate with c-Jun, phosphorylated c-Jun and c-Fos expression. No correlations between AR and c-Jun or c-Fos in AIPCs were detected. But a significant correlation was observed between AR and AP-1 (p=0.003, cc=0.460 for phosphorylated c-Jun) in androgen sensitive tumours (table 17). An increase in AP-1 formation (in excess of AR) may represent change from androgen mediated to androgen independent tumour growth, allowing AP-1 to function as an independent transcription factor.

The role of AP-1 is further complicated by the fact that c-Jun can act as AR co-factor. In addition to interactions between AP-1 and AR, there is evidence that c-Jun monomer could influence AIPC development independent of AP-1. c-Jun acts as AR co-activator by binding to N-terminal domain thus promoting AR dimerisation and gene transcription [193]. This action of c-Jun is independent of c-Jun phosphorylation, c-Fos, AP-1 DNA binding and AR ligand binding [193,207].

Ogreid et al [295] studied AR protein expression in prostate cancer biopsies before, after, during and after neoadjuvant androgen deprivation followed by radiotherapy. During androgen suppression and subsequent radiotherapy a gradual reduction in tissue immunoreactivity for AR was observed. The most striking observation was the complete lack of AR stainability after combined LHRH/radiotherapy. Of the 7 patients who had a long term follow-up after radiotherapy, 1 patient was cancer negative on biopsy and without AR stainability. Six patients with cancer-positive on final biopsies had regained AR stainability.

Gaston et al [296] reported racial differences in AR protein expression im men with clinically localized prostate cancer. In black compared with white men malignant nuclei were 27% more likely to immunostain for AR (P=0.005) and in immunopositive nuclei AR protein expression was 81% greater (p=0.002). Visual scoring of malignant nuclei revealed that AR immunostaining was significantly increased in black vs white men (171±40 vs 149 ±37, p=0.048).

Edwards et al [297] in their study on AR gene gene amplification and protein expression in hormone refractory prostate cancer reported a significantly higher AR protein expression in hormone resistant tumours compared to matched hormone sensitive tumours from the same tumour (mediam histoscore 130 vs 94.5, p=0.019) with or with out AR amplification.

4.2.3. Involvement.of AP-1(c-Jun, Phosphorylated c-Jun , c-Fos) in progression to AIPC

We observed in our androgen sensitive tumour cohort, c-Jun, phosphorylated c-Jun and c-Fos protein expressions correlated with each other significantly (table 14). c-Jun expression in ASPC correlated with phosphorylated c-Jun (p=0.008, correlation coefficient [cc]=0.396) and c-Fos expression (p=0.020, cc=0.343). Also c-Fos and phosphorylated c-Jun expression correlated in androgen sensitive group of tumours (p=0.027, cc=0.332). However with progression to AIPCs, c-Fos expression no longer correlated with either c-Jun (p=0.083, cc=0.274) or phosphorylated c-Jun (p=0.480, cc=0.113).

It is clear from the above data that c-Jun, phosphorylated c-Jun and c-Fos proteins are expressed by both hormone sensitive and refractory prostate cancers. Protein components of AP-1 (c-Jun, phosphorylated c-Jun and c-Fos) expression in hormone sensitive group correlated with statistical significance. This explains involvement of AP-1 activity in prostate tumours that are hormone dependent. In tumours that progressed to hormone independent status, still c-Jun and phosphorylated c-Jun had a correlation that was significant. But c-Jun and phosphorylated c-Jun expression did not correlate with c-Fos expression suggesting that, during development of AIPC, the balance between phosphorylated c-Jun and c-Fos has changed. This may be the result of c-Jun becoming phosphorylatedand hence more stable at relapse. Also a significant

proportion of tumours expressed high levels (histoscore >100) of AP-1 at relapse (c-Jun 68.6%[35/51]; phosphorylated-cJun 24%[24/51]; c-Fos 40%[20/51]).

We used AR expression data to correlate with c-Jun, phosphorylated c-Jun and c-Fos expression. No correlations between AR and c-Jun or c-Fos in AIPCs were detected. But a correlation was observed between AR and AP-1 ($p=0.003$, $cc=0.460$ for phosphorylated c-Jun) in androgen sensitive tumours (table 17). An increase in AP-1 formation (in excess of AR) may represent change from androgen mediated to androgen independent tumour growth, allowing AP-1 to function as an independent transcription factor. This would result in increased cell proliferation and survival [204].

AP-1 induces transcriptional activation by interaction with AP-1 DNA binding site (TRE) [201,282]. This AP-1 DNA binding site is recognized by c-Jun homodimers and Jun/Fos heterodimers formed by leucine zipper domain of both proteins [171,201,283] AP-1 activity is increased by TPA and AP-1 inhibits androgen induced PSA promoter activity in LNCaP cells. This was due to protein protein interaction between AR and AP-1 [204]. AR and AP-1 interaction prevents either protein from DNA binding and hence transcription, as both AR and AP-1 together form complexes and mutually inhibit each other [204]. Hence the effect of AP-1 on androgen regulated gene expression depends on the ratio of AR to AP-1 and the ability of AP-1 to bind to specific promoter regions in the androgen

regulated gene [110,206]. Such AP-1 and AR protein protein interaction could influence the ability of AP-1 to enhance androgen regulated gene transcription in the absence of androgens and hence might influence the development of AIPC [110,204]. *In-vitro* work on PC3 cells which are poorly differentiated prostate cancer cells and androgen independent demonstrated a 7 fold greater intracellular concentration of c-Jun than in LNCaP cells that are androgen sensitive [204]. This suggests that AP-1 may influence androgen escape in PC-3 cells.

Normal breast cells have high basal levels of AP-1, while breast cancer cells have low basal levels [284,285]. Ludes-Meyer's et al [285] investigated the role of AP-1 in controlling breast cell growth. They used normal breast cells, immortal breast cells and breast cancer cells to study the effect of AP-1 blockade on their growth using c-Jun dominant negative mutant, TAM-67. The study demonstrated highest basal transcriptional activity of AP-1 in normal and immortal human mammary cells and the growth of these cells was suppressed by AP-1 blockade. Therefore these cells require AP-1 transcriptional activity for their growth. Breast cancer cells had lower basal AP-1 transcriptional activity and hence were less sensitive to AP-1 blockade. Of the breast cancer cells tested, MCF7 cells were the most sensitive to growth suppressive effect of the AP-1 inhibitor. Where as other breast cancer cells_tested were resistant. These results demonstrate that the growth of some breast cancer cells is inhibited by AP-1 blockade and AP-1 is a promising target for treatment of breast

cancer. Similarly it may be possible to target patients with high levels of AP-1 expression in AIPCs .When c-Jun, phosphorylated c-Jun and c-Fos protein expression in the primary androgen sensitive tumours, was investigated, we were unable to demonstrate a significant correlation between the proteins investigated and time to relapse (table 9).

In our study 47% (24/51) of patients with AIPC had high levels of phosphorylated c-Jun expression (histoscore >100) in the tumours. This group of patients was noted to have a significantly shorter duration of survival (p=0.023) following development of hormone escape or relapse in comparison to those with low expression (figure 14A). The median duration of survival was 1.87 years (inter quartile range 1.1 years - 2.6 years) in the high expression group and in the low expression group it was 5.5 years (interquartile range 1.07 years - 9.9 years).

These observations support the hypothesis generated by cell line studies that increased AP-1 expression results in promotion of tumour growth [204,206] implicating AP-1 in tumour progression to androgen independent status. Therefore, inhibition of c-Jun activation may be a novel target for treating a subset of hormone refractory prostate cancers.

Gee et al [286] examined the activation of the AP-1 transcription factor in human breast tumours. Phosphorylated c-Jun expression in 78 primary breast tumours. They observed correlations between high phosphorylated c-Jun expression and decreased over all survival and presence of distant metastases. In addition, estrogen receptor positive tumours that

expressed high phosphorylated c-Jun expression developed progressive disease more rapidly than did tumours expressing low phosphorylated c-Jun. These clinical data suggest that in a subset of breast tumours, AP-1 transcription factor is activated and provide rationale to target AP-1 transcription factor In the prevention and treatment of breast cancer.

c-Jun expression and its relationship with clinical statistics was studied in laryngeal cancer by Que et al [287]. The immunohistochemical expression of c-Jun protein in laryngeal cancer (56.41+/-24.8%) was not only significantly higher than that in vocal cord (32.48+/-1.78%) and laryngeal tissue (no expression), but also was correlated with the degree of differentiation of laryngeal cancer and lymph node metastasis ($P<0.01$). However the expression of c-Jun was not associated with clinical staging ($P>0.05$). This study demonstrated, that c-Jun protein was significantly increased in laryngeal cancer cells and its expression may be considered as an indicator for differentiating degree of tumour grade, neck lymph node metastasis, and prognosis of laryngeal cancer.

Observations from our study and those from others suggest that AP-1 transcription factor expression is upregulated in a subset of patients promoting growth of tumours. Also AP-1 is a potential target for therapy in a sub group of patients.

4.2.4. Is PKC associated with c-Jun activation to AP-1 and progression to AIPC?

PKC is reported to phosphorylate and therefore activate c-Jun and also increase c-Fos expression. We therefore investigated PKC expression in relation to c-Jun, phosphorylated c-Jun and c-Fos expression in both Androgen sensitive and independent tumours (table 15). Pan PKC expression in androgen sensitive tumours was not significantly different from androgen independent tumours (p=0.125). In ASPCs no significant correlation was observed with c-Jun, phosphorylated c-Jun and c-Fos expression. However in AIPCs c-Jun expression and PKC expression correlated in a negative manner (p=0.012, cc= -.387).

In approximately 60% of tumours at relapse, pan PKC expression was high (histoscore >150) and in 28% its expression was increased. Another interesting observation was an increase in PKC protein expression in the transition from ASPC to AIPC resulted in a significant reduction in patient survival from time to relapse (figure 14). The median survival in the group with increased PKC expression was 1.61 years (interquartile range 1.4 years – 1.8 years) and in the group with a decrease or no change in expression it was 3.5 years (interquartile range 2.8 years – 4.1 years).

As we noted that patients with high phosphorylated c-Jun and an increase in PKC expression were both had shorter survival from relapse, we therefore combined both these cohort of patients. This combined cohort had survived for a significantly shorter period of time from relapse (p=

0.0036) compared to those with low phosphorylated c-Jun and no increase in PKC expression (figure 15B). Median survival changed from 5.5 years (interquartile range 3.99 – 6.38) to 1.62 years (interquartile range 0.99 – 2.24 years).

Powell et al [217] compared PKC mRNAs in cultured LNCaP and androgen independent prostate cancer cell lines DU 145, PC3 and PC3M. AIPC cell lines expressed more PKC mRNA (6-38 fold) and less PKCμ mRNA (4-16 fold) than LNCaP cells. PKCα expression was 3 fold higher in DU 145 cells than PC3 primary tumours that were formed by orthotopic implantation of the human cancer cells into SCID mice [215,288]. They demonstrated selective over expression of PKCα in AIPC cells.

Krongard and Bai [289] have shown that high levels of constitutive PKC activity in AIPC cells. In LNCaP cells phorbol ester TPA resulted in > 200 fold activation of the Fos promoter, and the cells exhibit negligible Fos expression in the absence of TPA treatment. These results provide a strong evidence that PKC is dormant in LNCaP cells and it could be activated by TPA. High constitutive PKC activity in AIPC cells may render these cells independent of growth factors and other mitogenic stimuli, there by offering the cells a selective growth advantage [215,289].

Chelerythrine is a highly selective PKC inhibitor [215,290]. Chelerythrine treatment has been shown to elicit an apoptotic response in four AIPC cell lines that express constitutively active PKC, but LNCaP cells were

resistant to treatment. These studies suggest the potential value of PKCα as a target of AIPC therapeutic options.

Currently little information is available whether *in-vitro* observation of increased PKCα in AIPCs reflects the PKCα expression in patients with prostate cancer Lahn et al [282] and Cornford et al [281] evaluated expression of different PKC isoforms expression in patients prostate biopsies from 23 men with early prostate cancer. PKCα and PKC γ were increased in comparison to benign prostate tissue. Most striking finding was a decrease in PKCβ and concomitant increase in PKCε. Because of the current limitation of reliable antibodies to detect activated/phosphorylated PKCα in paraffin embedded tumour tissue, more studies using larger number of tumour specimens are needed to investigate the distribution and variability of PKCα expression in prostate cancer. Studies differentiating the role of PKCα in ASPC and AIPC *in*-vivo would facilitate the development of selective or specific PKCα inhibitors [282].

Our study is the first to investigate pan PKC expression in prostate tumours that progressed to androgen refractory status. Our findings suggest that pan PKC is expressed in both hormone sensitive and resistant tumour groups. Increased PKC expression at relapse had a significant effect on survival. Increase in PKC together with high phosphorylated c-Jun expression appear to be poor prognostic ators. It

does appear from these observations that PKC has a significant role in progression of androgen sensitive tumours to androgen independence.

AP-1 transcription factor activity is modulated by growth factors, cytokines, oncogenes and PKC [199,204]. The AP-1 (Jun/Fos) transcription factor contains phosphorylation sites at its N-terminus and near the downstream DNA binding region. N-terminal phosphorylation by MAPK family members is involved in activation of c-Jun [291,292] Formation of Jun/Jun or Jun/Fos dimer requires phosphorylation of c-Jun by c-Jun N-terminal kinase (JNK), which is activated by either PKC / MAP kinase cascade [259]. Activation of c-Jun via PKC / MAP kinase pathway is reported to be involved in the development of AIPC [172,204,205]. In our study, we found an inverse correlation between PKC and c-Jun expression in AIPC tumours (Correlation coefficient=-0.387 and p=0.012). Neither phosphorylated c-Jun nor c-Fos expression correlated with PKC in androgen independent tumours. But both high phosphorylated c-Jun and increased PKC expression in patients with AIPC had a shorter survival from time to relapse. These results suggest that PKC has a role in the development of AIPC independent of c-Jun and AP-1 formation. When levels of phosphorylated c-Jun were combined with changes in PKC expression, the median survival fell from 5.5 years to 1.6 years. This evidence supports the conclusion that PKC plays an independent role in the development of AIPC.

The PKC family consists of at least 12 isoforms that have been reported to have different and opposing roles in cell growth and differentiation.[293]. As little information is available on the precise isoforms that mediate c-Jun activation, we used a pan PKC antibody to assess the role of PKC in the development of AIPC.

Tumour promoters, growth factors, oncogenes and cytokines stimulate COX-2 transcription via PKC and Ras mediated MAPK signalling. [294]. High COX-2 expression (histoscore >150) was observed in half of the androgen independent tumours evaluated. In another third (17/51) of AIPC tumours COX-2 expression was increased. COX-2 protein and PKC protein expression in both ASPCs and AIPCs correlated with statistical significance ($p=0.014$, $cc=0.387$ and $p=0.002$, $cc=0.451$). This was a significant finding. However COX-2 expression in AIPC had no significance to time to relapse (table 9). These observations suggest that both PKC and COX-2 have a significant role in progression to AIPC.

We investigated the role of AP-1 and PKC in prostate cancer patients who progressed to androgen independent prostate cancer. Our study was unique because we had opportunity to analyse paired prostate tumours. We demonstrated that AP-1 and PKC expression in both AIPCS and ASPCs. Both AP-1 and PKC appear to have significant roles in the development of AIPC. High phosphorylated c-Jun and increased PKC expression at relapse are poor prognosticators. Role of PKCα isoform in

human hormone sensitive and independent prostate cancer clinical material is an important area for further investigation.

4.2.5 Design of the study & Its Strengths and Weaknesses

As the study is retrospective, certainly the design of the study could be improved. A prospective study would help to recruit adequate number of patients from different centres. It would be more interesting if prostate biopsies are taken at three different time intervals from each patient studied. First biopsy taken at diagnosis, second biopsy taken when PSA level has reached nadir and finally the third biopsy taken at the time of relapse. This would help to study protein expression levels at diagnosis and then compare the levels of expression at response to hormonal treatment and also after the development of hormone relapse. This gives us a better understanding of protein changes taking place with progression of androgen sensitive prostate cancer to androgen independent prostate cancer. As the patients normally do not need biopsies once diagnosis is confirmed, this means that patients are subjected to prostate biopsies which is not free of complications such as sepsis and bleeding. This would need an informed consent from the patients to be studied. It would take longer time to complete the study as it takes years for the prostate cancer to develop androgen independence.

Protein expression was studied by immunohistochemistry which is qualitative strictly speaking. Hence quantitative protein estimation in addition to IHC could be carried out and it would be complementary to IHC.

The study has both strengths and weaknesses.

The strengths of the present study are

1. Patients were selection of based on strict inclusion and exclusion criteria.
2. Ethical approval was obtained from MREC and LRECs.
3. Paired prostate cancer tissue specimens were available at diagnosis and after the development of hormone relapse in each patient studied.
4. Six different proteins expressions were investigated and inter-observer variation was analysed using two statistical methods.
5. We demonstrated dysregulation of AP-1 related proteins with the development of AIPC. Differences in the levels of protein expression were observed as androgen sensitive prostate tumours progressed to androgen independent state.

   In androgen sensitive prostate tumours, we demonstrated a statistically significant correlation in expression of c-Jun, c-Fos and p-Jun proteins (table 14).

In androgen independent prostate tumours c-Jun expression correlated with phosphorylated c-Jun with statistical significance (p=<0.0001).

We also demonstrated that, high levels of phosphorylated c-Jun and an increase in Protein Kinase C expression at relapse were associated with a decrease in the duration of survival from relapse (p=0.003).

Protein Kinase C expression correlated with COX-2 expression in both androgen sensitive as well as in androgen independent prostate tumours (p=0.014 & 0.002 respectively).

A significant correlation was observed between AR and AP-1 (p=0.003, cc=0.460 for phosphorylated c-Jun) in androgen sensitive tumours (table 17). An increase in AP-1 formation (in excess of AR) may represent change from androgen mediated to androgen independent tumour growth, allowing AP-1 to function as an independent transcription factor.

There are weaknesses to the study. It is a retrospective study. A prospective power calculation for number of patients to be studied was not carried out, as time to complete the study was a limiting factor. It was very difficult to identify suitable patients retrospectively. Prostate cancer patients do not routinely undergo second biopsy (e.g. TURP) and even if they underwent TURP, the patients should have relapsed at the same time. Hence recruitment was the biggest problem. Many patients were excluded as they did not have second biopsy at the time of development

of AIPC or their tissue was not available for the study. Some had a second biopsy but they were still hormone sensitive at that time. So a significant number of patients were excluded.

Protein expression was studied by immunohistochemistry which is qualitative strictly speaking. Hence quantitative protein estimation in addition to IHC should have been carried out and it would have been complementary to IHC.

We were unable to demonstrate a significant correlation between PKC expression and c-Jun expression. This could be secondary to using pan PKC antibody rather than PKC α antibody.

4.2.6 Retrospective Power calculation

Based on the standard deviation of difference in histoscores between pre and post AIPC (c-Jun-64.9, p-Jun-47.4, c-Fos-62.5, PKC-39.7, COX2 - 45.9 and AR-61.8) for 51 paired sets of measurements, a generic power calculation was carried out to estimate the size of difference in protein expression between pre and post AIPCs that the study could be expected to detect.

Based on this calculation, a difference in histoscore between pre and post AIPC of 30 would be detected with a statistical power of 90%. If the difference in histoscore is 40, then the statistical power would be 99%.

Also a generic power calculation was performed to estimate the size of the difference in protein expression between pre and post AIPCs that the

study could reasonably be expected to detect by assuming that the standard deviation of the difference in histoscores was 50 units for all proteins (based on pilot data) and that statistical significance would be assessed at the 5% level using Student's paired t-test. This was calculated for varying number of paired samples (30, 40, 50, 60, 70, 80, 90 & 100),

For 40 paired samples, a difference in histoscore between pre and post AIPC of 30 would be detected with a statistical power of 95.9%. If the difference in histoscore is 40, then the statistical power would be 99.9%.

For 50 paired samples, a difference in histoscore between pre and post AIPC of 30 would be detected with a statistical power of 98.6%. If the difference in histoscore is 40, then the statistical power would be 100%.

In the present study, a total of 51 paired samples of prostate cancer patients were studied. Protein expression was defined as increased or decreased from pre to post AIPC if there was difference of 2 X mean inter-observer variation (c-Jun- 57.7, p-Jun-64.78, c-Fos-58.1, PKC-16.6,, COX-2 -33.4 & AR-29.98). Hence the statistical power would be 100%.for c-Jun, p-Jun and c-Fos proteins, 98.6% for COX-2 & AR, and for PKC-80%.

4.2.7 Hypothesis of the Study Proved or Disproved?

The hypothesis of the study was "AP-1 related transcription factors are dysregulated in the emergence of androgen insensitivity".

The study has shown that both androgen sensitive and androgen independent prostate cancers express c-Jun, c-Fos, phosphorylated c-Jun, PKC, COX-2 and AR proteins.

A significant proportion of tumours expressed high levels of AP-1 at relapse (c-Jun 68.6%, 35/51; P-Jun, 47%, 24/51; c-Fos 40%, 20/51). An increase or no change in AP-1 (c-Jun, P-Jun & c-Fos) was observed in >80% of AIPCs. Also An increase or no change in PKC (55%), COX-2(70%) and AR (90%) protein expression in AIPC was observed.

Differences in the levels of protein expression were observed as androgen sensitive prostate tumours progressed to androgen independent state.

In androgen sensitive prostate tumours, we demonstrated a statistically significant correlation in expression of c-Jun, c-Fos and p-Jun proteins (table 14).

In androgen independent prostate tumours c-Jun expression correlated with phosphorylated c-Jun with statistical significance ($p=<0.0001$).

We also demonstrated that, high levels of phosphorylated c-Jun and an increase in Protein Kinase C expression at relapse were associated with a decrease in the duration of survival from relapse ($p=0.003$).

Protein Kinase C expression correlated with COX-2 expression in both androgen sensitive as well as in androgen independent prostate tumours (p=0.014 & 0.002 respectively).

Also AR protein expression correlated with phosphorylated c-Jun expression in both ASPC and AIPC (p=0.003 & 0.07 respectively). This confirms that both the proteins are involved in the progression of ASPC to AIPC as demonstrated in cell line studies [110, 204].

The study has clearly shown that AP-1 related proteins are dysregulated with the emergence of androgen independent prostate cancer in a subset of patients thus proving the hypothesis.

Role of PKCα isoform in hormone sensitive and independent prostate cancer clinical material is an important area for further investigation.

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Appendix 1. Materials

1. Chemicals and reagents.

Chemical	Source
Absolute alcohol Hydrogen peroxide Xylene	Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK.
Avidin Biotin Calcium chloride Diaminobenzidine tetrachloride Silane Trypsin	Sigma chemical Co.Ltd; Poole, Dorset, UK.
DPX Ethylene diamine tetra-acetic acid disodium salt-EDTA Sodium chloride Tris (hydroxymethyl) aminomethane	BDH, Poole, Dorset, UK.

2. Antibodies

Antibody	Source
c-Jun monoclonal antibody	Novocastra laboratories Ltd, Balliol Bussiness Park West, Newcastle upon Tyne
Phosphorylated c-Jun monoclonal antibody	Santa Cruz Biotechnology, Inc. 2145 Delaware Ave, Santa Cruz CA 95060
c-Fos monoclonal antibody	Biogenex, San Ramon, CA 93583.
Protein Kinase c monoclonal antibody	Abcam Ltd,31 cambridge Science Park, Cambridge.
COX-2 monoclonal antibody	Parc d'Activités du Pas du Lac 10 bis avenue ampère F-78180 Montigny le Bretonneux FRANCE
AR monoclonal antibody	Novocastra laboratories Ltd, Balliol Bussiness Park West, Newcastle upon Tyne.
Dako Antibody diluent Dako Antibody diluent with background reducing components Horse serum PSA monoclonal Mouse IgG1 negative control	DAKO Ltd,Denmark House, Ely, Cambridge, UK.

3. Kits

	Source
DAB Substrate kit for Peroxidase Vectastatin Elite ABC kit Avidin/Biotin Blocking Kit	Vector laboratories, Inc, 30 Ingold Road, Burlingame, CA 94010
DAKO LSAB+ Kit, Peroxidase, Universal	DAKO Ltd,Denmark House, Ely, Cambridge, UK.

4. Miscellany

	Source
Cover slips Slides	BDH, Poole, Dorset, UK .
DAKO Pen	DAKO Ltd,Denmark House, Ely, Cambridge, UK.
Gloves	Kimberly –Clark Universal Hospital Supplies Enfield, EN3 7QJ London

5. Equipment

Equipment	source
Pipettes (Pipetman)	Anachem Ltd. Anachem House, Charles Street, Luton, Bedfordshire.
Weighing Scale (Mettler P1210) ph meter (Mettler Toledo MP 220)	Mettler Toledo Ltd 64 Boston Road, Leicester.
Microscope	Leitz in Wetzlar, Germany.
Microtome (Leica RM 2135)	Leica Instruments, Heidelberger Street, Germany.
Magnetic Stirrer (Variomag- R)	Jencons (Scientific) Ltd Stanbridge Road Leighton Buzzard, Bedfordshire.

Appendix II. Commonly used solutions

Name	Recipe
0.1% $CaCl_2$	0.08g $CaCl_2$ in 80 mls of distilled water.
0.3% Hydrogen peroxide	5mls of 30% H_2O_2 IN 500 mls of distilled water.
10 timesTBS	60 g TRIS base, 87.6g of Sodium chloride in 800 mls of distilled water, pH- 7.4, made upto 1litre.
TBS	100 mls of 10 times TBS in 900 mls of distilled water.
Tris EDTA buffer	0.37g Sodium EDTA, 0.55G Trizma base in 1L of distilled water.(pH 8.0)

Appendix III

c-Jun Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 1	AR 2	130	115
AR 3	AR 4	205	115
AR 5	AR 6	129	147.5
AR 8	AR 9	275	150
AR 14	AR 15	156	135
AR 16	AR 17	62	65
AR 18	AR 19	140	190
AR 23	AR 24	135	110
AR 29	AR 30	152.5	182.5
AR 31	AR 32	125	80
AR 33	AR 34	70	140
AR 35	AR 36	248	175
AR 37	AR 38	135	200
AR 41	AR 42	225	72.5
AR 43	AR 44	75	60
AR 45	AR 46	85	205
AR 47	AR48	260	175
AR 49	AR 50	190	120
AR 51	AR 52	120	152.5
AR 55	AR 56	168	205
AR 57	AR 58	180	172
AR 59	AR 60	198	200
AR 61	AR 62	185	155
AR 63	AR 64	155	181
AR 65	AR 66	246.5	125

c-Jun Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 67	AR 68	180	147.5
AR 69	AR 70	180	210
AR 72	AR 73	180	175
AR 74	AR 75	112.5	150
AR 76	AR 77	175	177.5
AR 78	AR 79	15	160
AR 80	AR 81	120	180
AR 82	AR 83	151.5	185
AR 84	AR 85	130	205
AR 86	AR 87	172.5	160
AR 88	AR 89	165	115
AR 90	AR 91	150.5	160
AR 92	AR 93	147	110
AR 96	AR 97	45	105
AR 100	AR 101	175	160
AR 102	AR 103	180	175
AR 104	AR 105	205	200
AR 107	AR 108	100	135
AR 109	AR 110	102.5	149
AR 111	AR 112	198	149
AR 114	AR 115	180	290
AR 116	AR 117	270	160
AR 118	AR 119	195	105
AR 120	AR 121	235	160
AR 122	AR 123	100	160
AR 124	AR 125	245	155

APPENDIX IV

Phosphorylated c-Jun Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 1	AR 2	77.5	117
AR 3	AR 4	135	110
AR 5	AR 6	100	90
AR 8	AR 9	119	115
AR 14	AR 15	77.5	50
AR 16	AR 17	35	0
AR 18	AR 19		95
AR 23	AR 24	50	115
AR 29	AR 30	95	119
AR 31	AR 32	110	5
AR 33	AR 34	55	35
AR 35	AR 36	135	100
AR 37	AR 38	16	135
AR 41	AR 42	132	0
AR 43	AR 44	40	85
AR 45	AR 46	96	125
AR 47	AR48	125	115
AR 49	AR 50	20	90
AR 51	AR 52	135	125
AR 55	AR 56	145	135
AR 57	AR 58	197.5	176.5
AR 59	AR 60	75	105
AR 61	AR 62	95	120
AR 63	AR 64	145	145
AR 65	AR 66	145	95

Phosphorylated c-Jun Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 67	AR 68	122	125
AR 69	AR 70	180	190
AR 72	AR 73	127.5	125
AR 74	AR 75	80	42
AR 76	AR 77	180	105
AR 78	AR 79	170	130
AR 80	AR 81	102.5	130
AR 82	AR 83	80	65
AR 84	AR 85	130	142.5
AR 86	AR 87	89.5	130
AR 88	AR 89	105	90
AR 90	AR 91	90	50
AR 92	AR 93	90	25
AR 96	AR 97	55	25
AR 100	AR 101	26	35
AR 102	AR 103	65	55
AR 104	AR 105	40	45
AR 107	AR 108	70	120
AR 109	AR 110	90	105
AR 111	AR 112	105	25
AR 114	AR 115	120	155
AR 116	AR 117	135	75
AR 118	AR 119	110	0
AR 120	AR 121	165	135
AR 122	AR 123	100	125
AR 124	AR 125	180	130

APPENDIX V

c-Fos Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 1	AR 2	72.5	40
AR 3	AR 4	15	115
AR 5	AR 6	65	100
AR 8	AR 9	77.5	140
AR 14	AR 15	50	120
AR 16	AR 17	74	0
AR 18	AR 19	119	95
AR 23	AR 24	62.5	95
AR 29	AR 30	115	52
AR 31	AR 32	85	50
AR 33	AR 34	5	125
AR 35	AR 36	40	35
AR 37	AR 38	55	155
AR 41	AR 42	100	0
AR 43	AR 44	0	0
AR 45	AR 46	25	37
AR 47	AR48	70	10
AR 49	AR 50	45	41
AR 51	AR 52	0	92
AR 55	AR 56	75	75
AR 57	AR 58	200	
AR 59	AR 60	155	97
AR 61	AR 62	160	76
AR 63	AR 64	107.5	85
AR 65	AR 66	125	115

c-Fos Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 67	AR 68	140	110
AR 69	AR 70	155	145
AR 72	AR 73	92.5	90.5
AR 74	AR 75	95	45
AR 76	AR 77	155	105
AR 78	AR 79	90	135
AR 80	AR 81	125	140
AR 82	AR 83	152.5	178
AR 84	AR 85	160	147.5
AR 86	AR 87	166	170
AR 88	AR 89	140	15
AR 90	AR 91	155	140
AR 92	AR 93	110	
AR 96	AR 97	50	30
AR 100	AR 101	75	177.5
AR 102	AR 103	125	115
AR 104	AR 105	152.5	137.5
AR 107	AR 108	70	65
AR 109	AR 110	147.5	195
AR 111	AR 112	210	172.5
AR 114	AR 115	135	190
AR 116	AR 117	157.5	155
AR 118	AR 119	130	0
AR 120	AR 121	180	20
AR 122	AR 123	130	120
AR 124	AR 125	200	77.5

APPENDIX VI

PKC Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR1	AR2	190	215
AR3	AR4	300	220
AR5	AR7	230	190
AR8	AR9	230	195
AR14	AR15	172.5	165
AR16	AR17	180	160
AR18	AR20	195	170
AR23	AR26	170	165
AR29	AR30	135	135
AR31	AR32	160	180
AR33	AR34	260	205
AR35	AR36	195	185
AR37	AR38	145	75
AR41	AR42	165	145
AR43	AR44	155	240
AR45	AR46	185	165
AR47	AR48	230	150
AR49	AR50	135	190
AR51	AR 52	155	180
AR55	AR56	150	140
AR57	AR58	140	220
AR59	AR60	125	135
AR61	AR62	155	130
AR63	AR64	135	155
AR65	AR66	125	150

PKC Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR67	AR68	175	200
AR69	AR71	180	170
AR72	AR73	135	160
AR74	AR75	160	145
AR76	AR77	190	135
AR78	AR79	200	200
AR80	AR81	130	112.5
AR82	AR83	115	115
AR84	AR85	105	135
AR86	AR87	130	160
AR88	AR89	150	115
AR90	AR91	157.5	140
AR92	AR93	150	150
AR96	AR97	185	160
AR100	AR101	200	162.5
AR102	AR103	185	160
AR104	AR106	125	200
AR107	AR108	175	155
AR109	AR110	175	172.5
AR 111	AR113	170	185
AR114	AR115	185	240
AR116	AR117	220	170
AR118	AR119	230	150
AR120	AR121	220	170
AR122	AR123	240	175
AR124	AR125	230	195

APPENDIX VII

COX-2 Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 1	AR 2	235	150
AR 3	AR 4	235	197.5
AR 5	AR 6	220	237.5
AR 8	AR 9	185	210
AR 14	AR 15	172.5	150
AR 16	AR 17	210	190
AR 18	AR 19	140	185
AR 23	AR 24	125	165
AR 29	AR 30	115	155
AR 31	AR 32	110	160
AR 33	AR 34	155	210
AR 35	AR 36	125	160
AR 37	AR 38	175	70
AR 41	AR 42	170	125
AR 43	AR 44	135	125
AR 45	AR 46	115	150
AR 47	AR48	235	145
AR 49	AR 50	105	152.5
AR 51	AR 52	135	160
AR 55	AR 56	185	140
AR 57	AR 58	120	165
AR 59	AR 60	162.5	125
AR 61	AR 62	175	160
AR 63	AR 64	140	130
AR 65	AR 66	95	147.5

COX-2 Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR 67	AR 68	135	175
AR 69	AR 70	145	195
AR 72	AR 73	152.5	155
AR 74	AR 75	135	130
AR 76	AR 77	185	150
AR 78	AR 79	195	160
AR 80	AR 81	175	170
AR 82	AR 83	95	160
AR 84	AR 85	115	145
AR 86	AR 87	152.5	155
AR 88	AR 89	132.5	165
AR 90	AR 91	120	112.5
AR 92	AR 93	180	190
AR 96	AR 97	140	70
AR 100	AR 101	140	75
AR 102	AR 103	140	130
AR 104	AR 105	80	160
AR 107	AR 108	155	80
AR 109	AR 110	190	135
AR 111	AR 112	92.5	155
AR 114	AR 115	170	205
AR 116	AR 117	180	185
AR 118	AR 119	115	40
AR 120	AR 121	105	120
AR 122	AR 123	160	175
AR 124	AR 125	150	170

APPENDIX VIII

AR Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR1	AR2	150	155
AR3	AR4	145	197.5
AR5	AR7	155	107.5
AR8	AR9	177.5	200
AR14	AR15	64.5	117.5
AR16	AR17	45	130
AR18	AR20	140	220
AR23	AR26	2.5	5
AR29	AR30	110	195
AR31	AR32	147.5	167.5
AR33	AR34	110	157.5
AR35	AR36	170	140
AR37	AR38	2.5	5
AR41	AR42	75	92.5
AR43	AR44	66	53
AR45	AR46	55	114.5
AR47	AR48	54	60
AR49	AR50	25	69
AR51	AR 52	105	118
AR55	AR56	74.5	55
AR57	AR58	94.5	192.5
AR59	AR60	27.5	167.5
AR61	AR62	125	217.5
AR63	AR64	60	42.5
AR65	AR66	1	10

AR Histoscores in ASPC And AIPC

AR NO	AR NO	ASPC Histoscores	AIPC Histoscores
AR67	AR68	95	155
AR69	AR71	167.5	
AR72	AR73	102.5	187.5
AR74	AR75	102.5	91
AR76	AR77	45	87.5
AR78	AR79	0	0
AR80	AR81	70	32.5
AR82	AR83	87.5	167.5
AR84	AR85	45	170
AR86	AR87	55	92.5
AR88	AR89	120	130
AR90	AR91	75	215
AR92	AR93	112.5	
AR96	AR97	75	155
AR100	AR101	57.5	132.5
AR102	AR103	110	
AR104	AR106	7.5	30
AR107	AR108	110	165
AR109	AR110	122.5	185
AR 111	AR113	92.5	32.5
AR114	AR115	115	175
AR116	AR117	180	125
AR118	AR119	120	135
AR120	AR121	125	210
AR122	AR123	160	190
AR124	AR125	115	125