Cranfield University

Cranfield Health

Emmanuel Okechukwu Nna (B.MLS, M.Sc)

Molecular Profiling of Prostate Cancer Patients

PhD Thesis

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Academic Years: 2005-2008

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Supervisors: Dr T A Bailey and Dr I E Tothill

June 2009

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Abstract

In the UK, more than 30 000 men are diagnosed annually with prostate cancer (PCa) and about 10 000 men die from it each year. Although several molecular markers have been associated with prostate cancer development and/ or progression, only few of them are used in diagnostic pathology. The current standard tests include serum PSA test, digital rectal examination and histology of prostate biopsy. Recently the PCA-3 molecular test was approved in the European Union, and it is now used in many laboratories. But these tests are not sufficient to molecularly characterise the behaviour of prostate cancer in many patients.

Through extensive literature review, a panel of sixteen molecular markers were selected for further evaluation in prostate cancer cases. They included KLK2, KLK3, MCM2, MCM5, TP53, Bcl-2, CD44, CDH1, AURKA, AURKB, and AURKC; ESRα, ESRβ, AR, FASN, TMPRSS2: ERG, and TMPRSS2:ETV1. The aim was to examine the link between development/progression of prostate cancer and the production of diagnostic/prognostic biomarkers. An *in vitro* model consisting of PC-3 and PNTIA, MDA PCa 2b prostate cell lines were used to investigate the influence of steroid hormones on these biomarkers using molecular and proteomic techniques. All the three cell lines expressed AR, ESRα, ESRβ and PSA at mRNA and protein levels. The AR expressed in PC-3 and MDA PCA 2b cells was 60 kDa while the PNT1A expressed a 90 kDa AR protein. The ESRβ was over-expressed in the MDA PCA 2b cells, and was also significantly up-regulated by 17β oestradiol treatment. At a concentration of 4.92 and 33.96μM 17β oestradiol inhibited the growth of 10 to 50% of PNT1A cell line and increased the doubling time three folds. Although the PC-3 cells expressed AR, it was still androgen insensitive and could not produce PSA in culture supernatants. AR and PSA were up-regulated in PNT1A cells in response to testosterone and dihydrotesterone treatment but were reduced in response to 17β oestradiol and Hydrocortisone treatment. All the molecular markers except the TMPRSS2: ERG and TMPRSS2:ETV1 were expressed in the cell lines. The MCM2 and MCM5 were not differentially expressed in response to hormonal treatment. However, the Aurora kinases A, B and C were up-regulated in response to steroid modulation. The KLK2 was only up-regulated by the androgens. Three candidate control genes: ABL1, GUS and G6PD were also evaluated in the cell lines and clinical samples; the ABL1 gene emerged as the most stably expressed house keeping gene and was subsequently used in the normalization of real time PCR assays (RQ-PCR).

Analysis of the sixteen biomarkers in prostate tissues and exfoliated urine cells of benign, prostate cancer and non-involved cases (n = 228) showed that seven of the molecular markers were significantly strongly associated with prostate cancer progression ($P<0.05$). The Aurora

kinases A and B were consistently significantly over-expressed in prostate cancer cases. The CD44 was also over-expressed in prostate cancer, and was associated with Gleason score. The TMPRSS2 fusion genes were detected in 15.6% of the prostate cancer cases. The TP53 was also over-expressed in prostate cancer, and significantly associated with tumour grade. The ESRβ was over-expressed in prostate cancer, and was significantly associated with high tumour grade. This implied a proliferative role for the ESRβ in prostate cancer progression, because the ESRα was not differentially expressed among the sample groups. Concomitantly, the AR was also over-expressed in same pattern with ESRβ. The combination of these biomarkers: AR, ESRβ, CD44, TP53, TMPRSS2 fusion genes, AURKA and AURKB could molecularly characterise most prostate cancers. Therefore 2 sets of pentaplex RQ-PCR assays including ABL1 for normalization would provide a cost-effective, flexibly high throughput assay for molecular grading of tissue sections in diagnostic pathology.

In addition to the gene expression studies, the genetic variation in KLK2 gene was further investigated by direct DNA sequencing, pyrosequencing and TaqMan allelic discrimination assay. Two SNPs in the gene were found significantly associated with prostate diseases. The T/T allele of rs198977 predicted the presence of prostate cancer at biopsy and was associated with high tumour grade. The A/A variant of rs2664155 was also significantly associated with the presence of benign nodular hyperplasia.

The combination of gene expression and genetic variation using real time PCR applications would provide an accurate, reproducible and cheap method for molecular profiling of prostate cancer patients.

An exploratory study of organic volatiles in urine of one prostate cancer patient and eight BPH patients using thermal desorption GC-MS showed that Ethanethiol, Dimethyl sulfide, Propyn-1-ol acetate, Nitro-2-propanone, pentane, Hydrazine and Nitrous oxide were differentially over-expressed in the prostate cancer patient compared to the benign cases. Further studies would be required to rule out possible contamination and drug metabolites.

Acknowledgements

Several people contributed to the success of this project. I would like, first of all, to thank my main supervisor, Dr Tracey Bailey, who initiated the project and was able to see to its completion. There were, like every other project, many difficult moments she had to navigate with me to bring this to fruition. I am greatly indebted to her. In no less measure, I must thank Dr Sam Tothill, for the energy and drive she brought into the project as the second supervisor. Her support, motivation and encouragement were enormous and very fantastic. I am also very grateful to Prof A C Woodman and Dr Karen Price, who at some points in time were co-supervisors of this project.

There would be many special thanks. Firstly, to the staff of Pathology department of the Royal Gloucestershire Hospital and Cheltenham General Hospital, especially to Prof Neil Shepherd, Dr Linmarie Ludeman and Mr Grant Newman. Dr Ludeman did a wonderful job in organizing the tissue blocks, re-assessment of histological slides and pathological reports. Secondly, many thanks to Dr M Wasil of the Research and Development Directorate of Bedford NHS trust for his assistance in the application for research ethics approval. Dr H Sharma was the collaborator who made it possible for us to gain assess to patients who attended the transrectal ultrasound guided biopsy sections at the Primrose Cancer unit, Bedford Hospital. Mrs Nora Smith, the Specialist Urology Nurse, was very helpful during sample collection. Many thanks are due to all the member of Primrose Cancer unit. My special thanks to Dr Margaret Wilkins and Mrs Gillian Flack, of the Cellular Pathology department, Bedford Hospital, for the provision of embedded prostate tissue blocks and pathology reports. Thirdly, I am very grateful to the staff of the Medical centre, Cranfield University for the assistance during sample collection from healthy participants.

In no particular order, all the members of the Cranfield health, from Silsoe campus to Cranfield campus, were very helpful. I am greatly indebted to the head of school, Prof Joe Lunec who assisted, in no little measure, to ensure that this project was completed. I mustn't fail to thank Dr Pascal Mavrommatis who helped me enormously. I am very grateful to Dr Chris Walton and Dr Michael Cauchi for their assistance on GC-MS analysis, and Dr Wayne Mitchell for his assistance on Flow cytometry. There were so many wonderful friends made during this project: Dmitris, Ewa, Samuel, Kemi, Emmanuel-my namesake and many others. Towards the completion of this project, Prof Letizia Foroni of Adult Leukaemia centre laboratory, Imperial College London was very helpful in so many ways.

I am greatly thankful to all subjects who participated in this study, without whom it would difficult to complete this study. I am equally indebted to the Cranfield Research Ethics Committee, Gloucestershire Research Ethics Committee and Hertfordshire Research Ethics Committee who gave the favourable opinion to commence this work.

Without the help and support of my wife, Mrs Nkem Nna, it would be absolutely impossible to complete this project. I am immensely grateful to her. It is very remarkable to reflect on the numerous supports from my mum, Mrs Victoria Nna, who has always been there for me. I am thankful to my senior brother, Samuel Nna, and members of our extended family who suffered my absence in many occasions.

Finally, it is 'not of him that wills nor of him that runs, but of God who shows mercy'. I am very happy that 'it came to pass'. The financial challenge involved in this project was enormous. The completion of this project was simply a miracle.

Dedication

This thesis is dedicated to all men who were victims of prostate cancer.

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Nomenclature/Abbreviations

ABL1	c-abl oncogene 1, receptor tyrosine kinase
AR	Androgen Receptor
AurkA	Aurora kinase A
AurkB	Aurora kinase B
AurkC	Aurora kinase C
Bcl-2	B-Cell CLL/Lymphoma 2
BPH	Benign Prostatic Hypertrophy (Nodular Hyperplasia)
CD	Cluster differentiation
CDH1	Epithelial Cadherin type 1
CDK	Cyclin dependent kinase
CEA	Carcinoembyronic antigen
CG	Control gene (endogenous reference gene)
DCC-FCS	Dextran charcoal treated foetal calf serum
DRE	Digital rectal examination
ELISA	Enzyme linked-immunosorbent assay
ESR	Oestrogen receptor
FASN	Fatty acid synthase
G6PD	Glucose 6 phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
GUS	Beta glucuronidase complex
hK2	Human glandular kallikrein 2 protein
hK3	Human glandular kallikrein 3 protein (PSA)
hK4	Human kallikrein 4 protein
ICC	Immunocytochemistry for cells
ICR	Institute for Cancer Research, UK.
IFA	Immunofluorometric assay
IHC	Immunohistochemistry for tissues
ISH	In situ Hybridization
KLK 2	Human glandular kallikrein gene
KLK3	Human kallikrein 3 gene (PSA gene)
KLK4	Human kallikrein 4 gene
LHRH	Lutenizing hormone releasing hormone
LI	Labelling index
MCM	Minichromosome maintenance protein
mRNA	messenger ribonucleic acid/transcript
PCA	Principal component analysis plot
PCa	Prostate Cancer
PCA3/DD3	Prostate cancer antigen 3
PCNA	Proliferating cell nuclear antigen
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate specific antigen
PSAP	Prostate-specific acid phosphatase
RQ-PCR / qPCR	Quantitative reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrilamide gel electrophoresis

Abbreviations Continued

SHR	Steroid hormone receptor
SIFT-MS	Selected Ion Flow Tube-Mass Spectrometry
SNP	Single nucleotide polymorphism
TMPRSS2	Transmembrane protease serine 2
TMs	Tumour markers
TP53	Tumour protein 53
TRUS	Transrectal ultrasonography
WHO	World Health Organization

Chapter 1- Introduction and Literature Review

1.1 Introduction to Molecular basis of Prostate Cancer

1.1.1 Prostate Cancer statistics

Globally, the incidence of prostate cancer has been on the increase in recent years. The World health organization (WHO) reported a steady increase in prostate cancer incidence which resulted in 239,000 deaths in 1998, 255,000 deaths in 1999 and 269,000 deaths in 2002 {WHO, 2004}.The mortality rate for prostate cancer, standardized to the world population, ranged from 1.3 to 22.1 per 100,000 men {Dijkman & Debruyne ,1996}.

It is estimated that 13% of all cancer deaths result from prostate cancer in most western countries {Droller, 1997}. The incidence and death rates for prostate cancer by country in the European Union in 1998 is shown in Table 1.1. In the United Kingdom, it is reported that more than 30,000 men are diagnosed annually with prostate cancer and about 10,000 men die from it each year; showing that prostate cancer has replaced lung cancer as the most common cancer in men {The Institute of Cancer Research UK, 2006}. In the United States of America, Prostate cancer (PCa) is the second most common type of malignancy and cause of death due to cancer among men {The Cancer Net, 2004}. In 2004 in the US, it was estimated that more than 200,000 males were diagnosed with prostate cancer, joining approximately 1.4 million men already diagnosed and about 30,000 died from it {Rodney *et al,* 2006}. For the Sub-Saharan Africa in 2002, a total of 26,800 new cases were estimated, comprising 10.6% of cancers of men {Dean *et al*, 2006}.

Most of the patients diagnosed with PCa have early-stage disease that is confined to the prostate gland, with less than 6% presenting with advanced, metastatic disease {Wilding et *al,* 2006}. Prostate cancer incidence increases with age more rapidly than any other type of cancer; less than 1% of it are diagnosed in men below 50 years of age {Isaac, 1997}. Ninety-five percent of cases of PCa are diagnosed in men between 45 and 89 years of age, with a median age at diagnosis of 72 years {Meikle & Smith, 1990}. It is often described as the disease of old age in men. However, it is presumed that PCa in younger men are often diagnosed in the metastatic stage at the time of clinical presentation, but there is no strong evidence to support this {Wilding *et al,* 2006}.

Table 1.1 Incidence and death rates from prostate cancer by country in EU in 1998.
(Source: Health Evidence Network, WHO Regional office for Europe 2004).

Population	Cases	Age-standardized Rate (per 100 000)	Deaths	Age-standardized rate(per 100 000)
European Union	144504	67.55	56035	25.55
Austria	3667	89.49	1139	27.21
Belgium	5566	95.34	1846	30.59
Denmark	1627	53.89	1009	32.11
Finland	3087	121.84	777	31.02
France	28 135	87.10	9239	27.08
Germany	30 911	77.21	11417	26.65
Greece	2823	41.00	1208	17.22
Ireland	1138	69.57	514	30.68
Italy	19 258	52.78	7109	19.12
Luxembourg	163	78.53	49	24.42
The Netherlands	6594	85.74	2383	30.25
Portugal	3210	55.23	1653	27.92
Spain	10659	45.33	5742	23.76
Sweden	6610	114.95	2480	37.71
United Kingdom	21056	60.97	9470	26.41

Racially, blacks have the highest prostate cancer incidence rates in the world compared to whites and Asians for all age groups {Meikle & Smith. 1990}. The lowest incidence rates observed in Asian populations in their native countries seem to change following migration to western countries {Dijkman & Debruyne, 1996}.

However, some reports have suggested under-estimation of prostate cancer incidence as the cause of purported low incidence rate in Asian population rather than a 'true natural low incidence' {Hong-Wen et *al*, 2005}. In the western countries, men of African descent still have the highest incidence and mortality rates in prostate cancer. The 5-year survival rate for prostate cancer diagnosed in the United States between 1974 and 1985 was 62% for African-Americans as compared with 72% for American whites. The racial/ethnic variations on incidence, mortality and survival rates of PCa may be contingent on socioeconomic status and health care delivery systems rather than genetic causes {Freedland & Isaacs, 2005}.The risk

factors of prostate cancer, which include age, environment, race, diet, family history and life style, are discussed in Section 1.2.3.

Even though the disease burden of prostate cancer is very significant in most countries, there are a few controversies in prostate cancer statistics. Prostate cancer incidence does not clearly correlate with its mortality. Until recent reports {Ling Zhang *et al*, 2009}, it was traditionally believed that the Chinese have the lowest prostate cancer incidence even though many cases were discovered at autopsy. But, the mortality to incidence rate (MR/IR) was 0.63 in the Chinese population, much higher than that of North America (MR/IR = 0.13) that has a higher incidence rate. Diagnostic strategy in China was deemed inadequate, many cases presented late at advanced stage and some died of it without diagnosis {Ling Zhang *et al*, 2009}. Another factor is that not all patients with prostate cancer die from it nor suffer symptoms from it during their life time. Some are not even diagnosed except during autopsy. But incidence rates are calculated from the numbers of patients who have a histologic diagnosis of the disease with or without any clinical or biologic significance {Meikle & Smith, 1990}. The age specific prevalence of histological prostate cancer, that is PCa diagnosed at autopsy and biopsy (accidental during TRUS resection), is uniform world-wide, and this frequency is many times greater than would be expected from the incidence and mortality of clinical prostate cancer {Dijkman & Debruyne, 1996}.

Screening and diagnostic criteria also vary among countries. In some countries, annual mass screening of men aged 50 years and above has been introduced; which is capable of detecting more cases of PCa (increase incidence rate up to 22%) {Andriole *et al,* 2009}. It is advocated in the United States that men with a positive family history of prostate cancer, after the age of 40 years, should undergo a digital rectal examination (DRE) and PSA monitoring yearly {Dijkman & Debruyne, 1996}. Results of the European Randomized study of Screening for Prostate Cancer (the largest screening of its kind) showed that mass screening using PSA test and DRE, followed by prostate biopsy for men with PSA > 3.0ng/ml yielded a 20% reduction in mortality from PCa (http://www.erspc-media.org/release090318.php). This result underpins the rationale why some countries are more aggressive in screening than others, leading to differential detection rates among countries. Furthermore, as the life expectancy of the male population increases over time, the incidence of clinical prostate cancer will also increase {Isaac. 1997}. It has been argued that the increase in incidence of PCa in many countries could be attributed to the availability of more frequent and better diagnostic tests, an aging population and increased awareness for both patients and physicians, and not necessarily a true rise in incidence {Andriole *et al,* 2009}. Another aspect of the controversy is the difficulty in accurately reporting mortality rates due to PCa because some prostatic

cancer patients die from other causes of death. Prostate cancer is a disease of old men, more of whom die with the disease than from the disease. This might explain the preference of incidence rate to mortality rates in reporting PCa cases.

1.1.2 Cellular and Molecular Basis of Cancer

The term cancer is synonymous with neoplasm and tumour. Human cancer is not a single disease rather a myriad collection of diseases with as many different manifestations as there are tissues and cell types in the body, involving numerous internal and /or external causative agents, and various disease mechanisms {Coleman & Tsongalis, 2002}. Willis (1967) defined a neoplasm as a mass, the growth of which is incoordinate with the surrounding normal tissues and that persists in the absence of the inciting stimulus {McKinnell RG, 2006}. At cellular level, neoplasm is characterized by unregulated cell growth, impaired differentiation, invasiveness (increased cell mobility and loss of contact inhibition) and metastatic potential (ability to spread to distant organs). These biological traits of neoplastic cells enable them to exert nutritional, spatial, signal and mobility pressures on normal neighbouring cells. The neoplastic cells also, in many cases, form structures and patterns that morphologically distinguish them from normal cells. The transformation process is accrual.

Neoplasms can be benign or malignant. Depending on cell and tissue of origin, the main classes (nomenclature) of neoplasm are:

i. Carcinoma which is the malignant form of cancer arising from the skin and epithelial lining of internal organs and surfaces. The benign tumours of epithelial origin have various names (depending on basic cell type) such as papilloma (from skin and urinary bladder), adenoma (solid epithelial organs, epithelia of gonads and bronchial epithelium), melanoma (skin pigments) and teratoma (germ cells);
ii. Sarcoma, which is malignant cancer of the mesenchyme (bone, fibrous tissue, fat, cartilage, muscle, blood vessels and lymph vessels) and shades of names for the benign forms (Osteoma, Fibroma, Lipoma, Chondroma, Leiomyoma and Haemangioma);
iii. Blastoma for the malignant cancers of the nervous system (neuroblastoma for nerve cells, Astrocytoma for astrocytes and Oligodendrocytoma for oligodendrocytes) while benign forms for meningeal cells are called Meningioma.
iv. Leukaemia for white blood cells, Erythroleukaemia for red blood cells, Lymphoma for the lymph nodes and reticulo-endothelial system. Cancer of the embryonic type tissues are known as teratocarcinoma {Franks & Teich, 2001}.

At molecular level, it is conceived that the accumulation of multiple mutations within genes of a single cell drive neoplastic transformation leading to cancer development {Coleman & Tsongalis, 2002}.Several agents cause these mutations including viruses, chemicals (carcinogens); radiations, bacterial and fungal activities, and the ageing process. In its simplest form, the human cancer is a genetic corruption of a normal cell. The multiple transforming mutations confer selective growth advantage to a cell by affecting three major classes of genes: proto-oncogenes, tumour-suppressor genes and DNA repair genes. These classes of genes are altered (activated and/ or inactivated) by a series of cellular and molecular events which include deletions, insertions, translocations (rearrangements), mutations, loss of heterozygosity, amplification (increase copy number), epigenetic changes and genomic instability (chromosomal instability and microsatellite instability){Lengaur *et al*, 1999}. These events result in aberrant genes encoding for oncoproteins and/ or abnormally expressed normal proteins, which drive neoplastic transformation; and the inability of the repair system to safeguard the genomic DNA. Therefore, the development of human cancers almost always includes certain patterns of abnormality: i) autonomy of cell proliferation-through loss of the normal extracellular regulatory activity and acquisition of dysregulated internal drive for proliferation, ii) escape from apoptosis, iii) evasion of senescence and iv) gain of angiogenesis {Hanahan & Weinberg, 2000; Wylie, 2008}.

Oncogenes are activated (frequently mutated) alleles of normally functioning wild-type genes (proto-oncogenes) that function in cell-cycle progression and cellular proliferation. Examples of oncogenes are the c-myc family, c-erbB family, the c-ras family, the c-abl; growth factors and their receptors: PDGF family, EGF family, Wnt family, the interleukins and the serine/tyrosine/threonine kinases (c-raf family, Protein Kinase C family, akt). Activated or mutated proto-oncogenes promote unregulated cell-cycle progression and cell proliferation, leading to cancer development {Coleman & Tsongalis, 2002}.

The tumour- suppressor genes encode proteins that inhibit, constrain or suppress cell proliferation. The tumour-suppressor (T-S) proteins function in parallel with the protein products of proto-oncogenes, but work instead to suppress cell proliferation through the regulation of signal transduction and nuclear transcription. Mutations in the T-S proteins (Table 1.2) lead to altered cellular morphology, loss of normal intracellular-signaling pathways and loss of normal intercellular interactions, all of which are features of neoplastic cells {Weinberg, 1991}. The loss of function of T-S proteins is as important as the gain in function of proto-oncogenes in the development of human cancers {Levine, 1993}.

Normal cells have the ability to repair damaged DNA; a process that helps to prevent permanent mutations during DNA replication. DNA repair systems involve four main processes:

i) recognition and removal of altered portion of a damaged DNA by DNA repair nucleases, ii) binding of DNA Polymerase to the 3'-OH end of the cut DNA to fill the gap by making a complementary copy of the information stored in the residual template strand, iii) proof reading of new DNA strands during replication by the 3'-5' exonuclease activity of DNA Polymerase and iv) post replication or mismatch repair {Coleman & Tsongalis, 2002}. Alterations of any these DNA –repair processes can result in a large increase in spontaneous mutations and predisposition to cancer development. Therefore, cancer cells may have a partially weakened DNA repair system. This phenomenon has become the basis of a new class of anti-tumour agents, which cause 'synthetic lethality' by destroying alternative DNA repair systems in cancer cells, predisposing them to death {Kaelin, 2005}. Just at the time of writing this thesis, the results were reported of phase 1 clinical trial of a new drug called Olaparib (AZD2281), which was designed based on synthetic lethality to inhibit the Polyadenosine triphosphate ribose polymerase (PARP) in prostate, breast and ovarian cancer patients with BRCA 1 or BCRA 2 mutations (DNA repair defect) {Fong *et al*, 2009}. The PARP1 enzyme plays a key role in the repair of DNA single-strand breaks through the repair of base excisions. The inhibition of PARPs leads to the accumulation of DNA single-strand breaks, which can lead to DNA double-strand breaks at replication forks. Normally, these breaks are repaired by means of the error free homologous-recombination double-stranded DNA repair pathway, key components of which are the tumor-

Table 1.2 Putative Tumour-Suppressor genes
{Coleman & Tsongalis, 2002}

Gene	Chromosomal Location	Inherited Cancer	Sporadic Cancer
Rb1	13q14	Retinoblastoma	Retinoblastoma, sarcomas,bladder,breast,oesophageal, and lung
p53	17p13	Li-Fraumeni cancer family syndrome	Bladder, breast, colorectal, oesophagus, liver, lung and ovarian carcinomas, brain tumours, sarcomas, lymphomas, and leukaemias.
DCC	18q21	-	Colorectal carcinomas
MCC	5q21	-	Colorectal carcinomas
APC	5q21	Familial adenomatous polyposis	Colorectal, stomach and pancreatic carcinomas.
WT1	11p13	Wilms tumour	Wilms tumour
WT2	11p15	Weidemann-Beckwith syndrome	Renal rhabdoid tumours, embryonal rhabdomyosarcoma
WT3	16q	Wilms tumour	-
NF1	17q11	Neurofibromatosis type 1	Colon carcinoma and Astrocytoma
NF2	22q12	Neurofibromatosis type 2	Schwannoma and Meningioma
VHL	3p25	von Hippel-Lindau syndrome	Renal cell carcinomas
MEN1	11q23	Multiple endocrine neoplasia type 1	Endocrine tumours such as pancreatic adenomas
Nm23	17q21	-	Melanoma, breast, colorectal, prostate, Meningioma, others
MTS1	9p21	Melanoma	Melanoma, brain tumours, Leukamias, sarcoma, bladder, breast kidney, lung and ovarian cancers.

suppressor proteins BRCA1 and BRCA2. A germ-line mutation in one *BRCA1* or *BRCA2* allele is associated with a high risk of the development of a number of cancers, including

breast, ovarian, and prostate cancer. It is reported that 2% of men with early onset prostate cancer harbour germ line mutation in BRCA2 gene {Edwards *et al*, 2003}. Cells carrying one functional copy of the BRCA gene can loose the wild type due to second hit mutation (Knudson theory of 2 hits) resulting in deficient homologous recombination DNA repair, which causes the genetic aberrations that drive carcinogenesis. This leads to the emergence of tumour cells that carry DNA repair defects which are not shared by normal cells of the patient. This tumour specific defect can be exploited using PARP inhibitors (Olaparib) to induce selective tumour cytotoxicity, sparing normal cells {Fong *et al*, 2009}.

1.1.3 Cancer Progression

Cancer development is a multi-step process through which cells acquire increasingly abnormal proliferative and invasive behaviours. The main trigger for tumourigenesis is mutation. It is now known that neoplastic cells also acquire multiple somatic mutations, which result in genetic variations and/ or abnormal gene expressions that enhance their ability to invade (migrate to) neighbouring normal tissues. This involves a clonal expansion of transformed neoplastic cells in a process of natural selection {Isaac JT, 1997}. Neoplastic cells often have a growth advantage that allows them to proliferate, loose contact inhibition and increase mobility; and invade adjacent tissues. In some organs, cancer progression is predictive, for example, in the colon, the transition from benign to malignant occurs in discernible stages: benign adenoma, carcinoma in-situ, invasive carcinoma; local and distant metastasis. Such progression is characterized by accumulation of multiple genetic alterations in the affected cells. However, in many others, the progression is unpredictable, for example, in the prostate gland, the development of pre-malignant lesions, collectively known as prostatic intraepithelial neoplasia (PIN) does not, in all cases, progress predictably to malignant lesions {Bostwick, 1996; Isaac, 1997}. Invasiveness describes the ability of cancer cells to penetrate basement membrane of underlying tissue stroma. This is caused by increased mobility of cancer cells. This is different from metastasis, which describes the spread of cancer cells to near and distant organs via blood and lymph vessels and neural networks. Both characteristics are indicators of aggressiveness of cancer cells. Invasive cancer cells require supply of nutrients, which often result from formation of new blood vessels (angiogenesis).

In cancer progression, specific genetic alterations have been shown to correlate with well-defined histopathologic stages of tumour development {Fearon & Vogelstein, 1990}. Figure 1.1 shows the morphologic continuum from normal prostatic epithelium through increasing grades of prostatic intraepithelial neoplasia to early invasive carcinoma, according to the disease-continuum concept {Bostwick, 1996}. These morphologic changes correlate, in many cases, with the genetic variations shown in Figure 1.2. In many cases of prostatic biopsy, multi-foci of different lesions (benign and malignant) often co-exist in the same sample. This heterogeneous phenotype corroborates with the notion that the development of a malignant prostate cancer from normal prostatic epithelium requires multiple transformation events {Isaac, 1997; Montironi et *al*, 2006}. Due to the multi-step nature of prostatic carcinogenesis, cells that have undergone some but not all of the transformation steps are present in pockets within the prostates of aging men, and the clonal expansion of these partially transformed cells produces morphologically detectable premalignant lesions in the gland {Bostwick, 1996; Isaac, 1997}.Molecularly, there is a prominent clustering of changes in expression for many biomarkers between benign epithelium and high grade PIN, indicating that this is an important threshold for carcinogenesis.

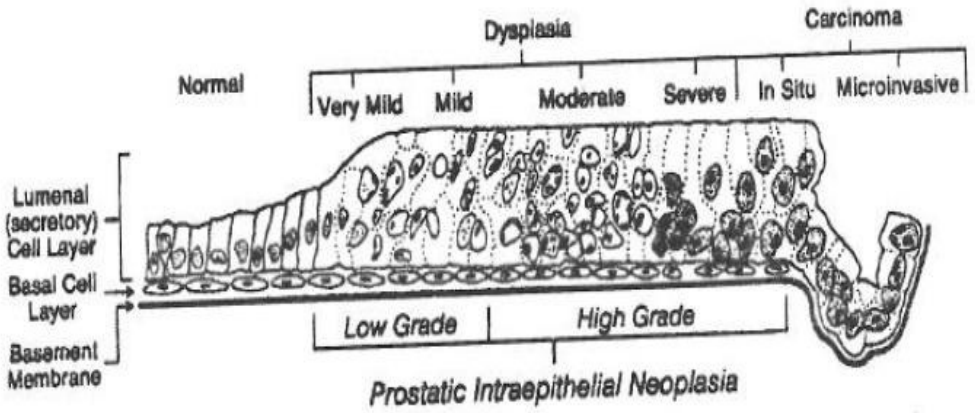


Figure 1.1 Morphologic continuums for Prostate cancer progression. This illustrates changes from normal prostatic epithelium through increasing grades of prostatic intraepithelial neoplasia to early invasive carcinoma, according to the disease-continuum concept {Bostwick, 1996}.

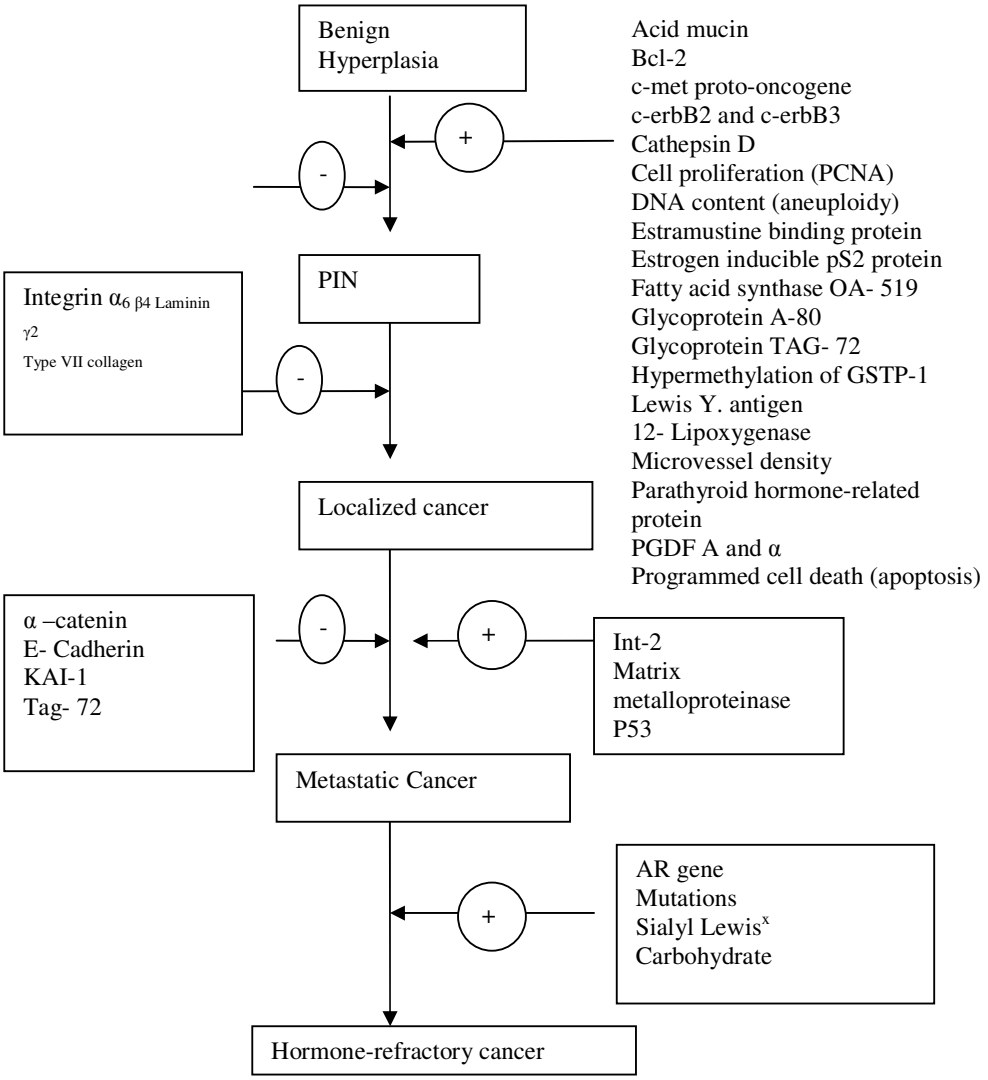


Figure 1.2 Genetic changes associated with prostate cancer progression. Some biomarkers show upregulation or gain (indicated by + sign), whereas others are downregulated or lost (- sign). There is a prominent clustering of changes in expression for many biomarkers between benign epithelium and high grade PIN, indicating that this is an important threshold for carcinogenesis {Bostwick, 1996}.

Another study {Mosquera *et al*, 2007} showed a correlation between gene rearrangements (TMPRSS2-ERG fusion on chromosome 21) and morphologic phenotypes of prostate cancer progression (high grade tumour). Five morphological features were associated with *TMPRSS2–ERG* fusion prostate cancer: blue-tinged mucin, cribriform growth pattern, macronucleoli, intraductal tumour spread, and signet-ring cell features. It is therefore believed that the biological effects of *TMPRSS2–ERG* over expression may drive pathways that favour these common morphological features {Mosquera *et al*, 2007}. In breast and colon cancers,

microsatellite instability from the BRCA1 gene mutations is known to drive cancer progression, inducing morphologically detectable lesions.

1.1.4 Pathways and Patterns in Carcinogenesis

One of the hallmarks of cancer development and progression is that neoplastic cells do not respond to normal regulatory pathways. Often regulatory networks are disrupted in tumours, through inactivating mutations and epigenetic suppression, by activating mutations, gene amplification and translocations, or as a result of alterations in micro RNA expression. The escape from normal regulation alters cell cycle and mitosis in neoplastic cells, causing abnormal patterns in cell proliferation, apoptosis, senescence and angiogenesis {Hanahan and Weinberg, 2000}. Implicitly, not all cells of the cancer population are 'exempt' from normal regulation, however, the 'cancer stem cells' are elusive from normal regulation as they provide the continuity in clonal expansion of neoplastic cells. Secondly, cancer cells do not necessarily cycle faster than normal cells; in most cases the difference is that many of the cancer cells are cycling at the same time compared to the normal population {McKinnell *et al*, 2006}. Figure 1.3 shows the cell cycle and the key regulators (the cyclins, cyclin-dependent kinases (CDKs) and their inhibitors in regulating the cell cycle, {Kumar *et al*, 2009}. Defects in these pathways (regulatory networks) may be genetic (directly affecting DNA sequence), or epigenetic (through alteration in gene transcript without change in DNA sequence), features of which are abundant in many human cancers. The key regulatory pathways and events in cell division cycles are as follows {Stein & Pardee, 2004; Wylie, 2008}:

i) **Cyclins and cyclin-dependent kinases (CDKs) form the basic clockwork of the cycle** {Hartwell & Kastan, 1994; Sherr CJ, 1996}, (Figure 1.3). The cell cycle is driven by a series of transient complexes each involving a cyclin protein, whose concentration varies with cell cycle phase, and CDKs, which are present throughout the cycle. The activity of CDKs is regulated by the cyclin partner, through a series of mutual phosphorylation reactions (a well defined example is Cyclin B and CDK1). The Cyclin B-CDK1 complex appears in G2, peaks in early metaphase and is destroyed in anaphase. On binding cyclin B, the ATP-binding site of CDK1 is blocked by phosphorylation. To initiate cell division (mitosis), the phosphate (P) must be removed. This is done by the phosphatase, CDC25, which is the critical trigger for mitosis. It opens the CDK1 active site and permits several simultaneous phosphorylation events responsible for nuclear envelope breakdown, chromosome condensation, spindle-

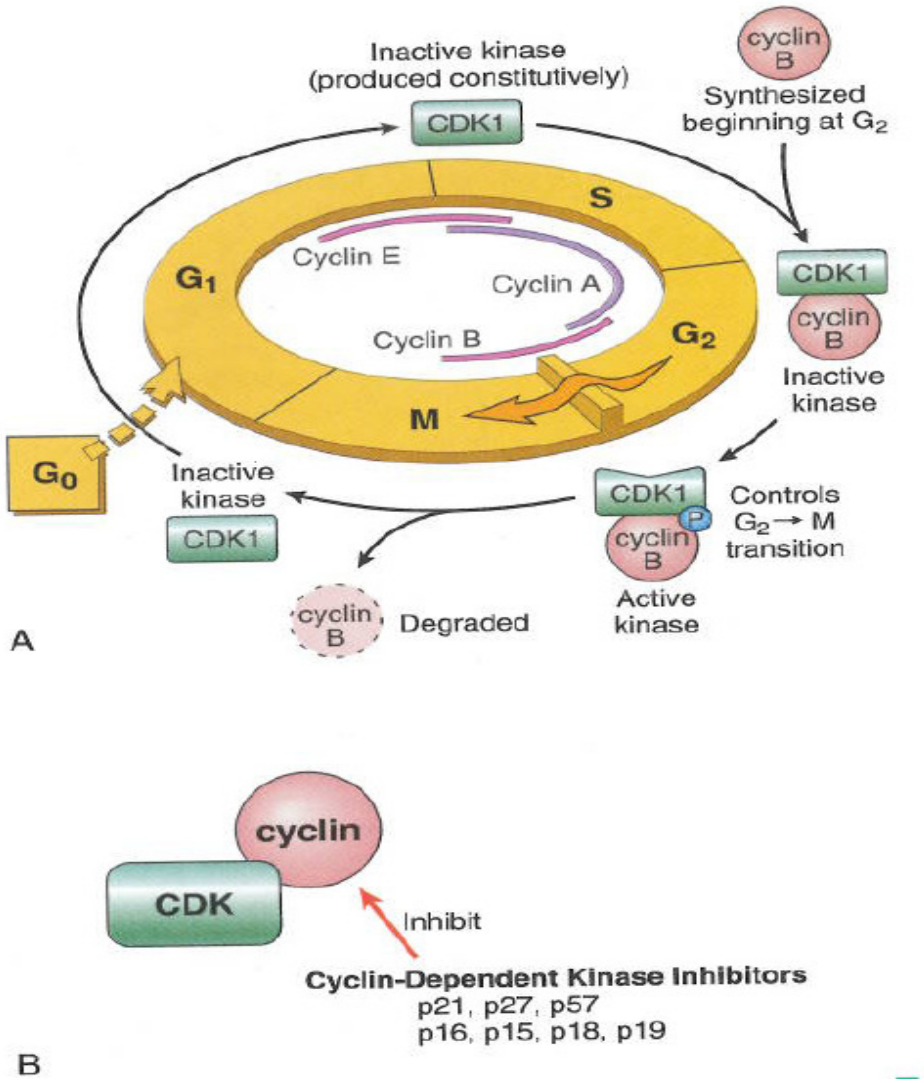


Figure 1.3 Regulators of cell cycle. This illustrates the role of cyclins A, B and E (classes of cyclins), cyclin-dependent kinases (CDKs), and inhibitors, in regulating the cell cycle {Kumar et *al*, 2009}.

Formation and fragmentation of the endoplasmic reticulum (ER) and Golgi appartus. A final substrate of CDK1 is the anaphase promoting complex, often called the cyclosome (APC/C), which, on activation, behaves as a ubiquitin ligase and targets cyclin B for degradation in the proteasome. Very similar transient complexes form between members of the cyclin and CDK families at other phases of the cell cycle: cyclins D1, 2 and 3 with CDK4 and CDK6 in G1, cyclin E with CDK2 at the G1-S transition, and cyclin A with CDK2 through S phase into G2 {Pardee, 1989}. Other kinases are also known to involve in driving the cell cycle: the Aurora Kinases (A, B and C) are involved in spindle and centriole function events {Scharer CD, et al, 2008}.

ii) **The retinoblastoma protein Rb is an important substrate of the cyclin D/ CDK4 complex** {Stein & Pardee, 2004}: The Rb is a nuclear protein which inhibits replication through sequestration of the transcription factor E2F-1 in a deep intra-molecular pocket. This binding (Rb/E2F-1) promotes chromatin condensation to a transcriptionally silent configuration. Cyclin D/CDK4 complexes hyperphosphorylate Rb, disrupting the binding to E2F-1. The activated E2F-1, in turn, activates transcription of a number of genes required for S phase entry, including the critically important oncogenes c-MYC {Kumar V *et al*, 2009} . The G1-S interface is very important in cell cycle because at that point the cell commits either to replication of the genome or to quiescence or differentiation or both {Sherr, 1996; Shapiro & Harper, 1999}. It is known as the G1 checkpoint. The Rb gene is mutated in a variety of cancers including inherited retinoblastoma and sporadic retinoblastoma and sarcomas.

iii) **APC/C regulates the exit from mitosis** (Figures 1.4 and 1.5) {Stein & Pardee, 2004; Blow & Tanaka, 2005}: During formation of the spindle, microtubules are polymerised from centres around the centrioles, radiating across the cell to anchor against the polar cell membrane, microtubules arriving from the opposite centriole, or the specialized region of the chromosomes called the kinetochore. This is a little platform, mounted on the pole-wards face of the centrosome (Figure 1.6), which captures the microtubules as they advance across the cell. The capacity of the microtubules to extend and shorten by alternating polymerisation and depolymerisation is called dynamic instability. Through a combination of the dynamic instability of microtubules and the action of molecular motors (kinesin-and dynein- like motors), the chromosomes gather at the cellular equator during metaphase (Figure 1.5). Remarkably, further progression through mitosis halts until the last kinetochore has achieved attachment to a microtubule (by the delay of APC/C activation). This is known as the Metaphase checkpoint. At that point, through molecular signals from kinetochore proteins (MAD2, Bub1, BubR1) that permit release of a critical cofactor (cdc20), the ubiquitin ligase activity of the anaphase promoting complex (APC/C) is switched on.

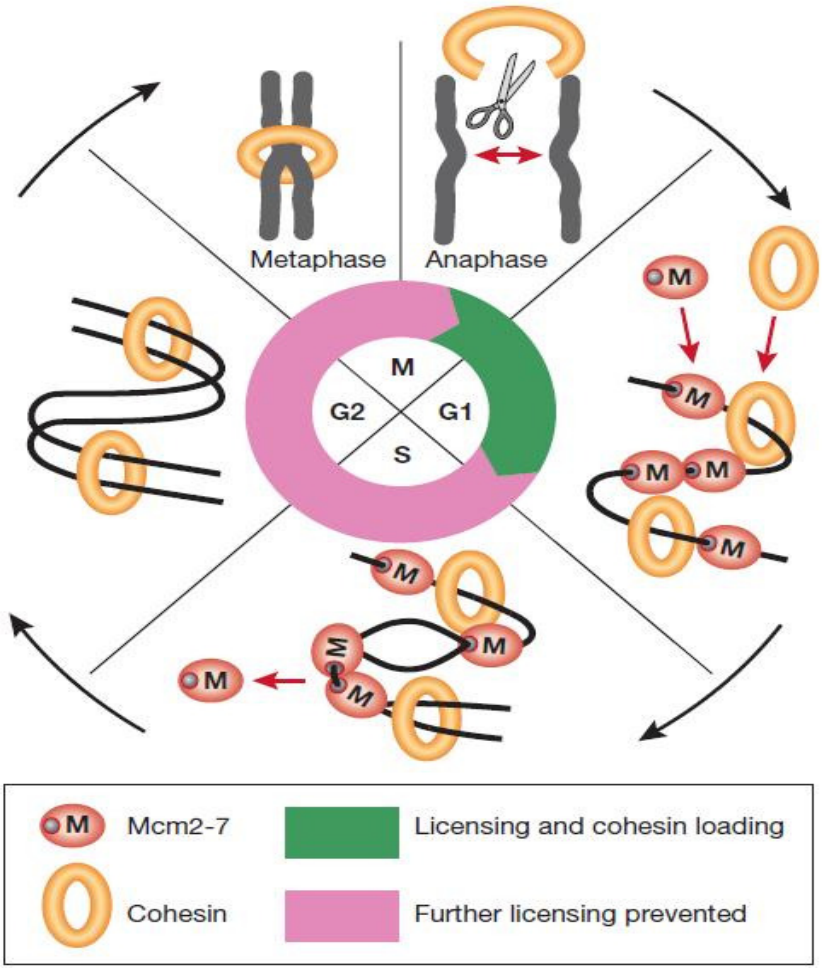


Figure 1.4 Overview of cell cycle licensing and mitosis. The MCMs and Cohesion proteins are very vital in marking DNA for replication and chromosomal segregation during mitosis {Blow & Tanaka, 2005}. G= Gap, S= synthesis, M=mitosis.

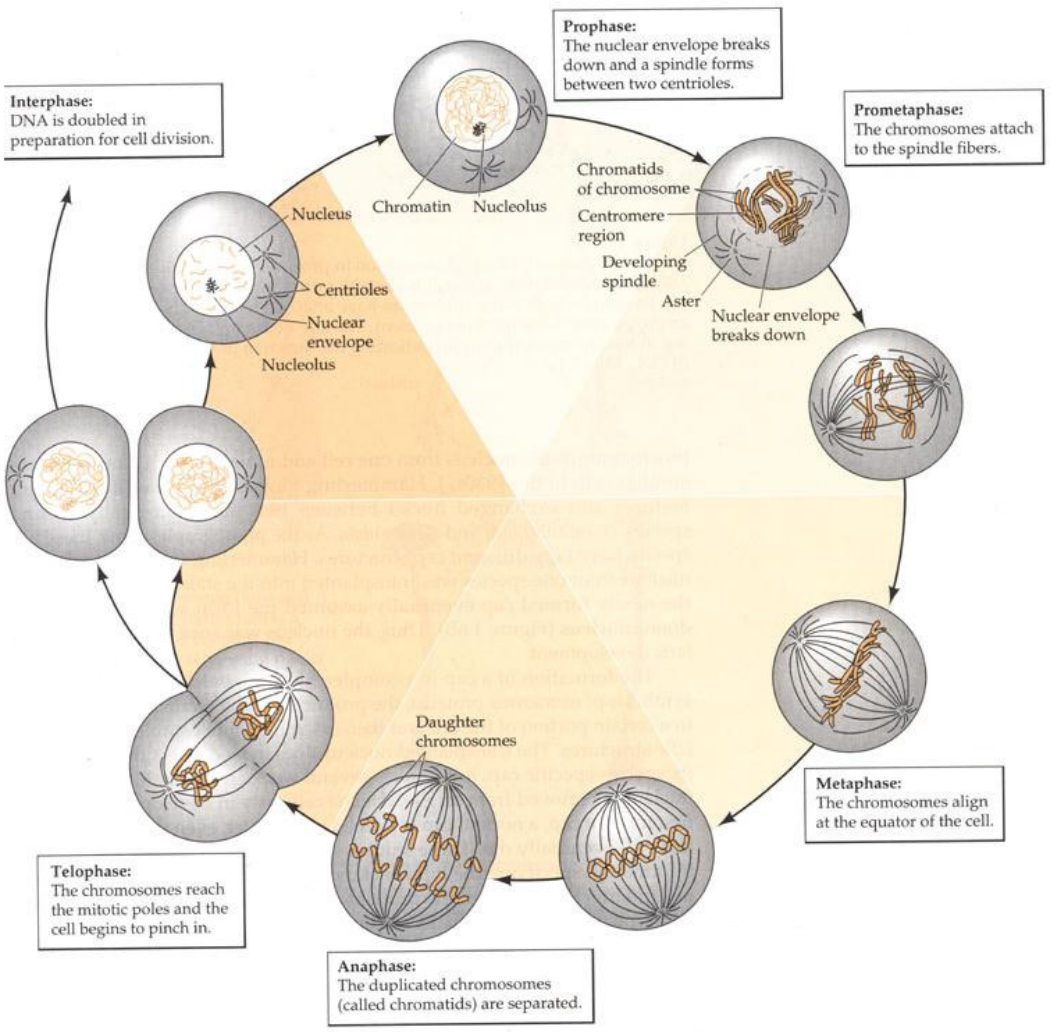


Figure 1.5 Mitosis in animal cells. During interphase (G1-S-G2 phases of cell cycle) DNA is doubled in preparation for cell division. During the prophase, the nuclear envelope breaks down and a spindle forms between the centrioles. https:/.../Katie+Mitosis+and+Meiosis+Lab.

Cyclin B is destroyed, leading to restoration of the nuclear envelope and chromatin decondensation. APC/C also digests a protease inhibitor called securin, releasing its partner, a protease called separase, to the proteolysis of a family of proteins called cohesins (members of the family include Smc1, Smc3, Scc1 and Scc3) (see Figure 1.4). The cohesins are responsible for binding the chromatids together. Their degradation permits chromatids to separate and anaphase to proceed.

iv) **MYC is an important regulator of the cell cycle** {Kumar V *et al*, 2009}. Myc proteins are transcription factors that ultimately promote synthesis of; a) the proteins required for DNA synthesis (examples: DNA polymerase, dihydrofolate reductase (DHFR), thymidine

kinase and origin of replication complex 1 (ORC1) and Minichromosome maintenance complex proteins (MCM 2-7), proteins of the DNA replication complex), b) proteins required for cell cycle progression (cyclins E and A, cdk2) and c) proteins that cause cycle arrest or apoptosis (p73 and p14ARF).

v) **DNA replicates only once per cell cycle** {Stein & Pardee, 2004}. Although thousands of replication forks are active throughout S-phase, each DNA sequence replicates only once in each S-phase {Blow & Tanaka, 2005}. As G1 proceeds, replication origins recruit several proteins from the MCM family. These are required to license DNA for replication (Figure 1.4), and their loading on to chromatin is facilitated by cyclin A {Tye, 1999}. They are displaced by the replication fork in S phase and destroyed by proteolysis, so they are not re-instated on the newly replicated DNA. On inception of the next S-phase, however, perhaps because of nuclear envelope breakdown in the intervening mitosis, MCM proteins from the cytoplasm reappear in the nucleus, so re-licensing it for replication {Stoeber *et al*, 2001}.

vi) **The cell cycle includes many different checkpoints** {Shapiro & Harper, 1999}. Notable checkpoints in the cell cycle are the G1/S checkpoint, the G2/M checkpoint and the metaphase checkpoint. They form part of a physiological regulatory system designed to restrain replication, or arrest it temporarily, perhaps to permit repair of damage. Cancer cells usually by-pass these checkpoints as they escape regulation. Several small proteins bind to and inhibit the cyclin-cdk complexes to enforce these checkpoints. Some of the inhibitors include p21, p27 and p57, some of which are activated by cytokines, but others are transcribed in response to DNA damage or hypoxia through the activation of the nucleoprotein p53. Incompletely replicated or damaged DNA arrests cell cycle progression at a further checkpoint just before entry to mitosis (the G2/M checkpoint). Here the signal is processed through the kinases CHK1 and 2, which inhibit the phosphatase cdc25.

vii) P53 causes cycle arrest at the G1/S checkpoint {Stein & Pardee, 2004}. The tumour suppressor protein, p53 is one of the most important regulators of the G1/S checkpoint. Its concentration in the nucleus is usually low, but is the result of a fine balance between continuous transcription, translation and nuclear import on one hand, and rapid proteolysis on the other. The balance is sustained through a negative feedback loop involving the protein MDM2- a transactivation product of p53 itself that binds to p53 and facilitates proteolysis. P53 stability can therefore be very rapidly altered, through modification of its binding to MDM2, and this can be achieved by p53 phosphorylation at the appropriate sites. DNA damage –particularly the formation of double-strand breaks-activates a protein kinase called ATM (because the inherited disorder Ataxia Telangiectasia results from its mutation) that

effects this phosphorylation. By this means, the concentration of p53 in the nucleus can increase many fold within a few minutes of DNA injury, thereby stopping G1-S phase progression to allow for DNA repair or apoptosis. A slower but probably longer-lasting dissociation of p53 and MDM2 is effected by production of the protein p14 ARF, which is translated from mRNA transcribed from the INK4 gene, but using an alternative reading frame.

viii) Entry into the cell cycle is initiated by extracellular signals {Hanahan & Weinberg, 2000}. Most cells in complex tissues are out of cycle altogether. To initiate replication, special signals are required, often specific for cell type and usually extracellular. These signalling molecules are often embedded in extracellular matrix (ECM) (having been secreted there by other cells) or may be on the surface of neighbouring cells. Examples of such growth factors are shown in Table 1.3. The receptor for many of these possess tyrosine kinase domains (often more than one) in their cytosolic moieties, that can thus relay complex multiple signals to the cell interior (Figure 1.6) through phosphorylation of tyrosine, serine or threonine amino acid residues of substrates {Coleman & Tsongalis, 2002}. It is important to note that some of these growth factors have oncogenic potential.

Table 1.3 Growth factors involved in cellular regulation {Coleman & Tsongalis, 2002}

Bone morphogenetic proteins (BMPs)
Epidermal growth factor (EGF)
Erythropoietin (EPO)
Fibroblast growth factor (FGF)
Granulocyte-colony stimulating factor (G-CSF)
Granulocyte-macrophage colony stimulating factor (GM- CSF)
Growth differentiation factor-9 (GDF9)
Hepatocyte growth factor (HGF)
Insulin-like growth factor (IGF)
Myostatin (GDF-8)
Nerve growth factor (NGF) and other neurotrophins (NT-3 and BDNF)
Platelet-derived growth factor (PDGF)
Thrombopoietin (TPO)
Transforming growth factor alpha (TGF-α)
Transforming growth factor alpha (TGF-β)
Vascular endothelial growth factor (VEGF)
Wnt family (Wnt-1 and Wnt-3)
Interleukins (IL-2 and IL-3)

ix) Ras activation is critical in pathway connecting receptor tyrosine kinases to reprogrammation of transcription {Stein & Pardee, 2004}**.** Generally, signal transducing proteins are widely grouped into two categories: a) non-receptor associated tyrosine kinases, for example c-abl (Abelson gene), cytoplasmic associated serine and threonine kinases; and b) receptor -associated GTP (guanidine triphosphate)-binding proteins (which include c-ras proteins, named after its first discovery in rat sarcoma). The mechanism of action of c-ras (which has three members: H-ras, K-ras and N-ras) is shown in Figure 1.7 {Coleman & Tsongalis, 2002}. The c-ras is active when complexed with GTP, and this interaction is facilitated by guanidine nucleotide-releasing or exchange factors (GEFs) in response to growth factor stimulation.

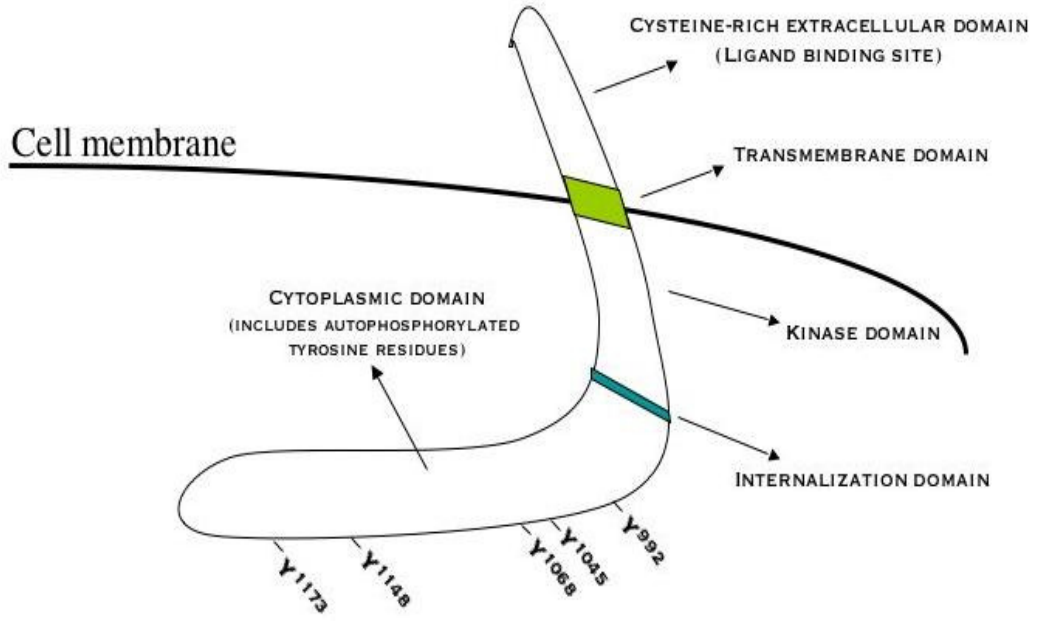


Figure 1.6 A schematic representation of transmembrane tyrosine kinase showing the extracellular domain, transmembrane domain and cytosolic domains.

The ras-GTP undergoes a conformational change that permits it to signal to the serine/threonine kinase, RAF and almost immediately activates its own GTPase function and hydrolyses the bound GTP to GDP. GTP hydrolysis is also stimulated by GTPase activating proteins (GAPs), such as neurofibromin. Downstream from RAF lies a cascade of phosphokinases (MEK and MAPK), whose ultimate substrate is the transcription factor AP-1, a heterodimer of two peptides, FOS and JUN. Phosphorylation enhances the activity of AP-1 and turns on the transcription of many immediate early response genes, including the

transcription factor, MYC. Mutated c-ras protein (as seen in cancer) has a decreased ability to hydrolyze GTP, or an increased rate of exchange of bound GDP for free GTP. By either mechanism, the result is increased activated c-ras.

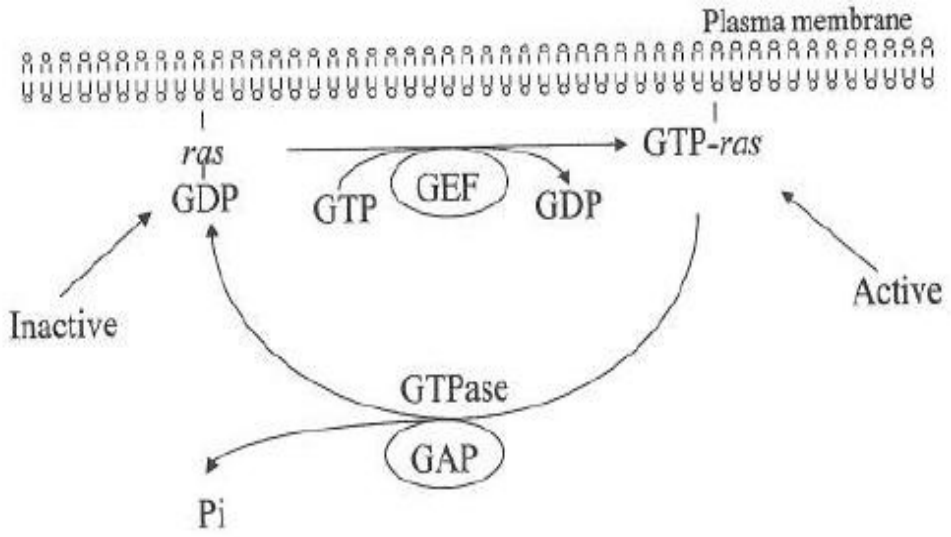


Figure 1.7 c-ras mechanism of action. The c-ras is active when complexed with GTP, and this interaction is facilitated by guanidine nucleotide-releasing or exchange factors (GEFs) in response to growth factor stimulation.

X) The P13 kinase pathway influences many growth-related cell functions {Kumar et *al*, 2009}. P13 is a lipid kinase that is activated by signals transmitted by many transmembrane receptors with protein kinase cytosolic domains (see Table 1.3 and Figure 1.6). This activation leads to production from membrane inositol of the inositol phospholipids: Ptdlns (3, 4, 5) P3 (PIP3) and Ptdlns (3, 4) P2. These in turn signal to a constitutive membrane threonine kinase, PDK-1, and a cytosolic protein kinase called AKT. This leads to the formation of an AKT/PDK-1 complex that phosphorylates many highly significant substrates including TOR (target of the drug rapamycin), S6kinase (an activator of protein translation) and protein kinase C (Figure 1.8). Other substrates are inhibited, including p21 (so releasing cells from G1 arrest), GSK3, which targets cyclin D for proteolysis and Bad, a pro-apoptotic protein. The activity of the AKT signalling pathway is inhibited through removal of the phosphate groups from the PIP3 by the PTEN (Phosphatase and Tensin homolog, which is same as 3' phosphoinositide phosphatase). The PTEN is one of the most commonly lost tumour suppressor genes in human cancers including prostate cancer, endometrial cancer and

Glioblastoma. PTEN mutations cause a variety of inherited predispositions to all sorts of human cancers.

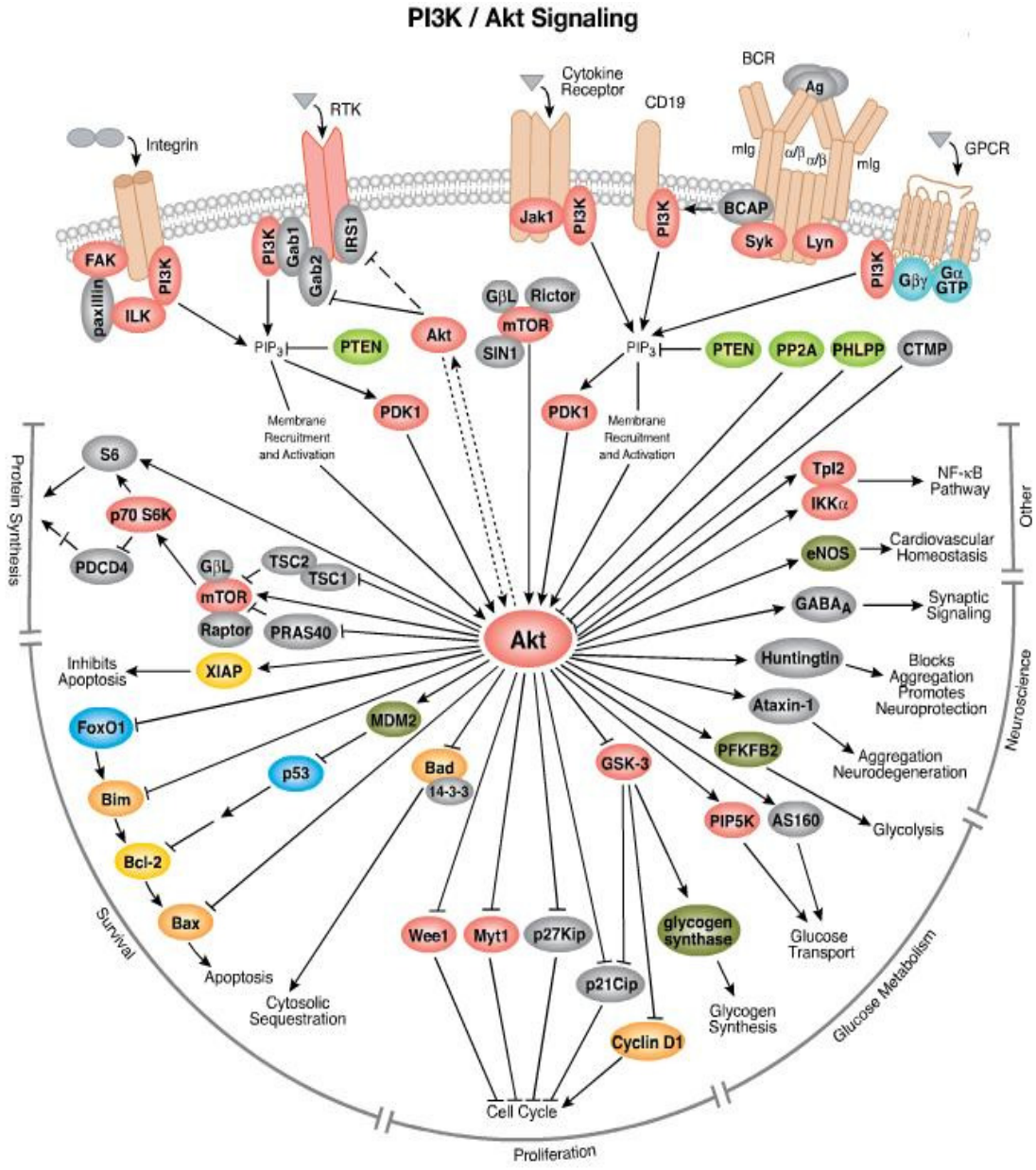


Figure 1.8 The P13K/ AKT signalling pathway. The AKT/PDK-1 complex phosphorylates many highly significant substrates including TOR (target of the drug rapamycin), S6kinase (an activator of protein translation) and protein kinase C. Other substrates are inhibited, including p21 (so releasing cells from G1 arrest), GSK3, which targets cyclin D for proteolysis and Bad, a pro-apoptotic protein. PTEN, commonly mutated in many cancers, inhibit the AKT pathway. http://www.cellsignal.com/pathways/akt-signaling.jsp.

X1) The Wnt pathway also connects external signals related to cell-to-cell contact with reprogrammation of transcription {Hanahan and Weinberg, 2000}. Wnt is a paracrine growth factor. When Wnt is bound to its receptor (called frizzled), the latter releases a cytoplasmic protein that inhibits the phosphokinase activity of GSK3. Under basal conditions, GSK3 forms a protein complex with the scaffold protein axin and the large regulatory protein APC (Adenomatous Polyposis Coli, the product of this tumour suppressor gene is mutated in most colon cancers). The complex (with APC in particular and the GSK3 kinase active) has the ability to entrap, phosphorylates and targets for proteasomal destruction of a substrate called β -catenin. The β -catenin can shuttle from the cell membrane, where it docks on the cytosolic domain of the cell-cell adherence protein E-cadherin (which is differentially expressed in prostate cancer), to the nucleus where it pairs with TCF (T cell factor) to form a heterodimeric transcription factor (Figure 1.9). Amongst the genes induced by β-catenin/TCF transcription factor is the MYC. Therefore, the Wnt stimulations lead to cell cycle activation provided the levels of APC do not rise at the same time.

XII) The notch pathway also mediates cell to cell interaction {Kumar et *al*, 2009} (Figure 1.10). Notch is a receptor for signals delivered by cell-bound ligands (Delta-like, serrated and jagged). When activated by ligand binding, the intracytoplasmic domain of notch is cleaved by a γ-secretase and becomes a nuclear –seeking transcription factor. Although the biology of notch is varied, it is essential for many developmental processes, notably the development of vascular networks.

XIII) The process that regulates telomere stability is frequently abnormal in cancers {Bodnar AG *et al*, 1998}. Telomeric DNA consists of repetitive TTAGGG sequences, without which the chromosome ends are unprotected from nuclease damage and the chromosomes themselves become unstable. Under normal conditions in most cells of most human tissues, one TTAGGG unit is lost from each DNA end in each cell replicative cycle. Certain cells, including stem cells, possess an enzyme called telomerase, which catalyses the extension of the telomeric tandem repeats. Telomerase is a protein complex that consists of a rate-limiting reverse transcriptase catalytic subunit called hTERT and an RNA template. Tumour cell populations often show reduced telomere length relative to normal tissues, together with activation of telomerase. The hTERT can be used to immortalize pre-senescent cells.

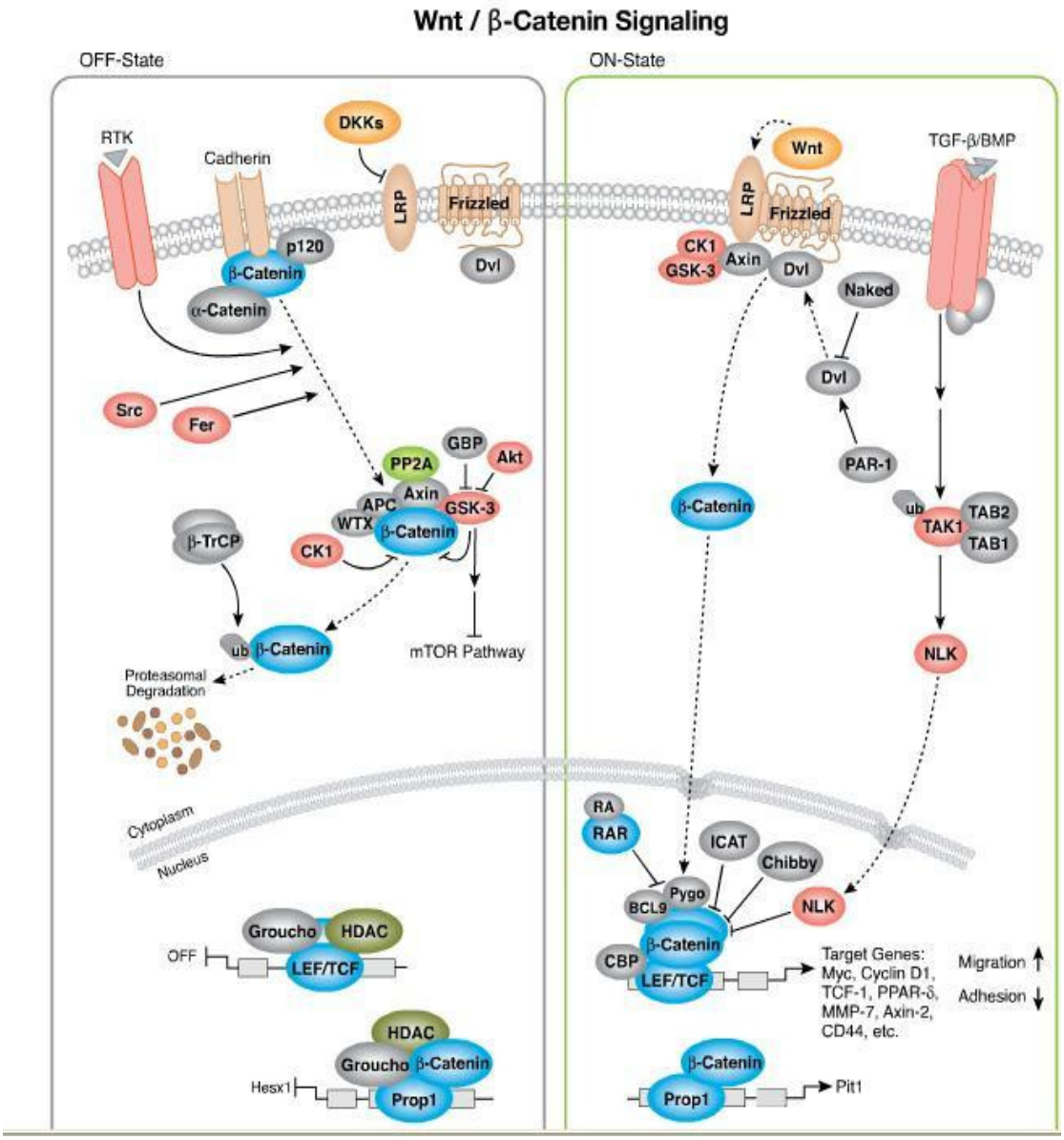


Figure 1.9 The Wnt/β-catenin signalling pathway. The β -catenin can shuttle from the cell membrane, where it docks on the cytosolic domain of the cell-cell adherence protein E-cadherin, to the nucleus where it pairs with TCF (T cell factor) to form a heterodimeric transcription factor. http://www.cellsignal.com/pathways/wnt-hedgehog.jsp.

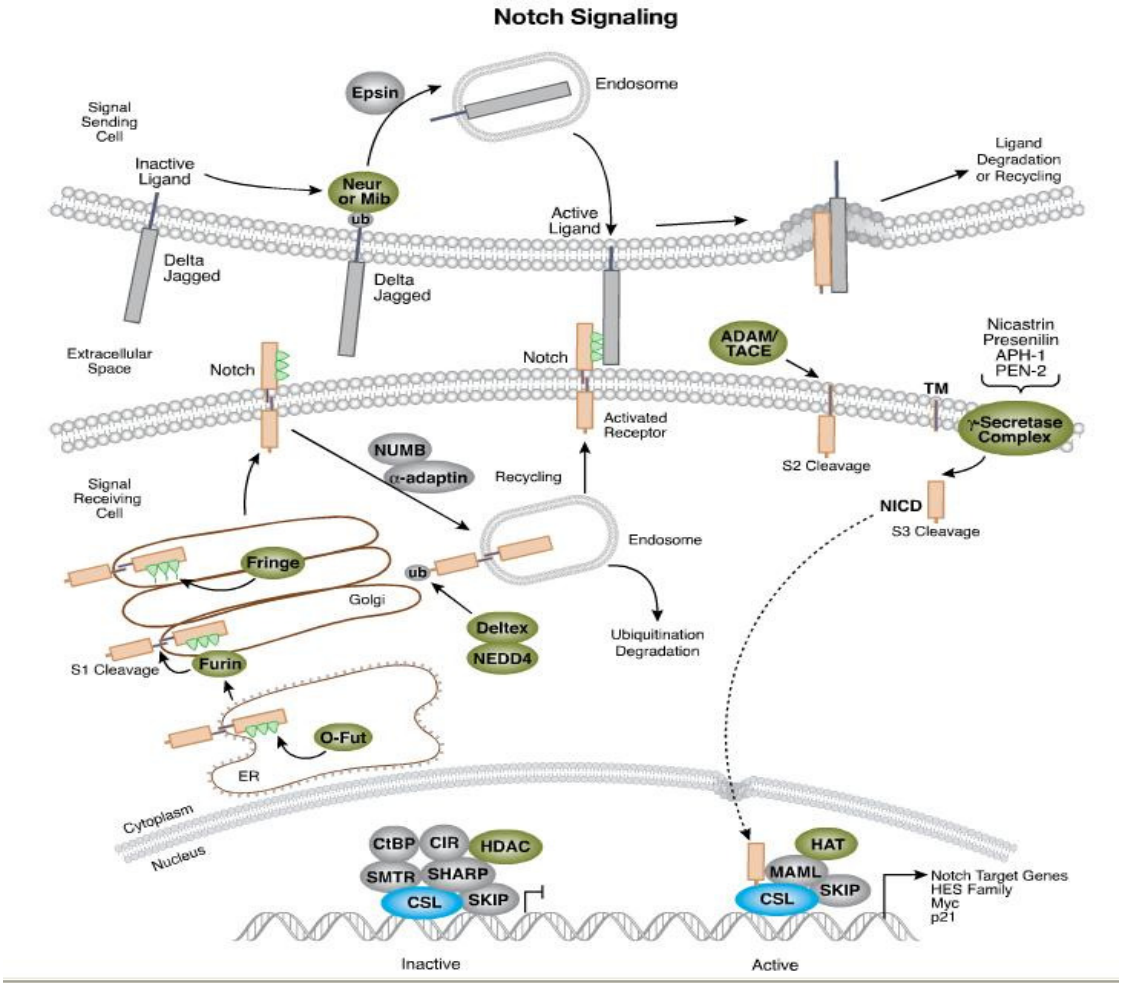


Figure 1.10 The notch signalling pathway. When activated by ligand binding, the intracytoplasmic domain of notch is cleaved by a γ-secretase and becomes a nuclear –seeking transcription factor

xiv) Regulatory RNAs: The discovery of RNA interference in 1998 by Andrew Fire and Craig Mello {Blow, 2009}; and the ubiquity of microRNAs in diverse biological systems have clearly established that novel species of RNAs regulate transcription and translation in hitherto unexpected ways {Stillman & Stewart, 2007}. Table 1.4 summarizes functions of currently known regulatory RNAs (double- and single-stranded non-coding RNAs).

The central theme of regulatory RNAs is that small RNAs (20-30 nucleotides long) and diverse non-coding RNAs (60 to several hundred nucleotides) can use their base-pairing potential to regulate the activity of DNA {Stillman & Stewart, 2007; Winter et *al*, 2009}. RNAs are also involved in catalysis as demonstrated by self-splicing introns and RNA cleavage. The entire field of regulatory RNAs is rapidly expanding, seeking answers to many questions such as how non-coding RNAs (RNAs other than the triumvirate of mRNA, rRNA and tRNA) dominate the regulatory circuitry of the cell; how these non-coding RNAs are

synthesized and processed in humans. It underpins the paramount importance of the interactome in the expression of cancer genes.

Table 1.4 Novel species of Regulatory RNAs

Types of RNA	Function
snRNA	Small nuclear RNAs Function in a variety of nuclear processes, including splicing of pre-mRNA
snoRNA	Small nucleolar RNAs Process and chemically modify rRNAs
scaRNA	Small cajal RNAs Modify snoRNAs and snRNAs
miRNA	Micro RNAs (20-30 nucleotides long) Regulate gene expression. Bind to 3'untranslated regions (UTR) of targets. About 1000 microRNAs are known. Block translation of selective mRNAs. Could be of diagnostic & therapeutic uses including understanding of cell: cell signalling.
siRNA	Small interfering RNAs Turn off gene expression Direct degradation Establishment of compact chromatin structure
Other non coding RNAs (ncRNAs)	Telomere synthesis X-chromosome inactivation Transport of proteins into the endoplasmic reticulum Anti-sense transcripts of unknown function.

In summary, carcinogenesis requires multiple events. It is associated, almost invariably, with abnormalities that indicate there have been a much larger number of events (cellular and molecular). Some of these events are 'drivers', directly responsible for the abnormal behaviour of the clonally expanding cancer cells, but many are probably 'passengers'. In reality, the regulatory pathways are interwoven in a complex network, which cancer cells tend to escape or modify. For example, aberrant K-ras and Wnt signalling have been reported to form synergy, accompanied by elevated androgen receptor, cyclooxygenase-2 and c-Myc levels in the acceleration of prostate tumourigenesis in mouse models {Pearson *et al*, 2009}.

1.2 Pathology of the Prostate gland

1.2.1 Physiologic Anatomy of the Prostate

The prostate is the largest accessory gland of the male reproductive system, measuring approximately 3cm in length, 20g in weight and is the size of a walnut in a normal adult {Kumar *et al*, 2009}. It lies underneath the urinary bladder with its base encircling the neck of the bladder (Figure 1.11). Embryologically, the glandular part is of epithelial origin (ectodermal) and comprises approximately two thirds of the prostate; while the other third is fibromuscular and mesenchymal.

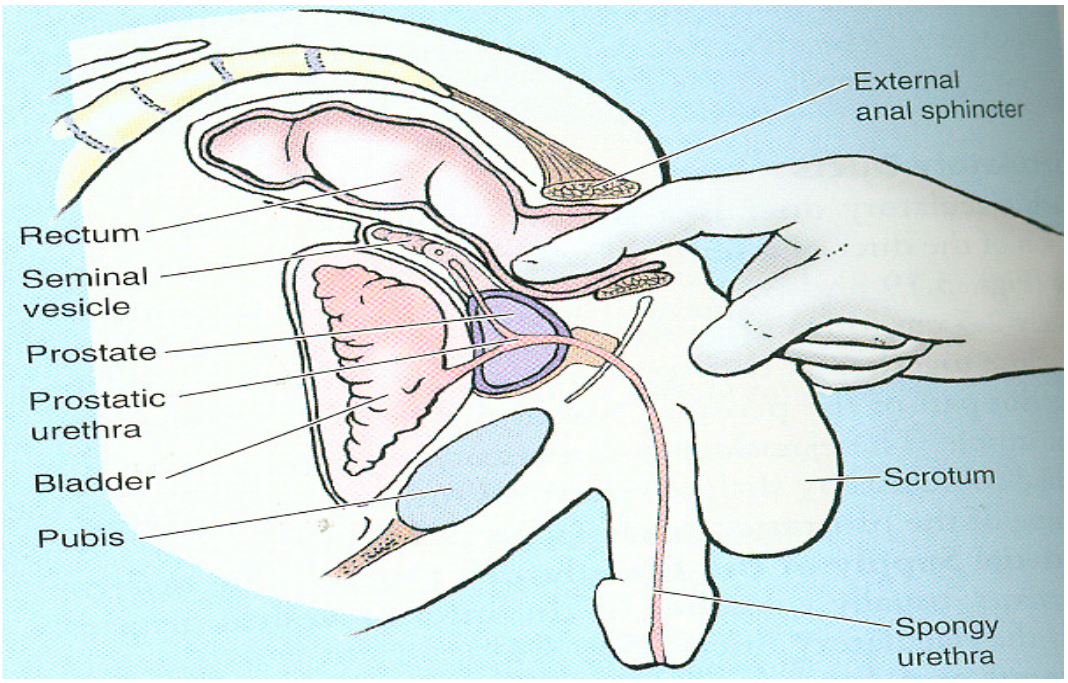


Figure 1.11 Posterior view of the prostate gland during digital rectal examination {Moore *et al, 1992*}.

Although not clearly distinct anatomically, the prostate consists of five lobes in relation to the urethra. The anterior lobe, or isthmus, lies anterior to the urethra and it is wholly fibromuscular. The posterior lobe lies posterior to the urethra and inferior to the ejaculatory ducts; it is readily palpable by DRE. The lateral lobes on either side of the urethra form the major part of the prostate. The middle (median) lobe lies between the urethra and the ejaculatory ducts and is closely related to the neck of the bladder. However, in the adult, prostatic parenchyma can be divided into four biologically and anatomically distinct zones or regions: the peripheral, central, and transitional and periurethral zones (Figure 1.12). The

central zone is comparable to the middle lobe. The types of proliferative lesions are different in each region.

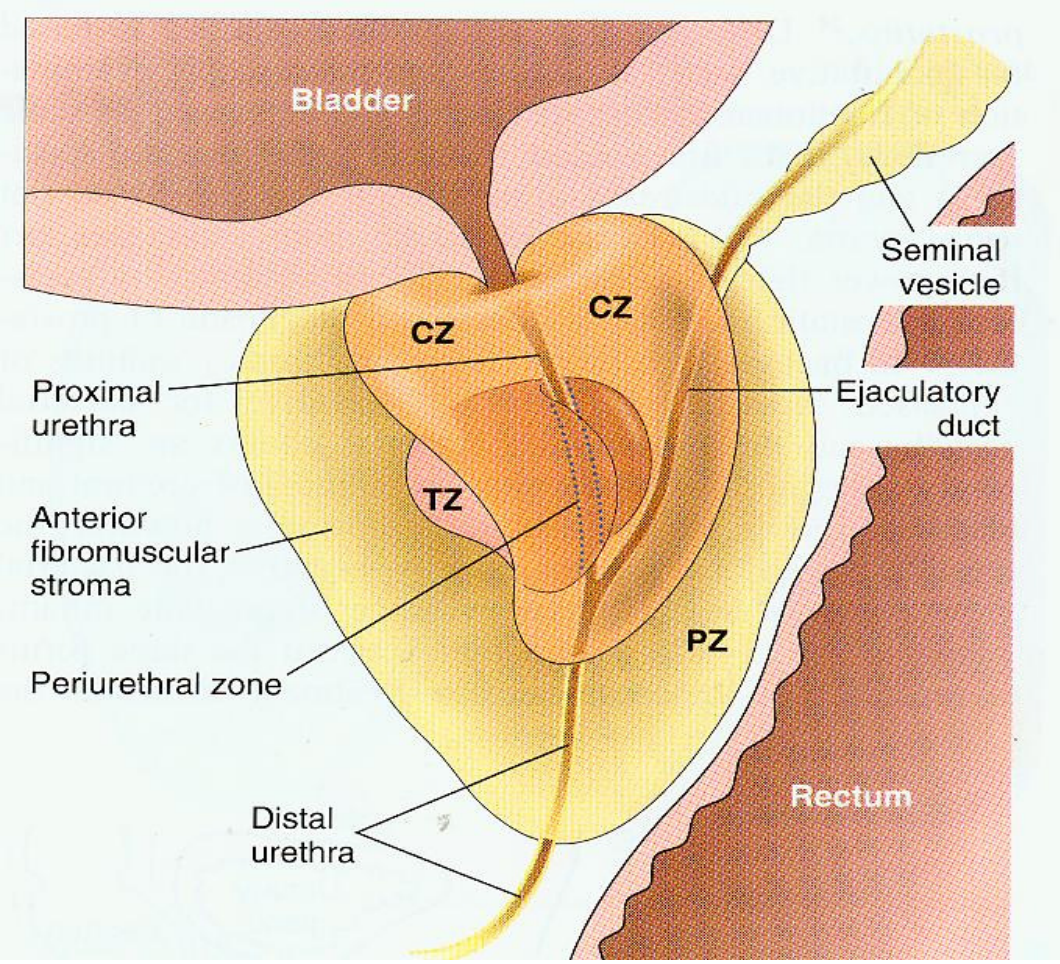


Figure 1.12 Anatomic zones of adult prostate gland. *CZ is central zone, PZ is peripheral zone, and TZ is transitional zone* {Kumar et *al*, 2009}. Most prostate cancers arise from the peripheral zone.

For example, most hyperplasias arise in the transitional and periurethral zones and are more likely to produce urinary obstruction earlier on than are carcinomas; whereas most carcinomas originate in the peripheral zone of the organ and are often palpable during digital rectal examination (DRE) {Kumar *et al*, 2009}.

Histologically, the prostate is a compound tubuloalveolar gland, which presents small to fairly large glandular spaces lined by two layers of epithelial cells: a basal layer of low cuboidal epithelium covered by a layer of columnar mucus-secreting cells. Molecularly, four cellular types exist within the prostate, which are identifiable by specific markers (basal cells, transient amplifying cells, luminal and neuroendocrine cells) {Agrawal & Dunsmuir, 2009}. It is suggested that the 'prostate stem cells' reside in the basal cell layer and may produce

intermediate transient amplifying cells, which exhibit both luminal and basal cell markers {Litvinov et al, 2006}. The glands all have a distinct basement membrane and are separated by an abundant fibromuscular stroma.

The prostate is a richly innervated organ, having parasympathetic fibres from the pelvic splanchnic nerves and sympathetic fibres from the inferior hypogastric plexus {Powell *et al*, 2005; Moore *et al*, 2006}.Prostatic neuroanatomy is important due to its relationship with prostate cancer in the process of perineural invasion, which constitutes the primary mechanism of metastasis more than direct extension{Powell *et al*, 2005}. Perineural invasion has been used as a prognostic marker, with the ability to predict progression, capsular extension, radiation therapy failure and PSA/biochemical recurrence {Powell *et al*, 2005}. The arterial blood supply to the prostate is by the prostatic arteries, which are mainly branches of the internal iliac artery including inferior vesical arteries, internal pudendal and middle rectal arteries. The veins that drain the prostate join to form a plexus around the sides and base of the prostate. This prostatic venous plexus, between the fibrous capsule of the prostate and the prostatic sheath, drains into the internal iliac veins. The prostatic venous plexus is continuous superiorly with the vesical venous plexus and posteriorly with the internal vertebral venous plexus {Moore *et al*, 2006}. The lymphatic vessels drain into the internal iliac and sacral lymph nodes, forming immediate access for metastasis.

The growth and function of the prostate is dependent on androgens. The two predominant androgens are testosterone, which is synthesized by Leydig cells of the testis from androstenedione and 5α-dihydrotestosterone (DHT), which is formed in the prostate from circulating testosterone by 5α-reductase type 2 {Vihko P *et al*, 2006}. DHT is the most active androgen in the prostate {Soronen *et al*, 2004}. Androgens are thought necessary for the initiation of prostate cancer because the balance between androgen induced proliferation and apoptosis regulates the growth of the normal and cancerous prostate{Vihko *et al*, 2005; Vihko *et al*, 2006}. The prostate secretes the prostatic fluid, which constitutes about 20% of the seminal plasma and is rich in PSA (prostate specific antigen). Normal prostate is a self-renewable tissue with low significant proliferative capacity; much similar to the mammary gland {Leblond, 1963}. The three main pathologic processes that affect the prostate gland are inflammation (prostatis), benign nodular enlargement, and tumours.

1.2.2 Diseases of the Prostate

a) Inflammations: Inflammation of the prostate is known as prostatitis, and has three categories: acute and chronic bacterial prostatitis and chronic abacterial prostatitis.

Differentiation among the three categories is based on quantitative bacterial cultures and microscopic examination of fractionated urine specimens and expressed prostatic secretions {Kumar *et al*, 2009}. In acute bacterial prostatitis, there is an acute focal or diffuse suppurative inflammation in the prostatic substance due to bacterial infection. Common pathogens are strains of *E.coli*, enterococci and staphylococci. Symptoms include fever, chills and dysuria; on DRE the prostate is exquisitely tender and boggy. Chronic bacterial prostatitis also results from bacterial infection mainly from recurrent urinary tract infections. It may present with low back pain, dysuria, perineal and suprapubic discomfort. Chronic abacterial prostatitis is clinically indistinguishable from chronic bacterial prostatitis; however there is usually no history of recurrent urinary tract infection. Diagnosis is based on presence of > 10 leukocytes per high field in prostatic secretions, but uniformly negative bacterial cultures. Likely pathogens include *C.trachomatis*, *U. urealyticum* and *M. hominis.* Proliferative inflammatory atrophy (PIA) co-exists in many foci of PCa biopsies and it is considered a precursor to prostate malignancy {Agrawal & Dunsmuir, 2009}.

b) Nodular hyperplasia. This is also known as benign prostatic hypertrophy or hyperplasia. This is a very common disorder in men over age 50 years. Histologic evidence of nodular hyperplasia can be seen in approximately 20% of men 40 years of age, a figure that increases to 70% by age 60 and to 90% by age 70 {Kumar *et al*, 2009}. It is estimated that more than 400,000 transurethral resections (for correcting nodular hyperplasia) are performed every year in the United States {Droller, 1997}. The disease is characterized by hyperplasia (increased number of cells) of prostatic stroma, predominantly smooth muscle cells and epithelial cells, resulting in the formation of large, fairly discrete nodules in the periurethral region of the prostate. It is believed to be caused by the continuous growth of the prostate gland throughout life {Coleman & Tsongalis, 2002}, a feature that requires minimally a supply of androgens {Vihko *et al*, 2006} and also the assistance of oestrogens {Droller, 1997}. Enlarged prostatic nodules compress and narrow the urethral canal causing partial or sometimes virtually complete obstruction of the urethra. This leads to difficulty in urination, retention of urine in the bladder with subsequent distention and hypertrophy of the bladder, infection of the urine and development of cystitis and renal infections. Patients experience increased frequency of need to urinate, nocturia, difficulty in starting and stopping the stream of urine, overflow dribbling and dysuria. Treatment includes catherization, surgical resection and antibiotics. Many studies have shown that nodular hyperplasia is however not a premalignant lesion, and therefore not a precursor to prostate tumours {Kumar *et al*, 2009}.

c) Prostate tumours. The most common malignant tumour of the prostate is the prostate adenocarcinoma (prostate carcinoma or prostate cancer). Although there are many dysplasias

of the prostate (with various microscopic descriptions), the generally recognized precursor of prostate carcinoma is the prostatic intraepithelial neoplasia (PIN){Bostwick 1996; Montironi *et al*, 2006}. PIN is '*associated with progressive abnormalities of phenotype and genotype which are intermediate between normal prostatic epithelium and cancer, indicating impairment of cell differentiation and regulatory control with advancing stages of prostatic carcinogenesis'* { Meikle & Smith. 1990; Bostwick 1996}. PIN and tissue hypoxia are also considered as precursors to prostate tumours. A rare carcinoma of the prostate known as small cell carcinoma of the prostate (SCPC) has also been described {Yao *et al*, 2008}. It can be found in both the pure form and mixed with conventional prostatic carcinoma. SCPC has typical histologic features of small cell carcinoma seen in the lung and other extra-pulmonary sites. Gleason grading does not apply to SCPC.

1.2.3. Aetio-Pathogenesis and Natural History of Prostate Cancer.

a) Aetiology: The exact cause of prostate cancer is not yet known. Conceptually, there are three approaches towards unravelling the causes of PCa. The first, which is most extensively reviewed in the literature is the epidemiological approach based on multi-factorial causes of PCa {Dijkman & Debruyne, 1996; Freedland & Isaacs 2005}. It is believed that prostate cancer is aetiologically heterogeneous, involving interplay of multi-step disease processes. Available data on the disease process of PCa suggest that genetic factors appear to be permissive, as are hormonal factors, which are partially regulated by genetics. Environmental factors (diet, pollutants and life style) appear to be promotional in genetically susceptible men {Meikle & Smith, 1990}.

The hormonal involvement in aetiology of prostate cancer is based on the fact that prostate epithelia possess androgen receptors, which are mutated and/ or aberrantly expressed in PCa. This results in abnormal androgen sensitivity to ligands, which is believed to drive prostate carcinogenesis. Several reports have produced strong evidence in favour of hormonal inducement of PCa disease process. For example, simultaneous oestrogen and testosterone treatment have been used to sensitize rodents to the development of prostate cancer {Prins GS *et al*, 2001}. It is also known that the increased level of oestradiol in aging men induces an increase in androgen receptors, rendering cells more susceptible to the action of dihydrotestosterone (DHT){Droller, 1997}. Androgen ablation therapy is another indication that hormonal inducement is paramount to the development PCa, although prostate cancer cells tend to develop resistance after a certain period of treatment. However, from embryological evidence of 'oestrogen imprinting' or 'oestrogenization', it is proposed that excessive oestrogen levels during prostatic development may contribute to the high incidence

of benign prostatic hyperplasia and prostatic carcinoma currently observed in the aging population {Sato *et al*, 1996}. The interactive effects of androgens, 17 β-oestradiol and their receptors are, therefore, very crucial in PCa pathogenesis.

The second approach, which essentially is also part of the first approach is the molecular genetics approach based on genetic mutation and inheritance as opposed to environmental determinants. In recent years much interest has been focused on the genetics and molecular pathogenesis of prostate cancer using analytical techniques such as array comparative genomic hybridization (aCGH), fluorescent *in situ* hybridization (FISH), gene expression profiling, loss of heterozygosity (LOH) analysis; and epigenetic studies such as DNA methylation. Molecular genetics approach asserts that cancer susceptibility genes are responsible for the development of PCa rather than hormones, which are only responsible for the maintenance of prostatic epithelium in both normal and cancerous cells. This approach speculates a possible germ line inheritance susceptibility gene mapped to chromosome 1q24-25 as causing prostate cancer irrespective of environmental exposure {Bostwick *et al*, 1996}. But this gene has not been identified. Several other gene loci have been associated with prostate cancer, for example, the 11q13, 8q24 and 17q12, these loci are known to harbour genetic variants responsible for prostate cancer risk {Zheng *et al*, 2009}. Several putative cancer-suppressor genes that are lost early in prostate carcinogenesis have also been mapped to chromosomes 8p, 10q, 12p, and 16q, of which E-cadherin has been found at location 16q22 {Standford *et al*, 2006}. Isaacs (1997) also reported a common loss of genetic material localised on chromosome 11p11.2, which is believed to suppress prostate cancer metastasis. But the identification of the principal susceptibility gene is yet to be reported. A study, based on genetic segregation analysis, suggested an autosomal dominant gene, a rare high risk allele to be responsible for the early onset of prostate cancer, but also agreed that chromosome 8p, 10q and 13q are frequently mutated or deleted in PCa {Haggman *et al*, 1997} .

The molecular mechanisms, exact roles of suspected genes and hereditary patterns likely to be involved are not yet elucidated; the cancer susceptibility gene model is still speculative. However, the discovery in 2005 by Tomlins *et al* of the gene fusion: TMPRSS2 and members of ETS gene family (ERG, ETV1 and ETV4) in more than 50% of prostate cancer cases has rekindled the investigation of molecular events underlying prostate cancer development {FitzGerald *et al*, 2008}. It is found that the promoter region of TMPRSS2, which is androgen-regulated, is fused with members of the ETS transcription factors (proto-oncogenes that are highly over-expressed in PCa). The oncoproteins encoded by the gene fusion is believed to drive prostate tumourigenesis. But some cases of TMRPSS2 -ERG or TMPRSS2-

ETV gene fusions were reported in benign, PIN and normal prostatic epithelia, once again a reminder of the heterogeneous aetiology of PCa.

The third approach is the virology approach, based on a suggestion of possible viral aetiology for prostate cancer, different from the well-trod cancer epidemiology. The discovery of a novel species of xenotropic murine retrovirus (XMRV) in prostate tumours of patients that have the R462Q variant of the RNASEL gene (which is involved in prostatic antiviral defence) has sparked the speculation that virus (es) may be involved in PCa aetiology {Urisman *et al*, 2006}. Mutations of the RNASEL gene were associated with 13% of all prostate cancers. Most of the mutated RNASEL prostate tumours had the XMRV (which is related to the xenotropic murine leukaemia virus), suggesting that viruses may be involved directly or indirectly in the mutation events and / or the development of the PCa especially in genetically susceptible males. After all, the involvement of viruses in human cancers has been known for decades; Harald zur Hausen received a Nobel Prize in 2008 for his work on the human papilloma virus in cervical cancer aetiology. Table 1.5 shows infectious agents that are implicated in human cancers {Bird, 2009}. Recently the Merkel cell polyoma virus has been associated with aetiology of the Merkel cell carcinoma, a rare but aggressive form of skin cancer, widely believed to be caused by sun exposure {Feng *et al*, 2008}. The viral involvement in PCa is currently very speculative, not much evidence to support it. However, it is worth investigating, knowing that the prostate gland is a rich haven for bacterial infection and may equally attract viruses.

Because the disease process in PCa involves a multi-step transformation of genetically altered prostate cells {Isaacs, 1997}, the epidemiological approach remains a preferred platform for most studies on aetiology of prostate cancer. From these, several risk factors that predispose humans to prostate cancer have been well- documented. These include family history, race; advancing age, environment and diet. It is important to point out that predisposition (risk factors) to a disease is not the same as causation.

b) Factors predisposing men to Prostate cancer:

i) Age: The majority of men with prostate cancer are aged 50 years and above {The Institute of Cancer Research UK, 2006}.This has formed the basis of a proposition by patient and physician groups, advocating annual mass screening of men aged 50 years {WHO, 2004}. Most public health authorities refute this idea on the basis of health cost and lack of controlled randomised clinical trial evidence to underpin additional health benefit through mass screening. However, several studies have shown that advancing age is the strongest predisposing factor to PCa {Meikle & Smith, 1990; Jemal, 2008}. It is believed that the

prostate gland grows steadily throughout life, a process that makes its prone to genetic alterations which could lead to prostate cancer or in most cases nodular hyperplasia {Coleman & Tsongalis, 2002}.

Table 1.5 Infections linked to human cancer.

{Bird, 2009}

Tumour type	Infectious cause
Some B-cell and T-cell lymphomas Burkitt's lymphoma Nasopharyngeal cancer Hodgkin's disease (30-40%) Gastric cancer (10%)	Epstein-Barr virus (EBV)
Cervical, anal and perianal cancers Vulval, penile and vaginal cancers Oropharyngeal cancer (25%) Specific squamous cell carcinomas of the skin	Various human papillomavirus (HPV) types
Hepatocellular carcinoma	Hepatitis B (HBV) and C (HCV) viruses
Adult T-cell leukaemia	Human T-lymphotropic virus (HTLV-1)
Seminoma (?)	Endogenous human retroviruses
Kaposi's sarcoma	Human herpes virus- 8 (HHV-8)
Gastric cancer and gastric lymphoma	*Helicobacter pylori*
Bladder and rectal cancer	*Schistosoma haematobium* and possibly other schistosoma species
Chlolangiocarcinoma	*Opisthorchis viverrin*i and O. felineus, *Clonorchis sinensis* and possibly Helicobacter bilis

ii) Family history/Genetics: Men with a brother or father who developed PCa at a young age have an increased risk of developing prostate cancer. Men with a family history of breast and ovarian cancers, Hodgkin's disease, leukaemia, liver cancer and melanoma are also at a higher risk of developing prostate cancer {Hemminki & Chen, 2005}. Monozygotic twins have double risks of predisposition to prostate cancer compared to dizygotic twins. At the moment, there is no single gene wholly responsible for predisposition of humans to prostate

cancer although involvements of possibly recessive or X- linked mechanisms have been suggested {Hemminki & Chen , 2005}. Other suggestions include mutation carriers of BRCA 1&2 genes, which encode proteins involved in double-stranded DNA repair and maintenance of chromosomal integrity. But inactivation of same genes has been reported in higher risk of pancreatic cancer; endometrial cancer is also related to BRCA 1 mutation and melanoma to BRCA 2 mutation. This plausibly explains some of the associations among cancers rather than causation. Another example is the occurrence of low penetrance genes such as RNASEL, MRS1 and the Toll-like receptor cluster in prostate cancer. These genes are also involved in inflammation and immunity {Sun *et al*, 2006}; which may explain the association of prostate cancer with non-Hodgkin's lymphoma and Hodgkin's disease. Several other chromosomal loci and genetic variants (mainly single nucleotide polymorphisms (SNPs)) have been associated with prostate cancer risk, including 11q13, 8q24 and 17q12; SNPs such as rs10896449, rs12418451, rs198977 and rs2664155 have been reported to show strong association with prostate cancer risk {Nam *et al*, 2006; Zheng SL *et al*, 2009}. The human genome diversity project (HapMap project) is aimed at unravelling genetic variants of humans in different geographical regions of the world, an effort that will help, among other things, map SNPs and haplotypes associated with disease risk http://www.hapmap.org/abouthapmap.html. There are about 10 million known SNPs in the human genome. Tags for SNPs (haplotypes) may help identify more genes and regulatory RNAs associated with prostate cancer development. Another genetic variant implicated in prostate cancer is microsatellite instability in the androgen receptor gene where a trinucleotide (CAG) polymorphic repeat length varies with prostate cancer risk. It was reported that patients with shorter CAG repeat length (usually below 22) tend to respond poorly to hormone treatment in metastatic PCa {Shimbo *et al*, 2005}.

iii) Race: PCa is more common in men of African descent than other ethnic/racial backgrounds. It is rare in Asians; although lack of adequate diagnostic strategy has been blamed for the reported low incidence rate in Asian population {Zhang *et al*, 2009}. Certain genetic factors associated with PCa have been reported to show ethnic/racial differences. For example, it has been reported that there was a significantly higher oestrogen receptor α expression in stroma of non-neoplastic prostate tissues from Hispanic and Asian men than those of Caucasian and African-American men, who are at higher risk for prostate cancer {Haqq *et al*, 2005}. Ethnic/racial biologic differences were reported on plasma levels of androgens {Ross R *et al*, 1986}, sensitivity of androgen receptors (due to reduced number of CAG or GGC repeats in exon 1) and altered insulin- like growth factor binding protein 3 (IGFBP-3) expressions in plasma {Haqq *et al*, 2005}. Freeman *et al* 1997 reported racial

difference in histological grade of prostate cancer, with American blacks having a significantly higher burden of high histologic grade than whites. However, a review suggested that ethnic/ racial differences in prostate cancer arose from multi-factorial causes spanning genetics, economics and sociology {Freedland SJ & Isaacs WB, 2005}. Data from the HapMap project is beginning to reveal the genetic variations associated with ethnic groups and disease risk; for example the risk allele (G allele in AG or GG genotypes) of the SNP rs10896449 localised on 11q13 is associated with aggressive prostate cancer and has the highest frequency in African ancestry (58-76%), followed by Caucasians (52-53%), Mexicans (32%), Asians in America (9-27%) and least frequency in Asians at their native home (3-8%). This information from the HapMap project is consistent with previous epidemiological observations {Dijkman & Debruyne, 1996}.

iv) Environment: Factors such as industrial pollution, occupational hazards and radiation exposures are environmental determinants, which have been implicated in the aetiology of prostate cancer. A very significant mortality rate from PCa was reported in rubber and tyre workers {Dijkman & Debruyne, 1996}. Farming and pesticide exposure were also weakly associated with PCa. Exposure (or dietary intake) of trace elements such as zinc and cadmium plus radiation exposure directly correlate with the mortality from prostate cancer {Meikle & Smith, 1990}. The fact that incidence of PCa tend to rise among those enjoying a low-incidence rate when they migrate to a high incidence locale is consistent with a role of environmental influences {Kumar *et al*, 2009}.

v) Dietary Factors: Based on epidemiological research and migrant studies, it is presumed that the development of prostate cancer is associated with some dietary components {Hebert *et al*, 1998}. Diets that contain high levels of animal saturated fat, low fibre content, reduced vitamins A and D levels are variously reported to increase risk of prostate cancer {Hebert *et al*, 1998 ; Dijkman & Debruyne , 1996}. It is generally thought that high fibre (for example grains, cereals and nuts) and low fat (fish) intakes are more likely to protect men from PCa. A recent dietary advice report recommends foods rich in vitamins, polyphenols, phytoestrogens, lignans, stilbens and carotenoids (lycophene) as being very protective; such foods can be sourced from vegetables, soy beans, cherries, strawberries, onions, nuts and grains, tomatoes and carrots {Thomas *et al*, 2006}. There are, however, some conflicting reports on the protective roles of Vitamins A and D. The trace elements: zinc, Cadmium and Selenium are also controversial in their roles in prostate tumourigenesis {Meikle & Smith, 1990}. However, there are several ongoing clinical trials on food and trace elements in prostate cancer management: i) Vitamin E supplement in PCa prevention, registration ID NCT00895115; ii) Cholecalciferol treatment on active surveillance patients, registration ID

NCT00887432 and iii) prostate and diet study: ISRCTN95931417 (http://www.controlled-trials.com/; http://apps.who.int/trialsearch . Dietary studies in prostate cancer prevention and management especially for high risk males and localized low grade prostate cancer patients on 'watchful waiting' has become important following reports of beneficial effects comparable to primary therapy{Thomas R *et al*, 2006; Bill-Axelson *et al*, A 2005}.

vi) Other Factors: Many other factors have been loosely associated with prostate cancer development. These include life-style (stress management and exercise), religion, and economic status; body size, physical activity and sexual activity. Data on these factors are conflicting and some are unconvincing in most cases. However, there are now more randomized controlled trials to evaluate most of these factors in relation to prostate cancer http://www.controlled-trials.com/.

c) Pathogenesis of Prostate Cancer. The molecular details of prostatic carcinogenesis have not been fully delineated, but it is clear that the development of a malignant prostate cancer cell from a normal prostatic glandular cell requires multiple transformation events {Isaacs, 1997}. The disease process is likely to have started from clonal expansion of genetically altered prostate epithelial cells. One hypothesis claims that the primary genetically altered cells reside in the basal layer of the prostate as ' Prostate stem cells', serving as a progenitor for terminally differentiated cancer cells {Agrawal & Dunsmuir, 2009}. This results in dysplastic and/or hyperplastic lesions in the prostatic epithelium that are, at the beginning, androgen dependent like the normal epithelia. The early stage genetic and morphological changes are very similar to those of PIN and BPH. There are many reported similarities between early prostate cancer and BPH: i) the parallel increase in prevalence with age; ii) both require androgens for growth and development, and iii) both respond to anti-androgen treatment regimens {Dijkman & Debruyne, 1996}, although advanced PCa tend to be hormone refractory in later stages.

Part of the multi-step transformation concept also implies that some of the cells may remain partially transformed and unable to progress to the next stage, accounting for the heterogeneity in the disease processs {Isaac, 1997}. The hyperplastic lesions are believed to slowly progress to prostatic intraepithelial neoplasia (PIN), which has low and high grade forms. PIN is characterized by progressive basal layer disruption (invasive), abnormalities in markers of secretory differentiation, increasing nuclear and nucleolar alterations; increasing cell proliferation, variation in DNA content (ploidy) and increasing genetic instability {Bostwick 1996; Haggman *et al*, 1997}. PIN is precancerous and is generally regarded as the precursor to localised prostate carcinoma {Agrawal S & Dunsmuir, 2009}. Within 5 to 10

years, some PINs progress to histologically detectable cancers, but others don't {Droller, 1997}. The ability of the neoplastic cells to invade the basement membrane of the gland is the key element in the disease progression. As the cancer cells expand, it is believed that newer cells are produced from the basal layer (cancer stem cells) {Quinn *et al*, 2005}. The tumour cells are mostly contained within the prostatic capsule; however the ability of the neoplastic cells to invade the prostatic capsule, spreading to the prostatic sheath and adjacent structures such as seminal vesicles, ejaculatory duct and the urethra is a mark of its aggressiveness.

The mechanisms of its spread include perineural invasion and lympho-haematogeous spread. In most cases the disease spread to the vertebrae as well as visceral organs such as liver and brain {Kumar *et al*, 2009}.The disease process is fairly correlated with Gleason scoring (morphological features of the prostate); poorly differentiated PCa tend to be more aggressive and lethal. However, the disease process is not well characterized molecularly. Although several molecular markers are prognostic of PCa in a multivariate model {Agrawal & Dunsmuir, 2009}, their use as single independent prognostic markers are limited. Molecular grading of prostate cancer could be invaluable for both prognosis and choice of patient-tailored therapy because the disease is seemingly uniquely different in each patient.

d) Natural History of Prostate Cancer: The natural history (clinical course) of PCa has a spectrum of duration and severity (WHO, 2004). In most cases, it has an insidious onset as a slow-growing, localised tumour (tumour confined within prostatic capsule, also known as histological tumour) strongly associated with advancing age. Like other malignant neoplasms, PCa is assumed to arise from a sequence of genetic (DNA damaging) events and is believed to present initially as a small focus {Gendler, 2001}. Autopsy studies showed that prostate glands in older men (glands considered to be clinically normal) contained foci of invasive cancer (histological cancer); the frequency of which rises from 10% in men in their fifties to 70% in men in their seventies and eighties (Droller, 1997). Figure 1.13 shows natural history of prostate cancer {Cross *et al*, 2008}. It is believed that more than 75% of PIN cases progress to histological detectable prostate cancers, which are either androgen dependent or independent. Some of the histological PCa progress to symptomatic forms (clinical PCa), while others asymptomatic. Histological PCa shows little geographical and ethnic variations {Meikle W & Smith JA, 1990} unlike clinical PCa.

There is no substantiated evidence to account for the disparity in capacity of some histological tumours to progress (and become symptomatic) or remain indolent (asymptomatic). Some progress further to metastatic PCa, which are more aggressive. Interestingly, some metastatic PCa are frequently asymptomatic. Epidemiological studies

have failed to explain this unpredictable natural history of prostate cancer. Moreover, it is not known if all histological prostate cancers (organ confined) have the capacity to progress to lethal forms and if they do, what are the stimuli to activate progression {Droller, 1997}. On the other hand, there are few biomarkers that can predict this capacity to progress. Morphologic examination of prostatic tissues often show mixed foci of inflammation, PIN, BPH or prostate carcinoma; sometimes very different pictures from both lobes. The aggressive forms of PCa tend to be hormone refractory, as they fail to respond to androgen ablation therapy in later stages. The molecular changes that characterize prostate cancer progression are beginning to emerge, for example the gene fusions (TMPRSS2-ETS), AR mutations and microsatellite polymorphism, p53 and Bcl-2 alterations, fatty acid synthase metabolic alterations and GSTP1 methylation {Benedettini *et al*, 2008}.

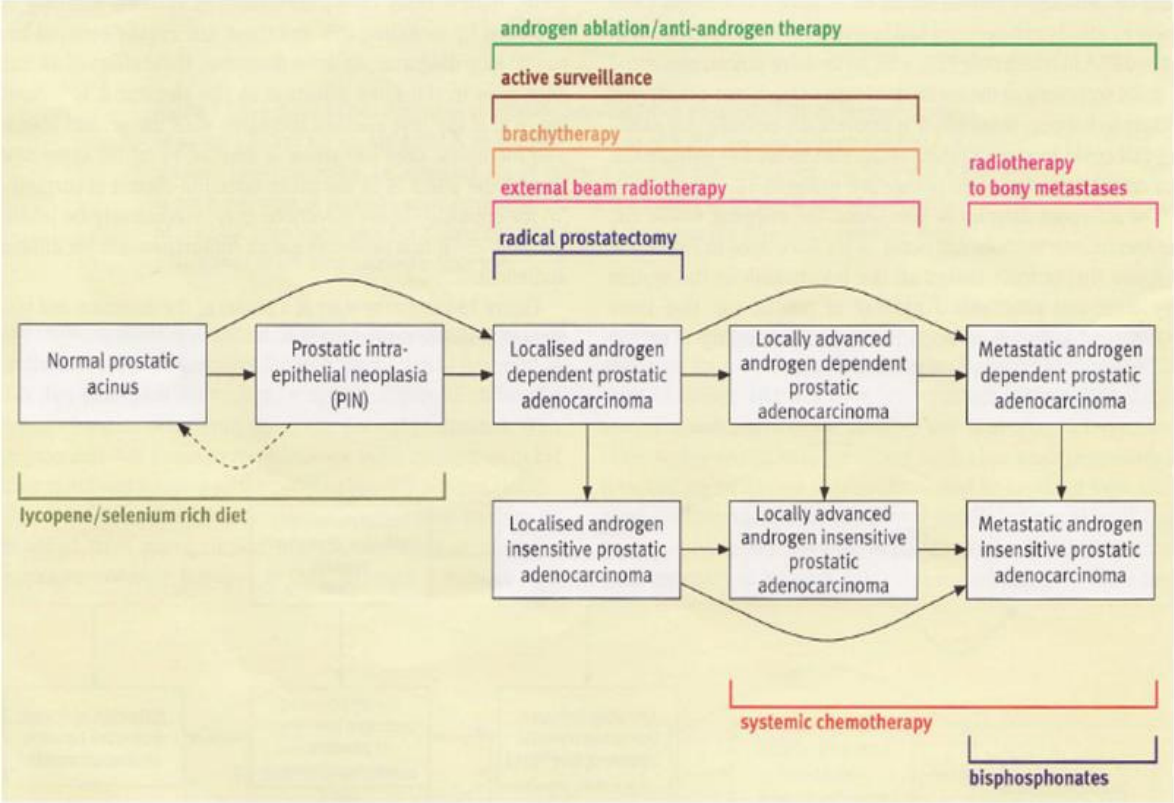


Figure 1.13 Natural course of Prostate cancer progression. Most prostate cancers arise from prostatic intraepithelial neoplasm (the precursor) and develop to organ-confined cancer. {Cross *et al*, 2008}

However, the correlation between the molecular markers and the currently used Gleason grading and pathology staging of prostate cancer is poor. Histological grading and staging still remains the most independent predictor of disease outcome and response to therapy. But intermediate grades (Gleason 5 and 6) behave unpredictably; requiring additional markers to define that group. One important fact about the natural history of PCa is that if offers a unique

opportunity for early detection by screening because of slow onset of disease process and long asymptomatic period.

1.2.4 Steroid hormones and receptors in prostate Cancer.

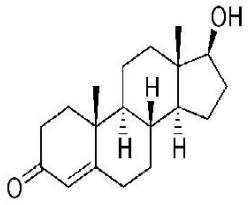
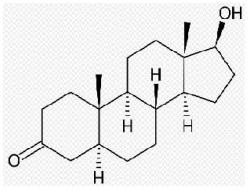
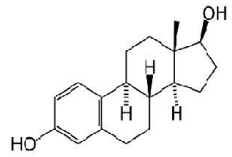
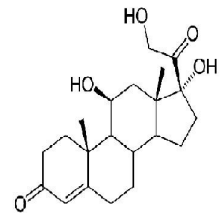
a) Steroid Hormones: Steroid hormones (SHs) are lipophilic molecules synthesized from cholesterol, and produced in the adrenal cortex (glucocorticoids, mineralocorticoids, and adrenal androgens), the testes (testicular androgens, oestrogen), and the ovary and placenta (oestrogens and progestagens or progestins). SHs are transported to their target cells through blood, where they are bound to carrier proteins, and because of their lipophilic nature they diffuse into cells {Soronen *et al*, 2004}. Within the cytoplasm of target cells, SHs bind to steroid hormone receptors (SHRs), which mediate the action of the hormones. The functions, characteristics and references ranges of some steroid hormones in humans are shown in Table 1.6. The growth and function of the prostate is controlled by androgenic SHs. The two predominant androgens are testosterone that is formed mainly in the testis from androstenedione(A-dione) and 5α-dihydrotestosterone (DHT), which is formed in the prostate from testosterone by 5α-reductases type 2 and is the most active androgen in the prostate {Scholzen & Gerdes, 2000}

The metabolism of steroid hormones in human prostate is shown in Figure 1.14. In normal conditions, a steady state exists between synthesis and inactivation of active androgens {Vihko *et al*, 2006}. A change in the balance (increased synthesis, decreased inactivation) can lead to androgen influence and increased cell proliferation. The majority of prostate tumours arise from the secretory, androgen-dependent epithelial cells, which forms the basis of hormonal therapy (ablation of androgens in the circulation and prostate tissue by surgical or chemical castration to treat PCa). Most prostate cancers are initially responsive to androgen withdrawal but become later refractory to the therapy and begin to grow androgen-independently {Soronen *et al*, 2004}. It is important to note that PCa that originate from neuroendocrine cells, the small cell carcinoma of the prostate do not respond to hormone therapy at all {Yao JL *et al*, 2008}

b) Steroid hormone receptors (SHR): SHRs are hormone- activated transcription factors. They belong to a sub-group of nuclear receptor super-family, which currently has 48 members in humans{Faus & Haendler, 2006}. The SHR sub-group is composed of the androgen receptor (AR), two oestrogen receptors (ERα and ERβ), the progesterone receptor (PR); the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). A variety of isoforms of SHRs have been identified; and these may be expressed in distinct cell types and

developmental stages, suggesting that they play specific physiological roles{Picard , 2006}. For example, the expression of oestrogen receptor α molecularly divides human breast cancers into ERα positive and ERα negative cancers; the ERα positives are mainly luminal breast cancers, which also express progesterone receptor and they appear to have the best outcome of therapy{Marchio & Reis-Filho, 2008}. The ERα negative breast cancers encompass the sub-groups of HER2 positive cancers, normal breast-like and basal-like cancers. This molecular classification based on ERα status and subsequent genetic signatures define choice of treatment and inform prognosis

Table 1.6 Functional characteristics and reference ranges of some steroid hormones in humans. {Zubay, 1996}

Steroid hormone	Characteristics	Functions	Reference ranges (in males)
Testosterone 	C_{19} steroid with unsaturated bond between C-4 and C-5, a ketone group in C-3 and a hydroxyl group in the β position at C-17. M_r is 288.4. It is both a hormone and a prohormone.	It promotes male sexual development; promotes and maintains male sex characteristics. It also influences erythropoiesis by stimulating renal production of erythropoietin. It influences neural development and activity.	In males, prepubertal (late) level in serum is 0.1-0.2 ng/ml; in adult the level is 3.0-10.0 ng/ml.
Dihydrotestosterone 	It is a metabolite of testosterone; derived in target cells, e.g. prostate gland, by the action of 5α-reductase type 2 enzymes. It has a ketone group at C-3 but no unsaturated bond between C-4 and C-5. M_r is 290.44.	Most potent form of testosterone, binds to AR with higher affinity than testosterone.	Varies depending on target cell reduction of circulating testosterone.
17 β oestradiol (E2) 	C_{18} steroid hormone with a phenolic A ring. It has two hydroxyl groups, one at C-3 and the other at C-17. M_r is 272.4. It is the most potent natural oestrogen, produced mainly by the ovary, placenta, and in smaller amounts by the adrenal cortex and the male testes. Chimeric forms of it exist.	It is responsible for sexual development in the female; promotes and maintains female sex characteristics.	In males oestradiol level is < 60 pg/ml. There is increased level at old age.
Hydrocortisone (cortisol) 	C_{21} steroid, also known as glucocorticoid (generic name). It has two ketone groups, one at C-3 and the other at C-20, an unsaturated bond between C-4 and C-5 and hydroxyl groups at C-17 and C-21. M_r is 362.47.	It promotes gluconeogenesis; suppresses inflammatory reactions	Concentration in peripheral plasma varies with day time: At 8 a.m, level is 16µg/100ml while at 4 p.m. the level 4µg/100ml.

Steroid receptors are mainly found, in the absence of hormone (ligand), in the cytoplasm complexed to chaperone proteins such as heat shock protein 90 (Hsp90). SHRs appear to rely on the Hsp90 machine for folding, regulation of the allosteric switch and recycling{Picard, 2006}, Figure 1.14

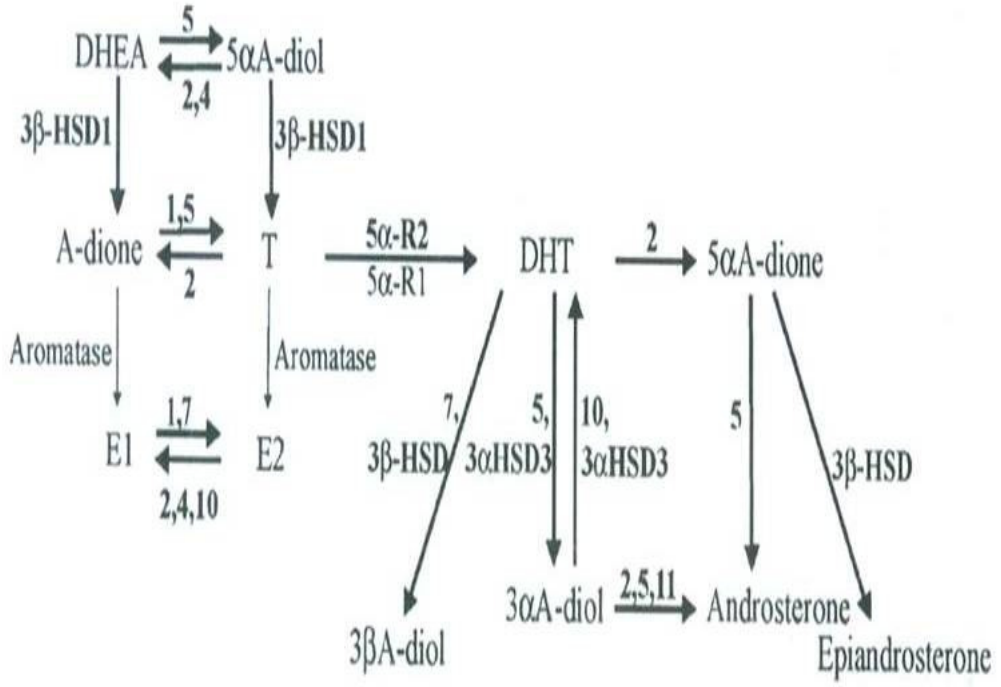


Figure 1.14 Steroid metabolism in human prostate tissue.1, 2, 4, 5,7,10 and 11 are different 17 HSD types; E1, oestrone, E2, oestradiol; A-dione, androstenedione; T, testosterone; DHEA, dihydroepiandrosterone; 5α A –diol, 5α-androstenediol; 5αA-dione, 5α-androstanedione, 5α-R1, 5α-reductase 1, 5α-R2, 5α-reductase 2; DHT, dihydrotestosterone; 3βA-diol, 5α-androstane-3β 17β-diol; 3αA-diol,5α-androstane-3α,17β-diol;3β-hydroxysteroid dehydrogenase type 1. Androgens and oestrogens are eliminated as sulphate or glucuronide derivatives.
{Soronen et al, 2004}.

Following ligand binding, SHRs undergo conformational changes and relocate to the nucleus where they activate or repress target genes as homodimers {Faus H & Haendler B, 2006}.SHRs are structurally organized in four main domains: an N-terminal region bearing important transactivation functions, a DNA-binding domain (DBD) composed of two zinc fingers, a hinge region harbouring the nuclear localisation signal and a ligand-binding domain (LBD) with additional transactivation functions {Vihko *et al*, 2006}.

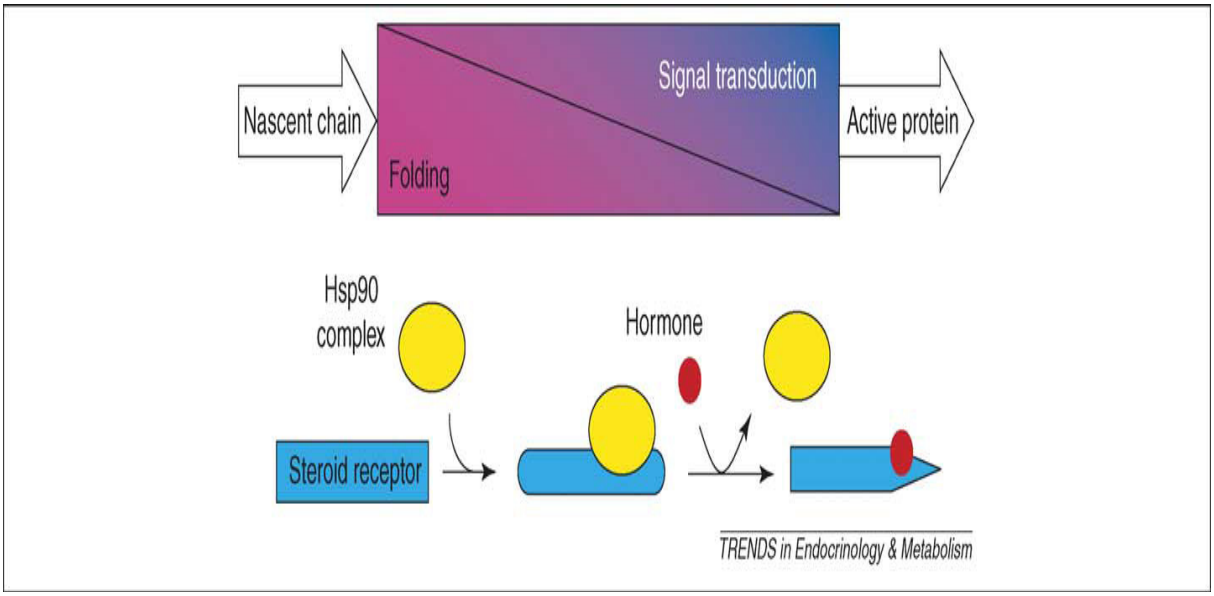


Figure 1.15. Folding and signal transduction overlap steroid receptors. Steroid receptors are assisted by the Hsp90 molecular chaperone machine, which acts both as a facilitator and repressor. The hormonal ligand triggers the completion of folding and maturation and pushes the equilibrium towards signal transduction {Picard, 2006}.

Binding of agonistic or antagonistic ligands leads to different conformational changes of SHRs making them capable of exerting positive or negative influences on the expression of target genes by different mechanisms: (i) after separation of chaperones, the hormone-SHR complexes can bind to chromatin organized DNA sequences in the vicinity of target genes, termed hormone response elements (HREs). The HRE-recruited hormone-receptor-complexes are then able to initiate chromatin remodelling and to relay activating or repressing signals to the target genes transcription machinery; (ii) through protein-protein interactions with other sequence-specific transcription factors; by this mechanism SHRs can also regulate the activity of many genes that are switched on, for instance, during stress or an inflammatory response; (iii) the SH response can also be integrated in the intracellular signalling network via cross-talk of SHRs with signal transduction pathways that transmit extracellular signals via membrane receptors and activation of protein kinase cascades to nuclear transcription factors that activate various target genes. By all these different mechanisms SHRs modulate numerous and specific responses in a large variety of cells, whereby their particular effect depends on the physiological, cellular and genetic context {Faus & Haendler, 2006}.

The recent discovery of oestrogen receptor beta (ERβ) and its possible role as an anti-proliferative agent has renewed interest in the role of steroid hormone receptors in prostate

cancer {Walton *et al*, 2009}. It has been reported that ERα, ERβ, PR and AR expressions were increased in male breast cancers compared to female breast cancers where ERβ expression was dramatically reduced {Murphy *et al*, 2006}. There are conflicting reports on ERβ gene expression in prostate cancer; majority of protein studies document a down-regulation of ERβ in PCA. However, a recent study using qRT-PCR analysis of SHRs gene expression in laser microdissected PCa specimens (11 samples) revealed an up-regulation of ERβ gene expression compared to benign controls {Walton *et al*, 2009}.The same study reported, arguably, no significant differences in AR, ERα or PSA gene expression between cancer groups and benign controls. In contrast, PR gene expression was significantly down-regulated in the cancer group. Understanding the distribution and expression patterns of SHRs in prostate cancer may offer a wide range of applications for both therapeutic and diagnostic interventions. In a very remarkable study reported in 1963, Leblond showed that the prostate gland and the breast share similar proliferative capacity; an observation that underpins the importance of SHRs in carcinomas of both organs.

c) Transformation to Androgen Insensitivity: The cause of androgen resistance in PCa treatment remains largely unknown. There are four different theories in the AR involvement in hormone insensitive (hormone refractory) status of prostate cancer cells. First, that prostate cancer cells loose the ability to express AR {Droller, 1997}. This hypothesis is the least favourable explanation of androgen insensitivity because it is now known that AR is more expressed in malignant than benign prostate cells {Rong Hu *et al* 2009}. Second, that prostate cancer cells express mutated AR, with ligand binding domain (LBD) unable to bind ligands (androgens and anti-androgens). The mutated AR lack regulatory activity, and have abnormal spatial conformation, which inadvertently allows it to bind other enhancers, for example, gastrin releasing peptide even in the presence of androgens {Yang et *al* 2009}. The AR is known to have numerous mutations, which makes this hypothesis worth further investigation. Third, that clones of androgen –resistant cells develop from mutated stem cells (cancer stem cells) *ab initio* and expand as androgen-sensitive clones die due to treatment. That is treatment selects mutant clones. Fourth, that clones of androgen-resistant cells evolve (by sporadic mutations, deletions, etc) and survive during androgen therapy {Droller, 1997}. Such adaptive responses will also include the expression of alternatively spliced AR variants that lack LBD, for example, the AR-V1 and AR-V7 described by {Rong *et al* 2009}; evolution of somatic mutations that confer resistance against anti-androgens {Hara *et al* 2005} and possible gene amplification (copy number variations). The AR is discussed further in chapter two. Another secondary explanation to androgen insensitivity in prostate cancer progression is the evidence from transformation studies on refractory phase of PCa in

LNCAP cell line. The study reported substantial changes in androgen and oestrogen metabolism in the cells during transformation suggesting oestrogen influence as the cause {Soronen *et al*, 2004}. This was supported by cDNA microarray screening results which showed over-expression of several genes up-regulated by oestrogen in the LNCaP cells. But this does little to explain how oestrogen influence can induce androgen insensitivity because oestrogens are known to stimulate AR synthesis {Droller, 1997}.

1.2 Diagnosis of Prostate Cancer

Current clinical diagnosis of prostate cancer involves six different tests: i) prostate specific antigen (PSA) test, ii) prostatic alkaline phosphatase (PAP) test, iii) Digital rectal examination, iv) Transrectal ultrasonography (TRUS), v) Prostate cancer antigen 3 (PCA-3) test vi) Histopathological examination of prostate biopsy. None of these tests can be substituted for the other; each provides additional information necessary for clinical decision. However the first five tests are primarily screening tests, histology is the definitive test for diagnosis. Of the first four screening tests, a randomized controlled trial carried in Rotterdam between 1994 and 1996, reported PSA as the strongest predictor for prostate cancer in favour of DRE and TRUS {Rietbergen *et al*, 1997}.

1.3.1 PSA testing

PSA is the most important and most widely used tumour marker for prostate cancer; useful for diagnosis, staging and monitoring of progression {Darson, 1997; Darson *et al*, 1999; Becker *et al*, 2000}. The PSA is a glycoprotein produced predominantly by the columnar epithelial cells of the prostate. It functions to liquefy the seminal coagulum, facilitating motility of sperm cells and fertilization. The molecular forms and physicochemical properties of PSA are discussed in Section 2.3. The PSA was identified and reported by several independent groups in the 1970s, and by the 1980s it became translated into clinical use {McCormack, 1995}. It is measured in serum or plasma using enzyme linked immunosorbent assay (ELISA), an immunometric sandwich assay in which specific anti-PSA antibodies are used to detect and quantify the marker photometrically. ELISA can detect as low as 0.1ng/ml of PSA in serum. Normal prostate epithelial cells produce more PSA glycoprotein than the malignant prostate tissue, and PSA mRNA is also expressed at higher levels in benign tissue than in malignant prostatic tissue {Panek et *al*, 1997}. It is believed that the PSA leaks into the blood stream as a result of deformations in the architecture of the prostate gland during trauma and or disease {McCormack, 1995}.

Large amounts of PSA (0.5mg/ml) are present in the seminal fluid (SF); while the serum/plasma level depends on the patient's age and prostatic volume. For men aged 50 years, the serum level is usually below 4.0ng/ml. It can increase up to 6.5ng/ml in men aged 70-79 years {Moul, 2000}. PSA is also detectable in urine but the urinary levels have no clinical significance {Panek et *al*, 1997}. There is a substantial overlap in serum PSA levels in men with BPH, PIN, acute prostatis; prostatic ischaemia, and men with PCa {Panek et *al*, 1997}.

There is a controversy on exact cut-off level for accepting normal PSA, but the European association of Urology (EAC) guidelines on prostate cancer recommends < 2.5-3.0ng/ml especially for younger men (< 50 years of age) {Heidenreich *et al*, 2008}. The reason for the controversy is that the sensitivity and specificity of PSA testing vary inversely at any chosen cut-off level (Table 1.7) {Djavan, 2004; Vickers *et al*, 2007}. In many countries 4.0ng/ml cut-off point is commonly used, giving sensitivity and specificity of ~ 20% and 94% respectively {Thompson *et al*, 2005}. The implication of this is that the PSA as a biomarker has a weak correlation with prostate malignancy. To improve the diagnostic utility of PSA testing, some other modifications of the test (ratiometric tests) have been evaluated: free to total PSA ratio, PSA velocity, PSA density, PSA doubling time and PSA and hK2 product. These modifications are discussed in Section 2.3. As a result of weak correlation with malignancy, elevated PSA is not on itself conclusive, further tests such DRE, PAP test, TRUS, PCA-3 are conducted before eventually carrying out a prostate biopsy.

Table 1.7 Sensitivity and Specificity for PCa by Cut points of PSA {Djavan, 2004}.

		Any Cancer (n=1225) vs. No Cancer (n=1362)
PSA, ng/ml	Sensitivity	Specificity
1.1	83.4	18.9
1.6	67.0	58.7
2.1	52.6	72.5
2.6	40.5	81.1
3.1	32.2	86.7
4.1	20.5	93.8
6.1	4.6	98.5
8.1	1.7	99.4
10.1	0.9	99.7

Despite the pitfalls (weak correlation and inconclusive outcome), PSA testing is widely used in mass screening exercises for early prostate cancer detection. The test is cost-effective, technically straight-forward and requires only a photometer to conduct the assay. Recent results of the European Randomized study of Screening for Prostate Cancer showed that mass screening using PSA test and DRE, followed by prostate biopsy for men with PSA > 3.0ng/ml yielded a 20% reduction in mortality from PCa {Andriole *et al*, 2009}. Other reports have also shown that PSA testing has increased the rate of prostate cancer detection, although with also an increase in the number of negative prostate biopsies {Welch *et al*, 2007}. The American Cancer Society recommends a yearly PSA testing for men aged 50 years, continuing until the man's life expectancy is less than 10 year; other studies suggest an initial PSA test at age 44-50 as the stronger predictor of long-term PCa risk {Vickers, 2007}.

1.3.2 Digital Rectal Examination

DRE is usually carried out by a urologist or ultrasonography technician. Any nodularity, induration or asymmetry is usually considered abnormal and may necessitate further testing (PSA and needle biopsy). However, DRE method has low specificity for PCa, since BPH causes most of the prostate enlargements. DRE supplements PSA testing.

TRUS is also performed by a urologist or an ultrasonography technician. Early studies suggested that prostate malignancies tend to be hyperechoic, but with improvements in high-frequency transducers and real- time imaging, it became evident that not all PCa exhibited

this characteristic and many were indeed hypoechoic {Tobocman *et al*, 1997}. It is now known that ultrasound reflections may fail to accurately reflect the histopathology of the prostate. TRUS also helps to determine prostate volume, the denominator in calculating PSA density.

1.3.3 Prostate cancer antigen 3 (PCA3) testing

First described by Bussemakers and colleagues in 1999, PCA3 formerly known as differential display 3 (DD3) is localised at chromosome 9q21 and encodes a prostate-specific mRNA that is highly overexpressed in PCa tissue compared with benign prostatic tissue {Marks et al, 2007}. The PCA3 does not encode a protein product. Its possible use as a urinary marker for PCa was suggested by de Kok *et al* in 2002. The clinic assay for PCA3, a real time PCR, was developed in the Nijmegen laboratory, the Netherlands; and results of controlled clinical trials in Canada and Austria confirmed the potential of the assay for PCa diagnosis {de Kok et al, 2002}. Following the acquisition of patent right from Nijmegen by DiagnoCure Company and later by Gen-Probe USA, a new quantitative molecular test based on target capture, transcription mediated amplification and hybridization protection for PCA3 received CE mark in 2008 and it is now used in many clinical laboratories in Europe {Torres & Marks, 2006} .

Biologically, the median upregulation of PCA3 from normal to tumour tissue was reported as 34- fold, increasing to 66-fold in tumour tissues containing more than 10% cancer cells {Hessels et *al,* 2003}. Although biologic function of the PCA3 is unknown, its upregulation in cancer tissues provided a basis for detecting the presence of the gene in tissues containing only a small number of cancer cells, against a background of low expression by many normal or BPH prostate cells, in tissue biopsies and bodily fluid {de Kok *et al,* 2002}. Thus, the importance of denoting PCA3 as a ratio with PSA mRNA (a surrogate for background prostate epithelial cell nuclear material) was established {Torres & Marks, 2006}. Equally important, a practical application was confirmed: the PCA3 ratio determined in voided urine, especially after light prostatic massage, or 'attentive' DRE was shown to be a sensitive and specific test for PCa{Hessels et *al,* 2003}. The validity of the assay depends very largely on informative specimen, that is, specimen with sufficient prostatic nuclear material measured by PSA mRNA. The result of the assay is expressed as a score (PCA 3 score) = (PCA3 mRNA/ PSA mRNA) x 1000.

The diagnostic reliability of the PCA3 score has been evaluated in several studies. Marks et *al* 2007 reported a sensitivity of 58% and specificity of 72% at the commonly accepted cut off score of 35 (Table 1.8). The PCA3 score of 35 means 35 mRNA copies of PCA3 per one

copy of PSA mRNA. Their report also showed an inter-run variation (precision) of about 20%. Fradet et *al* (2004) reported a sensitivity of 50% and specificity of 76% at the PCA3 cut off score of 35, with an area under the receiver operating characteristics (ROC) curve (AUC) of 0.68 compared to AUC of 0.54 for PSA test. The PCA-3 test was clinically superior to PSA test {Marks LS *et al*, 2007}.

Table 1.8 Sensitivity and Specificity of the PCA3 assay {Marks et *al*, 2007}.

PCA3 Score cut off	Sensitivity	Specificity	Odds ratio
10	87	28	2.5
35	58	72	3.6
50	47	81	3.7

A particularly important role of the PCA3 test appears to be in men with persistently elevated serum PSA levels (> 2.50ng/ml), but a negative initial biopsy {Torres & Marks, 2006} . In such men, who constitute a large problematic group, the odds ratio for the PCA3 test to predict cancer upon re-biopsy is 3.6, compared to only 1.2 for serum PSA testing {Marks *et al*, 2007}. At the cut off point of 35, the PCA3 could only predict a 30% positive biopsy, increasing to 50% at PCA3 score of 100 {Marks et *al*, 2007}.

Currently there is no clear-cut evidence that PCA3 score correlates with Gleason score; molecular grading would be invaluable in predicting the clinical outcome of intermediate Gleason score tumours (scores 6-7). Secondly, there is also the inverse relationship between sensitivity and specificity of the PCA3 score, much similar to that of PSA. At lower cut off points the sensitivity increases as the specificity drops (Table 1.8) compared to Table 1.7 for PSA. However, the factors that skew PSA results such as trauma, 5α- reductase inhibitors (finasteride and dutasteride), prostatic volume, age, inflammation and DRE do not affect PCA3 score {Torres & Marks, 2006}. The test uses 20-30ml of first catch urine after prostatic message. There are possibilities that the PCA3 test could be improved by addition of other tests such as the TMPRSS2: ETS gene-fusion and KLK2. Another important factor is that the regulation of the PCA3 gene is not clearly defined. The nucleotide sequence for the PCA3 RNA has several SNPs, more than 100 SNPs (using the UCSC Genome browser). More studies are still required to define the biologic role of this RNA.

1.3.4 Histopathological Examination

The histologic examination of prostate biopsy is regarded as the gold standard for definitive diagnosis of prostate cancer. By the EAC guideline, in routine clinical practice, elevated PSA levels, a free to total PSA of < 20% and a PSA velocity of > 0.75ng/ml/yr are valid parameters associated with high risk PCa, which facilitate the indication to perform a prostate biopsy. The number of biopsies required for the optimal detection of PCa is controversial. Ultrasound-guided transrectal, laterally directed 18G core biopsy has become the standard way to biopsy material for histopathological examination {Heidenreich *et al*, 2008}. The patients are given anaesthetics (locally via periprostatic injections or orally as effective analgesia) and antibiotics (usually Ciprofloxacin). The EAC guideline recommends a minimum of 10 systematic (5 from each side), laterally directed cores or the use of Vienna normograms. Many studies have shown that higher intensity biopsies (increased number of needle cores) improve cancer yield (proportion of men whose prostate biopsies are found to contain cancer){Durkan *et al*, 2002}; some urologists advocate 'saturation biopsy' (32 to 38 needle cores) claiming that micro cancers can still be found in many negative biopsies {Fleshner & Klotz, 2002}.Transition zone biopsies are not recommended in the first set of biopsies due to low detection rates {Heidenreich *et al*, 2008}. Currently the detection rate of PCa by biopsy is 30% {Welch *et al*, 2007}. The EAC guideline recommends one set of repeat biopsies for cases with persistent indication (abnormal DRE, elevated PSA or histological findings suggestive of malignancy at the first biopsy).

Several factors influence the detection of PCa in contemporary prostate needle biopsies (Table 1.9) {Bostwick & Meiers, 2006}. It is interesting to see that the low detection rate by prostate biopsy is contingent on several subjective factors; for example, false negative results from pathologists contributed between 0.6 to 1.0%. One of the anticipations of Bostwick and his colleague (2006) is that molecular diagnostics of needle biopsies will help improve the detection rate of PCa by biopsy. Often patients who have negative repeat biopsies face the difficult choice of a third biopsy; molecular testing may play a key role in reducing the number of negative prostate biopsies and associated risk (infection, bleeding and trauma).

The histopathological examination provides reports on macroscopic and microscopic changes on the prostate tissues. Key information includes the degree of architectural changes in the glandular pattern of the prostate (histopathological grading) and the extent of cancer spread to microenvironment and adjacent structures (pathological staging).

Table 1.9 Factors that influence the detection rate of cancer in y prostate needle biopsies {Bostwick & Meiers, 2006}.

Uncontrolled factors
Patient risk factors
Patient population (e.g., screening population vs. urologic practice)
Patient symptoms
Serum PSA
Clinical stage
Patient age
Patient race
Prior biopsy findings (e.g., PIN, ASAP)
Prostate-related factors
Prostate volume
TRUS and other imaging findings
Controlled factors
Urologist-controlled factors
Number of needle cores obtained
Method of biopsy (e.g., random, ultrasound guided, etc.)
Location of biopsy (e.g., laterally-directed biopsies vs. midline, etc.)
Amount of tissue obtained (e.g., biopsy ‘‘gun’’ employed; operator skill)
Pathologist-controlled factors
Histotechnologist’s skill in processing and cutting prostate biopsies
Number of needle cores embedded per cassette
Number of tissue cuts obtained per specimen
Pathologist’s skill in prostate biopsy interpretation

a) Gleason Score: This is the most commonly used pathological grading for prostate cancer. Named after Dr Gleason who first described this grading system, the Gleason grading stratifies prostate cancers into five grades on the basis of glandular patterns (architectural patterns of prostatic glands) and degree of differentiation (the resemblance of tumour gland to normal gland) as seen under low magnification {Kumar *et al*, 2009}. Figure 1.16 shows Dr Gleason’s own simplified drawing of the five Gleason grades of prostate cancer.

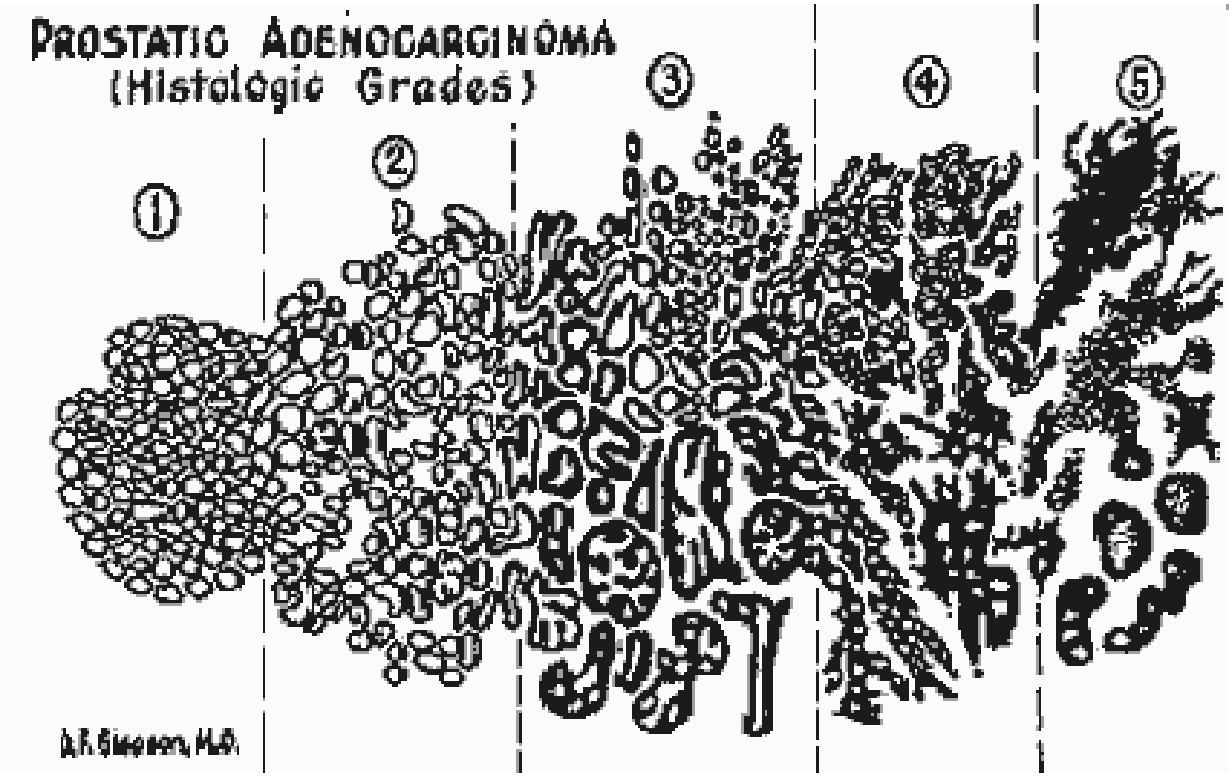


Figure 1.16 Dr Gleason's simplified drawing of the five Gleason grades. Grade 1 appears on the far left and grade 5 on the far right. Source**:**
http://www.phoenix5.org/Infolink/GleasonGrading.html#Grades

Grade 1 represents the most well differentiated tumours, in which the neoplastic glands are uniform and round in appearance and are packed into well-circumscribed nodules {Kumar *et al*, 2009}. They closely resemble normal prostate, seldom occur in the general population and have a better prognostic outcome. Grades 2 are similar to grade 1 but the tissues are loosely aggregated and some glands wander (invade) into the surrounding stroma (muscle). Grade 3 is also well differentiated (have glandular patterns) but more invasive (cancer cells wander into stroma). The cells are darker and the glands have more variable shapes. It is the most common grade by far {Kumar *et al*, 2009}. Grade 4 involves a huge disruption and loss of normal gland unit Figure 1.17.

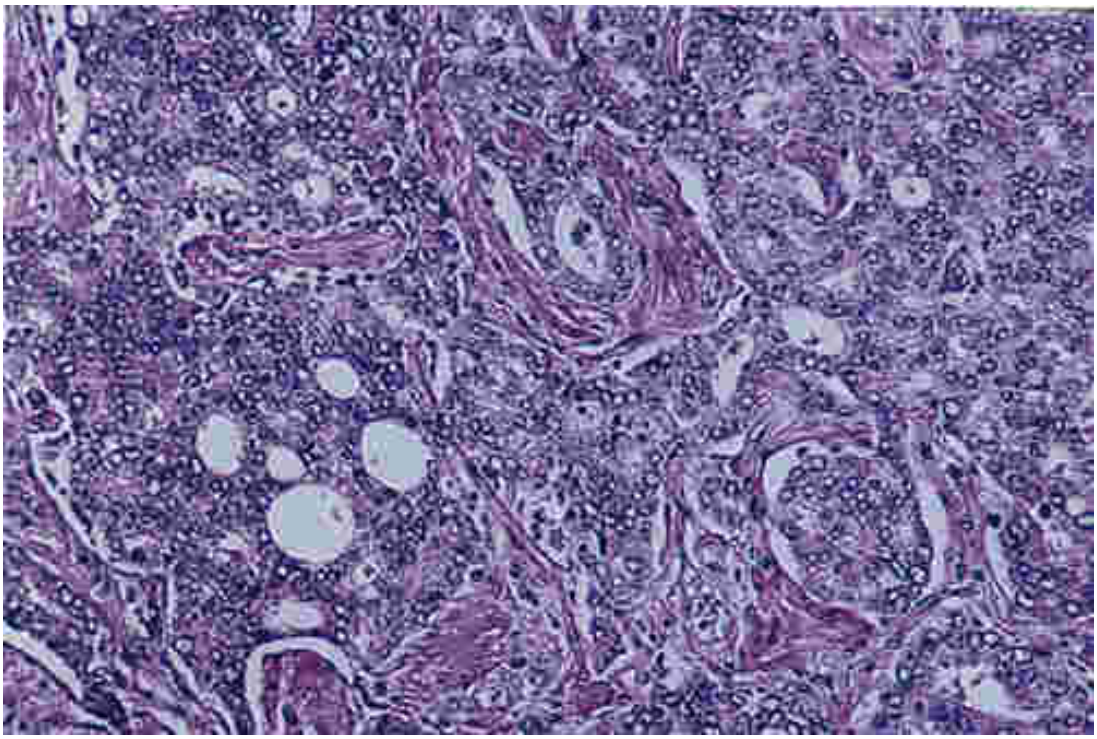


Figure 1.17 Grade 4 prostate carcinoma. There is a complete loss of glandular units. **http://www.phoenix5.org/Infolink/GleasonGrading.html#Grades**

The appearance of grade 4 varies in many cases due to the degree of loss of glandular pattern. By contrast Grade 5 tumours show no glandular differentiation, and the tumour cells infiltrate the stroma in the form of cords, sheets and nests {Kumar *et al*, 2009}. They are poorly differentiated.

Because most tumours contain more than one pattern, it is usual to assign a primary grade to the dominant pattern and a secondary grade to the subdominant pattern. The two numerical grades are then added to obtain a combined Gleason score or sum. For example, a Gleason score of 5 (2+3) means that the primary grade is 2 and the secondary pattern is 3. The lowest Gleason score is 2 and the highest is 10. In clinical practice, Gleason score of 5 and below (low grade) are rarely reported. The middle grade- Gleason scores 6 and 7 are the most abundant while the high grade consists of 8-10 (least or poorly differentiated) and are very aggressive. Grading (Gleason score) is of particular importance in prostatic cancer because there is in general fairly good correlation between the prognosis (disease outcome) and the degree of differentiation {Kumar V *et al*, 2009}. However the clinical behaviour (degree of aggressiveness) of intermediate Gleason scores (5 -7) is very unpredictable and may require additional prognostic factors for better assessment {Isaac, 1997}. The majority of PCa (76%) fall into the intermediate Gleason score category {Isaac, 1997}.

b) Staging of PCa: There are two different stagings of PCa: pathological (done by the pathologist) and clinical staging (done by the Urologists). Staging describes the anatomical extent of disease spread. The current system of staging is based on assessment of three components: T- the extent of the primary tumour, N- the absence or presence and extent of regional lymph node metastasis and M- the absence or presence of distant metastasis. This is known as the TNM system. Older systems of staging such as the ABCD (Jewett-Whitmore) system are still used by some Clinicians. Table 1.10 summarizes the staging of prostate adenocarcinomas using the TNM classification system {Wittekind *et al*, 2005}. Staging of prostate cancer is also important in the selection of the appropriate form of therapy and in establishing a prognosis {Kumar et *al*, 2009}.

The EAC guideline for the primary treatment of prostate cancer is based largely on staging of PCa. The combination of Gleason score and PCa staging is the strongest prognostic and predictive factors in the management of prostate cancer. The Partin coefficient tables (named after Dr Partin AW one of the authors) can be used to combine data on the PSA value, the Gleason score, and the clinical stage of a specific patient in order to predict the pathological grade of PCa tumour {Partin *et al*, 1997}. The table helps to assess the risk of a patient having organ-confined disease (curable by surgery), capsular penetration, spread to seminal vesicles or lymph nodes. Although the Partin coefficient table has not been validated through controlled clinical trials, the authors showed that the normograms correctly predicted pathological stages in 72.4% of all the cases.

Table 1.10 The TNM classifications for Prostate adenocarcinoma

{Wittekind *et al*, 2005}

Stage	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1a	Tumour in ≤5% of tissue (found incidentally)
T1b	>5% of tissue
T1c	Needle biopsy (due to elevated PSA)
T2	Confined within prostate
T2a	≤ half of one lobe
T2b	>half of one lobe
T2c	Both lobes
T3	Through prostatic capsule
T3a	Extracapsular
T3b	Seminal vesicle (s)
T4	Fixed or invades adjacent structures: bladder neck, external sphincter, rectum, levator muscles, pelvic wall
N	Regional lymph nodes
M1a	Non-regional lymph nodes
M1b	Bone (s)
M1C	Other site (s)

1.3.5 Supplemental and Future tests

Serum levels of prostatic acid phosphatase (PAP) had been used as a tumour marker of prostate cancer. The acid phosphatases are a group of enzymes capable of hydrolyzing esters of orthophosphoric acid in an acid medium {Igawa et *al*, 1995}. They are widely distributed in human tissues, with some isoenzymes that are tissue-specific. The human prostate is particularly rich in the PAP, a 100 kDa glycoprotein that exists in two forms: secretory and cellular forms, which are physico-chemically different. Although the serum PAP level is negligible in healthy individuals, its level is elevated in prostate cancer patients and correlates with the stage of prostate cancer {Veeramani *et al*, 2005}. The cellular form of PAP has been a useful marker for the detection of metastatic prostate cancer, due to its cell-specific expression {Veeramani *et al*, 2005}. Splice variants of the cellular PAP which are membrane-bound have been reported to have ecto-5'-nucleotidase activity (does not hydrolyze 5'-nucleoside monophosphates) and has been implicated in the formation of chronic pain in

patients with bone metastases of PCa {Zimmermann, 2009}. Generally, serum PAP (secreted form) level has low sensitivity and specificity for diagnosis and monitoring of PCa; it is unable to detect early tumour stage but markedly increases in patients with bone mestastases of PCa. Recent studies have shown that the cellular PAP plays a role in prostate carcinogenesis, which has a potential application for prostate cancer therapy. It is reported that cellular PAP functions as a neutral protein tyrosine phosphatase (PTP) in prostate cancer cells and dephosphorylates HER-2/ErbB-2/Neu (HER-2: human epidermal growth factor receptor-2) at the phosphotyrosine (p-Tyr) residues {Veeramani S et al, 2005}. Dephosphorylation of HER-2 at its p-Tyr residues results in the down-regulation of its specific activity, which leads to decreases in growth and tumorigenicity of those cancer cells. Conversely, decreased cellular PAP expression correlates with hyperphosphorylation of HER-2 at tyrosine residues and activation of downstream extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling, which results in prostate cancer progression as well as androgen independent growth of prostate cancer cells {Veeramani *et al*, 2005}. The expression of the cellular PAP decreases with progression of prostate cancer. However, these studies are in vitro; more clinical studies are required to confirm the activity of cellular PAP.

New tests for prostate cancer diagnosis, staging and monitoring are emerging. Recently, the result of metabolic profiling of 262- prostate related bio-specimens analyzed by liquid and gas chromatography-mass spectrometry techniques showed that sarcosine, an N-methyl derivative of the amino acid glycine was a potential marker for PCa {Sreekumar *et al*, 2009}. Sarcosine was identified as a differential metabolite that was highly increased during prostate cancer progression to metastasis and can be detected non-invasively in urine. It is known that AR and TMPRSS2: ERG gene fusion products co-coordinately regulate components of the sarcosine pathway {Tomlins *et al*, 2007; Sreekumar *et al*, 2009}. Although further clinical studies are required to establish the sarcosine urine test, it has a potential for development of biosensors that can be used for testing it at point of care.

Another metabolic tumour marker of PCa recently reported is the fatty acid synthase (FASN). The FASN is a 270 kDA cytosolic complex enzyme that functions as a homodimer, catalyzes the synthesis of palmitate by the condensation of malonyl-CoA and acetyl CoA {Benedettini *et al*, 2008}. FASN seems to play a crucial role in PCa by conferring growth and survival advantages to cancer cells, maintaining membrane biogenesis and membranous signalling proteins regulation, providing protein palmitoylation, which is a post-translational modification critical in cancer progression {Benedettini *et al*, 2008}. The over expression of

FASN in the prostate occurs during the earliest stages of neoplastic transformation (PIN lesions) and in nearly all invasive prostate carcinomas {Kuhajda, 2000}. FASN is a key player in lipid metabolism and tumour altered lipogenesis might represent a potential marker in tumour diagnosis (because it emerges early in carcinogenesis) and a target in treatment of advanced carcinomas {Benedettini *et al*, 2008}.

In addition to metabolic profiling, future tests for prostate cancer will also consist of molecular and genetic tests. A wide range of molecular markers have been reported, this is discussed in details in section 1.4.3. Genetic tests such as SNPs, DNA methylation and copy number variations are likely to be translated into clinical setting. This is discussed in section 2.8.

1.4 Molecular and Metabolic alterations in Prostate Cancer

1.4.1 Metastasis of Prostate Cancer

Metastasis (spread of cancer cells) is highly selective and consists of a series of sequential, interrelated steps that include growth, vascularisation, invasion, survival in the circulation, adhesion, extravasation, and proliferation at distant site {Isaacs, 1997}. In these seven steps, acquisition of metastatic ability by prostate cancer cells manifest not only by increased protein expression but also by decreased expression of metastasis- suppressor proteins through inactivation of their genes by either mutation, allelic loss, or epigenetic inactivation {Isaacs, 1997}. Any of these steps are potentially targets to serve as biomarkers of metastasis. For example, lack of metastatic ability in some prostate cancers is attributed to inadequate angiogenesis during prostatic carcinogenesis. Folkman *et al* (1989) demonstrated that the induction of angiogenesis is a critical step involving the conversion of hyperplastic lesions with low tumourigenic ability into cancerous lesions that can produce continuously growing tumours. Therefore, quantitation of angiogenesis was considered as a highly effective method for predicting aggressiveness of PCa. An example of such quantitation is the use of immunocytochemical staining with anti-factor -8- related antigen antibodies to identify endothelial cells, which represent vascularization {Weidner *et al*, 1993}. For PCa, the subject of tumour aggressiveness and metastasis is particularly important because not all histological PCa progress to aggressive forms that are life threatening. The ability to predict which tumours have the capacity to manifest aggressive behaviour requiring therapy becomes a critical issue as greater emphasis is currently placed on screening for earlier detection of PCa.

Several studies have examined various proteins, especially those involved in the seven steps of metastasis, as prognostic factors (disease-modifiers) for predicting outcome in prostate

cancer patients. In Table 1.11 Isaacs, 1997 reviewed several proteins including tumour suppressor proteins as possible prognostic factors. Freeman *et al* (1999) and Alison *et al* (2002) also reported possible prognostic utility of replicative proteins, the minichromosome maintenance proteins (MCMs). Vihko *et al* 2005 reported 17β Hydroxysteroid dehydrogenase (17HSD), a steroid hormone enzyme, as a possible marker of malignant transformation in prostate tumours. Quinn ID *et al* 2005 reviewed extensively the molecular markers of prostate cancer outcome; these include molecules involved in diverse processes such as cell proliferation, death and apoptosis; signal transduction, androgen receptor (AR) signalling, cellular adhesion and angiogenesis. Biomarkers identified to have potential prognostic value include p53, Bcl-2, $p16^{INK4A}$, $p27^{Kip1}$, c-Myc, AR, E-cadherin and vascular endothelial growth factor. Although a multivariate analysis on the expression of these proteins appears critical, at least to accurately predict the aggressive nature of individual prostate cancer {Isaacs, 1997}, it is possible that most of these proteins are differentially elaborated as a result of metastatic tumour biology and not drivers of metastases. The expression of most of these proteins may be suitably descriptive of metastasis rather than predictive. This may limit the ability of most of them to predict aggressiveness and therapy response. The TMPRSS2 gene fusion with members of the ETS family has also been identified as a predictor of PCa metastasis, with its occurrence correlating to worse outcome and high histological grades {Cross *et al*, 2008}.

When PCa spreads, it invades extra-prostatic tissues such as the seminal vesicles, ejaculatory ducts, the urethra and urinary bladder; it also seeds to the pelvic lymph nodes and in most cases will lead to bone metastases. It can also spread to visceral organs such as the liver and the brain. There is the need to establish molecular markers that can predict metastasis in locally confined PCa. Metastatic PCa is currently incurable.

Table 1.11. Possible Prognostic Factors for Identifying Aggressive Prostatic Cancers. {Isaacs, 1997}

Parameters in metastatic prostate cancers that are associated with	
increased expression include:	**decreased expression:**
bFGF	KAI-1
IL- 8	CD44 (standard form)
mdr-1[28]	E-cadherin
72-kd type IV collagenase	Normal p53 (wild type)
92-kd type IV collagenase	Angiostatin
Mutant p53	β_4 integrin subunit
Angiogenesis (factor-8-related antigen)	γ-2 laminin 5 subunit

1.4.2 Metabolic alterations in Prostate Cancer

The metabolic changes during development and progression of PCa have been investigated in recent years. The prostate cancer cells, like normal prostate cells, also require androgens for their growth and functions. Several *in vitro* studies have reported alterations in androgen and oestrogen metabolism during neoplastic transformation of prostatic cells {Vihko *et al*, 2005; Soronen *et al*, 2004}. For example, Vihko P *et al* (2005) reported a remarkable decrease in oxidative 17 β-hydroxysteroid dehydrogenase (17 HSD) type 2 activity during cellular transformation in the prostate. Testosterone and DHT are mainly inactivated in the prostate by 17 HSD type 2 to A-dione and 5α A-dione. Decreased inactivation of androgens in the prostatic epithelium could shift the balance toward an increase in the proliferative pressure of cells and lead to unregulated prostatic growth. This observation is further supported by the fact that the chromosome locus for the gene that encodes 17 HSD, which is 16q24.1-16q24.2, is often deleted in prostate cancer specimens. The report by Soronen *et al* 2004 showed that during neoplastic transformation of prostatic cells, oestrogen influence is increased in the cells, and several of the genes up-regulated by oestrogens are over- expressed.

Altered metabolism may not be the cause of prostate malignancies, although tumour cells may depend on altered metabolic pathways. Benedettini *et al*, 2008 reported that the lipogenic enzyme fatty acid synthase (FASN) seemed to play a crucial part in PCa by conferring growth and survival advantages to cancer cells. The FASN is a key metabolic enzyme that is responsible for the terminal catalytic step in *de novo* synthesis of fatty acids, which represents a common characteristic in human malignancies. Abnormal lipogenesis is a potential marker in tumour diagnosis and treatment. Cancer cells rely on anaerobic pathways to convert glucose to ATP, even in abundant oxygen, a phenomenon known as Warburg effect

{Benedettini *et al*, 2008}. Warburg in 1956 claimed that the first phase of cancer genesis is the irreversible injury to the respiratory chain. The anaerobic pathway is less efficient for energy supply than aerobic respiration which most of the normal cells use. In similar act, tumour cells produce fatty acids by *de novo* synthesis despite high levels of ambient fatty acids {Kuhajda, 2000}. Therefore over-expression of FASN in the prostate occurs during the earliest stages of neoplastic transformation (PIN lesions) and in nearly all invasive prostate carcinomas.

Recent studies have also shown that amino acid metabolism, nitrogen breakdown and methylation are enriched during prostate cancer progression {Sreekumar *et al*, 2009}. There is a significant increase in methyltransferase activity during PCa progression. For example, the enzyme, histone methyltransferase (EZH2) is significantly increased in metastatic PCa {Varambally *et al*, 2008}. Through metabolic profiling, also in the context of molecular alterations, sarcosine was identified as a key metabolite increased most robustly in metastatic prostate cancer and detectable in the urine of men with organ-confined disease {Sreekumar *et al*, 2009}. The transcriptional regulators of prostate cancer progression: AR and the ETS gene fusions seem to regulate directly sarcosine levels by means of transcriptional control of its regulatory enzymes (Glycine N-methyltransferase GNMT, sarcosine dehydrogenase SARDH and dimethylglycine dehydrogenase DMGDH). Therefore, 'components of the sarcosine pathway may have a potential as biomarkers of prostate cancer progression and serve as new avenues for therapeutic intervention' {Sreekumar *et al,* 2009}. Other metabolites associated with PCa were also identified in the study: uracil, glycerol-3-phosphate, leucine, proline and kynurenine.

The metabolism of the trace mineral selenium has also been associated with prostate cancer risk {Facompre & El-Bayoumy, 2009}. The report showed that the levels of selenium in blood and
prostate tissue is inversely correlated with prostate cancer isk and proposed for its use for chemoprevention.

1.4.3 Tumour markers of Prostate Cancer

Tumour markers (TMs) are generally biochemical indicators (biomarkers) of the presence and or progression of a tumour. They include cell surface antigens, nucleic acids, cytoplasmic and nuclear proteins, enzymes and hormones {Eissa & Shoman, 1995; Kumar *et al*, 2009). Tumour markers (biomarkers) can be categorized into the following:

a) Diagnostic markers (for detecting tumours for example PSA, PCA3).

b) Predictive markers (treatment modifiers because they help to match drugs with appropriate patients' populations or predict response to therapy; for example HER2, KRAS mutation).
c) Prognostic markers (correlate with disease outcome: invasiveness and metastasis, and can predict patients' survival; for example TMPRSS2: ETS gene fusion, TP53 mutation, pathological stage, PSA relapse).
d) Pharmacodynamic or therapeutic biomarkers (confirm biological activity; for example Bcr-abl1, EGFR, PARP; and they can be direct targets for drug actions).
e) Surrogate biomarkers (intended to substitute for clinical end points; for example Bcr-abl1)
f) Risk factor (cancer susceptibility genes that portend risk; for example genetic variants such as SNPs, copy number variations; they predict risk but may not be causative).

A single biomarker can belong to two or more categories mentioned above. Several molecular markers (biomarkers resulting from differential gene expression and genetic variations) of PCa have flooded cancer literature in recent years. Table 1.12 {Agrawal & Dunsmuir, 2009} summarizes the most documented molecular markers associated with PCa development and progression.

Table 1.12 Summary of molecular markers in prostate cancer
{Agrawal & Dunsmuir, 2009}

Marker	Action	Selected literature	Potential use
AKT and PTEN	Prostate-specific phosphatase and tension homologue (PTEN) loss of function induces AKT (protein kinase) inhibiting apoptosis and may cause tubule regeneration with PIN.	PTEN null mice develop high –grade PCa and metastasis. Genetic PTEN alteration seen in 10% primary PCa, > 30% metastases, PTEN loss induces p53 senescence. Possible use as gene therapy vector. See {Agrawal S & Dunsmuir WD, 2009}	Prognostic and therapeutic
AMACR	Alpha methlyacyl coenzyme A racemase protein voided in urine. Involved in fatty acid β-oxidation. Androgen-independent function as promoter of PCa	100% sensitive, 58% specific. Small study. Histopathological biomarker	Diagnostic
AR	Androgen receptor, nuclear transcription factor mediates Steroid hormones and stromal cell growth. AR Activation in luminal cells suppresses growth.	Stimulates early PCa growth, gene amplification in 30% AI tumours. AR mutations rare in untreated PCa. Vorinostat (histone deacetylase inhibitor) may reduce AR expression acting synergistically with Bicaltamide (AR antagonist) to inhibit PCa.	Prognostic and therapeutic
Bcl-2	B-cell CLL/lymphoma 2, antiapoptotic protein found in basal cells and stem cells. Loss of expression linked to PIN, progression and androgen independence.	Antisense oligonucleotides (Oblimersen) against Bcl-2 delay progression, improves chemo-sensitivity. Docetaxel Combination trial underway (NCT00085228).	Prognostic and therapeutic
BRCA2	Breast cancer type 2 susceptibility protein, tumour suppressor gene predisposes to Pca, chromosome 13q.	< 5% Familial/young-onset cases diagnosed < 55 years old .	Prevention
CgA	Chorionic gonadotropin alpha, neuroendocrine prehormone peptide. Unclear mechanism of action.	Limited use and reproducibility. Correlation with time to androgen independence and adverse outcome .	Prognostic
Cyclin D1	Role in cell cycle from G1- to S-phase.	Upregulation may be associated with androgen independence and poor prognosis.	Prognostic
E-Cadherin	Cell adhesion molecule. Down regulation/loss associated with invasion and metastasis.	Close univarate T and M stage and survival significance	Prognostic
EGFR (Erb B1) Her-2/Neu (Erb 2)	Activation associated with proliferation, malignant transformation, relapse, progression and androgen Independence [4]. Epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (Her2).	Higher levels in PCa than BPH. Monoclonal antibodies directed against specific binding domains, anti-EGFR, e.g. cetuximab, anti-HER2, e.g. trastuzumab [16]. Lack of significant role in PCa.	Prognostic and therapeutic

Table 1.12 Summary of molecular markers in prostate cancer (Continued)

Agrawal & Dunsmuir, 2009}

Marker	Action	Selected literature	Potential use
EN-2	Mouse engrailed-2 gene. Homoeobox-containing transcription factor/candidate oncogene, overexpressed in aggressive HRPC/PCa. May be positively modulated by PAX2.	En-2 down regulation by siRNA decreases PAX2 and decreases PCa cell proliferation. PAX2 has been postulated as essential for PCa cell survival.	Diagnostic and prognostic
EPCA 1 and 2	Early prostate cell antigen, nuclear matrix protein. Associated with proliferative inflammatory atrophy (PIA), PIN and PCa. Generally not-detected in noncancer cases.	No link to Gleeson score. Reported positive in BPH patients who subsequently developed PCa. EPCA 1 and 2, 92% sensitive, 94% specific. EPCA2 distinguishes localized *vs.* extracapsular disease ($P < 0.0001$). Awaiting larger trials.	Diagnostic and prognostic
ESR	Oestrogen receptor located in stroma. Role unclear.	Close univarate M stage and survival significance	Prognostic
EZH2	Enhancer of Zeste homolog, histone methyl transferase Upregulation.	Linked to high-grade PCa development	Prognostic
GSTP-1	Glutathione-S-transferase P1 protects DNA from free radicals (caretaker gene). Loss of gene expression due to hypermethylation associated with PCa	GSTP-1 hypermethylation: > 90% PCa, > 70% PIN, rare in benign disease. Urinary marker, may need prostatic massage. Small study. Diagnostic performance improved as a part of multiple gene panel	Diagnostic
IL-6	Interleukin-6: cytokine immunomodulator. Linked to AR cells and suppression of androgen-dependent cells	Elevated levels related to advanced disease but not prognostic. Possible role as combination markers	Prognostic
KLK3/PSA	Encodes PSA, a kallikrein-related peptidase (serine protease subgroup) on chromosome	Combination rV-PSA and fowl pox primed rF-PSA vaccines trialled. Phase II studies: median time to PSA progression increased (9.2–18.2 months) (study ECOG7897). Phase III PARADIGM trial underway	Prognostic and therapeutic
KLK2/hK2	Encodes hK2, a kalikrein-related peptidase (serine protease subgroup) on chromosome 19. Serum levels 1% of PSA and undetectable in healthy males	hK2 with free and total PSA significantly improved diagnostic sensitivity/specificity. Predicts prostate biopsy outcome ($P < 0.001$). Role in predicting recurrence/progression	Diagnostic and prognostic
MIB-1	Mindbomb homolog 1, monoclonal antibody and cell proliferation marker by Ki-67 antigen recognition	Close univarate T, M stage and survival significance	Prognostic

Table 1.12 is by no means exhaustive; many other molecular markers such as CD44, MCM proteins, FASN, Aurora kinases A, B and C {Scharer CD *et al*, 2008; Freeman A *et al* 1999}; p16 ^{INK4A,} c-Myc, VEGF, VEGF receptors, EGFR family {Quinn *et al*, 2005} and those mentioned in Table 1.11 {Isaac, 1997} etc have been reported in PCa. Some of these markers have not been tested in larger clinical studies.

1.5 Clinical Management of Prostate Cancer

1.5.1 Primary Treatment modalities

There are several options for primary treatment of prostate cancer. The EAC guideline for the primary treatment of PCa contains further details {Heidenreich *et al*, 2008}. The factors to be considered in choosing treatment options include: stage of PCa, patient's age, life expectancy and choice, Gleason score and cost of treatment. The treatment options include:

a. Watchful Waiting: This is also known as deferred treatment. It is a treatment strategy that includes an active standpoint to postpone treatment until it is required. In some cases there are proposals to encourage patients to participate in dietary and life style treatments. This is somewhat different from active surveillance which requires re-biospy of patients after 18 months period {Heidenreich *et al*, 2008}. This treatment option is suitable for low grade PCa (well differentiated) PCa in patients who have longer life expectancy and low risk of disease progression. Some studies have shown that watchful waiting has a similar outcome in PCa of men aged < 65yr and with Gleason score < 7 compared to surgical treatment in 12 years follow-up period {Albertsen , 2009}.

b. Surgery: This involves radical prostatectomy (RP) for clinical PCa that is still organ-confined and localised. Nerve-sparing RP is the approach of choice for all men with normal erectile function and organ.

c. Radiotherapy: This uses high energy rays (external beam) to destroy prostate cancer cells. At least 72 Gy is recommended for management of low risk PCa; dose escalation up to 81Gy may be used in intermediate and high risk PCa.

d. Brachytherapy: This treatment involves implantation of radioactive 'seeds' (iodine-125 or palladium-103) directly into tumour site to produce sustained-release of high energy for inactivation of cancer cells {Kirby & Xia, 2009}. Transperineal low-dose Brachytherapy is a safe and efficient technique that can be applied to patients with the following criteria: stage cT1b-T2a No Mo, a Gleason score ≤ 6 , initial PSA level ≤

10ng/ml, ≤ 50% of biopsy cores involved with cancer, and a prostate volume of < 50cm^3 and a good international Prostatic Symptom Score (IPSS) {Ash *et al*, 2000}.

e. Cryosurgical ablation: This involves the use of TRUS-guided transperineal freezing of the prostate using gas-based cryo-technology (cryo-probes), first applied in 1964 by Gonder et al {Langenhuijsen JF, 2008}. Patients ideally suitable for cryosurgical ablation of the prostate are those with organ-confined PCa, prostate size of ≤ 40ml, PSA serum level < 20ng/ml, and a biopsy Gleason score < 7 { Heidenreich *et al*, 2008}.

f. High- intensity focused ultrasound (HIFU): The HIFU treatment uses intersecting, precision focused ultrasound waves to raise the temperature of the prostate tissue to 80-90^{o}C in two to three seconds, effectively destroying the targeted cancerous tissues {Clement, 2004}. This does not involve incision (minimally invasive). It was approved by the National institute for clinical excellence (NICE) UK in 2005 for treatment of organ-confined PCa for research purposes only, and has achieved good biochemical outcomes, safety and efficacy in short-term evaluations{Ahmed *et al*, 2009}

In general all primary treatment modalities have various shades of side-effects including urinary incontinence, impotence, urethral sloughing, urinary tract infection and rectourethral fistulas.

1.5.2 Adjuvant and Salvage Treatments:

Hormone therapy (HT) is the main adjuvant or neo-adjuvant treatment in PCa management. The luteinizing hormone-releasing hormone (LHRH) agonists have become the standard of care in hormonal therapy because they avoid the physical and psychological discomfort associated with orchiectomy and the lack of potential cardiotoxicity associated with DES {Heidenreich *et al*, 2008}.Androgen ablation is also achieved by administration of steroidal or non-steroidal anti-androgens (for example Bicalutamide 150mg per day). Combined androgen blockade is a common practice compared to monotherapy. Intermittent androgen blockade has also been shown to preserve quality of life and reduce treatment cost compared to continuous ADT {Heidenreich *et al*, 2008}. Indications for HT include both symptomatic and asymptomatic M1, N+ and locally advanced Mo stages. However, the most appropriate time to introduce HT in patients with advanced PCa is still controversial due to lack of controlled randomized studies {Moul *et al*, 2004}. Radiotherapy and Brachytherapy can also be used as adjuvant therapies.

Follow-up treatment with curative intent (Salvage therapy ST) varies in PCa management:

a. In asymptomatic patients, a disease-specific history, PSA and DRE are the tests for routine follow up. The EAC guideline recommends these tests be performed at 3, 6 and 12 mo after treatment, then every 6 mo until 3 yr, and then annually.

b. Biochemical recurrence (BR): This broadly described as increase in serum PSA level after primary treatment. This could result from treatment failure (relapse) and or residual disease local or distant. The exact definition of BR varies depending on the primary treatment modality {Roberts & Han, 2009}. Secondly, the clinical significance of BR sometimes referred to as 'PSA only recurrence' to survival is controversial in the literature. A common consensus for BR definition after radical prostatectomy (RP) treatment is a serum PSA level equal or greater than 0.4 ng/mL {Roberts & Han, 2009}. However, the EAC guideline recommends a serum PSA level of equal or greater than 0.2ng/ml after RP as a BR.

For radiotherapy (RT) as a primary treatment, the definition of BR remains hugely controversial. In 1997, the American Society for Therapeutic Radiology and Oncology (ASTRO) convened and defined BR as 3 consecutive rises in the PSA with the date of failure calculated as the midpoint between the last non-rising PSA and the first of the series defining failure. In 2005 the Radiation Therapy Oncology Group (RTOG) in concert with ASTRO had a consensus definition of BR as a serum PSA rise of 2ng/ml above the nadir with the date of failure determined 'at call'. This is also known as the Phoenix criteria, because the meeting was held in Phoenix Arizona {Roberts & Han, 2009}. The ASTRO criteria were then reserved for RT delivered in the absence of hormonal therapy (HT). The EAC guideline defines BR after radiation therapy as 'a rising PSA level 2.0ng/ml above the nadir value, rather than a specific threshold value, is the most reliable sign of persistent or recurrent disease' {Heidenreich *et al*, 2008}. Several studies have evaluated these definitions {Thames *et al*, 2003}. However, the importance of BR is as a predictor of metastatic progression (MP) and prostate cancer –specific mortality (PCSM). It is reported that BR precedes MP and PCSM by a median of 8 years and 13 years, respectively following RP {Roberts & Han, 2009}. Therefore BR and other prognostic factors such as PSA kinetics (PSA doubling time and PSA velocity), Gleason score, pathologic stage and time interval between primary treatment and BR, are the key determinants of Salvage therapy options and survival outcomes {Roberts & Han, 2009, Heidenreich *et al*, 2008}.

c. Salvage therapy options: Timely initiation of salvage therapy may help to prevent further clinical progression and PCSM. Treatment options include radiation therapy, cryosurgical ablation, hormonal therapy, cytotoxic therapy and palliative management. Factors such as local or distant recurrence/ metastasis, hormone refractory status and side effects have to be considered. In patients with metastatic hormone refractory prostate cancer (HRPC), Docetaxel at 75mg/m^2 every 3wk results in a significant survival benefit and represents the reference treatment {Heidenreich et *al*, 2008}. In patients with symptomatic bone osseous metastasis due HRPC, Docetaxel with prednisone is the treatment option of choice. Newer treatment options such monoclonal antibody therapies may improve survival outcomes in HRPC cases. Zoledronate may be offered to patients with skeletal metastasis to prevent osseous complications.

d. Hormone-refractory prostate cancer (HRPC): The EAC criteria for defining HRPC include: i) serum castration levels of testosterone, ii) three consecutive rises of PSA 2 wk apart resulting in two 50% increases over nadir, iii) Anti-androgen withdrawal for at least 4 wk, v) PSA progression despite secondary hormonal manipulations and vi) Progression of osseous or soft tissue lesions. Common complications due to skeletal metastases include bone pain, vertebral collapse or deformity pathological fractures and spinal cord compression.

1.5.3 Advanced Therapy for PCa Management:

The term advanced therapy (AT) refers to the triad of gene therapy (GT), cellular therapy (CT) – stem and somatic cells and tissue engineered bio-products {Schneider, 2009}. AT is still at the infantile stage (not yet clinically used) but remarkable progress has been made.

a) Gene therapy: Several strategies for cancer gene therapy have been reported (Table 1.13). As at July, 2007 there were over 1340 gene therapy trials (including completed, ongoing or approved trials) world wide {Edelstein *et al*, 2007}. As at March 2009, there were 993 gene therapy protocols for cancer treatment http://www.wiley.co.uk/genmed/clinical/; although prostate cancer featured rarely. However, antisense oligonucleotides (AS) have been used to block Bcl-2 expression, inducing apoptosis in androgen-resistant prostate cancer cell lines. Blc-2 is anti-apoptotic, and its over-expression is associated with PCa aggressiveness. Mu *et al*, 2005 also reported that LNCaP and PC-3 prostate cell lines treated with AS were more sensitive to radiation therapy and showed increased p53 and p21 expressions.

Key challenges to cancer gene therapy include limitations in efficiency and targeting of gene-transfer vectors, incomplete understanding of control of gene transcription, long- term consequences of constitutive expression of a transferred gene , inflammatory responses and viral-vector tumourigenesis {Edelstein *et al*, 2007}.

Table 1.13 Strategies for cancer gene therapy. {Sikora & Pandha, 1997}.

Gene marking in BMT and to detect minimal residual disease.
Genetic immuno-modulation Cancer vaccines Polynucleotide immunization
Vectoring of bio-therapeutic genes to tumours
Increasing normal tissue tolerance
Selective drug activation
Somatic correction of genetic defect Antisense to mutant oncongene. Expression of tumour suppressor gene
RNA interference

b) Cellular therapy for PCa has been attempted in fewer preclinical studies, for example cellular vaccines expressing GM-CSF {McNeel, 2007}.

c) Tissue engineered bio-products: Preliminary results of Prostvac-VF, a vector based vaccine (recombinant vaccinia vector) targeting PSA in prostate cancer have shown negligible toxicity, and phase II trials have suggested a survival benefit after treatment of indolent prostate cancer cases and also possible combination with other treatment modalities {Madan, *et al*, 2009}. Tumour –associated antigens (TAA) have become targets for vector-engineered bio-products designed for cancer therapy, for example prostate stem-cell antigen, prostate-specific membrane antigen, MUC1, epidermal growth factor receptor, platelet-derived growth factor and its receptor, urokinase plasminogen activator and its receptor, and extracellular matrix metalloproteinase inducer are TAAs currently targeted for PCa treatment { Li Young *et al*, 2009}. Clinical trials with immune active agents (immunotherapy) reported in the past years have demonstrated efficacy in the treatment of PCa. These agents include immune modulators such as GM-CSF and anti-cytotoxic T-lymphocyte-associated antigen 4 monoclonal antibodies, antibody therapies targeting PSMA, and vaccines such as those targeting PAP, PSA, and cellular vaccines expressing GM-CSF { McNeel, 2007}.

The main regulatory challenge to AT development is the need to demonstrate that an advanced therapy is high quality, safe and efficacious, using methods that are applicable for each specific product {Schneider, 2009}. There are also challenges of clinical trial design models since most ATs are for disease conditions with fewer alternative therapies (they will lack effective comparators).

1.6 Trends in Prostate Cancer Research

1.6.1 Overview of Progress in PCa Research

There has been a remarkable advancement in prostate cancer research, especially in the PSA era (post 1980s). PSA measurement contributed tremendously to the increase in research activities in PCa. The key milestones include:

a) **The establishment of several prostate cell lines and xenografts** (this is discussed in Section 1.6.2). This enabled a wide range of *in vitro* and *ex vivo* mechanistic studies

b) **Advancement in molecular genomics**: Molecular tools such as array CGH interphase FISH, LOH analysis, PCR, DNA microarrays, bioinformatics and SNP arrays contributed immensely to prostate cancer research. For example the discovery of the TMPRSS: ETS gene fusion, a hallmark molecular event in PCa and premalignant lesions (PIN) by Tomlins *et al* in 2005 was largely due to insights from bioinformatics and the application of interphase FISH, Karyotyping and PCR {Cross et al, 2008}. The discovery of the PCA3 non-coding RNA by Bussemakers and colleagues in 1999 was another remarkable feat in PCa research, this time due to the application of Northern blotting and Phage Display techniques and RQ- PCR. The field of regulatory RNAs is burgeoning and may facilitate understanding of prostate tumourigenesis.

c) **The Human Genome project and the HapMap project**: These were large scale events that drove all aspects of Genomics and genetics of all diseases. Launched in 1990, the human genome project (HGP) was 'completed' in 2003 with > 95% of the human genome sequenced {Russell, 2007}. It created the physical map of the 24 human chromosomes (22 autosomes, X and Y), identified the entire set of genes and mapped them all to their chromosomes; determined the nucleotide sequence of the estimated 3 billion base pairs and analyzed genetic variations among humans http://www.ornl.gov/hgmis/project/timeline.html (assessed on July 2008). Started in

2002, the HapMap project was an international effort to 'identify and catalogue genetic similarities and differences in human beings' http://www.hapmap.org/thehapmap.html.en (assessed on June, 2009). It has been able to identify about 10 million SNPs in humans, which provides an invaluable platform for clinical evaluation of genetic variations in cancer risk. Currently, there is no clinical genetic test for predicting prostate cancer risk but many candidate SNPs are evaluated.

d) **Advancement in clinical management**: The introduction of robotic surgery to improve nerve-sparing in radical prostatectomy, advent of high intensity focussed ultrasound therapy and cyro-ablation gas-technology based on helium and argon gases have all improved the treatment of PCa and placed more demand on molecular characterization of the disease {Kirby & Xia , 2009}. Although more options are now available for treatment, the occurrence of HRPC remains the biggest challenge. More controlled trials are now conducted to evaluate various aspects of the disease management. There is optimistic prospect for tumour vaccine, gene therapy and targeted-therapy for PCa in the near future.

e) **Mass screening programmes**: Although still hugely debated in terms of cost-effectiveness, annual mass screenings of men in many countries have increased the public awareness for prostate cancer and improve early detection rate. Institutional funding for prostate cancer research has increased in the recent past {Kirby & Xia, 2009}. However, the reports of the long-awaited ERSPC (European Randomized Study of Screening for Prostate Cancer) and the PLCO (Prostate, Lung, Colorectal, and Ovarian (PLCO)) clinical trials have not helped matters. The ERSPC trial showed a 20% reduction in prostate cancer specific mortality (PCSM) as a result of early detection by mass screening. However, the PLCO trial showed no significant reduction in PCSM as a result of early detection by mass screening (Andriole *et al*, 2009).

f) **Novel technologies**: Biomarker discovery and validation using multiplex array systems such as surface-enhanced laser desorption/ionization (SELDI) technology and MALDI-TOF mass spectrometry and LC/MRM-MS are widening the frontiers of proteomic analysis for cancer research in general {Dutton G, 2009}. For example there is the development of Prostaplex, a new multiplexed real time immuno PCR (MRI-PCR) for 10 prostate cancer biomarkers (low abundance proteins associated with PCa development and progression {Bradford, 2006; Wright SC, 2008}; the Prostaplex TM project is sponsored by the NIH (www.novoseek.com). Such technologies will improve diagnostic reliability, reduce cases of negative biopsies and

ensure better monitoring of treatment. RQ-PCR can also used for gene expression and gene variation in the clinical laboratories. Samples such as urine, prostate biopsy and blood are evaluated in some of these technologies.

An emerging area of diagnostics for PCa is biosensing using techniques such as electrochemical, optical and mass –sensitive detection techniques to develop point-of-care testing devices {Tothill, 2009} . Such techniques could detect proteins and nucleic acids in treated samples. There is also possibility of multiplex assay formats. However, factors such as analytical reliability, reproducibility, cost, throughput and pre-analytical variables such sample treatment have to be considered.

Another area of interest in PCa diagnostics is analysis of volatiles in urine samples. The organic volatile constituents of biological fluids contain clinically useful diagnostic information for the recognition of metabolic disorders in man {Zlatkis *et al,* 1981}. For example, an investigation on lung cancer blood using SPME and GC-MS techniques, showed a much higher concentration of two aldehydes: hexanal and heptanal in lung cancer blood compared to control blood {Deng *et al*, 2004}. By comparison of these volatiles in breath and in blood, it was demonstrated that hexanal and heptanal in breath were originated from blood and screening of lung cancer by breath analysis might be feasible. The two aldehydes are regarded as biomarkers of lung cancer. By using SIFT-MS technique, Spanel *et al* 1999, reported a significant elevation of formaldehyde in the headspace of urine from bladder and prostate cancer patients of unknown clinical history. The compound acrolein (2-propenal) was measured in breast cancer cells *in vitro* using chemical ionization mass spectrometric technique; acrolein was considered a maker for sensitivity of breast cells to doxorubicin drug {Kato *et al*, 2002}.

1.6.2 Prostate cell lines for Research

There are over 200 prostate cell lines and sublines for PCa research {Sobel & Sadar 2005}. These are derived from different sources including murine tumours, prostate adenocarcinoma; bone, liver, lymph node, vertebral and brain metastases; insertion of transgenes, viral immortalization and xenografts. Most of these prostate cell lines have been characterized based on the expression of PSA, AR, cytokeratins and other molecular makers of PCa and the prostatic lineage of a given line. There is a freely accessible on-line database of prostate cell lines for research: http://www.CaPCellLines.com. Prostate cell lines mimic various aspects of human PCa and have proved useful in advancing prostate cancer research. However, it has been reported that most widely used prostate cell lines share common origins {van Bokhoven et *al*, 2001}. They share a high number of structural chromosomal abnormalities and nearly

identical DNA profiles. For example, ALVA-31, ALVA-41, PC-3, PC-3MM2, and PPC-1 all have a deletion of a C in codon 138 of the p53 gene and show almost identical DNA profiles. A study using Cytogenetics, mutational analysis and DNA profiling has shown that some of these cell lines could also be derivatives of one another or possibly contaminated by other cell lines {van Bokhoven *et al*, 2001}. This prompts necessity for proof of cellular origin of prostate cell lines used in research and for re-evaluation of results from cell line models.

1.7 Aims and Objectives of this project:

1.7.1 Aims of the Project: The overall aim of this research was to examine the link between development and progression of prostate cancer and production of diagnostic/prognostic molecular markers by manipulation of prostate cell lines in cultures supplemented with steroid hormones. The results from *in vitro* experiments were eventually compared with those of prostate tissues obtained from paraffin embedded sections, exfoliated urine cells and blood samples, all from prostate disease patients. There were five main research questions to address the overall aim of the project:

a) How does the gene expression of steroid hormone receptors (SHRs) change in prostate cell lines *in vitro* in response to hormone treatment?
b) How does the gene expression of putative molecular markers of prostate cancer progression change in prostate cell lines in vitro in response to hormonal modulation?
c) Can genetic variants of KLK2 gene predict prostate cancer risk (presence of prostate cancer at biopsy)?
d) Does gene expression of selected molecular markers correlate with clinico-pathological variables in prostate cancer patients?
e) Can organic volatiles in urine samples of prostate disease patients indicate prostate cancer?

1.7.2. Objectives of this project: To achieve the overall aim, specific objectives were designed as follows:

i. To evaluate gene expression of steroid hormone receptors(ER-α, ER-β and AR) in prostate cell lines stimulated with steroid hormones compared to untreated controls. Real time PCR (RQ-PCR), Immunocytochemistry (ICC) and western blotting (W/B) techniques were used.

ii. To determine diagnostic/prognostic value of SHRs expression using archived prostate sections and exfoliated urine cells of prostate cancer patients. RQ-PCR, IHC and W/B were also used

iii. To measure the gene expression of prostate specific kallikreins (hK2 and hK3) in prostate cell lines modulated with steroid hormones, and in clinical samples. ELISA, RQ-PCR, IHC and ICC techniques were used.

iv. To examine gene expression of molecular markers associated with apoptosis (p53 and Bcl-2) and cell proliferation (MCM2 and MCM5, Aurora kinases A, B and C) in hormonally treated prostate cell lines and in clinical samples using RQ-PCR, IHC and ICC.

v. To evaluate gene expression of putative prognostic molecular markers: TMPRSS2 gene fusions, and FASN correlation with clinical stage and histological grades. The RQ-PCR was used.

vi. To measure the gene expression of molecular markers associated with prostate cancer metastasis (CD44 and E-Cadherin) in treated cell lines and clinical samples using Immunoflourescence and RQ-PCR.

vii. To assess the frequency of genetic variants (SNPs) of KLK2 gene in predicting presence of prostate cancer at biopsy in both prospective patients and archived tissue materials. DNA direct sequencing, allelic discrimination assay and Pyrosequencing techniques were used

viii) To explore the expression of volatiles in urine samples of prostate disease patients using thermal desorption GC-MS technique.

ix) Extensive literature review of molecular markers associated with prostate cancer development and progression was performed.

1.7.3 Study Hypotheses

Key hypotheses in the study are as follows:

a. Differential gene expressions of SHRs are associated with prostate cancer outcome. Disease outcome is measured by known clinical variables such as clinical stage and histological grade. The null hypothesis is that there is no significant difference in gene expression of SHRs in various clinical stages and histological grades of prostate cancer at a P value of 0.05.

b. Frequency of SNPs in KLK2 gene predicts presence of prostate cancer at biopsy. The null hypothesis is that the occurrence of any SNPs is not significantly associated with presence of PCa at biopsy.

c. Molecular markers associated with cell proliferation (MCM2, MCM5 and aurora kinases A, B and C) are significantly over-expressed in high histological grades of prostate cancer, predicting worse outcome. Disease outcome is measured by known clinical variables such as histological grade and clinical stage. The null hypothesis is that there is no significant difference in the gene expression of the molecular markers in various histological grades and clinical stages at P value of 0.05.
d. Molecular markers associated with prostate cancer metastasis (CD44 and E-Cadherin) are significantly over-expressed in high histological grades compared to low histological grades. The null hypothesis is that there is no significant difference in the gene expression of these markers in various histological grades.
e. Gene expressions of TMPRSS2 fusion and FASN significantly correlate with prostate cancer progression (measured by increase in histological grade).
f. Gene expressions of TP53 and Bcl-2 are differentially expressed in prostate diseases.

Chapter 2: Molecular Markers of Prostate Cancer

2.1 Molecular markers of Signal Transduction in Prostate Cancer

2.1.1 Androgen receptor

Androgen receptor is the key regulator of prostatic tissue functions. Located on the long arm of X chromosome (Xq12), the 180kb gene has 8 exons (for the longest variant), 4314 bp mRNA and 2,762bp open reading frame (OFR) (Figure 2.1) and codes for a 99 kDa protein of 920 amino acid residues (longest isoform), which is found in both cytoplasm and nucleus of expressing cells {D'Antonio, 2008}. In the physical map of the human genome, the AR is localized on position 66680599 and ends at 66860844 bp from pter (based on HG 18, March 2006). In the RefSeq database (the NCBI database) two main transcripts are curated: i) NM000044.2 is for longest transcript variant of 4314bp, 8 exons encoding 920 amino acid residues (isoform 1) and ii) NM001011645.2 is a shorter transcript variant of also 8 exons but 1765bp transcript length encoding 338 amino acid residues (isoform 2). This variant is also known as the AR45 variant. The difference between both variants is in the length of the 5' UTR region and the coding region (CDS) particularly the transactivation domain. For the longest variant, the 5' UTR is 1.1 kb and the 3' UTR is 6.8 kb. Several novel transcript variants of AR have been reported. The Ensembl genome browser describes an AR variant (AR-202) of 2 exons, 204bp length encoding 68 amino acid residues http://www.ensembl.org/ ; the variant has a shorter N-terminal transactivation domain (NTTD) and DNA binding domain (DBD) without both the hinge region (HR) and the ligand binding domain (LDB). The longest transcript variant encodes a protein with all the four domains: NTTD, DBD, HR and LDB (Figure 2.1). Most of the novel variants differ by the absence of the DBD and/ or the length of the NTTD. For example Hu et al, (2009) reported 7 novel transcript variants of AR.

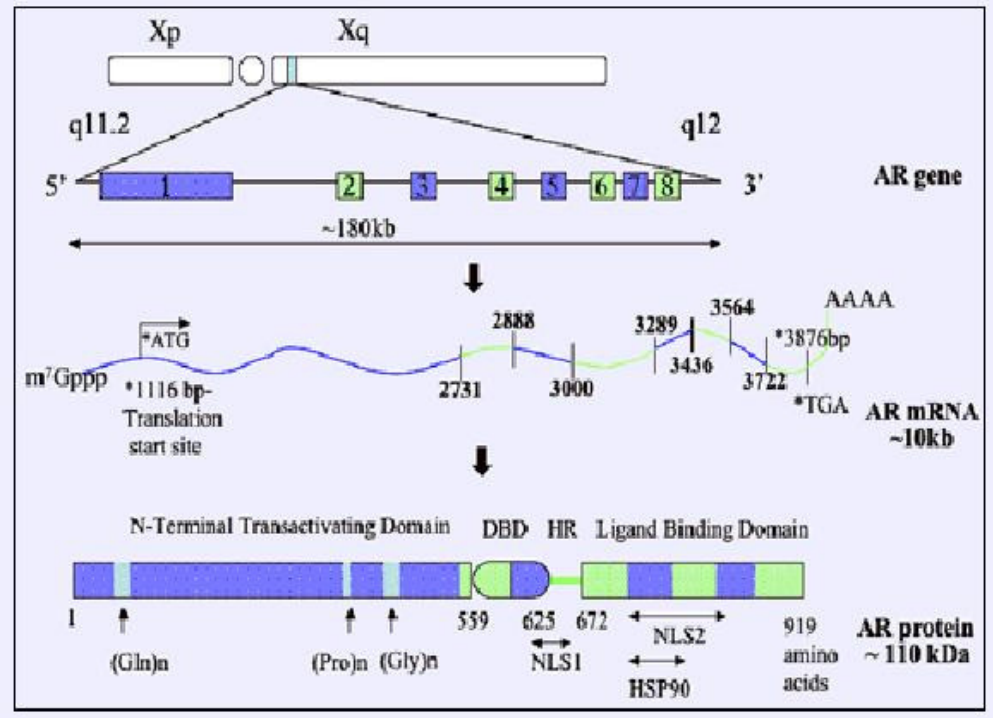


Figure 2.1 Structure of Androgen Receptor gene. The AR protein has four domains: N-terminal transactivation domain NTTD, DNA binding domain DBD, Hinge region HR and Ligand binding domain LBD. Many novel variants of AR lack the LBD. {Litvinov *et al*, 2003}.

associated with HRPC (hormone refractory status) Figure 2.2.They all lack the LBD due to splicing at the cryptic exon (CE) points within intronic sequences, resulting in the loss of exons 4-8 which encode the LBD. Ligands (androgen or anti-androgens) bind to the LBD, with the ligand-bound receptor moving to the nucleus to cause transcriptional regulation of androgen- responsive genes: KLK2, KLK3, TMPRSS2 etc. Hu and his colleagues (2009) showed that AR-V1 and AR-V7 were particularly associated with biochemical recurrence (BR) and HRPC status. The protein products of these novel AR species are truncated.

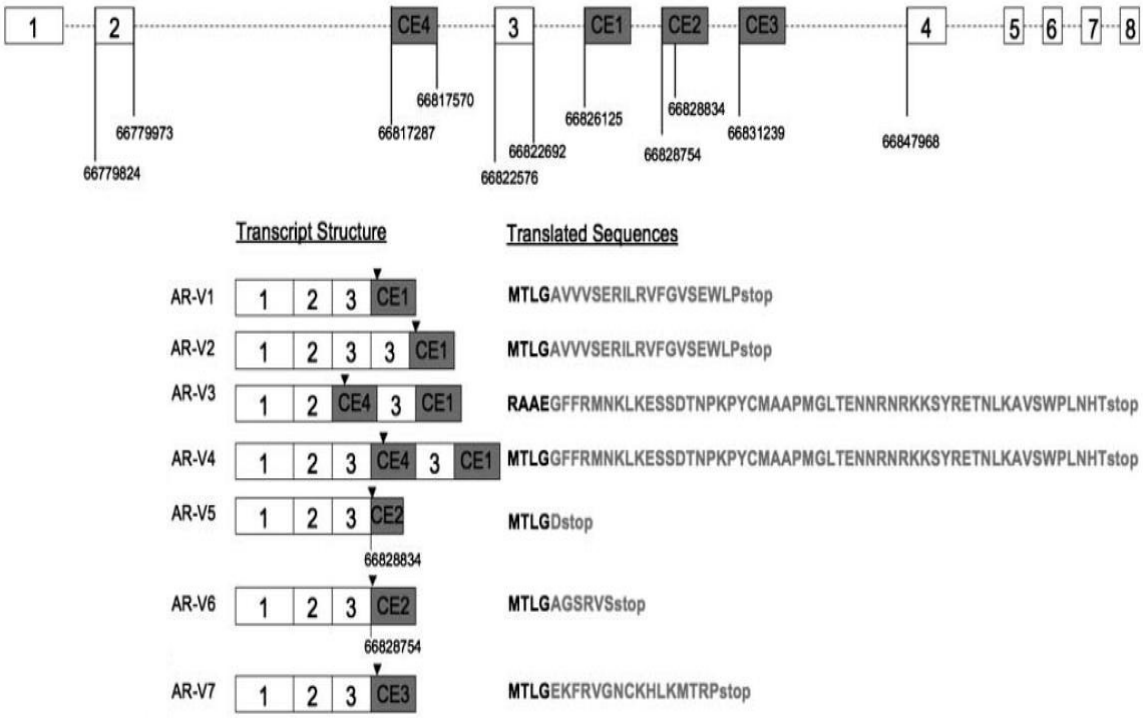


Figure 2.2 Gene structure of novel variants of AR. CE stands for cryptic exons, that is, exons within intronic sequences. Exons 4-8 that encode the LBD are spliced off due to splicing at CE points

Functionally, the AR is a nuclear transcriptional factor (mechanism of action was discussed in Section 1.2.4). As a result of its structure, certain ligands can activate it, especially in the absence of androgen or the LBD via the NTTD. For example, the neuropeptide, gastrin-releasing peptide (GRP) which is also implicated in HRPC, is reported to aberrantly stimulate AR via the NTTD {Yang *et al*, 2009}. Another important structural feature of AR is the poly-amino acid repeats (microsatellite polymorphism) in the NTTD (encoded by exon 1 and constitutes 60% of the protein); three different repeats are documented: i) the poly glutamine (CAG: Glu-Q), which is usually an average of 22 repeats (range 8 to 32); shorter (<18) length associated with increased AR transactivation and prostate cancer risk {D'Antonio, 2008}, and also associated with worse outcome of PCa {Shimbo *et al*, 2005} . CAG repeats in spinal and bulbar muscular atrophy patients range from 38 to 62 {D'Antonio, 2008}; ii) poly Proline (Pro-P) has an average of 8 (not yet associated with any disease outcome); and iii) Poly Glycine (GGC: Glycine-G), which has an average of 23 (range 10 to 31). The AR is also known to have several single nucleotide polymorphisms (SNPs) and mutations; there are over 605 reported mutations/SNPs of AR http://androgendb.mcgill.ca/map.gif. Inherited loss of function mutations of AR result in non-lethal loss of AR expression, a hallmark of androgen insensitivity syndrome (AIS), which leads to phenotypically female characteristics (Y

chromosome, functional testes but no male accessory organs) {D'Antonio, 2008}. The overall somatic mutation of AR in early PCa is estimated at < 10%.

AR plays several key roles as a transcription factor of targets such as KLK2, KLK3, TMPRSS2, PSCA, p27,VEGF and PSMA genes; involve in cell cycle regulation, intracellular signal transduction and cell – to- cell signalling.

AR expression is regulated by the stromal-epithelial signalling through paracrine actions of growth factors {Quinn, 2005}. The two prostatic cell types (stromal and epithelial) express AR but the epithelial cells express more AR than the stromal. Aberrant stromal-epithelial signalling (resulting in over-expressed AR in the epithelia and reduced AR in stroma) has been reported in many PCa {Hensall *et al*, 2001}. Over-expression of AR in the epithelia therefore leads to abnormal epithelial cell proliferation. However, the basal cells of the prostatic epithelium (believed to harbour 'prostate stem cells') do not express AR {Agrawal & Dunsmuir, 2009}. Another study also reported that AR activation in the luminal cells of the epithelium even suppressed prostatic growth {Dunsmuir, *et al*, 2000}. It is also known that androgens can stimulate epithelial cells to produce growth factors which act in both intracrine and autocrine fashions to increase AR expression. Although AR mediates the actions of androgens and anti-androgens, it is not clear as to what role does it play in the initiation of prostate malignancy. The general consensus is that increased AR expression in the epithelial cells leads to increased (abnormal) epithelial cell proliferation, a key feature of prostate malignancy.

In addition, the over-expression of AR could result in part 'from lack of response to homeostatic degradation mechanisms in cells with a mutated or amplified AR gene, while adjacent stromal cells have normal AR which is down-regulated' {Quinn et al, 2005}. This proposition brings another complexity to AR signalling. Early studies on AR mutations were very conflicting {Wallen, et al 1999}. Most studies reported AR mutations in both normal and malignant prostatic tissues, and therefore concluded that it was not important for the initiation of malignancy. The discrepancy in most of the studies was that different exons were interrogated for mutations in different studies. In some cases the studies were concerned with AR mutations that occur during HRPC rather than at the beginning of PCa. Some other studies evaluated the correlation between AR mutation and AR over-expression. It is now known that the overall AR mutation in early PCa is less than 10% {D'Antonio, 2008}, and therefore cannot account for the initiation of malignancy in most of the cases. The second variable is the AR gene amplification (more than 3-5 copies of AR in a cell). Gene amplification results in gene over-expression. AR amplification is poorly documented in early

onset of PCa rather there are reports showing that AR is amplified in more than 30% of PCa recurrent after androgen ablation {Visakorpi et al, 1995}. The overall picture is that AR signalling in early PCa is poorly understood. However, its prognostic significance in PCa progression is better understood and well documented. For example the phrase 'AR over-expression is a feature of progression, recurrence, lymph node metastases and / or androgen resistance in human prostate cancer' {Quinn et al, 2005} is a typical line of investigation in PCa literature, much more than early (onset) events. It is possible that in some PCa cases, AR may be amplified, over-expressed through epigenetic regulation and /or contain mutations that allow stimulation by a range of hormones and anti-androgens. The same mechanisms may also be applicable to AR signalling in PCa progression toward locally advanced or metastatic disease.

Another epigenetic event is the hypermethylation of AR in about 40% of PCa cases {Reibenwein *et al*, 2007}; generally there is global DNA hypomethylation and locus-specific hypermethylation in PCa. One of the consistently hypermethylated gene in PCa is the GSTP1 (in 75-95%) of PCa cases {Schulza & Hoffmannb, 2009}. The hypermethylated GSTP1 is a serum marker of PCa, also detectable in premalignant lesions such as high grade PIN but non-detectable in healthy subjects {Reibenwein *et al*, 2007}. But in the case of AR, hypermethylation was also found in the serum of healthy controls. Further more, hypermethylation of AR promoter region is believed to down-regulate AR expression in PCa progression {Reibenwein *et al*, 2007} , an observation that contradicts the recent finding of AR over-expression in advanced PCa {Hu *et al*, 2009}. It is also known that hypermethylation is associated with ageing process {Schulza & Hoffmannb, 2009}.There might be other events causing AR hypermethylation rather than prostate cancer development and progression. However epigenetic alterations may play crucial roles in many adaptative changes that accompany PCa progression; and therefore would become highly useful for diagnostics and therapy of prostate cancer.

Another important aspect of PCa progression is the involvement of AR in the development of hormone refractory status in prostate cancer. HRPC can occur in both locally advanced and metastatic PCa and it is very lethal. The overall theme is that the abnormality of AR signalling: over-expression, mutation, gene amplification, hypermethylation and/ or novel variants predict the occurrence of HRPC Hu *et al*, 2009; Schulza & Hoffmannb, 2009}. And that therapy (especially hormone therapy) could also select clones of AR expressed in HRPC status. Several mechanisms may be involved simultaneously; however, AR abnormality is certainly a consistent feature of PCa progression. The expression of steroid hormone

receptors (SHR) in general is prognostic in a number of endocrine tumours; for example in breast cancers where oestrogen receptor (ESR) positive cases predict better outcome and loss of ESR expression predicts a more aggressive disease and resistance to hormone therapy such as tamoxifen {Marchio & Reis-Filho, 2008). Some studies have demonstrated that ESR positive breast cancers were also significantly AR positive and that most progesterone receptor (PR) negative breast cancers were also AR negative {Isola, 1993}. And the prostate and breast tissues are in many aspects very similar {Leblond, 1963}.

2.1.2 Oestrogen receptors (ESR)

There are two types of ESR: ESR alpha (ESRα, officially known as ESR1) and ESR beta (ESRβ, officially known as ESR2). They are located on different chromosomes, differentially expressed in many prostatic cell types and are believed to perform different roles {Murphy *et al*, 2006}.

The ESR1 is localised on chromosome 6q24-q27 and has several transcript variants due to alternative splicing (http://www.ncbi.nlm.nih.gov/). The transcript variants differ in their 5 UTRs and use different promoters. The Ensemble Genome browser (http://www.ensembl.org) describes 14 transcript variants of ESR1, having 2 to 10 exons, 731 to 6,466 bp transcript length encoding 115 to 595 amino acid residues. Some of the transcript variants encode the same protein. The longest variant encodes a 66 kDa protein (ESR66), some variants encode a truncated form (lacking N-terminal transactivation domain) and has a molecular mass of 46 kDa (ESR46). TheSwiss-Prot http://www.uniprot.org/) has a detailed analysis of the protein domains and amino acid modifications of the ESR1. The ESR1 (ESRα) gene was first cloned in 1986 {Zhao *et al*, 2008}. The ESR1 has a wide tissue distribution and are involved in pathological processes including breast cancer, endometrial cancer, and osteoporosis {Marchio & Reis-Filho, 2008}.

The ESR2 is localised on chromosome 14q23.2, contains 8 exons and an opening reading frame of 1,593 bp (Figure 2.3) {Zhao et al, 2008}. The ESR2 was first isolated in rat in 1996, and also confirmed in humans in the same year {Kuiper *et al*, 1996}. It has two main curated isoforms: ESR beta1 (ESRβ1) and ESR beta 2 (ESRβ2) by alternative splicing. The ESRβ1 protein is 59.2 kDa and contains 530 amino acid residues while the ESRβ2 is 55.5kDa and contains 495 amino acid residues {Zhao *et al*, 2008; http://www.uniprot.org/ } . The ESRβ1 sequence is the canonical sequence. The ESRβ2 protein has a unique C-terminus, where the amino acids corresponding to exon 8 are replaced with 26 unique amino acids. Six more isoforms of ESRβ have also been described at protein level {http://www.uniprot.org/}. The

Ensemble genome browser described 6 transcript variants (mRNA) of ESRβ, with 6 to 9 exons, encoding 323 to 530aa residues. The ESRβ is expressed in many tissues: the ovary, the prostate, testis, bone marrow etc, and it is localised in the nucleus.

Both forms of oestrogen receptors (ESR) share a high degree of homology in their DNA-binding domains, a conservation of key amino acids within their ligand-binding domains and can form heterodimers with each other {Scobie *et al*, 2002}. Cellular signalling of oestrogens is mediated through the two receptors (alpha and beta types). Both receptors are nuclear transcription factors. They are essential for sexual development and reproductive functions, but also play a key role in other tissues such as bone {Dahlman-Wright *et al*, 2006}. Both are known to exhibit gene amplification (copy number variation) {Marchio & Reis-Filho, 2008}. However, there exact functions and distribution in distinct cell types vary; especially the function of ESRβ and its expression pattern are controversial. They are also known to regulate different sets of genes {Bardin *et al*, 2004}. They exhibit different affinities for some natural compounds {Zhao *et al*, 2008}.

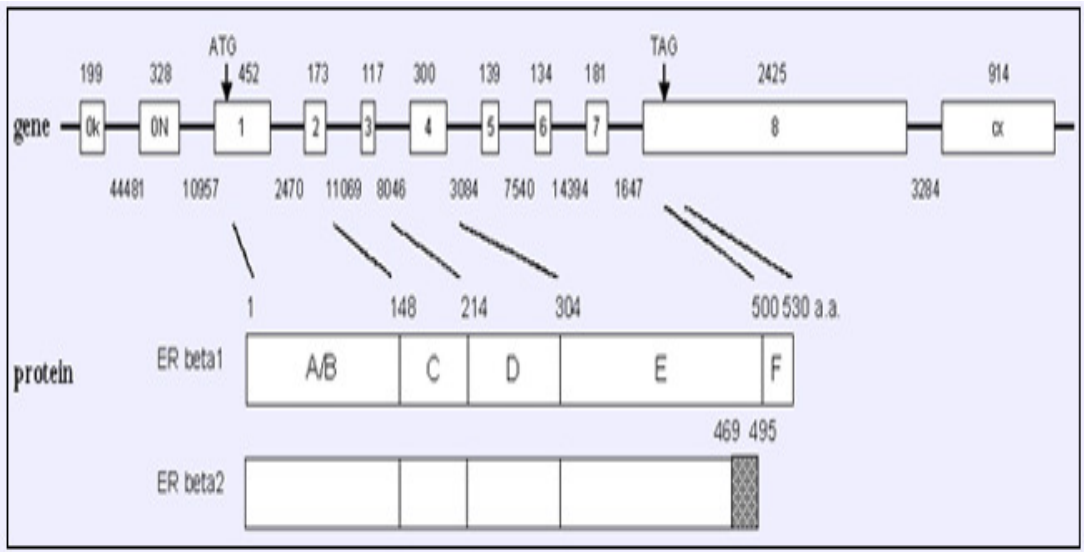


Figure 2.3 Genomic organization of human ER beta gene, protein and functional domains. Gene: exons are indicated with boxes and introns with lines. The numbers above each box indicate the size of the exons (bp); the numbers below each line designate the size of the respective introns (bp). Sectional lines between gene and protein point to protein domain junctions. Protein: numbers indicate the total size of the protein in amino acids. The shaded bar shows the divergent C-terminal regions between the isoforms. Amino acids 214 to530 (D-F) represent the steroid binding domain, C is the DNA binding domain, and AB is the transactivation domain. {Zhao *et al*, 2008}.

Binding of ligands (oestrogens and anti-oestrogens) activates ESR, by a mechanism that is similar to that of AR. However, oestrogen-modulated gene transcription is exerted via different mechanisms: the genomic and non-genomic pathways {Nilsson *et al*, 2001}. The representative model for ESR-mediated regulation of gene expression involves the direct binding of dimeric ESR to DNA sequences known as oestrogen response elements (EREs). The binding results in recruitment of a variety of coregulators to alter chromatin structure and facilitate recruitment of the RNA polymerase II transcriptional machinery. The transcriptional activity of ESRs can be altered by several post-translational modifications such as phosphorylation, acetylation, sumoylation, ubuquitination and methylation {Zhao, *et al*, 2008}.

In the prostate, the ESRs are expressed in both stromal and epithelial cells. The fibroblasts and the basal epithelial cells are known to express more ESR than other cell types; and it is believed that oestrogen enhances prostatic growth by direct effect on fibroblast and basal epithelial cells, and indirectly, as mediated by growth factor production {Droller, 1997}. Once again the stromal-epithelial signalling pattern involving paracrine mechanism of stimulation is hypothesized. However, the controversy as to which oestrogen receptor does what function, dates back to embryological development. Prins GS and colleagues in 2001 demonstrated in mice that ESRα was the dominant ESR form mediating the development of oestrogenization of the prostate gland, a process that defined morphology and adult behaviour of the prostate gland in mice. They concluded that if the ESRβ were involved, it would be a minor role and would require the presence of the alpha type. However, in humans, the ESR alpha is the most extensively studied. It is the type involved in breast cancer. Luminal breast cancer tumours express ESR alpha which predict better prognosis {Marchio & Reis-Filho, 2008}. In female breast cancers the ESR beta is often reduced. Murphy *et al* (2006) reported a concomitant high expression of ESR alpha and ESR beta in male breast cancers; with the report also showing increased expression of AR and PGR.

Immunolocalisation studies have consistently identified the majority of ESR alpha staining in the prostatic stroma rather than epithelial cells {Bonkhoff *et al*, 1999}, which conforms to the paracrine mechanism of stromal-epithelial regulation. These studies imply that the ESR alpha is proliferative (activate genes that result in epithelial cell replication and proliferation). Several IHC studies have shown reduced ESR beta expression in prostate cancer {Horvath, *et al*, 2001; Zhu *et al*, 2004}. In other studies ESR beta expression was increased in metastatic PCa {Fixemer *et al*, 2003}. These conflicting reports implied that the function of ESR beta is either proliferative or anti-proliferative. Walton *et al* (2009) used laser microdissection to

isolate 'pure' population of benign and malignant prostate epithelium and measured ESR beta expression using RQ-PCR. The report showed that ESR beta was significantly over-expressed in malignant group compared to benign controls. They concluded that ESR beta could be proliferative; however further studies with increased sample size are required to confirm this finding (because Walton's study examined only 17 specimens). It is known that oestrogens increase the levels of AR and stimulate prostatic basal epithelial cells to produce growth factors {Droller, 1997}. The emerging picture is that the stromal cells express more of ESR alpha while the epithelial cells express more of the ESR beta. In the healthy prostate, the predominant mechanism for regulating prostatic growth is the stromal-epithelial paracrine stimulation, but in the cancerous cells the epithelial cells are apparently independently regulated and may rely on their ESR beta for signal transduction (proliferation). This hypothesis is supported by the study of Walton *et al* (2009) which showed that there was no significant difference in AR and ESR alpha in both cancer and benign groups (because both receptors are involved the normal cellular proliferation). The implication of this hypothesis is that the ESR beta signalling could be the alternative driver of abnormal proliferation of prostate cancer cells; and it exerts proliferative pressure leading to abnormal AR production in advanced stage of the disease. Therefore ESR beta could be a therapeutic target in advanced PCa especially in the androgen insensitive stage. ESR beta over-expression could also predict poorer disease outcome. Torlakovic *et al* (2002) reported that ESR beta over-expression was positively associated with Gleason Score. More studies are required to confirm that ESR beta over-expression is consistently observed in high histological grade PCa and also associated with pathological stage.

2.2 Molecular markers of prostate cell proliferation

2.2.1 The Minichromosome maintenance proteins (MCMs)

DNA synthesis in eukaryotes is a complex, multi-step process that requires the participation of a number of proteins. The process involves the binding of Origin of Replication Complex (ORC) to replication origins, the recruitment of Cdc6 and MCM2-7 to form the pre-replicative complex (pre-RC) and the activation of pre-RC by Cdc7 and Cdc28 protein kinases to initiate DNA synthesis {Tye, 1999}. The MCMs were first revealed to be involved in DNA replication as the result of a genetic screen for mutants defective in minichromosome maintenance in Yeast cells {Lei & Tye, 2001}. Localised on different chromosomes, the MCM complex consists of 6 members (subunits) known as MCM2-7, a family of highly conserved proteins that are responsible for DNA licensing to initiate DNA synthesis in all

eukaryotes {Tye, 1999}. However, there are other proteins also known as MCMs, for example, MCM1 and MCM10, these do not bear sequence homology with MCM2-7 and they are not directly involved with DNA licensing and formation of the pre-RC.

The MCM2 gene is localised on chromosome 3q21.3, and has 16 exons, a transcript length of 3,432 bp encoding a 102 kDa protein of 904 amino acid residues {Ensemble Genome Browser, 2009}. There is no known transcript variant of MCM2 (previously known as BM28).

The MCM3 is localised on chromosome 6p12.1, and is currently known to have 4 transcript variants of 9, 17, 17 and 18 exons respectively; transcript length of 1030 bp to 3262 bp encoding a protein of 37 kDa {UCSC and Ensembl Genome browsers, 2009}. The MCM3 interacts with MCM5 and CDC46 in the formation of pre-RC during replication licensing. It is also known to be acetylated by MCM3AP, a chromatin-associated acetyltransferase {Tye, 1999}. The acetylation of MCM3 inhibits DNA licensing, disrupts initiation of DNA replication and halts cell cycle progression {Lei & Tye, 2001}.

The MCM4 is localised on chromosome 8q11.21, and has 3 curated transcript variants of 16, 17 and 18 exons, transcript lengths of 3.5, 3.4 and 3.3 kb encoding a protein of 93 and 97 kDa respectively {Ensemble Genome Browser, 2009}. The MCM4 was previously known as CDC21 and CDC54. Like MCM2, MCM 6 and MCM7, the MCM4 has a DNA helicase activity. Its binding to CDC2 protein kinase reduces the helicase activity and prevents it from binding to chromatin and MCM complex.

The MCM5 is localised on chromosome 22q12.3, and has 11 transcript variants (10 are known protein coding and one has no known protein); two of the protein coding have a nonsense_ mediated decay (the protein products are not stable) (Ensemble Genome browser & Swiss-Prot, 2009). The transcript variants have 2 to 17 exons, transcript lengths of 450 to 2545 bp, encoding protein isoforms of 7 kDa to 82 kDa (57 to 734 amino acid residues). The MCM5 was previously known as CDC46 {Tye, 1999; Lei & Tye, 2001}.

The MCM6 (also known as Mis5) is localised on chromosome 2q21.3, and has 2 transcript variants: 3 and 17 exons, 320 and 3,743 bp transcript lengths, encoding 10 and 93 kDa proteins of 83 and 821 amino acid residues respectively {Ensemble Genome browser, 2009}.

The MCM7 formerly known as CDC47 is localised on chromosome 7q22.1, and has 6 transcript variants, 2 to 15 exons, transcript lengths of 240 to 2,821 bp encoding protein isoforms of 7 to 81 kDa (64 to 719 amino acids). Cyclin D1-dependent kinase, CDK4, is

found to associate with MCM7, and may regulate the binding of this protein with the retinoblastoma protein (RB1).

Members of the MCM2-7 are all nuclear proteins that function together as a stable hexameric complexes involved in DNA licensing (MCMs bind to G1 phase chromatins to from replication fork) and cell proliferation. The biochemical, physiological, evolutionary conservation and structural models of the MCM2-7 proteins are extensively reviewed by Tye, 1999. Towards the beginning of the 21st Century, many studies reported the MCMs as pre-cancer biomarkers in different types of human cancers {Freeman *et al*, 1999; Stober et al, *1999*; Alison *et al*, 2002). Most of those studies reported the MCMs as markers of proliferation potential and showed that their aberrant over-expression was associated with malignancy. In particular, the MCM5 protein was strongly associated with urothelial cancers; an immunoassay for its detection in urine samples was reported by Stoeber *et al*, (1999). MCM2 and MCM5 over-expressions (immunohistochemically detected) were also reported in muscle invasive urothelial cancers; in which they correlated significantly with both Ki-67 proliferation- and p53 labelling-indices, and were associated with high-grade cases {Korkolopoulou *et al*, 2005}. The MCMs are down-regulated in cells undergoing differentiation-G2 and S phases or quiescence-G0 phase, and therefore are specific markers of proliferating cells {Freeman *et al*, 1999}.

As the MCMs became established as markers of proliferation potential, many studies evaluated their prognostic utility. Meng *et al* (2001) reported that 'prostate cancer patients with high MCM2 expression exhibited shorter disease-free survival', following radical prostatectomy for localised PCa. However, the MCM2 expression was not associated with any traditional clinical or pathological factors and therefore was considered as an independent predictor of survival in patients with localised PCa. Another study also showed that deregulation of MCM proteins was characteristic of dysplasia, which also indicated early events in the development of malignancy {Alison *et al*, 2002}. However, it is important to point out that both normal and dysplastic cells express MCMs, but there is over-expression in dysplasia (intermediate stage in the progression from normal tissue to neoplasia). And the over-expression may not be particularly tissue specific. Therefore, dysplasia in the prostate would result in MCMs over-expression in as much as it would in urothelial bladder cells. But the diagnostic value of some specific MCMs, for example, MCM5 in urothelial cancers (detected by urinary assays) would still require large-scale studies to prove discrimination against other urological cancers including PCa. There are also variants of MCMs that are not protein coding, and may have differential expression in malignancy.

2.2.2 The Aurora Kinases (A, B and C)

Cell cycle checkpoints have evolved to ensure the inheritance of undamaged DNA, and that each daughter cell receives the correct complement of chromosomes during mitosis {Scharer *et al*, 2008}. The Aurora family (with three members: A, B and C) of highly conserved serine/threonine kinases regulates cell entry into mitosis, centrosome maturation and the mitotic spindle checkpoint {Marumoto & Saya, 2005}.

The Aurora kinase A (AURKA) is localised on chromosome 20q13.2, and has 14 curated transcript variants, exons ranging from 5 to 10, transcript lengths of 584 to 2,238 bp; encoding protein isoforms of 9 kDa to 46 kDa (79 to 403 amino acid residues) {UCSC and Ensemble Genome browsers, 2009}. Pseudogenes of AURKA have also been located on chromosomes 1 and 10. AURKA is involved in centrosome separation, microtubule formation and stabilization at the spindle during mitosis {Glover *et al*, 1995}. Over-expression of AURKA is reported to confer resistance to the taxane paclitaxel (a mitotic inhibitor, which interferes with normal breakdown of microtubules during mitosis) {Scharer *et al*, 2008}; it is therefore suggested as a predictive marker for taxane resistant, ovarian cancer patients who are likely to benefit from a potential drug VE-465, still undergoing pre-clinical studies. It is generally believed that AURKA may play a role in tumour development and progression because of its critical role in mitotic entry as well as mitotic spindle checkpoint {Seki *et al*, 2008}. Two proteins known to bind and initiate activation of AURKA are TPX2 and Ajuba {Scharer *et al*, 2008}. Qu *et al* (2008) reported that DNAzyme (DNA molecules with enzymatic cleavage activity) could be used to target AURKA mRNA in prostate cancer cells as a potential therapeutic agent.

The Aurora kinase B (AURKB) is localised on chromosome 17p13.1 and has one transcript variant: 9 exons, transcript length of 1,245 bp encoding a 39 kDa protein of 344 amino acid residues {Ensemble Genome browser, 2009}. AURKB is a chromosomal passenger protein that begins mitosis localised to the centromeres but at the onset of anaphase relocates to the spindle equator {Keen & Taylor, 2004}. AURKB localizes to microtubules near kinetochores, specifically to the specialized microtubules called K-fibres; and it is known to regulate processes such as kinetochore and microtubule interactions, segregation of sister chromatids and cytokinesis {Scharer *et al*, 2008}. Immunohistochemical studies on prostate tissues and cell lines have demonstrated increased nuclear expression of AURKB in high Gleason score PCa compared to intermediate and low grades, as well as in all cancers compared to benign and normal glands {Chieffi *et al*, 2006}.

The Aurora kinase C (AURKC) is localised on chromosome 19q13.43, and has 3 transcript variants of 7 exons each, variable transcript lengths (1,108 to 1,249bp) encoding protein isoforms of variable molecular masses (32 to 36 kDa; 275 to 309 amino acid residues respectively). The AURKC is expressed specifically in the male testis and has meiotic functions {Scharer *et al*, 2008}.

Aberrant expression of the Aurora kinases can induce abnormal mitosis, centrosome dysfunction and chromosomal instability, which may lead to aneuploidy and neoplastic transformation {Lee *et al*, 2006}. Increased expression of AURKA and AURKB are reported in PIN and PCa compared to normal cells {Chieffi *et al*, 2006}. It is very likely that therapeutic agents that target the aurora kinases will be developed in near future to augment mitotic inhibitors like the taxanes (docetaxel and paclitaxel).

2.3 Prostate tissue specific molecular markers:

2.3.1 Prostate specific antigen (KLK3 gene)

The KLK3 gene (PSA gene) is a member of the human tissue kallikrein family of genes (with known 15 members), belonging to the S1A subfamily of the serine proteases {Obiezu & Diamandis, 2005}. The tissue kallikreins are tandemly co-localised on chromosome 19q13.4, occupying a genomic space of 300 kb without any intervening non-kallikrein gene {Grimwood *et al,* 2004}. They share many common characteristics, including exon/intron organization (coding sequence spanning 5 exons), conservation in coding exons and intronic phases, presence of 5'UTRs and 3'UTRs; conserved amino acid residues and the location of three catalytic residues His, Asp and Ser {Yousef *et al*, 1999; Obiezu & Diamandis, 2005}. They are expressed in several different organs, and their transcripts code for single chain serine protease pre-proenzymes of 248-293 amino acids {Obiezu & Diamandis, 2005}.

The KLK3, which encodes PSA (hK3, which is the official protein symbol) was first isolated from the seminal fluid (SF) in the 1970s by several independent groups {McCormack *et al*, 1995}. It was then thought to be produced exclusively in males by the columnar epithelial cells of the prostate, although it is now known that some other tissues like the breast and seminiferous tubules also produce minute quantities of PSA {Partin & Marks, 2001}. Currently, there are 11 known transcript variants of KLK3 (due to alternative splicing) {Kurlender *et al*, 2005}, ranging from 2 to 6 exons, with the longest variant (6 exons) encoding a 24 kDa protein of 220 amino acid residues {Ensemble Genome browser, 2009}.

The molecular mass of PSA varies depending on the method of detection and also the degree of glycosylation (N-linked oligosaccharide attached to Asp-46, which constitute about 8% of PSA) {McCormack *et al*, 1995}. By mass spectrometry the actual molecular mass of PSA was 28.5kDa {Belanger *et al*, 1995}; and by gel electrophoresis the apparent molecular mass of PSA ranges from 30 to 36 kDa, which could result from aberrant behaviour of glycoproteins in gel systems {McCormack *et al*, 1995}.

The primary biologic role of PSA is to cleave the major gel-forming proteins semenogelin I, II and fibronectin in SF into small peptides, resulting in increased sperm motility {Lilja *et al*, 1993}. The diagnostic value of PSA has been discussed in Section 1.3.1 of this thesis. In that section, it was pointed out that the PSA has a weak correlation to prostate malignancy, and therefore many ratiometric (modifications) tests were introduced to improve sensitivity and specificity. Some of these ratiometric tests are based on the molecular forms of PSA (Table 2.1). The PSA is capable of forming covalently linked complexes with certain serine protease inhibitors found in serum and these complexes are not dissociated by pre-analytical treatments{McCormack *et al*, 1995}. PSA is detectable in serum, urine and seminal fluid using immunometric assays such as ELISA.

The ratiometric PSA tests include:

a. Percent free PSA: this is the ratio of free- to- total PSA expressed in percent. In routine clinical practice, a free PSA ratio of < 20% in men with elevated total PSA levels (> 3.0ng/ml) is associated with a higher risk of PCa and facilitates the indication to perform a prostate biopsy {Heidenreich *et al*, 2008}. A cut off point of 18% (free PSA ratio) was reported to increase sensitivity and specificity of PSA testing to 71 and 95% respectively {McCormack *et al*, 1995}.
b. PSA velocity: this is the rate of change of PSA over time (expressed in ng/ml/year). A PSA velocity > 0.75 ng/ml/yr is an indicator for prostate biopsy {Heidenreich *et al*, 2008}. The PSA velocity is reported to improve sensitivity and specificity of PSA testing to > 80% {Djavan, 2004}.

c. PSA density (PSAD): this is the quotient of total serum PSA by prostate volume. A value of > 0.13 is considered abnormal {McCormack *et al*, 1995}.

d. A product of percent free PSA and hK2: the combination (hK2 x tPSA/fPSA) was reported to increase specificity to 90% and sensitivity to 55% {Becker C, 2000}. Mean values of the product in benign and cancer patients were reported 0.3 and 0.67 ng/ml respectively.

Table 2.1 Molecular forms and Complexes of PSA.
{McCormack *et al*, 1994; Obiezu & Diamandis 2005}

Formal Name	**Common Name**	**Description**
Free PSA	f-PSA	Non-complexed PSA; may be proteolytically active or inactive in seminal fluid, only inactive in serum.
Clipped free PSA	Clipped positions: 85-86,145-146 and 182-183.	Inactive forms of f-PSA; held together by internal disulphide bonds, co-migrates with active PSA as 30 kDa in non-reducing SDS PAGE but form smaller size fragments in reducing SDS PAGE.
Precursor forms	PreproPSA and ProPSA	PreproPSA is the proform with a signal peptide of 24 additional residues. ~ 261 aa residues on the whole. The proform is the inactive zymogen, ~244 aa residues.
Mature PSA	PSA	This is the extracellular PSA, enzymatically active and usually complexed.
PSA Complexes	PSA-ACT	PSA covalently bound to **α_1-antichymotrypsin inhibitor** in plasma. It is the major immunodetectable form in serum.
	PSA-MG	PSA covalently linked and encapsulated by **α_2-macroglobulin**; not immunodetectable, also known as occult PSA.
	PSA-PCI	PSA covalently bound to **protein-C inhibitor**; minor component in SF, not detectable in serum.
	PSA-AT	PSA covalently bound to **α_1-antitrypsin**; trace components in serum.
	PSA-IT	PSA covantly bound to **inter-alpha trypsin inhibitor**; trace components in serum.
Total PSA	t-PSA	All immunodetectable forms in serum, primarily f-PSA and PSA-ACT.

e. PSA doubling time (PSA DT): This is another measure of PSA kinetics, useful in follow-up to treatment. This is the time (usually expressed in months) it takes to reach twice the nadir value of PSA. For example PSA level after radical prostatectomy is expected to be zero because the prostate is removed. However, PSA relapse (biochemical occurrence) occurs in some patients, and a PSA DT of less than 10 months is a predictor of metastatic progression {Roberts & Han, 2009}.

PSA mRNA is also detectable in blood and urine samples, but no clinical significance has been associated with its levels {Corey *et al*, 1997; Rittenhouse *et al*, 1997}.

2.3.2 The human glandular kallikrein 2 (KLK2)

The KLK2, another member of the tissue kallikrein family of genes, encodes the hK2, which has about 80% homology with PSA. Both are up-regulated in response to androgens and progestins; however the KLK2 is very prostate tissue –specific {Obiezu & Diamandis, 2005}. Unlike the PSA, the hK2 is expressed at higher levels in prostate adenocarcinoma than in normal prostate epithelium {Becker, 2000}. There are 7 known transcript variants of KLK2 {Kurlender *et al*, 2005}, the longest variant encoding a 28.6 kDa mature protein of 237 amino acid residues {McCormack *et al*, 1995}. Unlike the PSA, the hK2 has trypsin-like protease activity, and it functions to cleave proPSA into active forms {Obiezu & Diamandis, 2005}.

There are now commercially anti-hK2 antibodies that do not cross-react with PSA, facilitating the development of immunometric assays for hK2 {Piironen *et al*, 1996}. By comparison, in the human prostate tissue, the expression of hK2 mRNA is about 10% to 50% of PSA mRNA {McCormack *et al*, 1995}. Median value of serum hK2 (measured by a research immunometric assay) in a population study was reported as 56 pg/ml compared to 3.1ng/ml for PSA {Partin *et al*, 1999} Like the PSA, hK2 mRNA is detectable in blood, seminal fluid and urine {Corey *et al*, 1997}; and no clinical significance has been associated with its urinary level {Panek *et al*, 1997}. The study by Becker *et al,* 2001 demonstrated that measurement of hK2 improves diagnostic value of PSA testing. The consensus opinion in most reports is that hK2 supplements PSA testing not a replacement {Partin & Marks, 2001; Obiezu & Diamandis, 2005}.

2.3.3 The TMPRSS2 gene fusions

The transmembrane protease, serine 2 (TMPRSS2) is a serine protease localised on 21q22.3, and currently has 6 known transcript variants (due to alternative splicing) encoding a 53.8kDa protein {Entrez gene browser}. The protein product has five domains: a type II transmembrane, a receptor class A, a scavenger receptor cysteine-rich and protease domains. The gene is up-regulated by androgens, and also involved in many physiological and pathological processes including prostate cancers (84% of all cases have over-expression) {Cross *et al*, 2008}

Tomlins *et al* in 2005 reported the recurrent gene fusion involving 5' UTR of TMPRSS2 and the ETS (E26 transformation-specific) family genes in prostate cancers. It was a landmark discovery in molecular pathology of prostate cancer; the gene fusion occurred in about 50% of localised prostate cancers {Cross *et al*, 2008}. Members of the ETS family of genes (transcription factors) involved in the gene fusion consist of ERG (localised on 21q22, ETV1 (7p21) and ETV4 (17q21). The protein products of ETS genes are transcription factors that activate or repress the expression of genes that are involved in cellular proliferation, differentiation, development, transformation and apoptosis {Cross *et al*, 2008}. The TMPRSS2: ETS gene fusions usually occur by deletion sometimes by chromosomal rearrangement {Tomlins *et al*, 2007}. The fusion leads to the juxtaposition of the androgen-responsive promoter region of TMPRSS2 and the proto-oncogenic region of ETS genes; and androgens are believed to drive the over-expression of ETS gene resulting in neoplastic transformation {Tomlins *et al*, 2007}. Clarke *et al* (2007) reported 14 transcript variants of the TMPRSS: ETS fusion genes: two encoding a normal full-length ERG protein, six encoding N-terminal truncated ERG proteins and one encoding a TMPRSS2: ERG chimeric protein. The TMPRSS2: ERG is the most common variant (occur mainly by deletion mechanism), followed by the TMPRSS2:ETV1 and TMPRSS2: ETV4 {Mehra et al, 2007}. The gene fusions are detectable by both FISH (using break-apart and fusion probes) and PCR techniques (multiplex RT-PCR and RQ-PCR).

Several studies have reported on PCa progression and clinical significance of the TMPRSS2: ETS gene fusion. The gene fusion occurs in high grade PIN, localised and metastatic PCa; not detectable in BPH {Cross *et al*, 2008}. The percentage of the fusions in high grade PIN is roughly half that of localised PCa {Hermans et al, 2006}. The report by Hermans *et al*, 2006

also showed that the effect of the gene fusion is bypassed in the late stage androgen-insensitive PCa, which corroborates with the fact that androgen regulation fails in the HRPC stage. Clark *et al* (2007) also reported that fusion transcript variants exhibit distinct patterns in the foci of PCa, suggesting that the fusions may arise independently in different regions of the same prostate.

The TMPRSS: ETS fusion is associated with high Gleason score (high histological grade) {Mehra *et al*, 2007; Tu *et al*, 2007}. The gene fusion is also associated with five morphological features in PCa: blue-tinged mucin, cribriform growth pattern, macronucleoli, intraductal tumour spread and signet-ring cell features {Mosquera *et al*, 2007}. Prostate cancers with TMPRSS2 fusions have a worse prognosis than those without {Cross *et al*, 2008}; Nam *et al* (2007) reported that the gene fusion is associated with tumour recurrence after radical prostatectomy for localised PCa. Currently TMPRSS2: ETS gene fusions have not been identified in other cancers {Yoo *et al*, 2007}. Using RQ-PCR the TMPRSS2: ERG transcripts have been detected in urine samples of men with known PCa following prostatic massage {Laxman *et al*, 2006}; therefore it could used be as a diagnostic test. However, Tomlins *et al* (2007) have shown that the ETV1 gene can fuse with other partners in the 5'UTR region, and that these fusions are also common in PCa (Figure 2.4). These findings add to the complexity underlying the molecular mechanism of chimeric oncogenic fusion products.

Studies have also shown that certain SNPs in the TMPRSS2 gene are associated with mechanism of gene fusion (deletion or translocation) and copy number; in particular the T allele of the SNP rs12329760 was identified with translocation fusions and multiple copies of gene fusion {FitzGerald *et al*, 2008}. Liu *et al* (2007) also reported that particular sequences (called the Alu family consensus sequences) were associated with the likelihood of TMPRSS:

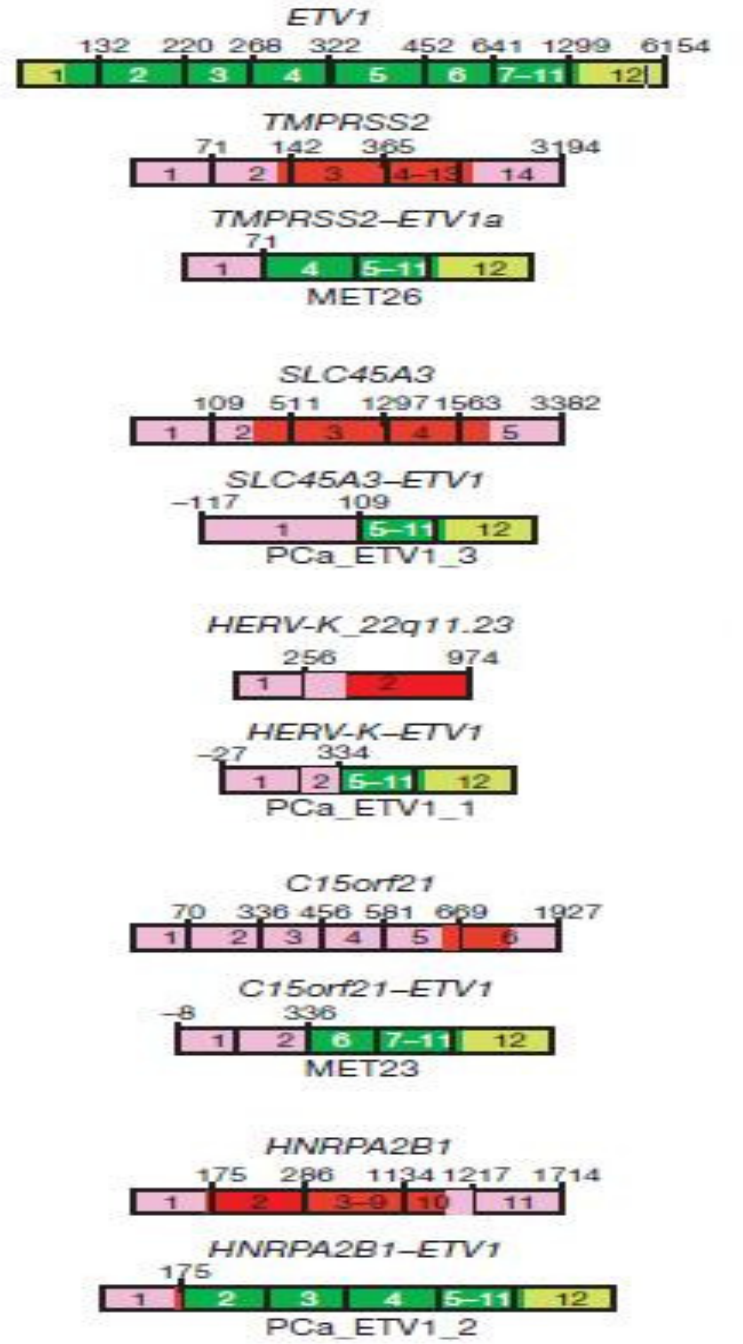


Figure 2.4 Prostate specific elements fused to ETV1. The 5' partners are androgen-responsive and they drive ETV1 over-expression. The numbers in the boxes represent exons. The numbers above the boxes indicate the last base of each exon. Untranslated regions are in lighter shades (pink and light green). {Tomlins *et al*, 2007}

ETS fusion and type of fusion transcripts that will occur. More studies are required to verify these sequences in both healthy and PCa men.

2.4 Molecular markers of apoptosis

2.4.1 The p53 gene

The tumour suppressor protein (TP53, commonly known as p53) regulates the transcription of genes involved in G1-phase growth arrest of cells in response to DNA damage {Agrawal *et al*, 1998}. The arrest enables repair or apoptosis of unrepaired cells, preventing passage of damaged DNA to daughter cells. Localised on chromosome 17q13.1, the p53 gene has currently 9 transcript variants with the longest variant (of 11 exons, a transcript length of 2,586) encoding a 43.6 kDa protein of 393 amino acid residues. It is also known to play roles in the mitotic spindle checkpoint, centrosome homeostasis and G2-M phase transition of cell-division cycle {Quinn *et al*, 2005}. Primarily, the p53 gene regulates apoptosis, a role in which it has been described as the 'the molecular policeman' {Stein & Pardee, 2004}.

TP53 has been associated with several human cancers. High penetrance mutations of TP53 are associated with hereditary breast cancers, which manifest at young age (Li-Fraumeni syndrome) {Marchio & Reis-Filho, 2008}. Sporadic mutations of p53 are uncommon in early PCa, but are independently prognostic in late-stage PCa {Agrawal & Dunsmuir, 2009}. Exons 5-8 are the predominant mutation sites in TP53 {Oden-Gangloff *et al*, 2009}, and have been associated with nuclear p53 accumulation in tumour cells {Bartek *et al*, 1990}. Isaacs (1997) reported that tumours positively staining for p53 harbour mutations and those p53 mutations appear to be more common with increasing tumour stage. Although the value of p53 nuclear accumulation (detected by IHC) as a prognostic marker in PCa is controversial, there is a consensus opinion that p53 mutation analysis and p53 gene expression in combination may more accurately define prognostically the importance of p53 dysfunction in PCa {Quinn *et al*, 2005}. Malins *et al* (2003) reported that concomitant homozygous PTEN-p53 inactivation led to PCa lethality in mice. Expression of p53 has been shown to predict overall survival in 'watchful waiting' patients; predict distant metastases in radiotherapy treated patients and also predict PSA recurrence in radical prostatectomy patients {Quinn *et al*, 2005}. It has also been implicated in hormone resistance during PCa progression. Recently, p53 mutation was reported as a marker of sensitivity to cetuximab (monoclonal anti-EGFR therapy) in KRAS wild-type patients of colo-rectal cancer {Oden-Gangloff *et al*, 2009}. TP53 expression is regulated by the Mdm-2 gene.

2.4.2 The Bcl-2 gene

The Bcl-2 (B-cell CLL/lymphoma 2) gene is another major apoptotic regulator. Localised on chromosome 18q21, the Bcl-2 is the prototype of a class of oncogenes that inhibit apoptosis (programmed cell death) {Haldar *et al*, 1989}. Other members of the Bcl-2 class include Bcl-xL and Mcl-1, both are inhibitors of apoptosis; but other members such as Bcl- xS, Bad, Bak, Bax and Bid are inducers of apoptosis. The Bcl-2 protein is expressed in the basal cells and stem cells of the prostate. Biochemically, apoptosis is the end point of a cascade of molecular events that are initiated by several stimuli and leads ultimately to the activation of proteolytic enzymes responsible for cell death {Kumar *et al*, 2009}. Through poorly understood mechanisms, the bcl-2 family of proteins, which are located in outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane, regulate the activation of these proteolytic enzymes (caspases); a process that involves interplay of factors such as p53, c-myc etc.

Bcl-2 expression increases with histological grade and clinical stage in PCa {Quinn *et al*, 2005}. Over-expressions of Bcl-2 confer androgen resistance, particularly in advanced stage PCa {McConkey *et al*, 1996}. Bcl-2 expression on biopsy, in advanced PCa is an independent predictor of response to radiotherapy. Anti-sense oligonucleotides targeted on Bcl-2 are known to increase sensitization of tumour cells to radiotherapy {Mu *et al*, 2005}. Reports on prognostic value of Bcl-2 on radical prostatectomy and PSA recurrence are conflicting {Agrawal & Dunsmuir, 2009}.

2.5 Molecular markers of metastasis

2.5.1 The CD44 gene

The CD44 also known as the Indian blood group is located on human chromosome 11p13, currently has 17 curated transcript variants (of variable number of exons: 4 to 18 due to alternative splicing), the longest variant encodes a 80.7 kDa protein of 734 amino acid residues {Entrez and Ensemble Genome browsers, 2009}. The protein is an integral transmembrane glycoprotein that participates in specific cell-cell interactions and cell-extracellular matrix interactions (cell adhesion and cell migration) {Isaacs, 1997}. It is a receptor for hyaluronic acid (HA), and can also interact with other ligands, such as

osteopontin, collagens, and matrix metalloproteinases (MMPs). It participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, haematopoiesis, and tumour metastasis {Madjd *et al*, 2009}. CD44 is normally expressed in the plasma membrane of prostatic epithelial cells (the cells of origin of PCa) {Isaacs, 1997}.

CD44 is believed to play a major role in tumorigenicity and metastasis in different types of tumour cells. Individual transcript variants and isoforms of CD44 differ in their ability to enhance or decrease tumorigenicity or metastatic potential {Isaacs, 1997}. Down-regulation of standard CD44 form at both mRNA and protein level correlates with metastasis (predicts aggressiveness of PCa) {Gao *et al*, 1997}.CD44 is a metastatic suppressor for PCa {Isaacs, 1997}. Conversely, over-expression of mutant CD44 could be associated with tumour invasiness {Isaacs, 1997}; however mutations of CD44 are never evaluated in routine IHC studies of CD44 expression. For example, Huh *et al* (2009) reported an immunohistochemical study in which 'standard' CD44 over-expression was associated with tumour stage and overall survival in colo-rectal carcinoma. In another study, Madjd *et al* (2009) showed that CD44 positive breast cancer stem cells over-expressed Bcl-2, implying that such cells could be chemo-resistant. It is very vital that mutant forms of CD44 are differentiated from other wild type transcript variants in mapping the prognostic value of CD44 expression. Recently, Desai *et al* (2009) reported increased expression of standard CD44 (sCD44) and transcript variants 4 to 10 (vCD44) in prostate cells that over-express osteopontin, and also concluded that the surface expression of CD44 and activation of MMP9 on the cell surface are interdependent. In addition to transcript variations and mutations, the degree of glycosylation of CD44 affects its ability to recognise ligands such the HA {Katoh *et al,* 1995}.

2.5.2 The E-Cadherin1 gene

The E-Cadherin type 1 gene, officially designated CDH1, previously known as CD324 is a classical cadherin from the cadherin super family {Du *et al,* 2009}. Located on chromosome 16q22, CDH1 encodes a 97.4 kDa calcium-dependent glycoprotein of 882 amino acid residues, comprised of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail {Du *et al,* 2009}. The protein is involved in the regulation of cell-cell adhesion and cell morphology through formation of complexes with other molecules particularly the catenins {Quinn *et al*, 2005}; the β-catenin signalling was illustrated in Figure 1.9 in Chapter one of this thesis.

Gene expression and mutation status of CDH1 have been associated with human cancers. Mutations in this gene are associated with gastric, breast, colorectal, thyroid, and ovarian cancers. Loss of function (by deletion or mutational inactivation) is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis {Du *et al,* 2009}. Early studies showed that benign prostate cells exhibit strong, uniform immunostaining for CDH1 at cell-to-cell borders, while prostate tumour cells showed reduced or absent E-Cadherin staining {Isaacs, 1997}. Aberrant CDH1 immunostaining correlated with high grade tumours (Gleason score), and was 'a powerful predictor of poor outcome, both in terms of disease progression and overall survival' {Isaacs, 1997}. Down-regulation of E-cadherin expression in localised PCa is associated with increased expression of other cadherin family members, particularly N-Cadherin {Quinn *et al*, 2005}. It is suggested that while CDH1 promotes epithelial cell-epithelial cell adhesion, N-cadherin promotes epithelial cell-stromal cell adhesion {Bussemakers *et al*, 2000}. Over-expression of CDH1 has also been reported in breast cancer metastasis, a paradox believed to be caused by 'epigenetic mechanism during invasion and diapedesis into vessel walls, only to be reactivated at the site of established metastases' {Bukholm *et al*, 2000}. It is possible that mutant forms of E-cadherin type 1 could be over-expressed in PCa metastasis. It is reported that transcript variants of CDH1 can arise from mutation at consensus splice sites {Du *et al,* 2009}. Many studies support the fact that reduced or aberrant E-cadherin expression is associated with PCa (especially clinically localised PCa) and its prognosis.

2.6 Sampling for Molecular Pathology of Pca

Molecular techniques offer highly sensitive, dynamic range detection and quantification assays. For example, the RQ-PCR could detect a cancer cell in a background of nearly one million normal cells {Kaeda *et al*, 2003}. Therefore, patients' samples that contain low level of prostatic cells could be analyzed using such techniques. There are three main types of samples for molecular testing for PCa: urine (with or without prostatic massage), blood and prostate biopsy samples.

a. **Urine and Prostatic Massage**: There are few prostatic cells in urine samples of healthy males {Panek *et al*, 1997}. In low grade, localised PCa there are also low infiltration of malignant cells in urine; however in metastatic PCa, more prostatic cells are found in urine {Agrawal & Dunsmuir, 2009}. Prostatic massage helps to increase the number of exfoliated cells in urine sediments.

b. **Blood samples**: PSA mRNA and KLK2 mRNA have been detected in blood of men with localised PCa {Corey *et al*, 1997}. However, in metastatic PCa, > 5 cells per 7.5ml of blood are common {Agrawal & Dunsmuir, 2009}, which could easily be detected by RQ-PCR.

c. **Prostate biopsy**: Prostate tissues, either from radical prostatectomy, resection or transrectal ultrasound (TRUS) guided biopsies are an invaluable source of diagnostic specimens. Frozen tissues, fixed and paraffin embedded tissues have all have been used in molecular testing. Gillio-Tos *et al* (2007) reported successful extraction of DNA from 25 years' old paraffin embedded tissue samples. Although fixation using anatomical fixatives cause tissue samples to defragment and in some cases degrade, short sequences (≤150bp) of nucleic acids could easily be obtained from more than 99% of archived embedded tissue samples {Antonov *et al*, 2005; Gillio-Tos *et al*, 2007}. And real time PCR applications for gene quantification and genotyping are optimally designed to amplify such short amplicons, enabling accurate and reproducible detection as well as quantification of assay targets. In addition, histology of prostate tissues can assist to pin-point foci of highest tumour load, facilitating analysis of 'pure cell types' involved in a particular aberration.

2.7 Genetic testing for prostate cancer risk

In addition to gene expression and epigenetic studies in PCa, genetic variations such as copy number variations (CNVs) and single nucleotide polymorphisms (SNPs) are strongly linked to prostate carcinogenesis. Defined as being larger than 1kb in size, CNVs involve gain or losses of genomic DNA that are either microscopic or submicroscopic and are, therefore, not necessarily visible by standard cytogenetic G-banding Karyotyping {Shlien & Malkin, 2009}. There are about 21, 000 CNVs in the human genome, some associated with inherited and acquired diseases {Shlien & Malkin, 2009}. For example, the BRCA2 gene associated with <5% of Familial/young-onset PCa (diagnosed in <55 years of age) is known to have germ line CNV {Agrawal & Dunsmuir, 2009}. Sporadic gene amplifications of AR and ESRs are associated with PCa progression {D'Antonio, 2008; Zhao *et al,* 2008}.

Through the International HapMap project about 10 million SNPs have been discovered in the human genome. SNPs (hereditary single base differences in humans) are the single largest

source of human genomic variation {Gibbs, 2005}. Mining data from genome wide association (GWA) studies have enabled the association of SNPs with clinical outcomes in patients. This has been facilitated by the advent of high-resolution, high through-put SNP arrays. For prostate cancer in particular, several SNPs have been associated with the presence/risk of prostate cancer at biopsy and PCa aggressiveness. Table 2.2 is a summary of selected literature on SNPs associated with PCa. Some of the SNPs have not been mapped to identifiable genes {Zheng *et al*, 2009}; others are contained in genes without known biologic (functional) influence on PCa development or progession. SNP studies are particularly important because of the potential to identify men with high risk of PCa as targets for annual screening. Secondly, SNPs can also predict disease outcome and therefore inform treatment approach at the onset of therapy. It is possible that individual SNPs or groups of clinically associated SNPs (haplotypes) can be tested in the near future using biosensor technologies at point-of-care. Information from such tests can inform likelihood of adverse reactions to drugs.

Table 2.2 SNPs associated with PCa risk/outcome

SNP ID	Gene/Locus	Clinical outcome	Reference
rs10896449	11q13	PCa risk/aggressiveness	Thomas, 2008; Zheng *et al*, 2009
rs12418451	11q13	PCa risk	Zheng *et al*, 2009
rs1447295	8q24	PCa risk	Pomerantz *et al*, 2009
rs13254738	8q24	PCa risk	Pomerantz *et al*, 2009
rs10486567	7p15	PCa risk	Wiklund *et al*, 2009
rs10993994	10q11	PCa risk	Wiklund *et al*, 2009
rs4962416	10q26	PCa risk	Wiklund *et al*, 2009
rs4430796	17q12	PCa risk	Wiklund *et al*, 2009
rs2735839	19q13	PCa risk	Wiklund *et al*, 2009
rs5945619	Xp11	PCa risk	Wiklund *et al*, 2009
rs1859962	17q24.3	PCa risk	Salinas *et al*, 2009
rs6983561	8q24	PCa risk	Salinas *et al*, 2009
rs6983267	8q24	PCa risk	Salinas *et al*, 2009
rs198977	KLK2	PCa risk	Nam *et al*, 2006
rs2664155	KLK2	PCa risk	Nam *et al*, 2006

Some of the SNPs show a demographic distribution pattern similar to that of incidence of PCa (from the HapMap project database in which 8 countries of the world participated in the SNP sequencing project). SNPs associated with diagnostic markers can be used to determine a genetically-specific cut off level, for example the case of PSA as reported by Wiklund *et al*, (2009). Currently, there are no clinical genetic tests for PCa risk; however efforts in SNPs studies could translate into useful clinical assays in near future.

2.8 Validation of Molecular markers

As new molecular markers emerge, often through new technologies, there is the challenge to validate these markers and the technologies before translating them to clinical use. For the molecular markers their diagnostic value has to be established in clinical cases. For the technologies their analytical reliability, applications and clinical utility should also be validated prior to clinical use. This is very important especially in targeted therapy, where a molecular marker is the target of therapy, for example the epidermal growth factor receptor (EGFR). The drugs erlotinib and gefitinib specifically target EGFR tyrosine kinase in variety of cancers. The copy number, expression level and mutation status of EGFR are therefore indicators of response to therapy {Dutton, 2009}. Another example is the Bcr-ABL1 gene fusion in chronic myeloid leukaemia and some cases of acute lymphoblastic leukaemia where the gene fusion product is a non-receptor associated tyrosine kinase that requires ATP for autophosphorylation and downstream effects {Foroni *et al*, 2009}. The drug imatinib specifically targets the gene fusion, inhibiting its ATP binding. These molecular markers confirm the pharmacodynamic activity of the drugs. The same case applies to molecular markers that predict sensitivity or resistance of cancer cells to therapy, for example the KRAS and TP53 mutation in advanced colorectal cancer treatment using Cetuximab (anti-EGFR monoclonal antibody). Chemoresitant patients who have advanced colorectal cancer with KRAS mutation do not respond to Cetuximab and therefore has no survival benefit for taking the treatment {Lievre & Laurent-Puig, 2009}. In another report, Oden-Gangloff *et al*, 2009 showed that TP53 mutation is a marker of sensitivity, particularly in patients without KRAS mutations who are on Cetuximab-based chemotherapy.

The process of validating molecular markers for clinical use will also consider assay formats: real time versus end-point, multiplex versus singleplex, high throughput or flexible throughput etc. Molecular markers often occur in complicated forms: transcript variants and

copy number variations. For example the TMPRSS2: ETS gene fusion in prostate cancer is known to have 14 transcript variants encoding different lengths of the chimeric proteins {Cross *et al*, 2008}. In such cases a multiplex assay is required to first identify transcript variant present in a sample before quantification. False negative results occur in cases where transcript variants are unknown due to rare gene fusion types unidentified prior to quantification. In many cases other assay types are required to establish the molecular abnormality, for example the use of Karyotyping and interphase FISH to identify gene rearrangements prior to molecular assays. In each assay type, the analytical sensitivity, specificity, quantifiable range (limit of detection) and reproducibility should be thoroughly evaluated during validation. The phenomenon of 'yet another prognostic factor' occurs in cancer literature where an unvalidated molecular marker is highly appraised, creating a hype of its potential clinical value prior to clinical assessment. Biomarker validation is the key towards translation of novel molecular markers and novel technologies to clinical use.

The field of clinical molecular diagnostics is rapidly expanding. In this context, molecular diagnostics refers to laboratory testing based on DNA or RNA analysis; even though proteins are the end products of RNA translation, proteomic analysis is not considered as molecular diagnostics. In 2001, Collins & McKusick estimated that 5% of all laboratory testing is based on molecular diagnostics. This proportion has increased tremendously over the years as many diseases now require molecular diagnostics for diagnosis, classification, choice of treatment options and monitoring. The PCA3 test is a molecular diagnostic test now approved for PCa diagnosis. Many molecular tests are likely to surface in the clinics in the near future. What makes a good molecular test are key parameters such sensitivity and specificity (diagnostic and analytical reliability), predictive value, cost, practical applicability (reproducible, user-friendly, interpretation, methodology) and clinical relevance. For example, sequencing the entire genome of a prostate cancer patient for about £2000 may yield a huge amount of information so difficult to make a meaning out of it, difficult to reproduce in other laboratories and requires huge electronic space to store the information; clinical relevance could also be difficult to discern. On the other hand, mutation analysis of exons 5 to 8 of p53 could offer reproducible and comprehensible information, which can predict disease outcome in PCa patients.

Chapter Three

In *vitro* studies on Prostate cell lines

Chapter 3: In *vitro* studies on Prostate cell lines

3.1 Introduction

In this chapter, three prostate cell lines: PNT1A, PC-3 and MDAPCA 2b were treated with four different steroid hormones: testosterone (T), dihydrotestosterone (DHT), 17 β- oestradiol (E) and hydrocortisone (H). The aim was to evaluate the influence of varying levels of steroid hormones on the gene expressions of putative molecular markers of prostate cancer development and progression. The transcript levels of androgen and oestrogen receptors were measured by real time polymerase chain reaction (RQ-PCR). Other prostate tumour markers including CD44, TMPRSS2: ERG and ETV1 fusion genes, p53, KLK2, KLK3, CDH1, FASN, Bcl-2, MCM2 and MCM5 were also evaluated. Three candidate endogenous control genes were also assessed for normalization of the RQ-PCR results.

3.2 Materials and Methods

Details of all equipment used, preparation of reagents and buffers; and list of suppliers and their contacts are contained in appendix A. Details of experimental protocols are documented in appendix B. All raw data and additional result presentations are contained in appendix C.

3.2.1 Cell lines and General Growth Requirements

Three prostate cell lines were used in this study: MDAPCa 2b was purchased from the American type culture collection (ATCC, US), PNT1A and PC-3 from the European collection of cell cultures (ECACC, UK). All the cell lines were epithelial in origin

The MDA PCa 2b cell line was established from a bone metastasis of a 63-year old black male with androgen-independent adenocarcinoma of the prostate {Navone *et al*, 1997}. The cell line was reported to expresses PSA and AR {Navone *et al*, 1997}. It is believed to mimic androgen sensitive progressive PCa. Passage number 33 was used at the beginning of cell expansion.

The PC-3 cell line was established from a bone metastasis of prostate adenocarcinoma grade 4 in a 62-year old male Caucasian {Kaighn *et al*, 1979}. It was reported as androgen-insensitive, and did not produce PSA {van Bokhoven *et al*, 2001; van Bokhoven *et al*, 2003}. It represents a hormone refractory metastatic PCa. Passage number 37 was used at the onset of cell expansion.

The PNT1A cell line was established by transfection of normal epithelial cells derived from a 35 year old male Caucasian post mortem with a plasmid containing SV40 genome with a defective replication origin. It was reported to express AR and PSA {ECACC, 2006}. It represents a 'normal' prostate epithelium. Passage number 56 was used for cell expansion.

The PC-3 and MDAPCa-2b cells were expanded in phenol-containing DMEM: F12 (Invitrogen, UK), supplemented with 2mM glutamine (Invitrogen UK), 10% (v/v) foetal calf serum(FCS) (Invitrogen, UK), 1% (v/v) Penicillin-Streptomycin mixture (5,000 units penicillin and 5mg streptomycin/mL) (Sigma, UK) and 0.1%(v/v) Amphotericin B 250 µg/mL (Sigma, UK). For stimulation experiments, the cells were grown in phenol-free DMEM: F12 supplemented with same additives except for FCS, which was replaced with dextran-charcoal treated foetal calf serum (DCC-FCS) (Perbio, UK). The PNTIA cells were grown in RPMI 1640 media (Invitrogen, UK) supplemented with same additives. DCC-FCS has a reduced level of steroids and proteins compared to normal FCS {Herbert *et al*, 1965; Luo *et al*, 2003}.

The protocols for routine cell cultures including thawing of frozen cells, feeding and splitting of cells are contained in appendix B (B.1-B.3)

The steroid hormones used for stimulation experiments were: Testosterone (T), 5α-Androstan-17β-3-one (dihydrotestosterone) (DHT), Hydrocortisone (H) and17β-Estradiol (E), all purchased from Sigma, UK. Dilutions were made as contained in appendix B.6.

3.2.2 Serum Supplementation experiments

After cell expansion, each cell line (2 x 10^5 cells per T75 flask) was weaned into culture media containing different sera (bovine growth serum (BGS) (Perbio UK), DCC-FCS or normal FCS) (Perbio, UK) at varying percentages: 10.0, 7.5, 5.0, 2.0 and 1.0 % (v/v). The aim of the experiment was to compare viable cell yield in different sera, to ensure that the DCC-FCS could support cell growth. The cultures were daily examined microscopically, and culture media were changed on day 3 post- incubation. The cells were harvested for viable cell count after 5 days of incubation at 37^{o}C with 5% CO_2 supply. Trypan blue staining and a manual haemocytometer were used for the cell count (appendix B.4). The experiments were performed in triplicate.

3.2.3 Determination of exponential growth phase of cell lines

The growth curves of cell lines were evaluated by two different methods: by Trypan blue cell count (appendix B.4) and by colorimetric Sulforhodamine B (SRB) assay as described by {Vichai & Kirtikara, 2006} contained in appendix B.5. For the Trypan blue method, 4 x 10^4 cells were seeded into micro-well plates (12-multi well tissue culture plates)-27 plates were seeded, and incubated at 37^{o}C with 5% of CO_2 supply. Three wells were harvested (trypsinized and counted-as in appendix B.4) at each time on days 1, 2, 3, 5, 7, 9, 11 and 13 post incubation. In another set of similar experiments, 27 plates were seeded but 24 hours later, only three wells were harvested, the remaining 24 wells were treated with 10^{-6}M concentration of steroid: dihydrotestosterone DHT, testosterone T, 17 β-oestradiol E, hydrocortisone H and ethanol (as a control because the steroids were dissolved in ethanol as contained in appendix B.6). The cells were later harvested, 3 wells at a time on days 1, 2, 3, 5, 7,9,11 and 13 post treatment (the harvest on the day of treatment was called day zero).

The aim of the experiment was to determine the log phase of the cell lines (period of exponential growth) and also the confluent phase (the plateau where there was no net increase in cell number).

In the colorimetric Sulforhodamine B assay (appendix B.5), 96- well plates were used. 10^3 cells were seeded per well. The experiment was done in 6 replicates (each column for a day). The SRB assay measured cellular protein content of viable cells stained with Sulforhodamine B, and results were read at 570nm in micro-plate reader (Dynex, UK). The SRB assay was amenable to high through-put, and subsequently used for cytotoxicity studies.

3.2.4 Change in population doubling/ doubling time

Two parameters that measure cell growth kinetics: doubling time and change in population doubling were determined for the prostate cell lines. The doubling time (DT) was defined as the time (in hours) required getting twice the number of viable cells seeded. Change in population doubling (ΔPD) was calculated from the formula:

$\Delta PD = \log (A/ (B*C))/ \log 2$

Where, A is number of harvested viable cells,

B is number of plated cells,

C is attachment efficiency, which was calculated as number of viable cells harvested after 15 hours of incubation divided by number of cells seeded (plated) {Korkolopoulou et *al*, 2005}.

About 2.0 x 10^5 cells (for each cell line) were seeded into a 6- multi-well tissue culture plates (triplicate was also set up) containing 3 ml of appropriate phenol-free media in a final concentration of 10^{-4} M of a hormone (DHT, or H, E, or T). Two control wells were set up: one with no hormone but culture media only added and another with 0.5ml of absolute ethanol added to 2.5ml of culture media .The plates were harvested after 5 days of incubation at 37^{o}C, 5% (v/v) CO_2 supply.

3.2.5 Growth inhibition tests

Growth inhibition assays (cell viability assays or cytotoxic tests or cell death assays) are increasingly used in laboratory determination of the effect of anticancer drugs on fresh tumour cells {Bosanquet, 2007}. Table 3.1 shows a summary of cytotoxic assays.

The SRB assay was used to determine inhibition concentration of steroids on cell lines. The protocol for SRB assay is contained in appendix B.5. Briefly, serial dilutions (1/10) of steroids (range: 10^2 µM to 10^{-3} µM) were prepared as described in appendix B.6. The steroids were diluted in culture media (phenol-free containing DCC-FCS at 10% (v/v)). About 10^5 cells were seeded to wells in a 96-micro well tissue culture plate. After 3 days of incubation at 37^{o}C, 5% $C0_2$ supply, the cells were tested by the SRB assay.

Table 3.1 Methodologies for cell death (viability) assays {Bosanquet, 2007}

Abbreviation	Name	Technology	Measured by
	Trypan blue	Cell membrane integrity	Dye exclusion
DiSC	Differential staining cytotoxicity	Cell membrane integrity	Dye exclusion, morphology
TRAC	Tumour response to antineoplastic compounds	Cell membrane integrity	morphology
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	Metabolic death	Dye reduction
ATP	Adenosine triphosphate	Metabolic death	ATP levels
FMC	Flourometric microculture cytotoxicity	Metabolic death	Metabolism of fluorescein diacetate
SRB	Sulforhodamine B assay	Cellular protein content	Protein stain update

3.2.6 Immunometric Assays

These are assays which rely on the use of antibodies specific to antigens in cells, cellular extracts and/ or fluids to determine the expression of proteins. In this study, they included immunocytochemistry (ICC), immunofluorescence (IF), enzyme linked immunosorbent assay (ELISA) and western blotting.

a) Immunocytochemistry and Immunoflourescence

Immunocytochemistry (ICC) is a technique for detecting cellular antigens by using specific primary antibodies that bind to them. Visualization is accomplished using labelling methods (detection systems). Broadly, detection systems can be enzymatic or non-enzymatic, requiring secondary antibodies that bind the primary antibodies to labels conjugated with linkers. The detection system used in this work is based on the biotin-avidin linker. Biotinylated horseradish peroxidase enzyme forms a complex with avidin reagent (AB complex), the complex combines with biotinylated polyclonal rabbit anti-mouse immunoglobulins (secondary antibody) forming efficient link to capture cellular antigen-primary antibody complex. The addition of a substrate chromogen (3, 3' diaminobenzidine DAB plus hydrogen peroxide or 3-amino-9-ethylcarbazole AEC), which is converted by the enzyme label into an insoluble coloured precipitate at the site of antigen localization, is used to visualize the detection.

However, in Immunofluorescence (IF) the primary antibody is conjugated with fluorescent dyes, for example, FITC; or the linker molecule for secondary antibody (streptavidin) is conjugated with a fluorescent dye, for example, AlexaFluor 555 (AF555), which was used in this study. IF was visualized using a confocal microscope.

Cells for ICC and IF were prepared by two methods: slide flasks and cytospin (protocols for cell preparation by both methods are contained in appendix B.7. While the methods for ICC and IF are contained in appendix B.8, the details of antibodies and dilutions are contained in appendix A.3; and reagents and buffers in appendix A.2 for both ICC and IF.

b) ELISA for PSA in culture supernatants

About 10^6 cells cultured as described in Section 3.2.1 and treated in 6-well tissue culture plates with IC_{50}, IC_{10} and half IC_{10} values of steroids. Culture supernatants were harvested at 24, 48 and 72 hours intervals, and concentrated using protocol described appendix B.9. Total PSA in culture supernatants was measured using Microwell total PSA ELISA assay

(Diagnostic Automation Inc, USA) according to the manufacturer's instructions (appendix B.10). The aim of the experiment was to assess the effect of the steroids on PSA secretion. For each concentration of steroid there were triplicate samples at any of the time intervals.

c) Western Blotting (W/B)

Western blotting (immunoblotting) was used to further confirm the expression of some of the cellular antigens identified by the ICC staining. The protocol for W/B is contained in appendix B.11, using some of the antibodies described in appendix A.3. W/B involves 3 steps: sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), blotting (transfer of proteins to membranes) and detection (incubation with primary and secondary antibodies, and chemiluminescent detection using ECL plus (GE Healthcare, UK). Details of the principle of W/B, and the protocol used in this study were described in {William *et al*, 2003}.

The cell cultures (10^6 cells) were harvested using radioimmuno-precipitation assay (RIPA) buffer (appendix A.2) and concentrated using the Eppendorf 5301 concentrator (appendix B.11).

3.2.7 Gene expression studies

a) Hormonal stimulation

Cell lines (10^6 cells) were stimulated in 12-well tissue culture micro well plates using IC_{10} and $1C_{50}$ values of steroids using the protocol described in appendix B.12. Cells were harvested at 12-, 24-, 48- and 72- hrs for RNA extraction. No treatment (culture media only) and DMSO-treated controls were included. The aim of the stimulation was to create sample heterogeneity and assess the effect of stimulation on gene expression of some selected molecular markers.

b) RNA extraction

The cells were trypsinized as described in appendix B.3, washed in sterile PBS and cell pellets lysed in 1.0ml of guanidine isothiocyanate (GITC) buffer; 350µl of which was used for RNA extraction using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instruction as described in appendix B.13. The remaining of the GITC lysate samples was stored at -20^{o}C. RNA was eluted in 60µl from the extraction columns; and 50µl was used for cDNA synthesis. RNA quality and concentration was checked using the BioRad automated electrophoresis system (BioRad, UK) and Eppendorf photometer (Eppendorf, Germany)

c) cDNA synthesis

The amount of RNA used for cDNA synthesis ranged from 0.7µg to 3µg. Equal volume of RNA elute was used in cDNA synthesis using the protocol in appendix B.14. The cDNA synthesis was done immediately after RNA extraction. The same batch of cDNA mix described in appendix B.14 was used through out the study.

d) Polymerase chain reaction (PCR) chemistries

There are various primer and probe design types (PCR chemistries) for RQ-PCR. They include SYBR green, TaqMan chemistry, Molecular beacons, BHQ plus probes, Scorpion primers, Amplifluor primers and Plexor primers. The choice of PCR chemistry depends largely on cost and the sequence detection platform for quantification. In this study, the SYBR green and TaqMan chemistry were used.

The SYBR green chemistry is based on the binding of SYBR green I dye on double-stranded (ds) DNA, the dye fluoresces upon binding to the ds PCR product. Emitted fluorescence is proportional to the amount of amplified product detected in every cycle.

The TaqMan chemistry consists of the conventional TaqMan and the minor groove binding (MGB) TaqMan probes. The conventional TaqMan probes are traditional linear, dual-labeled FRET (fluorescence resonance energy transfer) probes incorporating a fluorescent quencher and fluorophore reporter covalently linked to the 3' or 5' ends of an oligo typically 20 to 30 bases long. Fluorescence is released during the 5' exonuclease activity of Taq polymerase, which cleaves the fluorescent dye upon a probe's hybridization to its complementary sequence. Intact probe does not release reporter signal. In the TaqMan MGB probes, a non-fluorescent quencher replaces the traditional fluorescent quencher and there is stabilization of the 5-6 bp on the 3' end; the probe is therefore shorter and more specific for a given melting temperature.

The list of primers and probes for all the molecular markers measured in this study including the amplicon size and suppliers are contained in appendix A.5

e) 'Real time' quantitative polymerase chain reaction (RQ-PCR)

RQ-PCR offers a highly sensitive tool for accurate, reliable and reproducible quantification of gene expression {Beillard *et al*, 2003; Garbert *et al*, 2003}. It is now widely used in clinical molecular laboratories for diagnosis and monitoring of disease; and can detect a cancer cell in a background of 1 million normal cells {Kaeda *et al*, 2003}. Two methods of quantification are used: absolute quantification (requires plasmid or oligo calibrators for standard curve) and relative quantification (cDNA from cell lines were serially diluted in tRNA in TE buffer- as in appendix B.15).

The Applied biosystems 7900HT sequence detection system was used in all the RQ-PCR according to the manufacturer's instructions. All reactions including standards were set up in 96-well format using a standard mode thermal profile as described in appendix B.15.
For all the RQ-PCR assays, there were six technical parameters for accepting validity of results. The number of PCR cycles at which the relative fluorescent signal intensity of a sample or calibrator crosses a threshold line is known as the Ct value (Cycle of Threshold) and this value is inversely proportional to starting quantity of target transcript.

i. Slope of the standard curve, which determines the PCR efficiency (E) and is related by the formula $E= 10^{(-1/slope)}-1$.Theoretically, a slope of -3.30 corresponds to 100% PCR efficiency. The slope range adopted was -3.30 to -3.60 which corresponded to PCR efficiency of 100 to 90%.

ii. The correlation coefficient (R^2 value) which is a measure of the relationship between Ct value and log of starting quantity for a given standard curve. The R^2 value of > 0.98 was acceptable, although all obtained values were > 0.99.

iii. The Y-intercept of the standard curve, which measures the Ct value that corresponds to a copy of target molecule. The Y intercept was ≤ 40.

iv. The difference in Ct value of duplicate measures (**ΔCt**). Each sample was measured in duplicate and the ΔCt was < 1.0.

v. The relative fluorescence unit plateau value (RFU value) is the maximal normalized fluorescent signal at the PCR plateau. The value was consistently ≤ 1.

vi. Three negative controls**:** GITC treated as sample, water for elution treated as sample and no template control (NTC) which is a re-elution of RNA without the reverse transcriptase. These negative controls appeared undetermined (no reporter signal) for valid RQ-PCR.

For the RQ-PCR of each of the CGs and target transcripts to be acceptable, the six parameters must be fulfilled. The threshold value was set at 0.05, and baseline was auto set.

3.2.8 Statistical analysis

This was performed on results using GraphPad Prism software version 4.02 (www.graphpad.com). For all data sets, the D'Agositino and Pearson Omnibus normality test was performed to determine the distribution. For data sets involving more than two groups, analysis of variance (ANOVA) was performed using Kruskal-Wallis tests (for non-parametric, non-Gaussian distribution) and Dunn's multiple comparison tests. Where two data sets were compared, Mann-Whitney test was performed for unpaired, non-Gaussian distribution. A probability value of < 0.05 was considered statistically significant. Most graphical presentations were in logarithmic scale (power of 10). Where box plots are used, the box represents the upper and lower quartiles and the middle bar inside the box represents the median value. Where aligned dot plots are used, the bar across the plots represents the median value.

3.3 Results

3.3.1 Serum supplementation and Calculation of endogenous testosterone level

Figure 3.1 shows a representative result of the serum supplementation experiment. Results of replicates did not differ significantly from mean values.

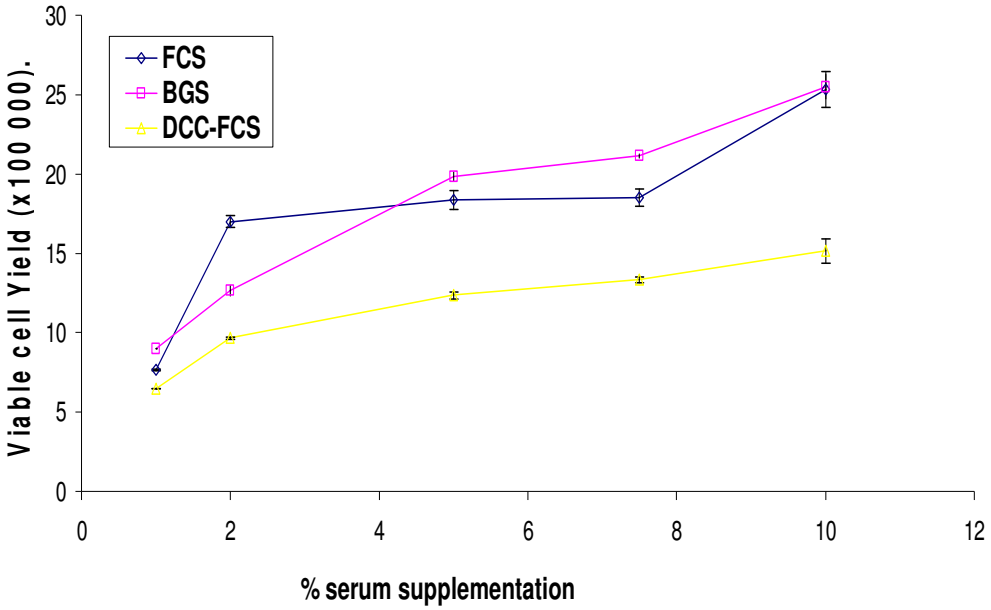


Figure 3.1 Viable PC-3 yields in different sera. There was no significant difference in viable cell yield when all the three serum types were compared using Kruskal-Wallis test (P value= 0.18). Similar results were obtained for PNT1A and MDAPCA 2b cells. Data points represented mean ± standard deviation of triplicate.

All the three serum types supported cell growth for the five days period. Although mean viable cell yields at 10% serum supplementation, for PC-3 cells were 25.4 x 10^5, 25.5 x 10^5 and 15.2 x10^5 for FCS, BGS and DCC-FCS respectively, there was no significant difference when all sera were compared for the entire serial dilution (1 to 10%). There were no detectable morphological changes observed in the daily microscopic examinations

Based on the data provided in the certificate of analysis of each serum from the manufacturer, the corresponding testosterone levels in the sera were calculated as shown in shown in Figure 3.2. At the 10% serum supplementation of culture media, the concentration of testosterone (by calculation) was 0.84, 3.03 and 0.30 ng/dl for FCS, BGS and DCC-FCS respectively.

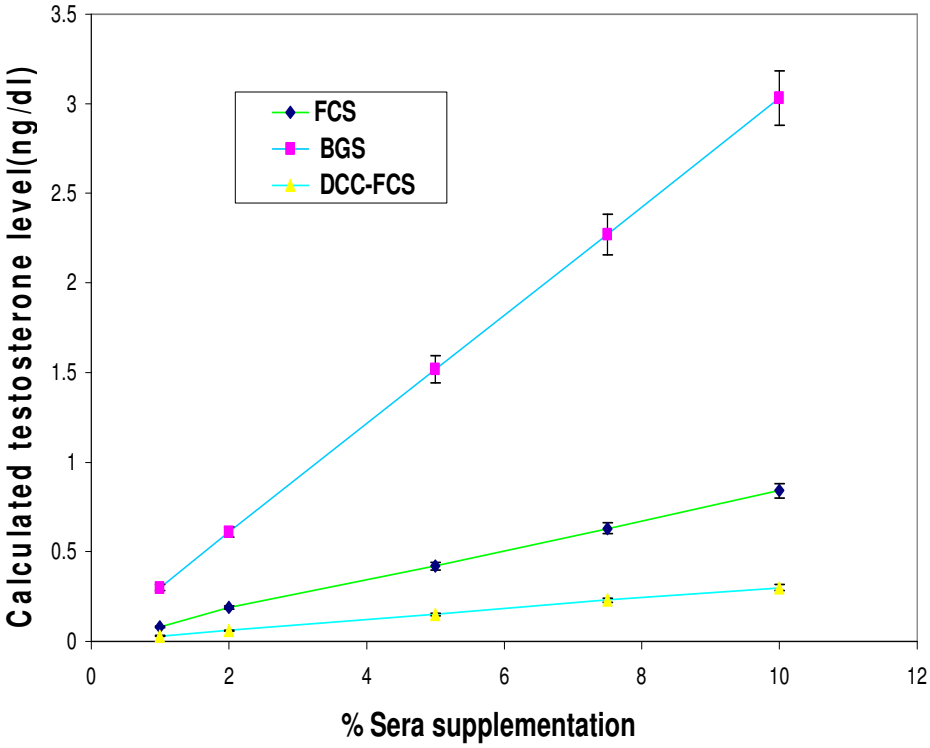


Figure 3.2 Calculated testosterone levels in different sera types. Calculation was based on certificate of analysis provided from the manufacturer for each particular serum type.

3.3.2 Determination of exponential growth phase

Figure 3.3 shows a representative growth curve of PC-3 cells for control group (no treatment), DHT- and ethanol-treated groups as determined by Trypan Blue cell count method. Cells were in exponential phase in days 3 to 8. Similar patterns of growth curves were observed for the cell lines in different steroid treatment at the same 10^{-6}M concentrations. The variations in manual cell count of triplicate (as shown by error bars) could be as much as 1000 cells.

Figure 3.4 shows a representative growth curve for PC-3 cells as determined by SRB assay. The cells were in exponential (log) phase in days 1 to 3 (similar result was obtained for MDAPCa 2b cells). For the PNT1A cells the log phase was 2 to 4 days.

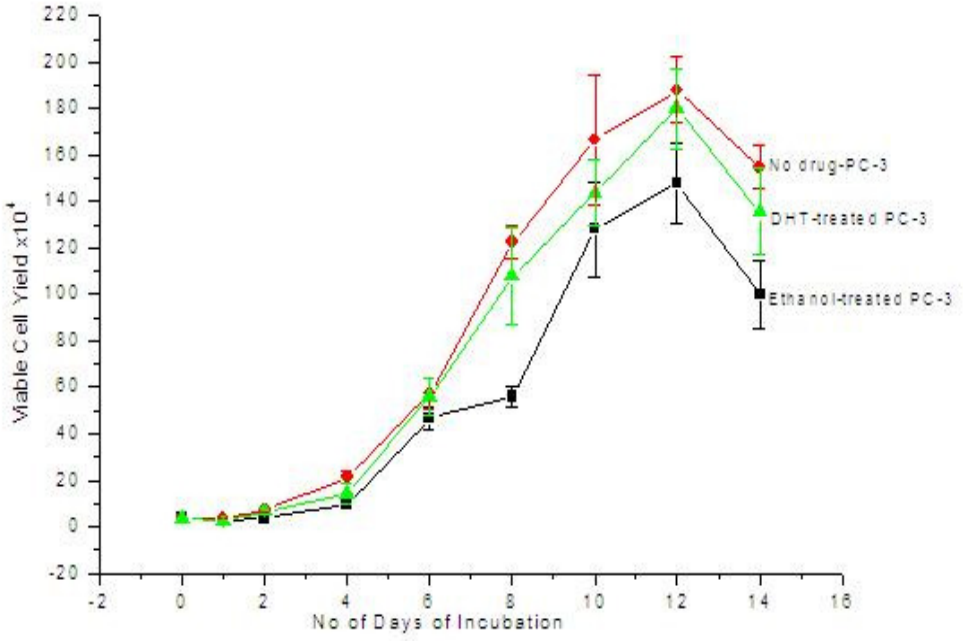


Figure 3.3 Growth curves of PC-3 cells in different treatments. The log phase was 4 to 8 days as determined by Trypan Blue cell count. Data points represent mean ± standard deviation of triplicate.

However, the essence of the growth curve experiments was to determine the log phase where the cells were actively dividing. Harvest of cells for down- stream experiments was done in the log phase.

Figure 3.5 shows a box plot comparison of the total viable yield of PC-3 cells in different steroid hormones over the 13 days period. The analysis of variance (ANOVA) by Kruskal-Wallis test showed no significant difference in the total cell yield of various treatments. The concentration used for these stimulations were low (10^{-6}M).

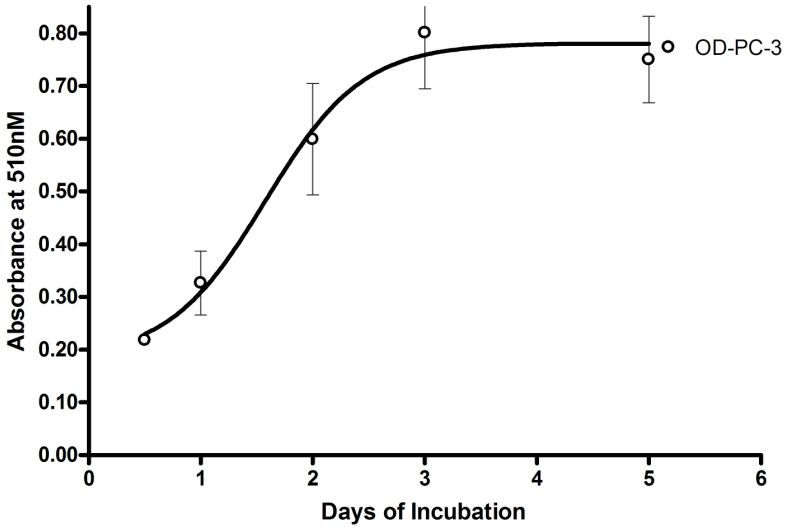


Figure 3.4 Growth curve of PC-3 cells determined by SRB assay. The cells were in log phase on days 1 to 3. Cells were harvested at days 0.5, 1, 2, 3, 5, 7, 9 and 11. Days 7-11 showed a reduction from the plateau, and therefore were truncated in the graph. The log phase for PNT1A cells was 2 to 4 days.

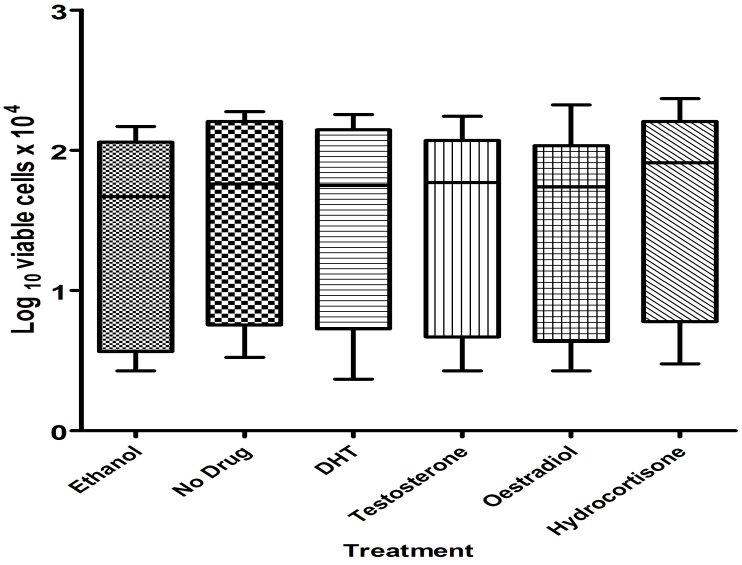


Figure 3.5 Box plots of total viable yield of PC-3 cells over 13 days period in different treatments. The horizontal line in box plots represents the median value. There was no significant different difference in the total cell yield of PC-3 cells under different treatments at same final concentration of 10^{-6}M (P = 0.91, Kruskal-Wallis test).

3.3.3 Change in population doubling and doubling time

Figure 3.6 shows the change in population doubling for various treatments of PNT1A cells. Oestradiol had the lowest change in population doubling, which implied inhibition of cell growth. Hydrocortisone showed a similar inhibitory effect, while the androgens caused increase in population doubling. For PC-3 and MDAPCa 2b cells, oestradiol and hydrocortisone produced similar results but DHT and testosterone did not differ from those of controls. The size of error bars reflected variations that could occur in manual cell count of triplicate over a period of time.

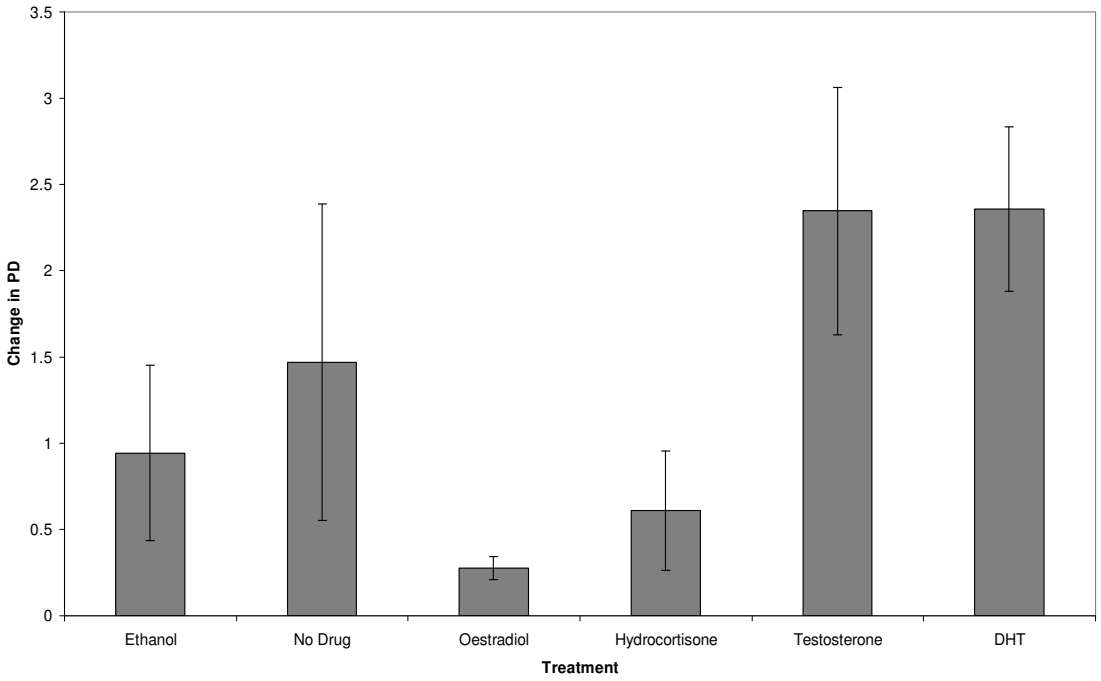


Figure 3.6 Change in population doubling of PNT1A cells. Oestradiol and Hydrocortisone caused a reduction in population doubling (growth inhibition) while the androgens promoted cell growth. But the androgens did not promote cell growth in PC-3 and MDAPCA 2b cells.

The Doubling time (DT) for PNT1A cells treated with oestradiol was 111.2 ± 5.87 hours compared to DTs of 52.60 ± 1.49, 63.8 ± 2.1, 47.2 ± 6.97 and 35.6 ± 4.1 hours for testosterone, dihydrotestosterone, ethanol and no treatment respectively. The DT values for oestradiol-treated PC-3 cells (71.98 ± 7.1 hours) and MDAPCA 2b cells (32.02 ± 6.2) were significantly lower than that of PNT1A cells ($P<0.05$). DT values are usually higher when frozen cells are seeded for expansion. However, cells harvested in log phase and re-seeded tend to have a reduced DT.

3.3.4 Growth inhibition

Figure 3.7 shows the % control growth for PNT1A cells, from which the inhibition concentrations (IC) were deduced. The IC_{10} and IC_{50} values for the steroids were significantly different.

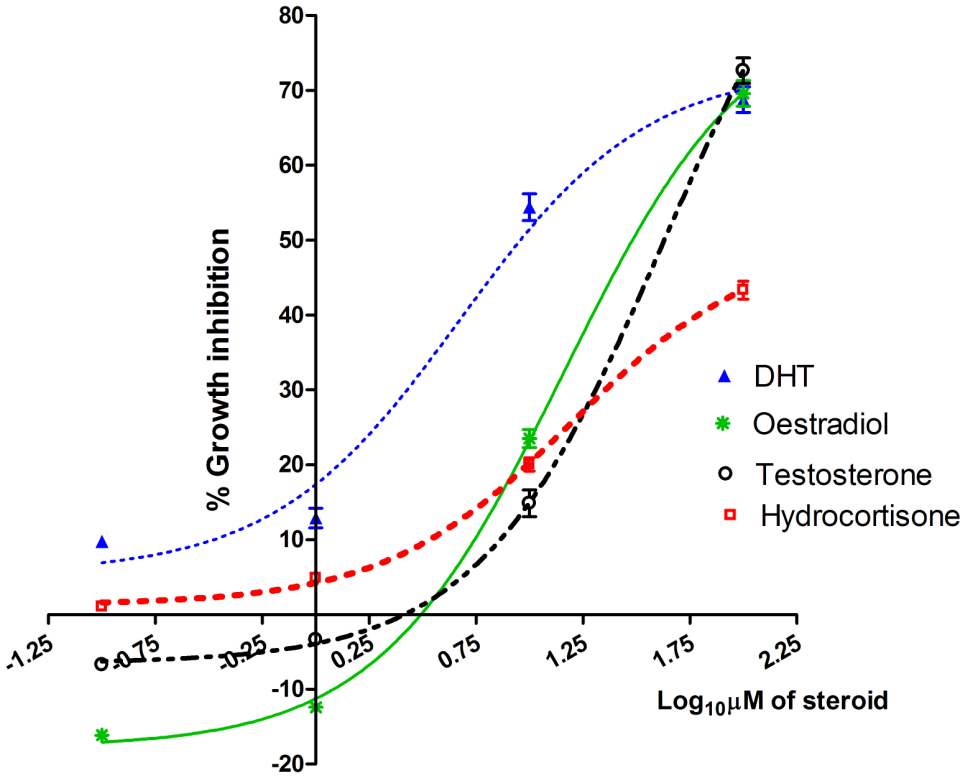


Figure 3.7 Percent control growth of hormones on PNT1A cells. From this curve, the IC_{10} and IC_{50} values were calculated.

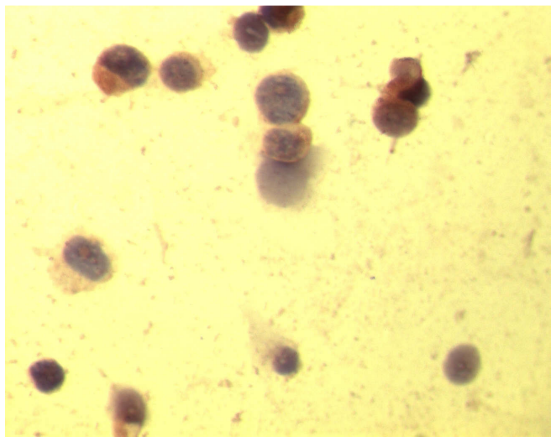
The IC_{10} and IC_{50} values for oestradiol were 4.92 and 33.96µM respectively; 0.86 and 8.44 µM for dihydrotestosterone (DHT); testosterone were 7.63 and 30.42 µM, and for hydrocortisone 4.64 and 261.9 µM respectively.

3.3.5 Immunometric assays

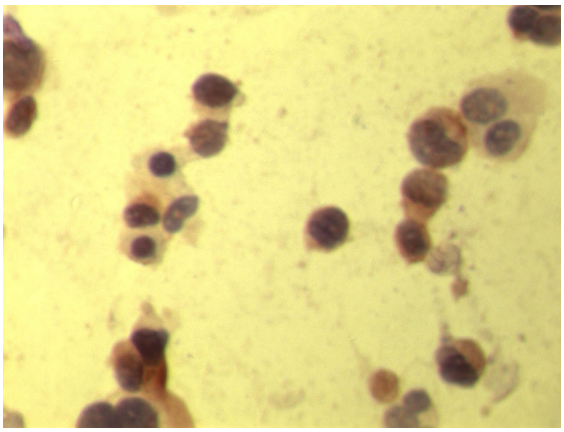
a) Immunocytochemistry and Immunofluorescence

Figures 3.8, 3.9 and 3.10 show representative photomicrographs for immunostaining of selected cellular antigens (more slides are contained in appendix C.7). PSA stained brown in the cytoplasm of all the cell lines. The intensity of PSA stain was strong and diffuse in the PNT1A cells but patchy in the PC-3 cells. The MDA PCA 2b cells also stained strong for PSA.

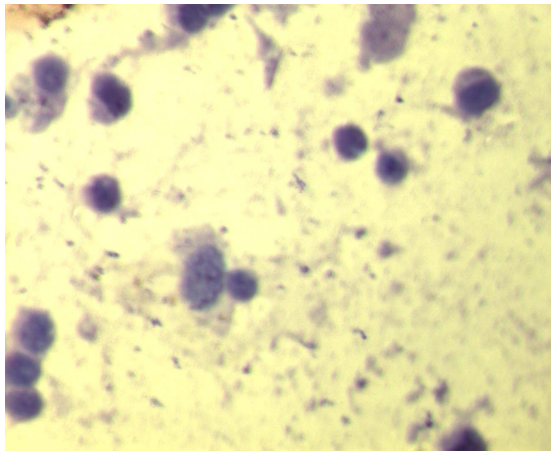
All the cell lines stained positive for AR, ESRα and ESRβ. The nuclei of PC-3 cells stained strongly and diffusely dark-brown for ESRβ; but weakly for AR. There was moderate staining for the ESRα in both MDAPCA 2b and PC-3 cells. The PNTIA stained strongly dark brown for AR more than PC-3 cells and MDAPCA 2b cells. All the cells stained positive for CD44 in the IF; the PC-3 cells stained stronger than the other two cell lines.



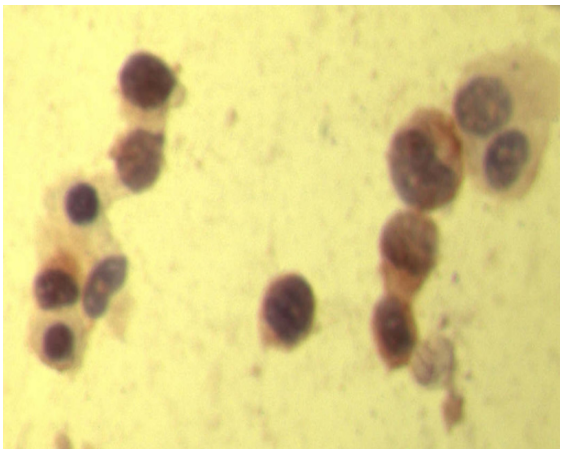
A) PSA positive PC-3 cells.



B) PNT1A: positive for PSA

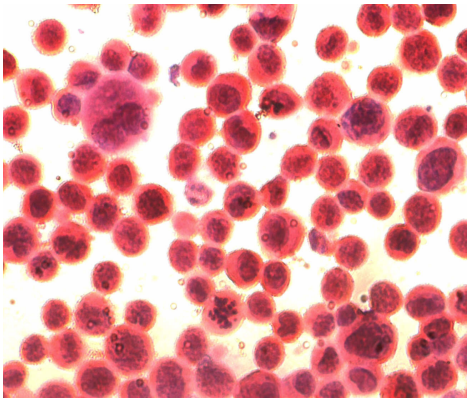


C) PNTIA: PSA negative control

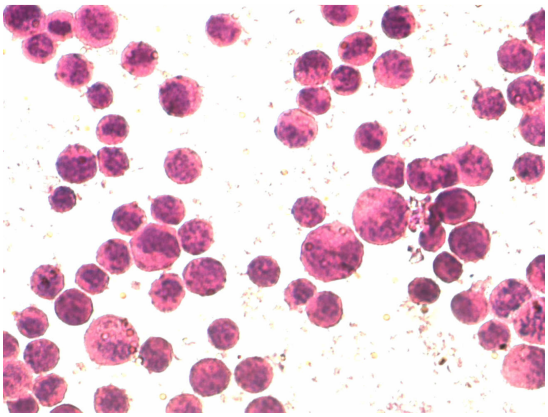


D) MDA PCA 2b: positive for PSA

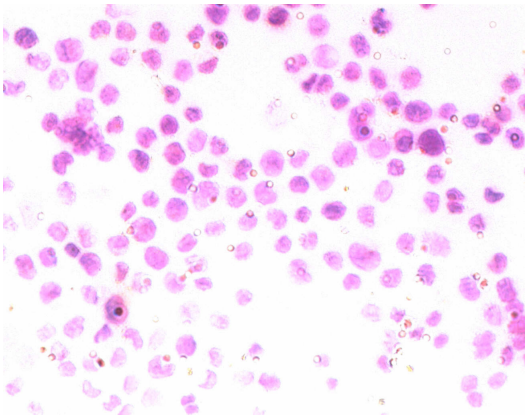
Figure 3.8 Immunolocalisation of PSA in prostate cell lines. A) Patchy cytoplasmic brown stain for PSA in PC-3 cells. B) PNT1A cells stained strong and diffuse for PSA. C) is a negative control slide for PSA stain. D) Moderate and diffuse cytoplasmic staining of PSA in MDAPCA 2b cells. Scale 1cm is 23µm (x 400 magnifications). Cells were prepared by cytospinning.



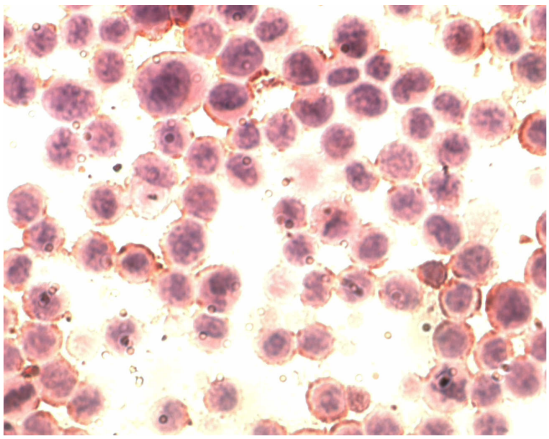
A) ESR beta2 in PC-3 cells



B) ESRα positive PC- cells

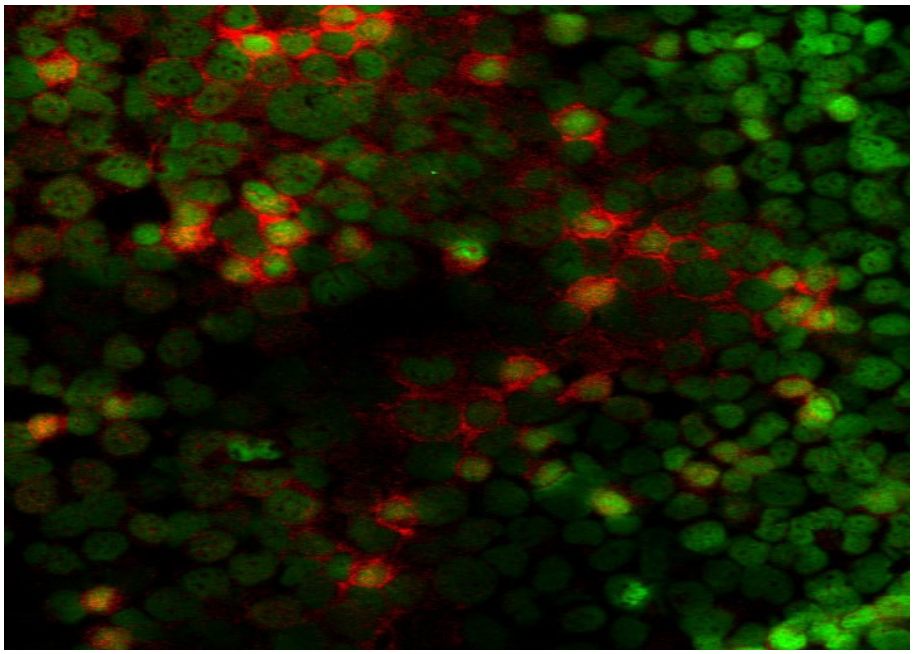


C) PC-3 negative control slide

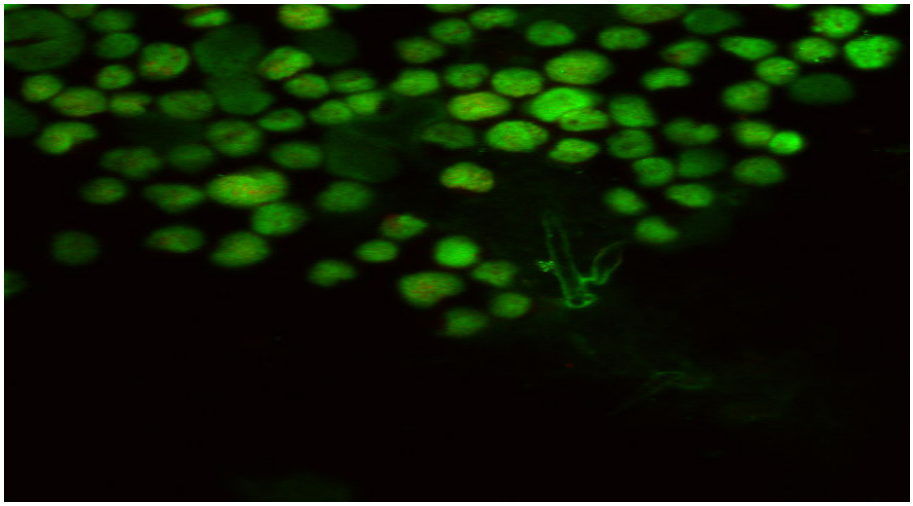


D) AR positive PNT1A cells

Figure 3.9 Immunolocalisation of oestrogen and androgen receptors in prostate cell lines. A = is ESRβ positive PC-3 cells, nuclei stained strongly dark- brown. B = ESRα- positive PC-3 cells, nuclei stained dark brown. C is PC-3 negative control slide. D = AR positive PNT1A cells, nuclei stained strongly dark brown. All the prostate cell lines expressed the three receptors; PC-3 cells stained weakly for AR receptors. Scale 1cm is 23µm. x 400 magnifications. Cells were grown on slide flasks.



A = CD 44 positive PC-3 cells.



B = PC-3 cells Negative control

Figure 3.10 Immunofluorescent localization of CD44 positive PC-3 cells. Alexa fluor 555 fluorophore was used. PC-3 cells expressed more CD44 in the cell membrane than the PNT1A and MDAPCA 2b cells.

b) PSA secretion in culture supernatants

Secreted PSA was not detectable in the culture supernatants of stimulated PC-3 and MDAPCA 2b cells. Figure 3.11 shows the PSA ng/ml in various steroid treatments in PNT1A

cells. In Figure 3.11b, DHT treatment did not produce a significant increase in PSA secretion (P = 0.20, Kruskal –Wallis test) for the 24-, 48- and 72- hours time intervals. However, there was a slight increase in the PSA content of cell lysates in PNT1A cells also treated with DHT (Figure 3.10c) compared to controls, but this was statistically non-significant (P = 0.09). Higher concentrations of oestradiol and hydrocortisone caused a reduction in PSA secretion (Figures 3.11d and e); although these were again statistically non-significant (P = 0.06). Because there were only three time points of measurement (24-, 48- and 72-hrs), the statistical analysis for the group would not be reliably robust.

In Figure 3.11f, low level of testosterone (3.82μM = 0.5 of IC_{10} value) caused an increased in PSA secretion, although statistically non-significant when compared with controls in that group (P= 0.18).

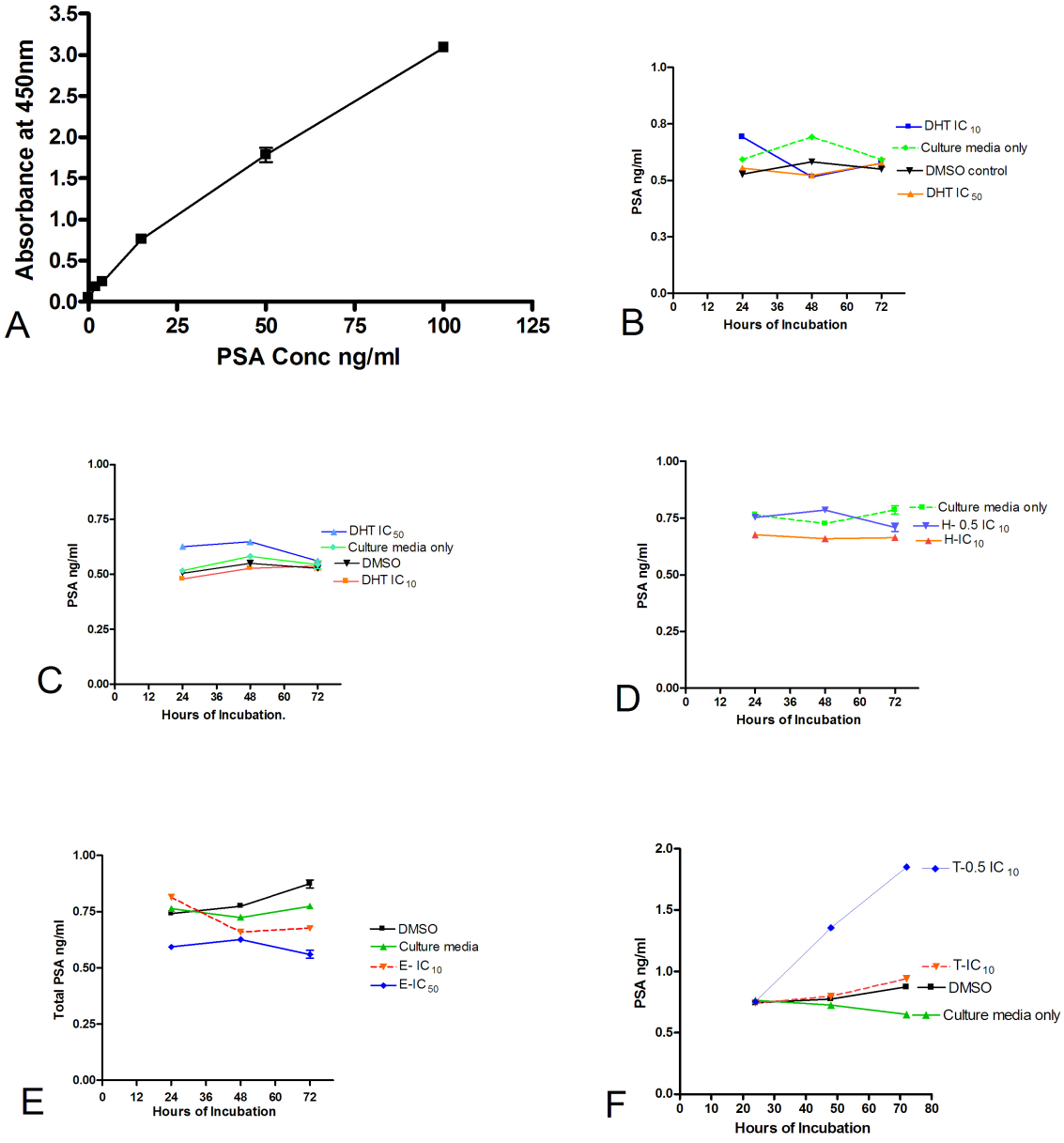


Figure 3.11 PSA levels in culture supernatants of treated PNT1A cells. A is the PSA standard curve; B is PSA DHT treated cells; C is PSA in cell lysate of DHT treatment; D = H- treated cells; E is oestradiol treated cells and F= T- treated cells.

c) Immunoblotting

Figure 3.12 shows the immunoblots for protein extracts from the cell lines. PC-3 cells had very weak expression of PSA and also expressed AR. Both MDAPCa 2b and PNT1A expressed PSA (size corresponding to 40 kDa in the blots). The AR expressed in PC-3 cells and MDAPCA 2b cells were 60 kDa, while the PNTIA expressed a 90 kDa AR protein. All the cells expressed ESRα, ESRβ1 and ESRβ2; the latter was over-expressed in the MDA PCA 2b cells.

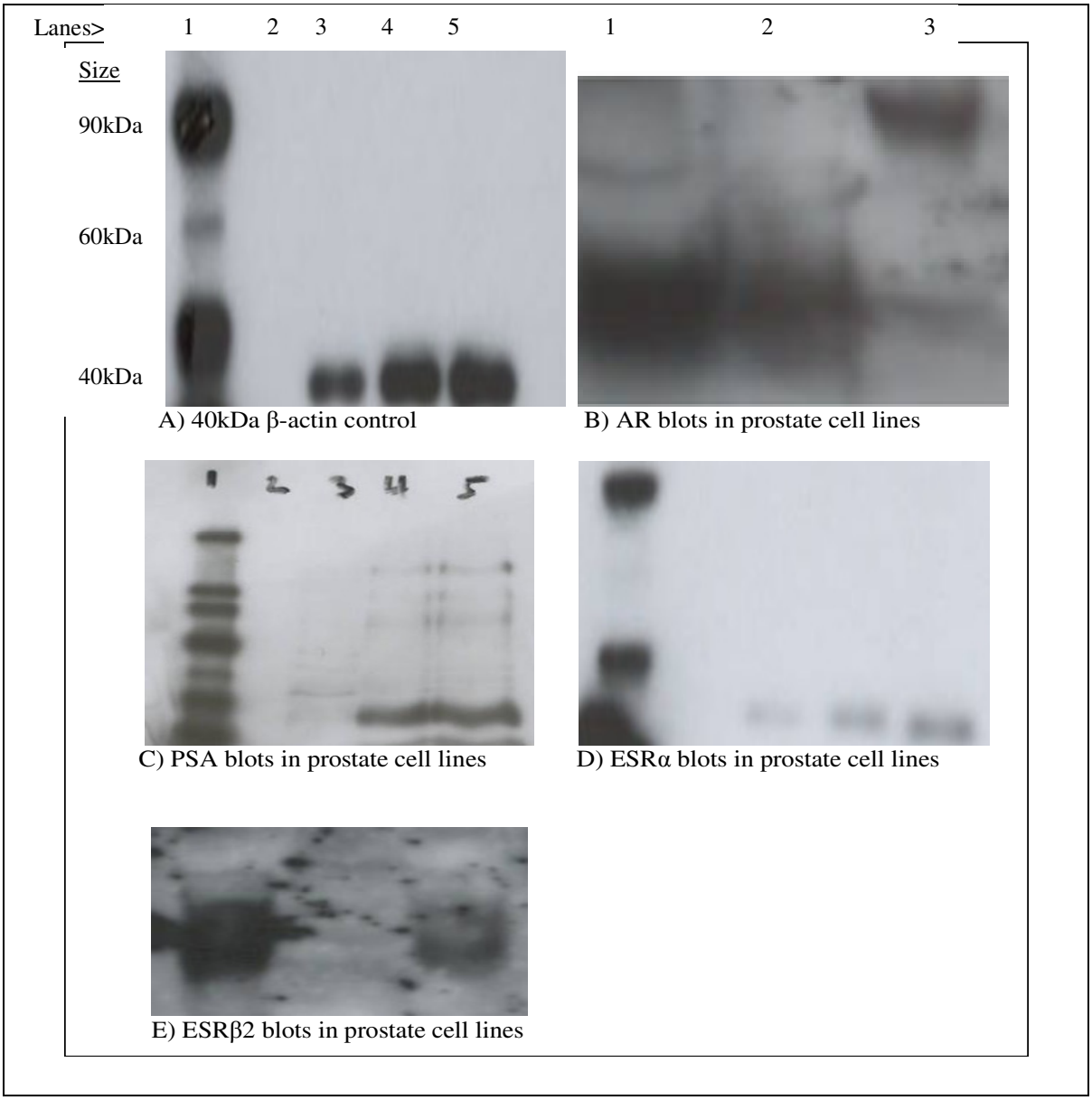


Figure 3.12 Immunoblots of protein extracts from prostate cell lines. A is β-actin blot as a control protein (Lanes 1-5 are ladder, no sample, PC-3, MDAPCA 2b and PNTIA cells respectively). B) AR blots which were about 60 kDa in MDAPCA 2b cells (lane 1) and PC-3 cells (lane 2) but 90 kDa in PNT1A cells (lane 3). C) PSA blots, PC-3 cells did express PSA (weakly) and D) ESRα expressed in all the cell lines; E) 60 kDa ESRβ2 expressed in MDAPCA 2b and PC-3 cells only.

3.3.6 Gene expression of molecular markers

Figure 3.13 shows a representative RNA chromatogram, the average 28S/18S ratio was 1.75 (range 1.61 to 2.01).

Figures 3.14 and 3.15 show representative amplification plot and standard curves. A ten fold serial dilution of plasmid calibrators (10^6 to 10^0) were used in generating standard curves. For each dilution point, the standards were measured in triplicate. From the amplification plot and standard curve, the six parameters of valid RQ-PCR were easily deduced. In place of plasmid calibrators, serial dilutions of cDNA from cell lines were used to construct a standard curve and determined PCR efficiency, especially in normalized relative quantification (NRQ).

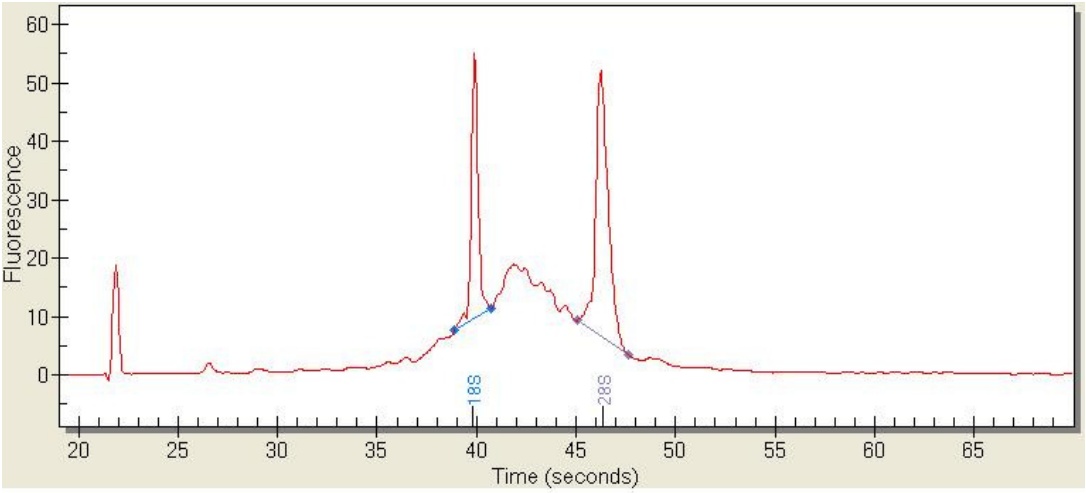


Figure 3.13 A representative RNA Electropherogram. Average 28S/18S was 1.75. Overall RNA quality was good for RQ-PCR.

Figure 3.16 shows the absolute gene expression of candidate endogenous control genes (CG): Abelson 1 gene (ABL1), beta-glucuronidase (GUS) and glucose 6 phosphate dehydrogenase (G6PD). The use of a control gene in RQ-PCR informs assay sensitivity, assesses sample quality and analytical reproducibility. This is achieved by parallel amplification of the target gene and one or more endogenous control genes (CGs).

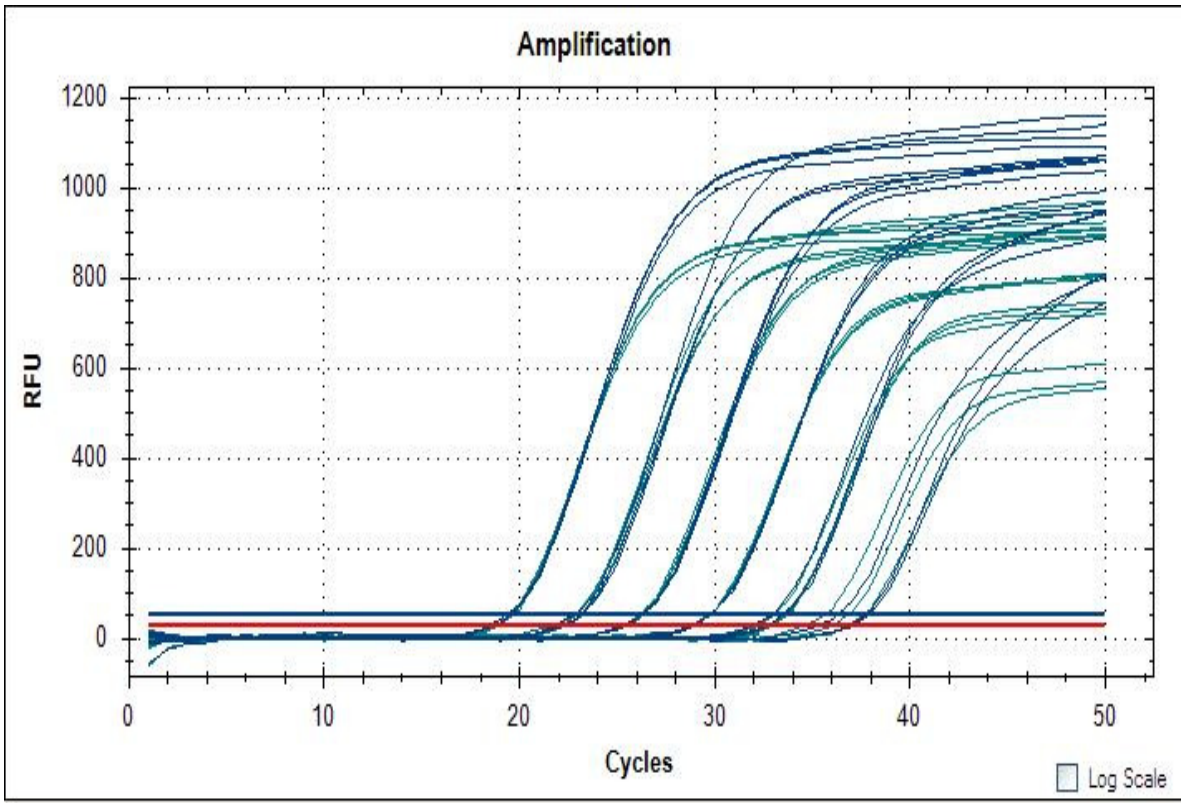


Figure 3.14 Amplification plot of a duplex real time PCR for ABL1 and G6PD.

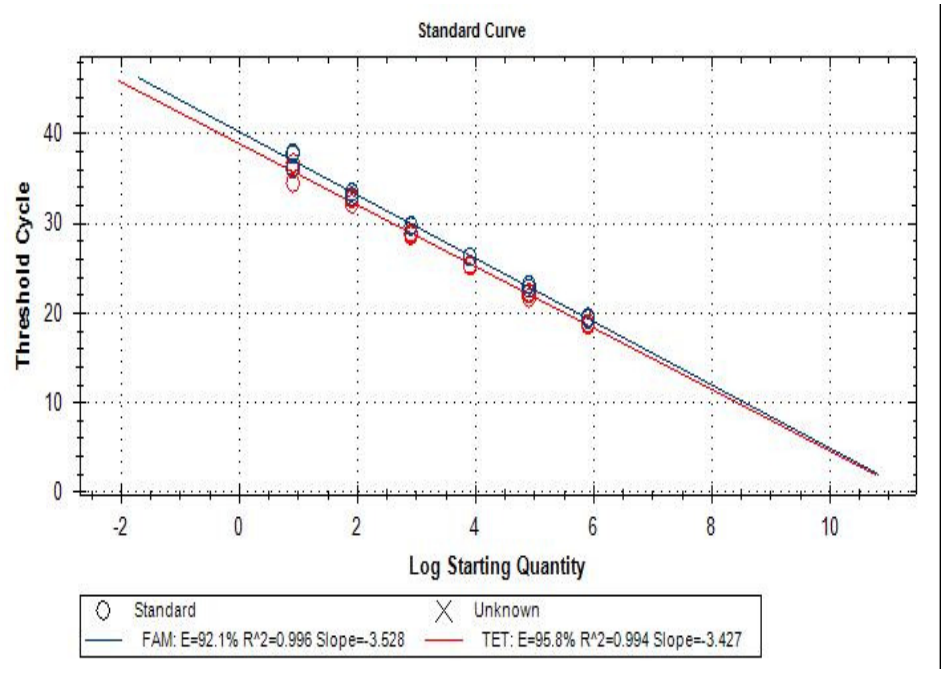


Figure 3.15 Standard curves for a duplex RQ-PCR for ABL1 and G6PD. ABL1 was detected by FAM and G6PD by TET. PCR efficiencies were within the 90 to 100% range (slope -3.3- to -3.60).

There are three main criteria for selecting control genes: absence of pseudogenes, good level and stability of expression, and little variation due to any pre-analytical treatment (e.g. GITC lysis of cells, RNA extraction etc). The three chosen candidate genes satisfied all the criteria.

In Figure 3.16 the median value of ABL1 copies (transcript number) was 1.72×10^4 (range: 3.62×10^0 to 2.22×10^5). There was no significant difference in the ABL1 transcript copies across the three cell lines.

In Figure 3.17 the median value of GUS copies was 1.44×10^4 (range: 6.15×10^0 to 1.56×10^5) in the cell lines; also no significant difference across the cell lines.

In Figure 3.18 the median value of G6PD transcript number was $4.76 \text{ X } 10^4$ (range: 1.71×10^0 to 5.91×10^5) in the cell lines; no significant difference across the cell lines.

The mean value of ABL1 transcript number related to that of GUS by a ratio of 1.19 and to that of G6PD by a ratio of 0.36 across the cell lines. Conversely, the mean value of G6PD transcript number related to that of GUS by the ratio of 3.30 and ABL1 by a ratio 1.25. Both median and mean ABL1 transcript numbers were in between that of GUS and G6PD.

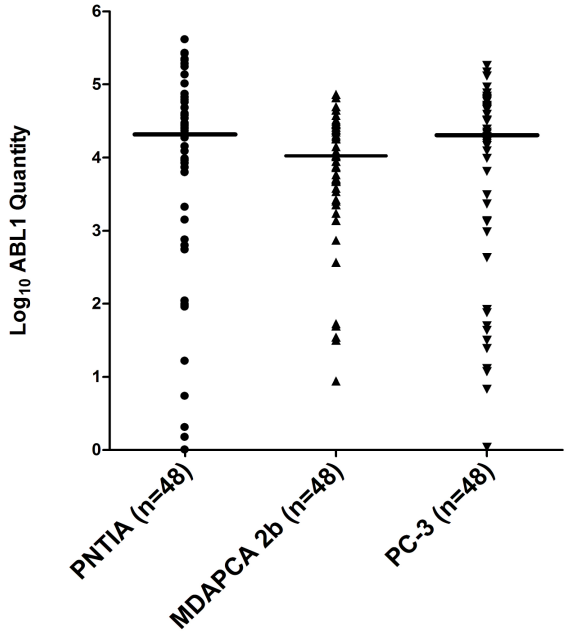


Figure 3.16 ABL1 transcript levels in the prostate cell lines. There was no significant difference in ABL1 transcript number across the cell lines.

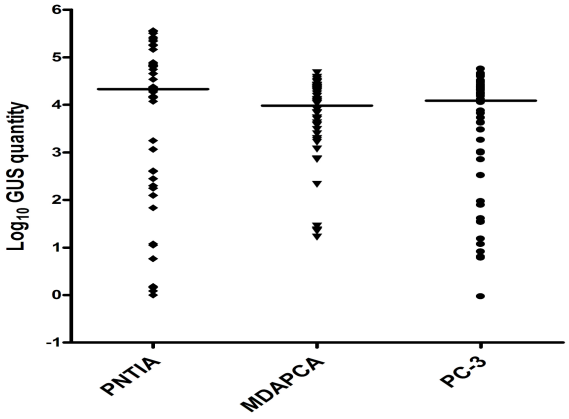


Figure 3.17 GUS transcript levels in the prostate cell lines. There was no significant difference in GUS transcript number across the cell lines.

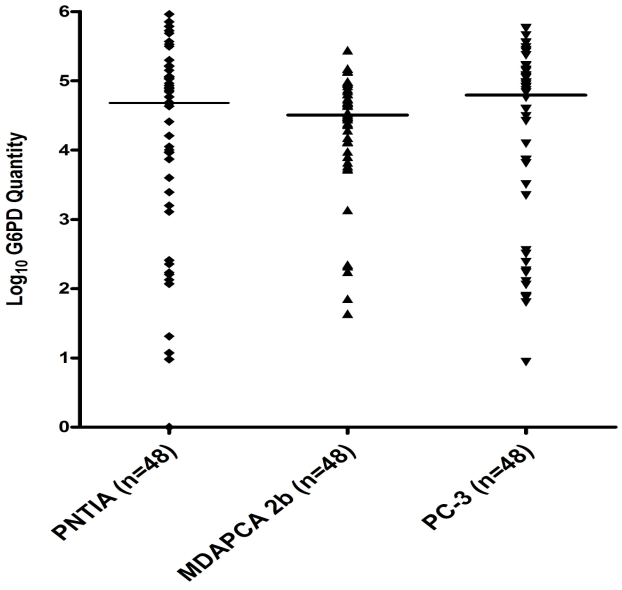


Figure 3.18 G6PD transcript levels in the prostate cell lines. There was no significant difference in G6PD transcript number across the cell lines.

However, in Figure 3.19, the candidate control genes were compared against one another and also against their geometric means (GM). The purpose was to find out if geometric mean of the three CGs

differed from their individual values because it is also a common practice for two or more CGs to be used in normalization of RQ-PCR. There was no significant difference between the GMs and ABL1 transcript number in all the cell lines ($P > 0.05$). There were significant differences between GUS and G6PD in PC-3 cells ($P < 0.01$), between G6PD in PC-3 and GUS in MDAPCA 2b ($P < 0.01$), G6PD in PNT1A and GUS in MDAPCA 2b ($P < 0.05$) and vice versa. There was no significant between either G6PD or GUS and ABL1 across the cell lines. ABL1 transcript was stably expressed across the cell lines.

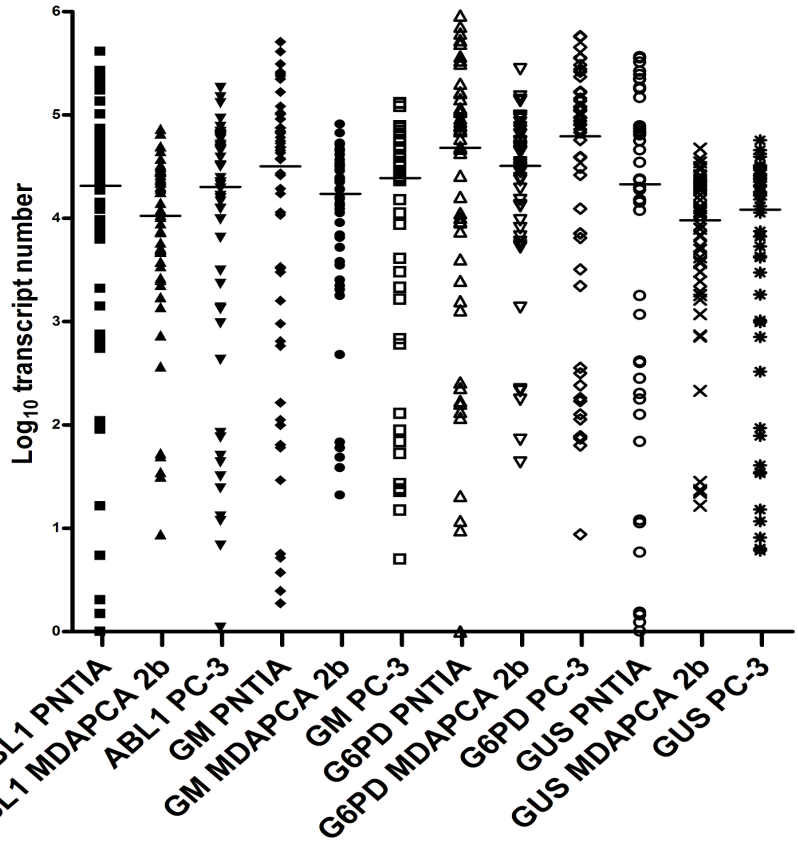


Figure 3.19 Comparison of all control genes with geometric means in the cell lines. There were significant differences between GUS and G6PD in PC-3 cells ($P < 0.01$), between G6PD in PC-3 and GUS in MDAPCA 2b ($P < 0.01$), G6PD in PNT1A and GUS in MDAPCA 2b ($P < 0.05$) and vice versa. There was no significant between either G6PD or GUS and ABL1 across the cell lines. ABL1 transcript was stably expressed across the cell lines.

Figure 3.20 shows the effect of steroid treatment on ABL1 transcript number. In Figure 3.20a, higher dose of 17β oestradiol caused a significant reduction in ABL1 transcript level. This was consistent with previous findings in which 17β oestradiol caused a reduction in population doubling and increased

doubling time of prostate cell lines. Similar variations were observed for G6PD and GUS. In figure 3.20b there was stable ABL1 expression in DHT-treatment.

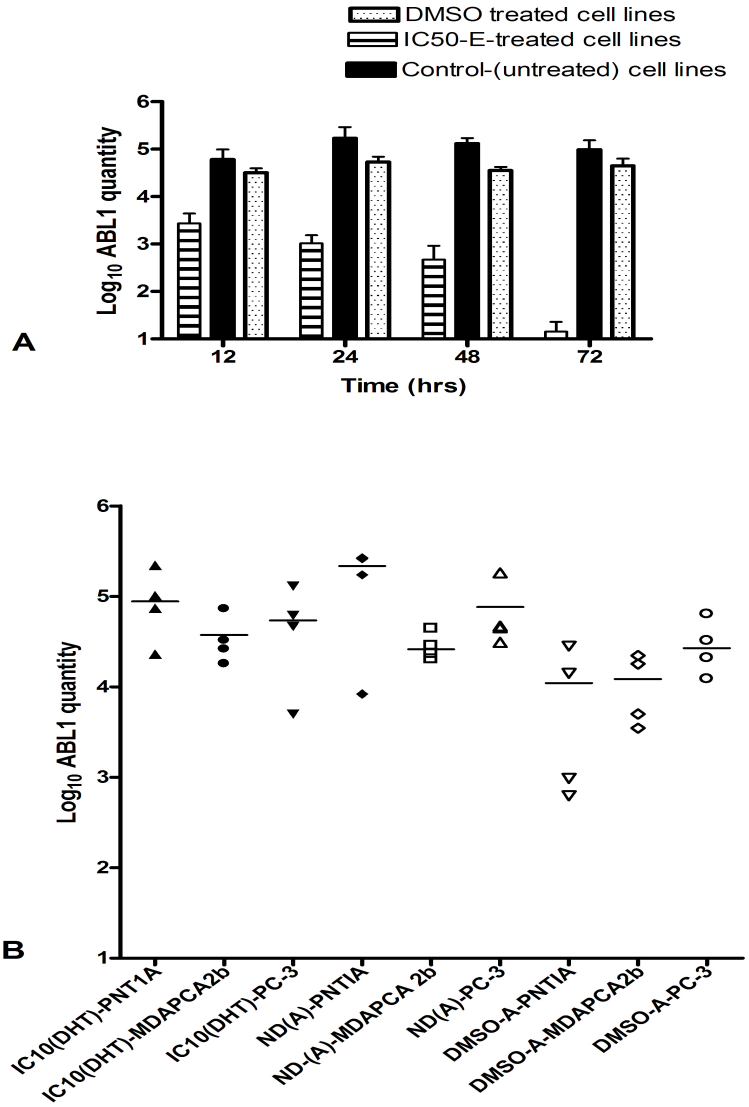


Figure 3.20 Effect of hormonal treatment on ABL1 transcript number. A is 17β oestradiol (E) treatment, B is DHT treatment; ND stands for no drug (culture media only); DMSO = dimethyl sulphoxide used as a solvent.

Figure 3.21 shows the normalized relative quantity (NRQ) of AR in all the prostate cell lines following treatment. The AR expression was calculated from NRQ= $E^{Ct\ AR}$/ $E^{Ct\ ABL}$ where E is the PCR efficiency of each target {Pfaffl, 2001}.

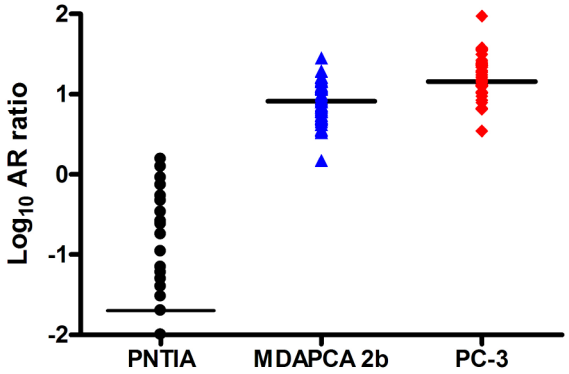


Figure 3.21 Normalized relative expression of AR in prostate cell lines. AR is significantly over-expressed in MDAPCA 2b and PC-3 cells compared to PNT1A cells.

Transcript number of AR was significantly higher in both cancerous cell lines compared to the 'normal' PNT1A cell line ($P < 0.0001$). The primers used in the RQ-PCR of AR were designed to amplify exons 2 and 3, which is preserved in both standard and novel variants of AR reported to date {Hu *et al*, 2009}.

Figure 3.22a shows the gene of expression of ESR beta (oestrogen receptor beta) while Figure 3.22b shows the variation in ESR beta due to 17β oestradiol treatment. ESR beta was significantly up-regulated in the cancerous cell lines compared to the 'normal' cell line (PNT1A). Both AR and ESR beta were significantly up-regulated in the two cancerous cell lines compared to the PNT1A cells. Unlike in the AR expression, the MDAPCA 2b significantly expressed more ESR beta than the PC-3 cells ($P < 0.001$).

17β oestradiol (E) treatment of the cells significantly increased ESR beta expression in surviving cells compared to control groups ($P < 0.05$).

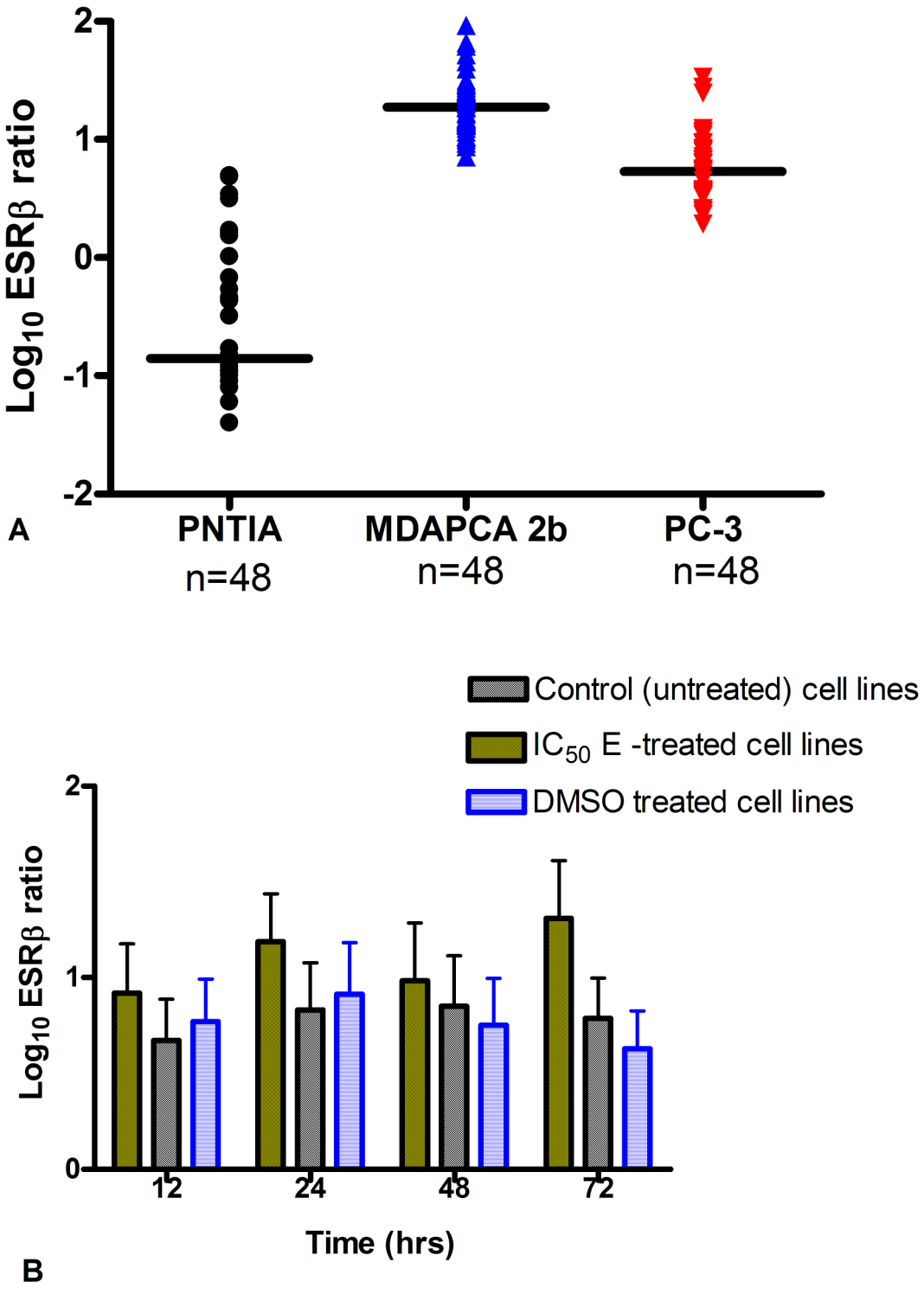


Figure 3.22 Modulation of ESR beta expressions in prostate cells. A is NRQ of ESR and B is variation due to oestradiol treatment.

In Figure 3.23 IC_{10} value of oestradiol did not produce any significant increase in ESRβ expression in the cell lines. Figure 3.24 shows the basal level of ESRβ in all the cell lines untreated for 12-, 24-, 48- and 72 hours.

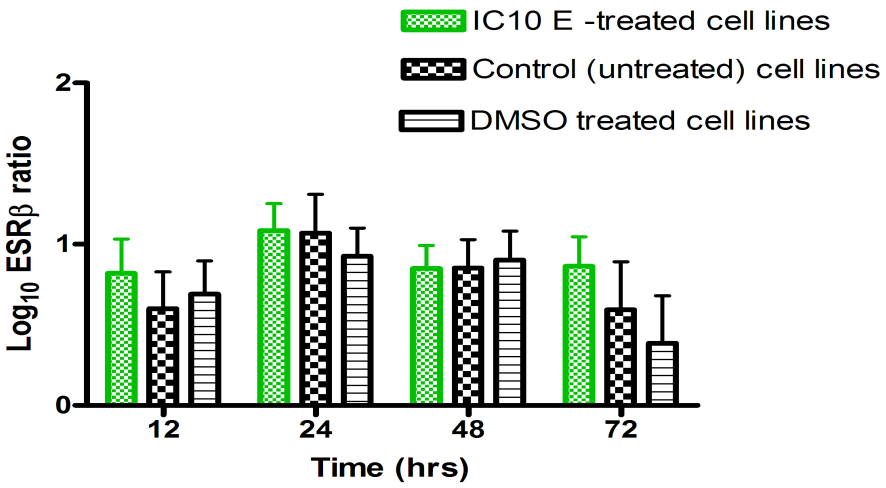


Figure 3.23 Effect of IC_{10} oestradiol treatment on ESRβ gene expression. There was a significant difference in transcript number only at the 72-hr harvest.

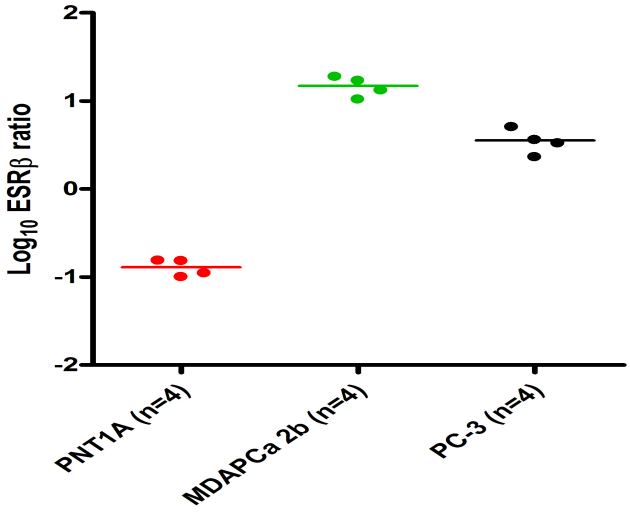


Figure 3.24 Basal level of ESRβ in untreated prostate cell lines. The MDAPCA 2b expressed a significantly higher levels of ESRβ for 12-, 24-, 48- and 72-hours period.

Figure 5.25 shows the effect of hydrocortisone at IC_{10} value on ESRβ transcript level.

Although hydrocortisone showed inhibitory effect on the population doubling in the previous section, it didn't reduce the transcript level of ESRβ. There were significant differences only between non-treated PNT1A cells and non-treated MDAPCA 2b cells ($P < 0.05$) and non-treated MDAPCA 2b and DMSO-treated PNT1A cells ($P < 0.05$). ESRβ transcript number was significantly highest in MDAPCA 2b cells. The androgens-testosterone and dihydrotesterone also did not alter the transcript level of ESRβ in the cell lines.

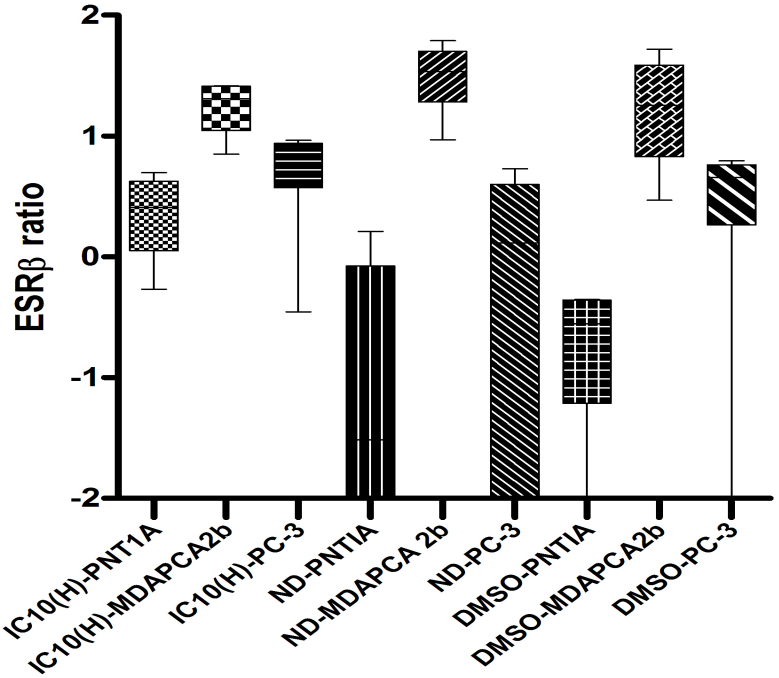


Figure 3.25 Effect of Hydrocortisone stimulation on transcript number of ESRβ Hydrocortisone did not significantly lower ESRβ transcript levels in MDAPCA 2b cells. ND stands for no drug treatment. H stands for Hydrocortisone. DMSO stands for dimethyl sulphoxide, which was used as a solvent.

Figure 3.26 shows that ESRβ transcript number also increased in testosterone treated PNT1A cells but not higher than the increase in the cancerous cell lines.

Figure 3.27 shows the gene expression of ESR alpha in the prostate cell lines. There was no significant difference in ESRα expression between MDAPCA 2b and PNT1A ($P > 0.05$); but PC-3 cells

significantly over-expressed ESRα ($P < 0.001$). ESRα was also up-regulated by the administration of 17β oestradiol (E).

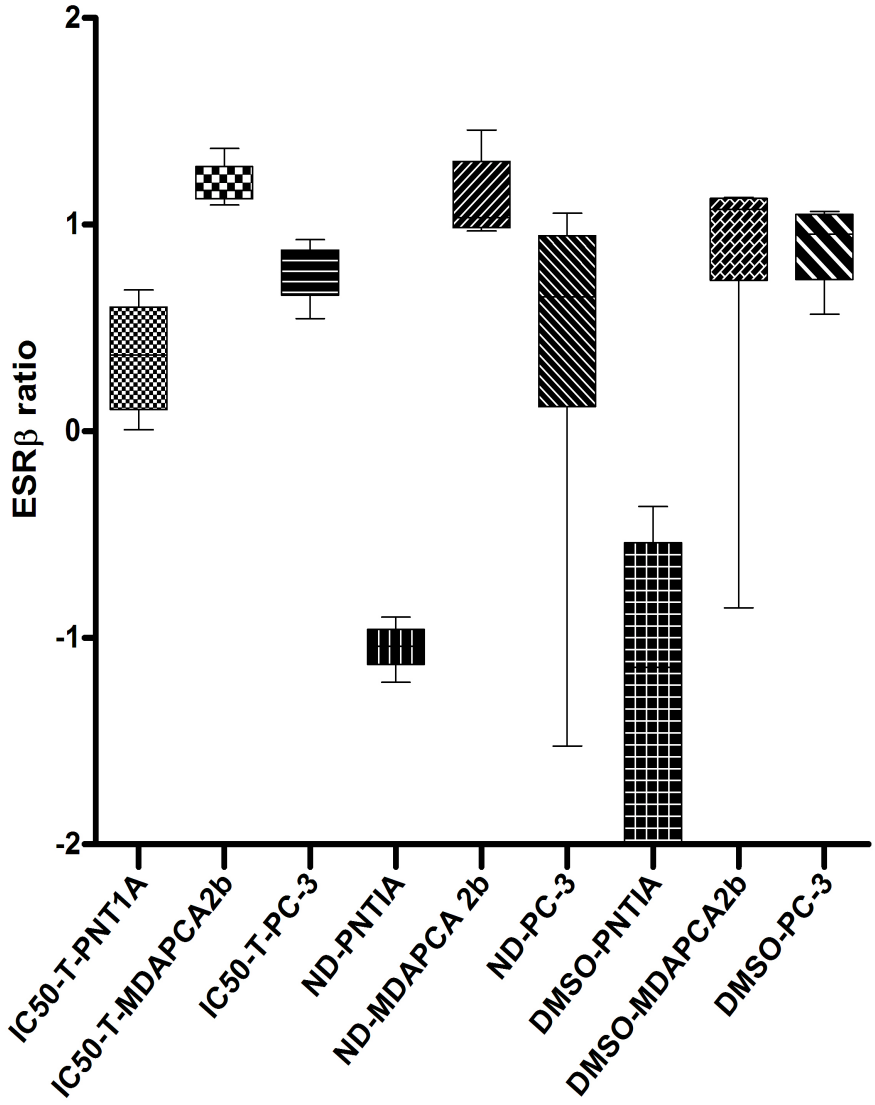


Figure 3.26 Effect of IC_{50} value of testosterone on ESRβ transcript number. PNT1A cells treated with testosterone expressed more ESRβ transcripts but not more than the cancerous cell lines.

Figure 3.28 shows normalized absolute quantification of KLK2 gene. A plasmid standard with KLK2 insert was prepared using the protocol described in appendix B.16. Absolute quantity of KLK2 was divided by that of ABL1, and the ratio expressed as a percentage.

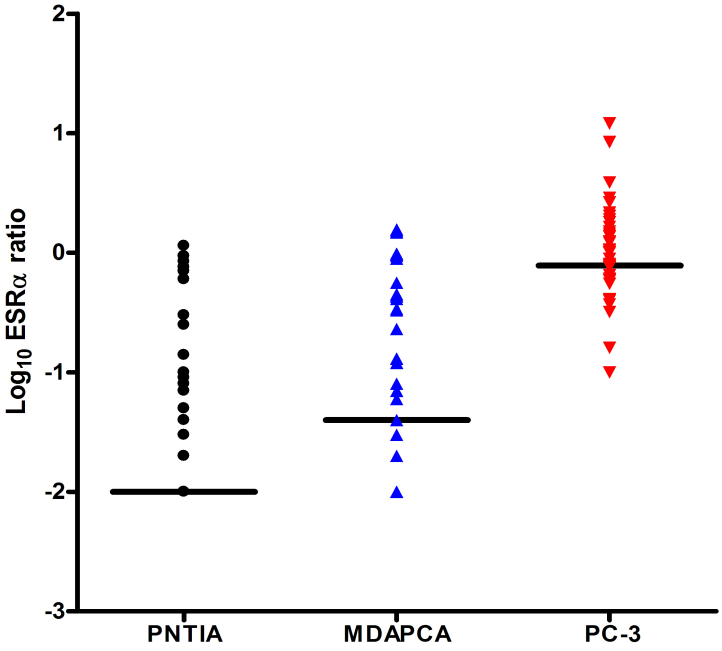


Figure 3.27 Normalized relative gene expression of ESR alpha in prostate cells. ESR alpha was significantly over-expressed in PC-3 cells compared to MDA PCA 2b and PNT1A cells.

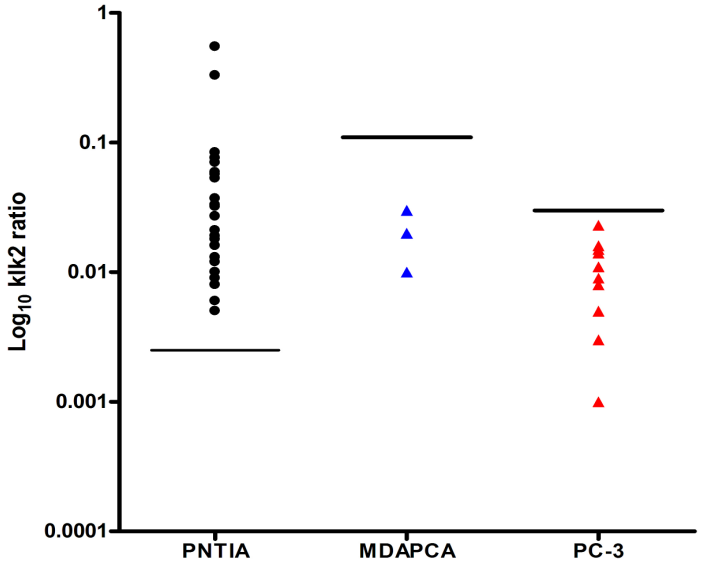


Figure 3.28 KLK2 expressions in prostate cell lines. PNT1A has a significantly higher expression of KLK2 transcripts compared to the cancerous cell lines ($P < 0.05$).

Figure 3.29 shows the expression of CD44 in the prostate cell lines. The cancerous cell lines significantly over-expressed CD44 compared to PNT1A cells.

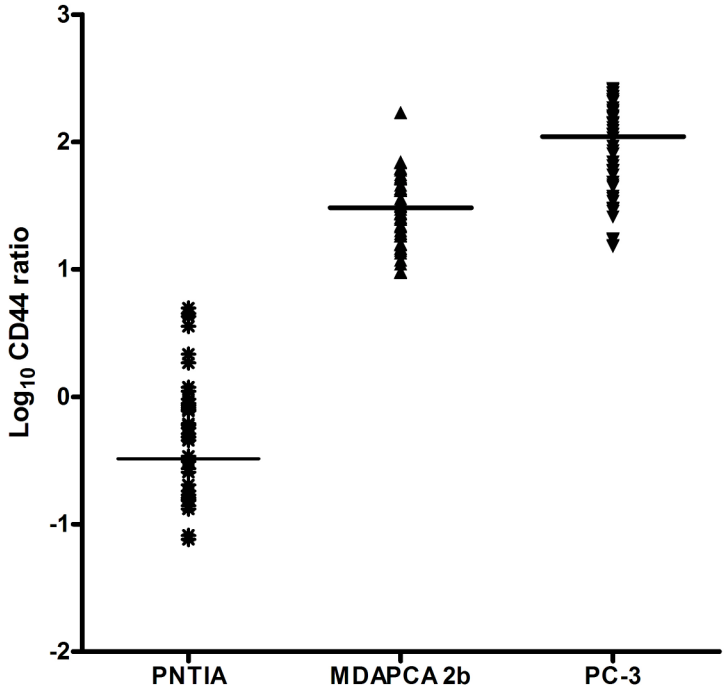


Figure 3.29 CD44 expressions in prostate cell lines. Cancerous cells over-expressed CD44 ($P < 0.05$).

Figure 3.30 shows the expressions of MCM2 and MCM5 replication licensing factors. There was no significant difference in the MCMs expressions in all the cell lines.

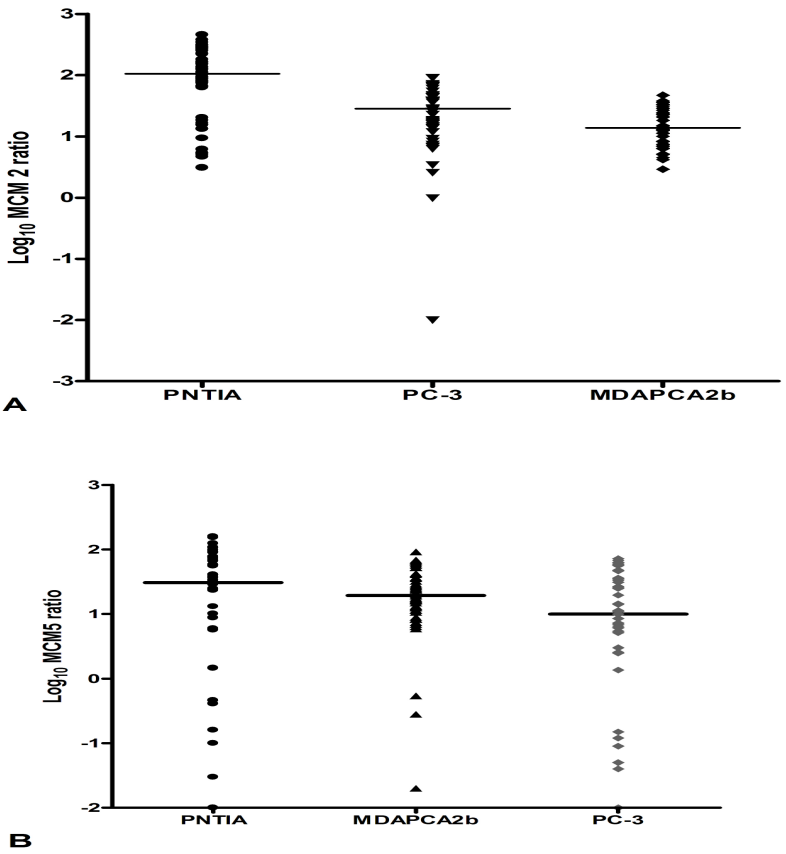


Figure 3.30 Transcript levels of MCM2 and MCM5 in prostate cells. A is the transcript number of MCM2 and B is the transcript level of MCM5 across the prostate cell lines. There was no significant difference in the expression of the MCMs ($P > 0.05$).

Figure 3.31a shows the E-Cadherin type 1 (CDH1) is significantly over-expressed in cancerous cell lines compared to the 'normal' cell line (PNT1A) ($P < 0.001$. In Figure 3.31b, TP53 transcript level was significantly higher in PNT1A cells compared to the cancerous cell lines ($P < 0.001$).

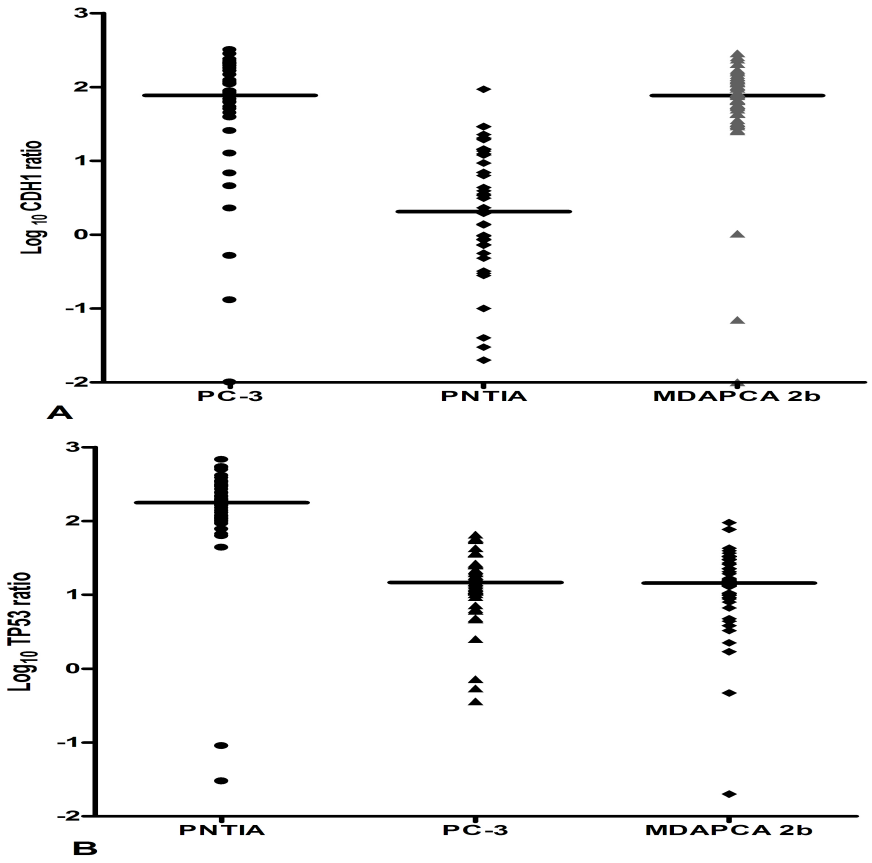


Figure 3.31 Gene expressions of CDH1 and TP53 in prostate cell lines. A shows CDH1 is significantly over-expressed in cancerous cells; B shows higher expression of TP53 in PNT1A cells.

Figure 3.32 shows KLK3 expression in prostate cell lines. There was no significant difference in KLK3 transcript number among the cell lines ($P = 0.24$).

Figure 3.33a shows Bcl-2 transcript levels in prostate cell lines. PC-3 has a significant over-expression of Bcl-2 compared to PNT1A cells ($P= 0.001$). Bcl-2 was also significantly higher in MDAPCA 2b compared to PNT1A cells ($P < 0.05$).

Figure 3.33b shows FASN transcript levels in prostate cell lines. FASN transcript number was significantly higher in MDAPCA 2b compared to PNT1A ($P < 0.05$) but not with PC-3 cells.

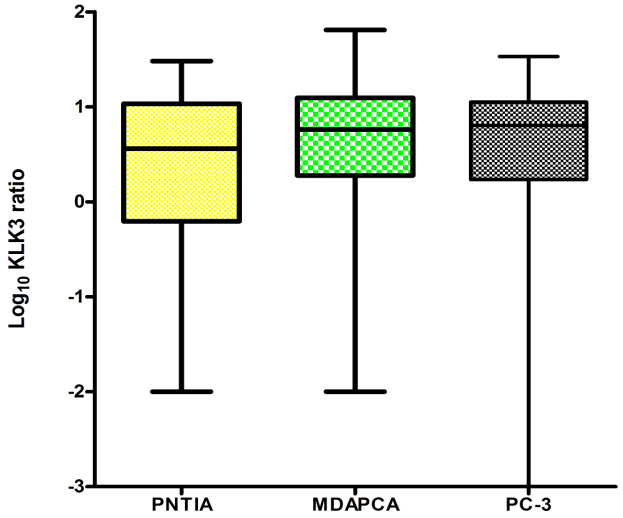


Figure 3.32 Transcript levels of KLK3 in prostate cell lines. There was no significant difference in KLK3 transcript levels in the cell lines.

The TMPRSS2 fusion genes (TMPRSS2: ERG and TMPRSS2:ETV1) were not detected in any of the three cell lines.

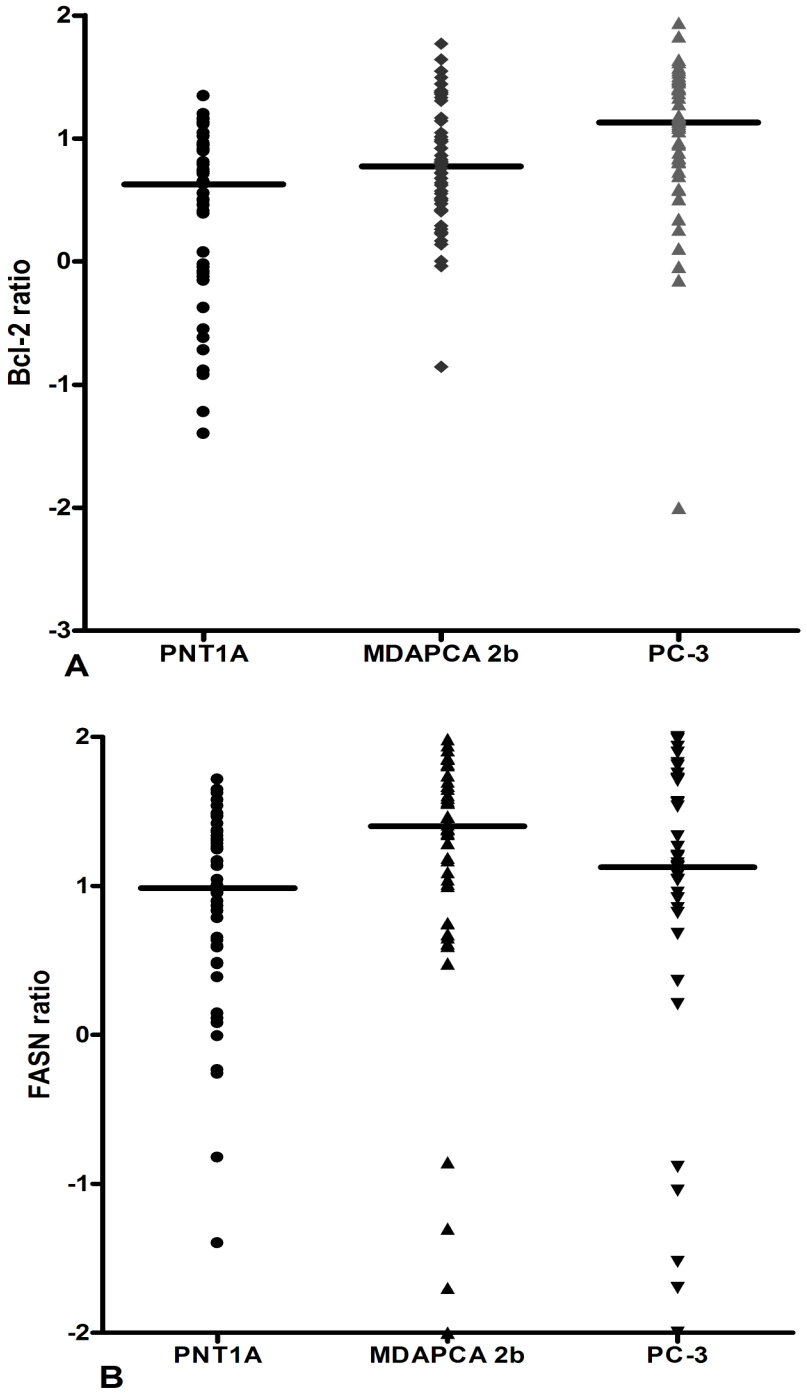


Figure 3.33 Gene expressions of Bcl-2 and FASN in prostate cell lines. A: Bcl-2 was significantly over-expressed in PC-3 cells; B: fatty acid synthase (FASN) was significantly over-expressed in MDAPCa 2b.

3.4 Discussion

3.4.1 Growth characteristics of cell lines under standard culture conditions

Although DCC-FCS supplemented culture media had reduced levels of steroid hormones and proteins, it could support the growth of the prostate cell lines without a significant reduction in viable cell yield. The reduced hormone level, for example testosterone in the DCC-FCS serum, would enable, without masking effect, the observation of stimulatory/inhibitory effects on the growth of prostate cell lines due to steroid treatment.

Two different methods gave comparable results on the growth phases of the prostate cell lines. The exponential growth phase was shorter in the SRB assay, which used the 96-well format compared to the 12-well tissue culture plate. The difference between the two methods could be a result of the effect of limiting surface area on cell growth (contact inhibition). In the SRB assay, 72 hours was optimal in the exponential (log) phase of the three cell lines. Therefore, cells were harvested after 72 hours for the cytotoxicity assays using the 96-well format. But for cell expansion to get sufficient number of cells for downstream applications, 5 days period was optimal for cells grown in the 12-well tissue culture plates and or T75 flasks. Low concentrations of the steroid hormones (10^{-6}M) did not alter significantly the growth curve characteristics of the cell lines. This provided the platform to observe the effects of higher doses' stimulation on the growth of the cells. The concentrations of steroids used in the growth curve experiments were intentionally kept very low to avoid growth inhibition. Yousef & Diamandis (1999) had shown that 10^{-6}M concentration of the steroids would not significantly inhibit growth of the cell lines, which was also confirmed in this study. The viable cell yield remained relatively the same in all the treatments for the 13 days period.

3.4.2 Growth inhibition of prostate cells

The ΔPD takes into account the epithelial nature of the cells as it measures their ability to form monolayer in tissue culture plates {Korkolopoulou et *al*, 2005}. The observed inhibitory effect of oestradiol has also been reported in other studies {Qiao *et al*, 2007; Arnold *et al*, 2007}. The doubling time for oestradiol-treated cells were the longest, confirming the inhibitory effect of oestradiol on prostatic growth In addition, the result also showed that hydrocortisone could significantly inhibit growth of prostate cell lines *in vitro*. Analogues of hydrocortisone are normally used in palliative care of patients with metastatic PCa {Droller, 1997; Heidenreich *et al*, 2008}. They inhibit synthesis of prostaglandins which mediate inflammation, thereby providing pain-relief. Cyclo-oxygenase-2 (COX-2), an enzyme involved in the synthesis of prostanoids including prostaglandins, is over-expressed in around 75% cases of PCa {Thomas *et al*, 2006}. Therefore the activity of hydrocortisone might be directed against COX-2 and similar mediators of inflammation.

Although androgens promoted growth of prostate cells, higher concentrations (more than physiological nM range) appeared to inhibit *in vitro* cell growth. According to Quinn *et al* (2005), dysfunction in the homeostatic breakdown mechanisms of steroids could affect their ligand activity. However, the mechanism of the observed *in vitro* androgen 'toxicity' (at higher micro molar concentrations) to prostate cells is unclear and has not been reported in the literature. But 17β oestradiol and hydrocortisone are known to inhibit prostatic cell growth *in vitro* {Droller, 1997; Thomas *et al*, 2006; Qiao et al; Arnold et al, 2007}. In the report by Qiao *et al* (2007), only 10 nM of DHT was used to induce cell growth while 10 μM of 17β oestradiol was used to inhibit prostatic cell growth in LAPC-4 prostate cells. In addition, the inhibitory range of 17β oestradiol observed in this study (4.92 to 33.96 μM) for inhibiting growth of 10 to 50% of prostate cell population *in vitro* could be useful in designing stimulation experiments with oestrogens. Direct inhibition of AR by oestrogens has also been demonstrated in prostate cells {Hedlund et al, 2003}. Therefore, in vitro studies to evaluate proliferative roles of AR and isoforms of ESR receptors in prostate cell lines would require oestrogen stimulation. These results would guide choice of concentrations for the four hormones studied.

3.4.3 Selection of housekeeping gene for normalization of real time PCR experiments

The overall quality of RNA obtained from the RNeasy mini spin columns was good for RQ-PCR. DNAse digestion was not used, but it could improve RNA quality {Burmeister and Reinhardt, 2008}. Although few studies have been conducted looking at the normalization of prostatic tumour markers, there are no validated control genes applicable for clinical use. In 2006 Schmidt *et al*, reported a quantitative multi- gene expression profiling of nine prostate tumour markers using 4 control genes (GAPDH, HPRT, PBGD and TBP) to normalize gene expression of target amplicons.The GAPDH gene is known to have 60 pseudogenes (www.pseudogenes.org/), which can result in the amplification of non-functional genomic DNA. The PBGD has alternative transcriptional start sites {Beillard *et al*, 2003}, which can produce undetectable transcript variants. The HPRT and TBP are also reported to have pseudogenes, and the TBP shows very low expression level which is tissue dependent {Garbert *et al*, 2003}. Nonetheless, in that study the TBP gene was chosen for normalization because it showed no differential expression between paired tumour and tumour- free samples. In another study, Antonov *et al* (2005) reported the RQ-PCR of degraded RNA of house keeping genes (endogenous control genes) in formalin fixed, paraffin embedded (FFPE) breast tissues. Although the report showed that gene transcript quantification could be done on RNA extracted from FFPE materials, the choice of controls genes like 18S rRNA which is known to have a very high expression in normal tissues {Beillard *et al*, 2003} and GAPDH also known to have several pseudogenes were inappropriate. It is still very common to see many gene expression studies that are normalized using beta-actin gene even though it is known to have 8 pseudogenes. Therefore, the choice of an endogenous control gene for normalizing transcript quantification of prostatic tumour markers is still a crucial issue and a consensus has yet to be found.
The results from the RQ-PCR in this study showed that ABL1 gene was the most stably expressed among the three candidate control genes. It has no known pseudogene, and it had also been evaluated in multi-centre studies for haematological malignancies {Garbert *et al*, 2003}. The ABL1 gene is not tissue specific; however, its expression was stable across different cell types. The stability in expression and absence of pseudogenes are the most important criteria for choosing an endogenous control gene for RQ-PCR normalization and sample quality check.

The reduction in ABL1 transcript number as a result of 17β oestradiol treatment of cells was a direct reflection of the reduction in number of viable cells. A good control gene varies according to cell number in samples. Although G6PD transcript number was also stable, it varied significantly with that of GUS, another commonly used house keeping gene. Good level and stability of expression was observed in the ABL1 transcript number. Therefore, ABL1 was used in the normalization of the

molecular markers. The notion of using two or more control genes for normalization, by taking their geometric mean, does not necessarily improve assay sensitivity. A single stably expressed control gene offers the advantage of multiplexing it with target transcripts for simultaneous quantification, which could reduce inter-run variation and assay cost while making analysis simpler without any change in sensitivity of the assay.

3.4.4 Influence of steroid hormones on expression of steroid receptors

The immunolocalisation of steroid receptors (AR, ESR alpha and beta) in the prostate cells and other endocrine tissues have been variously reported {Isola, 1992; Pelletier & El-Alfy, 2000; Murphy et al, 2006}. Isola, (1992) reported that most breast tumours that expressed ESR alpha also expressed AR. Walton *et al* (2009) also reported the immunopositivity of ESR beta in prostate tissue. The results from this study showed that AR, ESRα and ESRβ were expressed in the three prostate cell lines Contrary to the report by van Bokhoven *et al* (2003), the PC-3 cells stained positively for AR and PSA .The possible explanation to this observation could be a contamination with other cell lines over the previous years of passaging {van Bokhoven *et al* 2001}. Or sublines that express PSA and AR could have emerged over the years of passaging as a result of selective growth pressure.

Although PC-3 cell expressed AR and PSA in the immunocytochemistry and western blots, it did not produce detectable PSA in culture supernatants. The MDAPCA 2b cells also failed to secrete detectable PSA in the culture supernatants It had been reported that prostate cell lines which expressed AR could still be androgen insensitive due to lack of AR promoter function and inactivating mutations {Chlenski *et al*, 2001}.

The AR and ESRβ were significantly over-expressed in the cancerous prostate cell lines compared to the PNT1A; the ESRα was only significantly higher in the PC-3 cells. Their expressions were confirmed at both mRNA and protein levels. Although 17β oestradiol inhibited prostate cell growth, it up-regulated the expression of ESRβ in both time- and dose- dependent manner. This up-regulation suggested a proliferative role for ESRβ in PCa progression. The MDAPCA 2b cells mimic a progressive prostate cancer. It expressed the highest transcript levels of ESR β, which was increased only by 17β oestradiol stimulation. The treatment by hydrocortisone did not lower the expression of ESRβ; neither did the stimulation by androgens. In aging men, the level of oestradiol is increased. A possible hypothesis from these *in vitro* findings could be that oestrogen receptors, predominantly the ESRβ mediate the later stage proliferation of prostate cancer cells. This is consistent with the fact that the administration of DHT and testosterone did not alter the levels of AR in the MDAPCA 2b and PC-3 cells. The PNT1A cells

responded better to the androgens than the cancerous cells. However, the administration of oestradiol also increased the transcript levels of AR in the cancerous cell lines Arnold et al (2007) reported the induction of PSA production by the activity of oestradiol on both oestrogen receptors and AR receptors, and concluded that both receptors mediated the activity of the metabolite dihydroepiandrosterone (a precursor to both androgens and oestrogens). ESR stimulation in general is also known to induce production of AR {Droller, 1997}. It is also possible through the transactivation domain in all the receptors to activate them by common ligands {Hu *et al*, 2009}. Therefore, selective targeting of ESRβ in prostate cancer cells could offer therapeutic advantage in reducing cell proliferation.

The ESRα was only highly expressed in the PC-3 cells which mimic metastatic prostate cancer. Prins *et al* (2001) had shown that ESRα was the key mediator of oestrogenic activity in developing prostate gland of mice. The results from this study suggest a reversal of role for the ESRα. It appeared to play a minor role in proliferation in the later stage progression of cancer; its transcript level and protein expression was lower than those of ESRβ.

In summary, the steroid hormones caused a differential expression of the receptors especially the ESRβ receptor. In view of the overall aim of this study which is the examination of the link between development and progression of prostate cancer and production of diagnostic/prognostic molecular markers, this finding warrants a further investigation into the *in vivo* expression of these receptors in benign, non-involved and prostate cancer cases. The further objective was to find out if the up-regulation of ESRβ gene expression (observed in the cell lines) was associated with prostate malignancy and its phenotype.

3.4.5 Influence of steroid hormones on expression of PSA and KLK2.

Only the PNT1A cells secreted PSA in the culture supernatants; and low levels of testosterone induced the highest production of secreted PSA. Higher levels of DHT did not produce higher secretions of PSA, an observation that was congruent with the inhibitory effect of higher doses of androgens observed previously. Even the cell lysate of PNT1A cells treated with higher dose of DHT did not produce higher amount of cellular PSA compared to lower doses of testosterone. Similar result was observed at the transcript level. PSA mRNA level did not differ significantly in the three prostate cell lines. The immunoblots also showed that all the three cell lines expressed high PSA. However, there was a reduction in PSA secretion due to oestradiol and hydrocortisone treatment compared to testosterone treatment. This was contrary to the report by Arnold et al (2007) but in agreement with Qiao *et al* (2007)

who also reported a reduction in PSA level of culture supernatant as a result of 17β oestradiol treatment. PSA at both transcript and protein levels were inconsistent in correlation with malignancy (prostate cancer cell lines).

The KLK2 was significantly over-expressed in PNT1A cells compared to the cancerous cell lines. Down-regulation of KLK2 transcripts in prostate cancer cells had been reported by Magklara *et al*, (2001). This was contrary to the popular observations that KLK2 are up-regulated in PCa. On the other hand, the PNT1A cells in this study responded to androgen stimulation; and KLK2 is androgen-regulated {Diamandis & Obiezu, 2005}. However, the cancerous cell lines were less responsive to androgen stimulation as observed in lack of PSA secretion in culture supernatants compared to the PNT1A cells. The up-regulation of KLK2 and KLK3 in PNT1A cells is consistent with androgen regulation of human tissue kallikreins {Yousef & Diamandis, 2003}. Normal cells hardly harbour AR mutations, and therefore respond to androgen stimulations unlike prostate cancer cells where AR mutation inactivates its response to ligands. Further *in vivo* studies would still be required to assess the diagnostic/ prognostic value of KLK2 transcript levels in prostate malignancy since it was up-regulated *in vitro* in the prostate cell lines.

3.4.6 Influence of steroid hormones on expression of CD44 and E-Cadherin type 1

Several studies have reported over-expression of standard CD44 in prostate, colorectal and breast cancers {Jung *et al*, 2009, Madjd *et al*, 2009}. Early studies had suggested that only mutant forms of CD44 are over-expressed in prostate cancer {Isaacs, 1997}. In this study, the CD44 over-expression in the prostate cancer cell lines compared to the 'normal' cell line indicated an association with prostate malignancy. However, mutation analysis of CD44 was not part of this study; and it would be required to find out if CD44 over-expression was associated with mutations. The primers for CD44 RQ-PCR were designed to amplify all major transcript variants. It is known that transcript variants of CD44 might be differentially expressed in various tissue types. In this study, the overall CD44 transcript level was consistently significantly higher in the cancerous cell lines. A similar pattern of expression was observed for E-cadherin type 1 (CDH1); its transcript level was also consistently higher in the cancerous cell lines compared to the 'normal' cell line. Quinn *et al* (2005) reported over-expression of CDH1 in metastatic PCa. Isaacs (1997) suggested that over-expressed CDH1 in PCa could be mutated. Standard CDH1 is known to be down-regulated in PCa {Bussemakers *et al*, 2000}. Further in *vivo* studies would be required to confirm the over-expression of CD44 and CDH1 transcript levels in prostate malignancy and

its association with mutations. Their up-regulation in steroid stimulated prostate cancer cell lines indicated an association with malignancy.

3.4.7 Influence of steroid hormones on expression of MCM2 and MCM5.

MCMs are essential for cell replication. All proliferating cells express high levels of MCMs. All the cell lines involved in this study were actively proliferating, and therefore there was no difference in both MCM2 and MCM5 transcript levels.

MCM expression is not tissue specific, and therefore has been associated with many human cancers. For example, urothelial cancers significantly over-express MCM5 [Stober *et al,* 1999}. Prostate cancer cells have also been shown to over-express the MCMs {Freeman *et al*, 1999}. But this in vitro study has shown a very limited value of MCM transcript level in the diagnosis of prostate malignancy. Further *in vivo* study would be required to assess possible association of MCM transcript level with prognosis.

3.4.8 Influence of steroid hormones on expressions of TP53 and Bcl-2.

TP53 was significantly over-expressed in the 'normal' cell line compared to the cancerous cell lines. This suggested that the apoptotic role of TP53 was significantly functional in 'normal cells' compared to cancerous cells. TP53 mechanism is usually dysfunctional in PCa. It is known that TP53 nuclear accumulation in human cancers harbour inactivating mutations especially in exons 5-8 {Oden-Gangloff *et al*, 2009}.

However, the anti-apoptotic agent, Bcl-2 was significantly over-expressed in the cancerous cell lines. It was reported that the over-expression of Bcl-2 confer androgen resistance, particularly in advanced stage PCa {McConkey *et al*, 1996} and that Bcl-2 expression increased with PCa progression {Quinn *et al*, 2005}.

3.4.9 Influence of steroid hormones on expression of FASN and TMPRSS2 fusion genes.

FASN aberrant expression is believed to occur early in PCa development {Benedettini *et al*, 2008}. FASN transcript level was only significantly over-expressed in the MDAPCA 2b cells; there was no significant difference in the PC-3 cells which represents later stage malignancy. There was also no significant increase in FASN gene expression in the PNT1A 'normal' cell lines. This supported the hypothesis that FASN aberrant expression is an early stage event in prostate cancer development.

The TMPRSS2 gene fusions (TMPRSS2: ERG and TMPRSS2:ETV1) were not detectable in the three prostate cell lines. This could be due to complex re-arrangement of the ETS genes with several other 5'

partners, some times difficult to detect in prostate cell lines {Tomlins *et al*, 2007}. Such re-arrangements are detectable by interphase FISH, which was not included in this study.

3.5 Conclusions

- Prostate cell lines provide an *in vitro* model for manipulation of expressions of molecular markers using steroids. The summary of findings in this chapter includes:
- DCC-FCS (a steroid and protein depleted form of FCS) supported growth of prostate cell lines without a statistically significant reduction in viable cell yield. This would enable the observation of steroid stimulation effects on growth of cells and gene expression of molecular markers without 'masking effect'.
- 17β oestradiol and hydrocortisone reduced cell growth by increasing doubling time and reducing change in population doubling. The IC_{10} to IC_{50} range of 17β oestradiol observed in this study (4.92 to 33.96 μM) could be very useful in designing *in vitro* stimulation experiments with oestrogens. The observation also supported growth inhibition of prostate cancer cells by both agents, which could be exploited therapeutically.
- The gene expressions of AR, ESRα and ESRβ and PSA were confirmed at both protein and mRNA (transcript) levels in the PNT1A, MDAPCA 2b and PC-3 cells. They showed differential expression which should be investigated further in vivo model.
- The MDAPCA 2b and PC-3 cells failed to produce detectable levels of secreted PSA in culture supernatants. The PNT1A proved a better model for inducing PSA secretion *in vitro*.
- The PC-3 cells were not responsive to androgen stimulation *in vitro*.
- The KLK3 transcript level in the prostate cell lines was inconsistent with the protein expression and had limited association with prostate cancer cells.
- The KLK2 was up-regulated in PNT1A cells in response to steroid stimulation but down-regulated in cancerous cell lines. The transcript levels of both kallikreins showed little promise for diagnostic utility. The down regulation of KLK2 mRNA in prostate cancerous cells was inexplicable. It requires further investigation especially with regards to the role of regulatory RNAs (micro RNAs in particular) in prostate cancer.
- ESRβ gene expression was up-regulated by 17β oestradiol in both dose and time dependent manner.

- The expression of ESRβ transcripts in the prostate cell lines supported a proliferative role for late stage prostate cancer cell proliferation. Therefore selective targeting of the ESRβ could offer a therapeutic advantage in controlling late stage prostate cancer proliferation.
- The ESRα could play a minor role in late stage prostate cancer cells proliferation.
- The CD44 and CDH1 were significantly over-expressed in prostate cancer cells and could be associated with progression of prostate malignancy.
- The transcript levels of MCM2 and 5 showed a limited value for application to prostate cancer diagnosis.
- TP53 gene was over-expressed in normal cells but down-regulated in prostate cancer cells, while the Bcl-2 was over-expressed in the cancerous cell lines.
- The FASN transcript level supported an over-expression in early stage prostate cancer development
- The TMPRSS2 fusion genes were not detectable in the three prostate cell lines.

The findings from the *in vitro* studies provided answers to the overall research questions. The gene expression of some of the steroid hormone receptors were significantly altered by the hormonal stimulation, in particular the ESRβ .The gene expressions of some of the selected molecular markers were also significantly altered due to hormonal stimulation. Because the interplay of steroid hormones and gene expression are vital in the pathogenesis of prostate cancer, further *in vivo* studies were designed in subsequent chapters 4 and 5 to evaluate these markers in prostate diseases. The cell line model is still far from representing *in vivo* situations in human tissues.

Table 3.2 Summary of change in normalized transcript ratio of biomarkers in prostate cell lines.

Increased in prostate cancer cell lines compared to normal cell line	Decreased in prostate cancer cell lines compared to normal cell line	No significant change between prostate cancer cell lines and normal cell line
• **ESRβ** • **CD44** • **CDH1** • **Bcl-2** • **FASN**	• **KLK2** • **TP53**	• **KLK3** • **MCM2** • **MCM5** • **ESRα**

Chapter Four

Genetic variants of KLK2 gene in Prostate Cancer.

Chapter 4: Genetic variants of KLK2 gene in Prostate Cancer.

4.1 Introduction: Several single nucleotide polymorphisms (SNPs) have been associated with prostate cancer risk as previously discussed in Section 2.7 of this thesis. In this chapter, the frequency and association with prostate cancer risk of alleles of KLK2 gene was investigated. The KLK2 gene encodes the human glandular kallikrein.protein (hK2) which was discussed in Section 2.3.2. The aim of this chapter was to find out if SNPs in the KLK2 gene (alleles) could predict the presence of prostate cancer at biopsy, and if they had any association with the disease phenotype. SNPs in the entire KLK2 gene were investigated using three different techniques: Direct DNA re-sequencing, Allelic discrimination assay (TaqMan genotyping) and Pyrosequencing.

4.2 Materials and Methods

4.2.1 Ethical Approval: Thirty healthy male participants (with no history of prostate disease or current urinary symptoms) were recruited at Cranfield University, and favourable ethical approval was obtained from the Cranfield Health Ethics Research Committee. Sixty consecutive patients who attended the TRUS-prostate biopsy sections for prostate cores at the Primrose cancer unit, Bedford hospital, were recruited, and favourable ethical approval was obtained from the Bedfordshire Research Ethics Committee. All the participants gave their informed consent before sample collection. One hundred and thirty eight archived FFPE prostate tissue blocks were obtained from both Cheltenham and Gloucestershire hospitals, and favourable ethical approval was given by the Gloucestershire Research Ethics Committee. All the documents for ethics application and the approval letters are contained in appendix D.

4.2.2 Experimental design

The study on clinical samples were designed to sample urine, blood and formalin fixed, paraffin embedded (FFPE) samples from patients and controls with the exception of healthy controls from Cranfield University who were sampled for urine and blood only. Power analysis for sample size was calculated using the NCSS/PASS software {McGraw, USA}. Gene expressions were measured in both urine and FFPE samples. In two of the centres (Bedford hospital, n = 60; Cranfield University, n = 30), the sampling was prospective; and another centre (Gloucester and Cheltenham, n = 138 of FFPEs only) the sampling was retrospective. The clinical samples were evaluated for gene expressions of selected molecular markers; and genetic variations of KLK2 gene. Clinical data including patient's age, patitent's

ethnicity, PSA at referral, Gleason Score, Clinical stage and Prostatic volume were extracted from clinical notes. FFPE tissue samples were prepared from prostate cores; sections for molecular analysis were cut adjacent to those which had been used for histopathology diagnosis and grading.

4.2.3 Sample collection and Processing

a) Blood Sample: 5ml of venous blood was collected into K_3 EDTA tubes from each of the participants (healthy males (control group, n=30) and patients (n= 60) using standard venepuncture techniques. Plasma was harvested after centrifugation at 1800g for 5 minutes, and stored at -20^{o}C until required for use. The cell sediments were lysed and white cells harvested using the protocol in appendix B.17. White cell pellets were lysed in 350µl of GITC for genomic DNA extraction. Samples from healthy controls were not processed for genomic DNA extraction.

b) Urine sample: The participants voided 20ml of early morning urine. Within 6 hours of sample collection, the urine samples were centrifuged at 1800g for 10 minutes, decanted and cell sediments washed in 15ml of sterile PBS (Invitrogen, UK). Cell pellets were lysed in 350µl of GITC buffer for RNA extraction. The cell lysates were homogenized manually using 2ml syringes and blunt end needles. The decanted urine was stored at -80 ^{o}C for thermal desorption gas chromatography mass spectrometry (GC-MS) analysis.

c) Archived FFPE tissue sections: Tissue blocks (from 2006 to 2008 from Gloucester and Cheltenham, n = 138; and 2008 from Bedford n = 60) were chosen based on pathology reports (all Bedford patients were included because their urine and blood samples were collected). For Gloucester and Cheltenham, selected samples were 30 cases of nodular hyperplasia (BPH); 5 cases of Gleason score ≤ 5 (very low grade prostate carcinomas); 30 cases of Gleason score 6; 30 cases of Gleason score 7; 30 cases of Gleason score 8-10 (high grade) and 12 cases of non-involved prostate tissues (cases of chronic inflammation, no dysplasia, no carcinoma). Only tissue blocks with highest lesions of its category were sampled for microtomy. Two pieces of 25µM thick sections were cut using decontaminated microtome blades, and were aseptically picked into 2ml eppendorf tubes. The tubes were briefly centrifuged, deparaffinised in two washes of 1ml xylene for 10 minutes each; the xylene decanted and the tissue rehydrated by two washes in 1ml of 100% ethanol before allowing the pellets to dry for 5 minutes on a dry heat block kept at 37^{o}C. The tissue pellets were digested overnight in 540µl of ATL tissue lysis buffer and 60 µl of proteinase K (Qiagen, UK). The digest was centrifuged for 5 minutes and the

supernatant (containing nucleic acids) were collected. A 350µl of the supernatant was used for RNA extraction and 200 µl used for genomic DNA extraction.

4.2.4 Nucleic acids extraction and cDNA synthesis.

RNA was extracted from urine cell lysates and FFPE tissue lysates using RNeasy spin columns (Qiagen, UK) using the protocol described in appendix B.13; cDNA synthesis was done immediately by random hexamer priming using the protocol described in appendix B.14. Genomic DNA was extracted from white cell lysates and FFPE tissue lysates using QiaAmp DNA mini kits (Qiagen, UK) using the Qiacube automated DNA extraction system (Qiagen, UK) according to the manufacturer's instruction. DNA was eluted in 100µl. Genomic and complementary DNA was stored at -20^{o}C until required for analysis. Quality of gDNA from FFPE materials were checked by both bioanalysis using the BioRad automated electrophoresis system and by PCR amplification of 150bp G6DP.

4.2.5 DNA sequencing

The Big dye terminator direct sequencing technique was used to determine the nucleotide sequences of KLK2. The Big dye terminator sequencing is based on cycle sequencing in which successive rounds of denaturation, annealing, and extension in a thermal cycler results in linear amplification of extension products (Figure 4.1). The dideoxyribonucleotides (ddNTPs): A, C, T, and G are labelled with fluorescent dyes (four colours are generated). The fluorescent labelled DNA products (fragments) are then injected into a capillary for electrophoresis and subsequent detection. The ABI 3130 genetic analyzer platform (Figure 4.2) was used.

The KLK2 gene was divided into four PCR product sizes, amplified with primer sets shown in Table 4.1. First, a standard PCR reaction was carried out as described in appendix B.18; the quality of amplification was checked by 2% agarose gel electrophoresis and Syber safe staining (appendix B.19). Second, a big dye terminator reaction was set up in 96-micro well PCR plate (Applied biosystems, UK) using the protocol described in appendix B.18. Third, the PCR products were purified using the big dye x terminator purification kit (Applied biosystems, UK) as described in appendix B.18 according to the manufacturer's instructions. Fourth, the purified PCR products were loaded into the genetic analyzer, and run according to manufacturer's instructions. Fifth, after the run, the data were inspected, analyzed and transferred into a DNA sequence editing software (DNA star) which aligned sequences from all samples and identified nucleotide differences. Canonical sequences of KLK2 gene were copied from the

UCSS genome browser, which also highlighted known SNPs.Only gDNA from blood samples of Bedford patients (n = 60) were sequenced.

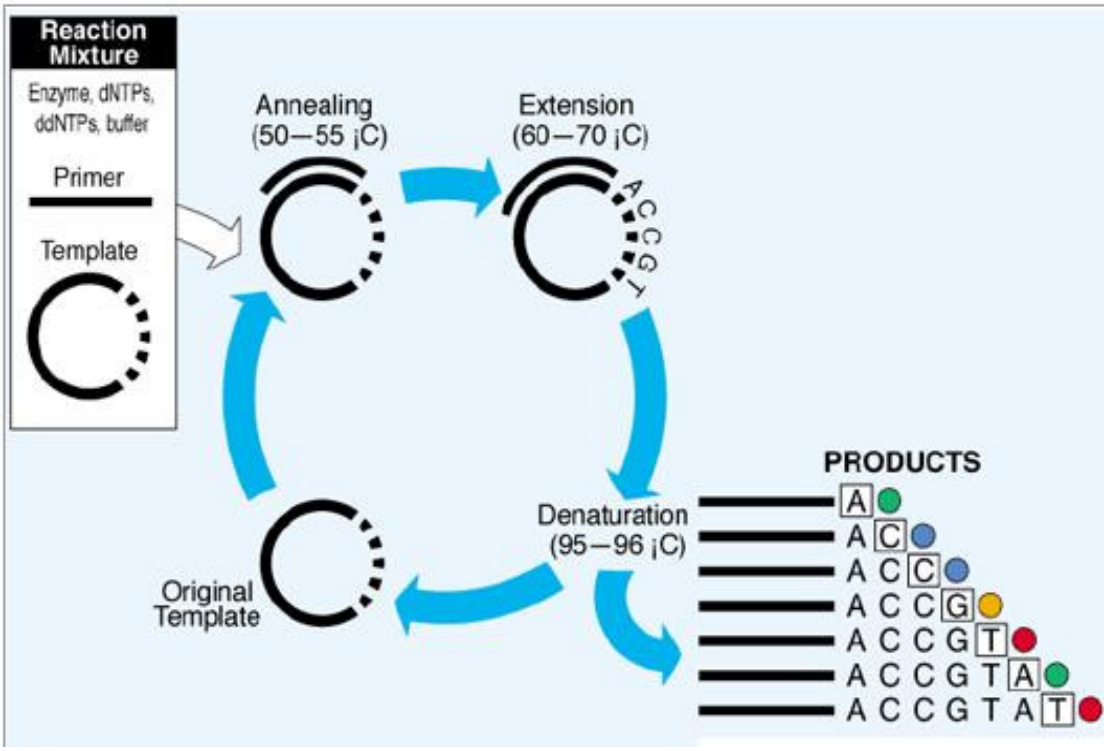


Figure 4.1 An illustration of big dye terminator sequencing reaction. Fluorescent dyes are used in labeling ddNTPs which give specific colours in the electropherograms. {Applied biosytems, 2009}.

Figure 4.2 The ABI genetic analyzer 3130. This is a four capillary electrophoresis sequencing platform. It uses the 96-well micro PCR plates.

Table 4.1 Sequencing design for the KLK2 gene {Nam *et al*, 2006}

Exon	Size (bp)	PCR product size (bp)	Primer name	Primer sequence
1	88	578	1F (forward)	CAGCATCTAGGTGCCAACAG
			1R (reverse)	CAGGGTTGAATGCTCACAGC
2	159	410	2F (forward)	CTACAGAATTGCCAGCCCTC
			2R (reverse)	AGCCTCACCTGAGAAGTGAC
3 + 4	424	664	3F (forward)	TGGAGTCTCCCTTATCCTCC
			3R (reverse)	CTTCACTCACCTTTCCCCTC
5	202	474	4F (forward)	TGTTCCCTCTGTTGGACTCC
			4R (reverse)	CACACCAGAAAGCACAGGTC

4.2.6 Allelic Discrimination Assay

The use of direct DNA sequencing for interrogation of genetic variants is limited by its cost and labour-intensiveness. Although a definitive method, the direct sequencing is also not suitable for genomic DNA from FFPE tissue materials due to defragmentation of such gDNA by fixation. However, most FFPE tissue materials could yield 100% on less than 200 bp gDNA, which would still be insufficient for most common sequencing techniques. A new technique for genotyping based on TaqMan chemistry real time PCR application was used to genotype gDNA from the FFPE tissue materials (n = 138).

This method of allelic discrimination (AS) is based on the use of two different dual-labelled TaqMan MGB probes (commonly FAM and VIC) to detect the alleles of a gene which differ in a nucleotide sequence. The primer sets would be the same but the probes would differ, targeting specific sequences of the alleles. A single nucleotide difference alters the melting temperature of the PCR products, resulting in fluorescent intensity that is indicative of a particular allele or its combination (See Figures 4.3 and 4.4 for illustration). This is a cheap, flexibly high throughput and reproducible technique for clinical genotyping. It uses the same platform for RQ-PCR (gene quantification), in this case the Applied biosystems 7900HT sequence detection system was used

The 60 samples (from Bedford patients) that were already investigated by direct DNA sequencing were also tested by the TaqMan genotyping assay for validation of the new assays prior to using the assays on DNA from FFPE tissue materials. Pre-designed TaqMan genotyping assay kits for SNPs rs198977 and

rs198972 were purchased from Applied biosystems, UK. The protocol for setting up the genotyping assay is described in appendix B.20.

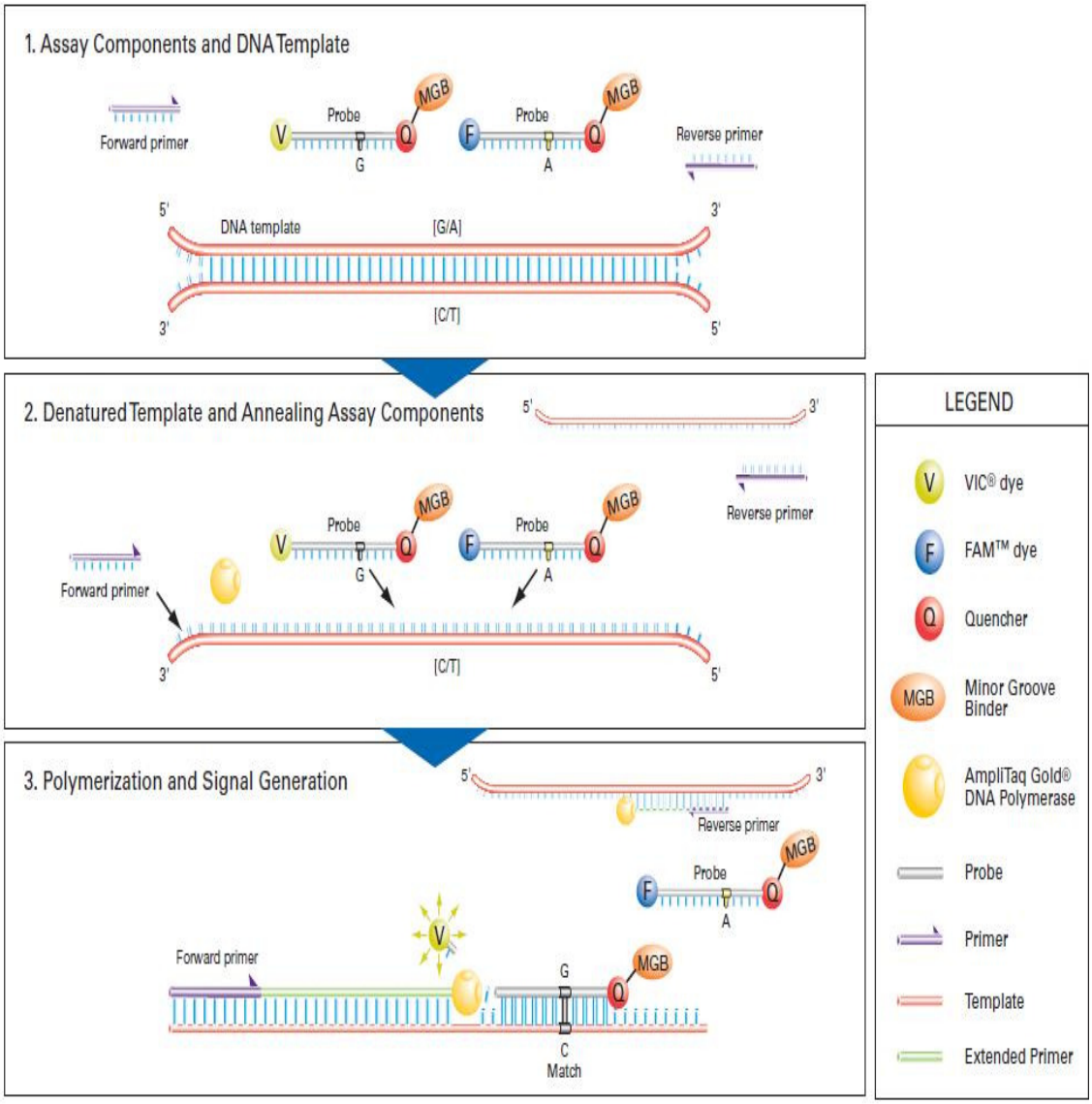


Figure 4.3: A schematic illustration of TaqMan allelic discrimination assay. FAM and VIC labelled probes are used to target alleles. {Applied biosystems, UK}.

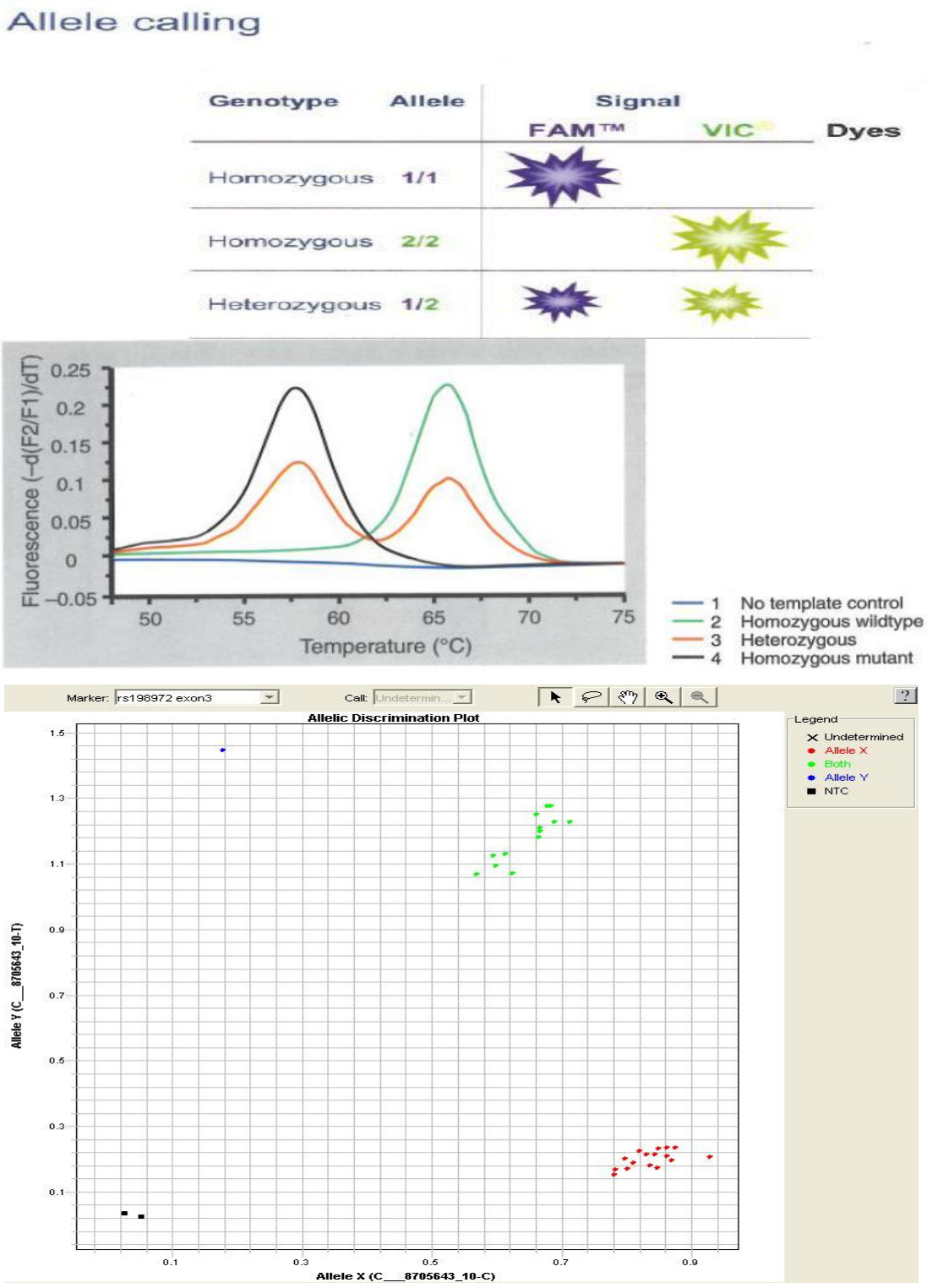


Figure 4.4 Signal detection in AS TaqMan genotyping assay. At user-interface, the cluster plot shows the alleles and control samples in quadrant pattern.

4.2.7 Pyrosequencing

Some allelic variants could be more than two forms, for example the SNP rs6072 located in exon 2 of KLK2 gene has A> C>G. Such variations are not suitably detectable by the TaqMan Genotyping assay. The technique of Pyrosequencing, first described in 1996 by Ronaghi *et al,* is a non-electrophoretic, real-time bioluminometric method for DNA sequencing, especially short segments (<150bp) {Csako, 2006}. It measures the formation of pyrophosphate, the by-product of DNA polymerization. The principle of pyrosequencing is illustrated in Figure 4.5. The instrument used for the pyrosequencing was the PyroMark-24 (Figure 4.6) (Qiagen, UK) according to manufacturer's instructions.

The rs6072 (A>C>G) and rs1064676 was further investi
gated in both Bedford patients and FFPE cases from Gloucester and Cheltenham. The primer sets for pyrosequencing are included in the list of primers and probes in appendix A.5. The protocol for setting up the assay is contained in appendix B.21.

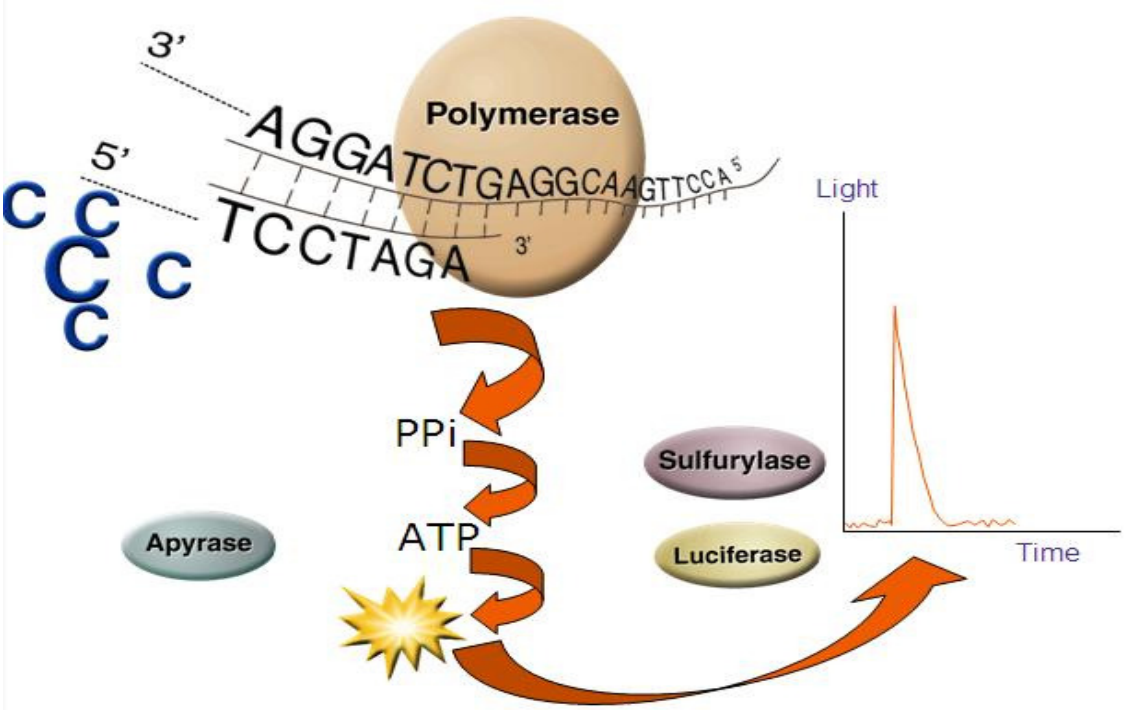


Figure 4.5 A schematic illustration of Pyrosequencing technique.{Biotage, 2008}.

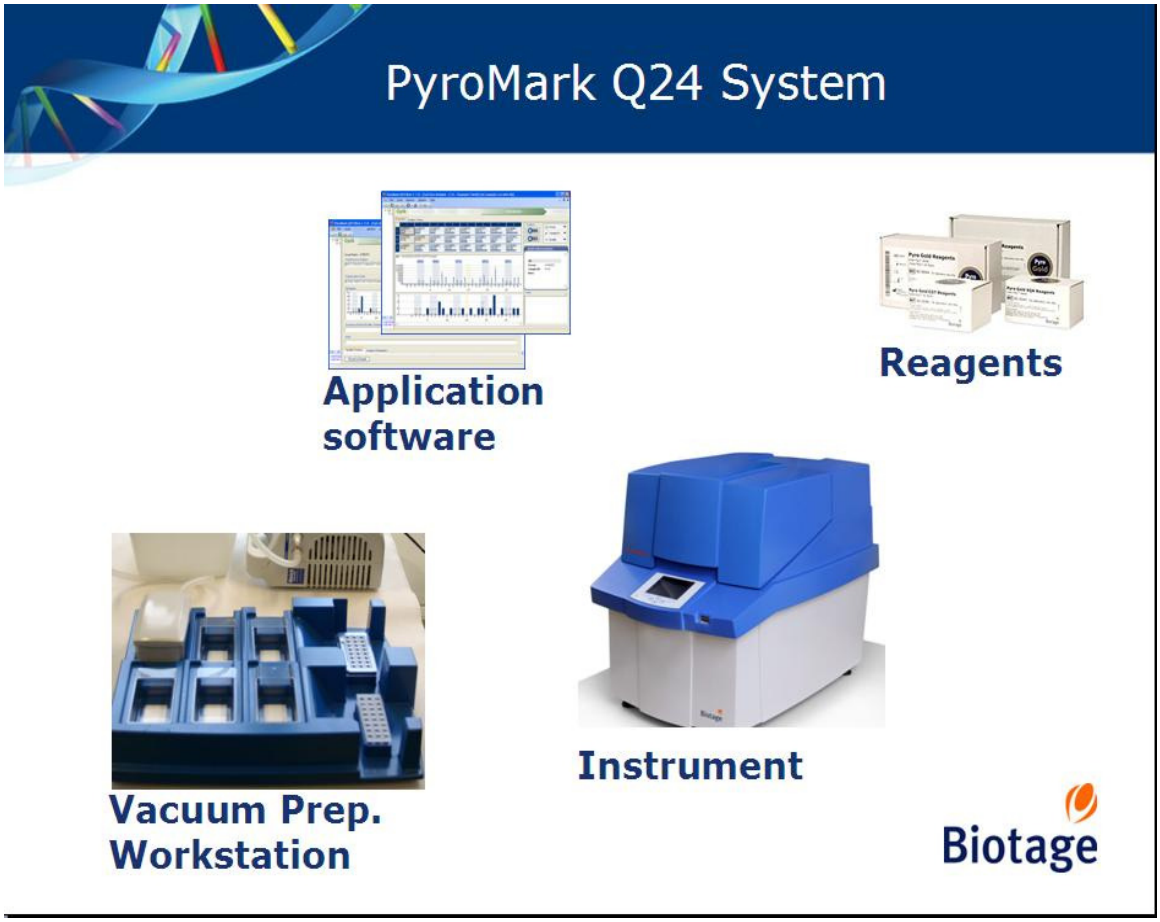


Figure 4.6 The pyrosequencing instruments and kits. {Biotage, UK}.

4.3 Results

4.3.1 Quality of genomic DNA

Figure 4.7 shows an agarose gel stain of gDNA from FFPE tissue materials. The quality of gDNA was good and concentration (average A260/A280 was 1.85) provided sufficient template for analysis.

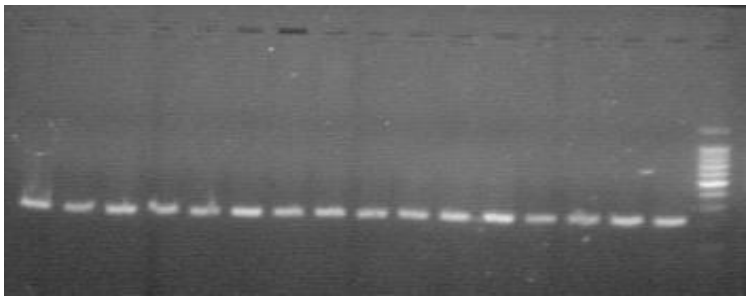


Figure 4.7 Sybr safe stain of 150bp G6PD in gDNA of FFPE samples.

Figure 4.8 shows a Sybr safe stain of PCR products prior to big dye terminator reaction. Single intensely stained bands of appropriate sizes for each exon was suitable for big dye terminator reaction (prevents sequencing of non-specific products).

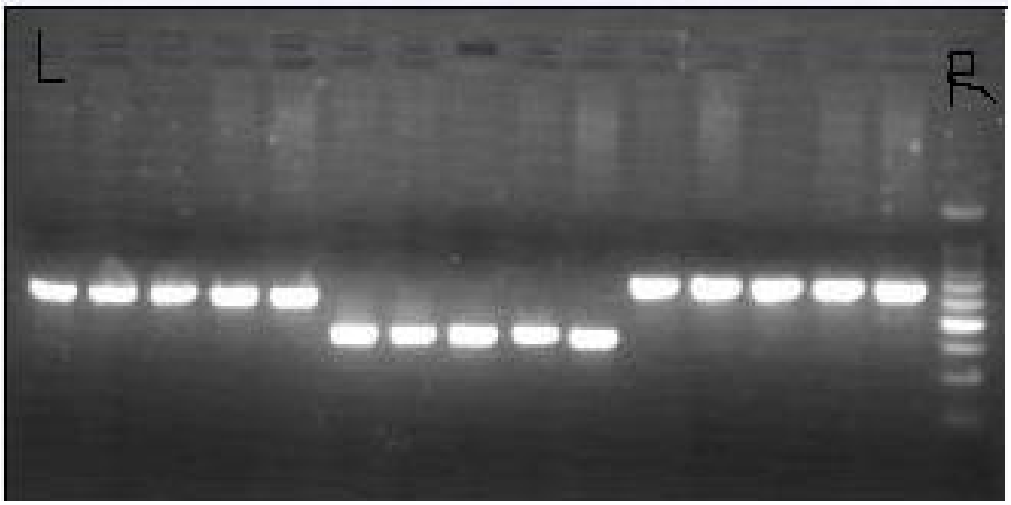


Figure 4.8 Sybr safe stains of KLK2 PCR products. L to R, the first five lanes were 578bp products (exon 1 of five samples), the next five were 410bp products (exons 2 of same five samples), and the next five on the right were 664bp products (exon 3+4 of same five samples). The letter R is on the 100bp DNA ladder lane.

4.3.2 Direct DNA sequencing

Figure 4.9 shows a representative SNP (C/T genotype) in an electropherogram while Figure 4.10 shows a homozygous wildtype. A heterozygous genotype will have two nearly equal chromatograms, one overlaid on the other with a letter of ambiguity (for example Y for C or T nucleotides; S for C or G nucleotides, M for A or C, W for A or T etc). A homozygous genotype will have a single chromatogram at that same position (Figure 4.10).

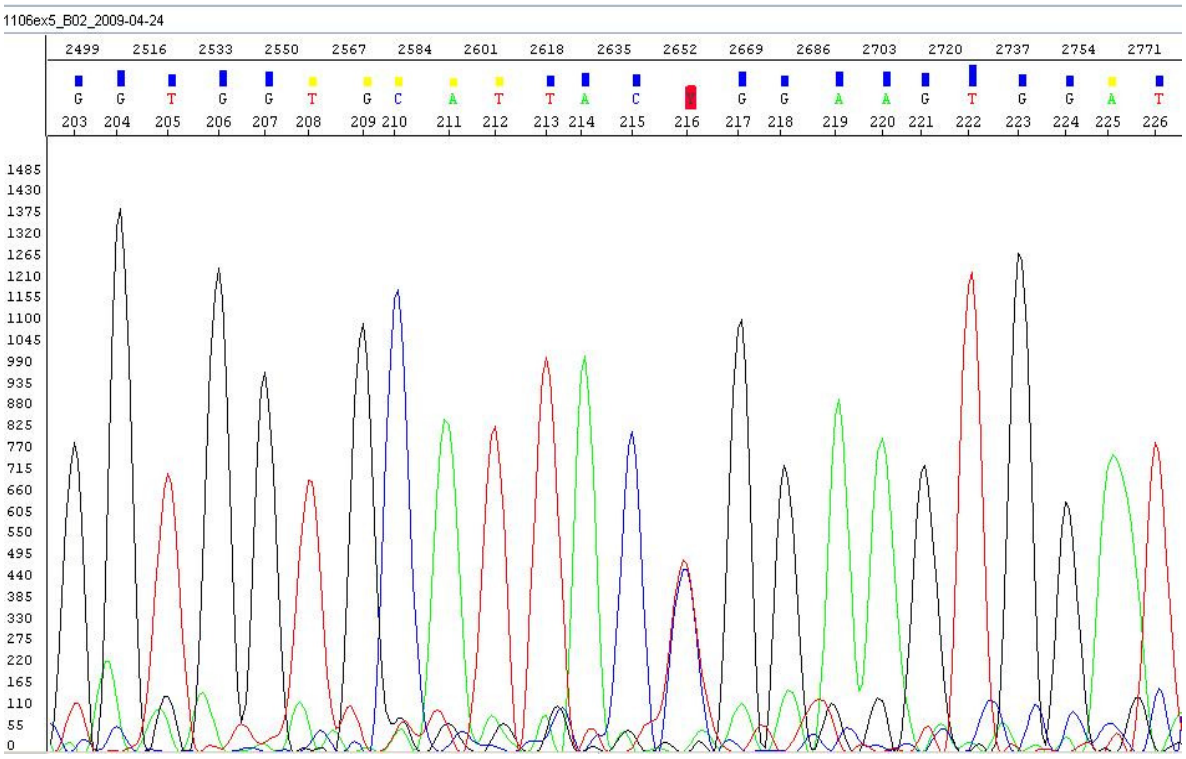


Figure 4.9 A representative DNA electropherogram. This was the chromatogram for participant number 1106 in this study, showing a heterozygous C/T genotype (depicted as Y in position 216 of the electropherogram-highlighted in red by the machine) in exon 5 for SNP rs11549921. On the top left hand corner shows the run ID of this participant including date and well number of the sample.

Figure 4.11 shows a representative view of Edit Sequence in DNA star software, which aligns all sequences and highlight SNPs (for example the letter S highlighted in red stands for C or G).

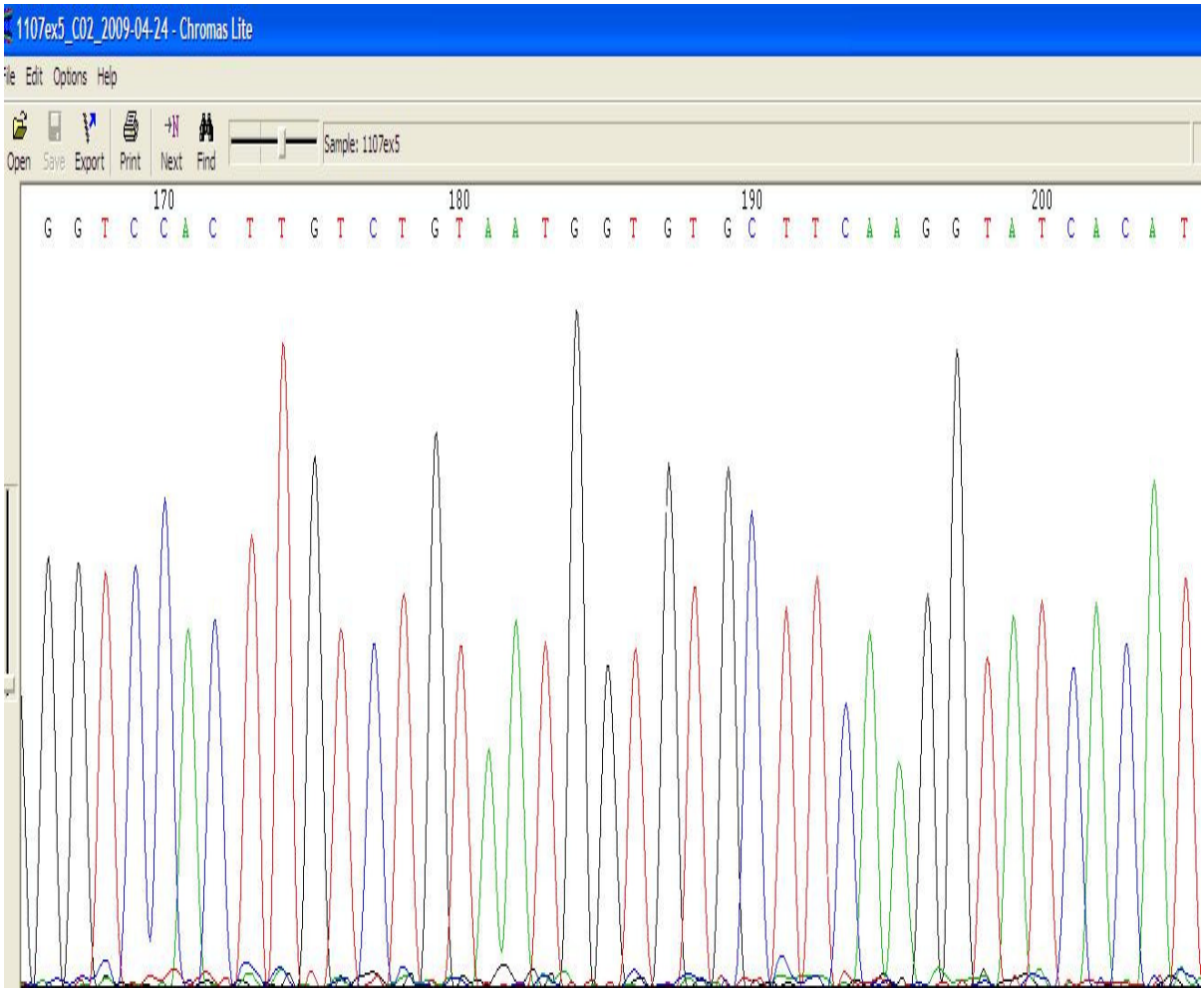


Figure 4.10 A representative DNA electropherogram for homozygous genotype. There was no letter of ambiguity shown in nucleotide sequence. On the top left hand corner is the participants study number 1107, well number and date of sequencing.

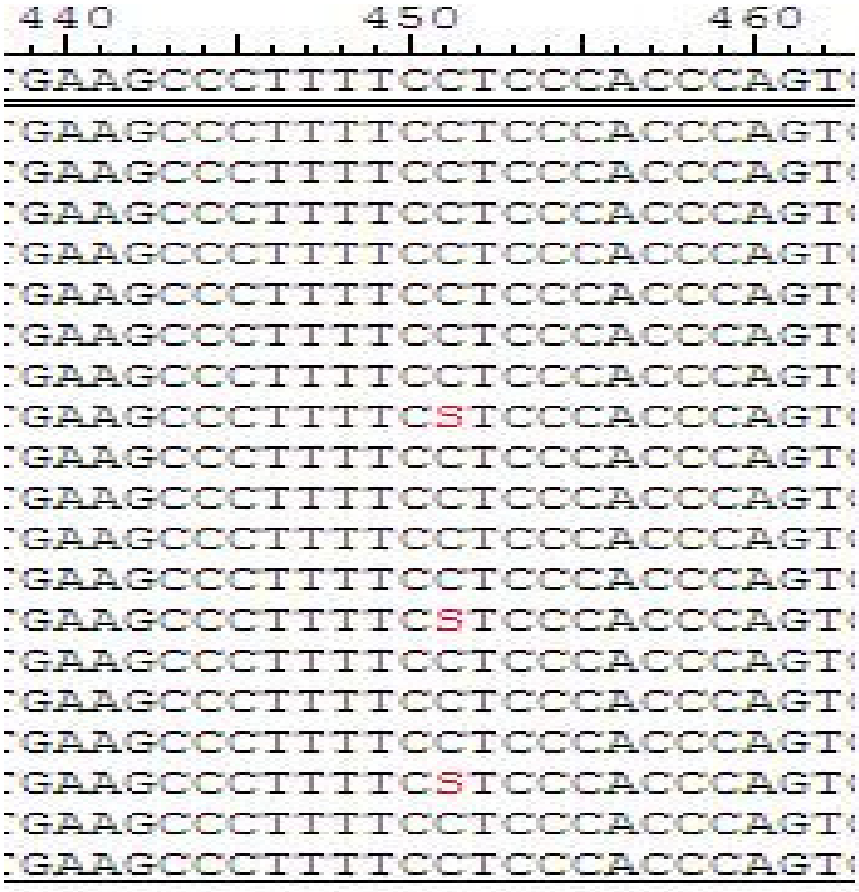


Figure 4.11 A representative view of Edit Sequence of DNA star software. It aligns all sequences and highlights the SNPs (in this case S for C/G genotype).

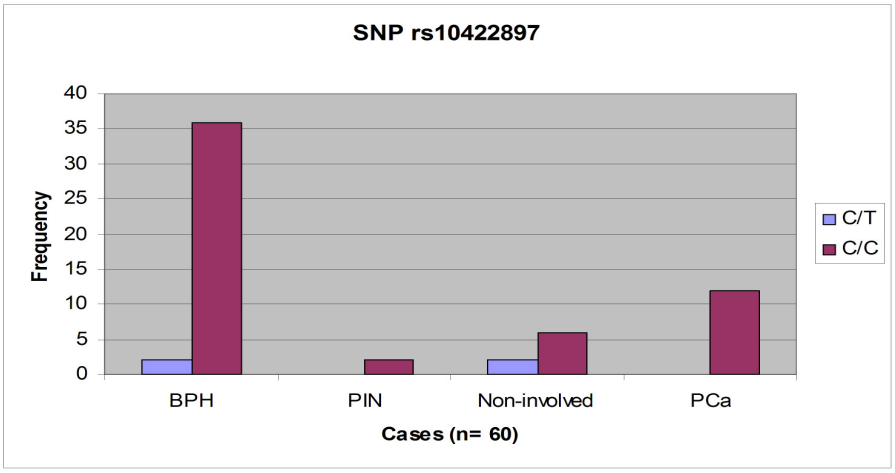
A total of 18 already curated SNPs were identified using the four primer sets covering exons 1 to 5 of KLK2 gene. Table 4.2 shows the frequency of all identified SNPs. Their SNP IDs were identified through co-aligning canonical sequences from UCSS genome browser with the sequencing from the patients' samples. The SNPs consisted of intronic, coding exons and untranslated region of the gene. 11 (61%) of the identified SNPs (highlighted in yellow) had 100% frequency of the reference allele; and therefore were not investigated further. The other 7 SNPs had a heterozygosity of 0.07 to 0.40 in the sample population (n = 60). Four of them were intronic SNPs; three were coding sequence (CDS) SNPs. Alleles of two of the 7 SNPs (rs2664155 and rs198977) had been previously reported as having strong association with prostate cancer risk {Nam *et al*, 2006}.

Table 4.2 Frequency distribution of identified SNPs in KLK2 gene

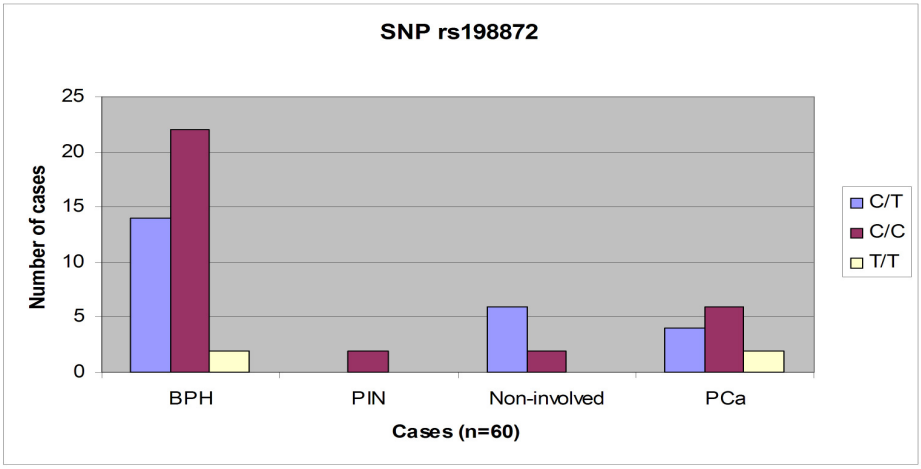
SNP ID	Molecular type	Genotypes and their frequency (n = 60)			Reference allele
rs62113073	intronic	C/G (17%)	C/C (83%)	G/G (0%)	C
rs2664155	intronic	A/G (37%)	G/G (60%)	A/A (3%)	G
rs2664156	intronic	C/T (13%)	C/C (77%)	T/T (10%)	T
rs34652810	5'UTR	Del/G (0%)	Del/del (100%)	G/G (0%)	del
rs2070854	intronic	A/G (0%)	A/A (100%)	G/G (0%)	A
rs198970	intronic	A/T (0%)	A/A (100%)	T/T (0%)	A
rs6072	CDS-misense	A/C/G (0%)	G/G (100%)	A/C/G (0%)	G
rs61750342	exon	C/T (0%)	C/C (100%)	T/T (0%)	C
rs10422897	CDS-misense	C/T (7%)	C/C (93%)	T/T (0%)	C
rs198972	CDS	C/T (40%)	C/C (53%)	T/T (7%)	C
rs1064676	CDS	C/T (0%)	C/C (100%)	T/T (0%)	C
rs6070	intronic	A/T (40%)	T/T (60%)	A/A (0%)	T
rs6071	intronic	A/G (0%)	A/A (100%)	G/G (0%)	A
rs1064703	CDS	C/T (0%)	T/T (100%)	C/C (0%)	T
rs198977	CDS	C/T (37%)	C/C (56%)	T/T (7%)	C
rs60268688	CDS	A/C (0%)	A/A (100%)	C/C (0%)	A
rs1059712	CDS	A/G (0%)	G/G (100%)	A/A (0%)	G
rs11549921	3'UTR	C/T (0%)	T/T(100%)	C/C (0%)	T

Three of the CDS located SNPs: rs10422897, rs198972 and rs198977 were in codons for amino acids: Arginine (R), Leucine (L) and Arginine (R) or Tryptophan (W) respectively. The association of the CDS- located SNPs with prostate cancer in the sample population (n= 60) is shown in Figure 4.12.

A



B



C

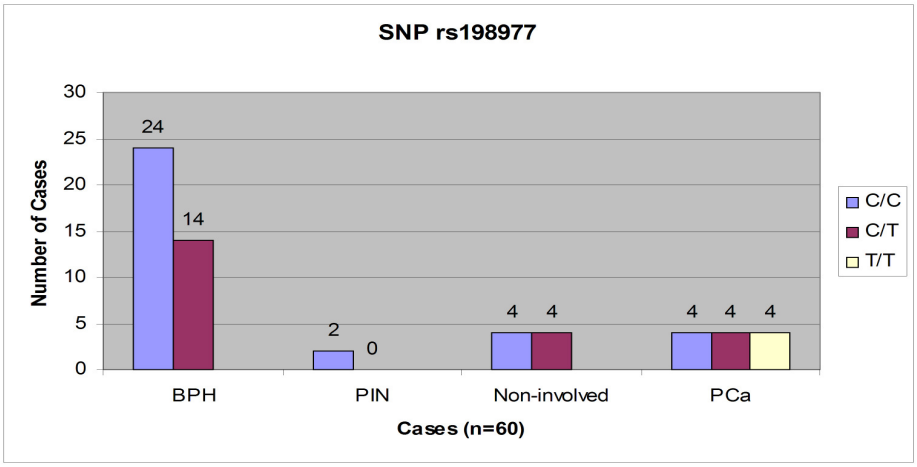
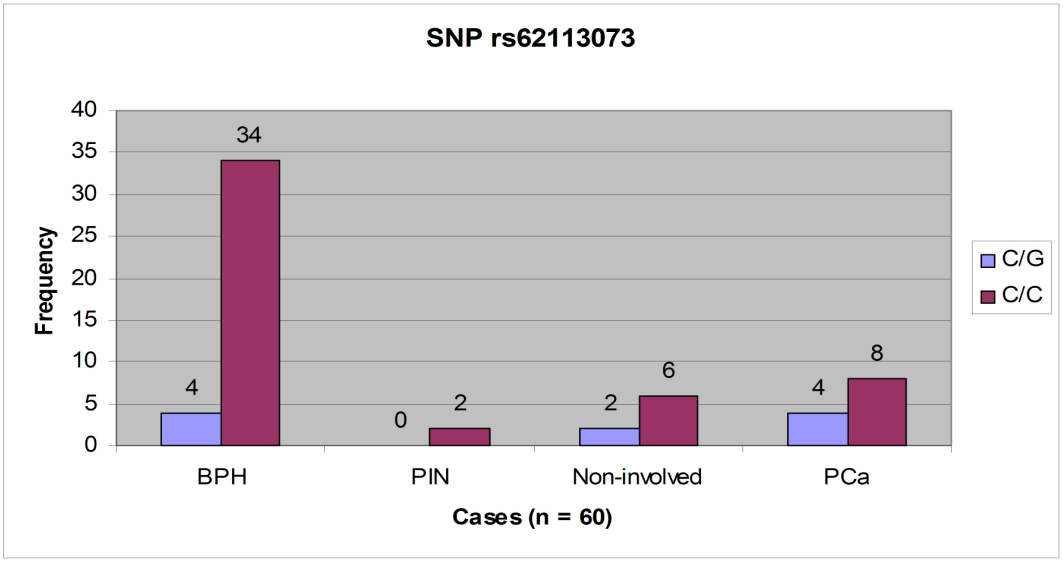


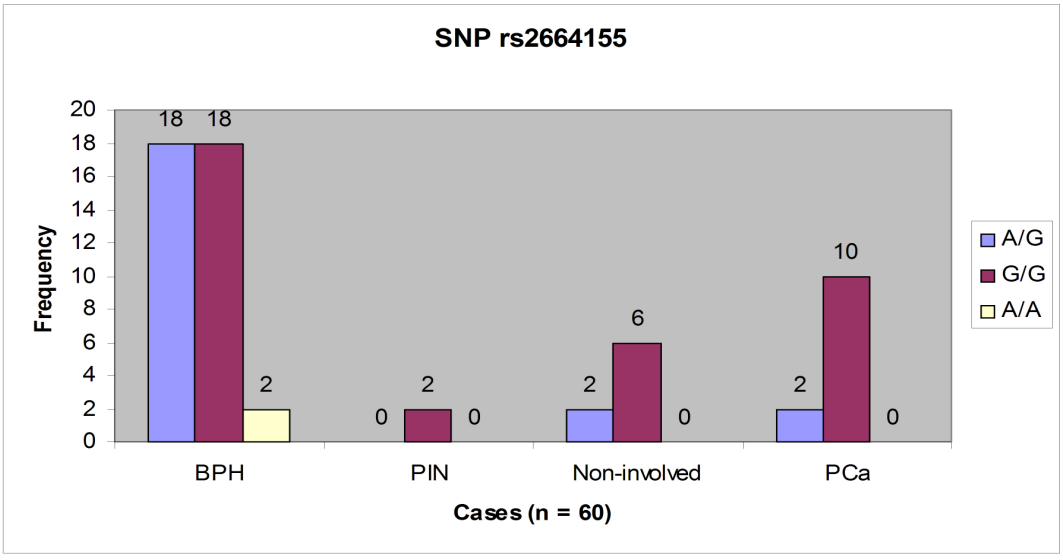
Figure 4.12 Association of cds-located SNPs with Prostate diseases. A is SNP rs10422897, B is rs198972 and C is 198977. All cases of T/T alleles of rs198977 had prostate cancer.

Four intronic SNPs: rs62113073, rs2664155, rs2664156 and rs6070 showed differential frequency distribution (Figures 4.13 and 4.14).

Figure 4.13 shows the frequency distribution of two intronic SNPs in KLK2 gene: rs62113073 and rs2664155. SNP rs62113073 showed no significant association with any particular prostate disease. However, the A/A genotype of SNP rs2664155 was only observed in benign hyperplasia cases. The A/A and A/G variants were also observed in both benign and prostate cancer cases with any significant differences.



A

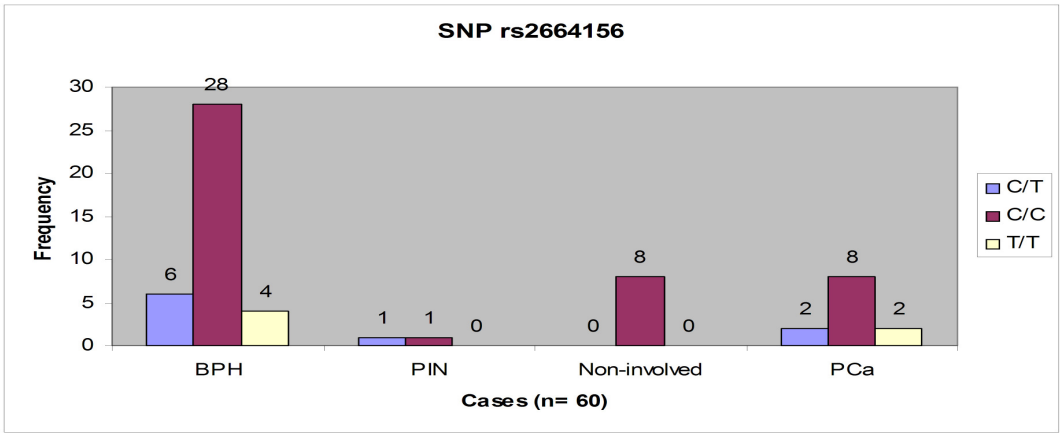


B

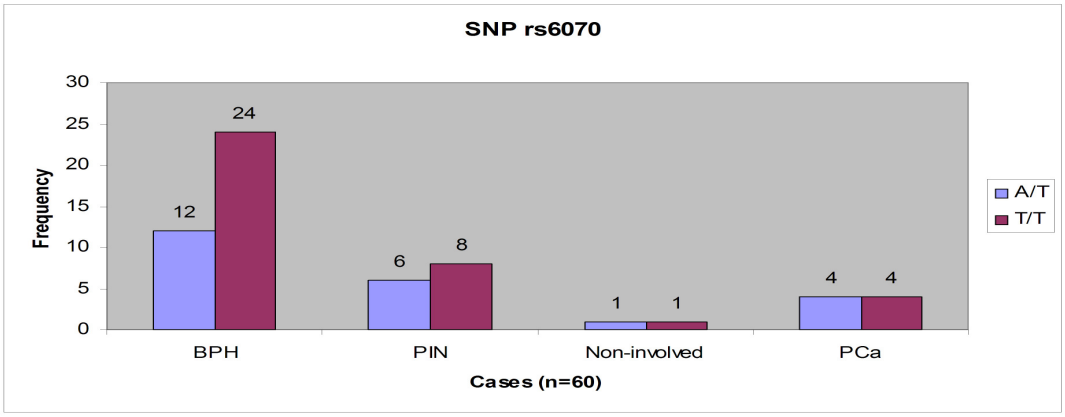
Figure 4.13 Frequency distribution of intronic SNPs in KLK2 gene. A is rs62113073 and B is SNP rs2664155. The A/A allele of rs2664155 occurred only in benign cases.

In Figure 4.14, SNPs rs2664156 and rs6070 showed no significant association with any disease status (P values 0.49 and 0.78, Chi-Square, respectively). The rs6070 had not been described in previous studies. The A/A allele was not observed in the sample population (though it was a small size).

Figure 4.13 Frequency distribution of intronic SNPs in KLK2 gene. A is rs62113073 and B is SNP rs2664155. The A/A allele of rs2664155 occurred only in benign cases.



A



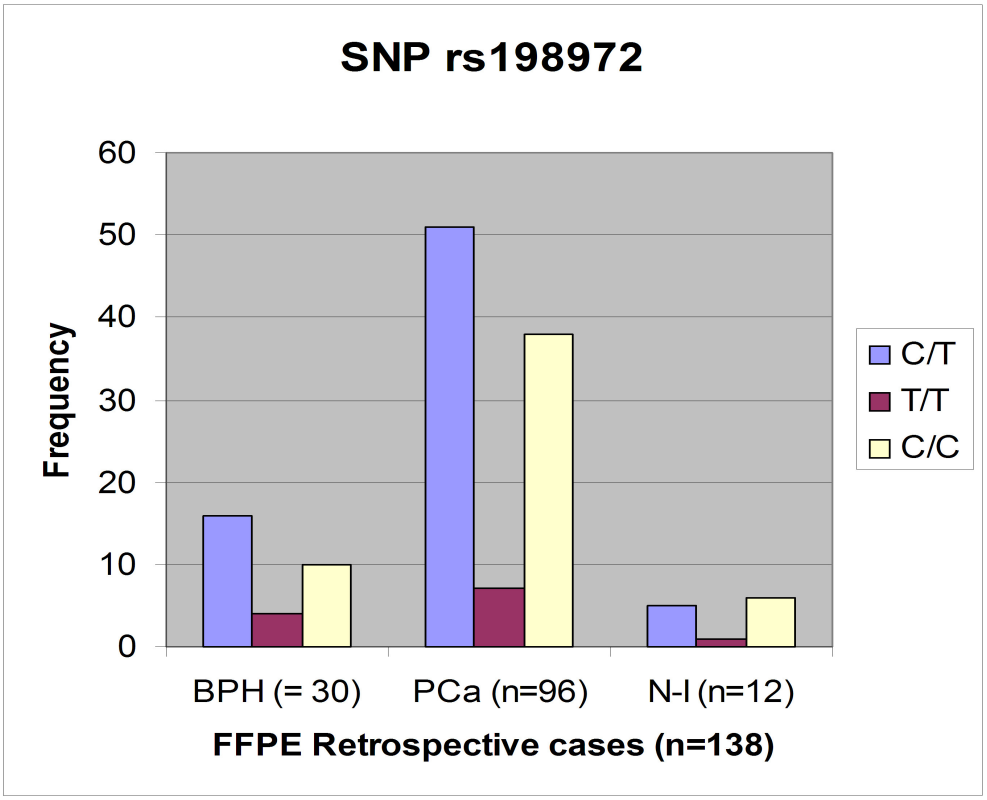
B

Figure 4.14 Intronic SNPs on KLK2 gene. A is rs2664156 (P value = 0.49, Chi-square) and B is rs6070 (P value = 0.78, Chi-square). No significant association with any disease status.

4.3.3 TaqMan SNP genotyping

The results of the previous 60 samples tested by TaqMan SNP genotyping were the same with those of direct sequencing, no discrepancy was observed. Figure 4.15 shows the results for rs198972 and rs198977 on retrospective archived FFPE tissue materials (n=138) determined by TaqMan SNP genotyping technique.

The samples, at 50 to 100ng per reaction yielded good quality signals. For the rs198972 there was no significant association with any prostate diseases. Sample (disease) groups had a proportional frequency of each genotype type. For the rs198977, the T/T genotype was found only in some PCa cases. Although the proportion was 5.2% (compared to the 33% in the Bedford patients, probably due to small sample size), all cases of T/T variant (rs198977) had PCa. Average heterozygosity for the C/T of rs198977 was 0.38 (it was comparable to previous results as well as the data from the HapMap project). The frequency of the reference allele (the C variant) was 0.96 (comparable to the previous results on 60 patients. Figure 4.16 shows the global distribution of the rs198977 allelic variants for various countries participating in the HapMap project.



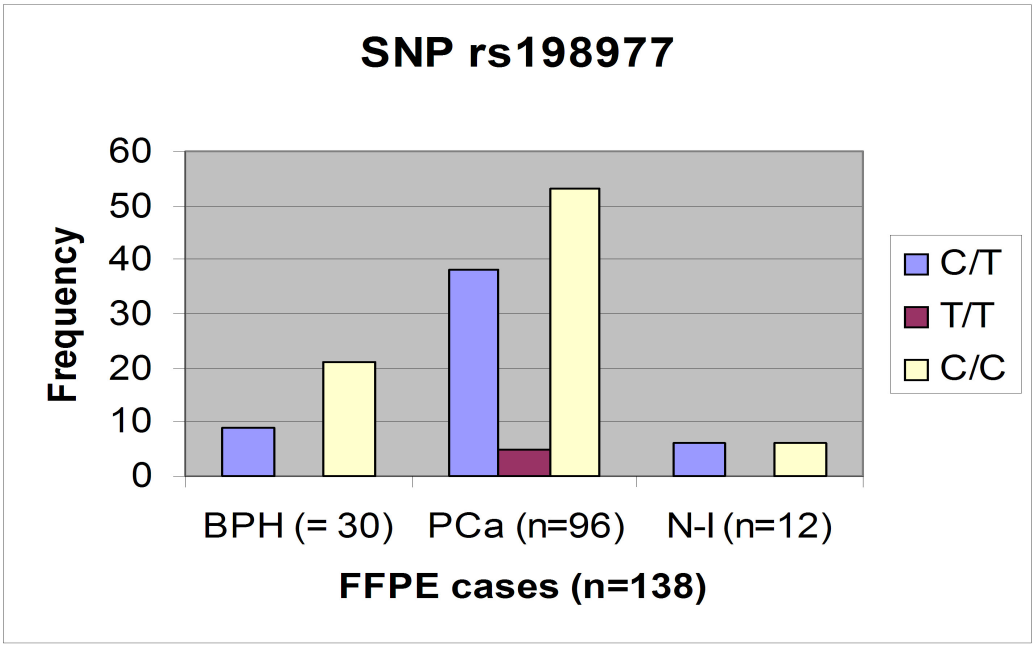


Figure 4.15 KLK2 SNPs in FFPE materials. T/T alleles of rs198977 were only found in PCa cases (5.2%) of the sample population.

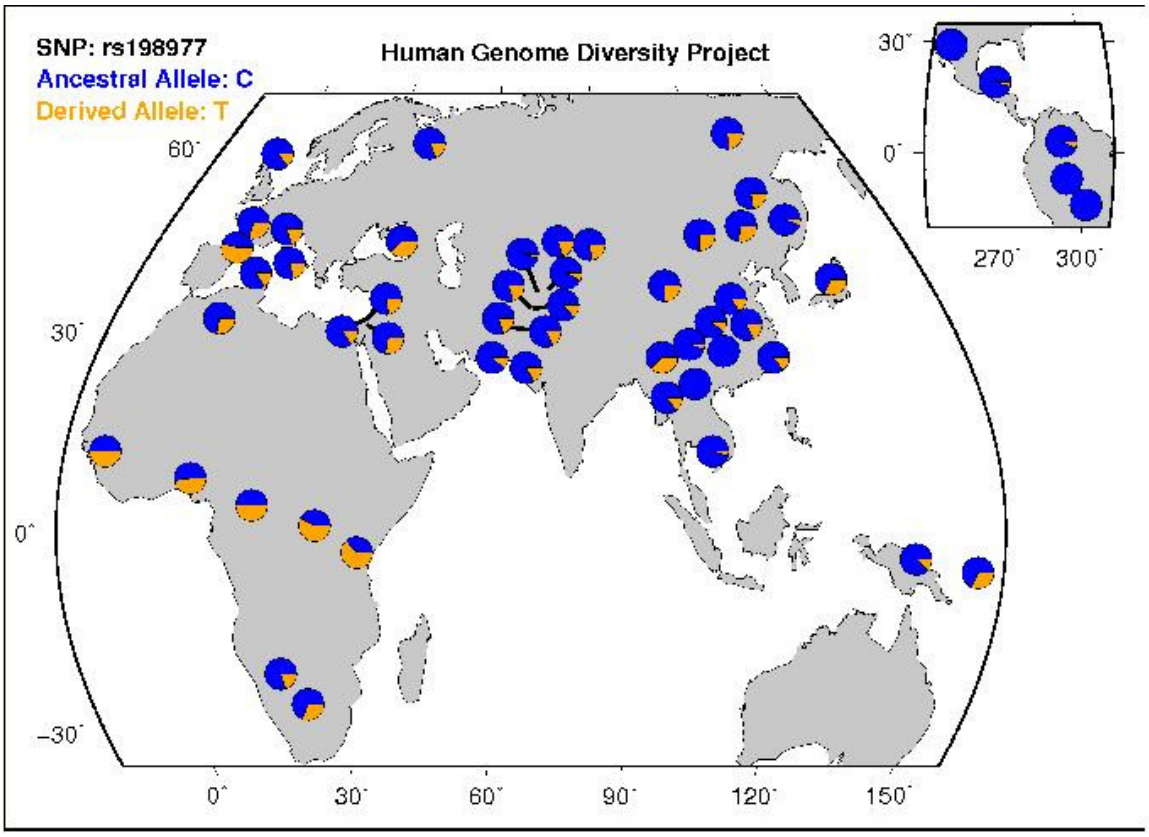


Figure 4.16 Global frequency distribution of the rs198977 allelic variants.
{HapMap International Consortium, 2009}.

4.3.4 Pyrosequencing

The results of rs6072 and rs1064676 obtained from pyrosequencing did not differ from those of direct DNA sequencing; therefore the method was validated for use in the FFPE cases. Figure 4.17 shows the rs6072 results in FFPE cases (n= 138), it had 100% of the reference allele G in the study population. Currently, the frequency distribution of rs6072 SNP from a British population is not documented in the HapMap project.

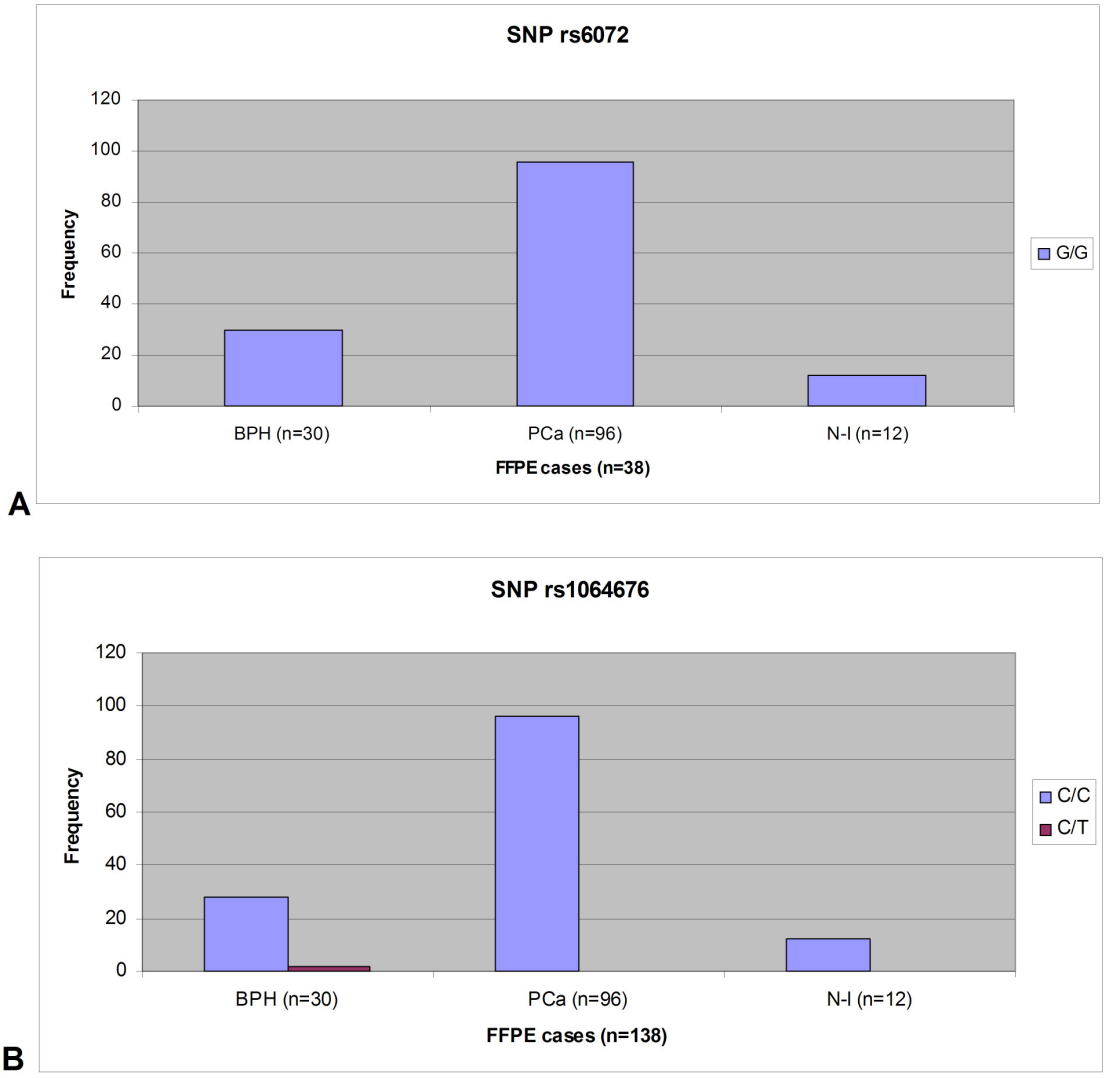


Figure 4.17 Frequency distribution of two SNPs determined by the pyrosequencing assay. A is the rs6072 which had 100% of the reference allele (G) in the study population; B is the rs1064676 which had 1.45% of the derived allele (T) in the study population.

The C/T variant of rs1064676 occurred only in BPH cases (~7% of the BPH); the frequency of the heterozygosity was 1.45% in the study population. Again there is no data on British population in the HapMap data base for comparison.

4.4 Discussion

4.4.1 CDS-located SNPs

From the simple frequency distribution tables, SNPs rs10422897 and rs198972 were not significantly associated to any particular prostate disease. However, the T/T homozygous allele of rs198977 was only found in prostate cancer cases. Although the sample size was small, the result was in agreement with the report of Nam *et al*, 2006. Unlike previous studies including Nam *et al*, 2006, BPH and PIN cases were included in this study, and the T/T alleles were only found in prostate cancer cases The first 60 cases in this study were prospective; and were also histologically established (at Bedford hospital). By implication (from this particular result), a proportion of prostate cancer patients will always have the T/T alleles of rs198977; or all males who have the T/T allele are very likely to suffer prostate cancer (larger validations studies are still required). The second implication is that genetic screening for T/T alleles in adult male populations could help select men with higher risk of developing prostate cancer and enroll them for annual screening (early detection). The stratum could be widened by considering males with T alleles (T/T and C/T) which accounted for 66% of all PCa in this study. However, the other 34% of men without the T/T alleles or men with only C/C genotypes could still be at risk of PCa.

Functionally, the C> T alleles of rs198977 are described as 'coding synonymous' {UCSS Genome browser} which means there is no change in peptide for allele with respect to reference assembly (whichever allele the patient has the amino acid at that position will still be Leucine). The average heterozygosity of the rs198977 from the HapMap project is 0.479 ± 0.101. From this study, the heterozygosity was 0.40; however the participants in this study are purely a British Caucasian population. After extensive literature search, there was no existing data for the British population on the frequency of the reference allele of this SNP (the C allele); from this study it was found to be 0.93, that of the French was 0.69 and Italians 0.62 {HapMap Project}.

Another important observation was that of the overall 7 cases of T/T genotype (rs198977), two of them had Gleason score 8 to 10; four cases had Gleason score 6 and one case had Gleason score 7. Although this was a small sample size, it did appear that the T/T genotype was associated with high grade prostate tumours (larger studies are required to confirm this trend).

The TaqMan SNP genotyping provides a fast and reliable method for genotyping FFPE tissue materials; the cost is estimated at less than a £1.30 per test done in duplicates. From the optimization data, it

required about 50 to 100ng of genomic DNA for optimal data collection. Also known (positive controls for all the allele variants) obtained by direct sequencing is very important for validation and quality control of the assay. The significance of the T/T allelic variant of rs198977 requires further large scale prospective studies in both blood and FFPE materials of patients.
The pyrosequencing technique, like the TaqMan genotyping, is cheap and flexibly high throughput; and both could be clinically used to genotype FFPE tissue materials without the labour- and capital-intensive direct DNA sequencing technique.

It is also conceivable that biosensor-based genotyping techniques could be available in the near future for genotyping at point of care. From results in this study, it was clearly demonstrated that FFPE materials could be successfully genotyped for clinically relevant SNPs.

4.4.2 Intronic SNPs in KLK2 gene

The biologic effects of intronic SNPs are very unclear especially variations that are not localised in the first two or last two bases of intron. The frequency distribution of the intronic SNP rs62113073 has no significant association with PCa risk (similar pattern of distribution was also found in non-involved cases). For the SNP rs2664155 located on intron 1 (near the promoter region) of the KLK2 gene, Nam *et al*, 2006 reported that the G allelic variants (G/G and A/G) were significantly associated with prostate cancer. As mentioned before, most previous SNP association studies did not include BPH as a separate case group, which is the most prevalent prostate disease. Contrary to previous reports, the G allelic variants (G/G and A/G) of rs2664155, were associated with almost equal chance to BPH and prostate cancer (Odds ratio (OR) = 0.58; 95% CI = 0.026 to 13.02); a participant who had G allelic variants was half a chance to suffer BPH or prostate cancer. On the other hand, the A/A genotypes of rs2664155 were exclusively found in BPH cases (larger studies are required to verify this). Previous studies also did not find any association between A/A genotype of rs2664155 and prostate cancer, Nam *et al* reported an odd ratio of 1.27 and P value of 0.22.

One of the reasons for the interest on KLK2 SNPs is that the protein product of this gene (hK2) is differentially expressed in prostate diseases (with an increased expression in PCa). Several reports documented the elevated serum levels of hK2 in PCa {Lintula *et al*, 2005}. Early genotyping studies indicated that SNPs rs2664155 and rs198977 were associated with both elevated serum hK2 and PCa

risk {Nam *et al*, 2006}.Genetic variants in the form of SNPs could help stratify patients into genetic risk groups and improve early detection programmes

4.4.4 Clinico-pathological features of sample population

Some clinical data of the sample population were collected and / or measured including age, serum PSA, prostatic volume, Gleason score and clinical stage of tumours.

a)Age and PSA distribution

Figure 4.18 shows the age and total PSA distribution of the n = 138 cases from Gloucester and Cheltenham. The mean ages for the BPH, Gleason scores (GS) 4-5, 6, 7, 8-10 and non-involved (NI) cases were 74, 66, 65, 64, 72 and 74 years respectively. Significant differences in age ($P < 0.05$, Kruskal-Wallis test) were observed between BPH and GS 6 cases; BPH and GS 7 cases; NI and GS 7 cases.

For total PSA ng/ml, the mean values for NI, BPH, GS4-5, GS6, GS7 and GS 8-10 were 4.88, 5.28, 8.95, 8.56, 8.00 and 14.64 ng/ml respectively. Significant differences ($P < 0.05$) were observed in total PSA levels between BPH and GS6; BPH and GS7; BPH and GS8-10; between NI and GS6; NI and GS 7; and NI and GS8-10. But there was no significant difference in total PSA between NI and BPH. The trend showed that total serum PSA could significantly discriminate between benign and cancer cases; and between non-involved cases (e.g. chronic inflammation etc) and cancer cases; but could not discriminate between BPH and non-involved cases. However, total PSA was not associated with histological grades (PSA had weak correlation with tumour grade).
All the cases had PSA value > 4.0ng/ml (commonly used cut-off point).

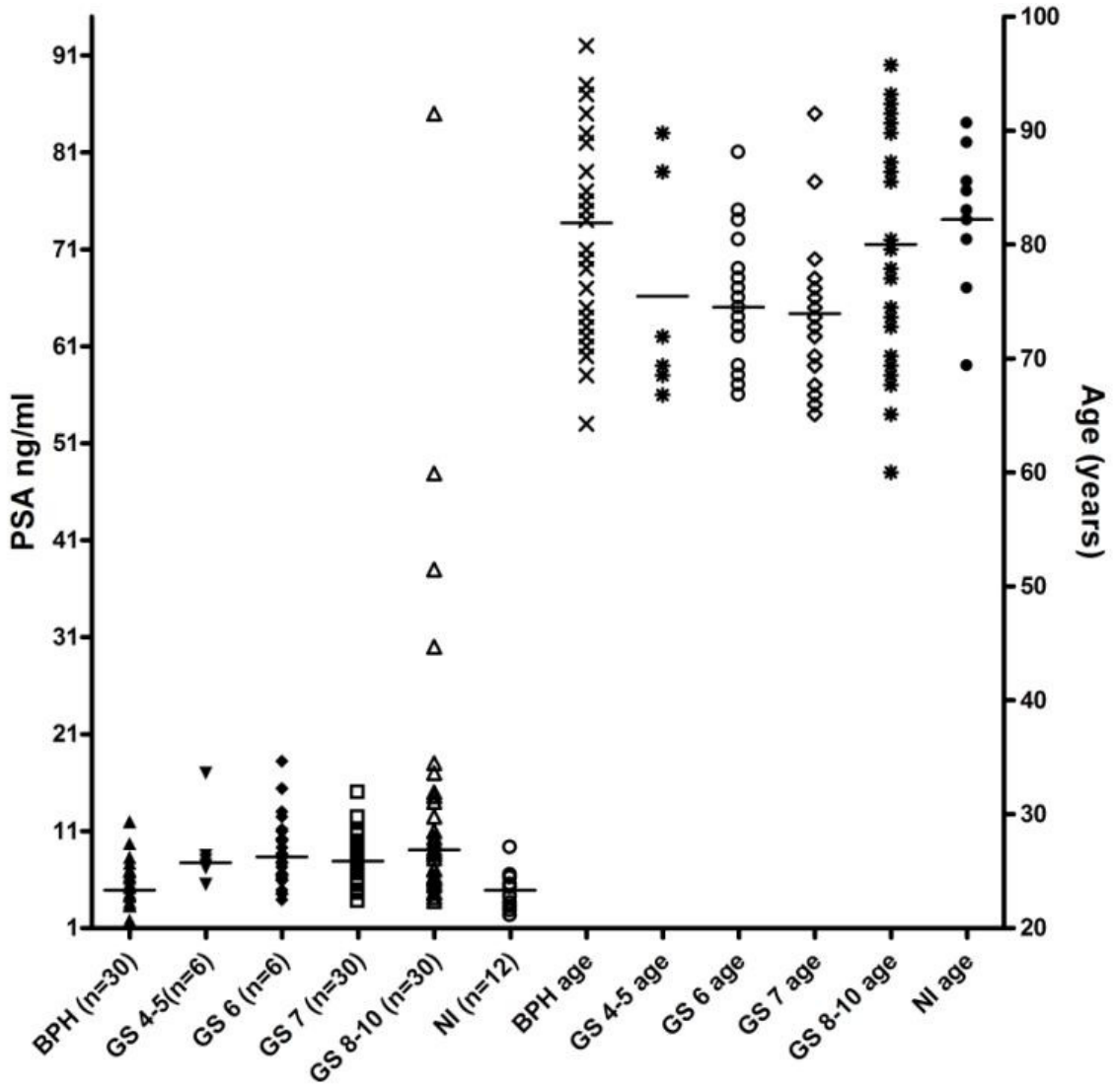


Figure 4.18 Age and PSA distribution in FFPE cases. NI stands for non-involved cases, GS for Gleason score, BPH for benign nodular hyperplasia.

For the Bedford patients (n= 60), there was no significant difference in age for PIN, BPH, NI and PCa cases (mean was 67 years, range 56 to 79 years). There was a significant difference in total PSA levels between NI and PCa cases ($P < 0.05$); the mean values for NI and PCa cases were 5.13 and 21.52 ng/ml respectively. In this cohort, PSA could only discriminate between NI and PCa cases. There were no significant differences in PSA density; mean values for BPH, NI, PIN and PCa cases were 0.22, 0.12, 0.10 and 0.54 respectively. A PSA density of > 0.13 would be considered abnormal. Overall, only 20% of the Bedford patients had prostate cancer; the rest were BPH cases (63%), NI cases (13%) and PIN cases (4%).

For the Cranfield health volunteers, the mean age was 51years (range 42 to 65 years); and the mean total PSA ng/ml was 0.70 (range was 0.50 to 1.91). Both age and the serum PSA levels were significantly lower in the Cranfield health volunteers compared to patient groups in Bedford, Cheltenham and Gloucester groups. Ageing is a strong predisposing factor for PCa.

b) Gleason Score and Clinical stage

For the Gloucester and Cheltenham cases, the design was that of a case- control study; the number of GS 6 , GS7 and GS 8-10 were 30 each (21.7% of the study population each); BPH cases were also 21.7%; NI cases were only 12 (9%) (cases available between 2006 and 2008); GS 4-5 cases were 0.2% (available within the study period). Of the 96 prostate cancer cases, 60 cases (62.5%) were T2 stage and 36 cases (37.5%) were T3 stage. 80% of the Gleason score 8-10 were T3 stage tumours.

For Bedford patients, 87.5% of the prostate cancer cases were intermediate grades (Gleason score 6-7), and all the intermediate grades were T2 stage. Only 6.25% of the PCa cases were GS 9; the remaining 6.25% were GS3.

4.5 Conclusions

In summary, two SNPs appeared very promising for detecting risk groups in prostate disease screening: the T/T variant of rs198977 for detecting prostate cancer risk, and the A/A genotype of rs2664155 for detecting BPH; and both could be genotyped in the same plate using the cheap and fast TaqMan genotyping assay.

The T/T allele was also significantly associated with high tumour grade. This could be very useful to clinical management of patients; indolent prostate cancer cases with the risk alleles of KLK2 would be considered for radical primary treatment on time before further spread of disease. However, further large scale studies would be required to examine the correlation between tumour stage and risk alleles of KLK2 gene and to rule out possible effects of genetic diversity on such results.

Chapter Five

Gene expression profiling of prostate cancer patients

Chapter 5: Gene expression profiling of prostate cancer patients

5.1 Introduction

This chapter describes the measurement of a panel of molecular markers at the mRNA (transcript) level using RQ-PCR technique. The markers included KLK2, KLK3, MCM2, MCM5, TP53, Bcl-2, CD44, CDH1, AURKA, AURKB, and AURKC; ESRα, ESRβ, AR, FASN, TMPRSS2: ERG, and TMPRSS2:ETV1. Three endogenous genes: ABL1, GUS and G6PD were also measured for normalization of the target gene expression. The aim was to find out the expression profile of these markers in FFPE tissue materials and urine samples of prostate cancer, non-involved and benign nodular hyperplasia patients. In chapter three of this thesis, the expression of some of these markers in cultured cell lines in response to hormonal modulation was reported. In this chapter, the level of expression of these markers in benign (BPH), non-involved (N-I) and prostate cancer cases were compared. Where it was significant, the expressions of these molecular markers were also compared with tumour grades. For urine samples, the aim was to find out if the molecular markers were detectable in exfoliated urine cells without prostatic massage (non-invasive sampling); and if there was a differential expression in detectable cases.

5.2 Materials and Methods

5.2.1 RNA extraction, cDNA synthesis and RQ-PCR: The methods for sample collection, RNA extraction and subsequent cDNA synthesis have been previously described in Section 4.1.2. The method of two-steps RQ-PCR used in gene expression profiling has also been described in Section 3.3.4. The list of primers and probes used in the RQ-PCR are contained in appendix A.5. The sample size (n) was 138 for FFPE prostate tissue cases (BPH= 30 cases, PCa = 96 cases and 12 non-involved cases); for urine samples, n = 83 (control group = 30, patients who attended TRUS-prostate biopsy sections = 53). The ethical approval processes have been previously described in Section 4.1. In the RQ-PCR, some markers were measured by absolute quantification using plasmid calibrators; and the results normalized and expressed as a ratio. Cases with higher ratios expressed more mRNA of the target gene than those with lower ratios. In other cases, normalized relative quantification (NRQ) was done without plasmid calibrators. The NRQ measures fold increase of a molecular target in a sample compared to another sample of a different group. Higher NRQ means lower expression of the target.

5.2.2 Immunohistochemical staining (IHC): IHC of PSA, AR, ESRα and ESRβ were performed on 30 cases from the Bedford samples (BPH cases = 12, PCa cases =12 and non-involved cases = 6).

Sodium citrate buffer in water bath was used for antigen retrieval in the tissue sections (appendix B.22). The method for IHC has been previously described in Section 3.2.6, and the protocol for IHC is also contained in appendix B.8. The aim of the IHC was to confirm the expression of these markers at protein level. There was no semi-quantitation of the degree/intensity of staining.

5.2.3 Statistical analysis: This was performed on results using GraphPad Prism software version 4.02 (www.graphpad.com). For all data sets, the D'Agositino and Pearson Omnibus normality test was performed to determine the distribution. For data sets involving more than two groups, analysis of variance (ANOVA) was performed using Kruskal-Wallis tests (for non-parametric, non-Gaussian distribution) and Dunn's multiple comparison tests. Where two data sets were compared, Mann-Whitney test was performed for non-Gaussian distribution. A probability value of < 0.05 was considered statistically significant. Most graphical presentations were in logarithmic scale (power of 10). Where box plots are used, the box represents the upper and lower quartiles and the middle bar inside the box represents the median value. Where aligned dot plots are used, the bar across the plots represents the median value.

5.3 Results

5.3.1 The endogenous control genes (CGs): The three candidate control genes reported in Section 3.3.4 for prostate cell line studies were also evaluated in the FFPE tissue materials and urine samples. Figures 5.1, 5.2 and 5.3 show the transcript levels of ABL1, GUS and G6PD in the FFPE materials. For ABL1 and G6PD, there was no significant difference in the expression levels in BPH, non-involved (N-I) and PCa cases ($P > 0.05$, Kruskal-Wallis test). But for GUS, there was a significant difference between N-I and PCa cases ($P < 0.05$). When the geometric means of all three CGs were compared to each of the CGs for all the cases (BPH, N-I and PCa), there was no significant difference observed ($P = 0.41$). The median value of ABL1 gene was related to those of G6PD and GUS by ratios of 1.03 and 0.82 respectively. Conversely, the median value of G6PD was related to those of ABL1 and GUS by 1.25 and 3.30 respectively.

Figure 5.4 shows the transcript levels of the candidate control genes in both patient group (Px) and control group (Cr) in exfoliated urine cells. There was no significant difference in ABL1 transcript number between the patient group and control group ($P = 0.13$, Mann-Whitney test); similar result was also obtained for G6PD ($P = 0.08$). But there was a significant difference in the GUS transcript number between patients and control group ($P = 0.0019$), which was similar to the result in FFPE tissue

materials. There was no significant difference between the geometric mean of ABL1 and G6PD compared to either ABL1 or G6PD (P = 0.34, Kruskal-Wallis test).

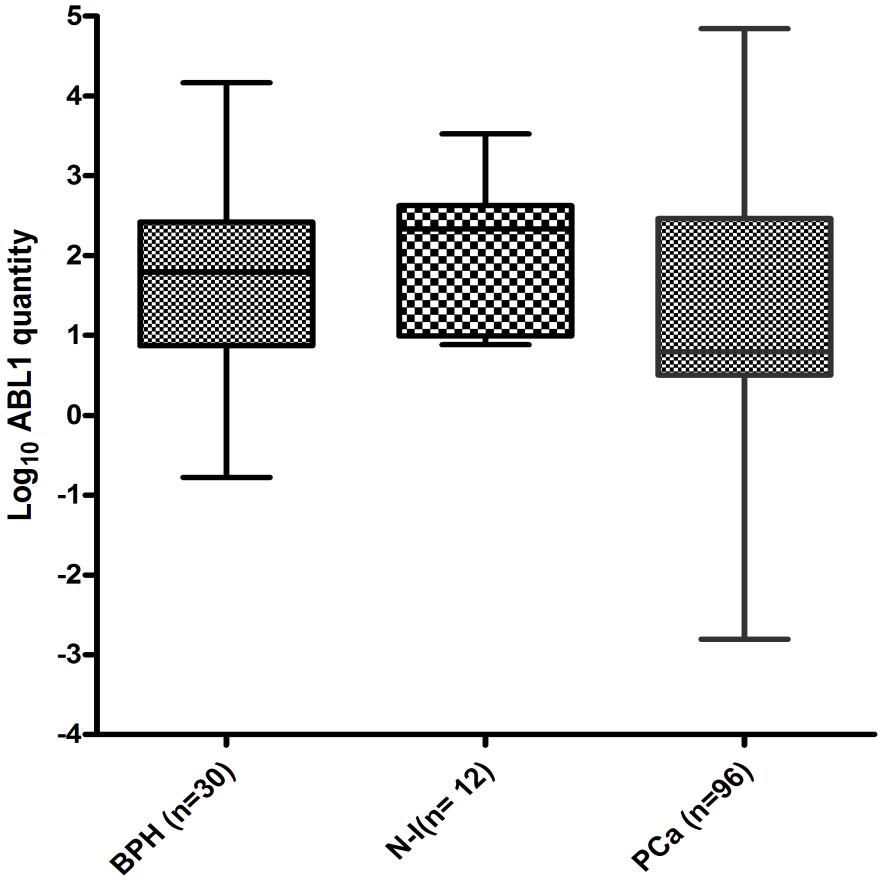


Figure 5.1 ABL1 transcript levels in FFPE tissue materials. There was no significant difference in the expression of ABL1 gene in benign (BPH), non-involved (N-I) and prostate cancer (PCa) cases (P > 0.05).

The median ABL1 transcript number in the urine samples was 1.40 logs compared to 1.99 logs in the FFPE tissue materials.

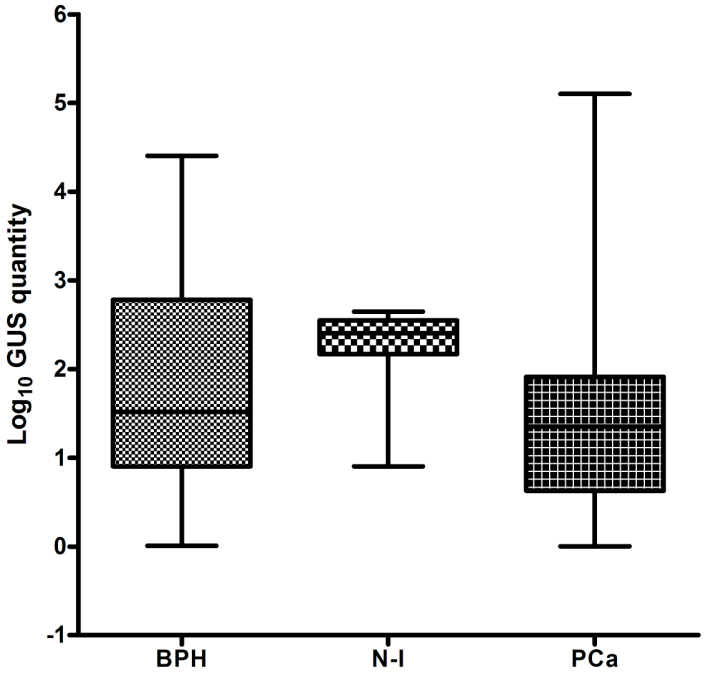


Figure 5.2 GUS transcript levels in FFPE tissue materials. There was a significant difference between N-I and PCa cases ($P < 0.05$).

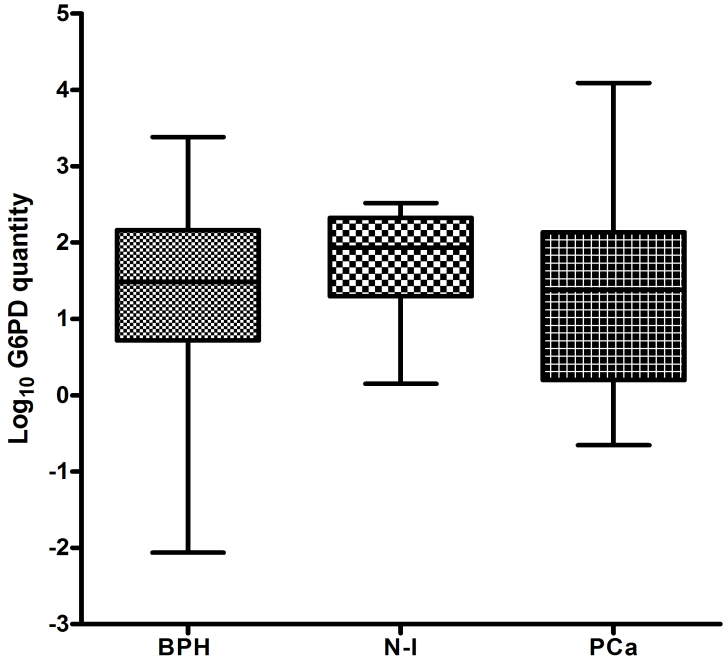


Figure 5.3 G6PD transcript levels in FFPE tissue materials. There was no significant difference among the group.

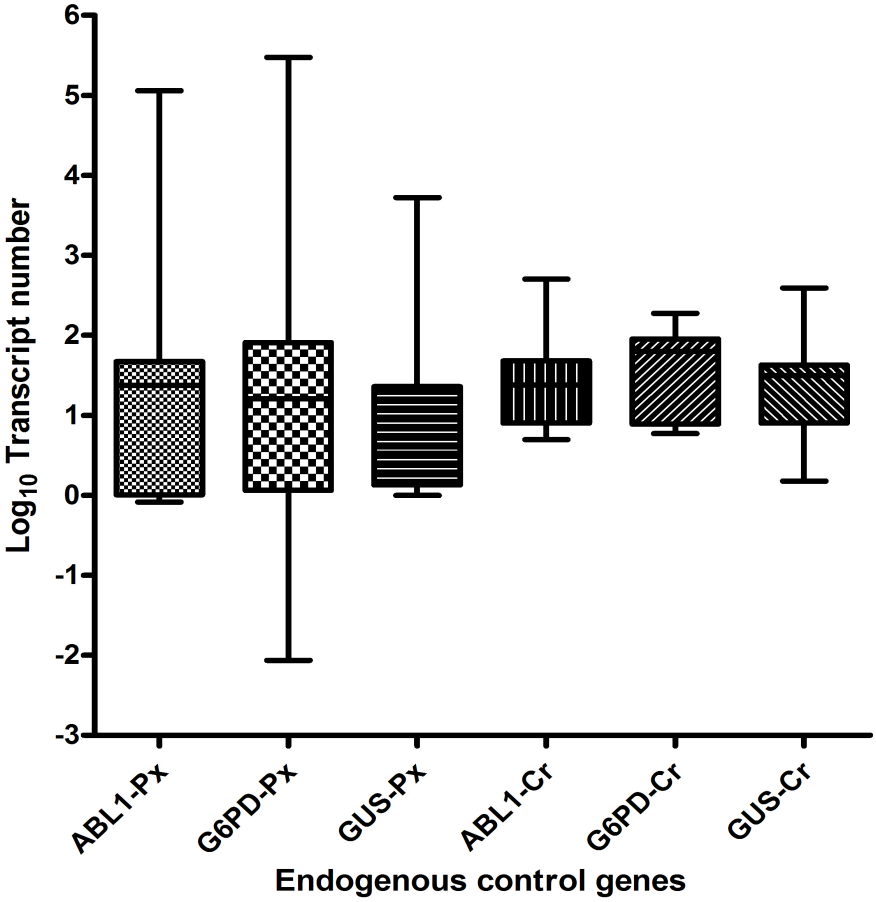


Figure 5.4 Transcript levels of endogenous control genes in exfoliated urine cells. GUS had a significant difference between patient group (Px), n = 53 and control group (Cr), n=. 30.

The effect of sample age on CGs, particularly for the FFPE tissue materials, was assessed. Figure 5.5 shows the variations in ABL1 transcript level in different ages of samples. There was a significant difference between 2006 and 2008 samples ($P < 0.001$); as well as 2007 and 2008($P < 0.001$), but no difference between 2006 and 2007 samples. Similar results were also obtained for G6PD and GUS. There was more than a log reduction in ABL1 level for greater than one year old samples. RQ-PCR sensitivity is reduced in aged samples. Of the 2008 samples, the most recently processed FFPE materials had ABL1 level in the range of 3 to 4 log (similar also in G6PD); and that range informs a better assay sensitivity.

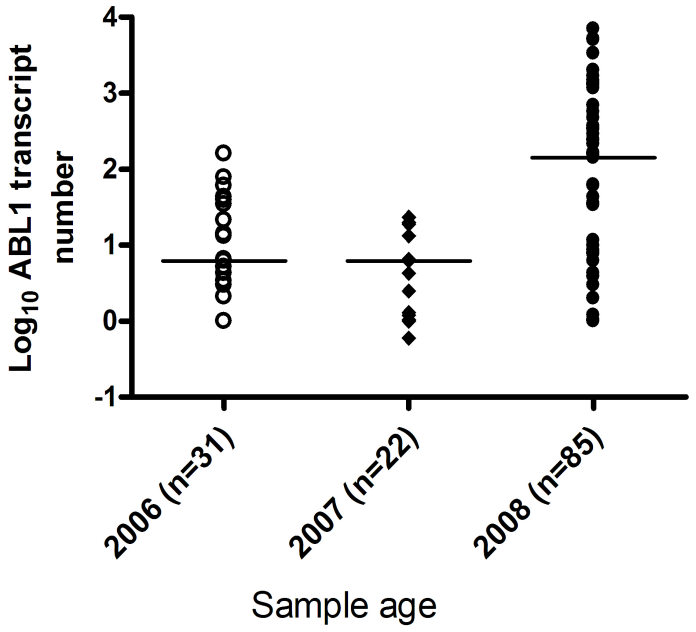


Figure 5.5 Effect of sample age on ABL1 transcript number. There was more than a log reduction in ABL1 in more than a year old samples. Most recently processed samples in 2008 group had ABL1 in the range of 3 to 4 log, which is better for assay sensitivity.

5.3.2 KLK2 transcript levels in FFPE prostate tissue materials and urine samples: The normalized relative quantity (NRQ) of KLK2 transcript number in FFPE tissue materials is shown in Figure 5.6. There were significant differences in NRQs of BPH and PCa cases ($P < 0.001$) and N-I and PCa cases ($P < 0.001$). The NRQ of KLK2 was significantly increased in prostate cancer cases compared to benign and non-involved cases; however there was no significant difference between benign and non-involved cases. That means that KLK2 transcripts are more expressed in benign cases than prostate cancer cases. However, the NRQ of KLK2 was not significantly different in various histological grades of PCa (Gleason Score -GS) ($P = 0.16$); low (GS 4-5), intermediate (GS 6-7) and high (GS 8-10) grades of PCa had similar expression level.

Figure 5.7 shows the NRQ of KLK2 in exfoliated urine cells. There was a significant difference between BPH and control (Cr) cases ($P = 0.0047$); between PCa and Cr cases ($P = 0.0115$). However, there was no significant difference between PCa and BPH cases ($P = 0.816$); non-involved and PCa cases ($P = 0.587$). The sample sizes for PCa, N-I and PIN cases were very small (these patients were sampled prospectively prior to histology examination).

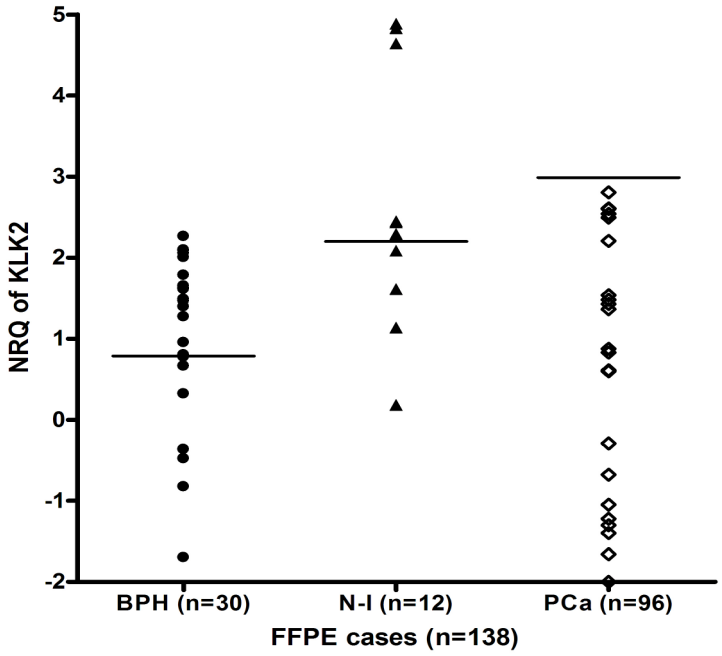


Figure 5.6 Normalized relative expression of KLK2 in FFPE tissue materials. The NRQ of KLK2 was significantly higher in PCa cases (reduced expression of KLK2 transcript in prostate cancer cases). The horizontal lines (bars) represent median values (the distribution was Non-Gaussian).

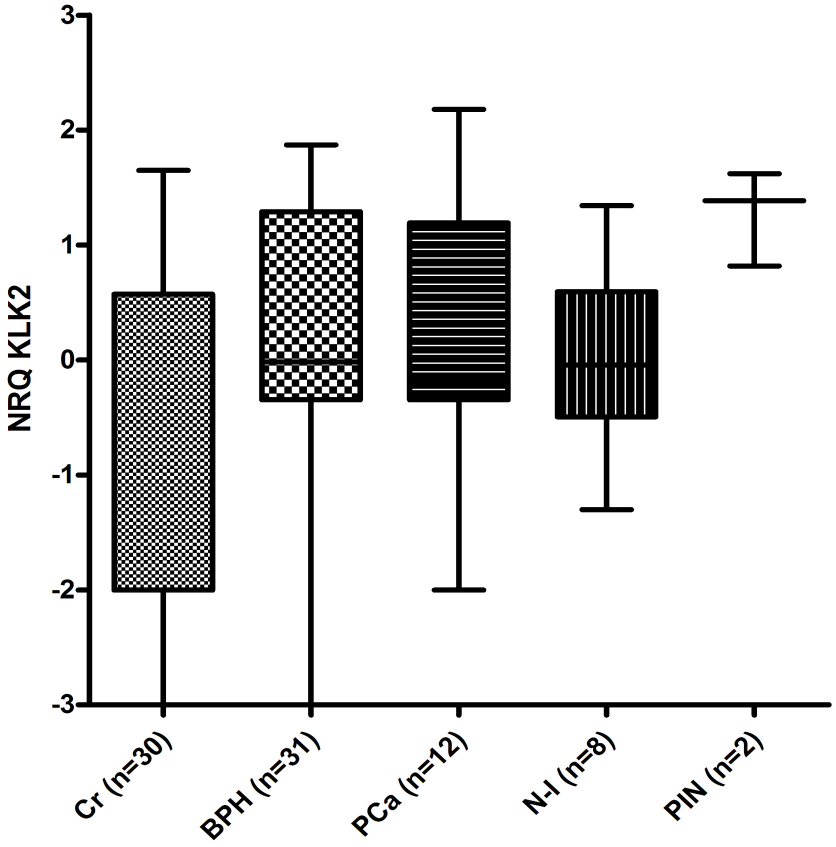


Figure 5.7 Box plots of KLK2 transcript levels in exfoliated urine cells. No significant difference between N-I and PCa cases. Cr stands for control group.

5.3.3 KLK3 gene expression in FFPE tissue materials and urine samples: Figure 5.8 shows the normalized relative quantity of KLK3 transcripts in FFPE tissue sections. KLK3 expression was significantly higher in prostate cancer cases than benign cases (P = 0.01). KLK3 expression was also significantly lower in non-involved cases compared to PCa cases (P <0.05). But there was no significant difference in KLK3 expression between BPH and N-I cases (P > 0.05).

Figure 5.9 shows the KLK3 transcript level in benign, non-involved and PCa cases of urine samples. There was no significant difference in the KLK3 normalized relative quantity (NRQ) in all the cases (P = 0.104)

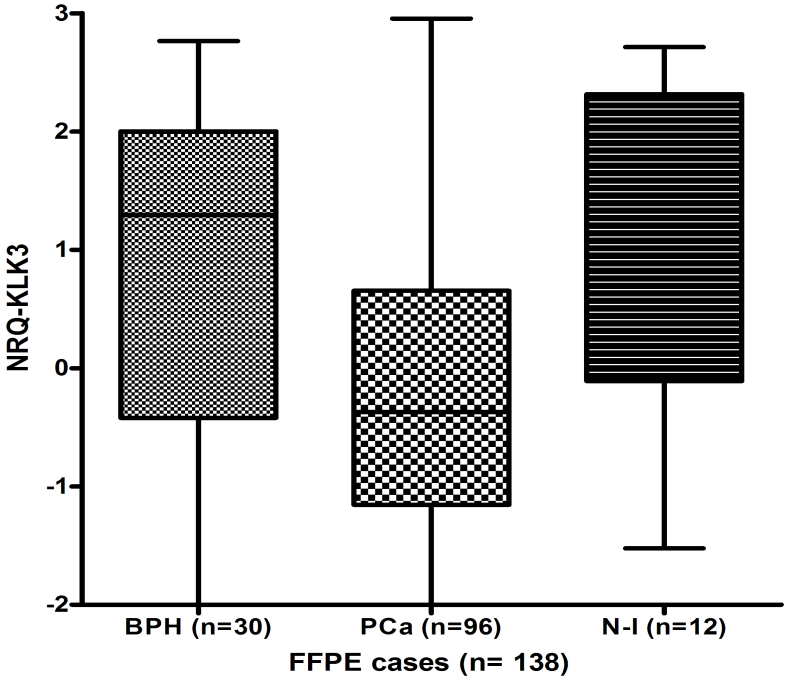


Figure 5.8 Box plots of NRQ of KLK3 transcripts in FFPE materials. KLK3 was significantly more expressed in prostate cancer cases than benign cases (P = 0.0003).

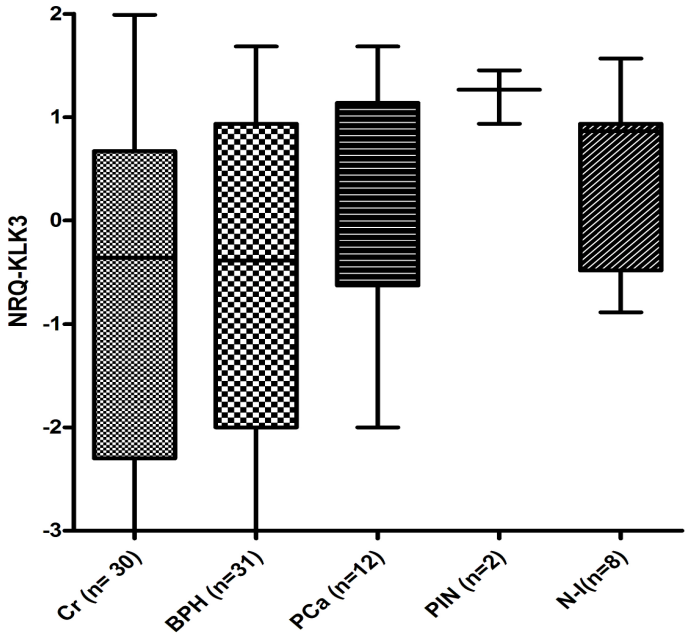


Figure 5.9 Normalized relative expression of KLK3 in urine cells. No significant difference among the group. Cr stands for control group.

5. 3.4 MCM2 and MCM5 gene expressions in FFPE tissue materials and urine samples:

Figure 5.10 shows the MCM2 transcript levels in healthy control group, benign, prostate and non-involved cases. There was a significant difference in MCM2 expression between the control group and BPH cases ($P < 0.01$). But there was no difference between the BPH and prostate cancer cases; no difference also between non-involved and prostate cancer cases.

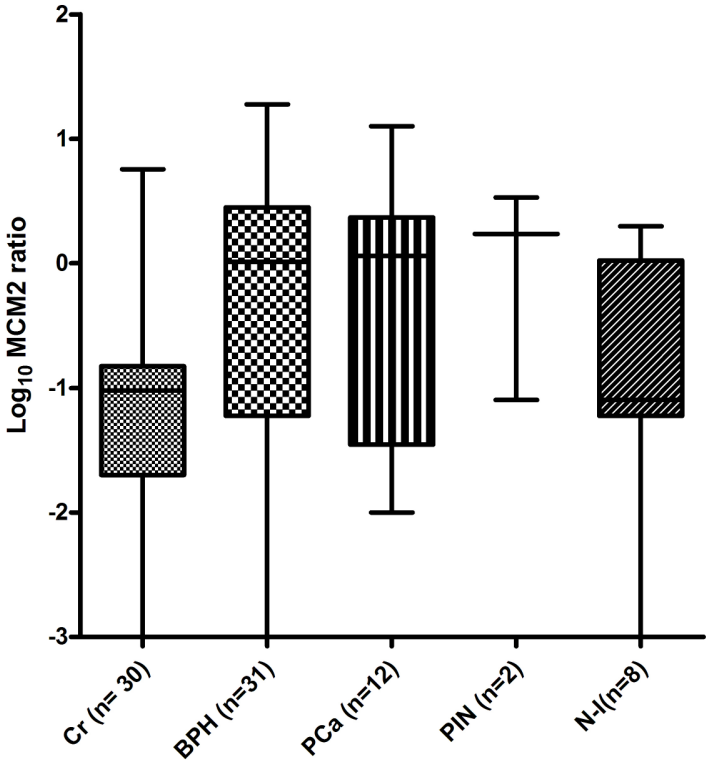


Figure 5.10 MCM2 transcript levels in exfoliated urine cells. There was a significant difference between only BPH cases and healthy control group.

Figure 5.11 shows the MCM2 transcript levels in FFPE tissue materials. There was a significant difference between BPH and PCa cases ($P < 0.01$); but no significant between non-involved (N-I) and PCa cases ($P > 0.05$). MCM2 was more expressed in the prostate cancer cases than in benign cases. When compared with Gleason Scores (GS), there were significant differences between GS 6 and GS7 ($P < 0.05$); GS6 and GS 8-10 ($P < 0.01$) (Figure 5.12).

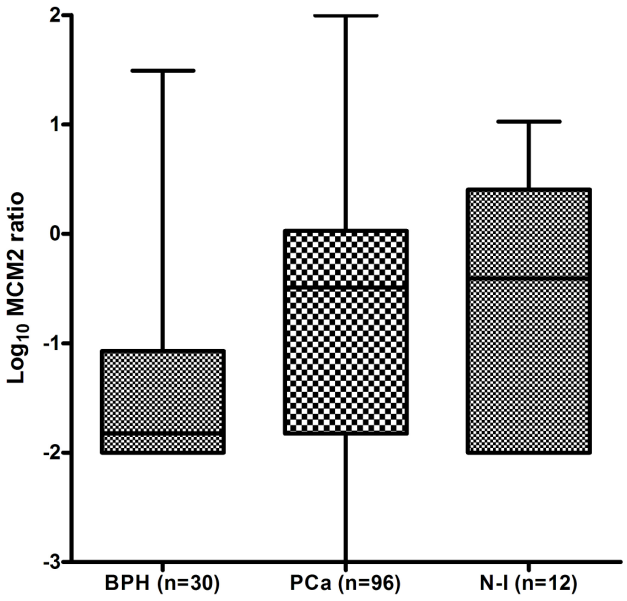


Figure 5.11 MCM2 transcript levels in FFPE tissue materials. There was a significant difference between benign (BPH) and prostate cancer (PCa) cases.

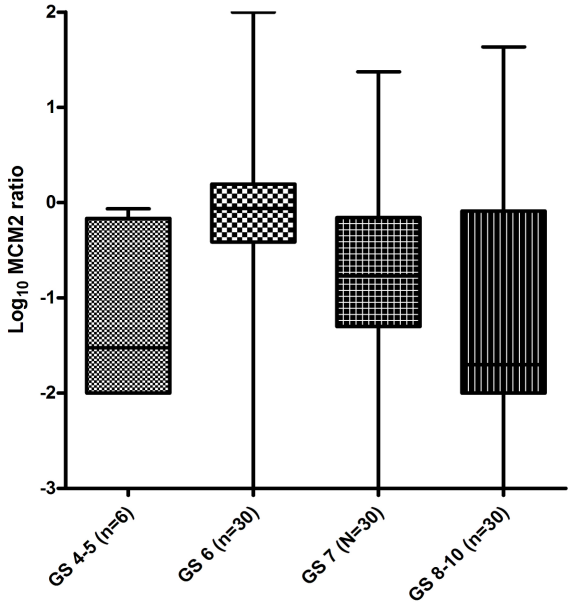


Figure 5.12 MCM2 expressions in various tumour grades. There were higher transcript levels in GS 6 than any other tumour grade.

Figure 5.13 shows MCM5 transcript levels in urine cells. There was no significant difference among the group (P = 0.056).

Figure 5.14 shows the MCM5 expression in FFPE tissue materials. There was also no significant difference in transcript level among the group (P = 0.17).

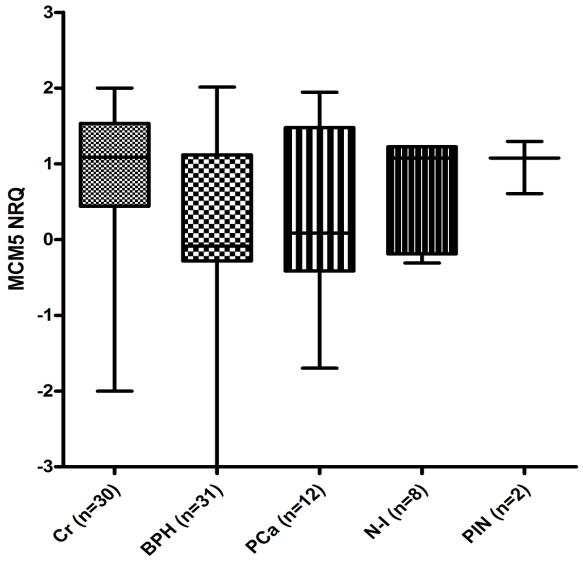


Figure 5. 13 MCM5 transcript levels in urine cells. There was no significant difference among the group.

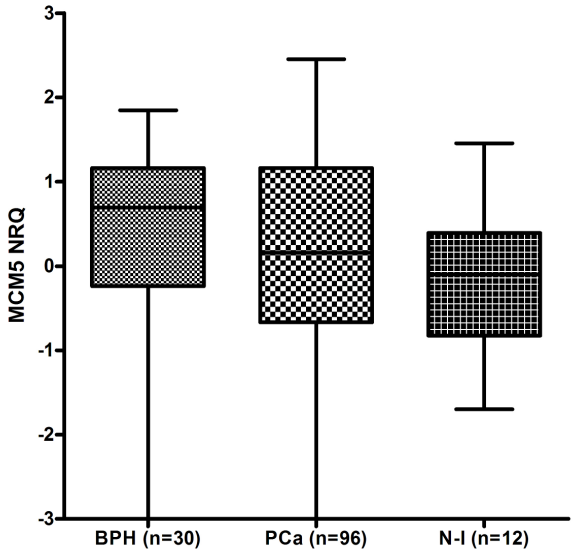


Figure 5.14 MCM5 transcript levels in FFPE materials. There was no significant difference among the group.

5.3.5 TP53 expression in FFPE tissue materials and urine cells:

Figure 5.15 shows TP53 expression in FFPE tissue materials. TP53 was significantly under-expressed in non-involved cases ($P = 0.02$). There was no significant difference between the benign and prostate cancer cases ($P > 0.05$).

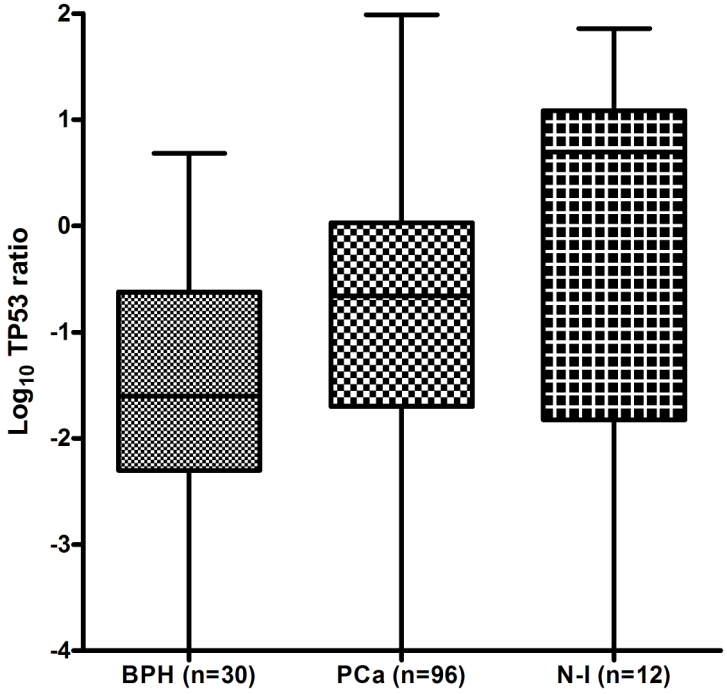


Figure 5.15 TP53 transcript levels in FFPE tissue materials. TP53 was significantly over-expressed in non-involved cases.

TP53 was also significantly over-expressed in GS 8-10 compared to GS 6 and GS 7 ($P < 0.01$).

Figure 5.16 shows the TP53 expression in exfoliated urine cells. There was a significant difference in TP53 transcript level between healthy group and benign cases ($P < 0.05$); also between healthy group and prostate cancer cases ($P< 0.05$). But there was no difference between benign and prostate cancer cases ($P > 0.05$) similar to what was observed in the FFPE tissue materials.

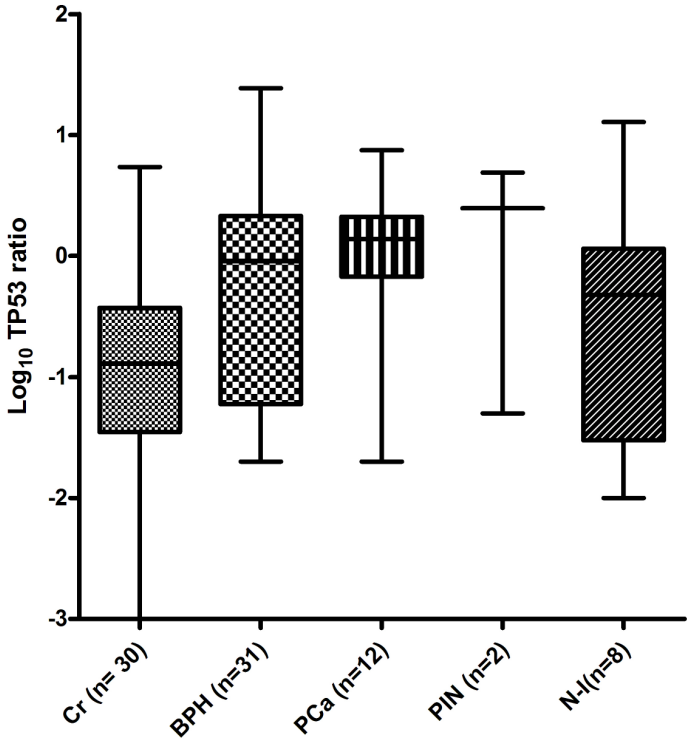


Figure 5.16 TP53 transcript levels in urine cells. There were significant differences between healthy group and benign cases; healthy group and prostate cancer cases.

5. 3. 6 TMPRSS2 fusion genes in FFPE and exfoliated urine cells:

The TMPRSS2: ERG gene fusion was not detected in the urine cells of healthy control group (n= 30). Only 3 cases were detected in the urine cells of patient group; and all the cases were prostate cancer patients (representing 25% of all cancer cases for the urine cell analysis). The mean Ct values for the 3 cases were 18.35, 17.53 and 16.65 respectively. Figure 5.17 shows the normalized relative quantity of TMPRSS2: ERG fusion gene in FFPE tissue materials. There were 2 cases of TMPRSS2: ERG gene fusion in BPH group, 13 cases in the prostate cancer group and 2 cases in the non-involved group. About 12.3% of the sample population was positive for TMRPSS2: ERG gene fusion; and 13.5% of the entire prostate cancer cases (n = 96) were positive for TMPRSS2: ERG. However, there was no significant difference in the NRQ of TMPRSS2: ERG positive cases (between BPH and PCa cases, N-I and PCa cases, BPH and N-I cases; P = 0.17). Of the prostate cancer cases, 3 were GS8-10, 4 cases were GS7, another 4 were GS6 and 2 cases were GS4-5.

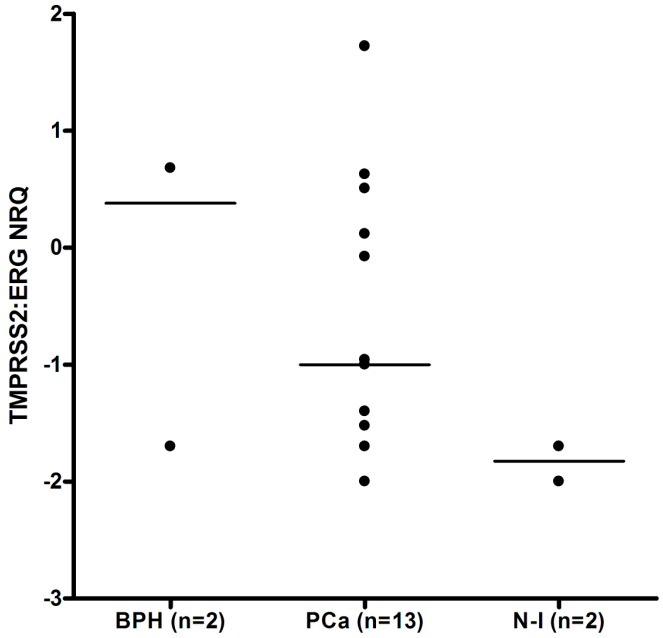


Figure 5.17 TMPRSS2: ERG transcript levels in FFPE tissue materials. There was no significant difference in the normalized relative quantity between the cases in BPH and prostate cancer cases. The gene fusion also occurred in two non-involved cases.

The TMPRSS2: ETV1 fusion type was not detectable in the urine cells of both healthy and patient groups. However, 2 cases of TMPRSS2: ETV1 fusion were detected in the FFPE tissue materials: one case was Gleason score 6 and the other was Gleason score 7.

In summary, a total of 15 cases of TMPRSS2 fusion genes were detected in the 96 prostate cancer cases (representing 15.6% of the cancer cases). The predominant fusion type was the TMPRSS2: ERG (87% of the positive cases).

5.3.7 Gene expressions of Aurora kinases (A, B, C) in FFPE tissue materials

Figure 5.18 shows the transcript levels of Aurora kinase A in FFPE tissue materials. There was a significant difference in the transcript number of AURKA between BPH and PCa cases ($P < 0.05$); AURKA expression was increased in PCa cases. Figure 5.19 shows significant differences between BPH and N-I cases ($P< 0.05$), PCa and N-1 ($P < 0.01$) in the transcript levels of AURKB; there was also increased expression of AURKB in prostate cancer cases (similar to AURKA expression).

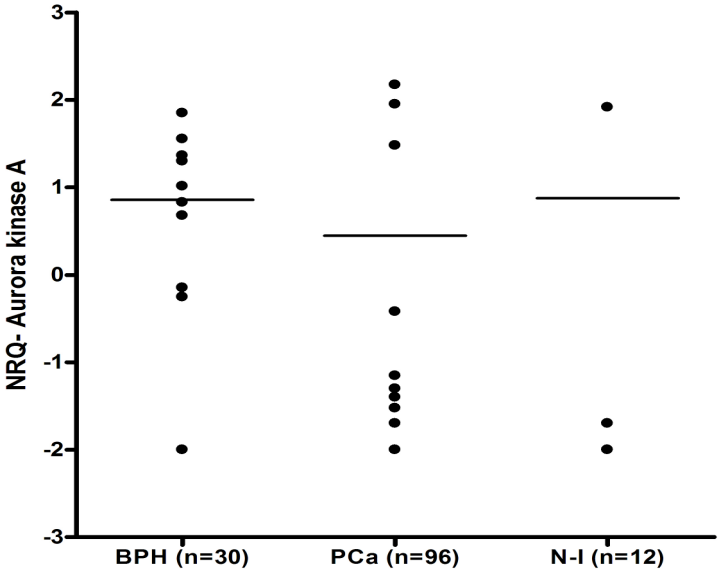


Figure 5.18 The gene expression of Aurora kinase A in prostate tissue sections. There was a significant increased expression of AURKA in prostate cancer tissues.

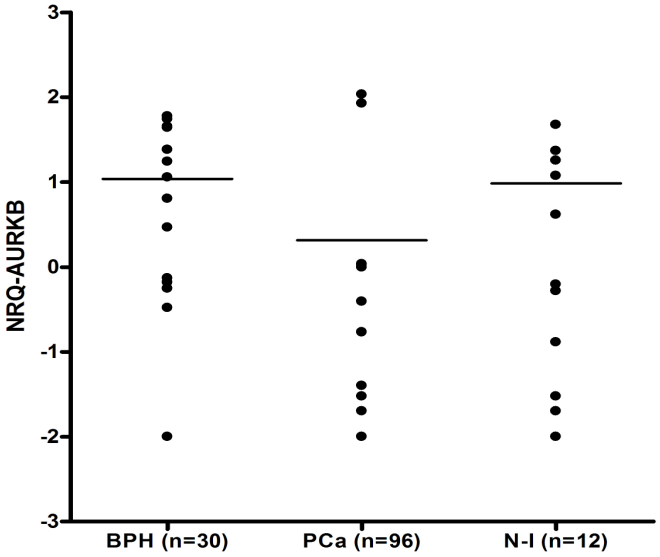


Figure 5.19 The gene expression of Aurora kinase B in prostate tissue sections. There was also a significant increased expression of AURKB in prostate cancer tissues.

Figure 5.20 shows a significant increase in the transcript level of AURKC in both benign and prostate cancer cases compared to non-involved cases ($P < 0.05$). There was no significant difference between BPH and PCa cases. Unlike the AURKA and AURKB, the AURKC transcript level was not significantly increased in prostate cancer compared to benign cases; but the levels in both benign and prostate cancer cases were higher than in non-involved cases.

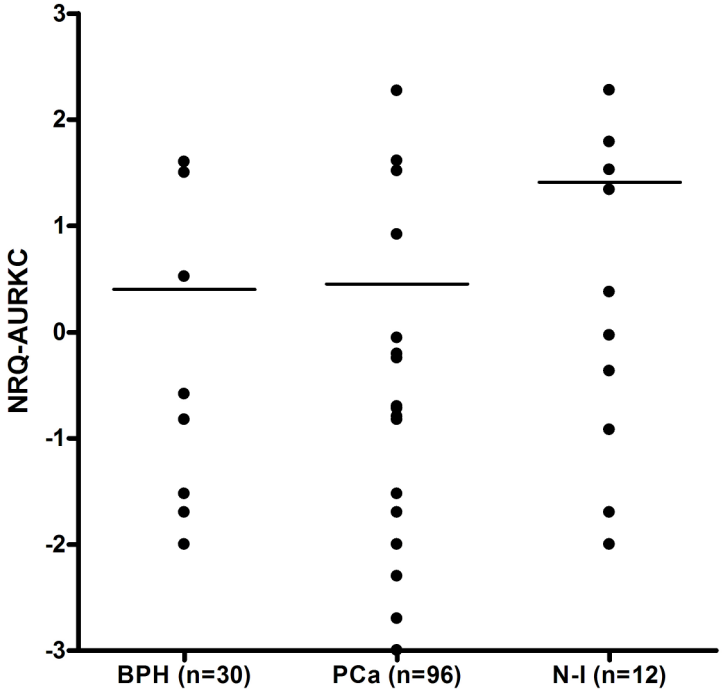


Figure 5.20 The gene expression of AURKC in prostate tissue sections. There was a significant reduction in AURKC transcript level.

5.3.8 Gene expressions of CD44 and CDH 1 in prostate tissue sections

In Figure 5.21a, the transcript levels of CD44 were significantly increased in prostate cancer cases compared to benign and non-involved cases ($P < 0.01$). And the CD44 transcript level in prostate cancer cases was also associated with tumour grade (Figure 5.21b). Higher tumour grades (Gleason score 8-10) expressed less CD44 compared to intermediate grades (Gleason scores 6 and 7). The lower grade (Gleason score 4-5) expressed more CD44 than higher grades.

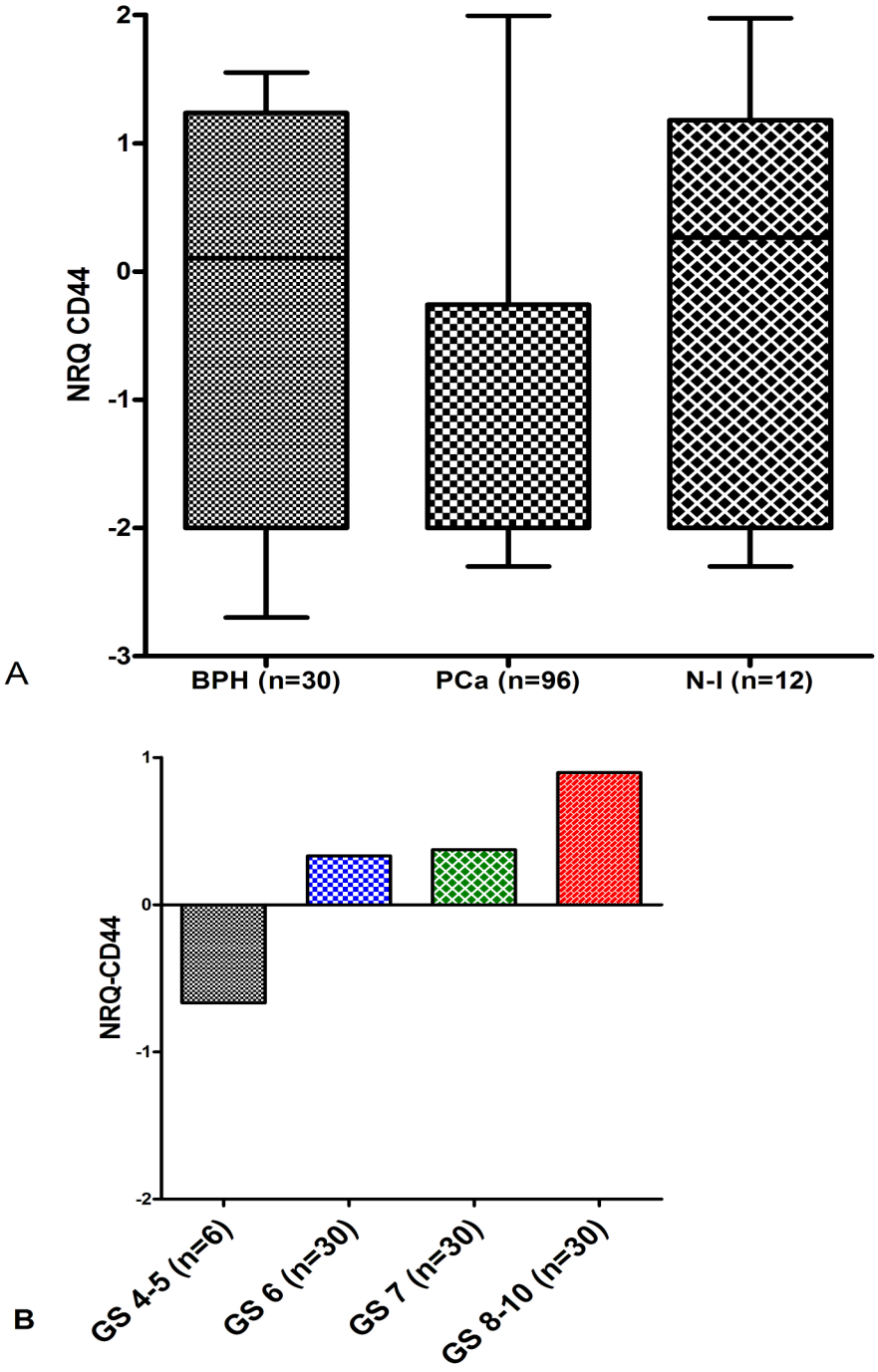


Figure 5.21 Gene expression of CD44 in prostate tissues. (A) CD44 mRNA level was significantly increased in prostate cancer cases compared to benign and non-involved cases. However, in (B) CD44 transcript level was associated with tumour grade.

Figure 5.22 shows no significant difference in the normalized relative quantity of CDH1 transcript level among benign, non-involved and prostate cancer cases (P = 0.77).

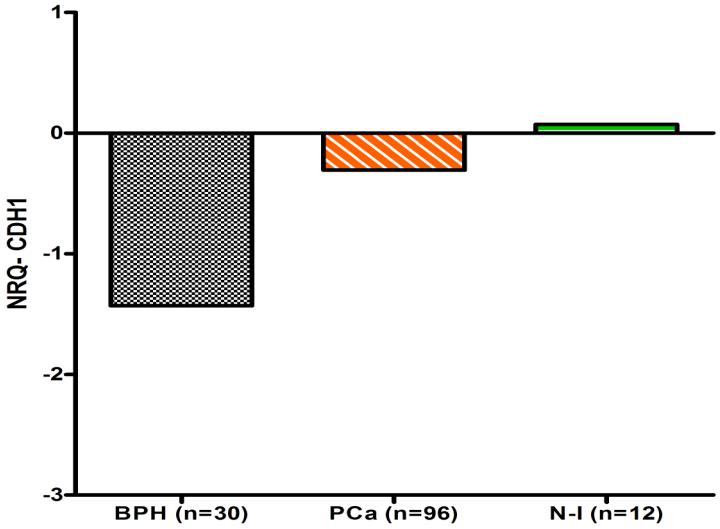


Figure 5.22 Gene expression of CDH1 in prostate tissues. There was no significant difference in transcript level.

5.3.9 Gene expression of FASN in urine cells and prostate tissues

Figure 5.23 shows no significant difference (P = 0.71) in the normalized absolute quantity of fatty acid synthase in exfoliated urine cells of benign, prostate cancer, non-involved and healthy controls.

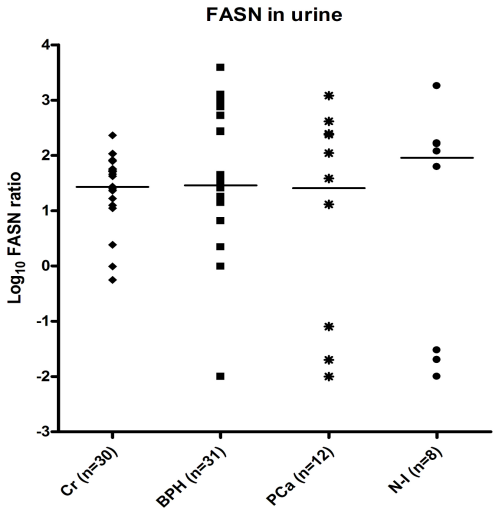


Figure 5.23 Absolute ratio of FASN transcript number in urine cells.

Figure 5.24 shows also no significant difference in the transcript levels of FASN prostate tissues of benign, non-involved and cancer cases (P= 0.17). Similar to the results from urine cells, the FASN transcript number was not differential among the prostate tissues.

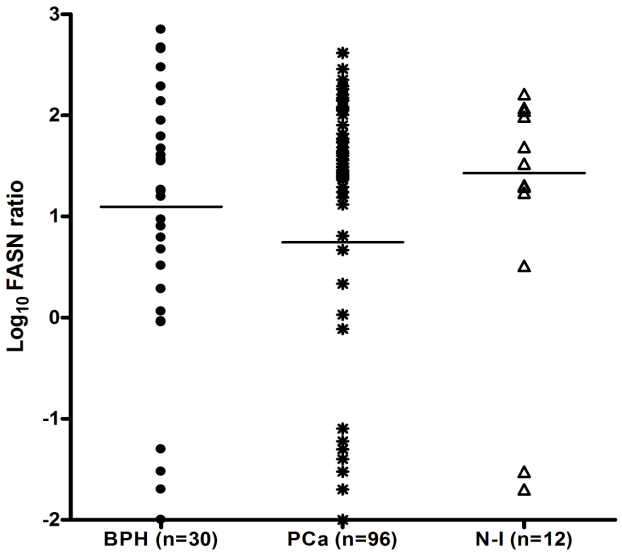


Figure 5.24 Transcript levels of FASN in prostate tissues. There was no significant difference.

5.3.10 Gene expression of Bcl-2 in FFPE prostate tissues

Figure 5.25 shows no significant difference in Bcl-2 transcript levels in FFPE prostate tissue of benign, non-involved and prostate cancer cases (P = 0.20). There was low level of Bcl-2 transcripts across the samples (most Ct values were around 37).

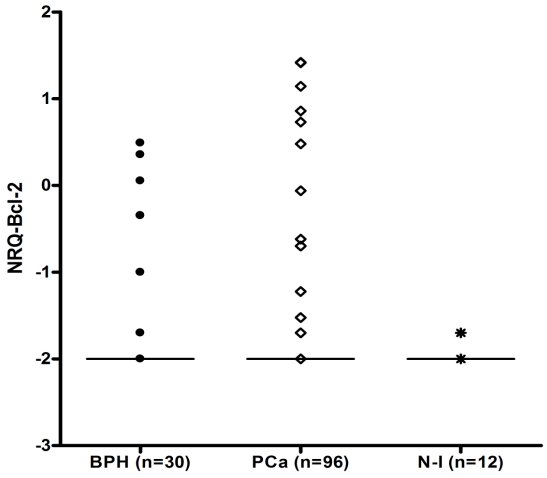


Figure 5.25 Transcript levels of Bcl-2 in FFPE tissues.

5.3.11 Gene expressions of steroid hormone receptors in prostate cancer

Figure 5.26 shows the transcript levels of ESRβ in prostate tissues. In Figure 5.26A, ESRβ was significantly over-expressed in prostate cancer cases compared to benign cases (P < 0.001). There was

also a significant increase in expression of ESRβ in non-involved cases compared to benign tissue ($P < 0.05$); non-involved cases include cases of chronic inflammation and dysplasia 'suspicious of malignancy' that were not histologically confirmed as malignancies. In Figure 5.26B, the ESRβ expression was significantly associated with Gleason score ($P < 0.0001$); there was over-expression in higher tumour grades compared to lower and intermediate tumour grades. However, within a tumour grade (Gleason score 8-10), the ESRβ expression was not significantly associated with pathological stage ($P = 0.65$, Mann-Whitney test).

Figure 5.27 shows no significant differential expression of ESRα in prostate cancer cases compared to benign and non-involved cases ($P = 0.33$).

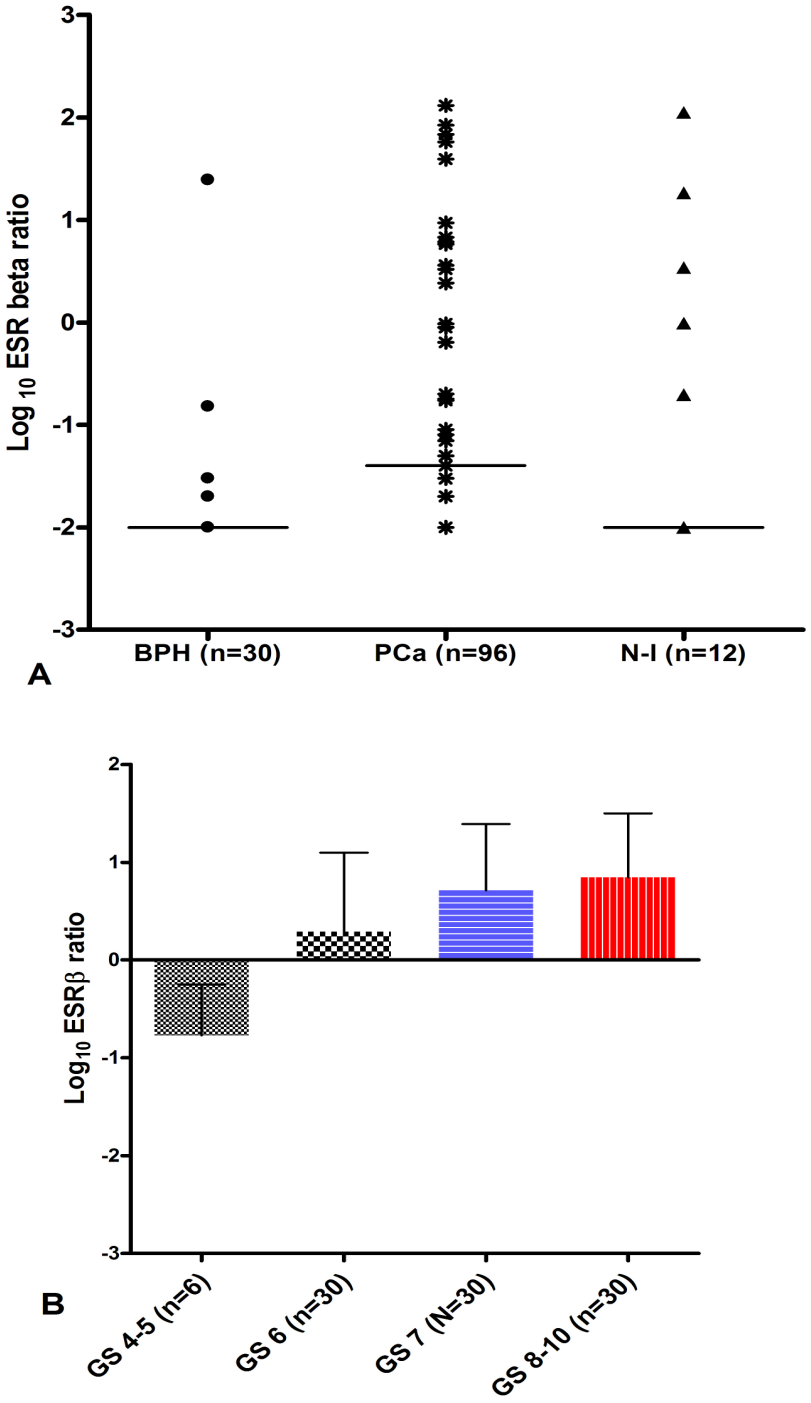


Figure 5.26 Gene expressions of ESRβ in prostate tissues. A is aligned dot plot showing a significant over-expression of ESRβ in prostate cancer cases. B a bar plot showing that ESRβ over-expression was associated with tumour grade.

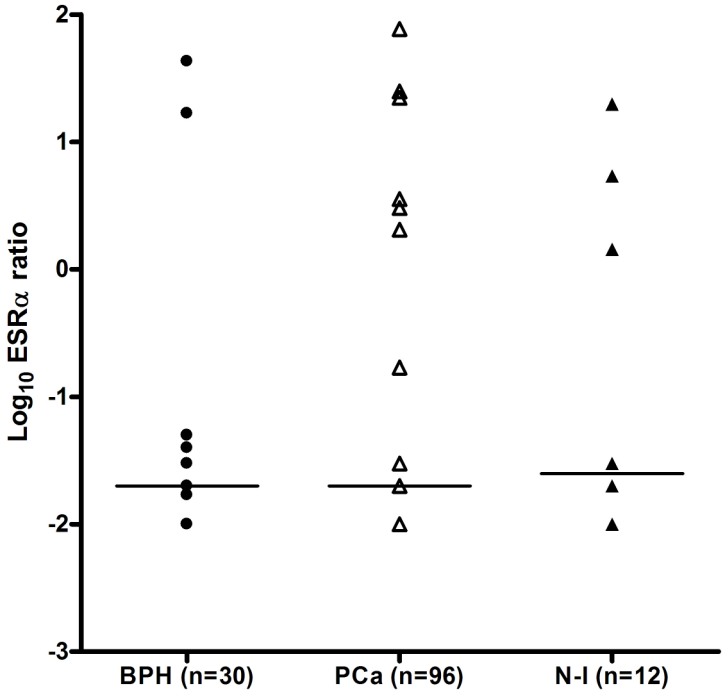


Figure 5.27 Transcript levels of ESRα in prostate tissues**.** There was no significant difference among the sample groups.

Figure 5.28 shows a significant over-expression of androgen receptors (AR) in prostate cancer cases compared to benign cases ($P < 0.01$). However, the increase was not associated with tumour grade and pathological stage.

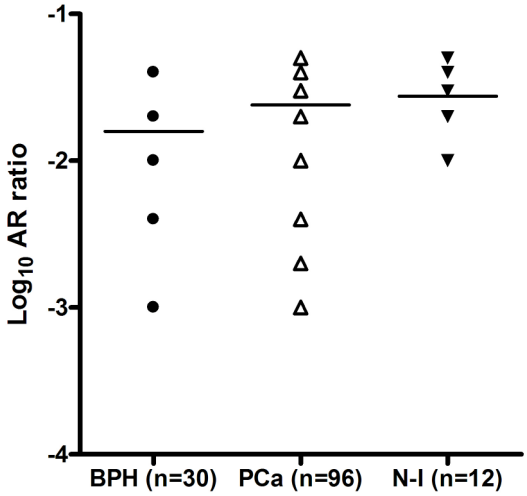


Figure 5.28 Transcript levels of AR in prostate tissues. There was a significant over-expression of AR in prostate cancer and non-involved cases.

5.3.12 Immunohistochemistry (IHC)

Figures 5.29 to 5.31 are a panel of representative photomicrographs of PSA. AR, ESRβ and ESRα staining in benign, non-involved and PCa cases; benign hyperplasia cases stained more strongly for PSA than PCa cases.

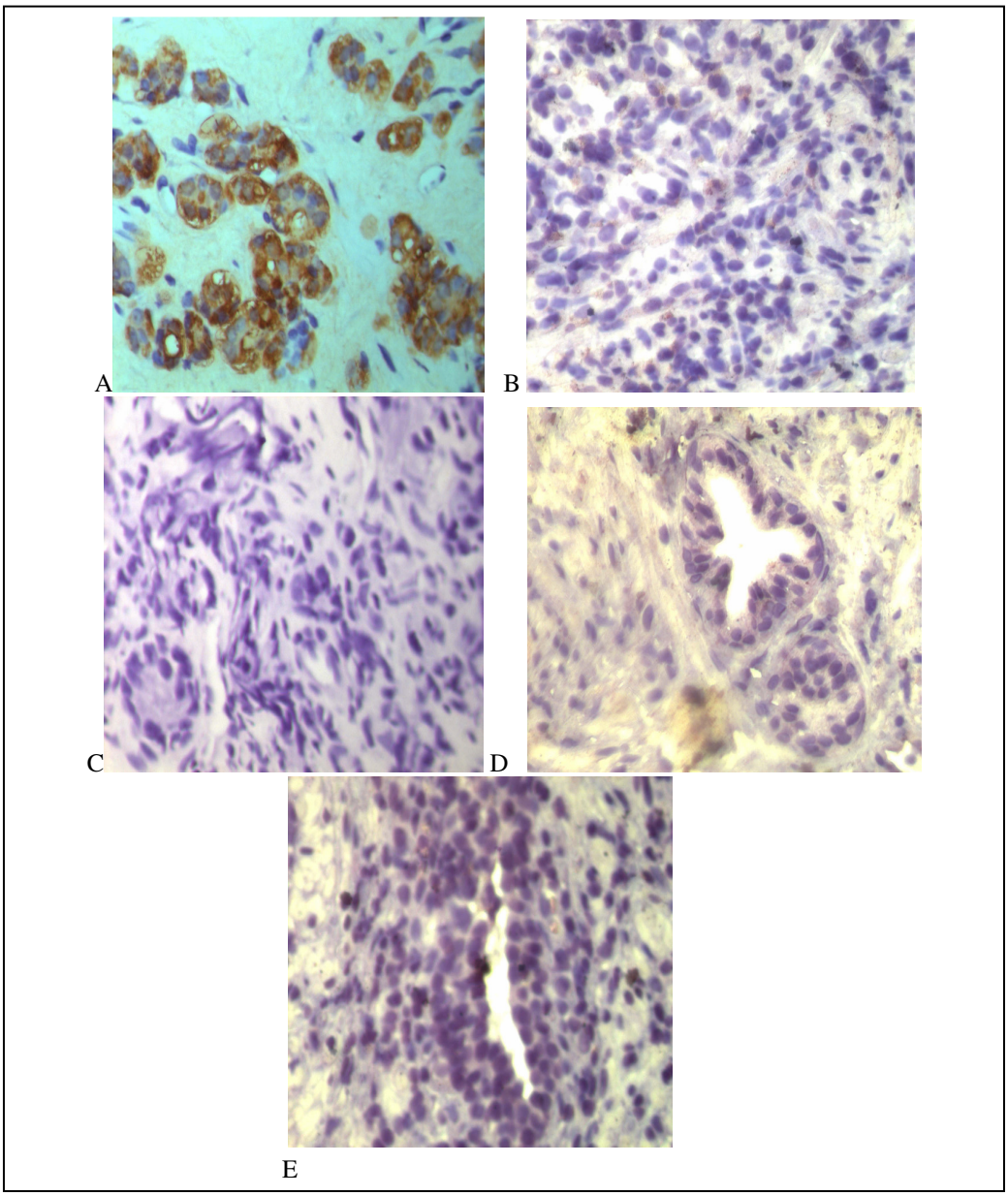


Figure 5.29 Representative photomicrographs of immunostained tumour markers. A is PSA positive PCa, B is ESRβ positive, C is a negative control slide; D is AR positive and E is ESRα positive slide. The SHRs stained dark-brown mainly in the nuclei while the PSA stained brown in the cytoplasm. Scale: 1cm = 23 μM (x 400 magnification).

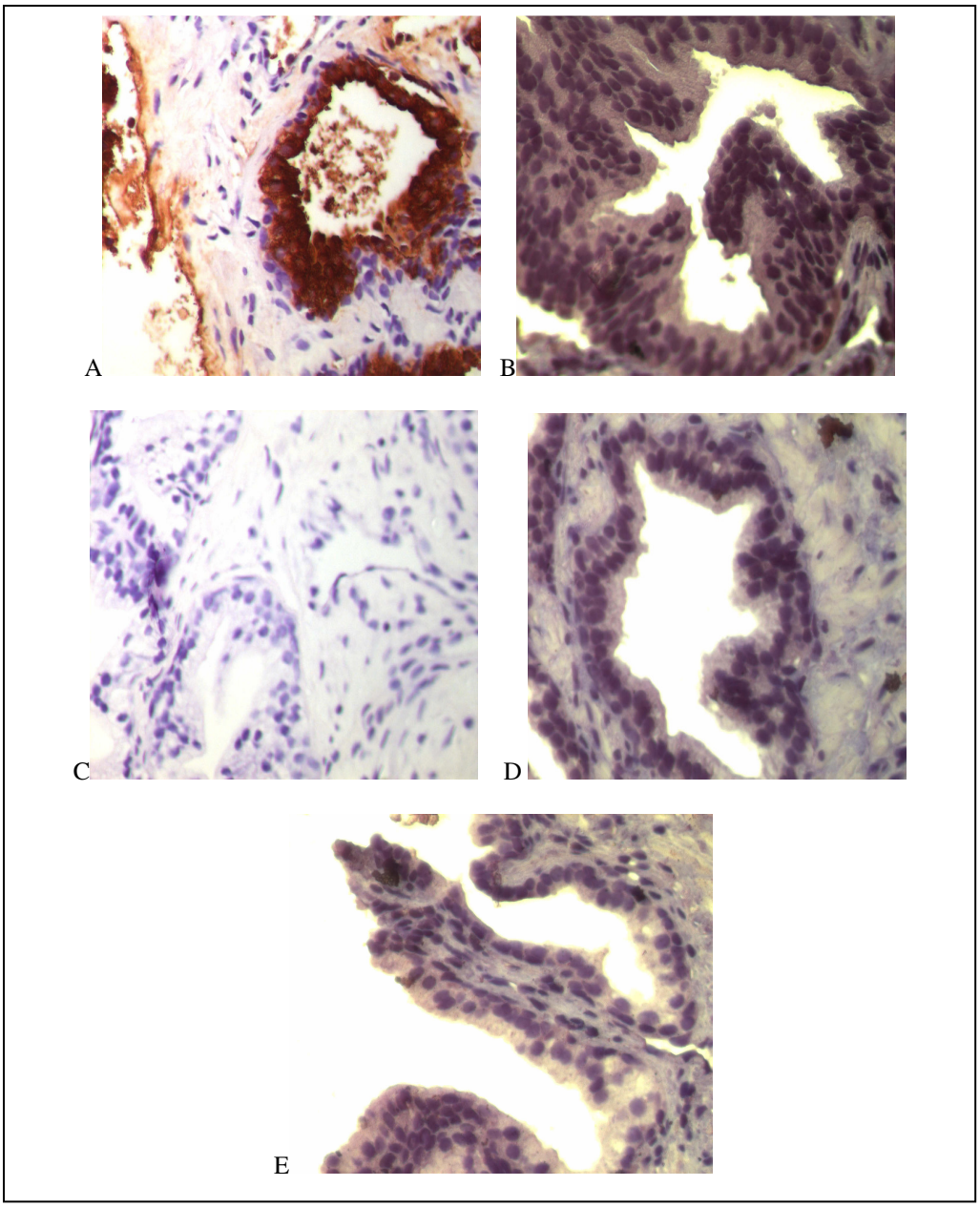
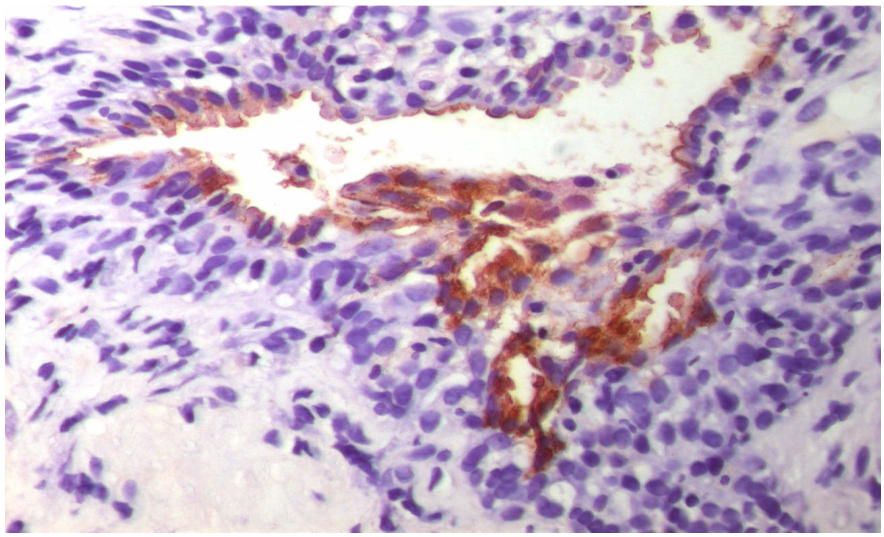
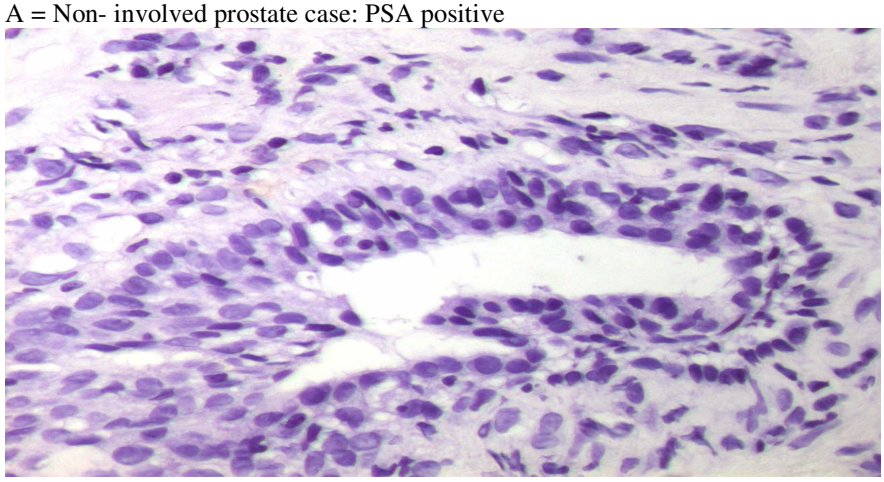


Figure 5.30 Representative photomicrographs of immunostained tumour markers in benign cases. A is PSA positive PCa, B is ESRβ positive, C is a negative control slide; D is AR positive and E is ESRα positive slide. The SHRs stained dark-brown mainly in the nuclei while the PSA stained brown in the cytoplasm. Scale: 1cm = 23 µM (in x 400 magnification).



A = Non- involved prostate case: PSA positive



B= Non-involved prostate case- PSA negative control.

Figure 5. 31 PSA immunostaining of non-involved prostate cases. X400 magnifications. Scale: 1cm = 23µm. PSA stained brown in the cytoplasm of epithelial cells (Moderate and patchy intensity was observed in non-involved cases).

5. 4 Discussion

5.4.1 Endogenous control genes: The choice of a single stably expressed control gene is very crucial to normalization of target transcripts of molecular markers. From the results, GUS was not stably expressed in both the FFPE tissue materials and exfoliated urine cells; and therefore not suitable for use in normalization of target genes. ABL1 and G6PD transcript levels emerged stable in all the samples groups; however the median ratios of ABL1 to GUS and G6PD remained steady compared to that of G6PD. In addition, both G6PD and ABL1 showed no difference when compared to the geometric mean of both. Therefore, ABL1 was used to normalize gene expression of all the target transcripts. Some multi-centre studies had also shown that ABL1 transcript levels were stably expressed in other cell types, especially in haematological malignancies {Beillard *et al*, 2003; Garbert *et al*, 2003}. The ABL1 gene has no known pseudogenes unlike many other CGs that had been used in prostate cancer gene expression studies {Schmidt *et al*, 2006}.

Both FFPE tissue materials and exfoliated urines offer the opportunity to profile prostate cancer patients molecularly. But the urine samples have an advantage of non-invasive collection. Most recently processed FFPE tissue materials yielded higher levels of CGs; aged samples would reduce RQ-PCR sensitivity due to degradation of RNA of target transcripts.

5.4.2 KLK2 gene expressions in FFPE tissue materials and urine cells:

In the FFPE tissue materials, the KLK2 transcript levels were differentially expressed (decreased in prostate cancer); this was similar to what had been reported in several studies {Kurlender *et al*, 2005; Diamandis & Obiezu, 2005}. But in the urine cells, the KLK2 transcript level was not differentially expressed; there was no significant difference between N-I and PCa cases contrary to the finding in FFPE materials. Similar finding was also reported by Panek *et al*, 1997 and Magklara *et al* (2000) who reported a decrease in the KLK2 transcripts in malignant compared to non-malignant tissues. However, several studies at protein level had shown that KLK2 protein product (hK2) was differentially expressed in prostate cancer (increased in prostate cancer) {Darson *et al*, 1999; Becker *et al*, 2000}. In addition, the results from this study show that KLK2 transcript level does not correlate with tumour grade.

5.4.3 KLK3 gene expressions in FFPE tissue materials and urine cells:

The findings in this study showed that KLK3 transcript level in prostate FFPE tissue sections would not be clinically useful for detecting or staging prostate cancer. The transcript level of KLK3 was higher in prostate cancer cases compared to benign cases. This was not comparable to the IHC results where benign cases stained intensely and diffusely for PSA compared with moderate staining in PCa cases. Previous studies had also reported that KLK3 transcript level in blood and urine samples were not diagnostically useful for detection and staging of prostate cancer {Corey et al, 1997; Becker et al, 2000}. However, some molecular tests, for example, the PCA-3 test rather use PSA transcript level as an endogenous, prostate specific, control gene for normalization of PCA-3 transcript level in urine samples. Although the PSA level did not vary in the urine samples, the reliability and robustness of PSA as a control gene compared to other endogenous control genes remained unclear. The PCA-3 molecular test was reported to have a moderate sensitivity and specificity; and relied on the 'tissue- specificity' of PSA. But there is also the problem of extra-prostatic PSA, contributing to the entire PSA pool.

5.4.4 MCM2 and MCM5 gene expression in FFPE tissue materials and urine cells:

The MCM2 and MCM5 are part of the MCM family of proteins involved in DNA licensing for cell replication. The transcript levels (expressed as absolute ratios) of MCM2 in FFPE tissue materials showed a significant difference between the benign and prostate cancer cases; MCM2 transcripts were over- expressed in PCa cases. Many other studies had reported increased MCM2 expression in human cancers {Freeman *et al*, 1999; Stober *et al*, 1999; Alison *et al*, 2002. However, the non-involved cases consisting of acute and chronic inflammation also showed over-expression of MCM2. Therefore, the MCM2 transcript level in FFPE tissue materials has a limited application for diagnosis. However, MCM2 transcript level showed an association with tumour grade; there was higher expression in Gleason score 6 grades than any other grade. Meng *et al* (2001) reported that prostate cancer patients with high MCM2 expression had shorter disease-free survival, following radical prostatectomy for localised PCa. Therefore, MCM2 could have a prognostic value in patients undergoing prostatectomy. The MCM2 transcript levels in exfoliated urine cells were also of limited diagnostic value. It could not differentiate between cancer and benign cases. The MCM5 transcript levels in both urine and FFPE tissue materials showed no diagnostic value in prostate cancer. However, some studies had shown that MCM5 expression was strongly associated with urothelial cancers {Stoeber *et al*, 1999}.

5.4.5 TP53 gene expression in FFPE tissue materials and urine cells:

In both urine cells and FFPE tissue materials, there was no significant difference in TP53 transcript levels between benign and prostate cancer cases. However, TP53 in prostate cancer cases (n=96) was over-expressed in higher tumour grades (GS 8-10). The prognostic value of TP53 gene expression has been controversial. Some studies reported increased nuclear accumulation of TP53 as a prognostic marker in PCa {Quinn *et al*, 2005}. Isaacs (1997) reported that increased positive staining for TP53 was associated with TP53 mutations and higher tumour grades.

5.4.6 TMPRSS2 fusion genes in FFPE and exfoliated urine cells:

The TMPRSS2 gene fusion was detectable in both urine and FFPE tissue materials. The predominant fusion type was the TMPRSS2: ERG. Contrary to the report by Mehra *et al* (2007), there was no significant association of the TMPRSS2 fusion genes with tumour grade (Gleason score) observed in this cohort. Only two common variant types of the fusion gene were tested for in this study; there are 14 known transcript variants of the gene fusion (Section 2.3.3). Secondly, the TMPRSS2: ERG gene fusion was also detected in benign and non-involved cases, contrary to the report by Cross *et al* (2008). However, Tomlins *et al* (2007) reported the fusion of ETV1 gene with other 5' partners in place of TMPRSS2 in many negative cases of TMPRSS2:ETV1 gene fusion. Studies on the TMPRSS2/ETV gene fusion would require interphase FISH technique and multiplex PCR approach to identify complex gene rearrangements and resulting fusion types.

5.4.7 Gene expressions of Aurora kinases (A, B, C) in FFPE tissue materials

The transcript levels of the Aurora kinases A an B were consistently increased in prostate cancer cases compared to non-involved and benign cases. Aberrant expression of Aurora kinases were associated with abnormal mitosis, the increased transcript levels of AURKA and B observed in this study was agreement with the report of increased expression by Chieffi *et al* (2006). It is possible that Aurora kinases could have increased transcript level and dysfunction in prostate cancer; a condition that could be exploited chemotherapeutically. Nevertheless, more studies would still be required to confirm the expression patterns of Aurora kinases in prostate cancer. Most recently processed FFPE tissue sections should be used to enhance the sensitivity of RQ-PCR. However, previous studies on Aurora kinases were immunohistochemical; mutant forms of aurora kinases could also contribute to increased abnormal expression of the Aurora kinases.

5.4.8 Gene expressions of CD44 and CDH 1 in prostate tissue sections

CD44 is a cell adhesion molecule. It was over-expressed in prostate cancer tissues compared to benign cases. Some studies have associated over-expression of CD44 in prostate cancer with mutation {Isaacs, 1997}; other studies have reported increased expression (detected by IHC) of the standard form of CD44 in human cancers {Hu *et al*, 2009}. The finding from this study showed that CD44 transcript level was over-expressed in prostate cancer cases. However, the mutation status of these transcripts was not investigated. Secondly, the primers used in the RQ-PCR were designed to amplify the common variants of CD44; it is possible that other novel variants of CD44 could occur in prostate cancer cases. Further studies, designed to investigate mutation status and frequency of transcript variants, would be required to elucidate the role of increased CD44 transcripts observed in this study. The transcript level was also associated with tumour grade. Higher Gleason score had lower CD44 transcript number. Although CD44 transcript level was significantly higher in prostate cancer cases, there was decreased transcript level with increasing tumour grade. Prostate lesions are often heterogeneous; foci of malignancy could co-exist with benign lesions. And without microdissection of particular cell types and lesions, the explanation of gene expression studies in prostate could be difficult.

The transcript levels of CDH1 did not significantly differ among benign, non-involved and prostate cancer cases. Some previous studies had reported reduced CDH1 immunostaining in prostate cancer, with benign cases showing a much uniform cell to cell border staining immunohistochemically. The finding from this study showed that the transcript level did not differ in all the cases. Factors such as post-translational modifications could alter protein products of genes in tissues. It is also becoming apparent that regulatory RNAs could alter the expression of gene transcripts in different tissues. At transcript level, CDH1 did not show any diagnostic value in prostate cancer cases evaluated in this study.

5.4.9 Gene expression of FASN in urine cells and prostate tissues

Fatty acid synthase (FASN) is a key enzyme in lipid metabolism. Its abnormal expression had been reported as an early event in prostate cancer development. Although it was detectable in urine cells; it showed no differential expression at transcript levels among the study groups. Similar results were obtained also for the FASN transcript ratios in FFPE tissue materials; there was no differential expression.

5.4.10 Gene expression of Bcl-2 in FFPE prostate tissues

The transcript levels of Bcl-2 were detectably low; and showed no significant variation among the groups. Using most recently processed FFPE tissue materials could improve the detection of Bcl-2. Previous immunohistochemical studies had shown that Bcl-2 expression increased with tumour grade. However, the transcript levels, from this study, were not differential between benign and prostate cancer cases.

5.4.11 Gene expressions of steroid hormone receptors in prostate cancer

The results from this study showed that ESRβ was over-expressed in prostate cancer tissues compared to benign tissues; and the over-expression was also significantly associated with tumour grade and not pathological/clinical stage. The role of ESRβ in prostate cancer progression has been controversial. Early studies reported a decreased expression of ESRβ (mainly by immunostaining) and thought it was anti-proliferative. However, some recent studies have shown that ESRβ was over-expressed in prostate cancer {Walton *et al*, 2009}. The results from this study show that ESRβ is over-expressed in prostate cancer, and probably for the first time, indicate that the over-expression is also associated with tumour grade. This implies a proliferative role for ESRβ. The results from prostate tissue were also similar to the *in vitro* results where ESRβ was over-expressed in MDA PCA 2b at protein and mRNA levels, a form of progressive prostate cancer cell line. These findings plus previous reports in this direction would require further studies on specifically targeting ESRβ in high grade tumours with selective oestrogen antagonists for therapeutic purposes.

Although no association with pathological /clinical stage was observed, there was lack of clarity in clinical notes on pathological versus clinical stage. The over-expression of ESRβ in prostate cancer

cases refutes the null hypothesis, which stated that there was no differential expression of ESRβ in prostate tissues.

On the other hand, ESRα was not differentially expressed among the sample groups. This reinforced the observation that ESRβ could be the key mediator of oestrogenic activity during prostate cancer progression. It does appear that there is a reversal of oestrogen receptor role in prostate cancer progression compared to the embryological development of the prostate, where Prins *et al* (2001) demonstrated in mice that ESRα was the key mediator of oestrogenic activity.
Androgen receptors AR, like the ESRβ were also significantly over-expressed in prostate cancer cases compared to benign cases. Increase synthesis of oestrogen receptors are known to induce concomitant production of androgen receptors {Droller, 1997}. Several immunohistochemical studies have shown that steroid hormones are often expressed in a similar pattern in most endocrine tissues {Quinn *et al*, 2005; Murphy *et al*, 2006}.

Results of immunohistochemical staining in this study also confirmed the expression of these steroid hormone receptors (SHR). However, the interesting observation is that ESRβ is the most predominant form of oestrogen receptors in prostate cancers and was associated with high tumour grade. In breast cancers, for example, the ESRα is the predominant form of oestrogen receptor that molecularly characterises the behaviour of the disease. It would be useful to investigate the prognostic and therapeutic value of ESRβ in prostate cancer.

5.5 Conclusions

The key findings in this chapter include:

- ABL1 transcript level is the most stably expressed endogenous control gene in both FFPE tissue materials and exfoliated urine cells. Therefore it was used to normalize gene expression of target transcripts. Each target transcript is expressed per copy of ABL1 in the tissue sample.
- Most recently processed FFPE tissue materials yielded better results; aged samples reduced RQ-PCR sensitivity. Aged samples (samples older than 1 year) had lower transcript number of ABL1, which implied degradation and loss of mRNAs including those of target transcripts.
- KLK2 transcript levels in FFPE tissue materials were decreased in prostate cancer cases and showed no correlation with tumour grade.
- KLK2 transcript levels in urine cells were not differential in both healthy and prostate disease cases.

- KLK3 transcript levels in urine were not clinically useful in detection or staging of prostate cancer. At mRNA level, KLK3 was more expressed in prostate cancer tissues compared to benign and non-involved cases; however at the protein level there was higher expression of PSA in benign tissues than prostate cancer and non-involved samples.
- The transcript levels of both KLK2 and KLK3 were not consistently reliable for diagnosis using urine samples.
- MCM2 transcript levels in exfoliated urine cells have a limited diagnostic value; it could not differentiate between cancer and benign cases. However, the MCM2 transcript number in FFPE tissue materials could have prognostic value; over-expression was associated with Gleason Score 6.
- MCM5 transcript level has no diagnostic value in prostate cancer.
- TP53 gene over-expression was associated with higher tumour grade.
- TMPRSS2: ERG was the most predominant variant of the gene fusion with ETS genes in prostate cancer.
- The TMPRSS2 gene fusions were also detectable in benign and non-involved prostate cases.
- Only 15.6% of the prostate cancer cases expressed the TMPRSS2 gene fusion in the sample population.
- However, some novel variants of the ETS gene fusions could have been undetectable by the two assays used in this study.
- Aurora kinases A and B were consistently significantly increased at transcript level in prostate cancer cases compared to benign and non-involved cases, but Aurora kinase C did not differ in both benign and prostate cancer cases.
- CD44 was over-expressed in prostate cancer tissues and also associated with tumour grade.
- Transcript levels of CDH1 did not show any differential expression among benign, prostate cancer and non-involved cases.
- Fatty acid synthase transcript numbers in urine cells and FFPE prostate tissue materials were not differentially expressed in benign, prostate cancer and healthy controls.
- Bcl-2 transcript levels in prostate tissues were not differentially expressed between benign and prostate cancer cases.
- ESRβ was remarkably over-expressed in prostate cancer cases, and was also associated with tumour grade.

- ESRα did not show any differential expression in prostate diseases.
- AR was also over-expressed in prostate cancer cases, but was not associated with tumour grade.

Overall, of the 16 molecular markers investigated, 7 of them showed significant association with prostate cancer progression: TP53, TMPRSS2 fusion genes, AURK A, AURKB, CD44, ESRβ and AR. Future studies would be required to establish the prognostic value of these seven molecular markers in prostate cancer. Most recently processed FFPE tissue materials, no longer than one year old should be used in designing RQ-PCR studies for markers. Also ABL1 gene emerged as the most stable candidate control gene for normalization of RQ-PCR.

Table 5.1 Summary of change in normalized transcript ratio of biomarkers

Increased transcript ratio in Prostate cancer compared to benign cases	Decreased transcript ratio in prostate cancer compared to benign cases	No significant change between prostate cancer and benign cases
• **MCM2** • **TP53** • **TMPRSS2:ERG** • **Aurora A** • **Aurora B** • **CD44** • **ESRβ** • **AR**	• **KLK2**	• **KLK3** • **MCM5** • **Aurora Kinase C** • **FASN** • **ESRα** • **CDH1**

Chapter Six

Exploratory GC-MS study on urine volatiles in prostate disease

Chapter 6: Exploratory GC-MS study on urine volatiles in prostate disease

6.1 Introduction

Organic volatile constituents of biological fluids could contain clinically useful diagnostic information for the recognition of metabolic disorders in man {Zlatkis et al, 1981}. A short review on the application of volatile gas analysis to cancer screening was described in Section 1.6.1f. In this study, urine samples of randomly selected ten patients from the Bedford Hospital cohort were analyzed by the method of thermal desorption gas chromatography mass spectrometry (GC-MS). The aim was to explore the presence and quantity of organic volatiles in the urine samples for possible identification of metabolic markers for prostate cancer.

6.2 Materials and Methods

The ethical approval and all documentations used in recruiting patients are contained in appendix D. The random selection of patients was based on the total PSA level at the time of referral to TRUS-prostate biopsy clinic. Three of the selected patients had PSA < 5.0 ng/ml; four patients had PSA between 6.0ng/ml to 13.0ng/ml and another three patients had PSA > 13ng/ml. The PSA class interval was arbitrary; and on the presumption that the three groups of patients would have non-involved, benign and malignant cases.

Decanted urine samples were stored at -80^{o}C until required for analysis. During preparation for analysis, the urine samples were thawed overnight at 4^{o}C; and 5ml was dispensed into sterile plastic bag for each sample, and air-tightly sealed with plastic clips. The sealed bags were heated in an enclosed thermal chamber at 40^{o}C for 5 minutes, enabling the headspace of each bag to be aspirated into a thermo-desorption (TD) tube. The TD tubes were then loaded into the carousel of an automatic thermal desorption system (ATD400) (Perkins Elmer, USA); and analyzed using Auto system XL Gas chromatograph linked to Turbomass mass spectrometer (Perkins Elmer, USA). A blank and two standards (calibrators with known separation time and peaks) TDs were also included. Set up details include auto injection at injection volume of 1ml at the rate of 1.56250 parts per second; run time was 30 minutes, and initial oven temperature was 50^{o}C for 20 minutes.

6.3 Results

Results of histological analysis from the Bedford hospital cellular pathology department later confirmed that eight of the selected patients had BPH, one had prostate cancer and one was non-involved case.

The GC-MS analysis through an in-built chemical search library identified, at > 85% homology, a total of 873 different organic volatiles in the urine samples. The chromatograms of the samples are shown in Figures 6.1, 6.2, and 6.3. A visual inspection of the chromatograms showed a high level of isothiocyanate in one BPH patient (Figure 6.2, sample ID: Nna011).

Principal component analysis (PCA) was carried out on the GC-MS data. Figure 6.4 and 6.5 show the three-dimensional PCA score plots.

Figure 6.6 shows the 2-D plot of the PCA score of the analytes. From these plots, eight analytes were found to be differentially expressed in the prostate cancer patient compared to the eight benign patients and non-involved case. These were Ethanethiol, Dimethyl sulfide, Propyn-1-ol acetate, Nitro-2-propanone, pentane, Hydrazine, Methanamine and Nitrous oxide. The quantity ranged from 0.01% to 5%. Of these eight, Methanamine is a urinary antiseptic and could have been taken by the patient in preparation for the TRUS- biopsy or as a result of urinary tract infection. The other 7 analytes are used in various complex mixtures in the laboratory but could also result from cancer metabolism.

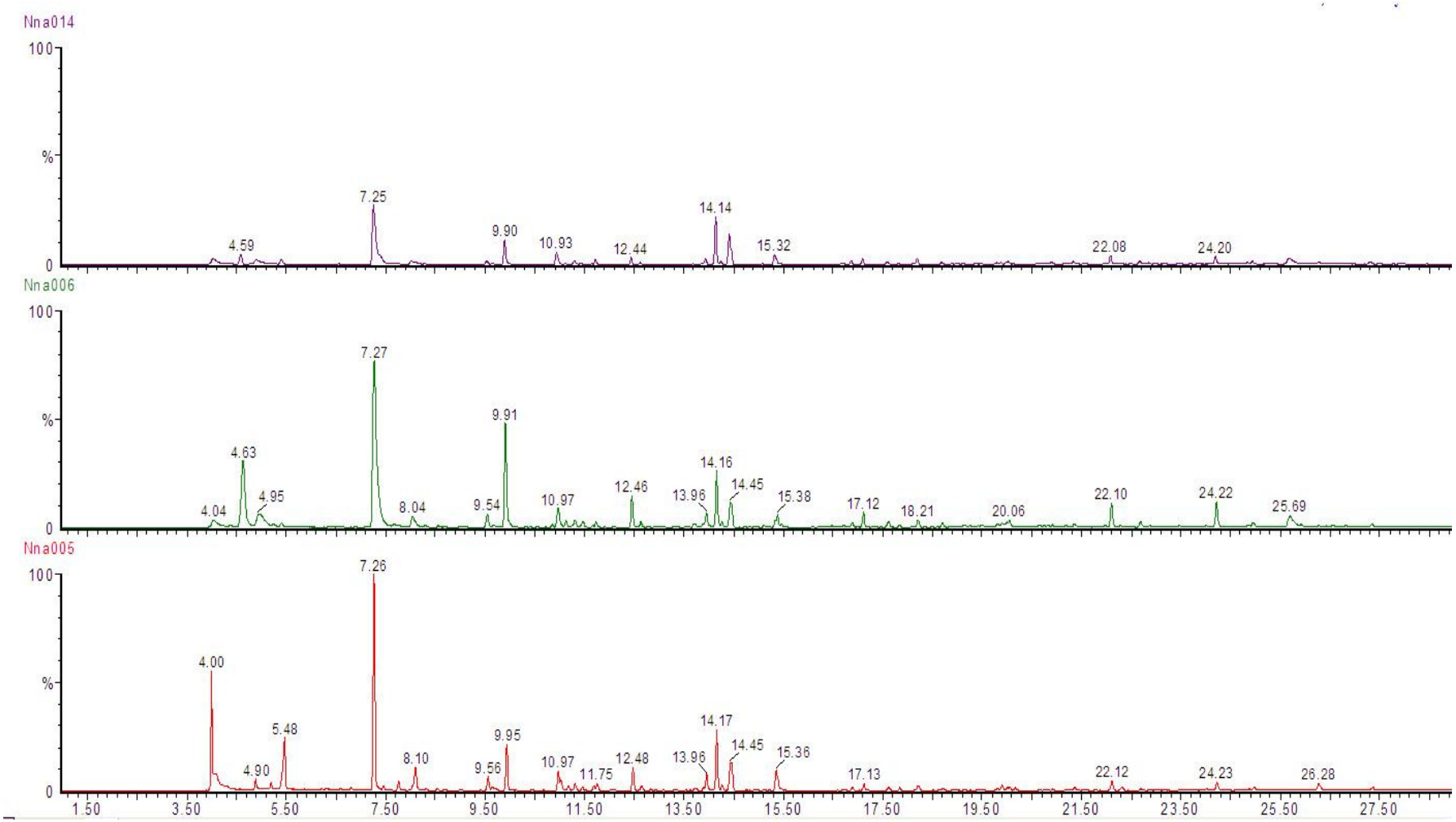


Figure 6.1 Chromatograms of volatiles from headspace urine analysis of high PSA patients. 873 different volatiles were identified but no differential trend in presence and quantity of the volatiles.

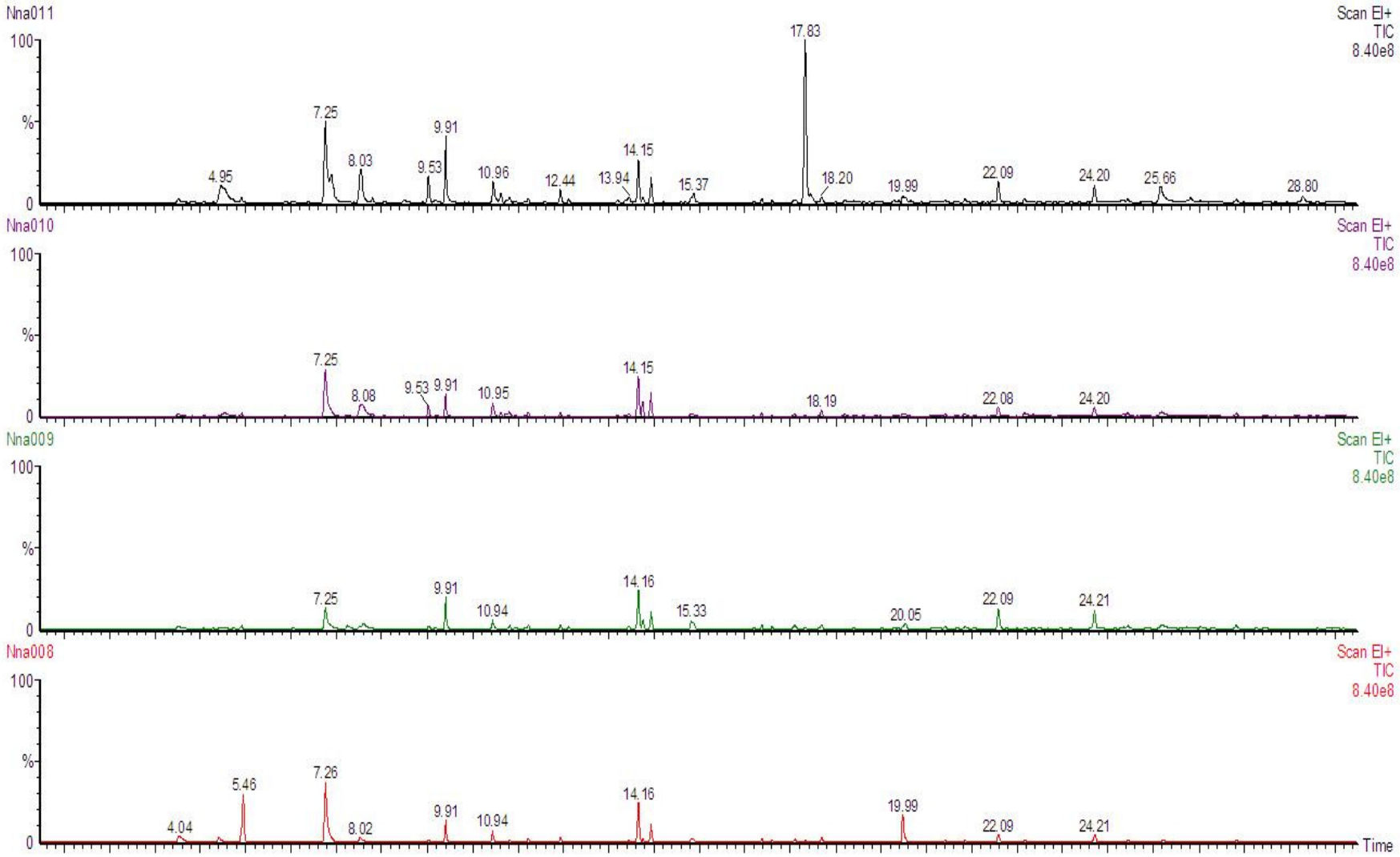


Figure 6.2 Chromatograms of volatiles from headspace urine analysis of medium PSA patients. 873 different volatiles were identified; isothiocyanate a drug metabolite (retention time 17.83) was found in large quantity in one of the BPH patients (sample Nna011), but no overall differential trend in presence and quantity of the volatiles.

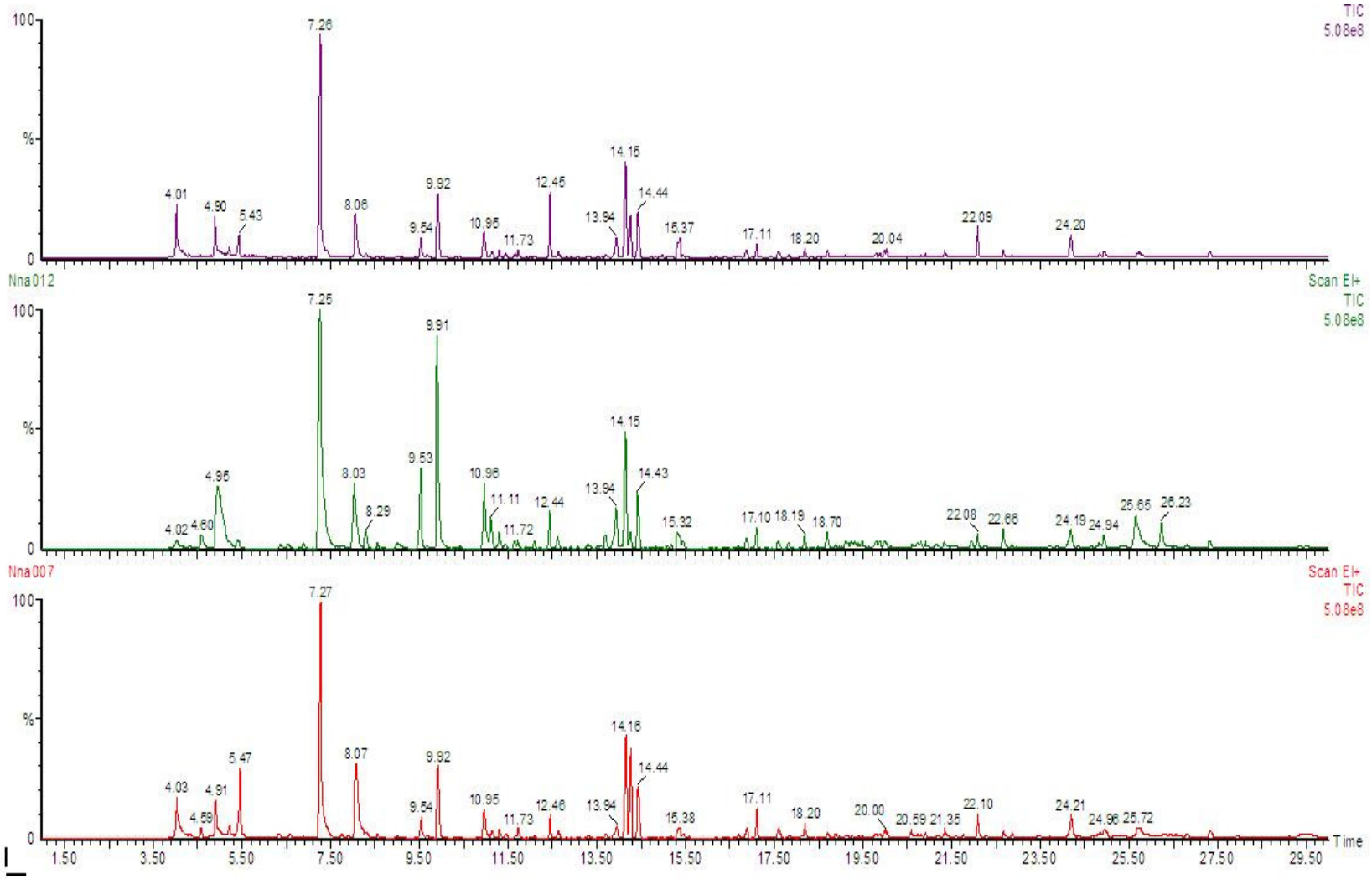


Figure 6.3 Chromatograms of volatiles from headspace urine analysis of low PSA patients. 873 different volatiles were identified but no differential trend in presence and quantity of the volatiles.

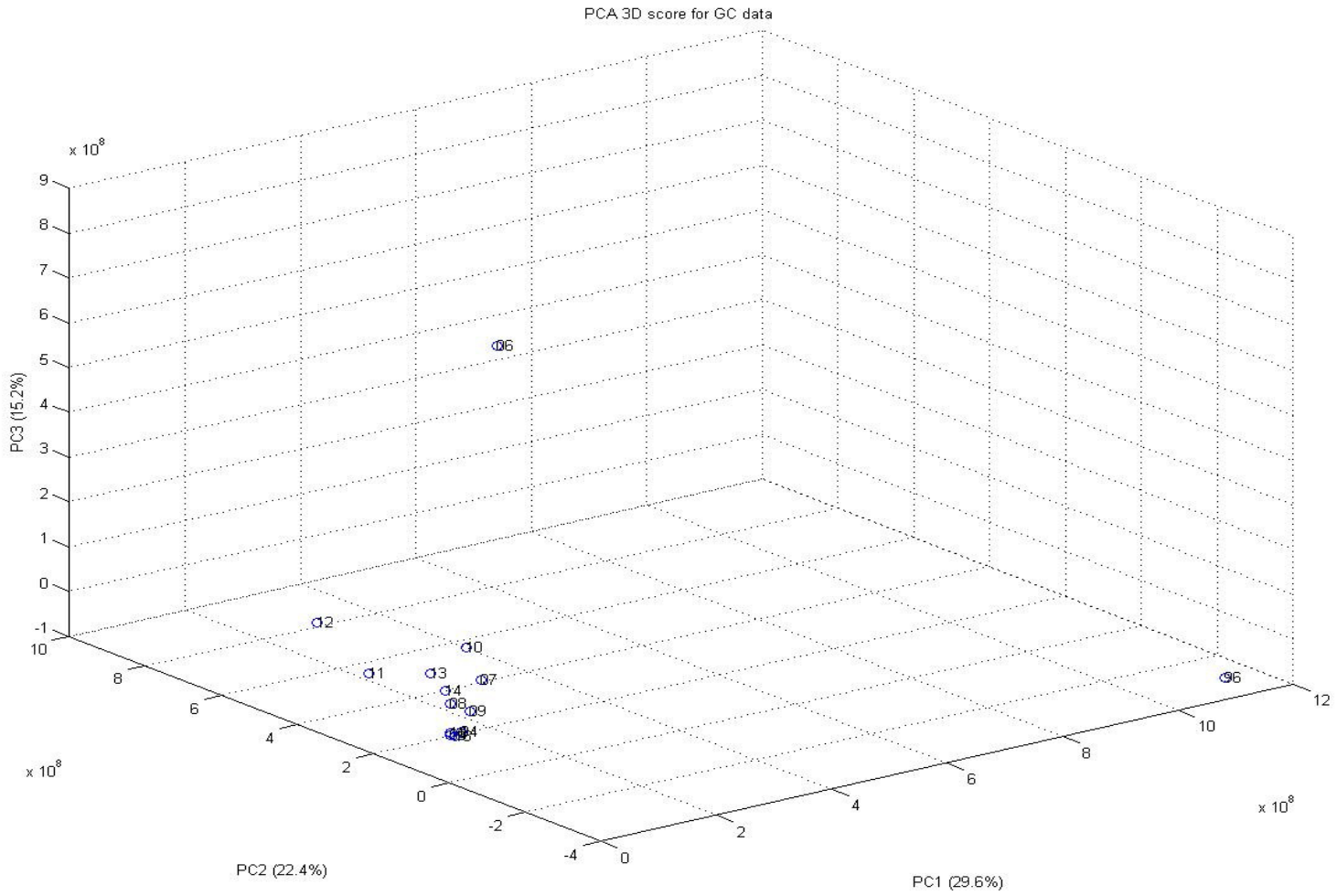


Figure 6.4 Three dimensional PCA score plot of the patients based on GC-MS data. The blue tagged numbers represent the patients ID. Patient 96 (the only prostate cancer patient) was significantly different from the rest on PC1 (29.6%).

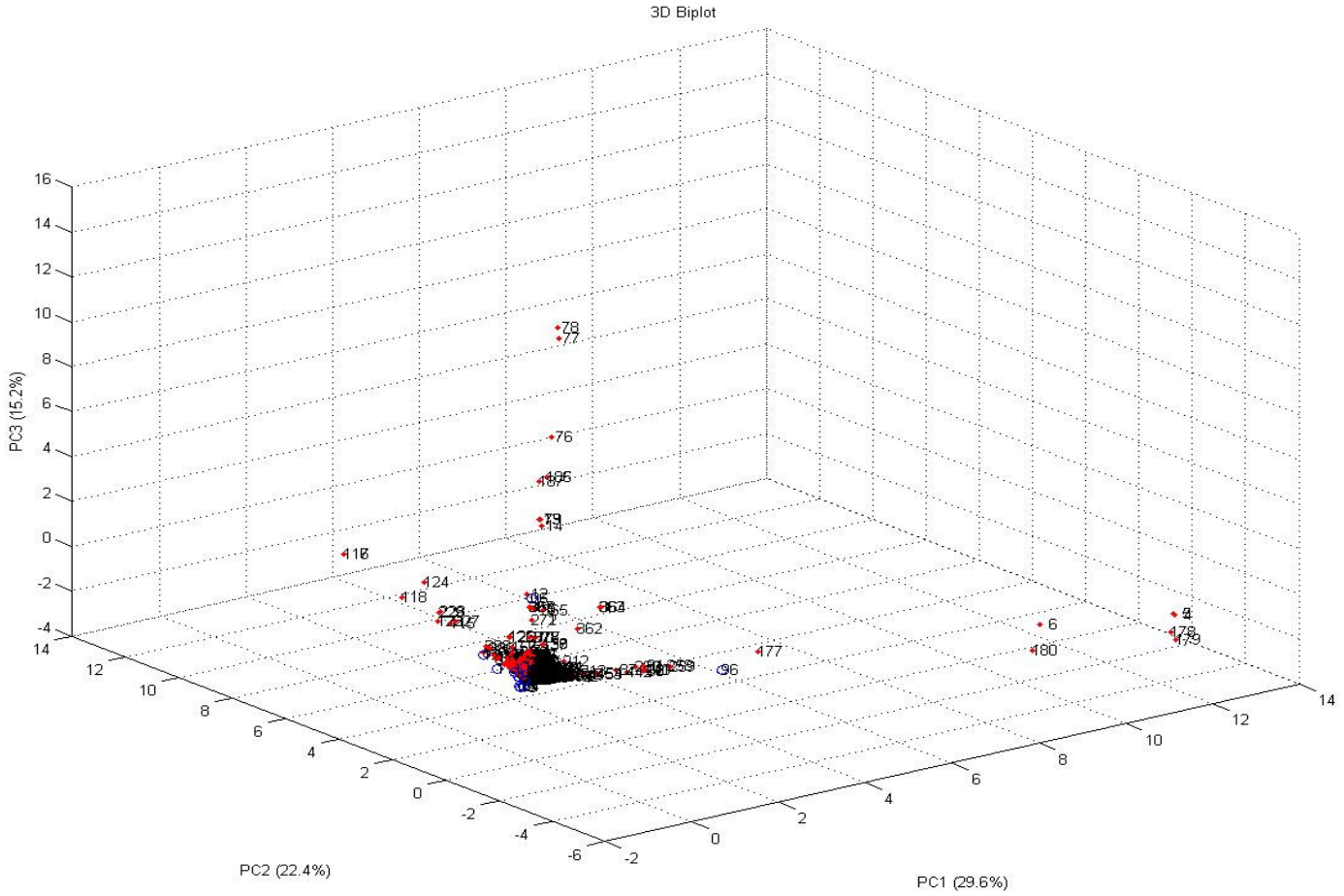


Figure 6.5 Three dimensional PCA score plot of both patients and analytes based on GC-MS data.

The red tagged numbers represent the ID of the analytes identified by GC-MS.

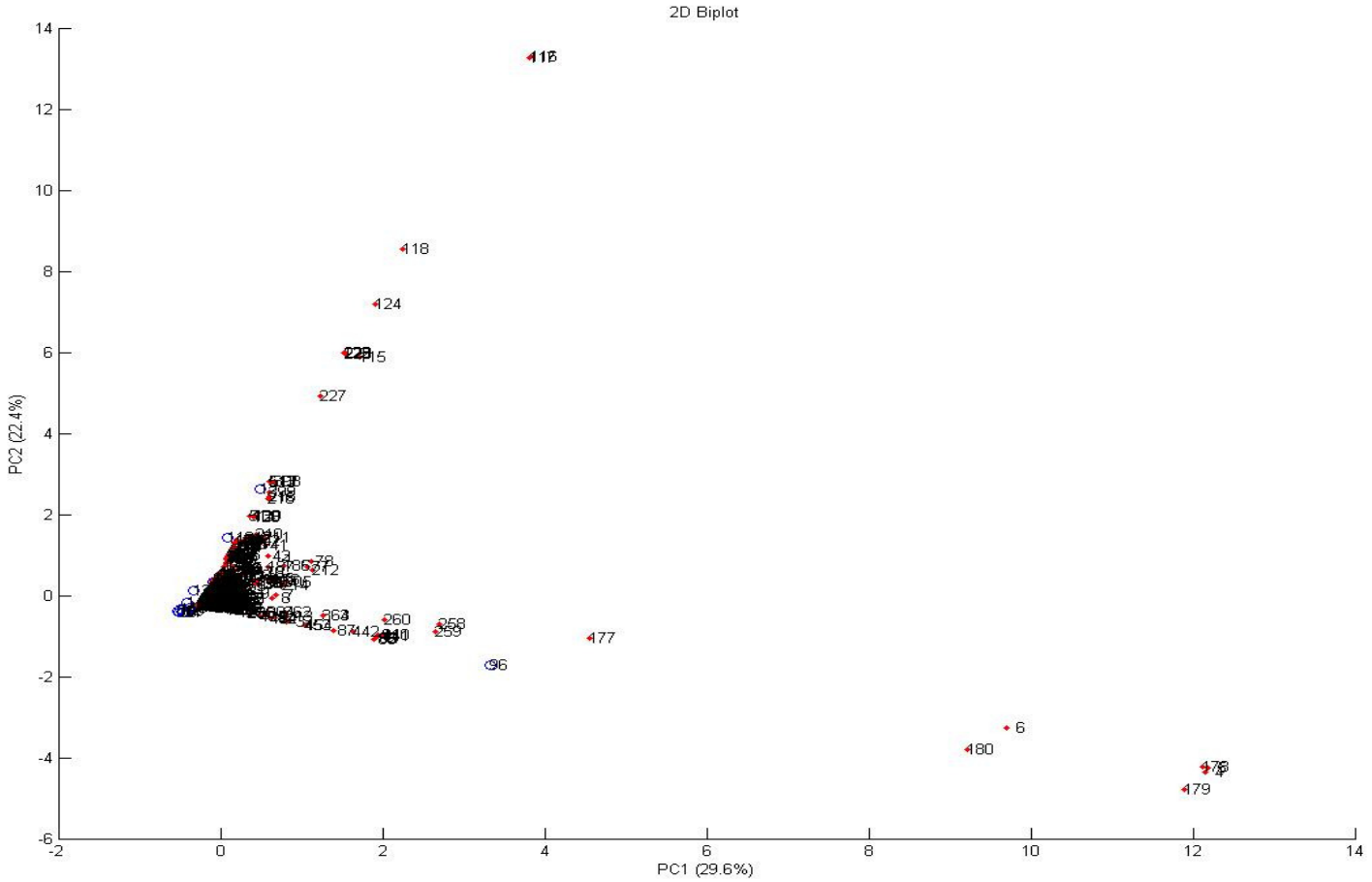


Figure 6.6 Two-D PCA plot of analytes and patients based on GC-MS data..

6.4 Discussion and Conclusion.

There was no remarkable change in the visual chromatogram pattern of the samples. Only one BPH patient had a high level of isothiocyanate, with 98% homology in the chemical search library. The retention time for the isothiocyanate was 17.83 minutes. Isothiocyanate is an anti-carcinogenic metabolite of several sulforaphane containing drugs or vegetables (for example broccoli) {Zhang *et al*, 1994}.

The principal component analysis (PCA) showed that eight analytes were differentially expressed in the prostate cancer patient compared to benign and non-involved cases. Further analysis would still be required to examine these individual volatiles. The first emphasis is to rule out that these analytes were not contaminants of any source. The second priority would be to rule out that they were not metabolites of drugs which the patients had taken prior to TRUS-prostate biopsy. However, some could be analytes that truly resulted from prostate cancer metabolism. In particular, pentane is known to be produced in oxidative stress. In comparison to lung cancer where two aldehydes: hexanal and heptanal are volatile markers and are also commonly used in the laboratory.

In summary, analysis of urine volatiles is a non-invasive technique, and may provide additional method for prostate cancer screening. Future studies are recommended to screen further seven of the analytes identified in this study. Care should be taken to recruit naive patients who are not on any sort of chemotherapy.

Chapter Seven

Discussion, Conclusion and Future Work

Chapter 7: Discussion, Conclusion and Future Work

7.1 Discussion

Over one hundred different molecular markers are associated with prostate cancer development and/ or progression. As new molecular markers emerge through new technologies, there is the need to validate both the biomarkers and the technologies through which they are discovered. In many cases most of these biomarkers fail to progress to clinical use. This is very typical of prostate cancer where little molecular grading/classification does exist. Despite the similarities of the prostate to the breast, molecular grading has become a standard practice in breast pathology and its clinical management but not the case with the prostate. The most commonly used tumour marker in prostate cancer, the prostate specific antigen PSA, has a weak correlation with the malignancy. The recently approved PCA-3 molecular test has also a modest diagnostic value. The definitive method of prostate cancer diagnosis which is histological examination has at best 30% detection rate at first biopsy for patients with elevated serum PSA and/ or abnormal digital rectal examination. All these necessitate the need for more molecular characterization of prostate diseases.

Through extensive literature review, sixteen different molecular markers were selected and evaluated in prostate cancer, benign and non-involved cases. The markers included KLK2, KLK3, MCM2, MCM5, TP53, Bcl-2, CD44, CDH1, AURKA, AURKB, and AURKC; ESRα, ESRβ, AR, FASN, TMPRSS2: ERG, and TMPRSS2:ETV1. The primary aim was to use molecular techniques to determine the gene expression of these markers in prostate cancer cases and to assess their association, if any, with tumour grade and pathological stage.

An *in vitro* model was designed using three prostate cell lines: PC-3, PNT1A and MDA PCA 2b to evaluate the expression of these molecular markers in response to steroid hormone treatment. The prostate gland depends on steroid hormones, mainly the androgens, for its growth and functions. Analysis of the transcript levels (mRNA) showed differential expression for most of the markers.

All the prostate cell lines expressed AR, ESRβ, ESRα and PSA at both mRNA and protein level. There had been previous reports that PC-3 cells do not express AR and PSA {van Bokhoven *et al*, 2003}. However, the results from this study showed that the PC-3 cells expressed AR and PSA. Alimirah *et al* (2006) also reported the expression of AR in PC-3 cells. However, on stimulation with androgens, the PC-3 did not secrete PSA that is detectable in culture supernatants by ELISA. But western blot of cell lysates showed cellular

PSA; immunocytochemistry also confirmed PSA expression. The transcripts of PSA in PC-3, detected by RQ-PCR did not show up-regulation by androgens. The western blot showed that both PC-3 and MDAPCA 2b expressed a 60 kDa AR while the PNT1A expressed a 90 kDa AR. Although the PC-3 expressed AR, it was still androgen-insensitive. There are several reasons for the expression of AR in PC-3 cells. It could result from contamination with other prostate cell lines over years of passaging. It could also result from evolution of sublines of the PC-3 cells that become AR positive in response to growth conditions. But the awareness that the PC-3 cells, regarded as one of the 'classical prostate cell lines' expressed these receptors would guide design of *in vitro* experiments in future. In addition, the MDAPCA 2b cells over-expressed ESRβ more than the PC-3 and the PNT1A cells; and it was up-regulated by the administration of 17 β oestradiol. The AR in PNT1A cells responded to androgen stimulation and produced secreted PSA detectable in culture supernatants.

Of the sixteen molecular makers, the TMPRSS2 fusion genes (TMPRSS2: ERG and TMPRSS2:ETV1) were the only markers undetected in the three prostate cell lines. The MCMs (MCM2 and MCM5) did not show any differential expression in response to steroid treatment in the prostate cell lines. The evaluation of three candidate endogenous control genes showed that the ABL1 gene was the most stably expressed house-keeping gene and could be reliably used to normalize RQ-PCR of prostate tumour markers. The expression of the ABL1 was stable in all the sample categories when benign cases were compared against prostate cancer and non-involved cases in both exfoliated urine cells and prostate tissues (FFPE materials).

Further analysis of the transcripts of the sixteen molecular markers in tissue materials showed that only seven of them were strongly associated with prostate cancer progression. These were TP53, TMPRSS2 fusion genes, AURK A, AURKB, CD44, ESRβ and AR. The TP53 over-expression was associated with tumour grade. The TMPRSS2 fusion genes (TMPRSS2: ERG and TMPRSS2:ETV1) were expressed in 15.6% of the prostate cancer cases. It is possible that other variants of the TMPRSS2 fusion genes could still be detectable from the sample population in this study. Interphase FISH method and multiplex RT-PCR could be used to investigate more of the TMPRSS2 fusion genes. However, the fusion gene was also detectable in two benign cases. The TMPRSS2: ERG was also detectable in exfoliated urine cells.

The Aurora kinases A and B were consistently significantly over-expressed in the prostate cancer cases compared to the benign cases. Because of the roles the play in cell mitosis, these

two kinases could be targets for therapeutics. However, the over-expression of AURK A and AURKB were not associated with tumour grade and pathological stage.

CD44 was over-expressed in the prostate cancer cases and was also associated with tumour grade. It is believed that CD44 over-expression in prostate cancer could result from mutant forms although the over-expression of standard CD44 has been reported in breast and colon cancer cases {Desai *et al*, 2009; Madjd *et al*, 2009}. It is also possible that transcript variants of CD44 could contribute to the over-expression in prostate cancer. The role of the CD44 protein is also affected by its degree of glycosylation.

The ESRβ was also over-expressed in prostate cancer cases. Because the ESRα did not show any differential expression in the study group, this finding strongly favoured a proliferative role for ESRβ. Remarkably, the androgen receptor AR was also up-regulated in those prostate cancer cases compared to the benign cases. It is strongly very likely that the ESRβ is the key mediator of oestrogenic activity in prostate cancer progression. Walton *et al* (2009) also reported over-expression of ESRβ in prostate cancer cases (although in 17 samples); but 96 prostate cancer cases were involved in this study. The over-expression of ESRβ, probably for the first time, was also associated with high tumour grade. Therefore selective targeting of ESRβ could provide a therapeutic means of slowing prostate cancer progression. Early studies on ESRβ had reported anti-proliferative role and therefore thought that it could be protective. But the finding from this study suggest otherwise. At both mRNA and protein levels, the ESRβ was over-expressed in prostate cancer and was significantly associated with high tumour grade and increased AR expression. In *vitro* studies also showed similar results; the ESRβ was also up-regulated by the administration of 17β oestradiol, a hormone whose level increases in ageing men.

The seven molecular markers would require further confirm studies using most recently processed FFPE tissue materials. Aged samples showed reduced RQ-PCR sensitivity; the levels of the control genes were reduced by more than a log in one year older samples.

The two kallikreins: KLK2 and KLK3 were not consistent in both urine and tissue samples; their transcript levels were not significantly associated with prostate cancer cases. The KLK2 transcript was reduced in prostate cancer cases in tissue samples while the KLK3 was significantly over-expressed. Both transcripts were not differentially expressed in urine cells. The PSA immunolocalisation on prostate tissues was stronger and more diffuse in benign

cases than prostate cancer cases. All these point to the fact that the two kallikreins have weak correlation with prostate malignancy especially at transcript levels.

Further genetic analysis on the KLK2 gene showed that two variants for SNPs rs2664155 and rs198977 had strong association with prostate diseases. The T/T genotype of rs198977 predicted presence of prostate cancer at biopsy and was also associated with high tumour grade. However, the A/A genotype of rs2664155 was found only in BPH cases. The association of both SNPs to prostate cancer had also been reported by Nam *et al* (2009); but contrary to previous reports, this study showed, for the first time, that the A/A genotype of rs2664155 was associated with benign nodular hyperplasia.

Combination of gene expression and genetic tests could assist in the risk stratification of patients for both screening and treatment options. The natural course of prostate cancer is hugely unpredictable. In some cases the disease could be undetectable, or treatment might be not necessary. And in many other cases treatment could be vital but may not necessarily prolong life of patients. Therefore molecular characterization of patients using gene expression and genetic studies could help identify high risk groups who might need annual screening for early detection, or determine those who might benefit from early radical treatment in comparison to those who possibly would not require the treatment. In addition, there are various treatment options, molecular grading could help in defining response to therapy and inform better choice of therapy. From this study seven already known molecular markers with a strong association to the disease were identified. The strength of gene expression techniques lies on ultra sensitivity, accuracy and reproducibility. They also provide multiplex formats for measuring several targets in one assay, which is highly cost-effective and flexibly high throughput. Therefore these seven markers could improve the molecular grading of prostate cancer.

An exploratory GC-MS study on ten patients suggested seven analytes that could be investigated further: Ethanethiol, Dimethyl sulfide, Propyn-1-ol acetate, Nitro-2-propanone, pentane, Hydrazine and Nitrous oxide. These analytes could be contaminants or metabolites of drugs or foods; however, they appeared differential on principal component analysis of urine samples of eight benign patients, a non-involved case and one prostate cancer case. Although in trace amounts, they were more expressed in the prostate cancer case compared to the benign and non-involved cases.

7.2 Conclusion

Seven molecular markers were strongly associated with prostate cancer progression: TP53, TMPRSS2 fusion genes, AURK A, AURKB, CD44, ESRβ and AR. They could help to molecularly characterize prostate cancer. The ESRβ in particular was found proliferative in prostate cancer cases and could be the key mediator of oestrogenic activity in prostate cancer progression. Therapeutic targeting of the aurora kinases-A and B involved in cell mitosis and the ESRβ could help slow down prostate cancer progression.

RQ-PCR technique provides a cost- effective, high through put multiplex format for quantifying these markers in prostate tissue sections and in exfoliated urine cells. Most recently processed tissue sections should be used to confirm these findings. Two genetic variants of the KLK2 gene were also predictive of prostate diseases: the T/T allele of rs198977 was found only in prostate cancer cases and was associated with high tumour grade. The A/A allele of rs2664155 was associated with benign nodular hyperplasia. A combination of gene expression and genetic variation, using the RQ-PCR application, could help improve diagnostic histopathology of prostate diseases. An emerging area of interest is the non-invasive analysis of urine samples using GC-MS technique to identify organic volatiles from prostate cancer cells' metabolism. Seven organic volatiles: Ethanethiol, Dimethyl sulfide, Propyn-1-ol acetate, Nitro-2-propanone, pentane, Hydrazine and Nitrous oxide were differentially expressed in a prostate cancer sample. Further studies would be required to rule out possible contamination or products of drug metabolism.

7.3 Future Work

- Further analysis is required on the GC-MS of urine samples.
- Further studies are required to verify mutation status of CD44 and TP53 expressed in prostate cancer.
- Further studies are also required to confirm the proliferative role of ESRβ
- Validation of a multiplex RQ-PCR assay in which TP53, CD44, AURKA, AURKB and ABL1 are measured in one reaction. This pentaplex assay could identify aggressive prostate cancer from indolent cases. Another pentaplex assay could measure AR, ESRβ, and TMPRSS2: ERG and TMPRSS2:ETV1 and ABL1. Therefore, establishing a baseline value in tissue sections would be necessary for clinical translation of these assays. The ABL1 would be used to normalize the results.

These two sets of pentaplex assays are capable of molecularly characterizing all prostate cancer cases.

- Large scale genotyping of archived FFPE tissue cases for rs2664155 and rs198977 using TaqMan SNP genotyping assays to confirm their association with prostate diseases.

The most promising single assay panel for molecular profiling of prostate cancer patients would consists of TMPRSS2:ERG, Aurora kinases A and B (AurkA & AurkB), Oestrogen receptor beta (ESRβ), Androgen receptor (AR) and CD44. Further study would be required to decide between choosing AurKA or AurkB in order to include a control gene (ABL1) for normalization. Analytical optimization and validation of a pentaplex real time PCR for these four markers using exfoliated urine cells would be clinically useful in diagnosis and prognostication of prostate cancer patients. Analytical optimization will involve mainly primer and probe concentration to achieve consistent and uniform amplification plots for all targets. Validation may also require preparing a single plasmid standard with inserts of all the five target markers for clinical laboratory assays.

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Appendix A: Equipment, Preparation of Reagents and Buffers

A.1 Equipment: Jencon's variable pipettes and tips: 2-20µl (lot no. E066044), 20-200µl (lot no. ER46745) and 200-1000 µl (Lot no. EN 3358); Sartorius analytical balance model MC22; Heraeus biological safety hood model H59 (level II containment); Tissue bank model RS 3000; Sanyo C02 incubator model MCO-17A1C, Olympus phase contrast microscope model TMS; Carl Zeiss Axioskop microscope and axiocam (camera); Beckman micro-centrifuge lite model 2A; Multispeed refrigerated centrifuge PK131R; Jouan refrigerated centrifuge model B41; WPA light wave UV/Visible spectrophotometer model S2000; 1.0cm light path cuvettes, pH meter model 410A; Grant water bath JB1 5PC; Heat stirrer model HB502; CFX96 Real time PCR system (BioRad, UK); ABI 7900HT Sequence detection system (Applied biosystems, UK); ABI Genetic analyzer 3130 (Applied biosystems, UK); Eppendorf concentrator 5301 (Eppendorf, Germany); BioRad power pack and Mini-protean III Electrophoresis system (BioRad, UK), Q-Mark 24 Pyrosequencing system (Qiagen, UK); MJ PTC 2000 thermal cycler (BioRad,UK), Accuri flow cytometry system (Accuri, UK); Gene Genius bio-imaging system (Syngene, UK); Carl Zeiss LSM 510 Meta Confocal microscope, and general laboratory glassware and plastics. The pipettes were calibrated according to manufacturer's standard procedures.

A.2 Reagents & Buffers:

i) Ammonia water: 1.4ml of concentrated ammonium hydroxide (Sigma UK) was added to 250ml of distilled water and mixed by swirling.

ii) DAB substrate chromogen buffer: 0.1g of 3, 3' diaminobenzidine (DAB) (Sigma, UK) was dissolved in 10ml of distilled water, and 250µl of 10M HCl added and mixed for 10 minutes (solution turned brown). Aliquots were stored at -20^{o}C until required for use. 100µl of 30% H_20_2 (Sigma, UK) was added to 10ml distilled water, mixed, aliquoted and also stored at -20^{o}C. For a working solution, 250µl of the DAB solution was added to 5ml of PBS (pH 7.2, Invitrogen, UK), mixed and 250µl of the H_20_2 dilution was added to the mixture (tube covered with foil. The mixture was used within less than 6 hours of preparation.

iii) Trypsin/ EDTA solution (TES) (Sigma UK): Commercially prepared 20 x solutions were diluted to 1x strength using PBS.

iv) TBS (pH 7.6): 6.06g of TRIS base and 8.7g of Nacl were dissolved in 400ml of distilled water; the pH adjusted to 7.6 using 0.1M HCL and the buffer made up to one litre with distilled water.

v) PBS: commercially prepared tablets were used: 2 tablets dissolved in 400ml of distilled water (dH20) and made up to 1.0 litre using dH20. PBS was aliquoted in bijou bottles and autoclaved at 121^{0} C for 15 min before use. Also commercially prepared sterile PBS solution (Invitrogen, UK) was used in cell cultures and IHC.

vi) RIPA buffer: It was made up of Sodium chloride 0.88g, Sodium deoxycholate 0.5g, Sodium dodecyl sulphate SDS 0.1g, Tergitol type NP-40 1.0g and 100ml of Tris-HCl (pH 6.8). It was properly mixed, filtered and stored at 4^{o}C. For cell pellets, 200 µl of RIPA buffer was used for protein extraction: cells were lysed for 10 minutes, centrifuged at 100g for 5 minutes and supernatants collected for analysis.

vi) Running buffer 5x: Glycine 144g, SDS 5g, Trizma 30g and 1litre of distilled water; properly mixed using a stirrer.

vii) Transfer buffer 10x: Glycine 144g, Trizma 30g and 1 litre of distilled water properly mixed using a stirrer.

viii) TBS 10x: Nacl 40g, KCl 1g and Trizma 15g; mixed properly and pH 7.4 using 1M HCl.

ix) Sample loading buffer for western blot: NUPAGE LDS sample buffer 4x (Invitrogen, UK) and NUPAGE sample reducing buffer 10x (Invitrogen, UK).

x) Separating gel for SDS PAGE: Shown in Table A.1

Table A.1 Composition of Separating gel for SDS PAGE

Separating Gel	7.5%	10%	12%
Tris-HCl pH 8.8 buffer	2.5ml	2.5ml	2.5ml
Bis-acrylamide (30%)(BioRad, UK)	2.5ml	3.3ml	4.0ml
Distilled water	5.0ml	4.2ml	3.5ml
10% APS(Fresh)	50µl	50µl	50µl
TEMED	33µl	33µl	33µl

xi) Stacking gel:

Tris-HCl pH 6.8 Buffer ……..1.2ml
Bis-acryl amide……750µl
Distilled H_20 ……...….3.0ml
10% APS…….....30µl
TEMED……..20µl

xi) Sodium Citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0): 2.94g of Tri-sodium citrate (dehydrate) in 1000ml of distilled water, mixed properly and pH adjusted to 6.0 using 1M HCl and then 0.5ml of Tween 20 added and mixed again. The solution was stored at room temperature and used within 3 months of preparation.

xii) Avidin-Biotin complex (Dako, UK). 100µl of solution A (Avidin) and 100µl of solution B (Biotin) were added to 5ml of TBS and mixed ready for use.

xiii) 10% rabbit serum: Normal rabbit serum (Dako, UK), 10ml of it added to 90ml PBS.

xiv) 0.057 %(wt/vol) SRB: 57g of SRB (Sigma, UK) in 1% (vol/vol) acetic acid.

xv) 10% (wt/vol) TCA: 10g of TCA in 100ml of distilled water (handled with extreme care in fume hood).

xvi) 10X Red cell lysis buffer (RCLB): Ammonium chloride (NH4Cl) (Merck, UK) 248.7g, EDTA 0.5M (Gibco, UK), and Potassium bicarbonate ($KHC0_3$) were fully dissolved in 2.5 litres of distilled water (contained in Winchester bottle) and stored at 4-8^{o}C.

xvii) 1x RCLB: 1 litre of 10x RCLB was added to 9litres of distilled water, kept overnight at 4-8^{o}C, and next day pH was adjusted to 7.4 with concentrated HCl. The 1x RCLB was stored at 4-8^{o}C.

xviii) Tris acetate EDTA buffer (TAE): 50X stock was prepared by adding 605g of Tris base, 36.5g EDTA and 125ml glacial acetic acid, making it up to 2.5litres using distilled water. The stock buffer was stored in a Winchester bottle at room temperature. To make 10 litres of 1x TAE, 1 litre of stock was made up to 10 litres with distilled water.

A.3 Antibody dilutions for ICS, IHC and IF

Antibody	Supplier	Parameters
Mouse anti-androgen receptor (AR) IgG.	Invitrogen, UK.	Monoclonal; Clone AR27; IgG1 isotype; antibody concentration 0.1mg/ml. 1 in 50 dilutions used (based on manufacturer's recommendation). 1% (v/v) normal rabbit serum in PBS was used as diluent in all commercial antibody dilutions.
Mouse anti-oestrogen receptor ($ER\beta_1$) IgG.	Serotec, UK	Monoclonal; clone number PPG5/10; IgG2 isotype; antibody concentration 1mg/ml. 1 in 40 dilutions used based on manufacturer's recommendation.
Mouse anti-oestrogen receptor (ERβ2) IgG.	Serotec, UK.	Monoclonal; clone number ID5; IgG1 kappa isotype. Antibody body concentration 1mg/ml. 1 in 40 dilutions based on manufacturer's recommendation.
Mouse anti-oestrogen receptor (ERα) IgG.	Serotec, UK.	Monoclonal; clone number 6f11; antibody concentration 1mg/ml, IgG1 isotype; 1 in 50 dilutions used based on manufacturer's recommendation.
Mouse anti-human p53 IgG (aa213-217).	Serotec, UK.	Monoclonal; clone number PAb240; antibody concentration 0.1mg/ml. It is IgG1 isotype. 1 in 40 dilutions used based on manufacturer's recommendation.
Mouse anti-human MCM 2 IgG.	Serotec, UK.	Monoclonal; clone number CRCT2.1, antibody concentration 1.0 mg/ml. IgG1 isotype. 1 in 50 dilutions used based on manufacturer's recommendation.
Biotinylated polyclonal rabbit anti-mouse IgG (secondary antibody for detection system).	Dako, UK.	Polyclonal (reacts with IgG subclasses and mouse IgM). 1 in 200 dilutions were used based on supplier's recommendation.
Mouse anti-human CD324 (CDH1)	Serotec, UK	Isotype IgG1, Clone 6F9; 1 in 40 dilution used.
Mouse anti-human CD44	Serotec, UK	Isotype IgG2a, Clone F10-44-2, 1 in 50 dilution used.
Mouse anti-human Bcl-2	Serotec, UK	Isotype IgG1, Clone 100, 1 in 50 dilution used.
Monoclonal anti-β-actin	Sigma, UK	Isotype: IgG2a, Clone: AC-74, 1 in 5,000 dilution; used as internal control for western blotting.
Mouse anti-human PSA	Zymed, UK	Isotype IgG2a, Clone Z009, prediluted.
Mouse anti-human hK2	Immuquest, UK	Isotype: IgG1, Clone 6B7, 1 in 50 dilution was used according to manufacturer's recommendation.
GAPDH (14C10) Rabbit monoclonal	Cell Signaling,USA	Isotype IgG1,Clone 14C10, 1 in 400 dilution for IHC, and 1 in 1000 dilution for W/B.

A.4 List of Suppliers and their contacts

1 Dako UK Ltd.
Denmark House
Angel Drove, Ely
Cambridgeshire, CB7 4ET
Tel: +44 (0) 1353 669911
Fax: +44 (0) 1353 668989
Email: info@dako.com
www.dako.co.uk

1 Sigma-Aldrich Company Ltd.

The Old Brickyard
New Road, Gillingham
Dorset, SP8 4XT
Tel: +44 (0) 1747 833000

2 Invitrogen Ltd

3 Fountain Drive
Inchinnan Business Park
Paisley, UK. PA4 9RF
Tel: 0141 8146100
Fax: 0141 8146260
Email: euroinfo@invitrogen.com

3 Serotec UK Ltd

22 Bankside, station approach
Kidlington, Oxford OX5 1JE,
United Kingdom
Tel: +44 (0) 1865 852700
Fax: +44 (0) 1865 852739
serotec@serotec.co.uk www.serotec.com

4 American Type Culture Collection

10801 University Blvd
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Manassas, VA 20108
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5 Perbio Science UK Ltd

Unit 9, Atley Way, North Nelson Industrial Estate.
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NE23 1WA
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6. Applied biosytems, UK

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T +44 1925 282588
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7. Diagnostic Automation /Cortez Diagnostics, Inc.

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9. GE Healthcare
GE Healthcare (UK) Ltd

Amersham HP7 9NA
GB
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10. ImmuQuest Ltd UK
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Ingleby Barwick, Cleveland, TS17 5AL
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A.5 List of Primers and Probes

Gene	Oligo	Sequence (5' to 3')	Exons	Amplicon (bp)
ESRα	F/P	TGAATCTGCAGGGAGAGGAGTT	6	77
	R/P	GGACAGAAATGTGTACACTCCAGAAT	7	
	Probe	[TET]TGTGCCTCAAATCT[TAM]	6&7	
ESRβ	F/P	TGATCCTGCTCAATTCCAGTATGT	7	86
	R/P	GTTCAGCAAGTGAGCCAGCTT	8	
	Probe	[6FAM]CCCTCTGGTCACAGCG[BHQ1]	7&8	
AR	F/P	TGGGAGCCCGGAAGCTG	3	66
	R/P	GGTGCTGGAAGCCTCTCCTT	4	
	Probe	[Cy5]AAGAAACTTGGTAATCTGAAACT[BHQ2]	3&4	
KLK2	F/P	TGCCCATTGCCTAAAGAAGAATAG	2	73
	R/P	CCTGTGTCTTCAGGCTCAAACA	3	
	Probe	[TET]CTGGGTCGGCACAAC[TAM]	2&3	
MCM2	F/P	CAGCATGCGCAAGACTTTTG	14	74
	R/P	TCAGTATGAAGAGCAACAGCTCATT	15	
	Probe	[FAM]CCGCTACCTTTCATTCCGGCGTG[TAM]	14&15	
ABL1	F/P	GATACGAAGGGAGGGTGTACCA	3	94
	R/P	CTCGGCCAGGGTGTTGAA	4	
	Probe	[FAM]GCTTCTGATGGCAAGCTCTACGTCTCCT[TAM]	3&4	
G6PD	F/P	GGCGATGCCTTCCATCAG	11	64
	R/P	CCAGGTCACCCGATGCA	12	
	Probe	[TET]CGGATACACACATATTC[NQF]	11& 12	
GUS B	F/P	GAAAATATGTGGTTGGAGAGCTCATT	2	101
	R/P	CCGAGTGAAGATCCCCTTTTTA	3	
	Probe	[FAM]CCAGCACTCTCGTCGGTGACTGTTCA[TAMRA]	2&3	
KLK3		ABI predesigned assay ID: Hs01105076_m1	2&3	65
CD44		ABI predesigned assay ID:Hs01081475_m1	5&6	82
CDH1		ABP predesigned assay ID: Hs00170423_m1	2&3	61
TP53		ABI predesigned assay ID: Hs99999147_m1	9&10	72
MCM5		ABI predesigned assay ID: Hs01052142_m1	16&17	70
Bcl-2		ABI predesigned assay ID: Hs99999018_m1	2& 3	76
AURKA		ABI predesigned assay ID: Hs0182073_m1	6&7	117
AURKB		ABI predesigned assay ID: Hs00177782_m1	2& 3	130
AURKC		ABI predesigned assay ID: Hs00152930_m1	4&5	91
PTEN		ABI predesigned assay ID:Hs02621230_s1	9&9	135
FASN		ABI predesigned assay ID:Hs00188012_m1	8&9	144
TMPRSS2:ERG		ABI predesigned assay ID: Hs03063375_ft	1&4	106
TMPRSS2:ETV1		ABI predesigned assay ID:Hs03024759_ft	1&4	123
rs1064676 SNP	F/P	[Biotin]AGCTTCCCACACCCGCTCTAC		228
	R/P	ATCTGGCCCAGGCGTACACT	3	
	SNP primer	GCTCCTGGGTGGGCA	3	
rs6072	F/P	TTGGGTCTGATCCCCCTGA	2	82
	R/P	[Biotin]CTCCCAGCCTCCCACAAT	2	
	SNP primer	ACCCCCTCCGCAGGT	2	

F/P = forward primer, R/P = reverse primer.

Appendix B: Protocols for experiments

B: 0 Protocol for cell cultures {Harrison & Rae, 2004; Freshney, 2005}.

B.1 **Expansion of prostate cell lines:**

- A water bath was set to 37°C, culture media containing 1.0 2.0, 5.0, 7.5 and 10.0 %(v/v) FCS or BGS or DCC-FCS were brought to room temperature; a waste beaker containing 2% (w/v) virkon disinfectant was prepared and culture hood disinfected using 70% (v/v) isopropanol (IPA) and allowed for 15 minutes. A small container was filled with liquid Nitrogen for thawing cells.
- Each vial of cell line was removed from the tissue bank, held in the small liquid nitrogen container with the lid of the vial covered in silver foil and then thawed in the water bath set at 37^0C. A vial of one cell line was thawed and plated at a time. Before thawing another different cell line, the hood was disinfected and allowed to clear for 15 minutes. All the details of each cell line, such as passage number and date frozen were logged in the laboratory notebook and tissue bank log book. Eye goggles were worn when handling liquid nitrogen.
- During thawing, vials were shaken gently to ensure that all cell lumps disaggregated.
- Vial was removed from water bath as soon as the last lump thawed, sprayed with 70% (v/v) IPA and placed in the hood.
- The contents of the vial were aspirated into a 15ml centrifuge tube and drops of culture media added using Pasteur pipette while gently swirling the tube for about 5 minutes (5ml of the culture media was added drop wise). Then another 5 ml of media was added with gentle swirling.
- The tube was centrifuged at 4^0C, 1300 rpm (100g) for 5 minutes.
- Cell pellet was resuspended in 1ml of media and added to T25 flask and supplemented with a further 6ml of media. Flasks were properly labelled.
- T25 flask was swirled gently to ensure even distribution of cells and incubated at 37^0C, with 5% (v/v) CO_2 supply.
- Flasks were checked routinely under the microscope and macroscopically; and culture media replaced during routine feeding.

Note: In the first thawing, cells were expanded in appropriate phenol-containing media supplemented with 10% (v/v) FCS, but in subsequent experiments, cells were weaned in the different sera types; and during hormonal stimulation appropriate phenol-free media were used.

B.2 Feeding prostate cell cultures:

Culture media were changed when they turned slightly yellow (from usual pink colour) or when cells were harvested for splitting, counting or further seeding for hormonal stimulation using the protocol below:

- Stored culture media were warmed to 37^0C ready to use.

- A waste beaker containing disinfectant and the culture hood were prepared ready for use as described in the protocol for thawing cells.
- A few flasks of a cell line were removed from the incubator at a time, sprayed with IPA and placed in the hood.
- Culture media in T25 or T75 was aspirated and replaced with new media, one flask at a time.
- Care was taken not to touch the neck of the flask and to carry the entire procedure aseptically.

B.3 Splitting prostate cell cultures by trypsinization:

Confluent cell cultures, in which cells cover more than 70-80% of the available flask surface, were split, counted and re-seeded for hormonal stimulation, cell expansion or frozen for storage. Hormonally-treated cells were also trypsinized for viability assessment and cell count. 1x of Trypsin/EDTA solution (10X 0.5% trypsin, 0.2% EDTA) (Sigma, UK) was used to detach the cells from the flasks using the protocol below:

- Trypsin/EDTA solution (TE), sterile PBS (pH 7.2) (Invitrogen, UK) and appropriate serum supplemented culture media were warmed to 37^0C ready to use.
- Culture media was aspirated from the flask to be split and the residual media rinsed away using sterile PBS.
- 5ml of TE was added into each T75 flask and placed in incubator for 1 minute; the flask was taped gently and examined under the microscope for cell detachment. This was repeated until all cells were detached and in suspension. For the 6 multi-well hormone stimulation experiment (section 2.1f) only 1ml of TES was used to trypsinize cells. In all trypsinization, the cells were never incubated for more than 10 min to reduce cellular damage.
- During the detachment, 5ml of culture media was added into 15 ml centrifuge tube and then the cell suspension was also transferred into the tube. The media helped to inactivate trypsin and prevent cell lysis. For the 6 multi-well hormone stimulation experiment (section 2.1f) only 1 ml of culture media was added into a 2 ml eppendorf tube before transferring the cell suspension.
- The tube was centrifuged at 1300 rpm (≈ 100g), at 4^oC for 5 minutes.
- The cell pellet was resuspended in desired volume of media for plating. In general, T25 flask was split 1:3 (pellet resuspended in 3ml of media) and T75 was split 1:6 (pellet resuspended in 6ml of media). For the 6 multi-well hormone stimulation experiment (section 2.1f), the cells were re-suspended in 1-3ml of PBS (depending on pellet size) and 100µl transferred to a 0.5ml tube for cell counting (see appendix B.3). The hormonally-treated cells were not re-seeded in any further experiment. Their remaining suspensions were discarded into Virkon disinfectant.
- 1ml of cell suspension (for the cells being expanded) was added to clean sterile flask (T75 flask), followed by 9ml of media, and then swirled gently to ensure adequate dispersion of cells.
- The flasks were incubated at 37^oC, with 5% CO_2 supply.

The protocols for thawing, feeding and splitting of prostate cell lines were used to expand the cell lines enough for the entire work, and many vials were stored for future use. The passage numbers for all the cell lines were recorded.

B.4 Cell Counting using Improved Neubauer Chamber and Trypan blue reagent:

Once cells were trypsinized, pellet was re-suspended in 1-4ml of PBS (depending on pellet size) and 100µl was transferred to a 0.5ml tube to assess viable cell yield. 100µl of Trypan blue (Sigma, UK) was added and the solution mixed together and left to stand for 1 min. The improved Neubauer haemocytometer was assembled as shown in figure B.1. The haemocytometer contained two identical grids one on the upper counting chamber and another on the lower counting chamber (Fig.B.1 and Fig.B.2). Each grid contained 9 squares, measuring $1mm^2$ each. Four corner squares (out of the 9 squares) were designated quadrants A, B, C and D. Each quadrant had 16 smaller squares. Cells were counted in these four quadrants except when cell number exceeded 200, in which case cells were counted in the central squares containing 25 smaller squares each (Fig.B.2) in both grids or cell suspension diluted further and counter on the 4 quadrants. A hand held tally counter was used in counting the cells (Figure B.3).

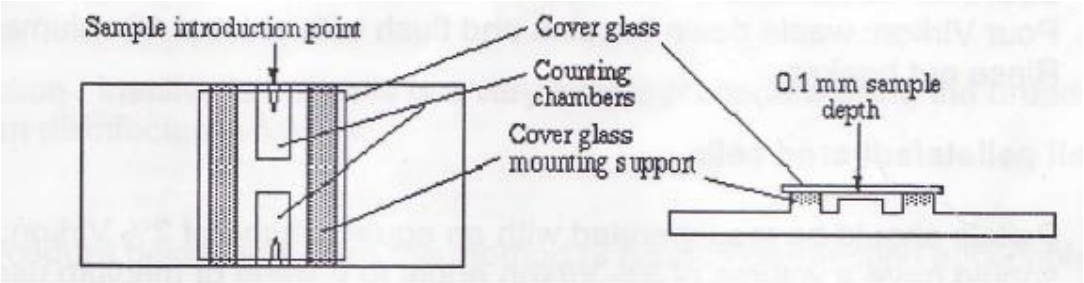


Figure B.1 Assembly of a haemocytometer. A cover slip is moistened and placed on the Haemocytometer covering the 2 central channels. Cell suspension got into the grid lines by capillary action. The depth between cover slip and grid was 0.1mm. {Harrison & Rae, 2004}

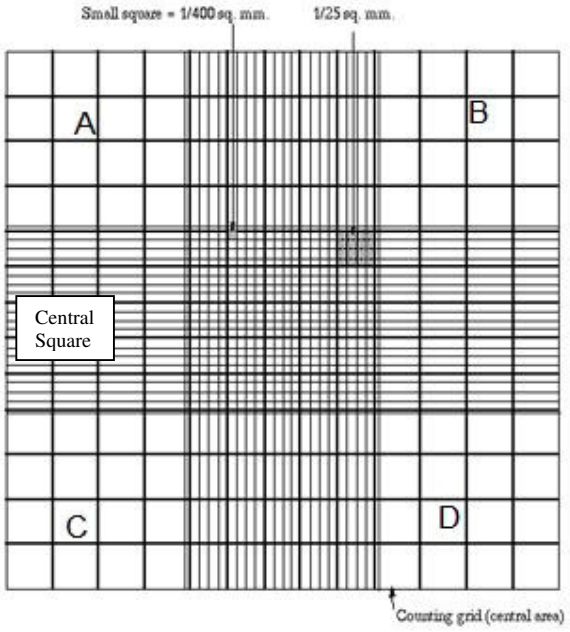


Figure B.2 Grid lines on haemocytometer. The chamber contained 9 squares (1mm x 1mm each), the four corners squares A, B, C and D contained 16 smaller squares each. Regular counting was done on the four quadrants. 20µl of cell suspension (mixed with Trypan blue reagent) was dispensed underneath the cover slip until the whole chamber was filled without spilling over to the side troughs.

Calculation of cell Count: An average of cell count in the four quadrants was calculated and the number of cells in, for example, 2ml suspension was calculated as follows:

Total number of cells = average cell count x 10,000* x 2 (dilution factor) x 2 (suspension volume).* 1ml = $1cm^3$ =$1000mm^3$.

Figure B.3 Hand held tally counter.

Cell viability, expressed in percentage, was obtained by counting dead or dying cells, which appear blue and viable cells, which remain clear.

$$\% \text{ of viable cells} = \frac{\text{total viable cells}}{\text{Total cell number (viable cells+ dead cells)}} \times 100$$

Note: Each quadrant had a volume of $0.1mm^3$ and that gives N number of cells per average quadrant. Therefore 1ml of diluted cell suspension ($1000mm^3$) would contain N x 10^4, that is per ml. But to account for dilution using Trypan blue reagent (1 in 2 dilution), the cell number per ml will be N x 10^4x2. For the entire cell yield in a suspension, the cell count per ml would be multiplied by the suspension volume.

B.5 Summarized Protocol for Sulforhodamine B assay {Vichai & Kirtikara, 2006}.

Cells in the log phase were harvested and seeded (10^5) per well in a 96-micro well tissue culture plate; appropriate volume of steroid hormone dilution (ranging from 10^{-4} to 10^2M final concentration) was also added. After 72-hrs of incubation at 37^{o}C with 5% $C0_2$ supply, the cells were fixed in 10% trichloroacetic acid (TCA) at 4^{o}C for 1 hr. The cells were then washed four times with slow-running tap water and completely dried using a blow dryer. 100µl of 0.057% Sulforhodamine B (SRB) was added to each well and incubated at room temperature for 30min. The SRB stain was washed off four times and wells dried. 200 µl of 10mM Tris base solution (pH 10.5) was used to solubilise the protein- bound dye; the plate was placed on gyratory shaker for 5min. Optical density (absorbance) was read photometrically at 510nm using the Varioskan Flash plate reader.

$$\% \text{ of control cell growth} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD negative control}} \times 100$$

Negative control is the well without any steroid treatment.

% growth inhibition = 100-% of control cell growth.

IC_{10} and IC_{50} values were derived from curve- fitting using the Origins Lab software.

For the determination of cell growth phase only using this method, steroid hormones were not used rather cell cultures in the micro well plate were harvested at days 1, 2, 3, 5, 7, 9, 11, and 13. The starting number of cells seeded was 10^3 per well.

This method was cheap, flexibly high throughput and very convenient to use (each test point was done in 6 replicates). Prior to the testing, cell density was optimized by seeding varying cell densities (10^3, 10^4, 10^5 and 10^6 per well) to assess OD values and linearity.

B. 6 Steroid Hormone dilution

Table B.1 Dilution of steroid hormones

All steroids were purchased from Sigma UK. Absolute ethanol was used as a diluent.

Hormone	Purity %	Molecular Weight	Dissolution
5α-Androstan-17β-ol-3-one (DHT)	97.5	290.40	Dissolve **0.297785**g in 10 ml of absolute ethanol to get 0.1M stock solution. 0.1ml of stock solution was added to 9.9ml of absolute ethanol to get 10^{-3} M solution for further dilutions.
Hydrocortisone	98	362.47	**0.369867**g was dissolved in 10ml of absolute ethanol to get 0.1 M stock solution. 0.1ml of stock solution was added to 9.9ml of absolute ethanol to get 10^{-3} M solution for further dilutions.
Testosterone	98	288.40	Dissolve **0.294286**g in 10ml of absolute ethanol to get 0.1M stock solution. 0.1ml of stock solution was added to 9.9ml of absolute ethanol to get 10^{-3} M solution for further dilutions
17β-Estradiol	98	272.39	Dissolve **0.138974**g in 10ml of absolute ethanol to get 0.05M stock solution and further dilutions made to get 10^{-3}M solution.

B.7 Slide flask method and Cytospin.

i) Preparing cells for ICS and IF by slide flask

Cells (10^5) were grown directly on slide flasks (Figure B.7) supplied by Fisher Scientific, UK. Cells were harvested after 5 days of incubation, washed in cold PBS thrice and snapped off the container according to manufacturer's instructions; fixed by immersion in cold methanol for 10 min, left to dry at 4^{o}C for 15 min and wrapped in foil; and stored at -20^{o}C until required.

Figure B.4: Tissue culture slide flasks.

ii) Preparing cells for ICS and IF by cytospinning:

Cytospinning is a method for preparing a monolayer of cells on a glass slide by centrifugation. Cells can then be used for ICS and/ or IF.

- Clean glass slides were first coated with poly-L-lysine by immersing the slides in 0.01% (v/v) poly-L-lysine solution (Sigma, UK) for 5 minutes and then allowed to air-dry over-night. Commercially coated slides (Sigma, UK) were also used.
- Cell lines in culture plates were trypsinized (appendix B.3)
- The cell pellets were resuspended in normal culture media (containing 10% FCS) for each cell line and counted using haemocytometer (appendix B.4)).
- Appropriate dilutions were made to ensure that cell suspensions for cytospinning were in the order of 10^5 cells per ml.
- The cyto-chamber was assembled according to manufacturer's instructions (Boeco-28, Germany) by placing a coated slide in the chamber, covered with filter card and then the chamber well.
- 100 µl of homogenous cell suspension (10^5 cells per ml) was dispensed into each chamber well and centrifuged at 3,000 rpm (≈800g) for 5 minutes.
- Once completed, the chamber well and filter cards were carefully removed to avoid scratching the monolayer of cells. Excess solution was blotted.
- The slides were allowed to air-dry completely before fixation in cold methanol for 10 minutes. The fixed slides were dried at 4^{o}C for 30 minutes before use or wrapped in foil and stored at -20^{o}C until required.

B.8 Protocols for ICS, IHC and IF.

i) Protocol for ICS using in- house reagents and DAB chromogen

- Slides were removed from the freezer (still wrapped in foil) and kept at room temperature for 10 minutes and then unwrapped. Cell sections were circled with diamond pencil (to form a well on slide for reagents).
- Three drops of 0.03% (v/v) H_2O_2 in methanol were added onto the slide and incubated for 5 min in a humid chamber at room temp. Three drops was ~ 150 µl. The slides were rinsed 3x in PBS with Tween 20, for 2 minutes each.
- Three drops of 10% (v/v) normal rabbit serum in PBS with Tween 20 were added and slides incubated for 15 min in humid chamber. Excess serum was tapped off and slides wiped carefully around the edges.
- Three drops of primary antibody were added to test slide, 3 drops of 1% (v/v) normal rabbit serum in PBS to control slide and all incubated in humid chamber at room temperature for 1hr min.Slides were washed 3x in PBS with Tween 20 for 2 min each.
- Three drops of diluted (1/200 dilution) secondary antibody (rabbit anti-mouse IgG-conjugated with biotin) were added and slides incubated for 30 minutes in humid chamber at room temperature. Slides were washed 3x in PBS with Tween 20 for 3 min each.

- Three drops of AB complex (avid-biotin conjugated with peroxidase) (Dako, UK) were added and slides incubated for 30 min in humid chamber at room temperature. Slides were rinsed in PBS with Tween 20 twice, 3 min each. Excess buffer was tapped off and slides carefully wiped around the edges.
- Three drops of substrate chromogen (DAB) (appendix A.3) were added and slides incubated for 3 min in humid chamber. Slides were washed 3x in PBS with Tween 20 for 2 min each.
- Slides were stained in Mayer's haematoxylin for 1 min and gently rinsed in slow-running tap water to avoid washing off specimens.
- One drop of Glycergel (Dako, UK), an aqueous mountant, was added and cover slip placed on top.

Slides were examined microscopically at x10 and x40 magnifications using Carl Zeiss microscope with Axiom camera, and photomicrographs taken.

ii) Protocol for ICS using Histostar kit (Dako, UK)

- Two drops of reagent A (serum blocking reagent) were added to completely cover slide section and incubated in a humid chamber at room temperature for 10 min. Slides were drained or blotted but never rinsed.
- Two drops of primary antibody was added and incubated in moist chamber for 45 min. Slides were rinsed three times in PBS for 2 min each time.
- Two drops of reagent B (biotinylated second antibody) was added and incubated for 10 min. Slides were rinsed in PBS for 2 min, 3x.
- Two drops of enzyme conjugate (reagent C) were added and incubated for 10 min. Slides were rinsed in PBS for 2 min, 3x.
- Two drops of cold AEC single solution was added and incubated for 10 min, and slides were rinsed in distilled water.
- Two drops of Mayer's haematoxylin was added and incubated for 2 minutes and rinsed gently in running tap water.
- A drop of glycergel was added and cover slip placed on top.
- Slides were examined microscopically at x40 objective, and photomicrographs taken.

iii) Protocol for IF staining

- Two drops (100µl) of *reagent A* (*serum blocking solution*) was added enough to completely cover cells on each section, and incubated in a humid chamber at room temperature for 10 minutes. The solution was drained or blotted off (not rinsed).
- Two drops of *Primary antibody* added to cover each section, and incubated for 45 minutes in a humid chamber at room temperature. Rinsed with PBS for 2 minutes, 3 times.
- Two drops Alex fluor 555-*conjugated secondary antibody* were added to each section, and incubated for 15 minutes. Rinsed with PBS for 2 minutes, 3 times.

- Two drops of Sytox Green nuclear counterstain at 500nM in DMSO (or enough to completely cover cells) were added on each section and incubated for 10 minutes.
- Gently rinsed, one drop of Dako fluorescent mounting medium was added and a cover slip placed on top. Slides were examined using Carl Zeiss LSM 510 Meta Confocal microscope according to the manufacturer's instructions.

B. 9 Concentration of culture supernatants: 2ml vivaspin (MW CO 20,000) (Vivascience, Germany) was used, according to the manufacturer's instructions, to concentrate culture supernatants before performing ELISA. Briefly, samples were added into vivaspin columns and assembled according to manufacturer's instructions. The columns were centrifuged at 10,000g for 10 min. The flow through discarded, the columns were reverse-centrifuged to obtained concentrated supernatants for PSA ELISA.

B.10 ELISA protocol: Total and free PSA (Cat No: 4222 and 4322 respectively) were measured using ELISA kits from Diagnostic Automation/Cortez Diagnostics, Inc USA according to the manufacturer's instructions. Briefly, the test was a sandwich assay in which a rabbit anti-PSA was already coated on the surface of microwells in 96 well plate. Samples were added to appropriate wells followed by the addition of anti-PSA monoclonal antibody labelled with horseradish peroxidase as a tracer. Unbound antibody-enzyme tracers were removed by washing. The peroxidase activity was measured (by the addition of a substrate chromogen (TMB)) colorimetrically (at 450nm using Varioskan Flash, Thermo Scientific, UK). Optical absorbance was proportional to concentration of PSA in serum/plasma.

B.11 Protocol for western blotting: The method of immunoblotting is described in details by Williams *et al*, 2003. Briefly, SDS-PAGE was done using the BioRad mini-protean electrophoresis system (Figure B.5) according to manufacturer's instructions. Run time was 1 hr at 100 volts. Samples were prepared in the NUPAGE LDS sample loading buffer (Invitrogen, UK). Wet blotting (liquid transfer) was done using PVDF membranes for 1hr at 100volts. Membranes were blocked in 5% non fat dry milk in 1x TBS with 0.1% (v/v) Tween 20, and incubated with primary antibody overnight at $4^{o}C$ in gyratory shaker; washed in TBS with Tween 20 for 3x, 5min each. Membranes were incubated in secondary antibody (1: 2000 dilution of HRP conjugated secondary antibody), washed 3x for 5 min each before chemiluminescent detection using ECL Plus system (GE healthcare, UK).

Components of the Mini-PROTEAN Tetra system. A, electrophoresis module, companion running module, tank, lid, and buffer dam; **B**, glass plates and combs*; **C**, sample loading guides**; **D**, casting stand and casting frames***.

Figure B.5 Mini-protean electrophoresis from BioRad.

B.12 Protocol for hormonal stimulation:

Cells harvested in the log phase were counted and seeded into a 12-well tissue culture plate. Phenol-free media of appropriate type supplemented with 10% DCC-FCS were used. After 6 hours of incubation at 37^{o}C with 5% $C0_2$, steroid hormones were added into appropriate wells (IC_{10} and $1C_{50}$ values) and the cells incubated for 12-, 24-, 48- and 72 hrs before harvesting the cells for RNA extraction. 10^6 cells were seeded per well. For each hormone, there were two control set ups: culture media only (no treatment) and DMSO treated cells for all the time intervals. For each cell line, there were 48 samples (covering the four time intervals, the two steroid hormone concentrations, four different steroids and two treatment controls).

B.13 RNA extraction:

The RNeasy mini kit (Qiagen, UK) was used according to the manufacturer' instructions. 350µl of GITC lysate of sample (cell pellet) was added to 350µl of 70% ethanol, and the mixture dispensed into the RNeasy spin column contained in a 2ml collection tube. The column was centrifuged at 8000g for 15 s and the flow through discarded. 650 µl of RW1 buffer was added to the RNeasy column and centrifuged at 8000g for 15 s. The flow through was also discarded. Then 500µl of RPE buffer was added to the spin column and centrifuged at 8000g for 15 s; the flow through discarded and the step also repeated but with 2 min centrifugation. The flow through was again discarded, the spin column was placed on a new 2ml tube and centrifuged at 10,000g for 1 min. Finally the spin column was placed on a 1.5ml tube and 60 µl of RNase-free water added into the column and centrifuged for 1 min at 10,000g. 50µl of the elute was used for cDNA snythesis; 5 µl was used for RNA quality and quantity check.

B.14 cDNA snythesis

A batch of cDNA mix was first prepared as shown in Table B.2. Each tube of the cDNA mix served for 10 samples. To each cDNA mix, kept on ice during set up, 12µl of RNasin (RNase inhibitor 40 u/µl)

(Promega, UK) and 24µl of M-MLV reverse transcriptase (200 u/µl) (Invitrogen, UK) were added and mixed by pipetting. 42 µl of the mixture was added into each RNA elute (50 µl), mixed by pipetting and incubated at 37^{o}C for 2hr in dry thermal block. The tubes were then transferred to another dry thermal block at 65^{o}C for 10min to inactivate the reverse transcriptase. The tubes were pulse-centrifuged, labelled and stored at -80^{o}C until required.

Table B.2 Preparation of cDNA mix

Reagent	**Volume**
5X Buffer	5.06ml
0.1M DTT	2.53ml
25 mM dNTP mix	506 µl
Random primers (hexamer) 3µg/µl	50µl
RNAse-free, DNAse-free water	3.354ml
Total	11.5ml
420µl aliquoted into 1ml sterile tubes and stored at -80^{o}C. This would serve for 250 reactions. 5X buffer, DTT and dNTP (Promega, UK); hexamer and water (Invitrogen, UK).	

B. 15 RQ-PCR set up.

For all RQ-PCR the 96-micro well PCR plates (Applied biosystems, UK) were used. Each set up required reagent a trough, a PCR plate, primer and probe mix, DNAse-free, RNase-free water, dedicated pipettes, microfilm, plate holder and a biological hood. In some cases, reactions were run in duplex format (two targets measured simultaneously in each well), and in many as singleplex format. Where primers and probes were designed, optimal primer and probe concentrations were determined prior to sample testing. For predesigned assays (Applied Biosystems, UK) manufacturer's recommendations were followed. Two platforms were used: ABI 7900HT sequence detection system was used for cell line studies; and the CFX96 Real PCR system was used for clinical samples. Results from both platforms were comparable. Total reaction volumes for the CFX96 system was 10µl while that of the ABI 7900HT was 25 µl. A typical reaction set up is shown in Figure B.3.

Table B.3 - Reaction Mix for control genes.

	Volume (µl) per 25µl for ABL1	Volume (µl) per 25µl for GUS	Volume (µl) per 25µl for G6PD
Forward primer	0.094 (300nM)	0.75 (300nM)	0.094(300nM)
Reverse primer	0.094 (300nM)	0.75(300nM)	0.094(300nM)
Probe	0.1 (100nM)	1.0 (100nM)	0.1 (100nM)
Universal master mix	12.5	12.5	12.5
Water (RNase-free, DNAse- free)	9.712	8.85	9.712
cDNA	2.5	2.5	2.5

For a predesigned assay on the CFX 96 system, a typical reaction would be Universal master mix = 5µl, primer and probe mix = 0.5 µl, water = 2.50 µl, cDNA= 2.0 µl. Where duplex format was used, for example, ABL1 and G6PD were run together with a FAM and TET dyes respectively, the TaqMan gene expression master mix was used in place of the Universal TaqMan master mix. Results of duplex did not differ from singleplex of each of the targets. Thermal profile was same for all reactions: **50**^{o}C for 2 minutes, initial denaturation at **95**^{o}C for 10 minutes; cycle denaturation of **95**^{o}C for 15 seconds, annealing and extension at **60**^{o}C for 1 minute for 40 cycles. Data was acquired at all stages. Calibrators were done in triplicates, 6 points of triplicate each (18 points overall) were used in generating standard curves; samples were measured in duplicates.

B.16 Protocol of KLK2 and TP53 plasmid cloning

Plasmid calibrators for KLK2 and TP53 were prepared using TOPO 10 cloning kit from Invitrogen UK, purified with EZ Maxi kit from VH Bio Ltd UK. Briefly, for the TOPO 10 cloning kit, target transcripts were amplified from the prostate cell lines using standard PCR method and PCR master mix from ROVLAB, sold by VH Bio Ltd UK. The PCR product was electrophoresed on 2% agarose gel stained with SYBR Safe (Invitrogen UK) to check for the quality. 4µl of the fresh PCR product was used for cloning the target sequence into the TOPO vector (pCR2.1-TOPO). 6µl of the TOPO cloning reaction was dispensed into a vial of one Shot *E.coli* (chemically competent), to transform the *E.coli* through heat-shock for 30 s at 42 ^{o}C. 250 µl of SOC medium was added to the cells, and mixed by shaking at 37^{o}C for 1 hour. 50 µl of the transformation was spread onto dry LB agar plates containing X-gal and 50µg/ml ampicillin and incubated overnight. About 5 white colonies where picked and emulsified in DNAse-, RNAse-free water and standard PCR performed to check for successful transformation. About 10 colonies were then emulsified in LB medium containing 50µg/ml ampicillin and incubated overnight at 37^{o}C, from which plasmids were harvested using the EZ Maxi kit (VH Bio, UK). The OD of plasmid was checked on Eppendorf photometer, and copies of DNA calculated using the calculator in the website http://www.uri.edu/research/gsc/resources/cndna.html . Six serial dilutions of plasmids were made to give a dynamic range of 10^{1} to 10^{6} copies per 2µl in tRNA in TE buffer. The plasmid calibrators were used to generate standard curves for absolute quantification of target genes. The plasmid calibrators for ABL1 and G6PD endogenous genes were a kind gift from the laboratory of Prof. Letizia Foroni, Imperial College London.

B.17 Red cell lysis:

The preparation of the red cell lysis buffer (RCLB) is described in appendix A.2. Participants' blood samples in K_3EDTA were carefully emptied into labelled Falcon's tubes (50ml) in a biological hood. 30ml of 1x RCLB was added to each tube, mixed by inversion and placed on ice for 10min. The tubes were centrifuged at 1800g for 7min; and supernatants carefully decanted into Virkon containing waste bucket. The step was repeated with 20ml of 1x RCLB, with the tubes vortexed to resuspend white cell pellets and placed on ice for 10min. The tubes were centrifuged at 1800g for 7min and supernatants carefully decanted. The cell pellets were then resuspended in 15ml of PBS (pH 7.2) by vortexing, and later centrifuged at 1800g for 7min. The supernatants were discarded and the tubes briefly drained on a clean blue towel. The white cells were then lysed in appropriate volumes of GITC.

B.18 PCR of KLK2, TP53 and other targets for Sequencing

Standard PCR was performed on all targets (cDNA or gDNA) for sequencing. All standard PCR used ROVLAB 2 X PCR Master mixes (VH Bio, UK) which contained all reagents required for PCR except primers and DNA. Set up for all PCR is shown in Table B.4.

Table B:4 Standard PCR set up

Reagent	For genomic DNA	Complementary DNA
2 x PCR Master mix	10 µl	10
Forward primer	0.6µl (300nM)	0.6µl (300nM)
Reverse primer	0.6µl (300nM)	0.6µl (300nM)
Water	7.8 µl	6.8 µl
Template	1 µl	2 µl
Total reaction volume	20 µl	20 µl

Primer concentration of 300nM was optimal for most standard PCR applications.

The thermal profile for most applications (annealing temperature determined by Gradient PCR) is shown in Table B.5.

Table B.5 Thermal profile for standard PCR.

Step	Time	Temperature	Cycles
Initial denaturation	2 min	94^{o}C	1X
Denaturation	1 min	94^{o}C	25 x
Annealing	30s	58 ^{o}C	
Extension	1 min	72 ^{o}C	
Final extension	7 min	72 ^{o}C	1x

For annealing temperature (Ta), the gradient PCR showed that the range 55 to 60 ^{o}C was optimal for all the reactions. Figure B.6 shows PCR products at Ta = 58 for KLK2 of two different sizes from many samples.

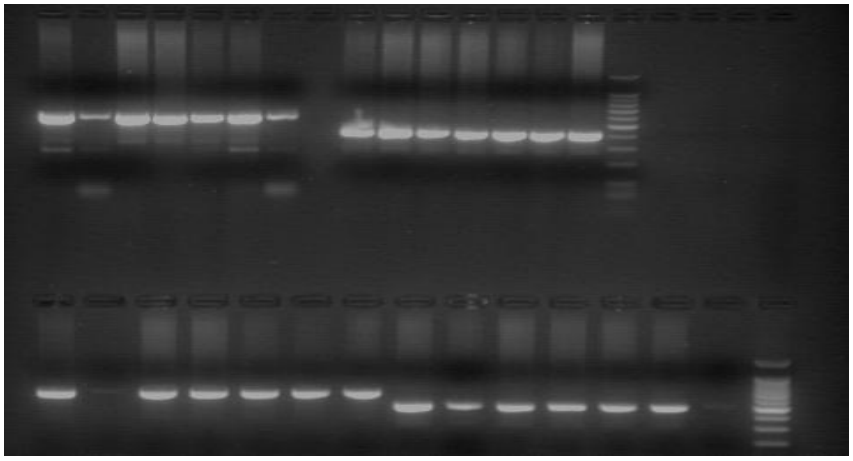


Figure B.6 KLK2 PCR products from a Ta of 58^{o}C. Two different product sizes were run at same Ta, yielding distinct bands.

To prepare for sequencing, 1 in 10 dilutions of the PCR products were made. A sequencing reaction was set up in a 96 well plate as follows

DNA template 1µl
Primer (F or R) 1µl
Big Dye v 1.1 0.5µl
Sequencing buffer 2µl
Water 5.5µl
Final volume was 10µl

The Big Dye and Sequencing buffer were purchased from Applied biosystems, UK. A larger reaction mix could be set up by scaling all other reagents appropriately except the DNA template. The plate was sealed with microfilm and pulse-centrifuged. Covered with a compression pad (to prevent evaporation), the PCR sequencing reaction was performed for 25 cycles using the thermal profile:

96^{o}C for 10s
50 ^{o}C for 5s
60 ^{o}C for 4 min

The Sequencing PCR product was then purified using the Big Dye X terminator purification kit:

To each well of PCR product, 45µl of SAM solution (Applied biosystems, UK) and 10 µl of Big Dye X terminator solution (Applied biosystems, UK) were added, the plate firmly sealed and mixed by vortex at 2000 rpm for 15 min. The plate was centrifuged at 1000g for 2 min and run on the Genetic Analyzer 3130 (Applied Biosystems, UK) according to the manufacturer's instructions using the protocol BDX_x36cm_POP7_SetE.

B.19 Agarose gel electrophoresis.

All agarose gel electrophoreses were done using Tris Acetate EDTA TAE buffer (appendix A.2). 2g of agarose gel was dissolved in 100ml of 1x TAE, heated for 3 min in microwave with occasional mixing. 10µl of Syber Safe (Invitrogen, UK) was added to the 100ml of molten agarose gel. After cooling for a few minutes, the gel was poured onto gel plate with combs inserted and allowed to set. BioRad horizontal electrophoresis system was used.

10 µl of PCR product and 2 µl of loading dye (Promega, UK) were loaded to each well accordingly. 8 µl of DNA ladder (mainly 100bp ladder, Promega, UK) mixed with 2 µl of loading dye was added to the ladder lane. Electrophoresis was run at 100v for 45-60min. After electrophoresis, gels were viewed in Syngene Gene Genius bio-imaging system.

B. 20 Protocol for SNP genotyping

The protocol for SNP genotyping using ABI predesigned TaqMan genotyping assays for allelic discrimination was basically the same with standard RQ-PCR except for a pre-read and post read scanning. Using the protocol in Section B.15 of RQ-PCR the reaction for SNP genotyping was set up, and standard format of allelic discrimination assay on ABI 7900HT platform was chosen. The first step was to pre-read the plate for background fluorescence, and then a standard RQ-PCR run was done; followed by post-read scan using the same pre-read window. The post read did the base calling and results were analyzed. The ABI predesigned SNP assays came with CD-ROM to enable faster loading of the assay run protocol and identity into the system.

B.21 Protocol for setting up Pyrosequencing assay

The Pyrosequencing assay had 5 main steps. Prior to these steps, the primers were designed using the PSQ assay designer software (Biotage, Sweden). The pyrosequencing (PS) assay required 3 primers, one of which must be biotinylated. The assay design software chose the primer to be biotinylated. All the primers for PS assay were purchased from Sigma, UK,

The first step was to perform a standard PCR using two of the primers, one of which was biotinylated. The reaction set up was in a 24-well plate as follows

PCR master mix (ROVLAB)	15µl
F-primer (biotinylated)	0.9 µl (300 nM)
R-primer	0.9 µl (300 nM)
Water	11.2 µl
Genomic DNA	2 µl
Final volume	**30 µl**

The standard PCR thermal profile described in B.18 was used to perform the PCR.

The second step was to bind the PCR products (biotinylated) to Sepharose beads containing Streptavidin for separation.

Binding buffer	40 µl
Water	28 µl

Sepharose beads	2 µl
PCR products	10 µl
Final volume was	**80 µl**

The Sepharose beads were purchased from GE Healthcare, UK. All other reagents and materials were purchased from Qiagen, UK. The mixture was vortexed for 30min at 2000rpm.

The third step was to anneal SNP primer (the third form of primer) to the biotin-streptavidin PCR product.

Annealing buffer	24.25 µl
10 µM SNP primer	0.75 µl
Final volume	**25 µl**

The fourth step involved the addition of the biotinylated PCR product to the 25 µl annealing mixture. Using the workstation shown in Figure 4.18 and in accordance with the manufacturer's instructions, the biotinylated DNA strand was washed in 70% ethanol, denatured in 0.2M NaOH and washed again in a Washing buffer, all using a vacuum pump. The biotinylated strand was then transferred to the annealing mixture in the Pyromark plate (24-well plate) and kept on a heat dry block at 80^{o}C for 2 min. Enzyme, substrate and nucleotides were mixed and dispensed into a cartridge according to pre-run calculations from the Pyromark 24 software (Qiagen, UK)

The fifth step: the plate was run in the PyroMark-24 machine (Qiagen, UK) using a run schedule from the Pyromark 24 software. It took about 13 minutes to run a 24 well plate on the machine. Results were analyzed on the PyroMark 24 software, giving the pyrograms for samples and reference calibrators.

B.22 Sodium Citrate buffer antigen retrieval method

The preparation of Sodium citrate buffer was described in Section A.2.

Tissue sections for antigen retrieval were already deparaffinised in 2 changes of xylene, 5 min each; hydrated in 2 changes of absolute ethanol for 3 min each, 95% and 80% ethanol for 2 min each and rinsed in distilled water. Slides were immersed in pre-heated water bath containing sodium citrate at boiling point of 100^{o}C for 30min. The slides were then removed and allowed to cool for 20 min, and rinsed twice in 2 changes of PBS with Tween 20 for 2 min each. Sections were then taken for IHC as described in Section B.8.

Appendix C: Raw Data and Additional Result Presentation

C.1 Raw data for serum supplementation experiment

% Serum level	Cell line	FCS		BGS		DCC-FCS	
		Mean Cell count	Std	Mean Cell count	Std	Mean Cell count	Std
	PNT1A	X10E5					
10		39.85	0.84	35.5	3.03	10.85	0.3
7.5		18.15	0.63	16	2.27	5	0.23
5		12.35	0.42	12	1.52	4.85	0.15
2		2.65	0.19	9	0.61	1.15	0.06
1		1	0.08	1.65	0.3	1	0.03
	PC-3						
10		25.35	0.76	25.5	1.13	15.15	0.76
7.5		18.5	0.12	21.15	0.53	13.35	0.17
5		18.35	0.29	19.85	0.59	12.35	0.23
2		17	0.32	12.65	0.36	9.65	0.06
1		7.65	0.02	9	0.06	6.50	0.00
	MDAPCa 2b						
10		30.28	0.96	30.00	0.20	22.40	0.17
7.5		25.6	0.17	25.72	0.06	21.08	0.06
5		24.8	0.10	13.20	0.35	18.80	0.52
2		20.92	0.06	9.88	0.06	14.68	0.06
1		8.66	0.03	7.87	0.02	7.98	0.04

C.2 PNT1A cell yield in varying percents of different sera.

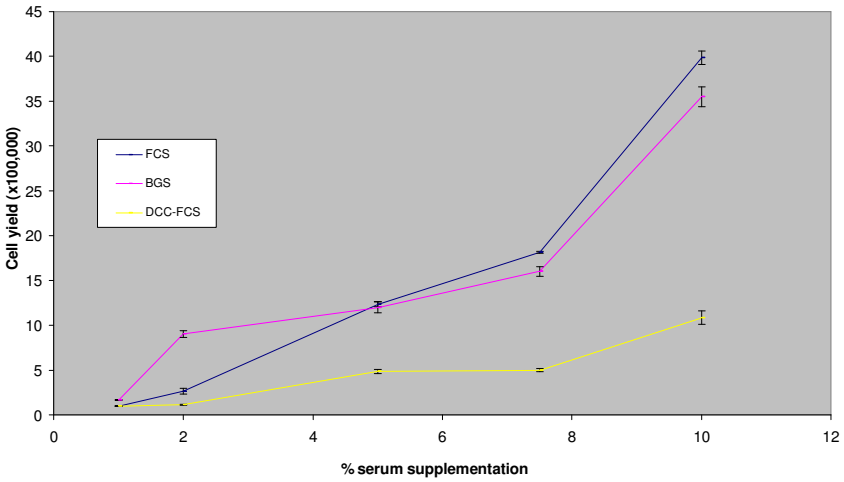


Figure C.1 PNTIA cell yield in different sera.

C.3 Raw data for growth curves

		PNT1A (X10e4)	Treatment Group					
	No drug		Ethanol		DHT		Oestradiol	
Days of Incubation	Mean	std	Mean	std	Mean	std	Mean	std
Di	8.00	0.00	8.00	0.00	8.00	0.00	8.00	0.00
D0	1.20	0.69	1.20	0.69	1.20	0.69	1.20	0.69
D1	8.67	1.80	2.27	0.23	0.53	0.23	0.47	0.12
D2	8.80	0.40	3.07	0.83	1.07	0.23	2.00	0.40
D3	6.93	1.67	5.73	1.01	0.80	0.40	0.93	0.46
D5	87.00	2.65	62.33	8.08	43.00	7.00	28.00	2.00
D7	52.40	7.99	27.60	3.17	27.40	3.62	54.73	3.80
D9	45.73	6.47	40.60	0.00	53.20	13.36	40.60	2.80
D11	27.20	5.00	20.80	3.46	28.80	8.40	49.93	4.87
D13	18.00	1.20	12.80	1.83	26.60	4.39	27.47	2.14
		PC-3 (X10e4)						
	No drug		Ethanol		DHT		Oestradiol	
	Mean	std	Mean	std	Mean	std	Mean	std
Di	4.00	0.00	4.00	0.00	4.00	0.00	4.00	0.00
D0	3.33	0.58	2.67	0.58	2.33	0.58	2.67	0.58
D1	7.33	1.15	3.33	1.15	6.67	1.15	4.67	1.15
D2	21.33	3.06	9.67	0.58	14.00	4.58	19.33	1.53
D3	57.33	8.33	46.67	4.62	56.00	8.00	61.33	8.33
D5	122.67	8.33	56.00	4.00	144.00	21.17	82.67	6.11
D7	166.67	34.02	128.00	20.78	108.00	14.42	133.33	12.22
D9	188.00	17.44	148.00	17.44	180.00	17.44	210.67	12.22
D11	154.67	11.55	100.00	14.42	136.00	18.33	54.67	8.33
D13	134.54	7.68	96.88	5.21	132.54	13.23	53.44	4.32
		MDAPCA 2b (x10e4)						
	No drug		Ethanol		DHT		Oestradiol	
	Mean	std	Mean	std	Mean	std	Mean	std
Di	2.00	0.00	2.00	0.00	2.00	0.00	**2.00**	**0.00**
D0	0.77	0.06	0.83	0.06	0.87	0.06	0.63	0.06
D1	6.67	1.15	2.67	1.15	4.00	0.00	5.33	1.15
D2	6.33	0.58	3.67	1.15	6.67	1.15	2.00	0.00
D3	38.67	8.33	26.67	6.11	14.67	4.62	21.33	4.62
D5	44.00	6.93	34.67	4.62	53.33	11.55	26.67	8.33
D7	50.67	8.33	70.67	14.05	74.67	16.17	40.00	4.00

D9	81.33	8.33	101.33	24.44	66.67	16.65	58.67	14.05
D11	58.67	11.55	98.67	12.86	56.00	6.93	64.00	14.42
D13	54.23	6.42	96.54	10.23	55.32	5.61	54.24	10.56

C.4 Raw data for change in population doubling.

Change in Population	Doubling (ΔPD)expt								
Drug	PC-3 count	AE	PC-3 count		MDA Pca 2b count	AE	MDA count		
	(16-hrs)x10 exp 4	For PC-3	(120 hrs)x10 exp 4	PC-3 Δ PD	(16-hrs)x10 exp 4	MDA Pca 2b	(120 hrs) x 10 exp4	MDA Pca 2b Δ PD	std
Ethanol	3	0.75	14.3	2.25	1.4	0.35	20	3.84	0.08
Dihydrotestosterone	2.5	0.95	33	2.94	1.4	0.35	33	4.56	0.11
Testosterone	3.8	0.95	29.3	2.38	1.4	0.35	30	4.42	0.06
Estradiol	3.8	0.95	7.3	0.94	1	0.25	10	3.32	0.04
Progesterone	2.6	0.65	20.3	2.97	1.3	0.33	30	4.53	0.12
Aldosterone	3.6	0.9	25.3	2.81	1.3	0.33	27	4.38	0.14
No drug	3.7	0.93	25	2.77	1.1	0.28	22	4.32	0.07
Hydrocortisone	2.5	0.63	4.3	0.78	0.8	0.2	8	3.32	0.06
seeding density	4x10exp4								
Volume of drug	25 microlitre								
Molar conc.	10 exp 4								
Micro-well volume	3.155 ml		3.217ml						
Suspension Volume	0.5ml		1.0ml						

C.5 Raw data for doubling time

Cell	Yield &	Doubling	Time (DT)	Stimulation	expt.	Result	
		1	2	3			
Sample	Mean DT	DT(hrs)	DT(hrs)	DT(hrs)	mean DT (hrs)	std	
PC-3 Negative	34.32	27.96	38.4	36.6	34.32	5.580967658	
PC-3 Alcohol	28.22	21.17	37.1	26.4	28.22333333	8.120014368	1st expt. Cell count x10exp5
PC-3 Oestradiol	71.98	64.55	72.8	78.6	71.98333333	7.060512257	10.3
PC-3 Testosterone	30.68	16.84	32.8	42.4	30.68	12.91120444	13.6
PC-3 Dihydrotestosterone	26.82	26.67	29	24.8	26.82333333	2.104194224	19.8
MDA Pca 2b Negative	25.05	27.96	24.8	22.4	25.05333333	2.788643637	17.1
MDA PCa 2b Alcohol	27.89	25.49	27.6	30.6	27.89666667	2.567884992	10.8
MDA Pca 2b Oestradiol	32.02	26.67	30.6	38.8	32.02333333	6.188992918	10.3
MDA Pca 2b Testosterone	23.47	26.92	23.8	19.7	23.47333333	3.621067982	11.3
MDA Pca 2b DHT.	25.3	27.69	24.6	23.6	25.29666667	2.132142897	10.8
							10.7
							10.4

DT PNTIA	DT (in hours)	std
PNTIA-Ethanol	47.21333333	6.976427
PNTIA-No Drug	35.60666667	4.1236068
PNTIA-DHT	63.76333333	8.3751557
PNTIA-Testosterone	52.63666667	1.4897091
PNTIA-Oestradiol	111.2233333	5.8736388
PNTIA-Progesterone	45.91333333	9.0712807
PNTIA-Aldosterone	40.62	4.2514468
PNTIA-Hydrocortisone	41.18	3.5125347

C.6 Raw data for % control growth of hormones on PNTIA cells

		Cell density optimization assay for MTT	Optimization assay fo					
PNTIA-72HR								
Cells/ml		Optical Density					Av.OD	STD
Blank	0.193	0.331	0.102	0.109	0.085	0.116	0.156	0.093552
1000	0.833	0.665	0.779	0.703	0.893	1.019	0.815333	0.129895
5000	0.309	0.382	0.252	0.596	0.372	0.626	0.422833	0.153399
10000	0.532	0.368	0.485	0.409	0.56	0.706	0.51	0.120325
50000	0.264	0.589	0.301	0.382	0.443	0.243	0.370333	0.130812
100,000	0.278	0.336	0.284	0.444	0.246	0.259	0.307833	0.073486
500,000	0.42	0.31	0.401	0.241	0.348	0.381	0.350167	0.066252
1,000,000	0.436	0.271	0.414	0.574	0.39	0.366	0.4085	0.099229

MDA-72HR								
Cells/ml		Optical Density					Av.OD	STD
Blank	0.057	0.05	0.049	0.05	0.053	0.052	0.051833	0.002927
1000	0.567	0.563	0.098	0.316	0.508	0.405	0.4095	0.181033
5000	0.366	0.51	0.606	0.478	0.47	0.628	0.509667	0.096436
10000	0.45	0.602	0.572	0.516	0.674	0.604	0.569667	0.077866
50000	0.531	0.507	0.485	0.568	0.53	0.546	0.527833	0.029075
100,000	0.372	0.579	0.558	0.545	0.566	0.528	0.524667	0.076816
500,000	0.288	0.414	0.514	0.481	0.601	0.629	0.487833	0.125579
1,000,000	0.178	0.297	0.728	0.576	0.445	0.483	0.451167	0.195785

SRB-PNTIA-Estradiol

Drug Con	OD													
µm	1	2	3	4	5	6	7	8	9	**Av OD**	Std OD	%CCG-mean	%gin	% cells killed
	0.28	0.25	0.23	0.24	0.29	0.31	0.25	0.26	0.27		0.0251	10.79184	89.208	89.204
100	5	4	8	6	8	4	6	4	5	**0.27**	45	616	15	32
	0.55	0.66	0.59	0.57	0.53	0.67	0.56	0.62	0.58	**0.5974**	0.0476	23.87867	76.121	76.111
10	9	6	2	2	9	6	8	3	2	**44**	97	484	33	78
		1.03	0.96	1.05	1.03	1.05	1.15	1.03	1.12	**1.0578**	0.0540	42.28173	57.718	57.701
1	1.07	1	7	5	8	3	3	2	2	**89**	66	017	27	36
	1.17	1.15	1.12	0.98	1.01	1.12	1.03	0.98		**1.0802**	0.0746	43.17434	56.825	56.808
0.1	7	6	5	6	2	3	5	8	1.12	**22**	72	941	65	39
				1.04	1.03	1.12		1.12	1.28	**1.1516**	0.0871	46.02984	53.970	53.951
0.01	1.19	1.25	1.11	2	4	4	1.21	3	2	**67**	23	279	16	75

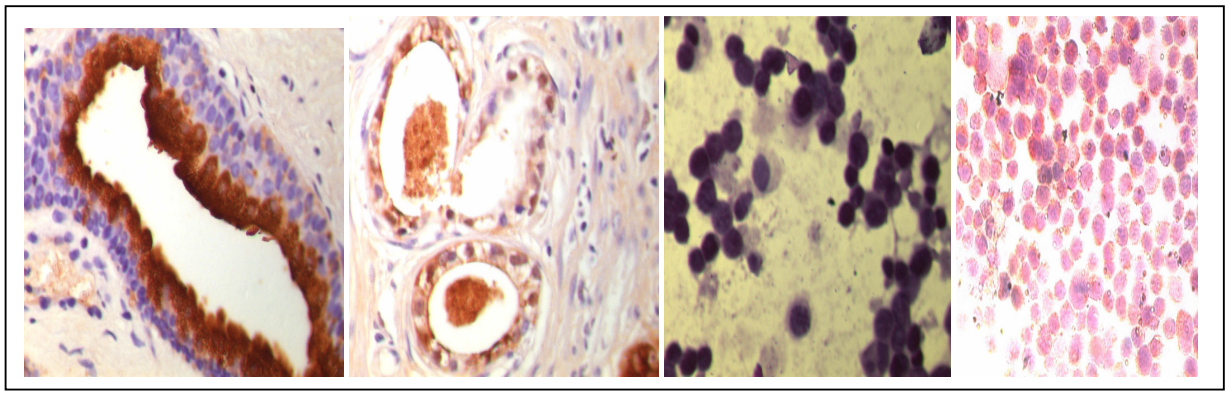
	1.404	1.867	1.593	1.517		1.648	1.539	1.725		**1.6436**	0.1441	65.69411	34.305	34.279
0.001					1.747				1.765	**67**	93	138	89	62
	1.439	1.996	1.928	1.836	1.709	1.857	2.072	1.938	1.367	**1.7935**	0.2444	71.68487	28.315	28.286
0.0001										**56**	61	432	13	46
	1.718	1.648	1.678	1.875	1.544	1.428	1.488	1.488	1.105	**1.5522**	0.2182	62.03925	37.960	37.935
0.00001										**22**	5	748	74	94
0.00000	1.339		1.128	1.228	1.177			1.315	1.371	**1.2968**	0.0964	51.83408	48.165	48.145
1		1.369				1.378	1.391			**89**	51	828	91	19
0.00000	0.681	0.663	0.866	0.879	0.715	0.703	0.668	0.672	0.879	**0.7473**	0.0969	29.86943	70.130	70.118
01										**33**	83	778	56	62
														-0.035
	2.183	2.097	2.433	2.601	2.693	2.603	2.663	2.637	2.607	**2.5018**	0.2185	99.99555	0.0044	
0										**89**	75	911	41	4

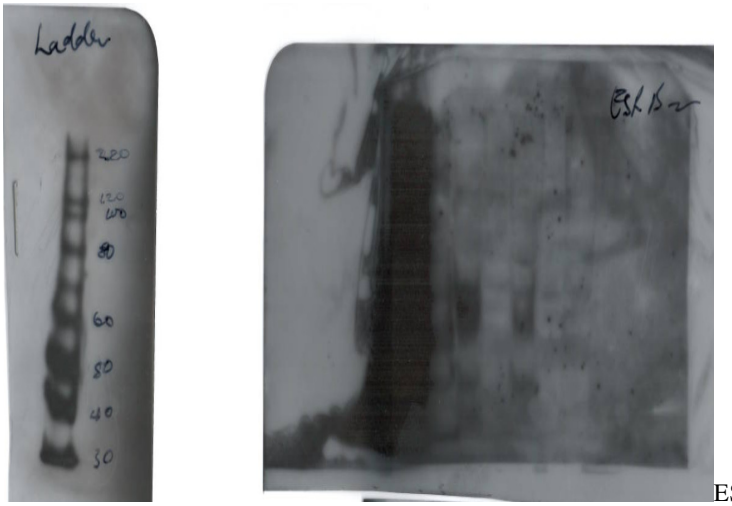
SRB-PNTIA-DHT

Notes	**100**	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**	**0.00001**	**0.000001**	**1E-07**	**Negative control**
	0.09	0.133	0.289	0.301	0.314	0.324	0.324	0.347	0.324	0.315	0.328
	0.094	0.147	0.29	0.303	0.327	0.33	0.329	0.354	0.33	0.332	0.332
	0.1	0.149	0.293	0.308	0.329	0.331	0.337	0.354	0.342	0.337	0.337
	0.101	0.151	0.311	0.311	0.338	0.335	0.339	0.355	0.345	0.342	0.343
	0.108	0.16	0.313	0.319	0.341	0.343	0.342	0.355	0.346	0.344	0.344
	0.112	0.165	0.314	0.322	0.341	0.349	0.345	0.328	0.347	0.347	0.357
	0.116	0.176	0.319	0.332	0.343	0.35	0.347	0.327	0.35	0.359	0.364
	0.133	0.185	0.319	0.338	0.344	0.354	0.354	0.345	0.356	0.362	0.371
	0.141	0.185	0.325	0.338	0.346	0.364	0.365	0.346	0.36	0.363	0.407
Average OD	0.110556	0.161222	0.308111	0.319111	0.335889	0.342222	0.342444	0.345667	0.344444	0.344556	0.353667
OD std	0.017205	0.018089	0.013743	0.014426	0.010493	0.013132	0.012411	0.011045	0.011447	0.015629	0.024688
%CCG mean	31.25979	45.58588	87.11899	90.22926	94.97321	96.76397	96.82068	97.73789	97.39231	97.42372	99.99991
%Growth inhibition	**68.74021**	**54.41412**	**12.88101**	**9.770742**	**5.026794**	**3.236032**	**3.173198**	**2.262109**	**2.607695**	**2.576278**	**9.43E-05**

Drug con	%CGG	%GI		IC10	1C50		
100	31.26	68.74		0.85µM	8.65µM		2
10	45.59	54.414					1
1	87.12	12.88					0
0.1	90.23	9.77					-1
0.01	94.97	5.027					-2
0.001	96.76	3.24					-3
0.0001	96.82	3.17					-4
0.00001	97.74	2.26					-5
0.000001	97.39	2.61					-6
0.0000001	97.42	2.58					-7

C.7 Additional ICS slides





ESRb1

Standard Curve for total PSA

Conc ng/ml	OD1	OD1	Av OD	Conc ng/ml	Av OD
0	0.049	0.047	0.048	0	0.048
2	0.182	0.177	0.1795	2	0.1795
4	0.231	0.257	0.244	4	0.244
15	0.718	0.8	0.759	15	0.759
50	1.696	1.872	1.784	50	1.784
100	3.039	3.138	3.0885	100	3.0885

		PNTIA	**Cell line**	ON Cellular lysate						
	DHTIC10		Av OD	Control1		Av OD	Control2		Av OD	DHT IC50
24hrs	0.047	0.079	0.063	0.048	0.06	0.054	0.054	0.054	0.054	
48hrs	0.048	0.046	0.047	0.053	0.046	0.0495	0.047	0.045	0.046	
72hrs	0.051	0.054	0.0525	0.05	0.039	0.0445	0.046	0.05	0.048	

Summary of DHT-PNTIA stimulation

	DHT IC10	DHT IC50	C1	C2
24hrs	0.063	0.0505	0.048	0.054
48hrs	0.047	0.0475	0.053	0.047
72hrs	0.0525	0.0525	0.05	0.046

PSA level in PNTIA treated with DHT (Lysate)

	DHT 10(ng/ml)	**DHT 50 (ng/ml)**	**C1 (ng/ml)**	**C2 (ng/ml)**
24hrs	**0.692307**	**0.5549445**	**0.527472**	
48hrs	**0.516483**	**0.5219775**	**0.582417**	
72hrs	**0.5769225**	**0.5769225**	**0.54945**	

PNTIA PSA secretion

	DHTIC10		Av OD	Control1		Av OD	Control2		Av OD	DHT IC50
24hrs	*0.047*	*0.04*	*0.0435*	*0.046*	*0.051*	*0.0485*	*0.047*	*0.051*	*0.049*	

hydrocortisone

Time	C1 OD			C2 OD			H IC10			H 0.5I C10		
24 hrs	0.064	0.071	0.0675	0.065	0.074	0.0695	0.0601	0.0602	0.0615	0.0073	0.0064	0.0685
48 hrs	0.079	0.062	0.0705	0.067	0.065	0.0656	0.0604	0.0556	0.0576	0.0074	0.0069	0.0715
72 hrs	0.08	0.079	0.0795	0.064	0.054	0.0589	0.0583	0.0603	0.0605	0.0065	0.0064	0.0645

summary of PSA secretion (ng/ml)

Time	C1 (ng/ml)	C2 (ng/ml)	HIC10	H0.5IC10
24 hrs	0.741758	0.763736	0.675824	0.7527465
48 hrs	0.774725	0.725274	0.65934	0.7857135
72 hrs	0.873626	0.648351	0.664835	0.7087905

Testosterone

Time	C1 OD			C2 OD			T10			T0.5		
24 hrs	0.064	0.071	0.0675	0.065	0.074	0.0695	0.0707	0.0602	0.0676	0.0073	0.0064	0.0685
48 hrs	0.079	0.062	0.0705	0.067	0.065	0.0656	0.08	0.0605	0.0725	0.0166	0.0081	0.1235
72 hrs	0.08	0.079	0.0795	0.064	0.054	0.0589	0.0901	0.0855	0.0855	0.0133	0.0207	0.1685

Summary of PSA secretion by PNTIA line treated with Testosterone

Time	C1 (ng/ml)	C2 (ng/ml)	T10 (ng/ml)	T0.5(ng/ml)
24 hrs	0.741758	0.763736	0.743254	0.7527465
48 hrs	0.774725	0.725274	0.796703	1.3571415
72 hrs	0.873626	0.648351	0.93956	1.8516465

PNTIA-free PSA (OD reading)

Free PSA	Time	C1OD	C2OD	E10	E0.5	H10	H0.5	T10	T0.5
	24 hrs	0.042	0.038	0.044	0.043	0.041	0.045	0.046	0.045
	48 hrs	0.042	0.039	0.043	0.044	0.039	0.048	0.068	0.064
	72 hrs	0.042	0.074	0.112	0.044	0.38	0.038	0.076	0.078

Free-PSA ng/ml for PNTIA line

Time	C1	C2	E10	E0.5	H10	H0.5	T10	T0.5
24 hrs	0.020916	0.018924	0.021912	0.021414	0.02041	0.02241	0.02290	0.02241
48 hrs	0.020916	0.019422	0.021414	0.021912	0.019422	0.023904	0.033864	0.031872

							0.01	0.03	0.03
72	0.020			0.021	0.18	892	784	884	
hrs	916	0.036852	0.055776	912	924	4	8	4	

C.10 Raw data on Gene expression studies.

ABL-PNTIA	GUS-PNTIA	G6PD-PNTIA	ABL-MDAPCA	GUS-MDAPCA	G6PD -MDAPCA	ABL-PC-3	GUS-PC-3	G6
2.71E+04	2.63E+05	1.63E+05	3.81E+03	3.71E+03	1.93E+04	5.80E+04	1.13E+04	
2.49E+04	2.36E+04	9.24E+04	2.95E+04	4.75E+04	9.64E+04	9.65E+02	7.09E+02	
2.20E+05	3.44E+04	6.14E+05	7.32E+04	2.61E+04	2.77E+05	1.30E+05	7.48E+03	
5.67E+04	1.87E+04	1.42E+05	1.41E+04	1.69E+04	9.86E+04	1.65E+04	6.72E+03	
2.67E+05	3.63E+05	7.15E+05	4.46E+04	7.94E+03	1.36E+05	1.82E+05	2.09E+04	
2.82E+04	6.96E+04	1.14E+05	1.79E+04	2.34E+04	9.16E+04	6.38E+04	4.57E+04	
1.85E+04	1.49E+04	7.24E+04	4.96E+04	2.53E+04	5.46E+04	5.17E+04	4.17E+03	
2.93E+04	1.42E+04	4.30E+04	1.94E+04	1.87E+04	3.30E+04	6.01E+04	1.66E+04	
1.02E+05	1.97E+04	1.66E+05	2.61E+04	4.98E+03	8.50E+04	4.68E+04	2.25E+04	
9.06E+03	1.50E+04	9.35E+03	1.08E+04	6.89E+03	4.89E+04	9.76E+03	1.80E+04	
2.61E+05	2.19E+05	5.29E+05	2.34E+04	4.18E+03	7.11E+04	4.49E+04	2.46E+04	
6.26E+02	2.00E+02	1.58E+03	5.02E+03	2.45E+03	2.33E+04	2.10E+04	1.52E+04	
7.47E+02	2.80E+02	1.29E+03	2.03E+04	2.04E+04	9.56E+03	1.94E+04	6.62E+03	
9.58E+03	4.04E+02	9.18E+03	3.12E+04	3.47E+04	3.05E+04	3.17E+04	2.56E+04	
7.38E+04	1.18E+04	1.07E+05	1.81E+04	1.25E+04	6.42E+04	5.01E+01	3.49E+01	
1.09E+02	1.19E+01	1.70E+02	8.78E+00	2.34E+01	4.35E+01	1.09E+00	9.37E-01	
8.34E+03	4.12E+02	1.12E+04	2.03E+04	1.31E+04	4.54E+04	3.17E+01	4.07E+01	
9.74E+01	1.19E+01	2.27E+02	3.50E+01	2.21E+01	7.19E+01	1.23E+04	4.28E+03	
9.58E+01	1.25E+02	1.17E+02	1.73E+03	7.15E+02	6.66E+03	1.32E+03	1.03E+03	
3.75E+04	6.83E+04	1.16E+05	5.35E+01	2.24E+01	1.75E+02	4.28E+02	3.29E+02	
6.66E+04	7.36E+04	8.60E+04	4.98E+03	3.82E+03	1.51E+04	2.23E+04	1.69E+04	
9.01E+01	6.85E+01	1.61E+02	3.43E+03	3.03E+03	1.34E+04	6.49E+03	5.34E+03	
4.11E+05	3.52E+05	9.17E+05	5.17E+03	1.96E+03	3.13E+04	9.21E+04	3.25E+04	
6.12E+04	1.46E+05	2.00E+05	2.23E+04	1.17E+04	5.03E+04	7.64E+04	3.06E+04	
1.49E+00	1.44E+00	9.56E+00	1.38E+03	7.35E+02	5.49E+03	2.44E+01	6.37E+00	
5.47E+02	3.99E+02	3.99E+03	2.26E+03	1.18E+03	5.89E+03	8.36E+01	3.41E+01	
3.41E+04	5.56E+04	5.89E+04	8.90E+03	8.62E+03	3.10E+04	1.37E+03	9.85E+02	
2.03E+00	5.82E+00	1.18E+01	2.45E+04	2.27E+04	5.60E+04	2.44E+01	8.13E+00	
1.91E+05	2.22E+05	3.15E+05	4.99E+04	2.08E+04	9.54E+04	1.48E+05	3.12E+04	
2.81E+04	7.79E+04	8.00E+04	2.80E+04	3.14E+04	2.80E+04	4.94E+04	2.35E+04	
1.00E+00	1.00E+00	1.00E+00	3.18E+01	1.65E+01	2.12E+02	6.80E+00	6.07E+00	
1.64E+01	1.12E+01	1.34E+02	4.97E+01	2.81E+01	2.22E+02	1.30E+01	1.17E+01	
9.79E+01	1.76E+02	2.57E+02	2.66E+03	3.04E+03	5.25E+03	4.34E+01	9.35E+01	
1.40E+03	1.16E+03	2.47E+03	3.68E+02	2.13E+02	1.37E+03	1.18E+01	1.52E+01	
2.14E+05	2.20E+05	3.69E+05	3.76E+04	6.70E+03	4.80E+04	3.87E+04	1.75E+04	
6.21E+03	2.38E+04	1.01E+04	6.61E+04	2.97E+04	1.51E+05	6.12E+04	2.03E+04	
7.28E+03	4.54E+04	1.61E+04	4.80E+03	1.13E+04	1.33E+04	6.87E+04	4.22E+04	
3.95E+04	1.77E+05	1.10E+05	4.72E+03	2.27E+04	2.44E+04	6.51E+04	3.22E+04	
2.31E+04	6.58E+04	7.75E+04	3.28E+04	1.96E+04	1.40E+05	6.16E+04	2.94E+04	
2.53E+04	6.35E+04	4.91E+04	7.38E+02	1.81E+03	8.04E+03	1.56E+04	1.79E+04	
1.73E+05	1.83E+05	4.88E+05	2.87E+04	3.79E+04	1.36E+05	4.70E+04	2.60E+04	
1.41E+04	7.79E+04	4.72E+04	2.22E+04	2.15E+04	6.51E+04	3.25E+04	2.68E+04	

5.44E+00	1.22E+00	1.00E+00	5.82E+03	1.06E+04	2.91E+04	2.32E+03	1.82E+03
2.09E+03	1.77E+03	7.42E+03	1.03E+04	1.51E+04	4.36E+04	7.56E+01	7.85E+01
1.21E+04	2.32E+04	2.57E+04	2.53E+03	1.63E+03	1.31E+04	1.45E+04	1.31E+04
1.00E+00	1.52E+00	2.05E+01	1.21E+04	2.16E+04	4.39E+04	3.11E+03	2.99E+03
1.35E+05	2.44E+05	3.38E+05	7.57E+03	5.34E+03	3.47E+04	3.87E+04	3.95E+04
4.76E+04	3.22E+05	6.97E+04	7.32E+03	2.37E+04	7.57E+04	2.45E+04	5.68E+04
2.08E+04	***2.15E+04***	***4.82E+04***	***1.06E+04***	***9.61E+03***	***3.22E+04***	***2.02E+04***	***1.22E+04***
5.56E+04	***7.32E+04***	***1.29E+05***	***1.68E+04***	***1.27E+04***	***4.91E+04***	***3.38E+04***	***1.46E+04***

ABL-PNTIA	***GUS-PNTIA***	***G6PD-PNTIA***	***ABL-MDAPCA***	***GUS-MDAPCA***	***G6PD - MDAPCA***	*ABL-PC-3*	*GUS-PC-3*	***G6PD-PC-3***
4.432969	5.419955748	5.21143725	**3.580925**	**3.569374**	**4.2857642**	4.763428	4.053078	4.972811
4.396199	4.372912003	4.96561726	**4.469822**	**4.676694**	**4.9842691**	2.984527	2.850646	2.500023
5.342423	4.536558443	5.78786359	**4.864511**	**4.416641**	**5.4430246**	5.113943	3.873902	5.548174
4.753583	4.271841607	5.15212689	**4.149219**	**4.227887**	**4.9937043**	4.217484	3.827369	5.040215
5.426511	5.559906625	5.85420034	**4.649335**	**3.899821**	**5.1348763**	5.260071	4.320146	5.761695
4.450249	4.84260924	5.05812001	**4.252853**	**4.369216**	**4.9617467**	4.804821	4.659916	5.159488
4.267172	4.173186268	4.85994577	**4.695482**	**4.403121**	**4.7375598**	4.713491	3.620136	4.756956
4.466868	4.152288344	4.63344704	**4.287802**	**4.271842**	**4.5183182**	4.778874	4.220108	4.831513
5.0086	4.294466226	5.21915977	**4.416641**	**3.697229**	**4.9291773**	4.670246	4.352183	5.441913
3.957128	4.176091259	3.97075982	**4.033424**	**3.838219**	**4.689249**	3.98945	4.255273	4.904573
5.416641	5.340444115	5.72344135	**4.369216**	**3.621176**	**4.8517923**	4.652246	4.390935	5.217684
2.796574	2.301029996	3.19957144	**3.700704**	**3.389166**	**4.3675222**	4.322219	4.181844	4.587737
2.873321	2.447158031	3.10960969	**4.307496**	**4.30963**	**3.9806693**	4.287802	3.820858	4.42005
3.981366	2.606381365	3.96287367	**4.494155**	**4.540329**	**4.4843449**	4.501059	4.40824	4.592579
4.868056	4.071882007	5.03006699	**4.257679**	**4.09691**	**4.8075747**	1.699838	1.542825	2.26043
2.037426	1.075546961	2.23048136	**0.943495**	**1.369216**	**1.6383556**	0.037426	-0.02826	0.940995
3.921166	2.614897216	4.05078857	**4.307496**	**4.117271**	**4.6568852**	1.501059	1.609594	2.05448
1.988559	1.075546961	2.35614264	**1.544068**	**1.344392**	**1.8568344**	4.089905	3.631444	4.092865
1.981366	2.096910013	2.06890345	**3.238046**	**2.854306**	**3.8234093**	3.120574	3.012837	3.851754
4.574031	4.834420704	5.06270838	**1.728354**	**1.350248**	**2.2421196**	2.631444	2.517196	3.345095
4.823474	4.866877814	4.93431472	**3.697229**	**3.582063**	**4.1795187**	4.348305	4.227887	4.977683
1.954725	1.835690571	2.20591184	**3.535294**	**3.481443**	**4.1285477**	3.812245	3.727541	4.593562
5.613842	5.546542663	5.96233417	**3.713491**	**3.292256**	**4.4960508**	4.96426	4.511883	5.756595
4.786751	5.164352856	5.30171204	**4.348305**	**4.068186**	**4.7017736**	4.883093	4.485721	5.077894
0.173186	0.158362492	0.98055928	**3.139879**	**2.866287**	**3.7398372**	1.38739	0.804139	1.868197
2.737987	2.600972896	3.6006937	**3.354108**	**3.071882**	**3.7698588**	1.922206	1.532754	2.382874
4.532754	4.745074792	4.7701049	**3.94939**	**3.935507**	**4.491375**	3.136721	2.993436	3.504751
0.307496	0.764922985	1.07268284	**4.389166**	**4.356026**	**4.748137**	1.38739	0.910091	1.800727
5.281033	5.346352974	5.49895333	**4.698101**	**4.318063**	**4.9795828**	5.170262	4.494155	5.654916
4.448706	4.891537458	4.90287306	**4.447158**	**4.49693**	**4.4474683**	4.693727	4.371068	5.369835
0	0	0	**1.502427**	**1.217484**	**2.3271217**	0.832509	0.783189	1.891013
1.214844	1.049218023	2.12863897	**1.696356**	**1.448706**	**2.34674**	1.113943	1.068186	2.104553
1.990783	2.245512668	2.40945412	**3.424882**	**3.482874**	**3.7197714**	1.63749	1.970812	2.231508
3.146128	3.064457989	3.39283302	**2.565848**	**2.32838**	**3.13767**	1.071882	1.181844	1.878011

5.330414	5.342422681	5.5665169	4.575188	3.826075	4.6815062	4.587711	4.243038	5.225566
3.793092	4.376576957	4.00461341	4.820201	4.472756	5.1786385	4.786751	4.307496	5.555235
3.862131	4.657055853	4.20620917	3.681241	4.053078	4.1249905	4.836957	4.625312	5.074539
4.596597	5.247973266	5.04181051	3.673942	4.356026	4.3872995	4.813581	4.507856	4.942882
4.363612	4.818225894	4.88941572	4.515874	4.292256	5.1456642	4.789581	4.468347	5.417788
4.403121	4.802773725	4.69071388	2.868056	3.257679	3.9051123	4.193125	4.252853	4.860029
5.238046	5.26245109	5.68849706	4.457882	4.578639	5.1336551	4.672098	4.414973	5.137189
4.149219	4.891537458	4.67433833	4.346353	4.332438	4.813251	4.511883	4.428135	5.053399
0.735599	0.086359831	0	3.764923	4.025306	4.46417	3.365488	3.260071	3.810311
3.320146	3.247973266	3.87047123	4.012837	4.178977	4.6395273	1.878522	1.89487	2.552249
4.082785	4.365487985	4.41004945	3.403121	3.212188	4.1168251	4.161368	4.117271	4.911182
0	0.181843588	1.31190467	4.082785	4.334454	4.6424051	3.49276	3.475671	3.856794
5.130334	5.387389826	5.52867222	3.879096	3.727541	4.5409021	4.587711	4.596597	5.483416
4.677607	5.507855872	4.84309867	3.864511	4.374748	4.8789172	4.389166	4.754348	4.488174

AR-ncn PNTIA	AR-ncn MDAPCA	AR-ncn-PC-3	ESRa-ncnPNTIA	ESRa-ncn MDAPCA	ESRa-ncn PC-3	ESR2 ncn PNTIA	ESR2 ncn MDAPCA	ESR2 ncn PC-3
1.24	19.46	12.64	0.7	0.94	1.51	4.82	23.3188	8.44612
1.56	12.26	13.21	0.76	0.97	1.44	4.97	25.70077	3.51976
0.05	12.12	16.37	0.02	0.03	1.25	0.14	27.40354	5.56257
0.06	8.96	10.30	0.03	0.98	2.64	0.15	30.04784	12.36811
0.04	9.95	12.68	0.01	0.02	1.65	0.09	28.69739	6.33103
0.02	8.03	9.42	0.04	1.57	2.16	0.14	13.57854	11.59218
0.74	11.85	8.41	0.94	0.45	1	3.15	12.4204	5.61723
0.91	9.57	15.35	0.85	0.43	1.38	3.44	7.11229	8.17015
0.06	15.02	16.46	0.02	0.02	2.04	0.17	18.88656	9.38641
0.00	6.05	10.55	0	1.47	3.86	0.12	16.3791	24.63279
0.02	12.97	12.75	0	0.02	1.81	0.13	9.99015	11.33312
0.00	16.51	10.64	0.3	1.52	1.9	0.00	13.16619	10.80388

0.00	3.65	6.53	0.6	0.41	0.6	1.02	14.29058	6.61392
0.54	4.95	7.84	1.14	0.34	1.01	0.54	26.00272	9.23107
0.03	7.17	8.36	0	0.06	2.88	0.08	45.54109	27.73654
0.00	0.00	0.00	0	0	8.43	0.00	92.23482	0
0.34	5.13	6.70	0.02	0.03	0	0.09	11.65644	33.60938
0.00	14.44	3.49	0	0	1.44	0.00	13.63009	7.16211
0.00	5.04	21.55	0	0.12	0.41	1.62	39.27896	5.35338
0.02	1.50	36.70	0.07	0	0	0.12	29.11203	6.25246
0.01	4.82	12.47	0.02	0.08	0.61	0.14	21.80403	3.52153
0.00	5.39	36.10	0	0	0.55	1.62	23.28677	5.41625
0.03	19.63	37.98	0.01	0.04	0.4	0.15	16.95131	3.29867
0.11	28.39	26.57	0.05	0.56	0.81	0.43	22.25542	1.93424
0.00	6.74	0.00	0	0	0	0.00	28.95761	0
0.00	4.17	16.40	0	0.12	0	0.44	52.20274	5.33692
0.02	7.22	13.02	0.02	0.33	0.89	0.17	24.7352	5.40709
0.00	10.01	0.00	0	0.06	0	0.00	31.52673	0
0.01	16.07	23.88	0.01	0.07	0.37	0.11	18.88144	2.31044
0.26	12.62	24.24	0.09	0.23	0.41	0.46	14.08207	2.45299
0.00	0.00	0.00	0	0	12.11	0.00	61.24656	0
0.00	0.00	0.00	0	0	0	0.00	25.28149	0
0.00	5.58	25.24	0	0.89	0	0.00	65.79886	7.86377
0.00	3.53	0.00	0	0	0	0.11	19.95838	0
0.02	14.87	25.23	0	0.06	0.76	0.15	13.18779	5.05712
0.47	8.66	22.81	0.25	0.13	0.1	0.32	8.706	3.76148
0.25	6.46	14.67	0.1	0.02	1.04	1.53	14.85831	3.49578
0.24	11.54	12.86	0.14	0.06	0.79	1.70	15.05144	7.14546
0.07	4.55	19.66	0.02	0.01	0.59	0.15	13.12238	4.50114
0.02	14.64	18.76	0.01	0	1.37	0.04	14.76198	8.12869
0.03	6.96	23.27	0.01	0.01	1.06	0.06	9.32315	2.59944
0.18	3.33	17.45	0.08	0.01	0.77	0.43	10.60641	3.68905
0.00	8.33	37.77	0	0.02	0	0.00	21.58103	3.35555

0.00	**7.35**	**94.13**	**0**	**0**	**0**	**0.13**	14.43578	2.48581
0.00	**6.15**	**19.24**	**0**	**0**	**0.65**	**0.08**	15.16477	6.71554
0.00	**11.92**	**31.39**	**0**	**0**	**0.16**	**0.00**	18.41154	3.41438
0.05	**11.63**	**35.13**	**0.01**	**0.02**	**0.32**	**0.10**	10.39552	3.61406
0.18	**12.33**	**14.10**	**0.07**	**0.04**	**1.22**	**0.68**	14.19437	3.42224
0.02	***8.18***	***14.38***	***0.01***	***0.04***	***0.78***	***0.14***	***18.65***	***5.35***
0.16	***9.12***	***17.55***	***0.13***	***0.25***	***1.30***	***0.62***	***23.32***	***6.51***

Patient Information Sheet

1. **Study Title**

   Indicators of Prostate disease.

2. **Invitation Paragraph**

   You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

   For further advice you may contact **Patient advice and liaison services (PALS)** and **Independent Complaints Advocacy Services (ICAS)** through your local hospital or by phoning NHS direct line: 08454647 or by visiting their website: http://www.pals.nhs.uk/ . Contact details for ICAS: telephone 08454561082, e-mail: dhmail@dh.gsi.gov.uk; fax : 02072105454 or write : Veronica Fraser, Head of Complaints and Public Enquires & Customer Service Centre, Department of Health, Richmond House, 79 Whitehall, London, SW1A 2NS. You can seek further advice from INVOLVE by visiting their website: http://www.invo.org.uk

3. **What is the purpose of the study?**

   The natural course of prostate disease is unpredictable. Some people have prostate problems but without any signs and symptoms. Others show symptoms. However, prostate diseases can progress from one stage to the other without noticeable symptoms. There are little or no factors to discriminate between 'good' and 'bad' prostate disease. This study examines the expression of several types of disease markers (indicators) which are involved in prostate disease development and progression. Knowledge of these indicators will help improve diagnosis and treatment of prostate diseases. Those who have raised PSA and or enlarged prostate, which are indicators of prostate problem are invited to participate in this study.

4. **Why have I been chosen?**

   As you are aware, your clinician has informed you that a biopsy is going to be taken from your prostate as you have been shown to have a raised PSA and / or an enlarged prostate. If you consent to taking part, urine and blood samples will be collected before your biopsy. Once your biopsy has been examined, any remaining tissue will be allocated to the study.

5. Do I have to take part?

It is up to you to decide whether or not you wish to take part. If you decide to take part, you are still free to withdraw at any time without any reason. This will not affect the standard of care you receive.

6. What happens to me if I take part?

If you decide to take part, you will be asked to sign a consent form for this study. Before your biopsy is taken, you will be asked to give a urine sample and a blood sample will also be collected by the hospital phlebotomist. When the biopsy has been looked at for your diagnosis, part of the remaining tissue will be allocated for this study.

7. What do I have to do?

We ask you to carefully read this information and take your time in deciding if you want to be part of this study. If you agree to take part, you'll be asked to sign a consent form and give a urine sample and a blood sample. When the routine biopsy is taken the normal procedure is performed and part of any remaining tissue is allocated for this study.

8. What is the drug or procedure that is being tested?

No drug or procedure is being tested.

9. What are side effects of taking part?

There are no risks associated with collection of urine and blood samples. The biopsy has no additional side effects apart from those normally associated with the clinical care operation.

10. **What are the possible disadvantages and risks of taking part?**

There are no possible disadvantages or risks associated with taking part in this study.

11. **What are the possible benefits of taking part?**

There is no direct benefit to you but this study may help improve diagnosis and future treatment of prostate disease. However, results of this study will be made available to you through your Clinician in the Bedford Hospital.

12. **What happens when the research study stops?**

All tissue allocated for this study will be destroyed and the results will form the bases of a scientific thesis and may generate scientific publications.

13. **What if something goes wrong?**

If you are harmed by taking part in this research, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspects of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism may be available to you.

14. **Will my taking part in this study be kept confidential?**

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised. All samples will be analysed anonymously.

15. **Who is organising and funding the research?**

The research is being organised and funded by Cranfield University.

16. **Who have reviewed the study?**

Cranfield health local ethics committee, Bedford Hospital NHS trust research and development unit and Hertfordshire research ethics committee

17. **Contact for further information:**

Dr. M Wasil
Department of Research and Development
Bedford Hospital, Kempston Road,
Bedford, MK42 9DJ.
Tel: 01234 355122 Ext. 5855
Mohammad.Wasil@bedfordhospital.nhs.uk

Outline of methodology and Subject materials.

STUDY PROTOCOL: Hormonal modulation of putative tumour markers in Prostate cancer and microarray based sensor for prostate cancer diagnosis.

Twelve different tumour markers are under investigation in this study. These are androgen receptor (AR), oestrogen receptors(ER-α, ER-β1 and ER-β2); minichromosome maintenance proteins (MCM 2 and MCM 5); p53 and Bcl-2, prostate specific kallikreins (PSA and hK2); E-cadherin and CD44.

Sample Collection: 100 patients will be involved. Patients who have endorsed the consent form will be asked to give blood and urine samples. 10ml of venous blood will be collected into a plain tube (without anticoagulant) from each patient by a nurse. The patient will be given a sterile universal container to void 20ml of urine. Both urine and blood samples will be collected in the Urologic clinic before prostate biopsy. The consultant Urologist (Dr H Sharma) will collect the prostate biopsies as part of normal patient clinical care. The biopsies are usually sent to Histopathology laboratory. Surplus tissue will be assigned to this study.30mg of each surplus tissue will be placed into a 2ml sterile eppendorf tube for RNA extraction. Power analysis for sample size was done using both NCSS/PASS 2000 software and Origins Lab 2007 software. Each sample will be assigned a serial number in a log book. And from each formalin fixed paraffin embedded block (prepared by the histopathology laboratory), 12 slides of 4μm thick section per slide will be cut and labelled at the histopathology laboratory. Tissue blocks that do not have enough tissue to be kept in the archive after sample collection will be excluded. The slides, urine, blood and tissue in eppendorf tubes will be transported to Cranfield University molecular biology laboratory for analysis. See the chart on page three.

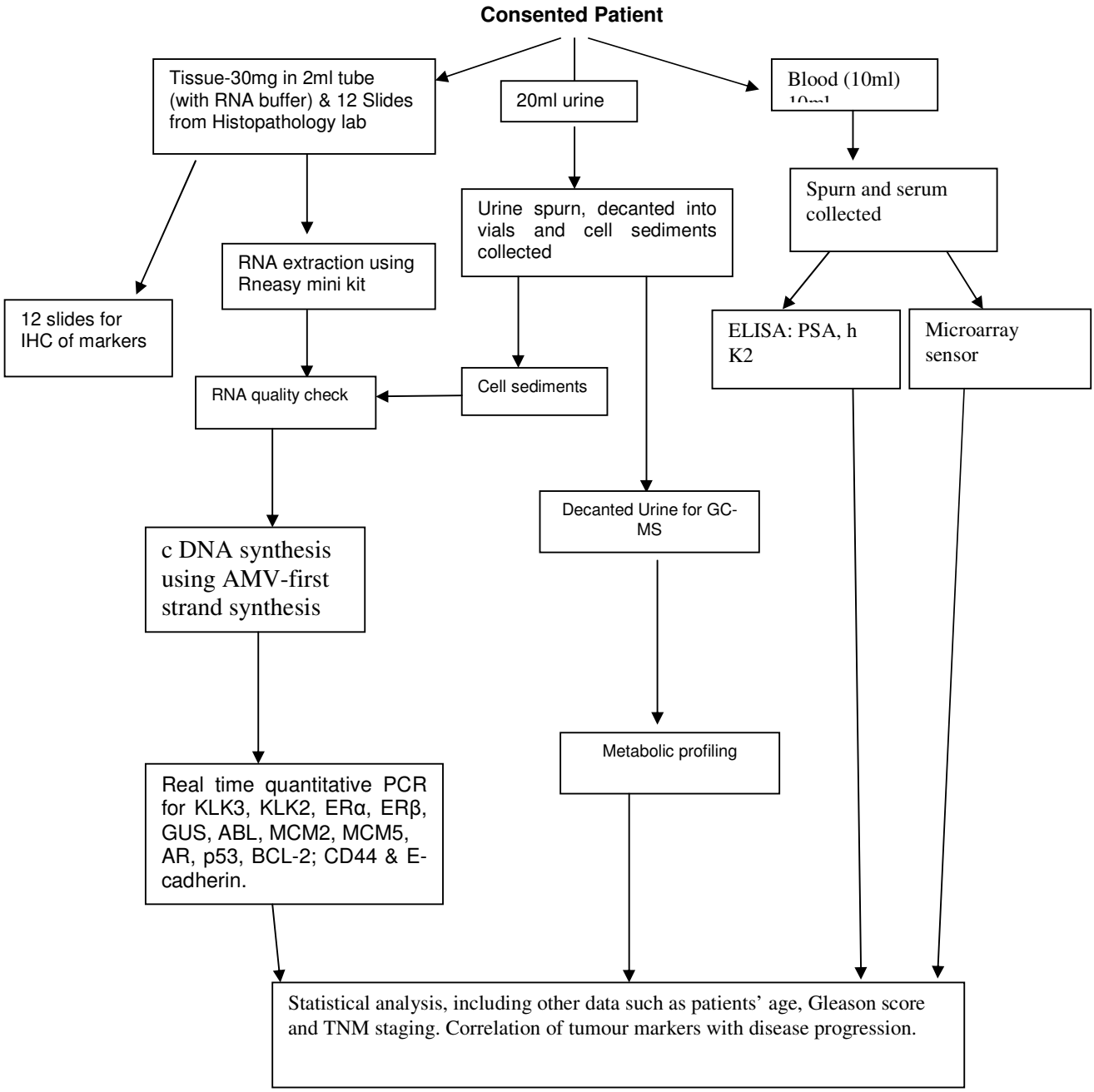
Analysis: 12 slides from each tissue block will be used for immunohistochemistry to assess protein expression for each marker. 30mg tissue in the 2ml eppendorf tube (containing RNA stabilizing buffer) will be used for total RNA isolation using RNeasy Mini kit (QIAGEN, UK).Isolated RNA will be used for cDNA synthesis using AMV first – strand cDNA synthesis kit (Invitrogen, UK). Real time quantitative Polymerase chain reaction (PCR) will be performed using ABI sequence detection system (7900HT model), using TaqMan primer and probe sets as well as SYBER green 1 kits to assess gene expression of selected markers. Urine samples will be spurn, decanted into 20ml headspace vial and capped, and exfoliated cell sediments collected for RNA extraction using same

technique for the 30mg tissue sample. The decanted urine will be analysed using headspace sampling GC-MS for metabolic volatiles. Clotted 10ml blood samples will be spurn and serum harvested. The serum will be tested for PSA and h K2 using both microarray based sensor and Enzyme linked immunosorbent assay (ELISA).

Results: Results from laboratory assays will be statistically analysed and used in writing up PhD thesis and will also be published in peer-reviewed journal.

Anonymity: No patient-identifiable information is required at any stage of this work. No genetic testing will be done. The log book for sample collection and results of laboratory analysis will be stored in a pass word protected computer.

Sample Collection and Analysis flow chart.



Centre: **Bedford Hospital NHS trust**
REC Reference Number: **08/H0311/90**
Patient identification Number for this study:.....

CONSENT FORM

Title of Project: *Hormonal modulation of putative markers in prostate cancer and microarray based sensor for prostate cancer diagnosis*.

Name of Researchers: *Emmanuel Nna & Yildiz Uludaq.*

(Please initial box)

1. I confirm that I have read and understood the information sheet dated 12/06/08, version 01/08 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from Cranfield University, from regulatory authorities or from NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study ☐

5. I agree to take part in the above study.

.....	.....	.....
Name of patient	Date	Signature
.....	.....	.....
Researcher	Date	Signature

Letter of invitation

Dear Participant,

We invite you to participate in a study on prostate disease. This study looks at some indicators of prostate disease development and progression. Our aim for the study is to examine the link between the development of prostate disease and the production of these indicators, also known as disease markers. Results from this study may help improve diagnosis, treatment and management of prostate disease in the future.

As you are aware, your clinician has informed you that a biopsy is going to be taken from your prostate as you have been shown to have a raised PSA and / or an enlarged prostate. The biopsy is part of your clinical care and will be used by the hospital laboratory to find out if you have prostate problem. The left over tissue will be used for this study. If you consent to taking part, urine and blood samples will be collected from you before your biopsy.

More details of this study and your participation are contained in an information sheet also given to you. Please take time to read the information carefully and discuss with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. There is a letter for your GP, informing him or her about your participation in this study. Please kindly give the letter to you GP. Summary of results from this study will be made available to you and your GP.

You are also given a consent form. If you decide to take part in this study, you will be asked to sign a consent form for this study. Before the Urologist takes your biopsy, you will be asked to give a urine sample and a blood sample, which will be collected by a nurse.

We thank you for taking time to read this.

Yours Sincerely,

Emmanuel Nna

Cranfield Health
Building 52,

Cranfield University
Bedfordshire,
MK43 0AL.
15-08-08.

Dear GP,

Hertfordshire REC Reference Number: 08/H0311/90

Study Title: Indicators of Prostate Disease

Your patient, MR…… …… is invited to participate in a study on '**indicators of prostate disease'**. The study examines disease markers that can be used to identify 'bad' prostate disease. As you are aware, your patient has raised serum PSA level and or enlarged prostate gland and has been scheduled to undergo prostate biopsy. If he consents to this study, blood and urine samples will be collected from him before clinical biopsy.

Find enclosed a copy of patient information sheet, patient invitation letter and consent form, which are also given to your patient.

Results of this study will be made available to you and your patient through the collaborating clinician at the Bedford hospital (Dr.H Sharma). The results of this research will also be used in writing a PhD thesis, which will be published on line in the Cranfield University website: www.cranfield.ac.uk

If you have any concern, please do not hesitate to contact me on phone: 01234 75 5561 or e-mail: e.o.nna.s05@cranfield.ac.uk .

Yours Sincerely,

Emmanuel Nna

Submitted Manuscripts