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PROSTATE CANCER PROGRESSION TO ANDROGEN INDEPENDENCE – DO CHANGES IN THE ANDROGEN RECEPTOR CAG REPEAT NUMBER PLAY A ROLE?

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MB., BCh., BAO, BMedSc (Hon), M.R.C.S.I.

A thesis submitted for the degree of Doctor in Medicine (M.D.),
University of Dublin, Trinity College, Ireland

2004-2006
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Signed: [Signature]

Rustom Pervez Manecksha, MB., BCh., BAO, BMedSc (Hon), MRCSI

Dated: 2/10/2007
Dedicated to Venita
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Finally, I acknowledge the Irish Cancer Society for supporting this project and for their continued support of ongoing prostate cancer research within the Prostate Cancer Research Group.
List of abbreviations

μl Microlitre
μm Micrometer (also known as micron)
ACTH Adrenocorticotropic hormone
AIPC Androgen-independent prostate cancer
AR Androgen receptor
bp Basepair
BPH Benign prostatic hyperplasia
CK Cytokeratin
Cₚ Threshold cycle
DBD DNA-binding domain
DES Diethyl-stilboestrol
DHT Dihydrotestosterone
dNTP Deoxyribonucleoside triphosphate
EDTA Ethylenediaminetetraacetic acid
FCS Foetal calf serum
FRET Förster or Fluorescence resonance energy transfer
g Acceleration of gravity
GSTP-1 Glutathione S-transferase pi-1
H&E Haematoxylin and eosin
HRE Hormone-response elements
HRPC Hormone-refractory prostate cancer
IGF-1 Insulin-like growth factor
IR Infrared
LBD Ligand-binding domain
LCM Laser capture microdissection
LH Luteinizing hormone
LHRH Luteinizing hormone-releasing hormone
mA Milliamp
MgCl₂ Magnesium chloride
ml Millilitre
mM Millimole
mRNA Mitochondrial ribonucleic acid
msec Millisecond
mV Millivolt
mW Milliwatt
ng Nanogram
NTC No template control
OD Optical density
PBS Phosphate-buffered saline
PCR Polymerase chain reaction
pmol picomole
PSA Prostate-specific antigen
RPMI Roswell Park Memorial Institute
RT-PCR  Real-time polymerase chain reaction

SBMA  Spinal bulbar muscular atrophy

TURP  Transurethral resection of prostate
Abstract

PROSTATE CANCER PROGRESSION TO ANDROGEN INDEPENDENCE – DO CHANGES IN THE ANDROGEN RECEPTOR CAG REPEAT NUMBER PLAY A ROLE?

Rustom Pervez Manecksha, MB., BCh., BAO, B.Med.Sc.(Hon), M.R.C.S.I.

Prostate cancer is the commonest non-cutaneous male malignancy and is the second commonest cause of cancer-related deaths in many Western countries. Approximately half of all men diagnosed with prostate cancer will have incurable advanced disease. Most of these men are treated with and initially respond to androgen-ablative therapy. However, the duration of response is variable, and most patients will eventually progress to androgen-independent disease. The mechanisms that drive prostate cancer progression are not clear, although the androgen receptor appears to play a key role. The androgen receptor gene contains polymorphic CAG and GGC trinucleotide repeats. CAG repeat length has been inversely correlated with prostate cancer risk and androgen receptor transcriptional activity. This study aimed to examine the role of the CAG repeat in prostate cancer progression to androgen-independence. This study firstly examined the CAG repeat number in a range of tumorogenic and non-tumorogenic prostate cell lines. In particular, the study compared the CAG repeat number of the androgen-dependent LNCaP cell line with that of the androgen-independent sub-line of LNCaP, the LNCaP-HOF cell line. There was a shortening of 3 CAG repeats in the LNCaP-HOF cell line compared with the parental LNCaP cell line. The second part of this study examined CAG repeat number in patient prostate tissue, with specific comparison of CAG repeat numbers between tissue that had not been subjected to androgen deprivation and tissue that had been exposed to androgen ablation therapy. Nineteen patients that had at least two prostate transurethral resections and had been treated with androgen ablation therapy in between the two operations were recruited. Using formalin-fixed, paraffin-embedded histological material, benign and malignant prostate epithelium were selectively microdissected using laser capture microdissection. DNA was extracted from the prostate tissue and the CAG repeat fragment was amplified by PCR. The PCR products were subjected to fragment analysis using a Genetic Analyzer and GeneScan software. Seven of the 19 patients (36.8%) had CAG shortening following androgen ablation therapy (p=0.008). There was no association between CAG shortening and age at diagnosis, pre-treatment Gleason score, pre-treatment PSA levels, PSA nadir, PSA levels at androgen-independence or the time interval between the two operations. Patients with high a post-androgen ablation therapy Gleason score (8-10) were more likely to have CAG shortening (p=0.016). In conclusion, CAG shortening occurred in a significant number of patients following androgen ablation therapy. CAG shortening may have conferred a growth advantage to these prostate cancers, driving progression to androgen-independence.
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CHAPTER 1

Introduction
Chapter 1

1.1 ANATOMICAL CONSIDERATIONS

The prostate is a small gland, normally about 20-30 grammes in size, located in front of the rectum, just below the bladder and it surrounds the urethra (Figure 1-1). McNeal described 3 anatomical zones: the peripheral zone, the transition zone and the central zone (Figure 1-2) (McNeal 1968). The peripheral zone makes up about 70-75% of the total glandular volume and the central and transition zones make up the remaining 25%.

The clinical significance of the zonal anatomy of the prostate is reflected in the development of both benign prostatic hyperplasia (BPH) and prostate cancer. Nodules of BPH usually originate within the transition zone whereas the majority of cancer (>70%) are believed to originate in the glands of the peripheral zone. About 25% of prostate cancer arises from the transition zone and less than 5% of prostate cancer arises from the central zone (McNeal 1988; McNeal, Redwine et al. 1988).
1.2 EPIDEMIOLOGY OF PROSTATE CANCER

1.2.1 Incidence

Prostate cancer is the commonest non-cutaneous male cancer in Europe, North America and some parts of Africa (Gronberg 2003). Prostate cancer is
the commonest non-cutaneous male malignancy in Ireland, with over 1,900 new cases diagnosed annually (Table 1-1) (Campo, Comber et al. 2004).

There has been a huge global rise in the incidence of prostate cancer over the past 20 years (Hsing, Tsao et al. 2000). Increases in the incidence are probably related to increased public awareness, improvement in the diagnostic tools and the widespread determination of prostate-specific antigen (PSA) (Bono 2004). The incidence of prostate cancer demonstrates wide geographical variability worldwide, with a higher incidence in certain parts of North America (e.g. Los Angeles) and lower incidence in China and other Asian countries. The incidence and prevalence of prostate cancer vary with race and ethnicity also, being highest amongst African-American men and lowest in Orientals and other Asians (Figure 1-3) (Farkas, Marcella et al. 2000), (Gilligan, Wang et al. 2004).

![Figure 1-3. The incidence of prostate cancer by racial background. Adapted from Farkas A et al. Ethn Dis 2000.](image-url)
1.2.2 Mortality

In the European Union, over 100,000 men are found to have prostate cancer and 35,000 die from the disease every year. It is the third commonest cause of male cancer deaths in Ireland, claiming about 726 lives per year, third only to lung cancer, which claims about 1430 lives per year and colorectal cancer, which claims about 842 lives per year (Table 1-1) (Campo, Comber et al. 2004).

<table>
<thead>
<tr>
<th>Site</th>
<th>Average annual cases</th>
<th>% of all cancers</th>
<th>Average annual deaths</th>
<th>% of all cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>1934</td>
<td>19.9</td>
<td>726</td>
<td>12.4</td>
</tr>
<tr>
<td>Lung</td>
<td>1541</td>
<td>15.9</td>
<td>1430</td>
<td>24.4</td>
</tr>
<tr>
<td>Colorectal</td>
<td>1488</td>
<td>15.3</td>
<td>842</td>
<td>14.4</td>
</tr>
<tr>
<td>Bladder</td>
<td>464</td>
<td>4.8</td>
<td>156</td>
<td>2.7</td>
</tr>
<tr>
<td>Stomach</td>
<td>444</td>
<td>4.6</td>
<td>322</td>
<td>5.5</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>426</td>
<td>4.4</td>
<td>213</td>
<td>3.6</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>320</td>
<td>3.3</td>
<td>202</td>
<td>3.4</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>277</td>
<td>2.9</td>
<td>122</td>
<td>2.1</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>275</td>
<td>2.8</td>
<td>132</td>
<td>2.3</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>265</td>
<td>2.7</td>
<td>288</td>
<td>4.9</td>
</tr>
<tr>
<td>Pancreas</td>
<td>244</td>
<td>2.5</td>
<td>266</td>
<td>4.4</td>
</tr>
<tr>
<td>Melanoma of the skin</td>
<td>154</td>
<td>1.6</td>
<td>48</td>
<td>0.8</td>
</tr>
<tr>
<td>Brain</td>
<td>215</td>
<td>2.2</td>
<td>157</td>
<td>2.7</td>
</tr>
<tr>
<td>Testis</td>
<td>164</td>
<td>1.7</td>
<td>10</td>
<td>0.2</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>154</td>
<td>1.6</td>
<td>117</td>
<td>2.0</td>
</tr>
<tr>
<td>Larynx</td>
<td>153</td>
<td>1.6</td>
<td>65</td>
<td>1.0</td>
</tr>
<tr>
<td>Liver</td>
<td>70</td>
<td>0.7</td>
<td>128</td>
<td>2.2</td>
</tr>
<tr>
<td>Breast</td>
<td>64</td>
<td>0.7</td>
<td>8</td>
<td>0.1</td>
</tr>
<tr>
<td>Thyroid</td>
<td>35</td>
<td>0.4</td>
<td>8</td>
<td>0.1</td>
</tr>
<tr>
<td>All other sites</td>
<td>--</td>
<td>9.8</td>
<td>625</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Table 1-1. Average new cancer cases and average number of cancer deaths in male patients in Ireland. Data from All-Ireland Cancer Statistics 1998-2000. (Campo, Comber et al. 2004). Prostate cancer data are highlighted above.
1.2.3 Risk Factors

Prostate cancer is a heterogeneous disease in terms of its causes and progression. Furthermore, the incidence of prostate cancer varies widely between ethnic populations and countries (Farkas, Marcella et al. 2000). The reason for these differences is probably multifactorial. Genetic factors have been implicated, and since risk of prostate cancer also varies between populations that are ethnically similar but living in different locations, and since nutritional and hormonal evidence have been associated with changes in risk, environmental factors probably also have a contributory role (Greenlee, Murray et al. 2000).

1.2.4 Genetic factors and family history

The clustering of prostate cancer in families can be because of genetic susceptibility, exposure to common environmental factors, or chance alone since prevalence of this cancer is so high. 10-15% of patients with prostate cancer (regardless of ethnicity) have at least one relative who is also affected (Hayes, Liff et al. 1995; Whittemore, Wu et al. 1995) and first-degree relatives of patients with prostate cancer have a two-fold to three-fold increased risk for developing this disease (Carter, Bova et al. 1993).

In familial prostate cancer, the age of onset is usually younger than 55 years, and patients often have several first-degree relatives who have been affected by prostate cancer (Gronberg, Xu et al. 1997). Familial types of
prostate cancer account for about 40% of patients who present younger than 65 years and for up to 9% of those presenting at 85 years or older (Carter, Bova et al. 1993; Gronberg, Xu et al. 1997).

Genome-wide linkage analyses of familial prostate cancer have mapped susceptibility loci for prostate cancer to chromosomes X, 1, 8, 17 and 20 (Smith, Freije et al. 1996; Berthon, Valeri et al. 1998; Gibbs, Chakrabarti et al. 1999; Gibbs, Stanford et al. 1999; Berry, Schaid et al. 2000; Berry, Schroeder et al. 2000; Xu, Zheng et al. 2001). Hereditary prostate cancer 1 (HPC1), which is located at chromosomal region 1q24-25, was the first prostate cancer locus to be reported, and in one study, was linked with prostate cancer in 6% of families investigated (Xu, Zheng et al. 2001). Other putative prostate cancer susceptibility genes have also been identified, including BRCA1 (Langston, Stanford et al. 1996) and HPCX (Xu, Meyers et al. 1998).

1.2.5 Age

Prostate cancer is strongly related to age. Every decade of aging nearly doubles the incidence of prostate cancer – from 10 percent in men in their 50s to 70 percent in men in their 80s (Scott, Mutchnik et al. 1969), (Sheldon, Williams et al. 1980), (Sakr, Haas et al. 1993), (Majeed, Babb et al. 2000), (Pu, Chiang et al. 2004). Most prostate cancers are slow-growing and from epidemiological studies it is clear that more men die with prostate cancer than from the malignancy. However, current investigational methods do not allow
clinicians to differentiate with certainty between tumours that will progress and those that will remain quiescent. Because of increasing life span and an aging male population in Ireland, and indeed globally (figures 1-4 and 1-5), prostate cancer is undoubtedly a major public health problem, compounded by many controversies surrounding its management.

**Figure 1-4.** Percentage of global population aged 65 and over in 2000. *Adapted from Kinsella, K et al. (Kinsella and Velkoff 2001).*
1.2.6 Diet

Diet has been shown to play a role in the pathogenesis of prostate cancer and is suspected to be at least part of the explanation for the variation in prostate cancer incidence between Western men and Asian men. Major East-West differences in animal fat and soy consumption are known to exist (Clinton and Giovannucci 1998), (Moyad 1999), but a clear linkage relating diet to carcinogenic change in prostatic tissue has not yet been established. A recent study compared dietary influences on prostate tissues of Japanese men who spent their life in Japan versus other Japanese men who spent their life in the U.S. The Western diet, relatively rich in animal fat and poor in soy, was found to exert cancer-causing influences that could be traced directly into prostate tissues (Marks, Kajima et al. 2004).
Other dietary risk factors with a positive association with prostate cancer include alpha-linolenic acid, red meat (Kolonel 2001), dairy food (Giovannucci, Rimm et al. 1998), height, abdominal obesity (Severson, Grove et al. 1988), elevated intraprostatic androgens (Gann, Hennekens et al. 1995) and elevated insulin-like growth factor (IGF-1) (Pollak 2001). Selenium (Clark, Dalkin et al. 1998), (Klein, Thompson et al. 2001), lycopene (tomato foods) (Gann, Hennekens et al. 1995), Vitamin E supplements (Heinonen, Albanes et al. 1998) and legumes (including soya) (Parnes, House et al. 2004), on the other hand, are inversely associated with the risk of prostate cancer.

1.3 STAGES & TREATMENT OPTIONS

1.3.1 Organ-confined disease

Organ-confined prostate cancer is treated either by radical prostatectomy or radiation therapy, with curative intent. However, by the time many patients are diagnosed, the disease has already spread beyond the prostate gland, making the disease incurable. In Europe approximately 50% of men presenting to the urologist with prostate cancer will have metastatic disease (Schroder 1993) although with the increasing use of prostate specific antigen (PSA) and increasing patient awareness, more patients are now being diagnosed with early prostate cancer. Furthermore, about one-third of patients that are
assumed to have gland-confined disease experience relapse of disease despite radical prostatectomy or radiation therapy (Gittes 1991; Khan, Han et al. 2003).

<table>
<thead>
<tr>
<th>Prostate – TNM staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
</tr>
<tr>
<td>Not palpable or visible</td>
</tr>
<tr>
<td>T1a ≤ 5%</td>
</tr>
<tr>
<td>T1b &gt; 5%</td>
</tr>
<tr>
<td>T1c Needle biopsy</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>Confined within prostate</td>
</tr>
<tr>
<td>T2a One lobe</td>
</tr>
<tr>
<td>T2b Both lobes</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>Through prostatic capsule</td>
</tr>
<tr>
<td>T3a Extracapsular</td>
</tr>
<tr>
<td>T3b Seminal vesicle(s)</td>
</tr>
<tr>
<td>T4</td>
</tr>
<tr>
<td>Fixed or invades adjacent structures, bladder neck, external sphincter, rectum, levator muscles, pelvic wall</td>
</tr>
<tr>
<td>N1</td>
</tr>
<tr>
<td>Regional lymph node(s)</td>
</tr>
<tr>
<td>M1a</td>
</tr>
<tr>
<td>Non-regional lymph node(s)</td>
</tr>
<tr>
<td>M1b</td>
</tr>
<tr>
<td>Bone(s)</td>
</tr>
<tr>
<td>M1c</td>
</tr>
<tr>
<td>Other sites</td>
</tr>
</tbody>
</table>

Table 1-2. Histological TNM staging of prostate cancer.

1.3.2 Advanced prostate cancer

Androgens, which exert their effects via the androgen receptor (AR), are essential for preserving the normal function and structure of the prostate. As is the case with normal prostate development, the growth of prostatic neoplasms is generally dependent on androgens, especially on 5α-dihydrotestosterone (DHT). Men castrated when young (eunuchs) or men with inherited deficiency of 5α-reductase (type 2) do not develop prostate cancer (Wu and Gu 1991). Since Huggins & Hodges described their observations in 1941 (Huggins and Hodges 1941), androgen withdrawal, either by chemical or surgical castration, remains the treatment of choice for advanced prostate cancer (see Figure 1-6). Initially,
almost all patients respond to androgen ablation (Palmberg, Koivisto et al. 1999). Studies suggest initial response rates of between 72-85% (Bolla, Gonzalez et al. 1997; Furuya, Akakura et al. 1999). The clinical response to this treatment is unfortunately transient, and eventually, the tumour reaches a rapidly proliferating, hormone-independent state, which leads to clinical progression of the disease. For patients with advanced prostate cancer being treated with androgen ablation, the median progression-free survival is 18-20 months and the median overall survival is 24-36 months (Robinson, Smith et al. 1995).

Prostate cancer that recurs after androgen ablation therapy has been termed hormone-refractory or androgen-independent prostate cancer (AIPC). This term is appropriate because only a small fraction of these tumours respond to secondary hormonal therapies, and the responses are generally modest and of short duration. The biggest challenge remains the search for an effective treatment option for such hormone-refractory disease.
1.4 DEVELOPMENT AND PROGRESSION OF PROSTATE CANCER

The precise mechanisms of development and progression of prostate cancer are not well understood. In general, it is thought that malignant transformation and cancer progression are driven by an accumulation of genetic changes that affect the expression and function of critical genes (Elo and Visakorpi 2001). Genetic aberrations affect genes that are commonly
categorised as oncogenes, tumour suppressor genes and mutator genes. In a subset of cancers, some of these changes may be inherited. The majority of alterations are, however, acquired as somatic alterations (Kallioniemi and Visakorpi 1996). The most common chromosomal aberrations in prostate cancer include deletions at chromosome regions 6q, 8p, 9p, 10q, 13q and 18q and gains at 7p, 7q, 8q and Xq (Joos, Bergerheim et al. 1995; Visakorpi, Kallioniemi et al. 1995; Nupponen, Kakkola et al. 1998). Gain of the whole long arm of chromosome 8 is the most common aberration in hormone-refractory prostate cancer (Nupponen, Kakkola et al. 1998) and it is associated with an aggressive phenotype of the disease (Alers, Rochat et al. 2000).

Possibly the most common genetic event in prostate cancer is hypermethylation of the glutathione S-transferase (GSTP1) gene promoter (Lee, Morton et al. 1994). More than 90% of prostate carcinomas show extensive hypermethylation of the 5'-CpG island of the gene. In addition, 70% of high-grade PIN lesions contain hypermethylation, whereas it is very rare in normal prostate tissue (Lee, Isaacs et al. 1997; Brooks, Weinstein et al. 1998).

1.5 ANDROGEN-INDEPENDENT PROSTATE CANCER

Almost all men with prostate cancer who are deprived of androgens, either by medical or surgical (bilateral orchidectomy) approaches, ultimately progress to an androgen-independent phase where the initial androgen deprivation regimen no longer controls the tumour (Oh and Kantoff 1998). Androgen-independent prostate cancer (AIPC) is the term used to describe
failure of initial androgen deprivation, and AIPC progresses and invariably leads to death. At present there is no effective therapy for AIPC, although a minority of men who have progressive disease after initial androgen deprivation respond to additional hormonal treatments. These responses, however, are modest and short-lived. Prostate cancer that no longer responds to any hormonal treatment is referred to as hormone-refractory prostate cancer (HRPC).

There are several pathways by which AIPC can develop (Feldman and Feldman 2001). Knowledge of these pathways provide insights into the mechanism of androgen action and schemes by which cancer cells subvert normal growth control and escape attempts to treat the cancer. Recent investigations support the theory that androgen ablation therapy provides selective pressure to target the androgen signalling pathway (Taplin, Bubley et al. 1995; Taplin, Bubley et al. 1999). The potential mechanisms by which AIPC can develop have been categorized into five pathways (Feldman and Feldman 2001) (Table 1-4).

One possible mechanism by which prostate cancer becomes refractory to androgen ablation is by increasing its sensitivity to very low levels of androgens. This mechanism is referred to as AR hypersensitivity (Table 1-3). Therefore, these prostate cancers are not considered androgen-independent; rather, they have a lower threshold for androgens. This may be achieved by
increasing the expression of the AR itself. About 30% of tumours that become androgen-independent after androgen ablation therapy have amplified the AR gene, resulting in increased AR expression, whereas none of the primary tumours from the same patients before androgen ablation had an AR gene amplification (Visakorpi, Hyytinen et al. 1995; Koivisto, Kononen et al. 1997). Tumour cells could become hypersensitive to low concentrations of circulating androgen, as suggested by animal model studies in which the concentration of DHT required for growth stimulation in AIPC cells (LNCaP-C4-2 and CWR-R1 cell lines) was four orders of magnitude lower than that required for androgen-dependent LNCaP cells (Gregory, Johnson et al. 2001).

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Ligand dependence</th>
<th>Mechanism</th>
</tr>
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<tbody>
<tr>
<td>AR Hypersensitivity</td>
<td>Androgen dependent</td>
<td>AR amplification Increase in AR sensitivity to androgen Increased androgen</td>
</tr>
<tr>
<td>AR Promiscuity</td>
<td>Pseudo-androgens</td>
<td>Widened AR specificity Ilicit stimulation by non-androgens</td>
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<td></td>
<td>Androgen antagonists</td>
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<td></td>
<td>Corticosteroids</td>
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<tr>
<td></td>
<td>Coregulator mutations</td>
<td>'Flutamide withdrawal' (i.e. antagonists acting as agonists)</td>
</tr>
<tr>
<td>Outlaw AR</td>
<td>Androgen independent</td>
<td>Mutant PTEN gene</td>
</tr>
<tr>
<td></td>
<td>Ligand independent</td>
<td>Amplified HER-2/neu gene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activated mitogen-activated protein kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutant coregulators</td>
</tr>
<tr>
<td>Bypass AR</td>
<td>Androgen independent</td>
<td>Parallel or alternative survival pathways e.g. overexpression of bcl-2 or activation of other oncogenes</td>
</tr>
<tr>
<td>Lurker cells</td>
<td>Androgen independent</td>
<td>A subpopulation of malignant AIPC cells exists even before initiation of androgen ablation therapy, lurking in the background, and continues to proliferate after androgen ablation.</td>
</tr>
</tbody>
</table>

Table 1-3. Five possible mechanisms of development of AIPC (adapted from Feldman BJ and Feldman D (Feldman and Feldman 2001).
There is some evidence to support the promiscuous pathway as a means of developing AIPC (Table 1-3). Whilst a proportion of these tumours, at least initially, have adapted to the low-androgen environment, others acquire mutations that allow them to escape the control of androgens on their normal growth. Studies by Taplin et al (Taplin, Bublely et al. 1995) and subsequently by Marcelli et al (Marcelli, Ittmann et al. 2000), suggest that there is an increased incidence of AR mutations in metastatic prostate cancer. It seems likely that the frequency of mutations in the AR is significantly increased in tumours after androgen ablation therapy, and that the acquisition of mutations in the AR is likely to be one mechanism by which AIPC develops (Feldman and Feldman 2001).

In a cell line model, Culig et al established an androgen-independent subline of the LNCaP cell line by growing the parental cell line in androgen-deprived medium over many passages until the variant cell line emerged (Culig, Hoffman et al. 1999). This cell line, named LNCaP-abl (HOF), had a 4-fold increase in AR protein expression and 30-fold higher basal AR transcriptional activity as compared with its parental cell line (Culig, Hoffman et al. 1999). In the LNCaP cell line, bicalutamide functions as an AR antagonist, but in the LNCaP-abl (HOF) cell line, bicalutamide functions as an agonist, indicating that an AR mutation had taken place as a result of androgen deprivation.
‘Outlaw’ receptors refer to steroid hormone receptors that are activated by ligand-independent mechanisms (Table 1-3). Craft et al suggests that the AR can be turned into an outlaw receptor by the over-expression of the HER-2/neu receptor tyrosine kinase, which indirectly leads to phosphorylation and activation of the AR in the absence of androgens (Craft, Shostak et al. 1999).

1.6 MANAGEMENT OF ANDROGEN-INDEPENDENT DISEASE

1.6.1 Maximal androgen blockade

Cases of hormone resistance often require the employment of second-line therapy. Although the prognosis in these circumstances is generally still poor, there are a number of approaches that may provide benefit.

For patients who have been managed initially by monotherapy (either by orchidectomy or luteinizing hormone-releasing hormone analogues), the addition of an anti-androgen may offer some benefit, although the effects, if any, are short-lived. Another hormonal treatment strategy is to introduce an oestrogen such as diethyl-stilboestrol (DES). Evidence for the efficacy of DES in this context, however, is virtually anecdotal.
1.6.2 Cytotoxic agents

Two randomized trials have been instrumental in shifting the paradigm relating to activity of chemotherapy in HRPC. Tannock and colleagues compared prednisone alone to the combination of mitoxantrone and prednisone in 161 symptomatic men with HRPC (Tannock, Osoba et al. 1996). The palliative response rate and duration of palliation were significantly improved with mitoxantrone/prednisone compared with single-agent prednisone (Tannock, Osoba et al. 1996). Kantoff and colleagues and later, Berry and colleagues looked at survival as an end point in a study comparing mitoxantrone/hydrocortisone combination with single-agent hydrocortisone, but no appreciable survival benefit was seen in either study (Kantoff, Halabi et al. 1999; Berry, Dakhil et al. 2002).

Other agents that have been investigated include paclitaxel and docetaxel. These cytotoxic agents were used as monotherapy or in combination, but the results have been generally modest with high treatment-related toxicity and complications including neutropenia and deep venous thrombosis (Roth, Yeap et al. 1993; Hudes, Nathan et al. 1997; Haas, Roth et al. 2001; Sinibaldi, Carducci et al. 2002; Gravis, Bladou et al. 2003).
For bone-targeted therapies, surgery or palliative radiation therapy can be considered for localized lesions and bisphosphonates may be considered for reducing skeletal-related events (Saad, Gleason et al. 2002).

1.7 THE ANDROGEN RECEPTOR (AR) GENE

The androgen receptor gene, located on at Xq11.2-q12, (Lubahn, Joseph et al. 1988; Brown, Goss et al. 1989) is a member of the steroid hormone receptor family of genes which includes the oestrogen receptors alpha and beta, progesterone receptor, vitamin D receptor, retinoic acid receptors and thyroid hormone receptors (Evans 1988) (Figure 1-7). Like the other members of this family of transcription factors, there are functionally distinct regions of the protein. The first exon codes for the N-terminal domain that is the transcriptional regulatory region of the protein. Exons 2 and 3 code for the central DNA-binding domain. Exons 4 to 8 code for the C-terminal ligand-binding domain (Gelmann 2002) (figure 1-8).
Figure 1-7. Location of the Androgen Receptor gene on the long arm of the X chromosome at Xq11.2-q12.

Figure 1-8. The exonic organization of the AR gene with the 3 functionally distinct regions shown above. The hinge region connects the DNA-binding domain (DBD) to the ligand-binding domain (LBD).

The central DNA-binding domain (DBD) is responsible for targeting the receptors to their hormone response elements (HRE). The ligand-binding domain (LBD) participates in several activities including hormone binding.
dimerization, formation of the heat-shock protein complex and transcriptional activation and repression. The binding of hormones induces conformational changes that seem to control these properties and influence gene expression.

Androgen, acting through the AR, helps to maintain the normal function and structure of the prostate (Dorkin and Neal 1997). When androgens bind to the AR, it is activated, dimerizes, and localizes in the nucleus, where it attaches to specific sequences in the regulatory regions of target genes. The AR functions include transcriptional activation of other genes and the transport of the androgen hormone (Ross, Pike et al. 1998).

The production of prostate-specific antigen (PSA) in most tumours is initially androgen-regulated and declines sharply following androgen ablation. However, when the tumour progresses to its invariable androgen-independent state, serum PSA levels rise and may eventually surpass the pre-treatment value (Gleave, Goldenberg et al. 1996). The androgen-independent regulation of PSA gene expression is a transcription-related event as demonstrated in the LNCaP tumour model of human prostate cancer by Sato and colleagues (Sato, Gleave et al. 1996). The fact that PSA production ultimately increases in an androgen-deprived environment suggests that other factors not directly related to androgens, but possibly acting through the AR, become paramount, leading to androgen-independent induction of PSA mRNA.
The AR gene is known to be polymorphic, having a highly variable trinucleotide microsatellite of CAG-repeats in exon 1 (Figure 1-9). Another trinucleotide repeat that resides within exon 1 is the GGC repeat. These polymorphisms are described in more detail later in this chapter.

![Diagram of the AR gene with the CAG repeat segment within exon 1.](image)

**Figure 1-9.** Diagrammatic representation of the AR gene with the CAG repeat segment within exon 1.

### 1.8 ALTERATIONS OF THE AR GENE

Like the benign epithelium from which they arise, prostate cancer cells retain responsiveness to and dependence on androgens. It has been known for over sixty years that prostate cancer in most cases retains androgen responsiveness and will undergo regression in response to androgen deprivation (*Huggins and Hodges 1941; Huggins, Stevens et al. 1941*). Over 85% of men with metastatic prostate cancer will show some clinical response to androgen ablation. However, there is no way to predict which patients will not respond or
how long the responding patients will benefit from androgen control of their prostate cancer. Although the median duration of response to hormonal ablation is less than 3 years, response durations range from several months to many years. Androgen responsiveness in prostate cancer does not correlate with either the presence or the levels of androgen receptor in cancer tissues (Trachtenberg and Walsh 1982; Sadi, Walsh et al. 1991).

Typically, the AR gene is expressed in primary prostate cancer at levels akin to normal prostate. However, following androgen ablation therapy, a number of AR gene alterations may occur. These alterations lead to increased sensitivity of the receptor to low levels of circulating androgens and also to the receptor’s ability to recognize a broadened spectrum of ligands (e.g. progesterone, oestrogen, adrenal androgens and the anti-androgen hydroxyflutamide) (Veldscholte, Ris-Stalpers et al. 1990; Fenton, Shuster et al. 1997) as potent agonists for AR action.

Prostate cancer cells respond to androgen ablation therapy by amplifying AR gene copy number in approximately 25% - 30% of patients who experience relapse (Visakorpi, Hytinen et al. 1995). The presence of AR gene amplification may reflect an adaptation of the cancer cells to castrate levels of circulating androgens.
Mutations in the AR occur in a subset of recurrent prostate cancers, perhaps at a frequency as high as 40% to 50% (Taplin, Bubley et al. 1995; Bentel and Tilley 1996). Most of these mutations result in the acquisition of gain of function properties for the AR, usually manifested by reduced discrimination for ligand-dependent activation. There is evidence that the type of androgen ablation therapy used may provide selective pressure for specific mutations. In one study, mutations in the AR were found in 5 of 16 recurrent tumours following therapy with flutamide, allowing flutamide to act as an agonist of the AR (Taplin, Bubley et al. 1999).

1.9 CAG REPEATS

1.9.1 Overview

The AR gene contains two regions of repetitive DNA sequences, namely the CAG and GGC triplet repeats. The CAG triplet repeat is perhaps the most relevant as a potential biomarker for disease development. It begins at codon 58 and extends for an average of 21 ± 2 repeats (La Spada, Wilson et al. 1991). The number of CAG-repeats varies greatly in the population with normal numbers ranging from as low as 8 and up to 35 (average ≈ 21) (Caskey, Pizzuti et al. 1992; Edwards, Hammond et al. 1992). Also, the number of CAG repeats may vary somewhat with ethnicity and race (Sartor, Zheng et al. 1999). Like other genes with trinucleotide repeats, the length of the repeat is highly polymorphic because of slippage of DNA polymerase on the multiple CAG
nucleotides in the DNA template when copying DNA resulting in variability in the final number of CAG-repeats copied during DNA replication. The CAG repeat number has been associated with prostate cancer risk and is described in detail later in this chapter. A number of studies have examined the effect of CAG repeat number on AR transcriptional activity. Some of these have demonstrated reduced in vitro transcriptional activation of an androgen responsive reporter construct by an AR with a pathologically expanded CAG repeat sequence (Mhatre, Trifiro et al. 1993; Chamberlain, Driver et al. 1994; Jenster, de Ruiter et al. 1994; Kazemi-Esfarjani, Trifiro et al. 1995; Nakajima, Kimura et al. 1996). Two studies specifically examined the effect of CAG variation within the normal range on androgen receptor transcriptional activity (Kazemi-Esfarjani, Trifiro et al. 1995; Tut, Ghadessy et al. 1997). These studies demonstrated an inverse relation between CAG repeat number and AR transactivation.

The number of CAG repeats have been shown to correlate with a number of androgen-related clinical conditions; specifically, a high number of repeats appears to adversely influence fertility and spermatogenesis (Yoshida, Yano et al. 1999) and fewer repeats are associated with increased risk of male-pattern baldness (Saway and Shalita 1998) and benign prostatic hypertrophy (Giovannucci, Platz et al. 1999). Expansion of the CAG tract to over 40 repeats leads to spinal bulbar muscular atrophy (SBMA, also known as Kennedy disease), an adult onset neurodegenerative disease that also presents with low
virilization and spermatogenetic defects (*La Spada, Wilson et al. 1991; Wieacker, Knoke et al. 1998*).

**1.9.2 CAG repeat and prostate cancer risk**

There have been several case-control studies that have observed an increase in prostate cancer risk associated with short CAG alleles (*Irvine, Yu et al. 1995; Giovannucci, Stampfer et al. 1997; Ingles, Ross et al. 1997; Hsing, Deng et al. 2000; Hsing, Gao et al. 2000; Xue, Irvine et al. 2000*) although other studies did not find such an association (*Bratt, Borg et al. 1999; Edwards, Badzioch et al. 1999; Miller, Stanford et al. 2001*). Chamberlain and colleagues (*Chamberlain, Driver et al. 1994*) demonstrated using an *in vitro* system that the elimination of the CAG repeats in both human and rat AR resulted in a marked elevation of transcriptional activation activity. This functional difference might be the factor associated with increased prostate cancer risk, since reduced androgen sensitivity of the long AR variants may be protective to the prostate, whereas the increased responsiveness of the short variants might predispose the prostate to chronic androgen overstimulation and a higher risk of developing cancer (*Coetzee and Ross 1994; Irvine, Yu et al. 1995*).

Several observations suggest indirectly that variation in the AR polyglutamine length, by modulating androgen activity, influences prostate carcinogenesis. African-Americans, who have generally shorter CAG-repeat lengths in the AR (*Coetzee and Ross 1994*), have a higher incidence of and
mortality rate from prostate cancer. Irvine et al found that the mean number of CAG-repeats was smallest in African-Americans, intermediate in Caucasians, and largest in Asians, who respectively, have a high, intermediate and low incidence of prostate cancer (Irvine, Yu et al. 1995) (Figure 1-10).

Some investigators found that a shorter CAG repeat sequence was associated with earlier age at diagnosis (Bratt, Borg et al. 1999; Beilin, Harewood et al. 2001). Other investigators have found that polymorphisms in the AR gene were not only associated with prostate cancer risk but also were significant predictors of relapse and survival after androgen ablation (Suzuki, Akakura et al. 2002; Powell, Land et al. 2005). Both CAG and the GGC repeat lengths in the AR gene were associated with poor response to androgen ablation (Tilley, Buchanan et al. 1996).
A possible mechanism for prostate cancer to gain growth advantage in the presence of near castrate levels of testosterone would be shortening of tumour CAG repeat lengths. Understanding the changes in AR signalling in the development of AIPC is critical in our aim to improve overall survival of patients with advanced prostate cancer.

1.9.3 GGC repeat

Exon 1 of the AR also contains another polymorphic region of repetitive DNA sequence of GGC triplet repeats. The polyglycine tract is encoded by the GGC repeat and is localised in the NH₂-terminal transactivation domain of the AR protein (Edwards, Hammond et al. 1992; Sleddens, Oostra et al. 1993). The length of the GGC tract varies from about 10 to 30 repeats. The effect of the variation in the length of the GGC tract on AR activity is unclear. Some studies have shown that deletion of the GGC tract resulted in increased AR transcriptional activity and others have shown no alteration in AR transcriptional activity (Jenster, de Ruiter et al. 1994; Gao, Marcelli et al. 1996). Moreover, there have been varying results from epidemiological studies that examined the association between GGC repeat numbers and prostate cancer risk, with certain studies showing an increased risk of prostate cancer with shorter GGC repeats (Hakimi, Schoenberg et al. 1997; Stanford, Just et al. 1997; Platz, Giovannucci et al. 1998; Hsing, Gao et al. 2000) while other studies that found no association between GGC repeat number and prostate
cancer risk (Miller, Stanford et al. 2001; Chen, Lamharzi et al. 2002). The role of GGC repeats was not examined in this study.
CHAPTER 2

Hypothesis & Objectives
Chapter 2

2.1 Overview

To improve overall survival of patients with advanced prostate cancer, we need to determine:

(a) How to prolong the androgen-dependent state,
(b) The precise mechanisms by which androgen-independent prostate cancer develop, and
(c) Novel treatments for androgen-independent prostate cancer based on these molecular mechanisms.

Because prostate cancer is a heterogeneous disease, several mechanisms must account for its development and progression.

2.2 Hypothesis

Changes in CAG repeat number occur within the AR gene as a result of androgen ablative therapy, which could explain the development of androgen-independent prostate cancer in certain patients.

2.3 Objective 1

To characterise the CAG repeat number in a panel of prostate cell lines, and to compare the CAG repeat number of an androgen-dependent prostate cancer cell line with that of its androgen-independent sub-line.
2.3.1 **Significance of objective 1**

By comparing the CAG repeat number of an androgen-dependent prostate cancer cell line with its androgen-independent subline, this will establish if a change CAG repeat number plays a role in the development of androgen-independence.

This will enable the researcher to optimise DNA extraction, PCR and GeneScan techniques prior to commencing work on patients’ prostate tissue in objective 2.

2.4 **Objective 2**

To establish if changes in CAG repeat number occur in prostate cancer patients who initially respond to hormone treatment, but subsequently become hormone-refractory.

2.4.1 **Significance of objective 2**

The significance of the second objective will be to utilise patients’ prostate tissue to validate the theory that change in CAG repeat number plays a role in the development of androgen-independence.
This could provide an explanation for the androgen-dependent to androgen-independent (AI) transition in certain prostate cancer patients, as the mechanisms underlying this transition remains to be delineated. Determining the mechanisms by which disease progression occurs could potentially lead to the development of a predictive marker of relapse. These findings may also be useful in determining how to prolong the androgen-dependent state and to develop novel treatments for androgen-independent prostate cancer based on these molecular mechanisms.
CHAPTER 3

Materials and methods
Chapter 3

3.1 STUDY OF CAG REPEAT NUMBER IN PROSTATE CELL LINES

3.1.1 Source of cell lines

The ten prostate cell lines that were examined in this study are outlined in Table 3.1. A number of the ten prostate cell lines utilised in this study were obtained from commercial sources and others as gifts from collaborating laboratories. The LNCaP, DU-145, PC-3 and 22Rv-1 cell lines were obtained from ATCC-LGC Promochem (ATCC-LCG Promochem, Middlesex, UK). The PWR-1E, RWPE-1, PNT-2, HPr-1, HPr-1AR and LNCaP-HOF cell lines were gifts from Dr. William Watson’s prostate cancer research group at the Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland. The PWr-1E, RWPE-1 and PNT-2 cell lines are commercially available from LGC Promochem (ATCC-LCG Promochem, Middlesex, UK). The HPr-1 and HPr-1AR cell lines were obtained by the group from the originator of the cell lines (Dr. Chee-Keong Choo, Department of Pathology, University of Hong Kong, Pokfulam Road, Hong Kong, China) (Choo, Ling et al. 1999; Ling, Chan et al. 2001).
3.1.2 Characteristics of cell lines

The origin of each cell line is described in the subsequent paragraphs. Before a cell line can be accepted as having prostatic epithelial origin, some basic characteristics must be established. This is because other cell types are also present in prostate tissue (e.g. fibroblasts, smooth muscle and stromal cells). Epithelial origin is established by examining cytokeratin expression, namely cytokeratin 8 and 18 (Webber, Bello et al. 1996). Prostatic origin is demonstrated by examining growth response to androgens, induction of prostate-specific antigen (PSA) expression in response to androgen exposure and expression of androgen receptor (AR). The characteristics of each of the five prostate cancer cell lines and the five non-tumorogenic prostate cell lines are detailed in sections 3.1.3 and 3.1.4 and summarized in Table 3-1.

3.1.3 Prostate cancer cell lines

3.1.3.1 DU-145

The DU-145 cell line, established and characterized in 1978, is one of the earliest described prostate cancer cell lines (Stone, Mickey et al. 1978). The tumour was obtained in 1975 from a 69 year old Caucasian man with widespread metastatic carcinoma of the prostate (Stone, Mickey et al. 1978). It was derived from human prostate cancer brain metastases and is now commercially available from the American Type Culture Collection, ATCC-LGC Promochem, Middlesex, UK. It is of epithelial origin as confirmed by its cytokeratin expression, CK-8 and CK-18 (Mickey, Stone et al. 1980; Webber,
Chapter 3 Materials and methods

Bello et al. 1997). Its prostatic origin was demonstrated by its positive but weak nuclear immunostaining for the androgen receptor (AR) in 75% of DU-145 cells (Brolin, Skoog et al. 1992) and its tumorigenicity in nude mice (Stone, Mickey et al. 1978). It is, however, PSA negative (Mickey, Stone et al. 1980).

3.1.3.2 PC-3

Metastatic tumour tissue obtained from a lumbar vertebra was used to establish the PC-3 prostate cancer cell line (Kaighn, Narayan et al. 1979). The tumour sample was derived from a 62 year old Caucasian man and the pathological diagnosis for both the primary and the skeletal metastases was ‘poorly differentiated prostatic adenocarcinoma’ (Kaighn, Narayan et al. 1979). PC-3 cells did not express PSA and were shown to be hormone unresponsive (Kaighn, Narayan et al. 1979). Similar to the DU-145 cell line, the PC-3 cells are CK-8 and CK-18 positive (confirming their epithelial origin) and there were histological similarity between tumours grown in nude mice and the parent tumour (supporting its prostatic origin). PC-3 cells do not express the AR.

3.1.3.3 22Rv1

22Rv1 is a human prostate carcinoma epithelial cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft (Sramkoski, Pretlow et al. 1999). In nude mice, the line forms tumors with morphology similar to that of the xenografts, and like the parental CWR22 and
CWR22R xenografts, this cell line expresses PSA and AR (*Sramkoski, Pretlow et al. 1999*). Its growth was slightly stimulated by dihydroxytestosterone (DHT). When adult prostatic epithelial cells are placed in culture and grow in the absence of androgen yet retain the ability to respond to androgen, they are classified as being androgen responsive but not androgen dependent. The 22Rv1 cells are, therefore, classified as androgen responsive (*Sramkoski, Pretlow et al. 1999*).

### 3.1.3.4 LNCaP

LNCaP cells were derived in 1977, from fragments of a biopsy of a lymph node metastasis of a 50 year old Caucasian man with a moderately differentiated prostate cancer diagnosed one year earlier (*Horoszewicz, Leong et al. 1980*). The cells stain positive for CK-8 and CK-18, proving their epithelial origin. They also express PSA and AR and grow in response to androgen (*Berns, de Boer et al. 1986; Langeler, van Uffelen et al. 1993*). LNCaP cells form tumours in nude mice and grow faster in male mice compared with female mice, again confirming their androgen responsiveness (*Berns, de Boer et al. 1986*).

### 3.1.3.5 LNCaP-HOF

The LNCaP-HOF cell line is a subline of the LNCaP established by Klocker and colleagues (*Culig, Hoffman et al. 1999*). LNCaP cells were initially grown in androgen-depleted medium (10 percent charcoal-stripped...
FCS). An intermediate subline named LNCaP-abl was established after 41 passages. This intermediate subline underwent a total of 87 passages before it was grown as xenografts in castrated nude mice. It was noted that these LNCaP xenotransplanted tumours grew equally well in castrated mice as in intact mice, proving to be genuinely androgen-independent. This subline was named LNCaP-HOF (Culig, Hoffman et al. 1999; Nessler-Menardi, Jovota et al. 2000).

3.1.4 Non-tumorigenic prostate cell lines

3.1.4.1 PNT2

This cell line was established by immortalisation of normal adult prostatic epithelial cells by transfection with a plasmid containing SV-40 genome with a defective replication origin. The primary culture was obtained from a prostate of a 33 year old male at post mortem. It is now commercially available from CAMR Centre for Applied Microbiology & Research (ECACC, Salisbury, Wiltshire. PNT 2 cells possess a well differentiated morphology with the expression of cytokeratin 8, 18 and 19 with the latter being a feature of differentiated luminal cells of the glandular prostate. PNT2 has been shown to express PSA and grows in response to androgen (Lang, Sharrard et al. 2001) and the cells are non-tumorigenic in nude mice (Berthon, Dimitrov et al. 1995; Lebeau, Gerbault-Seureau et al. 1995; Berthon, Waller et al. 1997).
3.1.4.2 PWR-1E

Epithelial cells were isolated from the prostate of a Caucasian man undergoing a radical cystoprostatectomy and cells were immortalized by infection with the adenovirus 12-simian virus 40 hybrid virus (AD12-SV40) (Webber, Bello et al. 1996). The cells are positive for cytokeratin 8 and 18, confirming their epithelial origin and show positive immunostaining for PSA and AR. They also show a growth response to 5α-DHT and were non-tumorigenic in nude mice (Webber, Bello et al. 1996).

3.1.4.3 RWPE-1

This cell line was derived from cells isolated from a normal radical prostatectomy specimen and was transfected with HPV-18 DNA contained in liposomes (Weijerman, König et al. 1994; Bello, Webber et al. 1997). The origin of these cells was established as epithelial on the basis of their positive staining with cytokeratin 8 and 18 and their prostatic origin was confirmed on the basis of positive immunostaining for PSA and AR. Like the PWR-1E cell line, these cells grow in response to androgen and do not form tumours in nude mice.

3.1.4.4 HPr-1 and HPr-1AR

The HPr-1 and HPr-1AR cell lines (the latter being a subline of HPr-1) were established by Choo and Ling et al (Choo, Ling et al. 1999; Ling, Chan et al. 2001). The HPr-1 cell line was immortalized from normal human prostate
epithelial cells, recovered from a 17-year old Hispanic male at autopsy, by the introduction of HPV16 E6/E7 (Choo, Ling et al. 1999). It was found to be non-tumorogenic but does not express AR and does not respond to androgen stimulation. The reason for the lack of AR expression is uncertain. AR was subsequently stably transfected into HPr-1 cells by replication-defective retrovirus, resulting in the establishment of the HPr-1AR cell line (Ling, Chan et al. 2001).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Site of origin</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td><strong>Malignant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU 145</td>
<td>Brain metastasis</td>
<td>AR not expressed; androgen-independent</td>
</tr>
<tr>
<td>PC-3</td>
<td>Bone metastasis</td>
<td>AR not expressed; androgen-independent</td>
</tr>
<tr>
<td>22Rv1</td>
<td>Primary prostate cancer, xenograft</td>
<td>AR expressed; androgen-sensitive</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Lymph node metastasis</td>
<td>AR expressed; androgen-dependent</td>
</tr>
<tr>
<td>LNCaP-HOF</td>
<td>Subline of LNCaP</td>
<td>AR expressed; androgen-independent</td>
</tr>
<tr>
<td><strong>Non-tumorogenic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNT 2</td>
<td>Normal human prostate epithelial cells</td>
<td>AR not expressed; androgen dependen</td>
</tr>
<tr>
<td>PWR-1E</td>
<td>Normal human prostate epithelial cells</td>
<td>AR expressed; androgen dependent</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>Normal human prostate epithelial cells</td>
<td>AR expressed; androgen dependent</td>
</tr>
<tr>
<td>HPr-1</td>
<td>Normal human prostate epithelial cells</td>
<td>AR not expressed; androgen-independent</td>
</tr>
<tr>
<td>HPr-1AR</td>
<td>Subline on HPr-1</td>
<td>AR expressed (transfected AR); androgen-dependent</td>
</tr>
</tbody>
</table>

Table 3-1. Characteristics of prostate cancer and non-tumorogenic prostate cell lines.
3.2 STUDY OF CAG REPEAT NUMBER IN PATIENTS WITH ANDROGEN INDEPENDENT PROSTATE CANCER

3.2.1 Patient selection

The pathology laboratories in St. James’s Hospital and AMNCH have a combined archive of prostate tissue extending back over 40 years, comprising of transurethral resections of prostates (TURPs), radical prostatectomies and prostate biopsies. Patients who had 2 or more TURPs between January 1990 and December 2004, with a histological diagnosis of prostate cancer, and had androgen-ablative treatment in the intervening period between TURPs were identified. Androgen-ablative treatment was administered either by surgical castration (bilateral orchidectomy) or by medical means, typically involving the use of either luteinizing hormone-releasing hormone (LHRH) analogues or anti-androgens.

The initial TURP would have been performed typically because of bladder outflow obstructive symptoms, or complications of bladder outflow obstruction (e.g. acute urinary retention, bilateral hydronephrosis or renal impairment). The TURP chips were referred for histological analysis, and confirmed a diagnosis of prostate cancer. These prostate chips were formalin-fixed and paraffin-embedded. The original haematoxylin and eosin (H&E) stained slides and their corresponding paraffin blocks were archived in the pathology department.
The patients selected for this study would have then been commenced on androgen-ablative treatment, and response to treatment was confirmed by a fall in the prostate-specific antigen (PSA) level to its nadir*.

At some subsequent time point, varying between three and 41 months, these patients re-presented with recurrence of symptoms, representing clinical progression of their disease. This was confirmed biochemically, by a rise in PSA compared to the PSA nadir, indicating that the prostate cancer was now refractory to the androgen-ablative treatment (androgen-independent prostate cancer). To relieve their symptoms these patients had a subsequent channel TURP. These TURP chips were also formalin-fixed, paraffin-embedded, sectioned, mounted on glass slides, stained with H&E and analysed histologically to confirm the presence of further prostate cancer.

Medical notes of all recruited patients were retrieved from the medical records departments in St. James’s Hospital and the Adelaide and Meath Hospitals, incorporating the National Children’s Hospital (AMNCH) and reviewed. Clinical information was recorded including patients’ age, dates of prostate operations, details of androgen-ablative treatment, PSA results and histology reports (which included diagnosis, percentage of chips involved, Gleason grades and score).

* The PSA nadir is the lowest PSA reading achieved after any treatment for prostate cancer.
3.2.2 Storage of information

All clinical information was stored in a Microsoft Excel spreadsheet which was password protected.

3.2.3 Slides – review and sectioning

All original slides were retrieved from the pathology departments and reviewed by consultant pathologists. Areas of prostate cancer and adjacent areas of benign prostate tissue were identified and marked on the cover slip. The corresponding paraffin blocks were then retrieved and 6 consecutive 5 μm sections from each block were obtained using a microtome. All sections were mounted on uncoated and uncharged glass slides, and stained with H&E. For each set of six sections, the first slide was cover-slipped and the subsequent 5 were left uncovered. The cover-slipped slides were marked in the areas corresponding to those marked on the original slides.

3.2.4 H&E staining of sections

Most of the sections were stained in the histology laboratory using an automated staining machine. Some sections were stained manually using a 16-step protocol. Slides were placed into metal slide holders and sequentially placed in basins as outlined below:

- First xylene wash (5 minutes),
• Second xylene wash (5 minutes),
• 100% ethanol wash (15 seconds),
• 95% ethanol wash (15 seconds),
• 70% ethanol wash (30 seconds),
• Distilled water (15 seconds),
• Mayers Haemotoxylin Solution (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) (30 seconds),
• Distilled water (15 seconds),
• Blueing Reagent (Thermo Electron Corporation, Anatomical Pathology International, Cheshire, UK) (15 seconds),
• 70% ethanol wash (15 seconds),
• Eosin solution (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) (15 seconds),
• 95% ethanol wash (15 seconds),
• A further 95% ethanol wash (15 seconds),
• 100% ethanol wash (15 seconds),
• A further 100% ethanol wash (15 seconds),
• Final xylene wash (3 minutes).

Slides were allowed to air dry in a fume hood. Slides were then stored in slide boxes until required for laser capture microdissection.
3.2.5 Laser Capture Microdissection

Laser capture microdissection (LCM) is a technique that permits the rapid and reliable procurement of pure populations of cells from tissue sections, in one step, under direct microscopic visualisation. In this project, LCM was used to selectively isolate pure populations of malignant and benign prostate epithelial cells. The LCM workstation (Arcturus PixCell II, Arcturus Engineering Incorporated, Mountain View, California, USA) comprises a computer with a monitor, a laser electronics box, a microscope and a viewing box (Figure 3-1).

![Arcturus PixCell II Laser Capture Microdissection workstation.](image)

The principle underlying LCM is illustrated in figure 3-2. A histological section is placed on the stage of the specially designed microscope and the area of interest is selected. A transparent ethylene vinyl acetate layer, which sits
under a CapSure™ LCM cap (CapSure™ Macro LCM Caps, Arcturus Bioscience, Mountain View, CA, USA) (figure 3-3), is apposed to the section and a low power infrared laser beam is directed at the cells of interest. When the operator activates the laser beam, the film directly above the targeted area transiently melts and the long chain polymers in the film surround and tightly hold the cells, which remain embedded in the film after its removal from the section. In the current commercially available version of the LCM, the size of the laser beam spot can be selected at 30 μm, 15 μm and 7.5 μm, facilitating the dissection of groups of cells or even single cells. This technique can be used on formalin-fixed, paraffin-embedded sections, allowing the use of archival pathological material. The technique causes no apparent alteration to the morphology of the chosen cells, which can be directly visualised after microdissection. In order to heat the polymer (containing a naphthalocyanine near IR absorbing dye) to its melting point (80°C), the melted polymer expands downward making contact with the cell surface and flowing into microscopic air spaces in the desiccated tissue section. At the end of the pulse within a couple of milliseconds, the heat is rapidly conducted away in the glass slide, causing the cooled polymer to rapidly resolidify and bond to the targeted tissue. The strong bond formed allows the bonded cell to be ripped out of the surrounding tissue when the film is subsequently lifted off the tissue. By focusing the laser to 7 μm spot and using a pulse of 0.8 msec (or less) the region of polymer melting, expansion and bonding can be confined to an individual cell.
Chapter 3 Materials and methods

Transfer film on backing

Tissue

Selected cell(s)

Laser pulse

Vacancy following selective procurement

Transfer of selected cell(s)

Figure 3-2. This figure illustrates the principle of LCM. The laser beam transiently melts the thermoplastic film coating the cap, causing the film to adhere to the chosen cells. On removal of the cap from the slide, the chosen tissue remains adherent to the cap (Liotta and Petricoin 2000).

Figure 3-3. The CapSureTM Macro LCM caps are held on plastic or glass slides. The slides were mounted on a holder and one cap is lifted off at a time.
Selecting the smallest diameter laser, the laser was first focussed, using a clean slide. The size of laser used for LCM was 15 micron shot size, the duration of each laser pulse was 2.0 milliseconds and power of the laser was set at 70 mW.

An uncovered slide was mounted on the microscope stage, and fixed in place by a vacuum. A cap was engaged and positioned over an area on the slide corresponding to an area on the marked slide. The visualizer (a filter) was engaged in order to view the slide with near coverslip quality and malignant glands were identified from within the marked area. The visualizer was then disengaged and the stage moved with the joystick so that the cells to be captured underlie the laser. A pre-LCM photograph was obtained for each specimen (Figure 3-4). The laser was pulsed, causing the polymer to melt as described earlier. When the desired cells were procured, the cap was removed from the slide, and a post-LCM photograph was obtained of the remaining slide after the captured tissue was removed (Figure 3-5). A photograph of each cap was also obtained of the captured tissue (Figure 3-6). These were saved as JPEG images onto a Zip diskette. The cap was then placed facing up until tissue lysis and DNA extraction was performed.
3.2.6 Optimising number of LCM shots

To determine the number of shots required to obtain a sufficient amount of DNA, a shot curve was performed. This involved obtaining tissue by LCM with 10 shots, 50 shots, 100 shots, 200 shots, 500 shots and 1000 shots. On the basis of the shot curve, 1000 shots were used for all LCM with the size of the laser configured at 15 microns, the duration of the laser pulse was 2.0 milliseconds and power of the laser was 70 mW.

3.2.7 Preparation of slides for LCM

In both our experience and the finding of others, LCM works best with stained and dehydrated sections. Any moisture, even a trace amount, appeared to inhibit transfer of cells to the transfer film (see figures 3-7 to 3-9). As such, our protocol was to immerse the slides in a jar containing 99.9% molecular grade ethanol for 30 seconds each, followed by immersion in a jar containing xylene for 5 minutes. The slide was then air dried for 10 minutes in the fume
hood and then kept in an air tight jar with silica gel and used for LCM on the same day as preparation.

Figures 3-7 is a photograph taken of benign prostatic epithelium (stained with haemotoxylin and eosin) before LCM was attempted. Figure 3-8 is a photograph taken after LCM and figure 3-9 is a photograph of the cap and tissue adherent to it after LCM. The pick-up was extremely poor – the photograph of the cap shows melted polymer with almost no tissue adherent to it. This occurs when the tissue is not completely dehydrated before LCM. The tissue appears atypically coloured for H&E staining because these slides were not cover-slipped. Figures 3-4 to 3-6 are examples where the pick-up yield of tissue was good.

3.3 CELL CULTURE

All cell lines were grown as monolayer cultures in RPMI 1640 medium (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) supplemented with 10 percent foetal calf serum (FCS), streptomycin and penicillin antibiotics. The cells were grown to confluence in 75 cm² flasks and incubated at 37°C with 5% carbon dioxide (Thermo Forma Steri-cycle CO₂ Incubator with HEPA filter, model number 381).
Figure 3-10. LNCaP cells as seen under microscopy showing low density confluence (left) and high density confluence (right). Cells were harvested when approximately 75-90% confluent.

When the cells were approximately 75-90% confluent, they were either harvested or passaged (Figure 3-10). Harvesting was performed by removing the growth medium from the flask with a pipette and applying 2.5 ml of the enzyme trypsin per 75 cm$^2$ flasks in order to tease cells away from the base of the flasks. The cells were trypsinised for 5 minutes at 37°C. To prevent further digestion by the enzyme, the trypsin was then neutralized by adding 2.5ml of the old medium to the flask. A cell scraper was used to ensure that all cells were freed from the base of the flask (if necessary). A further 5 ml of old medium was added and the entire volume then transferred into a sterilin and centrifuged at 1600 rpm for 6 minutes. The supernatant was discarded and the remaining precipitated cells were kept on ice prior to performing DNA extraction. If the precipitated cells were for further growth, they were re-suspended in an appropriate volume of new medium and transferred to a new 75 cm$^2$ flask. Twenty millilitres of new medium was added to the cells and the flask was
replaced in the incubator, as described earlier. Medium was changed every 4-5 days and cells were passaged by trypsinization once a week.

3.4 DNA EXTRACTION

3.4.1 Cell line DNA extraction

DNA was extracted from cell lines using a DNA mini extraction kit (Qiagen, UK) according to the manufacturer’s guidelines. Samples were equilibrated to room temperature and the cell pellet was resuspended in Dulbecco’s PBS (Gibco™, Invitrogen Corporation, UK) to a final volume of 200 μl in 1.5 ml eppendorf tubes. 20 μl of protease and 20 μl of RNaseA (20mg/ml) were then added, before adding 200 μl of Buffer AL (lysis buffer). This was mixed by pulse-vortexing for 15 seconds. Following this, the mixture was incubated on a heating block at 56°C for 10 minutes to achieve maximum DNA yield. The mixture was then centrifuged briefly to remove drops from inside the lid. 200 μl of 100% ethanol was added, and the mixture pulse-vortexed for 15 seconds (the volume of ethanol is increased proportionally if the initial sample volume exceeded 200 μl). Upon adding ethanol, it was usually possible to see the DNA. The mixture was carefully transferred (using a Gilson pipette) from the eppendorf tube to the QIAamp spin column (in a 2 ml collection tube), taking special care not to wet the rim. With the cap closed, the spin column was centrifuged at 8000 rpm for 1 minute. The QIAamp spin column was then transferred to a new 2 ml collection tube and the previous tube
containing the filtrate was discarded (if the lysate did not completely pass
through the column after centrifugation, it was centrifuged again at a higher
speed until the column was empty). 500 μl of Buffer AW1 (Wash Buffer 1) was
then added and the tube centrifuged at 8000 rpm for 1 minute. The QIAamp
spin column was again transferred to a new 2 ml collection tube. 500 μl of
Buffer AW2 (Wash Buffer 2) was added to the spin column, the cap closed and
centrifuged at 14,000 rpm for 3 minutes. The QIAamp spin column was
similarly transferred to a new collection tube. The tube was centrifuged at
14,000 rpm for a further 1 minute. The QIAamp filter was transferred to a 1.5
ml microcentrifuge tube this time and 200 μl of Buffer AE (Elution Buffer) was
added to the filter and left to incubate at room temperature for 5 minutes before
centrifuging at 8000 rpm for 1 minute. A second elution step with a further 200
μl of Buffer AE increases DNA yields by up to 15 percent, according to the
manufacturers booklet, and therefore, this extra step was performed. The final
product (cell line DNA) was stored at -20 degrees Celsius until required for use.

3.4.2 Prostate tissue DNA extraction

The PicoPure™ DNA Extraction Kit (Arcturus Engineering, Inc.,
Arcturus Ltd. UK) was used for extraction of DNA from LCM tissue. The
protocol detailed in the kit’s user guide used.
155 μl of Reconstitution Buffer was added to one vial of Proteinase K powder with a pipette. The mixture was vortexed gently to mix the reagents and the tube was immediately placed on ice. The reagents were completely dissolved, avoiding excessive mixing which can denature the Proteinase K.

50 μl of Proteinase K Extraction Solution was pipetted into 0.5 ml microcentrifuge tubes.

The CapSure Macro LCM Cap with LCM captured cells was then inserted into the microcentrifuge tubes which were then inverted. The 50 μl of Proteinase K Extraction Solution was then shaken down until it completely covered the inside surface of the CapSure Macro LCM cap.

The inverted tubes were incubated at 65 °C for 48 hours. After incubation, the tubes were removed from the incubator and placed in a microcentrifuge and centrifuged for one minute at 1,000 x g. After centrifugation, the CapSure Macro LCM caps were removed and the microcentrifuge tubes containing the extract were heated to 95 °C for ten minutes in a heating block (MJ Research) to inactivate to Proteinase K. The tubes were then cooled to room temperature. The samples were stored at -20°C until required for PCR analysis.
3.5 QUANTIFICATION OF CELL LINE DNA

DNA was quantified using the Amersham Pharmacia Biotech GeneQuant II RNA/DNA Calculator (Amersham, Bucks, UK). DNA was diluted one in a hundred (5 μl DNA plus 495 μl distilled water) to a total volume of 500 μl. After calibrating the machine with 500 μl of distilled water, 500 μl of the diluted DNA was pipetted into the cuvette. An average of 3 readings was obtained of absorbance (in nanometres), ratio (OD260/OD280) and DNA concentration (ng/μl). The cuvette was cleaned with distilled water between samples.

3.6 POLYMERASE CHAIN REACTIONS (PCRs)

3.6.1 Primer design

The CAG repeat region resides within exon 1 of the androgen receptor gene. Giovannucci et al (Giovannucci, Stampfer et al. 1997) described primers for the isolation of the CAG repeat in 1997, and since then, numerous studies of the CAG repeat have used these primers with success. The primers described by Giovannucci were forward primer 5′-TCC AGA ATC TGT TCC AGA GCT GGC TGC-3′ (codon 146-169) and reverse primer 5′-GCT GTG AAG GTT GCT GGT GTT CCT CAT-3′ (codon 404-427).

As an exercise in primer design, alternative primers flanking the CAG repeat segment were designed “in house”, complying with the universal primer
design criteria listed in Table 3-3. An additional set of primers were designed in house to flank the CAG segments as follows: Forward primer 5’−TCC AAG ACC TAC CGA GGA GCT T−3’ (codon 123-144) and reverse primer 5’−ATC CAG GAC CAG GTA GCC TGT−3’ (codon 386-405).

<table>
<thead>
<tr>
<th>CRITERIA</th>
<th>RECOMMENDED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>Ideal size: 20-25 nucleotides long. Generally: 18-30 nucleotides long</td>
</tr>
<tr>
<td>3’ end base</td>
<td>Should be a G or a C</td>
</tr>
<tr>
<td>Melting temperatures (Tm)</td>
<td>50-65 °C</td>
</tr>
<tr>
<td>GC content</td>
<td>40-60%</td>
</tr>
<tr>
<td>Self-complimentary</td>
<td>Should be avoided</td>
</tr>
</tbody>
</table>

Table 3-2. Universal criteria for designing primers.

The “in house” designed forward primer was 22 nucleotides long with a 55% GC content and the reverse primer was 21 nucleotides long with a GC content of 57%. Although both primers ended with a T base, this did not seem to affect the efficiency of the primers.

Both sets of primers, ones described by Giovannucci and ones designed in house, were obtained commercially from MWG-Biotech (UK) Ltd (Milton Keynes, England). Forward primers were obtained unlabelled and fluorescent-labelled with FAM (6-carboxyfluoresceine). Temperature and magnesium chloride curves were performed to determine the optimum annealing temperature and magnesium chloride concentration for PCR amplification.
3.6.2 Androgen receptor gene

The first exon (bp 79—1691) and intron (bp 1692—1753) of the androgen receptor gene (Lubahn, Joseph et al. 1988) as sourced from the National Library of Medicine Entrez Genome website is shown in figure 3-2.

The bases in green and red correspond to the forward and reverse primers respectively (Giovannucci, Stampfer et al. 1997). The CAG segment in the androgen receptor gene is coloured blue (Figures 3-11 and 3-12).

```
1 agcctgttgga actcttctgaa gcacaagaag gggaggcggg gtaaggggaag taggtggag
dl attcagccaa gctcaaggat gsaagtcag ctagggctgg gaagggtcta cctcggccg
dd 1 ccgrtccaaga cctaccgagg agctttccag aatctgttcc agagcgtgcg cgaagtgatc
dd 1 cagaacccgg gccccaggca cccagaggg gcagcgccag cacctccgcgg gcagcgcttt
dd 241 tctgtctgctc acgcagcgca gcacgagcag cagcagcagc gcagcagcagc gcagcagcag
dd 301 cagcagcagc aagagactag cccaggcaagac acagaggtta gtagtggct tggtggtcct
dd 361 ccccaaggccc atcctaggcc gcacccagagg ccatctggct cggaggttca ccagggttca
dd 421 tccacgcgctc gtcgccctctc gccggagagc cctgccctgc cggaggttca ccagggttca
dd 481 ggcgagtcgg gcagcgccaa ggggtgctgc gcagcgccag cacctccgcgg gcagcgcttt
dd 541 gaactcgagtgc cccacattcc cggcagctgg ccctggccca ctttccccgg cttaagcagc
dd 601 tgcagcggtgc gggacagcagc gcacccagagg ccatctggct cggaggttca ccagggttca
dd 661 cagcagcagc aagagactag cccaggcaagac acagaggtta gtagtggct tggtggtcct
dd 721 gctccactgcc cctctcagga caattactta gcacccaggct gacgccatgg gcacccaggct
dd 781 aaggagttgt gtaagccagt gtctgccgttc atgggctgcgg tggsgctgcc gtggagcttgc
dd 841 ctgagctcgct tgggagctct tgcctcgtgt cggggggaag ccctggcggc cggggggaag
dd 901 cccgctgtgcgt cccctcaggg ccctggcggc cggggggaag ccctggcggc cggggggaag

dd 961 gacgccgccct cgggccggtgt cgggccggcg cgggccggcg cgggccggcg cgggccggcg
dd 1021 aaccggcgct cggccccgca tagagagttc tgtgtgtgtt gcacccaggct gacgccatgg
dd 1081 ggccagcttg aacctgcttc gccctcgtct cctctcaggg cggcagcggt ggcacccaggct
dd 1141 gtcgctcgcc agctctgctga ccattaatgg ttccttcaggg cggcagcggt ggcacccaggct

dd 1321 ggcgttcggcc ccgctcgttc gccctgccgc cggctgccgc cggctgccgc cggctgccgc

dd 1381 actctctcct cagccagagag gcagtcggag aatggtcgcc ggtgccgggg ggtgccgggg

dd 1441 gagcgctggc ggagccaggg gcaggaggg gcgcgggagg gcgcgggagg gcgcgggagg

dd 1501 ggagctggc ggcggggcgg ggcggggcgg ggcggggcgg ggcggggcgg ggcggggcgg

dd 1561 gactctggag cccccgctcg cggcctgctc tgggtccttg cggcctgctc tgggtccttg

dd 1621 actctctcct cagccagagag gcagtcggag aatggtcgcc ggtgccgggg ggtgccgggg

dd 1681 gggagctgcc gcagcatttt ctccgtcgca aatgtgcgttc cggcagcagc ggcacccaggct

dd 1741 cctctcaggg cggcagcggt ggcacccaggct gacgccatgg
dd
```

Figure 3-11. First exon and intron of the androgen receptor gene with published forward and reverse primers (Giovannucci, Stampfer et al. 1997) identified in green and red respectively. The CAG repeat segment is highlighted in blue.
**3.6.3 Optimisation of PCR for cell line DNA amplification**

Temperature and magnesium chloride (MgCl$_2$) curves were performed to optimise polymerase chain reaction (PCR) conditions. Fifty microlitre PCRs were set up with MgCl$_2$ concentrations of 1.0, 1.5 and 2.0 millimole (mM). The reaction tubes contained GeneAmp 10X PCR Buffer II (5 µl per reaction), 1.0, 1.5 and 2.0 mM MgCl$_2$ each, AmpliTaq DNA polymerase (0.2 µl per reaction), 20 pmol Forward primer (1 µl), 20 pmol Reverse primer (1 µl) and 10 mM deoxyribonucleoside triphosphate (dNTP) (1 µl). Three microlitres of cell line DNA (approximately 200 ng) was added and the volume was adjusted to 50 µl with molecular biology grade water (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany). Two PCRs were set up in a thermal cycler (MJ Research PTC-200 DNA Engine Peltier Thermal Cycler), one with an annealing temperature of 59°C and the other of 60°C (guided by the melting temperatures of the primers as estimated by the manufacturer). PCR conditions were an
initial denaturation step for 1 minute at 94°C, 34 cycles at 94°C for 30 seconds, 59 (and 60) °C for 30 seconds and 72°C for 30 seconds with a final elongation step at 72°C for 7 minutes. Amplified DNA was stored at 4 °C temporarily and at -20 °C long-term. Similar curves were designed to optimise the self-designed primers (MgCl₂ concentrations 1.0, 1.5 and 2.0 mM and annealing temperatures 58, 59 and 60 °C). All reactions were performed in triplicate.

3.6.4 PCR amplification of cell line DNA

The CAG segments were amplified using the previously optimised MgCl₂ concentration and annealing temperature as determined by the curves. A total volume of 50 µl reaction mixture contained 200 ng DNA, PCR Buffer II (Applied Biosystems), 1.0 mM MgCl₂ (Applied Biosystems), 1 µl (10 mM) dNTP (Promega, UK), 1 µl (20 pmol) of each primer (MWG-Biotech) and 0.2 µl AmpliTaq DNA polymerase (Applied Biosystems). PCR reaction conditions were as described for temperature and MgCl₂ curves.

All PCRs were performed using positive (known cell line) and negative controls (molecular grade water).

3.6.5 PCR amplification of prostate tissue DNA using Nested PCR

Nested PCR is a variant of the standard PCR technique described in the cell line work. It utilises two sets of primer pairs, an outer set and an inner set, designed to amplify a single locus (Figure 3-13). Using nested primers for PCR
increases PCR yields and increases amplification specificity. The quantity of DNA extractable from laser capture microdissected tissue is very small; therefore, this technique was very useful for amplification of the CAG segment.

3.6.5.1 First round (outer) PCR

For the first round PCR (outer PCR), an in house-designed forward primer, 5’-TCC AAG ACC TAC CGA GGA GCT T-3’ and reverse primer described by Giovannucci, 5’-GCT GTG AAG GTT GCT GTT CCT CAT-3’ (Giovannucci, Stampfer et al. 1997) were employed.

A 20 μl reaction was set up comprising 2.0 μl 1X PCR Buffer II (Applied Biosystems), 0.8 μl magnesium chloride (Applied Biosystems), 0.4 μl
dNTP (MWG-Biotech), 0.2 μl (20 pmol) of each primer, 0.2 μl Amplitaq DNA polymerase (Applied Biosystems) and 2 μl DNA.

The PCR was performed in a thermal cycler (MJ Research PTC-200 DNA Engine Pelter Thermal Cycler). The samples were preheated to 94 °C for 1 minute then 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 1 minute and a final elongation step at 72 °C for 30 minutes. The samples were then cooled to 20 °C for 30 minutes then cooled further to 4 °C. Extending the final elongation step to 30 minutes and avoiding a sudden ramp-down from 72 °C to 4 °C minimised the additional +A nucleotide to the end of PCR products from falling off, which otherwise resulted in split peaks.

3.6.5.2 Second round (inner) PCR

The first round PCR products were diluted 1:10 and were used as the template DNA for the second round PCR (inner PCR). The forward primer used was the forward primer described by Giovannucci, 5'–TCC AGA ATC TGT TCC AGA GCG TGC–3' (Giovannucci, Stampfer et al. 1997) and the reverse primer was designed in house, 5’–ATC CAG GAC CAG GTA GCC TGT–3’. The samples were preheated to 94 °C for 1 minute then 20 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, extension at 72 °C for 1 minute and a final elongation step at 72 °C for 30
minutes. The samples were then cooled to 20 °C for 30 minutes then cooled further to 4 °C and stored at this temperature until analysis by GeneScan.

3.7 GEL ELECTROPHORESIS

The PCR amplified cell line DNA was resolved in a 2% agarose gel. The appropriate weight of Agarose D-1 LE (Low EEO) powder (Laboratorios Conda, S.A.) was placed in a beaker and the corresponding volume of 1X Tris-Acetate-EDTA (TAE) buffer added to it. The powder was dissolved by heating the mixture and intermittently swirling until a crystal clear solution was achieved. The solution was cooled slightly prior to adding ethidium bromide (2 μl per 100 ml gel). The gel was poured into a plate and allowed to set at room temperature. A 2% Metaphor gel was used instead of the agarose gel in order to obtain better resolution of the migrating bands.

Seven microlitres of 50bp DNA step ladder (Promega Corporation, Madison, WI, USA) and 2.5 μl of 6X Blue/Orange loading dye (Promega Corporation) was loaded into the first well. The same volume of loading dye and 15 μl of PCR product were placed into each subsequent well. The gel was run for a varying length of time (1-2 hours) at approximately 80 millivolts and 120 milliamps.
3.8 FRAGMENT ANALYSIS

3.8.1 Optimisation of fragment analysis for cell line PCR products

Initially, a dilution curve was performed to determine the optimum concentration of amplified DNA to be used in the genetic analyzer (ABI Prism 3100, Applied Biosystems, Warrington, UK) (Figure 3-14). Amplified cell line DNA from 4 cell lines (PC-3, PNT-2, PWr-1E and RWPE-1) were diluted 1:25, 1:50, 1:100 and 1:200.

Figure 3-14. ABI 3100 Genetic Analyzer workstation.

3.8.2 Fragment analysis of cell line PCR products

After the optimum DNA concentration was ascertained, that concentration was used for all subsequent cell line fragment analyses. Materials required were GeneScan™ 350 ROX size standard (ABI Prism™, Applied Biosystems), 3100 POP-6™ polymer (ABI Prism™, Applied Biosystems), Hi-Di™ formamide (Applied Biosystems), 10X Genetic Analyzer EDTA buffer
(ABI Prism™, Applied Biosystems), 96-well plate (ABI Prism™, Applied Biosystems) and 36cm Capillary Array (Applied Biosystems). All Applied Biosystems products were obtained from their UK/Ireland office, Warrington, UK. The 10X Genetic Analyzer EDTA buffer was diluted 1:10.

The volumes of GS350-ROX size standard, diluted amplified DNA and Hi-Di™ formamide are detailed in Table 3-4. A master-mix comprising of the appropriate volume of GS350-ROX size standard and Hi-Di™ formamide was prepared. Nine microlitres of master-mix and 1 µl of amplified DNA were mixed into 0.5ml eppendorf tubes. Each sample was then loaded into the 96-well plate (12 columns and 8 rows), making note of the well number for each sample. The plate was loaded column by column, from A1 to H12. Unused wells were filled with Hi-Di™ formamide to complete multiples of 16 and remaining wells were left empty.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size standard (GS-350 ROX)</td>
<td>0.5</td>
</tr>
<tr>
<td>Diluted amplified DNA</td>
<td>1.0</td>
</tr>
<tr>
<td>Hi-Di™ formamide</td>
<td>8.5</td>
</tr>
<tr>
<td><strong>Total volume per well</strong></td>
<td><strong>10.0</strong></td>
</tr>
</tbody>
</table>

*Table 3-3. Volume of reagents per well for fragment analysis.*

The genetic analyzer was set up according to the manufacturer’s instructions. The 36 cm capillary array was installed and the reservoirs and
syringes were filled with 1X EDTA buffer and POP-6™ polymer respectively. The polymer blocks were primed and all bubbles removed. The plate was heated to 95 °C for 2 minutes using a thermocycler to denature the DNA and then chilled on ice until the plate was ready to be loaded. The plate was assembled and placed onto the autosampler tray. A new plate record was created and linked to the plate, causing the plate position indicator to change from yellow to green. The run was commenced, and data saved to the computer hard-disk drive and then on CD-ROM. The data were analysed using ABI Prism™ GeneScan Software Version 3.7 (Applied Biosystems).

3.8.3 Fragment analysis of prostate tissue PCR products

The materials required were the same as described earlier for analysis of CAG repeat number in prostate cell lines. The concentration of DNA from the laser capture microdissected tissue was far less compared with that of the cell lines, and therefore, the second round PCR product was used undiluted. 1 μl of the final round PCR product was mixed with 0.5 μl of size standard GS-350 ROX and 8.5 μl HiDi formamide. The 10 μl mixture for each sample was loaded into each column of a 96-well plate from A1 to H12. The ABI 3100 Genetic Analyzer was set up and the plate was prepared and mounted as described earlier. The data were analysed using ABI Prism™ GeneScan Software Version 3.7 (Applied Biosystems).
3.9 CALCULATING CAG REPEAT LENGTH AND NUMBER

3.9.1 Calculating the CAG repeat length and number of cell line DNA

The allele size of the PCR amplified product was determined by the GeneScan software. The CAG repeat length (the only variable part of the amplicon) was calculated by subtracting from the allele size, the sum of basepairs of the forward and reverse primers and the basepairs on either side of the CAG repeat segment (the constant part of the amplicon, determined to be 222 basepairs) (Figure 3-12). The number of CAG repeats was a third of the CAG repeat length.

3.9.2 Calculating CAG repeat length and number of tissue DNA

The allele size of the PCR amplified was determined by the GeneScan software. The CAG repeat length was calculated by subtracting from the allele size, the constant region (determined to be 200 basepairs) on either side of the CAG repeats (Figure 3.15). The number of CAG repeats was a third of the CAG repeat length.
3.10 SPECIMEN CONTROL PCR

The Specimen Control Size Ladder, a PCR-based assay (InVivoScribe Technologies, Marseille, France) amplifies multiple target genes and generates a series of amplicons of 100, 200, 300, 400 and 600 base pairs to ensure that the quality and quantity of input DNA is adequate to yield a valid result. The Specimen Control Size Ladder was used to demonstrate DNA degradation in samples from St. James’s (fixed in unbuffered formalin) and to demonstrate the quality of DNA from AMNCH samples (fixed in buffered formalin). The PCR protocol followed for this procedure was as described by InVivoScribe technologies. For each PCR 22.5μl FAM (fluorescent) labelled specimen control size ladder mix (InVivoScribe technologies), 0.125μl Amplitaq gold and 2.5μl of LCM DNA was used. Detection of the amplicons was performed with ABI 3100 Gene Scan analyser using Rox 350 standard.
3.11 PATIENT EXCLUSION

Thirty-one patients, which had initially met the inclusion criteria as described above, were subsequently excluded from this study. They were excluded because of degradation of DNA and inability to amplify the CAG segment from their archived material.

Laser capture microdissection techniques were repeatedly altered, including altering the shot size, the power of the laser beam and the duration of each laser pulse.

DNA extraction techniques were varied and several DNA extraction kits were tried, including the QIAmp DNA mini kit (Qiagen, West Sussex, UK), the PureGene DNA isolation kit (Gentra, Minneapolis, USA) and the PicoPure DNA Extraction Kit (Arcturus Engineering).

Standard PCR was substituted with PCR using nested primers on the assumption that the quantity of DNA obtained from laser captured tissue was minute and the DNA quality was poor.

It eventually became apparent that the lack of experimental success was as a result of slides obtained from blocks archived in St. James’s Hospital. All specimens prior to 1999 were fixed in unbuffered formalin. This had evidently
led to significant degradation of DNA. The amplicon of interest in this study was between 250-300 basepairs, but as a result of DNA fragmentation and degradation, it was not possible to amplify the CAG repeat segment. A literature search yielded a number of papers that concurred with this conclusion (Legrand, Mazancourt et al. 2002; Zsikla, Baumann et al. 2004). Kosel et al showed that DNA extraction from tissue fixed in buffered-formalin resulted in greater yields, the molecular weight of the isolated DNA was higher and PCR was more successful (Kosel, Grasbon-Frodl et al. 2001).

Samples from the Adelaide and Meath Hospitals incorporating the National Children’s Hospital (AMNCH) were all fixed with saline-buffered formalin, and the DNA from these samples was better preserved, allowing amplification of the CAG repeat segments.

The exclusion of most patients from St. James’s meant that the number of patients that were available for analysis was reduced to 19.

3.12 STATISTICAL ANALYSIS

The signed rank test was used to compare changes in CAG repeat number between the pre-treatment and post-treatment samples. The Kruskal-Wallis test (non-parametric) was used to analyse associations between the various clinical parameters and CAG shortening (Appendix 3).
CHAPTER 4

Analysis of CAG Repeat Number in
Prostate Cell Lines
Chapter 4

4.1 OVERVIEW

Initial objectives of the analysis of CAG repeat number in prostate cell lines were to optimise the techniques required for cell culture, DNA extraction, PCR and fragment analysis using GeneScan software for this study. These techniques were then used to determine the CAG repeat number in the panel of cell lines and specifically to compare the CAG number of the LNCaP cell line with that of the LNCaP-HOF cell line.

A panel of ten prostate cell lines were selected for this part of the study. The cell lines comprised of malignant prostate cell lines and non-tumorogenic prostate cell lines derived from a variety of sites of origin with a range of characteristics as described in 3.1.3 and 3.1.4.

4.1.1 DNA extraction

DNA was extracted from prostate cell lines and DNA concentrations were quantified as described in Chapter 3. The GeneQuant II RNA/DNA Calculator determines the UV absorbance of the cell line DNA solution and displays the calculated DNA concentration. DNA absorbs ultraviolet light, and therefore, DNA may be easily quantified in a UV photospectrometer. Pure solutions of DNA are quantified by reading the absorbance at 260 nm. Typically, 1 OD$_{260}$ (i.e. a solution having an absorbance of one unit at 260 nm
with a path length of 1 cm) corresponds to a single-stranded DNA concentration of 30-37 µg per ml and a double-stranded DNA concentration of 50 µg per ml.

An absorbance ratio of 260 nm and 280 nm gives an estimate of the purity of the solution. A pure DNA solution has an OD260/OD280 value of 1.8. Table 4-1 outlines the absorbance, the optical density ratio (OD_{260}/OD_{280}) and the DNA concentration of each of the ten prostate cell lines studied.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Absorbance*</th>
<th>OD_{260}/OD_{280}</th>
<th>DNA concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU 145</td>
<td>0.009</td>
<td>1.81</td>
<td>47.8</td>
</tr>
<tr>
<td>PC-3</td>
<td>0.014</td>
<td>1.82</td>
<td>121.1</td>
</tr>
<tr>
<td>22Rv1</td>
<td>0.024</td>
<td>1.77</td>
<td>89.4</td>
</tr>
<tr>
<td>LNCaP</td>
<td>0.023</td>
<td>1.81</td>
<td>289.3</td>
</tr>
<tr>
<td>LNCaP-HOF</td>
<td>0.008</td>
<td>1.79</td>
<td>112.8</td>
</tr>
<tr>
<td>PNT 2</td>
<td>0.011</td>
<td>1.79</td>
<td>68.4</td>
</tr>
<tr>
<td>PWr-1E</td>
<td>0.058</td>
<td>1.83</td>
<td>124.3</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>0.041</td>
<td>1.75</td>
<td>147.1</td>
</tr>
<tr>
<td>HPc-1</td>
<td>0.006</td>
<td>1.84</td>
<td>187.4</td>
</tr>
<tr>
<td>HPc-1AR</td>
<td>0.013</td>
<td>1.88</td>
<td>74.4</td>
</tr>
</tbody>
</table>

*Table 4-1: Readings from the photospectrometer are tabulated above. An average of 3 readings for each parameter was taken. *Absorbance (nm) and $^\dagger$DNA concentration (ng/µl). OD = optical density.

4.1.2 Optimisation of published primers

Primers to amplify the CAG repeat region of the AR gene were described by Giovannucci (Giovannucci 1997), forward primer 5’-TCC AGA
ATC TGT TCC AGA GCG TGC–3’ (codon 146-169) and reverse primer 5’–
GCT GTG AAG GTT GCT GTT CCT CAT–3’ (codon 404-427) were obtained
from MWG-Biotech (UK) Ltd. Additionally, a further set of primers were
designed in house: forward primer 5’–TCC AAG ACC TAC CGA GGA GCT
T–3’ (codon 123-144) and reverse primer 5’–ATC CAG GAC CAG GTA GCC
TGT–3’ (codon 386-405). Both sets of forward primers were labelled with
FAM. Using PC-3 cell line DNA, reaction conditions for both sets of primers
were optimised. The optimal annealing temperature was determined as was the
optimum magnesium chloride (MgCl₂) concentration by performing PCR
reactions at a range of temperatures and MgCl₂ concentrations as described in
Section 3.6.3. PCR was performed as described in Section 3.6.4 and the
products were resolved in a 2% agarose gel (Figure 4-1). Results indicate the
strongest band appearing around the expected 300 basepair region in the lane
corresponding to 1.0 mM MgCl₂ and an annealing temperature of 60°C and
these parameters were used for amplification of all subsequent samples.
Figure 4-1. Gel photograph showing published primers optimised at 1.0 mM of MgCl2 and annealing temperature of 60°C. A 50 basepair ladder was used (first lane) and the second lane, labelled 'C' represents the negative template control.

4.1.3 Optimisation of in house designed primers

The primers designed in house were determined to show optimum amplification at 1.0 mM MgCl2 and at an annealing temperature of 58°C as represented by the strongest band (circled) in the 300 basepair region (Figure 4-2) and these parameters were used for amplification of all subsequent samples.
4.1.4 Gel Electrophoresis of Prostate Cell Line DNA

The PCR products of all cell lines were resolved on a 2% agarose or metaphor gel to confirm the presence of amplified product. Figure 4-3 shows the presence of amplified product in the region between 250 and 300 basepairs, consistent with the region within which one would expect to find the CAG repeats.

The HPr-1 cell line does not express the AR and therefore, a subline was established by Choo and Ling et al by stably transfecting this cell line with an additional AR gene. The resultant cell line was named the HPr-1AR cell line. The gel photograph (Figure 4-3) shows two bands in the lane labelled HPr-
1AR, representing the amplified CAG region within the native AR gene as well as that of the transfected AR gene.

The LNCaP cell line is a commercially available cell line that was established from a lymph node metastasis of a patient with prostate cancer. It expresses AR and is androgen dependent. The LNCaP-HOF cell line is an androgen-independent subline of the LNCaP and was established by Klocker and colleagues as described in Chapter 3. The LNCaP-HOF band is visibly lower on the gel photograph compared to the parental LNCaP (Figure 4-3) indicating a shorter length. However, in order to precisely determine the sizes of the different repeats, it was necessary to electrophorese the samples in a polyacrylamide gel matrix and use automated fragment analysis software to ascertain fragment size.

![Gel photograph of cell lines](image)

**Figure 4-3.** Gel photograph of the 5 non-tumorogenic and the 5 prostate cancer cell lines flanked by a 50 basepair ladder on the left and a 100 basepair ladder on the right. The white arrows are at 300 basepairs. These samples were resolved on a 2% metaphor gel.
4.1.5 CAG repeat lengths of cell line DNA – Fragment analysis results

The PCR products of the ten cell lines were diluted 1:25 and were analysed on the Genetic Analyzer using GeneScan software. The GeneScan data was generated and length of the amplicon was determined by the software, which generated an electropherogram with a peak corresponding to the total number of basepairs in the amplified segment.

The human AR gene sequence obtained from the National Centre for Biotechnology Information (NCBI) GenBank, as cloned by Lubahn et al (Lubahn DB, Joseph DR, Sar M et al. Molecular Endocrinology 1988; 2(12):1265-1275), was used initially to determine the number of basepairs flanked by the forward and reverse primers (Figures 4-4 and 4-5). In the cloned sequence, the total length of the segment from the start of the forward primer to the end of the reverse primer was 288 basepairs and the number of CAG repeats was 22. Therefore, the basepairs outside of the CAG segment totalled 222.

**Figure 4-4.** The 1st 540 basepairs of exon 1 of the AR gene including the CAG segment (coloured blue). The forward and reverse primers (published) are indicated in green and red respectively.
4.1.6 **Calculating the CAG repeat number**

To determine the length of the unknown CAG repeat fragments, 222 basepairs are subtracted from the allele size of the amplified region, as determined by the GeneScan software. The number of repeats was determined using the formula below:

\[
\text{number of repeats} = \frac{(\text{fragment length} - \text{constant region})}{3}
\]

4.1.7 **GeneScan electropherograms**

The analyzed data is provided in the form of an electropherogram and in numerical form. The red dye represents the size standard peaks for the GeneScan size standard, GS-350 ROX (Figure 4-6). The PCR products are then sized by extrapolation to this in-sample size standard. The resolution of the
polymer used to separate the PCR products is 1 nucleotide for sizes ranging from 20-250 bp and 2 nucleotides from 250-350 bp.

Figure 4-6. The GS-350 ROX size standard has peaks at 35, 50, 75, 100, 139, 150, 160, 200, (~250), 300, 340, and 350 bp. Peaks in red are the known size standard peaks that the GeneScan software uses to draw a standard curve.

The GeneScan electropherograms for the 10 prostate cell lines studied are shown in Figures 4-7 to 4-16. The CAG repeat numbers for the 10 prostate cell lines are summarized in Table 4-1.
PNT-2

*Figure 4-7.* The GeneScan electropherogram for PNT-2 cell line. The blue peak represents the CAG repeat fragment and corresponds to 16 CAG repeats.
Figure 4-8. GeneScan electropherogram for PWr-1E. The blue peak represents the CAG repeat fragment and corresponds to 23 CAG repeats.
Figure 4-9. The electropherogram for the RWPE-1 cell line. The blue peak represents the CAG repeat fragment and corresponds to 22 CAG repeats.
HPr-1

Figure 4-10. The electropherogram for the HPr-1 cell line. The blue peak represents the CAG repeat fragment and corresponds to 21 CAG repeats.
Chapter 4  Analysis of CAG Repeat Number in Prostate Cell Lines

HPr-1AR

Figure 4-11. The electropherogram for the HPr-1AR cell line which had two androgen receptor genes and therefore, had 2 CAG repeat fragments (indicated by the two blue peaks). The transfected gene had 10 CAG repeats (taller blue peak) and the AR of the parental line had 21 CAG repeats.
PC-3

Figure 4-12. The PC-3 cell line electropherogram. The blue peak represents the CAG repeat fragment and corresponds to 23 CAG repeats.
Figure 4-13. The GeneScan electropherogram for DU 145 cell line. The blue peak represents the CAG repeat fragment and corresponds to 15 CAG repeats.
22Rv-1

Figure 4-14. The GeneScan electropherogram for the 22Rv-1 cell line. The blue peak represents the CAG repeat fragment and corresponds to 20 CAG repeats.
LNCaP

Figure 4-15. The LNCaP cell line GeneScan electropherogram is shown above. The blue peak represents the CAG repeat fragment and corresponds to 24 CAG repeats.
Figure 4-16. The LNCaP-HOF cell line GeneScan electropherogram is shown above. This androgen-independent subline of the LNCaP has only 21 CAG repeats (the CAG repeat fragment peak is indicated in blue) compared to its parental cell line's 24 CAG repeats.
Chapter 4 Analysis of CAG Repeat Number in Prostate Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CAG repeat numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-tumorogenic</strong></td>
<td></td>
</tr>
<tr>
<td>PNT 2</td>
<td>16</td>
</tr>
<tr>
<td>PWr-1E</td>
<td>23</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>22</td>
</tr>
<tr>
<td>HPr-1</td>
<td>21</td>
</tr>
<tr>
<td>HPr-1AR</td>
<td>10 &amp; 21*</td>
</tr>
<tr>
<td><strong>Malignant</strong></td>
<td></td>
</tr>
<tr>
<td>DU 145</td>
<td>23</td>
</tr>
<tr>
<td>PC-3</td>
<td>15</td>
</tr>
<tr>
<td>22Rv1</td>
<td>20</td>
</tr>
<tr>
<td>LNCaP</td>
<td>24</td>
</tr>
<tr>
<td>LNCaP-HOF</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 4-2. The CAG repeat numbers of the 10 prostate cell lines. *The HPr-1AR cell line has two AR genes and therefore has 2 separate CAG repeat segments; the segment with 10 CAG repeats is from the transfected AR gene and the segment with 21 CAG repeats is from the parental cell line, HPr-1.

4.2 DISCUSSION

4.2.1 The rationale for cell line selection

There were several rationales for studying a range of prostate cell lines. It allowed optimisation of the techniques required for subsequent analysis of prostate cancer samples. Furthermore, using a range of cell lines, it was possible to demonstrate the polymorphic nature of the CAG repeats, with repeat numbers ranging from 10 to 24 (table 4-1). It was also apparent that the techniques employed were able to consistently and reliably isolate and amplify the CAG segment as well as determine the CAG repeat numbers for each sample.
LNCaP, DU-145 and PC-3 are probably the best known and most widely studied prostate cancer cell lines in prostate cancer research. They were developed between 1977 and 1980, and have greatly contributed to our current understanding of prostate cancer. Since then, and especially in the past ten years, numerous new cell lines have been developed. While the validity of the extrapolation of in vitro studies to the in vivo human situation has, at times, been questioned, it cannot be denied that major strides have been made in the areas of aetiology, detection, diagnosis, prevention, and treatment of cancer using cell culture models.

Many fundamental questions on growth regulation, cell differentiation and cell structure and behaviour, which are necessary for our understanding of malignant transformation, cannot easily be investigated in vivo because of the inherent complexities of the system. In vitro systems provide the opportunity to dissect and separate the various actions and interactions and enable the specific actions on the target cell to be pinpointed.

The four prostate cancer cell lines and one subline have been well characterized and until recently, the LNCaP cell line was the only human prostate cancer cell line to demonstrate androgen dependence.

The five non-tumorigenic prostate cell lines, comprising of the RWPE-1, PWr-1E, HPr-1, HPr-1AR and PNT-2, were included as additional sources of
prostate cells and allowed comparison in relation to the distribution of CAG repeat numbers. These cell lines were obtained as gifts from colleagues in a collaborating laboratory.

4.2.2 DNA extraction

The quality of nucleic acid extraction from the ten cell lines was judged by the absorbance ratio. The DNA obtained was reasonably pure, with ratios ranging from 1.75 to 1.88 (a pure DNA solution has an OD$_{260}$/OD$_{280}$ value $\geq$ 1.8).

4.2.3 CAG repeat number of cell Line DNA – results of fragment analysis

From the GeneScan electropherograms, and using the GeneMapper software (Applied Biosystems), the CAG repeat allele was identified without difficulty in all the ten cell lines.

4.2.4 HPr-1 and HPr-1AR cell lines

The presence of 2 CAG repeat fragments in the HPr-1AR cell line was an expected result because the additionally transfected AR gene in this cell line meant that there were two AR genes and, therefore, two CAG repeat fragments. The larger CAG repeat (21 repeats) of the HPr-1AR cell line corresponded with the CAG repeat number of the parental cell line, HPr-1, confirming the authenticity of the result.
4.2.5 LNCaP and LNCaP-HOF cell lines

The comparison of CAG repeats between the parental LNCaP cell line and the androgen-independent subline, LNCaP-HOF, is the most significant result of the cell line work. The LNCaP-HOF subline became androgen-independent following growth in an androgen-deprived environment, thus mimicking the in-vivo scenario of patients with prostate cancer being treated with androgen-ablative therapy, with the eventual progression to androgen-independent, hormone-escape disease.

The shortening of CAG repeats in the androgen-independent subline represents an acquired mutation as a direct consequence of androgen-deprivation. It validated the hypothesis that CAG shortening occurs as a result of androgen-ablation.

This finding offered a possible explanation for the mechanism by which prostate cancer progresses to androgen-independence and provided the basis for examining the CAG repeat number in patients who had developed androgen independent prostate cancer.
CHAPTER 5

Analysis of CAG Repeat Number in Patients with Androgen Independent Prostate Cancer
Chapter 5

5.1 OVERVIEW

Having established and optimised the various techniques for DNA extraction, PCR amplification and GeneScan short tandem repeat (STR) analysis using cell line DNA, and having shown that CAG shortening occurred as a result of androgen-ablation in the cell lines LNCaP and LNCaP-HOF, the next objective was to determine if CAG shortening occurred in patients’ DNA samples after a period of androgen ablation therapy.

5.2 PATIENT SELECTION

The pathology laboratory databases and the medical records were reviewed and patients that met the inclusion criteria as outlined in Chapter 3.2.1 were recruited. At the outset, 50 patients met the inclusion criteria and were recruited. Thirty-one of these patients were subsequently excluded because of degradation of DNA (see Section 3.11). The expected amplicon size for the CAG repeat fragment was between 200-300 basepairs. Therefore, it was not possible to amplify the CAG repeat fragment from archival samples with severely degraded and fragmented DNA. DNA degradation was confirmed using a specimen control size ladder, an unlabelled PCR-based assay (InVivoScribe Technologies, Marseille, France) which targeted multiple genes and generated a series of amplicons of 100, 200, 300, 400 and 600 base pairs to ensure that the quality and quantity of input DNA was adequate to yield a valid
result. Figure 5-1 is an electropherogram of a sample with good quality DNA, with peaks at 100, 200, 300 and 400 basepairs. Figure 5-2 is an example of an electropherogram of a sample with degraded and fragmented DNA with a peak at 100 basepairs only.

**Figure 5-1.** An electropherogram of a sample with good quality DNA. Amplicons of 100, 200, 300 and 400 basepairs were generated by the size standard control ladder (as indicated by blue coloured peaks at the respective points on the x-axis).
Figure 5-2. An electropherogram of a sample with degraded and fragmented DNA such that the only size that could be amplified using the control PCR was 100bp and no evidence of peaks at the other sizes.

The exclusion of patients whose material was unsuitable resulted in the final patient number recruited to the study being reduced to 19 patients. The demographic and clinical data of these patients were collected and are
summarised in Table 5-1. All 19 patients were Caucasian, and all patients were treated with androgen ablation in the form of an initial four to six week period of anti-androgen, during which time, a depot (either monthly or three-monthly) luteinizing hormone-releasing hormone analogue (LHRHa) was commenced.

The mean, median and ranges for all clinical parameters are summarised in Table 5-2.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age (years)</th>
<th>Surgical interval (months)</th>
<th>PSA pre-treatment</th>
<th>PSA nadir</th>
<th>PSA post-treatment</th>
<th>Therapy</th>
<th>Race</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68</td>
<td>16</td>
<td>32</td>
<td>14</td>
<td>104</td>
<td>LHRHa</td>
<td>Caucasian</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>3</td>
<td>26</td>
<td>6</td>
<td>44</td>
<td>LHRHa</td>
<td>Caucasian</td>
</tr>
<tr>
<td>3</td>
<td>73</td>
<td>31</td>
<td>40</td>
<td>7.6</td>
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<td>LHRHa</td>
<td>Caucasian</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>37</td>
<td>18</td>
<td>3.9</td>
<td>89.4</td>
<td>LHRHa</td>
<td>Caucasian</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>12</td>
<td>31</td>
<td>7.2</td>
<td>150</td>
<td>LHRHa</td>
<td>Caucasian</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>7</td>
<td>30.5</td>
<td>27.8</td>
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<td>LHRHa</td>
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<td>16.1</td>
<td>7.4</td>
<td>61.2</td>
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<td>5</td>
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<td>12.2</td>
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<td>9</td>
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<td>4.3</td>
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<td>28</td>
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<td>LHRHa</td>
<td>Caucasian</td>
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</table>

Table 5-1. Demographic and clinical data are summarized in this table. Age = age of patients (years) at diagnosis; Surgical interval = time interval (months) between two TURPs; PSA pre-treatment = PSA level (ng/ml) prior to commencement of androgen-ablation therapy; PSA nadir = lowest documented PSA level (ng/ml) following commencement of androgen-ablation therapy; PSA post-treatment = PSA level (ng/ml) when patient refractory to androgen-ablation just prior to second TURP; Therapy = choice of androgen-ablation therapy; LHRHa = luteinizing hormone-releasing hormone analogue.
Chapter 5 Analysis of CAG Repeat Number in Patients with Androgen Independent Prostate Cancer

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<th>Median</th>
<th>Range</th>
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</thead>
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</tr>
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<td>PSA post-treatment</td>
<td>86</td>
<td>74</td>
<td>22 – 209</td>
</tr>
</tbody>
</table>

Table 5-2. Mean, median and range of the various demographic and clinical parameters listed in Table 5-1. PSA levels in ng/ml.

5.3 LASER CAPTURE MICRODISSECTION

All histology slides were prepared as described in Chapter 3 and areas with prostate cancer and areas with benign prostate glands were marked on the cover-slipped slide by a pathologist. The uncovered slides were used for LCM, and malignant glands and benign glands were selectively dissected from corresponding areas as marked in the cover-slipped slide. A representative set of photographs of the slides used are shown in Figure 5-1. All other available photographs are provided in Appendix 1.

Figure 5-3. All three photographs are from Patient Number 1, from the TURP prior to androgen ablation therapy (pre-treatment). Photograph A was the slide before laser capture.
microdissection was performed. Photograph B was the slide with the remaining tissue after the captured material was selectively microdissected and removed. Photograph C is of the LCM cap with the captured material that was subsequently used for DNA extraction.

5.4 CALCULATING CAG REPEAT NUMBER

The CAG repeat length was calculated by subtracting the constant region (200 basepairs) from the total allele length (Figure 3.15). The CAG repeat number was a third of the CAG repeat length (Table 5-3).

5.5 FRAGMENT ANALYSIS

GeneScan software (version 3.7) was used to analyse the PCR amplified products. Electropherograms were generated by the software – the size standard peaks appeared in red and the sample peaks appeared in blue. Primer dimers, a side effect of nested PCR, appeared in the 0-50 basepair range, and were recognised by the software as being artefacts (Figure 5-4).
5.6 **3'-A NUCLEOTIDE ADDITION**

Many DNA polymerases catalyse the addition of a single nucleotide (usually an adenosine) to the 3' ends of the two strands of a double-stranded DNA fragment. This non-template complementary addition results in a denatured PCR product that is one nucleotide longer than the target sequence. A PCR product containing the extra nucleotide is referred to as the “plus-A” form.

This “plus-A” form may have been present in small quantities, accounting for some of the tiny peaks visible immediately adjacent to the main...
peak (Figure 5-5). However, the software was able to make a consistent allele call for each of the samples studied.

Figure 5-5. An example of an electropherogram showing tiny “plus-A” peaks adjacent to the main peak (at the 269 basepair mark). The main (tallest) peak in the region between 250-300 basepairs is that of the CAG repeat fragment.

5.7 STUTTER PRODUCTS

During PCR amplification of dinucleotide, trinucleotide and tetranucleotide microsatellite loci, minor products that are 1-4 repeat units shorter than the main allele are produced. The minor product peaks are referred to as “stutter” peaks (Figure 5-6). Stutter peaks might be caused by polymerase slippage during elongation.
Chapter 5 Analysis of CAG Repeat Number in Patients with Androgen Independent Prostate Cancer

Figure 5-6. An example of an electropherogram showing "stutter products" adjacent to the main blue peak in the 250-300 basepair range.

The longer the length of the repeat unit, the less stutter product is made. Among microsatellite loci with the same number of repeat units, the percentage of stutter is dinucleotide loci > trinucleotide microsatellite loci > tetranucleotide microsatellite loci > pentanucleotide microsatellite loci (Walsh, Fildes et al. 1996).

The stutter peaks were also visible in some of the electropherograms of the samples studied, but again, the software was able to reliably and consistently distinguish stutter peaks from the allele itself.
Chapter 5 Analysis of CAG Repeat Number in Patients with Androgen Independent Prostate Cancer

5.8 CAG SHORTENING OBSERVATIONS & DATA ANALYSIS

Seven of nineteen (36.8%) patients (Patient numbers 1, 3, 7, 9, 12, 14 and 19) had shortening of the CAG repeat number in post-androgen ablation therapy (post-treatment) malignant tissue as compared with tissue prior to androgen ablation (pre-treatment) (see Table 5-4). Figures 5-7 and 5-8 are examples of the electropherograms demonstrating CAG repeat shortening. Using a non-parametric signed rank test, the number of patients in which CAG shortening occurred is statistically significant (p=0.008). Four patients had CAG shortening of two repeats and one patient each had CAG shortening by one, three and ten repeats (Table 5-4). Where benign tissue was available, the CAG repeat number of the benign tissue was compared. For several patients (Patient numbers 1, 4, 5, 6, 7, 9, 10, 11, 12, 14, 16 and 18), benign tissue was not available (marked ‘x’ in Table 5-3) because all the prostate chippings were completely replaced with tumour.

There was no shortening in either the pre-androgen ablation benign tissue or post-androgen ablation benign tissue. Furthermore, there were no differences between the CAG repeat numbers of the pre-androgen ablation malignant tissue compared with the corresponding benign controls.
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<th>Patient Number</th>
<th>Treatment status</th>
<th>Histology</th>
<th>Allele length (basepairs)</th>
<th>CAG repeats</th>
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*Table continues on next page.*

107
### Table 5.3

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<th>Histology</th>
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<tr>
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<td>Malignant</td>
<td>269</td>
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</tbody>
</table>

Table 5.3: The allele size for each sample as determined by GeneScan. X = no benign tissue available. ‘Pre’ treatment status = before the introduction of androgen-ablative therapy; ‘Post’ treatment status = androgen-independent samples after the introduction of androgen-ablative therapy. Patients showing CAG shortening are shaded in grey.
Chapter 5 Analysis of CAG Repeat Number in Patients with Androgen Independent Prostate Cancer

Figure 5-7. The electropherogram (zoomed) for Pre-androgen ablation therapy malignant tissue of Patient number 9. The size standard peaks are shown in red and the allele peak (263bp, 21 CAG repeats) is shown in blue (marked with an arrow). The blue peak adjacent to the allele peak represents a stutter peak.

Figure 5-8. The electropherogram (zoomed) for Post-androgen ablation therapy malignant tissue of Patient number 9. The size standard peaks are shown in red and the allele peak is shown in blue (233bp, 11 CAG repeats).
Table 5-4. This table summarizes the CAG repeat numbers for the 7 patients with CAG shortening. n/a = not available. CAG number Pre-treatment = CAG triplet repeat number before the introduction of androgen ablation therapy. CAG number Post-treatment = CAG triplet repeat number at androgen-independent/hormone-refractory stage, following androgen ablation therapy. For each group, pre-treatment and post-treatment, the CAG repeat number is listed for benign tissue (controls) and prostate cancer tissue (malignant). CAG shortening = Pre-treatment CAG repeat number – Post-treatment CAG repeat number.

5.8.1 CAG shortening and age

The data was sub-analysed to investigate the association between CAG shortening and age (Table 5-5). The median and mean age at diagnosis was 76 years (range 51 to 88). The Kruskal-Wallis test showed that there was no statistically significant association between the age of the patients at initial diagnosis and CAG shortening (p=0.97).
5.8.2 CAG shortening and Gleason score

An association between CAG shortening and Gleason score was also assessed (Table 5-6). There was no statistically significant association between CAG shortening and pre-androgen ablation therapy Gleason score (p=0.22). There was, however, a statistically significant association between CAG shortening and the Gleason score of malignant tissue following androgen ablation therapy (p=0.016). Patients with a high Gleason score following androgen ablation therapy (Gleason score 8-10) were more likely to have CAG shortening. There was no statistically significant association between CAG
shortening and the change in Gleason scores from pre-treatment and post-
treatment tissue (p=0.65).

<table>
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<th>GS Post-Tx</th>
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Table 5-6. CAG shortening and Gleason score. GS Pre-Tx = Gleason score of malignant tissue prior to commencement of androgen ablation therapy. GS Post-Tx = Gleason score of malignant tissue at androgen-independence, following androgen ablation therapy. Patients with CAG shortening are shaded in grey.

5.8.3 CAG shortening and PSA levels

The PSA values at the time of diagnosis, the PSA nadir and the peak PSA just prior to undergoing TURP for symptom relapse (with clinical and biochemical evidence of androgen-independent disease relapse) were analysed (see Table 5-7). There was no statistically significant association between CAG shortening and the PSA at initial diagnosis (p=0.40), CAG shortening and the
Chapter 5 Analysis of CAG Repeat Number in Patients with Androgen Independent Prostate Cancer

PSA nadir (p=0.33) and CAG shortening and the PSA following androgen ablation therapy (p=0.20).

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<td>7.4</td>
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<td>12.2</td>
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<tr>
<td>9</td>
<td>22</td>
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<td>94</td>
<td>10</td>
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<tr>
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<td>5.2</td>
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</table>

Table 5-7. CAG shortening and PSA values (ng/ml). PSA pre-Tx = PSA at diagnosis of prostate cancer prior to commencement of androgen ablation therapy. PSA nadir = lowest recorded PSA following commencement of androgen ablation therapy. PSA post-Tx = PSA following androgen ablation therapy, prior to undergoing subsequent TURP (androgen-independent disease relapse). Patients with CAG shortening are shaded in grey.

5.8.4 CAG shortening and time to androgen-independence

The mean time to androgen-independence was 18.2 months (3-41 months). There was no statistically significant association between CAG
shortening and the time interval between the two TURPs (p=0.10) (see Table 5-8).

<table>
<thead>
<tr>
<th>Patient number</th>
<th>interval between 2 TURPs</th>
<th>CAG shortening</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
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<tr>
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<td>2</td>
</tr>
<tr>
<td>4</td>
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</tr>
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<tr>
<td>19</td>
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</table>

Table 5-8. CAG shortening and interval between the 2 TURPs (months). Patients with CAG shortening are shaded in grey.
5.9 DISCUSSION

5.9.1 Patient Selection

Fifty patients met the inclusion criteria and were recruited to the study. However, formalin-fixed, paraffin-embedded samples for 31 patients were unsuitable because their DNA was fragmented into fragments of less than 200 basepairs. Therefore, it was not possible to amplify the CAG repeat fragment, where the expected amplicon size was between 200-300 basepairs. A major problem with any fixed and processed solid tissue is the degradation of nucleic acids which could obscure its use for molecular analysis. In formalin-fixed tissue, several factors affect degradation of DNA including the duration of fixation, pH, salt concentration and temperature (Ben-Ezra, Johnson et al. 1991; Karlsen, Kalantari et al. 1994; Srinivasan, Sedmak et al. 2002).

Unbuffered formalin oxidises to formic acid and the resultant acidic environment causes degradation of nucleic acids because the β-glycosidic bonds in the purine bases are hydrolysed at pH 4. Furthermore, unbuffered formalin is known to break DNA into small fragments compared with buffered formalin (Ben-Ezra, Johnson et al. 1991; Karlsen, Kalantari et al. 1994; Frayling 2002; Srinivasan, Sedmak et al. 2002). The archival materials for all the excluded 31 patients were processed prior to 1999, when unbuffered formalin was used routinely in the pathology laboratory. Subsequent to that era, the pathology laboratories in St. James’s and AMNCH used buffered formalin to facilitate the use of molecular pathology analysis in routine biopsy material.
All samples (19 patients) that were processed after 1999 and fixed in buffered formalin were suitable for DNA extraction and PCR amplification.

The small sample size of 19 patients is recognized as a limitation of this study; however, the use of laser capture microdissection facilitated the enrichment of cells of interest from the tissue sections and went some way to offset this limitation. Extrapolating from the results from this study to the general population is made cautiously, recognizing the limitation of the small sample size.

5.9.2 CAG Shortening

CAG shortening occurred in a significant number of patients (9/17 patients, 36.8%) following androgen ablation therapy. It has been observed that the incidence of prostate cancer in African-Americans, Caucasians and Asians inversely correlate with the average CAG repeat lengths for these ethnic groups (Caskey, Pizzuti et al. 1992; Irvine, Yu et al. 1995). African-Americans have the highest prostate cancer risk and have shorter CAG repeat lengths, whilst the reverse is true for Asians (Hayes, Liff et al. 1995; Farkas, Marcella et al. 2000; Bennett, Price et al. 2002). In this study, there was no shortening in either the pre-androgen ablation benign tissue or post-androgen ablation benign tissue, which supports the hypothesis that the decrease in CAG repeat number represents a mutation in the AR gene in prostate cancer cells as they progress to
androgen independence. Furthermore, there were no differences between the CAG repeat numbers of the pre-androgen ablation malignant tissue compared with the corresponding benign controls. This suggests that any shortening observed in the post-androgen ablation malignant samples were related to androgen ablation therapy. The finding of this study of CAG repeat length shortening in patients suggests that this trinucleotide repeat also plays a role in disease progression.

Several studies have shown that shorter CAG repeat lengths correlated with greater AR transcriptional activity (Chamberlain, Driver et al. 1994; Choong, Kemppainen et al. 1996). A limitation of this study is the lack of confirmation that the CAG shortening observed here resulted in an increase in AR transcription. This was an assumption made on the strength of conclusive reports from other studies (Chamberlain, Driver et al. 1994; Choong, Kemppainen et al. 1996). Allowing for that assumption, it is possible that CAG repeat shortening following androgen ablation therapy, as observed in this study, conferred a survival advantage to prostate cancer cells that had become androgen independent.

5.9.3 Association Between Clinical Parameters And CAG Shortening

Analysis of the data showed that age at diagnosis, Gleason score prior to commencing androgen ablation therapy (pre-treatment), PSA level at the time
of diagnosis, the PSA nadir, PSA at the time of androgen-independence and the
time to progression to androgen-independence were not associated with CAG
shortening. The absence of association between these clinical parameters and
CAG shortening removed them as confounders and further supports the
hypothesis that the observed CAG shortening was as a result of androgen-
ablation. The time interval between the two TURPs reflected the rate of
development of androgen-independence because recurrence of bladder outflow
obstructive symptoms that warranted a re-do TURP heralded re-growth of
prostate tissue. For re-growth to occur despite androgen ablation therapy
suggested that re-growth of the prostate was occurring in an androgen-
independent manner. The Gleason score of androgen-independent prostate
cancer tissue was associated with CAG shortening in that patients with a higher
Gleason score (Gleason score >8) were more likely to have undergone CAG
repeat contraction (p=0.016). However, this observation may be biased because
it has been shown that androgen ablation therapy causes histological changes
that mimic those of high-grade disease (Smith and Murphy 1994; Civantos,
Soloway et al. 1996; Bostwick 1998; Yang, Lecksell et al. 1999).
CHAPTER 6

Summary & Future Directions
Chapter 6

6.1 OVERVIEW

One of the most important areas in prostate cancer is the terminal end of the disease spectrum, i.e. androgen-independent disease. Whilst the disease is being detected at an earlier stage in more men, a significant proportion of men will have advanced disease at diagnosis. Furthermore, about a third of men that have apparently early prostate cancer, and are treated with curative intent, will relapse and eventually progress to end-stage prostate cancer. Most prostate cancers will respond to hormonal manipulation, at least initially. However, progression to androgen-independence is inevitable. Because the survival of patients after hormone escape is poor and second line therapies are largely ineffective and short lived, identification of the molecular mechanisms associated with disease progression is an important goal. To this end, several hypotheses of treatment failure have been proposed over the years, involving both androgen-dependent and androgen-independent mechanisms. This study focused on exploring the androgen-independent mechanism, in particular, the AR as a possible target for a molecular event that might trigger the transition to a hormone-refractory state. The working hypothesis was that androgen ablation causes shortening of the CAG repeat number within the AR.
6.2 CHARACTERISING THE CAG REPEAT NUMBER IN A PANEL OF CELL LINES

The aims of including a panel of cell lines were to optimise the techniques required for cell culture, DNA extraction, PCR and fragment analysis, which were then utilised to determine the CAG repeat number in the assembled panel of cell lines. To this end, ten prostate cell lines were selected, comprising five malignant cell lines and five non-tumorogenic cell lines.

Using a panel of cell lines rather than merely the two cell lines of primary interest (LNCaP and LNCaP-HOF), the polymorphic nature of the CAG repeats was demonstrated, with repeat numbers ranging from 10 to 24. Analysis of this panel also served to demonstrate that techniques employed were reliable in isolating and amplifying the CAG segment, as well as the results being consistent and reproducible.

6.3 CAG SHORTENING IN LNCaP-HOF CELL LINE

The LNCaP-HOF cell line is an androgen-independent subline that was derived from the more well-known androgen-dependent LNCaP cell line. Having been established by growth of LNCaP cells in androgen-deprived medium through many passages, the eventual LNCaP-HOF cell mimics in-vivo androgen-independent prostate cancer cells that emerge after androgen ablation therapy.
The LNCaP-HOF cell line had a fourfold higher expression of AR and a 30-fold increase in AR transcriptional activity compared with the parental LNCaP line (Culig, Hoffman et al. 1999).

The LNCaP-HOF line demonstrated a shorter amplicon both visibly by gel electrophoresis and analytically through GeneScan analysis. This shortening translates to a difference of three CAG trinucleotide repeats. The finding of CAG shortening in the LNCaP-HOF line compared with the parental LNCaP line was significant in validating the hypothesis of the study that CAG shortening occurs as a result of androgen ablation. It validated pursuing the second phase of the study, which was to explore this phenomenon in patients, in search for an explanation for the development of androgen independence in some patients.

6.4 CAG SHORTENING AND PROGRESSION TO ANDROGEN INDEPENDENCE

The CAG repeat length was shortened following androgen ablation therapy in a statistically significant number of patients in this study (7/19 patients; i.e. Patient Numbers 1, 3, 7, 9, 12, 14 and 19; p=0.008). None of the clinical parameters, age at diagnosis, various Gleason scores and PSA levels at the various time-points, were associated with CAG shortening. They did not
influence the change in trinucleotide repeat number, validating the hypothesis that CAG shortening occurred as a consequence of androgen ablation.

The CAG microsatellite has been the focus of many studies that examined its role in prostate cancer risk. However, the role of the CAG microsatellite in disease progression had not been previously reported. It is yet to be established what the role of the androgen receptor is in the acquisition of the androgen-independent phenotype. However, this study has shown that contraction of the CAG microsatellite may be responsible for disease progression in a subgroup of patients.

Under androgen-deprived conditions associated with the hormonal manipulation for prostate cancer, the AR is present in an unliganded form, and therefore, is unable to give the appropriate growth-stimulation signal, but instead, brings about cell death by apoptosis. In the hormone-refractory state, the apoptotic signals no longer occur, with resulting tumour relapse and the inevitable demise of the patient. Several potential pathways by which the AR can directly influence the switch to androgen-independence in prostate cancer have been summarised in Table 1-3. The results of this study support the pathway of an alteration in the AR. Clearly if AR mutations, such as the one described here, appear in advanced prostate cancer, there is potential for these cells to have a growth advantage within the androgen-deprived environment generated by that treatment.
6.5 LIMITATIONS AND STRENGTHS

A number of limitations of this study have been recognised. The small sample size of 19 patients precludes the application of this study to the general population. The difficulties encountered with regards the exclusion of 31 patients because of unusable DNA were discussed in 5.9.1. The constraints of time did not allow for prospective recruitment of further patients to increase the sample size. Nonetheless, the results achieved statistical significance with a sample size of 19 patients.

No data on serum testosterone levels was available, and therefore, it was not possible to confirm that serum testosterone was at castrate levels in all patients. However, all patients were treated with maximum androgen ablative therapy.

A further limitation of this study is the lack of confirmation that the CAG shortening observed here resulted in an increase in AR transcription as discussed in 5.9.2. Time constraints forced an assumption to be made on the strength of conclusive reports from other studies (Chamberlain, Driver et al. 1994; Choong, Kemppainen et al. 1996).

The use of prostate tissue subsequent to the proof of principle established with cell lines confers significant weight to the study. Furthermore,
this is the first study evaluating the role of CAG repeat length in disease progression, and in particular, in an Irish population.

The use of laser capture microdissected material ensured that the tissue procured was pure cell populations of benign and malignant prostate epithelial tissue, while maintaining cellular and nucleic acid integrity necessary for analysis.

Furthermore, the use of in-situ controls (i.e. each patient’s own benign prostate tissue) eliminated the chance that the CAG shortening were due to factors other than androgen ablation.

6.6 FUTURE DIRECTIONS

The precise molecular mechanisms that drive prostate cancer progression remain unclear, and identification of these pathways will continue to be an important goal.

The AR remains a fundamental mediator of growth in AIPC. Further understanding of the role of the AR could lead to the development of strategies to prolong the response period of androgen-ablation, the cornerstone of treatment of advanced prostate cancer. Until more effective treatments are developed or second-line therapies with sustainable survival advantages are
designed, prolonging the androgen-responsive state is paramount. The platform for these strategies lies in understanding the mechanism that propels AIPC. Defining the pathways to progression and understanding the mechanisms that drive it could also facilitate the development of alternative treatment pathways and more effective treatments for this group of patients.

There are various mechanisms proposed for the development of AIPC. This study suggests one such mechanism, i.e., shortening of the CAG repeat length may have driven disease progression to the androgen-independent state and conferred a growth advantage in the seven patients in this study.

Although the inverse relationship between CAG repeat length and AR transcriptional activity has been established in other studies, future work could aim to verify this observation in the cohort of patients from this study.

Further work stemming from this study should be to examine other trinucleotide repeats, e.g. the GGC triplet repeat, that lies closer to the DNA binding domain of the AR, and codes for the amino acid glycine. The transactivation domain of the AR clearly has an important function in modifying androgen receptor activity. Further understanding of this domain and to possible naturally occurring mutations and polymorphisms is invaluable toward the development of better therapeutic strategies.
On a wider scale, there are a number of areas for future work on the androgen-independent end of the cancer spectrum. These include the role of tumour hypoxia in cancer progression may emerge as an important area, as hypoxic tumours have elevated rates of mutagenesis and genetic instability. Alternative pathways for disease progression, could be a focus of future work, including the 'promiscuous receptor' which allows the AR to be activated by nonandrogenic steroids, or activation of downstream signalling of the AR by ligand-independent mechanisms.

Increasing the survival in patients with AIPC is the ultimate aim. Identifying and understanding the different mechanisms by which prostate cancer cell become resistant to androgen ablation will lead to a multifaceted approach to developing novel therapies for the treatment of AIPC.


Berns, E. M., W. de Boer, et al. (1986). "Androgen-dependent growth regulation of and release of specific protein(s) by the androgen receptor
containing human prostate tumor cell line LNCaP." Prostate 9(3): 247-59.

Berry, R., D. J. Schaid, et al. (2000). "Linkage analyses at the chromosome 1 loci 1q24-25 (HPC1), 1q42.2-43 (PCAP), and 1p36 (CAPB) in families with hereditary prostate cancer." Am J Hum Genet 66(2): 539-46.


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prostate cancer: a Canadian randomized trial with palliative end points."


Appendix 1

Laser Capture Microdissection Images
LASER CAPTURE MICRODISSECTED IMAGES

Microscopically magnified photographs of histology slides stained with H&E stain that were used for LCM were taken for a number of patients to illustrate the concept of LCM. Photographs were taken of benign and malignant glands on the slide that were selectively microdissected using LCM. A photograph was taken of the slide prior to LCM (labelled before LCM), of the remaining slide after the selected area had been microdissected off (labelled after LCM) and of the material laser captured on the LCM cap (labelled captured material). Benign (where available) and malignant tissue was obtained from slides of TURPs prior to commencement of androgen ablation therapy (labelled pre-treatment) and from slides of TURPs following androgen ablation therapy (labelled post-treatment).

Before LCM  After LCM  Captured material

Patient number 1. Pre-treatment malignant tissue.
Appendix 1

**Patient number 1.** Post-treatment malignant tissue.

**Patient number 8.** Pre-treatment benign tissue.

**Patient number 8.** Pre-treatment malignant tissue.

**Patient number 8.** Post-treatment benign tissue.
Appendix 1

Patient number 19. Post-treatment malignant tissue.
APPENDIX 2

Electropherograms
### Appendix 2

| Patient 1 | Pre-treatment malignant tissue | PCR reference = 2657T | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph](image-url)
Appendix 2

| Patient 1 | Post-treatment malignant tissue | PCR reference = 6201T | Amplicon size = 254 bp | Number of CAG repeats = 18 |
| Patient 2 | Pre-treatment benign tissue | PCR reference = 3907B | Amplicon size = 266 bp | Number of CAG repeats = 22 |

![Graph showing the data provided in the table.](image-url)
| Patient 2 | Pre-treatment malignant tissue | PCR reference = 3907T | Amplicon size = 266 bp | Number of CAG repeats = 22 |

![Graph showing gene expression analysis](image-url)
| Patient 2 | Post-treatment benign tissue | PCR reference = 6695B | Amplicon size = 266 bp | Number of CAG repeats = 22 |

![DNA sequencing result](image-url)
### Appendix 2

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<table>
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![Graph showing data](image)
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</table>

![Graph showing genetic analysis](image-url)
Appendix 2

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</thead>
</table>

![Graphical representation of data](image)
## Appendix 2

| Patient 4 | Pre-treatment malignant tissue | PCR reference = 5640T | Amplicon size = 269 bp | Number of CAG repeats = 23 |

![Graph showing the analysis of the pre-treatment malignant tissue with PCR reference, amplicon size, and number of CAG repeats.]
**Appendix 2**

| Patient 4 | Post-treatment malignant tissue | PCR reference = 8556T | Amplicon size = 269 bp | Number of CAG repeats = 23 |

![Graph showing a peak at 70 cycles with a molecular weight of 269 bp and 23 CAG repeats.](image-url)
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![Graph showing genetic data](image-url)
| Patient 5 | Post-treatment malignant tissue | PCR reference = 2997T | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph showing DNA profiling results](image-url)
Appendix 2

| Patient 6 | Pre-treatment benign tissue | PCR reference = 3436B | Amplicon size = 272 bp | Number of CAG repeats = 24 |

![Graph](image-url)
Appendix 2

| Patient 6 | Pre-treatment malignant tissue | PCR reference = 3436T | Amplicon size = 272 bp | Number of CAG repeats = 24 |

![Graph showing the pre-treatment malignant tissue analysis](image)
## Appendix 2

<table>
<thead>
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![Graph](image-url)
### Appendix 2

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</tr>
</thead>
</table>

![Graph showing allele peaks](image-url)
| Patient 7 | Pre-treatment malignant tissue | PCR reference = 4090T | Amplicon size = 269 bp | Number of CAG repeats = 23 |

[Diagram of a gel electrophoresis result showing bands at positions 250, 300, 350, 400, and 450 bp.]
### Appendix 2

| Patient 7 | Post-treatment malignant tissue | PCR reference = 4652T | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph showing the results of PCR analysis for Patient 7's post-treatment malignant tissue.](image-url)
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</table>

![Graph showing PCR results with peaks at different amplicon sizes.](image)
Patient 8  Pre-treatment malignant tissue  PCR reference = 11435T  Amplicon size = 242 bp  Number of CAG repeats = 14
Appendix 2

| Patient 8 | Post-treatment benign tissue | PCR reference = 2136B | Amplicon size = 242 bp | Number of CAG repeats = 14 |
### Appendix 2

| Patient 8 | Post-treatment malignant tissue | PCR reference = 2136T | Amplicon size = 242 bp | Number of CAG repeats = 14 |

![Graph](image-url)
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<th>Patient</th>
<th>Pre-treatment benign tissue</th>
<th>PCR reference = 967B</th>
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</tr>
</thead>
</table>

![Graph showing a peak at around 275 bp, with a smaller peak at 300 bp and a baseline at 400 bp.](image-url)
| Patient 9 | Pre-treatment malignant tissue | PCR reference = 967T | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph showing DNA analysis results](image-url)
### Appendix 2

<table>
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<tr>
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![Graph](image-url)
<p>| Patient 10 | Pre-treatment benign tissue | PCR reference = 3276B | Amplicon size = 254 bp | Number of CAG repeats = 18 |</p>
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![Graph showing DNA analysis results]
| Patient 10 | Post-treatment malignant tissue | PCR reference = 3801T | Amplicon size = 254 bp | Number of CAG repeats = 18 |

![Graph showing the data for Patient 10.](image-url)
Appendix 2

<p>| Patient 11 | Pre-treatment malignant tissue | PCR reference = 516T | Amplicon size = 257 bp | Number of CAG repeats = 19 |</p>
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![Graph showing DNA analysis results.

186
Appendix 2

| Patient 12 | Pre-treatment benign tissue | PCR reference = 8447B | Amplicon size = 266 bp | Number of CAG repeats = 22 |

[Graph showing genetic analysis results]
<table>
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<th>Patient 12</th>
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![Graph showing results of PCR analysis](image_url)
| Patient 12 | Post-treatment malignant tissue | PCR reference = 3424T | Amplicon size = 260 bp | Number of CAG repeats = 20 |

![Graph showing genetic analysis results](image-url)
Appendix 2

| Patient 13 | Pre-treatment benign tissue | PCR reference = 8794B | Amplicon size = 254 bp | Number of CAG repeats = 18 |
Patient 13 | Pre-treatment malignant tissue | PCR reference = 8794T | Amplicon size = 254 bp | Number of CAG repeats = 18
| Patient 13 | Post-treatment benign tissue | PCR reference = 6398B | Amplicon size = 254 bp | Number of CAG repeats = 18 |

![Graph showing the results of the PCR analysis for Patient 13.](image-url)
| Patient 13 | Post-treatment malignant tissue | PCR reference = 6398T | Amplicon size = 254 bp | Number of CAG repeats = 18 |

![Graph showing DNA analysis results](image-url)
| Patient 14 | Pre-treatment malignant tissue | PCR reference = 113T | Amplicon size = 269 bp | Number of CAG repeats = 23 |
Patient 14 | Post-treatment benign tissue | PCR reference = 10771B | Amplicon size = 269 bp | Number of CAG repeats = 23
| Patient 14 | Post-treatment malignant tissue | PCR reference = 10771T | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph showing DNA sequence analysis with peaks at specific nucleotide positions.](image-url)
| Patient 15 | Pre-treatment benign tissue | PCR reference = 5515B | Amplicon size = 257 bp | Number of CAG repeats = 19 |

---

![Graph showing the number of CAG repeats](image-url)
| Patient 15 | Pre-treatment malignant tissue | PCR reference = 5515T | Amplicon size = 257 bp | Number of CAG repeats = 19 |

![Graph showing genetic analysis results for Patient 15](image-url)
### Appendix 2

<table>
<thead>
<tr>
<th>Patient 15</th>
<th>Post-treatment benign tissue</th>
<th>PCR reference = 6742B</th>
<th>Amplicon size = 257 bp</th>
<th>Number of CAG repeats = 19</th>
</tr>
</thead>
</table>

![Graph showing the analysis of DNA samples](image-url)
| Patient 15 | Post-treatment malignant tissue | PCR reference = 6742T | Amplicon size = 257 bp | Number of CAG repeats = 19 |

![Graph showing molecular analysis results]
Appendix 2

| Patient | Pre-treatment malignant tissue | PCR reference = 12242T | Amplicon size = 263 bp | Number of CAG repeats = 21 |
| Patient 16 | Post-treatment malignant tissue | PCR reference = 6216T | Amplicon size = 263 bp | Number of CAG repeats = 21 |
| Patient 17 | Pre-treatment benign tissue | PCR reference = 1284B | Amplicon size = 263 bp | Number of CAG repeats = 21 |
### Appendix 2

| Patient 17 | Pre-treatment malignant tissue | PCR reference = 1284T | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph showing DNA analysis results for Patient 17](image)
| Patient 17 | Post-treatment malignant tissue | PCR reference = 9882 | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph showing post-treatment malignant tissue analysis]
| Patient 18 | Pre-treatment malignant tissue | PCR reference = 5995T | Amplicon size = 263 bp | Number of CAG repeats = 21 |

![Graph showing genetic analysis results for Patient 18.](image-url)
| Patient 18 | Post-treatment malignant tissue | PCR reference = 5835T | Amplicon size = 263 bp | Number of CAG repeats = 21 |
Patient 19 | Pre-treatment benign tissue | PCR reference = 6692B | Amplicon size = 269 bp | Number of CAG repeats = 23
| Patient 19 | Pre-treatment malignant tissue | PCR reference = 6692T | Amplicon size = 269 bp | Number of CAG repeats = 23 |

![Graph showing DNA sequencing data with peaks at specific bases. Peaks are labeled with DNA base sequences.](image-url)
<table>
<thead>
<tr>
<th>Patient 19</th>
<th>Post-treatment benign tissue</th>
<th>PCR reference = 459B</th>
<th>Amplicon size = 269 bp</th>
<th>Number of CAG repeats = 23</th>
</tr>
</thead>
</table>

![Graph showing genetic analysis results](image-url)
| Patient 19 | Post-treatment malignant tissue | PCR reference = 459T | Amplicon size = 266 bp | Number of CAG repeats = 22 |

![Graph](image-url)
Appendix 3

Statistical data
Appendix 3

CAG SHORTENING OBSERVED IN 7 OUT OF 19 PATIENTS.

Distributions
CAG shortening

<table>
<thead>
<tr>
<th>CAG shortening</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.5</td>
</tr>
</tbody>
</table>

Test Mean = value

<table>
<thead>
<tr>
<th>Hypothesized Value</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual Estimate</td>
<td>1.15789</td>
</tr>
<tr>
<td>Df</td>
<td>18</td>
</tr>
<tr>
<td>Std Dev</td>
<td>2.36322</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>t Test</th>
<th>Signed-Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Statistic</td>
<td>2.1357</td>
<td>14.000</td>
</tr>
<tr>
<td>Prob &gt;</td>
<td>t</td>
<td></td>
</tr>
<tr>
<td>Prob &gt;</td>
<td>t</td>
<td></td>
</tr>
<tr>
<td>Prob &gt;</td>
<td>t</td>
<td></td>
</tr>
</tbody>
</table>

CAG shortening occurred in 7/19 (36.8%) of patients (p=0.008). Using a signed rank test a p-value of 0=0.008 means that the number of patients in which CAG shortening occurs is statistically significant.
ANALYSIS OF ASSOCIATION BETWEEN CAG SHORTENING AND AGE AT DIAGNOSIS.

One way analysis of age at diagnosis by CAG shortening

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>75.3333</td>
<td>2.4887</td>
<td>70.083</td>
<td>80.584</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>76.8571</td>
<td>3.2585</td>
<td>69.982</td>
<td>83.732</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>121</td>
<td>10.0833</td>
<td>0.042</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>69</td>
<td>9.8571</td>
<td>-0.042</td>
</tr>
</tbody>
</table>

2-Sample Test, Normal Approximation

| S     | Z     | Prob>|Z| |
|-------|-------|------|
| 69    | -0.04246 | 0.9661 |

The Kruskal-Wallis test showed that there was no significant association between age at diagnosis and CAG shortening (p=0.9661).
ANALYSIS OF ASSOCIATION BETWEEN PRE-TREATMENT GLEASON SCORE AND CAG SHORTENING.

One way analysis of pre-treatment Gleason score by CAG shortening

Means and Std Deviations

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Std Err Mean</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>7.250</td>
<td>1.215</td>
<td>0.35086</td>
<td>6.4778</td>
<td>8.0222</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>8.000</td>
<td>1.528</td>
<td>0.57735</td>
<td>6.5873</td>
<td>9.4127</td>
</tr>
</tbody>
</table>

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>105.5</td>
<td>8.7917</td>
<td>-1.231</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>84.5</td>
<td>12.0714</td>
<td>1.231</td>
</tr>
</tbody>
</table>

2-Sample Test, Normal Approximation

| S  | Z     | Prob>|Z| |
|----|-------|------|----|
| 84.5 | 1.23113 | 0.2183 |

The Kruskal-Wallis test showed that there was no statistically significant association between pre-treatment Gleason score and CAG shortening (p=0.22).
ANALYSIS OF ASSOCIATION BETWEEN POST-TREATMENT GLEASON SCORE AND CAG SHORTENING.

One way analysis of post-treatment Gleason score by CAG shortening

![Box plot showing distribution of Gleason scores by CAG shortening]

### Means and Std Deviations

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Std Err Mean</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>7.7500</td>
<td>1.13818</td>
<td>0.32856</td>
<td>7.0268</td>
<td>8.4732</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>9.1428</td>
<td>0.69007</td>
<td>0.26082</td>
<td>8.5047</td>
<td>9.7811</td>
</tr>
</tbody>
</table>

### Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>93</td>
<td>7.7500</td>
<td>-2.411</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>97</td>
<td>13.8571</td>
<td>2.411</td>
</tr>
</tbody>
</table>

### 2-Sample Test, Normal Approximation

| S     | Z     | Prob>|Z| |
|-------|-------|------|-----|
| 97    | 2.41066 | 0.0159 |

The Kruskal-Wallis test showed that there was a statistically significant association between pre-treatment Gleason score and CAG shortening (p=0.016). Patients with a high Gleason score post-treatment (8-10) were more likely to have CAG shortening.
ANALYSIS OF ASSOCIATION BETWEEN THE INCREASES IN GLEASON SCORE (FROM PRE-TREATMENT TO POST TREATMENT) AND CAG SHORTENING.

One way Analysis of increase in Gleason score by CAG shortening

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>107.5</td>
<td>8.9583</td>
<td>-1.110</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>82.5</td>
<td>11.7857</td>
<td>1.110</td>
</tr>
</tbody>
</table>

2-Sample Test, Normal Approximation

| S     | Z     | Prob>|Z| |
|-------|-------|------|
| 82.5  | 1.1040| 0.2668 |

1-way Test, ChiSquare Approximation

<table>
<thead>
<tr>
<th>ChiSquare</th>
<th>DF</th>
<th>Prob&gt;ChiSq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3379</td>
<td>1</td>
<td>0.2474</td>
</tr>
</tbody>
</table>

The Kruskal-Wallis test showed that there was no statistically significant association between increases in Gleason score and CAG shortening (p=0.25).
ANALYSIS OF PRE-TREATMENT PSA AND CAG SHORTENING.

One way analysis of pre-treatment PSA by CAG shortening

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>109.5</td>
<td>9.1250</td>
<td>-0.846</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>80.5</td>
<td>11.5000</td>
<td>0.846</td>
</tr>
</tbody>
</table>

2-Sample Test, Normal Approximation

| S     | Z     | Prob>|Z| |
|-------|-------|-----|-----|
| 80.5  | 0.8467| **0.3974** |

The Kruskal-Wallis test showed no statistically significant association between pre-treatment PSA and CAG shortening (p=0.40).
ANALYSIS OF ASSOCIATION BETWEEN PSA NADIR AND CAG SHORTENING.

One way analysis of PSA nadir by CAG shortening

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>132</td>
<td>11.0000</td>
<td>0.973</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>58</td>
<td>8.2857</td>
<td>-0.973</td>
</tr>
</tbody>
</table>

2-Sample Test, Normal Approximation

| S   | Z    | Prob>|Z| |
|-----|------|-------|
| 58  | -0.97278 | 0.3307 |

The Kruskal-Wallis test showed no statistically significant association between the PSA nadir and CAG shortening (p=0.33).
ANALYSIS OF ASSOCIATION BETWEEN PSA PEAK AT ANDROGEN-INDEPENDENCE AND CAG SHORTENING.

One way analysis of PSA peak at androgen independence by CAG shortening

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>104.5</td>
<td>8.7083</td>
<td>-1.268</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>85.5</td>
<td>12.2143</td>
<td>1.268</td>
</tr>
</tbody>
</table>

2-Sample Test, Normal Approximation

| S     | Z     | Prob>|Z| |
|-------|-------|------|
| 85.5  | 1.26829 | 0.2047 |

The Kruskal-Wallis test showed no statistically significant association between the peak PSA at androgen independence and CAG shortening (p=0.20).
ANALYSIS OF ASSOCIATION BETWEEN TIME INTERVAL BETWEEN THE TWO TURPs AND CAG SHORTENING.

One way analysis of interval between 2 TURPs (months) by CAG shortening

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

<table>
<thead>
<tr>
<th>Level</th>
<th>Count</th>
<th>Score Sum</th>
<th>Score Mean</th>
<th>(Mean-Mean0)/Std0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>100</td>
<td>8.3333</td>
<td>-1.652</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>90</td>
<td>12.8571</td>
<td>1.652</td>
</tr>
</tbody>
</table>

2-Sample Test, Normal Approximation

| S     | Z     | Prob>|Z| |
|-------|-------|------|----|
| 90    | 1.65168 | 0.0986 |

The Kruskal-Wallis test showed no statistically significant association between the time interval between the two TURPs and CAG shortening (p=0.10).