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INVESTIGATION OF GENE

AMPLIFICATION AND EXPRESSION IN

PROSTATE CANCER
Declaration

a) This thesis has not been submitted previously for a degree at this or any other university.

b) This thesis is my own work however the contribution of Dr. Amanda Murphy who constructed the tissue microarrays must be acknowledged.

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Caroline Hughes
Summary

The purpose of this thesis was to identify significant genetic amplifications and protein expression of a gene or genes that have not been recognised as overamplified to date in prostate cancer.

Methods used:

1) Investigation of possible gene amplifications in prostate cancer:

Laser capture microdissection was used to identify/capture malignant glands from three tissue samples of prostate cancer and benign glands from one case of benign prostatic hyperplasia. By utilising this technology we could ensure that we were examining genetic changes in epithelial cells only and thus avoid the background noise of genetic changes within the stroma.

DNA was extracted from the microdissected tissue and amplification of the extracted DNA was performed by DOP-PCR.

CGH microarray analysis was used to detect possible gene amplifications within the DNA of the samples.

Major findings in the investigation of possible gene amplifications in prostate cancer using CGH microarray analysis:

The gene Topoisomerase II alpha gene which is involved in cell replication was significantly amplified in all our prostate cancer samples. This gene was amplified to the greatest extent in the case of advanced hormone insensitive prostate cancer.

2) Identification of protein overexpression of Topoisomerase II alpha gene in prostate cancer:
The second major area of investigation of this thesis was to identify whether there was significant protein overexpression of Topoisomerase II alpha gene in a larger cohort of prostate cancer patients. This was achieved by an immunohistochemical study using 100 prostate cancer samples and 42 controls (benign prosatic hyperplasia).

The results of this study were analysed by the SPSS statistical package using the U Mann Whitney test.

Major findings of the immunohistochemical study:

There was no statistically significant difference between the groups benign prostatic hyperplasia and prostate carcinoma Gleason Score 6 (P=0.245). The difference between the group’s prostate carcinoma Gleason Scores 6 and 7 was statistically significant (P<0.008). In addition there was a highly statistically significant difference between the groups prostate carcinoma Gleason Score 7 and Gleason scores 8,9 and 10 (not selected for hormonal status), (P=0.000).

When the category of Gleason score 8,9,10 was analysed as regard to hormone treatment and insensitivity (n=15) and cases with no hormone treatment (n=34) the difference in Topoisomerase indices approached statistical significance (P=0.081).

Conclusion:

There was statistically significant overexpression of Topoisomerase II alpha gene in the high Gleason score prostate cancers compared to lower Gleason score prostate cancers and benign prostatic hyperplasia. These findings indicate that Topoismerase II alpha gene is a potential gene target for chemotherapy in high Gleason score prostate cancers.
CHAPTER ONE

GENERAL INTRODUCTION
1.1 Introduction

This study investigates gene amplification and expression in prostate cancer.

In this introductory chapter we shall review what is known regarding genetic changes in prostate cancer.

Prostate cancer is the second leading cause of cancer deaths in men. It is not invariably lethal, however and is a heterogeneous disease ranging from asymptomatic to a rapidly fatal systemic malignancy. The prevalence of prostate cancer is so high that it could be considered a normal age related phenomenon. 33% of males in their 8th decade had evidence of prostate cancer at autopsy and died with the disease but not from it in a Spanish study. This study examined Caucasian Mediterranean males and data from American autopsy studies show an even higher prostate cancer prevalence rate.[1] In recent years there have been big increases in the five year survival rates for prostate cancer with a 5 year relative age standardised survival rate of 65% in England and Wales for the years 1996-1999. This was the third highest survival rate of all cancers over this time period with only testicular cancer and melanoma having superior survival rates and was around 11% higher than that for patients diagnosed during 1991-1995.[2] This improvement does not unfortunately reflect better treatment for prostate cancer. It largely reflects an increasing number of men being diagnosed with very early stage prostate cancer as a result of widespread use of prostate specific antigen (PSA) testing. PSA is a protein produced by both normal and cancerous prostate cells and a high PSA level can be a sign of cancer. Most men diagnosed at a very early stage will die with
prostate cancer but not from it, therefore the survival rate has increased. The American Cancer Society recommends in its' prostate cancer screening guidelines that men be informed on what is known and what is uncertain about the benefits and limitations of early detection of prostate cancer so that they can make an informed decision about testing. Therefore the early diagnosis of prostate cancer through screening creates difficulties in predicting the outcome of individual patients. The difficulty is in distinguishing between clinically indolent prostate cancers, which will be asymptomatic, and aggressive prostate cancers with the potential to kill the patient. Gleason grading on histopathological examination is the best prognostic indicator to date in prostate cancer however inter-observer variation can occur, grading on biopsies may not correlate with the prostatectomy specimen due to sampling problems and cases of morphologically identical prostate cancer can behave differently.

1.2 New Technologies

This is an exciting era with the emergence of new investigative tools such as DNA micro array technology and the application of the field of proteomics to the study of human cancers. Knowledge of genetic changes underlying initiation, development and progression of prostate cancer is accumulating rapidly. With increasing knowledge it may be possible to distinguish indolent from aggressive prostate tumours by molecular fingerprinting. A clinical application of this knowledge would be that radical treatment and its' associated morbidity could be avoided in prostate cancers unlikely to progress. Resources and radical treatment could be focussed on prostate cancers with poor prognostic indicators. Within this general introduction chapter we shall discuss the
most consistently reported molecular pathological findings in prostate cancer together with new concepts and technologies.

1.3 Hereditary Prostate Cancer

Prostate cancer can be divided epidemiologically into hereditary and sporadic forms[3] but it is not possible to distinguish these 2 groups on a molecular level. Highly penetrant inherited genes conferring the prostate cancer phenotype have not been identified.

Linkage studies using genetic markers to search for chromosomal regions that show excessive sharing of inherited alleles in cancer families have been helpful in identifying important cancer susceptibility genes in other cancers. However similar studies using prostate cancer families have not yielded the same success. Although possible inherited prostate cancer susceptibility genes have been identified such as ELAC2, RNASEL, MSR1, NSB1 and CHEK2 genes in some families, the proportion of cases of hereditary prostate cancer attributable to germline mutations in these loci is small. Many studies have not supported the role of these genes in hereditary prostate cancer. Mutations of these candidate genes have also been identified in sporadic prostate cancer. As prostate cancer is a common cancer it may be difficult to distinguish clustering of sporadic prostate cancer within families from true hereditary prostate cancer. This difficulty may have hindered research in hereditary prostate cancer to date. Alternatively the failure to identify highly penetrant genes in hereditary prostate cancer may be due to the fact that multiple genes with small to moderate effect are involved in hereditary prostate carcinogenesis. The risk of disease in the presence of
a susceptibility gene might be substantially increased only in the appropriate genetic, dietary and environmental background. We will briefly outline the most significant hereditary prostate cancer susceptibility genes to date.

1.3.1 Possible Cancer Susceptibility Genes

ELAC2

ELAC2 was the first identified possible hereditary prostate cancer gene. The function of ELAC2 is not definitively known and it has been proposed as a metal dependent hydrolase. Association of ELAC2 genotypes with familial prostate cancer have been reported. However multiple large subsequent studies have not provided confirmatory evidence of this association. Overall it appears that if ELAC2 plays a role in prostate cancer it is a weak role.

Host Response to Infection Genes:

RNASEL

RNASEL is a ribonuclease that degrades viral and cellular RNA and can produce apoptosis on viral infection. Mutations in the RNASEL gene have been identified in familial and sporadic prostate cancer in multiple studies. Other studies have not supported these findings. Overall there is strong support that RNASEL is the most important hereditary prostate cancer gene identified to date.
MSR1

MSR1, encodes a macrophage scavenger receptor responsible for cellular uptake of molecules including bacterial cell wall products. The importance of MSR1 as a prostate cancer susceptibility gene in hereditary prostate cancer is controversial. Germline MSR1 mutations have been linked to prostate cancer in some families with prostate cancer and in sporadic prostate cancer.[15][16] However a recent report, which investigated 163 families with familial prostate cancer, did not provide confirmatory evidence of the role of MSR1 in familial prostate cancer.[17]

Possible mechanisms of action by which mutations of these host response to infection genes increase the risk of prostate cancer is that they may predispose to chronic inflammation due to failure of viral RNA and bacterial degradation. There is accumulating knowledge supporting the role of inflammation in prostate cancer, which we will refer to again later in the article.

Cell Cycle Checkpoint Genes:

NBS1

The rare human genetic disorder, Nijmegen Breakage Syndrome, is characterised by radiosensitivity, immunodeficiency, chromosomal instability and an increased risk for
cancer of the lymphatic system. The NBS1 gene which is involved in this human genetic disorder, codes for a protein, nibrin, involved in the processing/repair of DNA double strand breaks and in cell cycle checkpoints.[18] Mutations in the gene for the Nijmegen breakage syndrome (NBS1) have been identified in both sporadic and familial cases of prostate cancer and are associated with a small increased risk of prostate cancer.[19]

CHEK2

The CHEK2 gene is an upstream regulator of p53 in the DNA-damage-signalling pathway. CHEK2 mutations have been identified in both sporadic and familial cases of prostate cancer and are associated with a small increased risk of prostate cancer.[20][21]

NBS1 and CHEK2 genes have only recently been identified as possible prostate cancer susceptibility genes. ELAC2 was the first hereditary prostate cancer susceptibility gene identified and subsequent studies have not provided confirmatory evidence of its’ role in prostate cancer. Therefore it is not possible to comment on the importance of these two genes in hereditary prostate cancer until additional confirmatory studies have been performed.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal locus</th>
<th>Putative function</th>
<th>Status in prostate cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELAC2</td>
<td>17p</td>
<td>Metal dependent hydrolase</td>
<td>Unknown</td>
</tr>
<tr>
<td>RNASEL</td>
<td>1q</td>
<td>Ribonuclease that degrades viral and cellular RNA and can produce apoptosis on viral infection</td>
<td>Deleted</td>
</tr>
<tr>
<td>MSR1</td>
<td>8p</td>
<td>Encodes a macrophage scavenger receptor responsible for cellular uptake of molecules including bacterial cell wall products</td>
<td>Deleted</td>
</tr>
<tr>
<td>NBSI</td>
<td>5p</td>
<td>Encodes for a protein, nibrin, involved in the processing/repair of DNA double strand breaks and in cell cycle checkpoints</td>
<td>Deleted</td>
</tr>
<tr>
<td>CHEK2</td>
<td>22q</td>
<td>Upstream regulator of p53 in the DNA-damage-signalling pathway</td>
<td>Deleted</td>
</tr>
</tbody>
</table>
The study of hereditary prostate cancer genes is in its' infancy and the challenge for the future will be to detect genes of small to moderate effects. Advances in statistical methods to amplify signals from susceptibility genes in the presence of heterogeneous factors are required in order to decipher the genetics and molecular pathology of hereditary prostate cancer.

1.4 Sporadic Prostate Cancer

The vast majority of prostate cancer is sporadic. In our discussion of the molecular pathology of sporadic prostate cancer we will discuss the evidence to date under the following categories: polymorphisms associated with increased prostate cancer risk, somatic genetic changes and factors involved in the progression of prostate cancer such as the androgen receptor, growth factors and invasion and metastasis genes. We will discuss separately recent findings of gene over and under-expression by micro array technology. The application of the field of proteomics to the study of prostate cancer and current theories regarding the role of inflammation in prostate cancer will also be discussed.

1.4.1 Polymorphisms Associated with Increased Prostate Cancer Risk:

A polymorphism is a genetic variant that appears in at least 1% of the population. These common genetic polymorphisms are likely to have small relative risks, yet large population attributable risks due to their high frequencies.
TLR4

TLR4 encodes a receptor, which is a central player in the signalling pathways of the innate immune response to infection by Gram-negative bacteria. A TLR4 sequence polymorphism is associated with a small increased risk of prostate cancer.[22] This is in keeping with the current hypothesis of inflammation having a role in prostate carcinogenesis. We will discuss the current hypothesis of the role of inflammation in prostate cancer later in the article.

CDKN1B (p27)

The loss of cell cycle control is believed to be an important mechanism in the promotion of carcinogenesis. CDKN1B (p27) belongs to the Cip/Kip family and functions as an important cell cycle gatekeeper. A recent study has revealed a significant association between a single nucleotide polymorphism of CDKN1B (p27) and prostate cancer.[23] There is also an association between CDKN1B (p27) and another Cip/Kip family member, CDKN1A (p21) and advanced prostate cancer.[24]
Growth of prostate cells depends on androgens. Genes that encode products, which play a role inducing androgen stimulation of the prostate gland, are very significant. The androgen receptor (AR) is currently a therapeutic target for the treatment of prostate cancer. Other genes involved in androgen stimulation of the prostate such as SRD5A2 and CYP17 also hold potential as future therapeutic targets.

Androgen Receptor (AR)

The androgen receptor contains polymorphic polyglutamine (CAG)$_n$ trinucleotide repeats. It has been reported in the past that shortening of these repeats are associated with increased prostate cancer risk.[25]

Short CAG length has also been correlated with high grade, high stage, metastatic and fatal prostate cancers. A hypothesis that has been proposed for the influence of the short CAG repeat on prostate carcinogenesis is that due to its role in androgen receptor function it causes an increase in activation of androgen dependent genes.[26]

Other groups have not identified CAG repeats as a risk factor for prostate cancer and a recent significant study and epidemiological review article have demonstrated that this risk factor is less important than it has been regarded previously.[27][28]

CYP17

CYP17 encodes cytochrome P-450c17α, an enzyme responsible for the biosynthesis of testosterone. A variant CYP17 allele is associated with both hereditary and sporadic
prostate cancer.[29] This allele is hypothesized to increase the rate of gene transcription, increase androgen production and thereby increase the risk of prostate cancer.[30]

SRD5A2

SRD5A2 encodes the predominant isozyme of 5-α-reductase in the prostate, an enzyme that converts testosterone to the more potent dihydrotestosterone. The alleles that encode enzymes with increased activity have been associated with an increased risk of prostate cancer and with a poor prognosis for men with prostate cancer.[31][32]
Table 2  Polymorphisms Associated With Increased Prostate Cancer Risk

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal locus</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>9q</td>
<td>Encodes a receptor which is a central player in the signalling pathways of the innate immune response to infection by Gram-negative bacteria</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>12p (p27)</td>
<td>Belongs to the Cip/Kip family and functions as an important cell cycle gatekeeper</td>
</tr>
<tr>
<td>AR</td>
<td>Xq</td>
<td>May cause activation of androgen dependent genes</td>
</tr>
<tr>
<td>CYP17</td>
<td>10q</td>
<td>Enzyme responsible for the biosynthesis of testosterone.</td>
</tr>
<tr>
<td>SRD5A2</td>
<td>2p</td>
<td>Converts testosterone to the more potent dihydrotestosterone</td>
</tr>
</tbody>
</table>

1.4.2 Polymorphisms associated with Advanced Sporadic Prostate Cancer:

Vitamin D Receptor

13
Physiologic levels of vitamin D promote the differentiation and growth arrest of prostate cancer cells in vitro.[33] The precise mechanism through which vitamin D mediates this effect is unknown however it is most likely through its’ effect on cell growth proteins. Allelic differences in the vitamin D receptor (VDR) gene result in variation in VDR activity.[34] VDR alleles have been significantly associated with prostate cancer and this association was stronger with advanced prostate cancers.[35] As discussed previously polymorphisms in CDKN1A (p21^{OP}) and CDKN1B (p27^{kip}) are associated with advanced prostate cancer.[24] In addition polymorphic variants of a number of other genes have been proposed as possible contributors to the risk of prostate cancer.[36]

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Polymorphisms Associated With Advanced Prostate Cancer Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Chromosomal locus</strong></td>
</tr>
<tr>
<td>Vitamin D receptor</td>
<td>13q</td>
</tr>
<tr>
<td>CDKN1A (p21)</td>
<td>6p</td>
</tr>
<tr>
<td>CDKN1B (p27)</td>
<td>12p</td>
</tr>
</tbody>
</table>
1.5 Somatic Genetic Changes

The number of genetic loci involved in prostate carcinogenesis is large and the mechanisms are complex and not fully understood. A compilation of the most commonly reported chromosomal abnormalities in sporadic prostate cancer together with the putative genes involved at these chromosomal sites is presented in Table 4.[37][38]
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7p</td>
<td>EGFR</td>
<td>Growth factor</td>
<td>Amplified</td>
<td>39</td>
</tr>
<tr>
<td>7q</td>
<td>CAV 1</td>
<td>Structural protein of caveolae membranes in fibroblasts and endothelia</td>
<td>Amplified</td>
<td>40</td>
</tr>
<tr>
<td>8p</td>
<td>MSR</td>
<td>Encodes a macrophage scavenger receptor responsible for cellular uptake of molecules including bacterial cell wall products</td>
<td>Deleted</td>
<td>41</td>
</tr>
<tr>
<td>8p</td>
<td>NKX3-1</td>
<td>Tumour suppressor gene</td>
<td>Deleted</td>
<td>42</td>
</tr>
<tr>
<td>8q</td>
<td>c-myc</td>
<td>Transcriptional activator</td>
<td>Amplified</td>
<td>43</td>
</tr>
<tr>
<td>10q</td>
<td>PTEN</td>
<td>Tumour suppressor gene</td>
<td>Mutated</td>
<td>44</td>
</tr>
<tr>
<td>13q</td>
<td>Rb</td>
<td>Tumour suppressor gene</td>
<td>Deleted</td>
<td>45</td>
</tr>
<tr>
<td>16q</td>
<td>E-CAD</td>
<td>Adhesion molecule</td>
<td>Deleted</td>
<td>46</td>
</tr>
<tr>
<td>Xq</td>
<td>AR</td>
<td>Androgen receptor</td>
<td>Amplified</td>
<td>47</td>
</tr>
</tbody>
</table>
Although these are the most common areas of chromosomal loss and gain, prostate carcinogenesis is complex and multiple genes from other chromosomal loci are also thought to be involved.

1.5.1 Tumour Suppressor Genes and Loss of Heterozygosity

Tumour suppressor genes are likely to be involved in the prostate carcinogenesis pathway. Loss of tumour suppressor genes was initially proposed to occur by loss of function of two alleles (the “two-hit hypothesis”) by mutation or deletion.[48] This model has been revised to include epigenetic modification by (a) inactivation of one or both alleles by DNA methylation of CpG sites in gene promoters or (b) function heritably downregulated or (c) otherwise compromised in a clonal fashion.[49] The change can be by mutation, methylation of the promoter or by some other modification of the protein product and must be coupled with evidence that the normal (wild type) gene does suppress growth of tumour cells.[50]

Glutathione S-Transferase Gene (GSTP1)

Glutathione S-Transferase Gene (GSTP1) is emerging as one of the most important tumour suppressor genes in prostate cancer. GSTP1 can detoxify environmental electrophilic carcinogens and oxidants and may have a genome caretaker role by preventing oxidant and electrophilic DNA damage.[51] This gene has been shown to be inactivated by hypermethylation of the promoter region in prostate tumours.[52][53] Hypermethylation of the glutathione S-transferase gene (GSTP1) is the most common
(greater than 90%) reported epigenetic alteration in prostate cancer. It occurs early in cancer progression and it is a promising marker for detecting organ-confined disease. Quantitation of GSTP1 hypermethylation was found to accurately detect the presence of cancer even in small, limited tissue samples. It may represent a promising diagnostic marker that could be used as an adjunct to tissue biopsy as part of prostate cancer screening.[54]

Aberrant DNA methylation patterns may be the earliest somatic genome changes in prostate cancer. A recent study found that CpG islands were hypermethylated in >85% of prostate cancers and cancer cell lines but not in normal prostate cells and tissues. CpG island hypermethylation patterns in prostate cancer metastases were very similar to the primary prostate cancers and tended to show greater differences between cases than between anatomical sites of metastasis.[55]

PTEN

PTEN is an important tumour suppressor gene in prostate cancer and influences the levels of CDKN1B (p27) another important tumour suppressor gene. The gene for phosphatase and tensin homologue (PTEN), encodes a phosphatase active against both proteins and lipid substrates and is a common target for somatic alteration during the progression of prostate cancer.[56][57] PTEN is present in normal epithelial cells and in cells in prostatic intraepithelial neoplasia. In prostate cancers the level of PTEN is frequently reduced, particularly in cancers of a high grade or stage. In prostate cancers that do contain PTEN, a considerable heterogeneity in levels, with regions devoid of PTEN has been described.[44] The mechanism by which PTEN might act as a tumour suppressor gene in the prostate may involve the inhibition of the phosphatidylinositol
3'-kinase-protein kinase B (P13K-Akt) signalling pathway that is essential for cell cycle progression and cell survival.[58]

CDKN1B (p27)

CDKN1B (p27) is an important tumour suppressor gene in prostate cancer. Reduced levels of p27, a cyclin-dependent kinase inhibitor encoded by the CDKN1B gene, are common in prostate cancers and particularly in prostate cancers with a poor prognosis.[59][60] The somatic loss of DNA sequences at 12p12-1, encompassing CDKN1B, has been described in 23% of localised prostate cancers, 30% of metastases of prostate cancer in regional lymph nodes, and 47% of distant metastases of prostate cancer.[61] Levels of p27 are suppressed by the P13K-Akt signalling pathway.[58] By inhibiting P13K-Akt, PTEN can increase the levels of CDKN1B messenger RNA and p27 protein.[62] For this reason, low p27 levels may be as much a result of the loss of PTEN function as of CDKN1B alterations.

NKX3.1

Loss of 8p appears to be an early event in prostate cancer and the most promising candidate tumour suppressor gene at this site is NKX3.1. NKX3.1 encodes a prostate specific homeobox gene that is likely to be essential for normal prostate development. NKX3.1 binds DNA and represses expression of the PSA gene.[63] Loss of NKX3.1 expression does appear to be related to the progression of prostate cancer. One study found that NKX3.1 was absent in 20% of lesions of prostatic intraepithelial neoplasia, 6% of low stage prostate cancers, 22% of high stage prostate cancers, 34% of androgen independent prostate cancers and 78% of prostate cancer metastases.[42] The loss of
this gene is of particular interest as when normally present it represses expression of the PSA gene and the loss of NKX3.1 may be involved in increased levels of PSA with prostate cancer progression.

KLF6

Kruppel-like factors (KLFs) are a group of transcription factors that appear to be involved in different biological processes including carcinogenesis. Significant genetic alterations of KLF6 have been reported including deletions and loss of expression in a minority of high-grade prostate cancers.[64][65]

KLF6 and NKX3.1 have not been reported as frequently as the tumour suppressor genes previously discussed and have been identified due to the fact that they are within areas of frequent allelic loss in prostate tumours.

Retinoblastoma (Rb)

Retinoblastoma (Rb) has been reported as an important tumour suppressor gene in many human cancers and prostate cancer is no exception. The disruption of the normal retinoblastoma regulatory pathway is associated with the pathogenesis of many human cancers. The retinoblastoma gene plays an important role in the G₁ phase of the cell cycle. It binds tightly to the E2F family of transcription factors. When phosphorylated, the Rb protein releases the E2F proteins causing transcriptional activation of a variety of genes involved in cell growth.[45] [66][67] Inactivation of Rb appears to be important in neoplastic transformation, because expression of wild type Rb in Rb-negative prostate cancer lines results in loss of tumourigenicity.[68] The predominant
mechanism of Rb inactivation involves allelic loss or mutation but decreased transcription of Rb has also been reported.[69][70]

p53

Mutations in p53 are common in human neoplasms, but in prostate cancer it occurs with a low frequency of mutation. However p53 has an important role in prostate cancer progression as abnormal p53 expression is associated with bone metastases and the development of androgen independent disease. Abnormal p53 expression correlates with high histological grade, high stage and clinical disease progression.

The p53 tumour suppressor gene product restricts entry into the synthetic phase of the cell cycle and promotes apoptosis in cells that are disorganised or have damaged DNA.

Loss of normal p53 function results in uncontrolled cell growth.[71]

The analysis of p53 expression can be difficult. The mutated p53 gene product has a longer half life thus rendering it detectable by immunohistochemistry. However sensitive immunohistochemical techniques may detect over-expressed normal p53. It is therefore more reliable to detect mutations in p53 by molecular techniques. Abnormal p53 expression is correlated with reduced survival after radical prostatectomy.[72]

1.5.2 Oncogenes

c-myc and bcl-2 are well known and important oncogenes not only in prostate cancer but in many human cancers.
c-myc

Several studies have demonstrated increased myc expression in prostate cancer and a significant correlation of myc overexpression with Gleason grade. The myc oncogenes are members of the basic helix-loop-helix-leucine zipper (bHLHZ) family of transcription factors. Myc proteins act as transcriptional activators or repressors through dimerization or with other bHLHZ family members.[43][70][73][74][75]

bcl-2

The bcl-2 family of genes are commonly expressed in primary and metastatic prostate cancers. bcl-2 is not expressed in the normal prostate. Proteins expressed by the bcl-2 family have a crucial role in the regulation of apoptosis and the bcl-2 gene inhibits programmed cell death.[76][77][78] bcl-2 has also been implicated in the development of androgen-independent prostate cancer due to its' high levels of expression in androgen-independent cancers in the advanced stages of disease.[79][80]

Other oncogenes have only recently been recognized but may well emerge as novel targets for molecular genetic intervention or through modified expression may allow accurate prediction of the manner in which a neoplasm is likely to progress.

c-Kit /Tyrosine Kinase Receptor

c-Kit (tr-Kit) is a strong activator of the Src-family tyrosine kinases. In a recent study it has been reported that human tr-Kit mRNA and protein are expressed in prostatic
cancer cells. It also describes for the first time the existence of a truncated c-Kit protein in primary tumours and show a correlation between tr-Kit expression and activation of the Src pathway in the advanced stages of the disease.[81]

Stat5

A signal transducer and activator of transcription 5 (Stat5) has been identified as a critical survival factor for prostate cancer cells.[82] Activation of Stat5 is also associated with high histological grade of prostate cancer.[83]

1.5.3 Telomerase and Telomere Shortening

Telomere length has been found to be strikingly shorter in prostate cancer including prostatic intraepithelial neoplasia (PIN) than in normal prostate. Telomeres stabilize and protect the ends of chromosomes, but shorten because of cell division and/or oxidative damage. Critically short telomeres, in the setting of abrogated DNA damage checkpoints, have been shown to cause chromosomal instability leading to an increase in cancer incidence as a result of chromosome fusions, subsequent breakage and rearrangement. In normal cells, successive cycles of cellular replication result in progressive loss of telomeric sequences. Normal cells sense very short telomeres as evidence of DNA damage and exit the cell cycle.[84] Telomerase is an enzyme that maintains telomere length by adding repetitive telomeric sequences to chromosome ends. Telomerase activity is present in the majority of prostate cancers and not in normal prostate epithelium.[85][86]
1.5.4 Androgen Receptor (AR)

The androgen receptor has a critical role in prostate cancer. Androgen receptor blockade can delay the progression of prostate cancer and is used to treat patients unsuitable for radical surgery or with cancer that has spread beyond the prostate. It has been extensively studied in prostate cancer due to the fact that androgens are required for the development of both the normal prostate and prostate cancer. Initially the majority of prostate cancers are sensitive to androgen deprivation.

However in patients with advanced disease, most tumours progress to an androgen independent state with proliferation of cells that do not require androgens for growth. The mechanism of acquired androgen insensitivity is unknown and has been the subject of much research, as androgen insensitive prostate cancers can no longer be treated with endocrine therapy.

Mutations, amplifications and deletions of the androgen receptor itself and structural change in the androgen receptor protein, have been postulated as causing androgen insensitivity.[47][87][88][89][90][91]

In an analysis of 44 mutant androgen receptors from prostate cancers, 16 per cent had loss of function, 7 per cent maintained wild type function, 32 per cent demonstrated partial function and 45 per cent displayed a gain in function.[92]

Structural change of the androgen receptor however has only been identified in a minority of androgen insensitive prostate cancers so therefore other factors must also be involved in this phenomenon.
Growth factor stimulation may sensitize the androgen receptor transcriptional complex
to sub physiological levels of androgen.[93] We will refer to this topic once again when
we discuss the role of growth factors in prostate cancer.

1.7 Invasion and Metastasis Suppression Genes

For cancer cells to spread to distant sites they must invade the stroma, penetrate the
vasculature and implant at distant sites, and be able to survive there. Changes of
adhesion to the substratum are crucial for tumour cell invasion and distant metastasis.
Several genes encoding proteins involved in invasion and metastasis in prostate cancer
have been identified.

E-cadherins

The cadherins are membrane glycoproteins that play an important role in cellular
differentiation by mediating cell-cell recognition and adhesion. Reduction of e-cadherin
expression occurs is a common occurrence in prostate cancer, and has been reported to
correlate with tumour grade, stage and survival.[46] [94][95][96] However the degree
of E-cadherin expression in prostate cancer remains controversial. Normal expression
of e-cadherin was found in most prostate carcinoma cases examined in an
immunohistochemical study which systematically evaluated E-cadherin expression in a
broad range of formalin fixed prostate tissue.[97]
Integrins

Normal basal epithelial cells in the human prostate express integrins however the expression is abnormal or absent in the majority of prostate cancer.[98][99][100]

C-CAM

C-CAM is expressed on the surface of normal prostate epithelium however is absent in most prostate cancers.[101] Loss of C-CAM1 expression occurs early in the development of prostate cancer, suggesting that C-CAM1 may help maintain the differentiated state of the prostate epithelium. Re-introduction of C-CAM1 into cancer cells can reverse their cancerous growth.[102]

KAII/CD82

Metastasis suppressor genes are defined as genes that do not affect cell growth of primary tumour cells but can inhibit development of distant metastases.[103] Cancer metastasis suppressor KAII/CD82 belongs to the tetraspanin superfamily and inversely correlates with the metastatic potential of a variety of cancers including prostate cancers. CD82 expression is reduced or absent in most primary prostate cancers and in more than 90% of metastatic prostate cancers.[104][105] It is thought that the mechanism of KAII/CD82-mediated metastasis suppression involves a cell-surface protein physically associated with KAII/CD82 named KASP.[106]
CD44

CD44 is another metastasis suppressor gene for prostatic cancer and CD44 expression is inversely correlated with histological grade, ploidy, and distant metastases.[107][108] Additional candidate metastasis suppressor genes have been identified for prostate cancer – NME23, mapsin, BRMS1, KISS1 and MAP2K4.[103]

The identification of invasion and metastasis suppression genes has potential clinical applications. Prostate cancers with loss of these genes may represent cancers with a potentially metastatic phenotype. These cancers may require more aggressive treatment in contrast to cancers, which have retained expression.

In our opinion the most promising genes, which could be used as specific targets for the detection, diagnosis and treatment of prostate cancer, include the tumour suppressor genes GSTP1, NKX3.1, PTEN and p27. NKX3.1, PTEN and p27 genes also involve growth factor signalling pathways, which have potential for molecular genetic intervention. Genes, which play a role inducing androgen stimulation of the prostate gland such as AR, SRD5A2 and CYP17, are also potential targets for gene therapy in the future.
CHAPTER 2

MATERIALS AND METHODS
2.1 Introduction

This is an exciting era for molecular pathology as powerful new investigative tools are being used now in research. Comparative genomic hybridisation allows the screening of the entire genome for chromosomal aberrations by comparing fluorescence ratios for normal and tumour DNA. CGH microarrays are a modification of this technique where genes are spotted onto a microarray as in Figure 1. Tumour DNA and normal reference DNA are hybridised to the microarray and gene amplifications can be detected by comparing fluorescence ratios for normal and tumour DNA.

CGH microarray technology was used in this work in conjunction with laser capture microdissection (LCM) which facilitates the investigation of a pure epithelial population. As the technique of laser capture microdissection is extremely selective the quantity of DNA is smaller using this technique therefore DOP-PCR was used in conjunction with LCM to amplify the quantity of DNA obtained from samples.

Low stringency PCR conditions allow random priming of DOP primer throughout the genome which results in amplification of the whole genome as opposed to a specific target.

These techniques were used to identify genetic amplifications in our prostate samples and are outlined in more detail below (2.1-2.3).

Amplification of Topoisomerase II alpha gene was identified in our prostate samples using the CGH microarray technology.

Immunohistochemistry was used to investigate whether increased expression of Topoisomerase II alpha gene was present in a larger cohort of prostate cancer and
benign prostatic hyperplasia cases and the details of how this was carried out is also outlined below (2.4-2.5).

2.1 CGH Microarray Analysis

2.1.1 Samples:

We analysed 4 prostate samples using the Vysis GenoSensor. 2 of the prostate samples were from autopsy cases of advanced prostate cancer. One of these cases (Gleason Score 9) had been treated with anti-androgens and was known to be hormone resistant. The second case was a case of metastatic prostate cancer which had not been treated by anti-androgens. The third prostate sample was a TURP surgical specimen with involvement of prostate chippings by Gleason Score 7 prostate adenocarcinoma. The fourth sample was a TURP surgical specimen with benign prostatic hyperplasia (BPH) and no evidence of prostatic carcinoma.
2.1.2 Laser capture microdissection:

Laser capture microdissection (LCM) permits the rapid and reliable procurement of pure populations of cells from tissue sections, in one step, under direct microscopic visualization while maintaining nucleic acid and protein content. LCM is a powerful upstream tool facilitating the generation of informative data from downstream technologies such as CGH arrays, cDNA arrays and Taqman PCR. Laser capture microdissection was performed on each of the cases as previously described.[109] Briefly a laser microdissection system (Arcturus Pix Cell, Arcturus Engineering) was used to isolate prostate epithelial cells from each specimen according to the manufacturer’s instructions. First the laser was used to remove the surrounding stromal cells from an area of interest. Then the target cells were attached to the cap by firing the laser. The cap was transferred to a sterile microcentrifuge tube containing 40μl proteinase K extraction buffer (10mM Tris HCL, pH 8.0, 1mM EDTA, proteinase K 40μl/ml, 1% Tween 20, pH 8). The tubes were inverted at 55°C overnight. The supernatant containing the DNA (approx. 350μl from laser microdissected tissue) leaving behind the precipitated protein pellet was poured into a clean 1.5ml microfuge tube which contained 300μl 100% Isopropanol and 35μl 3M sodium acetate. As the DNA yield was expected to be low due to the use of microdissected tissue we added 1μl of glycogen solution as a DNA carrier. The sample was mixed by inverting gently 50 times. It was then centrifuged at 16,000xg for 5 minutes. The supernatant was poured off and the tube was drained on clean absorbent
paper. 300μl 70% ethanol was added and the tube was inverted to wash the DNA pellet. The sample was then centrifuged at 16,000xg for 1 minute. The ethanol was carefully poured off. The tube was inverted and drained on clean absorbent paper and allowed to air dry for 10-15 minutes and dissolved in 6μl of water. The sample was incubated overnight at room temperature. The DNA was then stored at 4°C.

Figure 1: Example of laser capture microdissection on benign prostate tissue
Figure 2: Example of laser capture microdissection on prostate cancer

Before microdissection

After microdissection
2.2 DOP-PCR and Nick Translation

Figure 3: Diagramatic representation of DOP-PCR

DS Genomic DNA

Low stringency PCR conditions allow random priming of DOP primer throughout the genome

DOP-PCR allows Random Amplification of Multiple loci and amplification of the entire genome
Several authors have reported the feasibility of combining laser capture microdissection with DOP-PCR and CGH.\[110\] The DOP-PCR was performed using the DOP-PCR master kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. 1ng of male reference DNA and Cosh DNA also under 2 rounds of DOP-PCR amplification. Briefly in a total volume of 50μl using 5μl of test DNA template or 5μl of reference containing 1ng of male reference DNA the sample was heated to 94°C followed by 5 cycles of 94°C for 1 minute; 30°C for 1.5 minutes; and a ramp of 30°C-72°C at 23°C/second for 3 minutes. Then 25 cycles followed at 94°C for 1 minute; 62°C for 1 minute and 72°C for 3 minutes +10 seconds per cycle. After a final extension period of 10 minutes at 72°C, the sample was cooled to 4°C. 5μl of the DOP-PCR product was used as a template for the 2\textsuperscript{nd} round DOP-PCR reaction, which was performed at temperature, and time settings identical to the 1\textsuperscript{st} round DOP-PCR. 10μl of the 2\textsuperscript{nd} round DOP-PCR product were used to check the fragment size on a 2% agarose ethidium bromide gel. Samples were taken into analysis if a smear could be detected after the second round of DOP-PCR. DNA labelling was performed by nick translation. 10μl of the DOP-PCR product was used as a template for the reaction. The reaction was performed in a total volume of 50μl with the following reagents: 2μl of 1mM Alexa-488 (Green), 17μl of dNTP mix, 5μl 10X nick translation buffer, 10μl nick enzyme mix and 6μl of water. A similar reaction using 10μl of the DOP-PCR product of 1ng of male reference DNA and Cosh DNA which had undergone 2 rounds of DOP-PCR amplification and 2μl of 1mM Alexa-594 (Red) was used instead of 2μl of 1mM Alexa-488 (Green) for reference cases.
2μl of the nicked DNA (tests and references) were used to check the fragment sizes on a 2% agarose ethidium bromide gel.

Figure 4: 2nd round DOP-PCR products of the 4 prostate samples (sample 1-4). Sample 5 represents a positive control as does the lane immediately to it’s right. DNA ran below the 300 nucleotide marker with tails extending up to about the 600 nucleotide marker. A negative control is present in the extreme right hand lane.
We applied a commercially available genomic DNA microarray kit (Vysis Genosensor) and hybridised the nicked products from our 4 prostate samples and 4 references to Amplionc 1 DNA microarrays containing 57 oncogenes as per instructions supplied by the manufacturer. Briefly the labelled DNA (4μl of green test and 4μl red reference were mixed with 22μl microarray hybridisation buffer (Vysis) containing Cot-1 followed by incubation at 80° for 10 minutes to denature the DNA. 30μl of hybridisation mixture containing denatured DNA was dropped on the chip slide following incubation of the microarray at 37° for at least 18 hours in an incubator. After hybridisation the slides were washed in 3 series of washing solutions (50%
formamide/2XSSC) with incubations at 40° (10 minutes each), and 4 series of washing solutions (1X SSC) at room temperature and counterstained with 18µl 4'6-diamidinophenylindole (DAPI) IV solution. (Vysis).

The hybridised microarray slides were analysed with microarray reader and analysis software (Genosensor Array 300). Green (test) to red (reference) (G/R) ratios were automatically determined for each sample and the normalised G/R ratio was taken to represent the relative average number of copies of the sequence for those spots that were selected as controls. Spots with G/R ratios more than the mean plus three standard deviations (~1.3) were considered as amplifications or gains of the indicated copy number.

A control experiment was run in tandem with the experiment using cell line DNA (Cosh) mixture comprising 3 cell lines) provided by the manufacturer with known documented amplifications of a variety of oncogenes. Our results were exactly concordant with those described by Vysis.
Figure 6: Hybridisation of tumour and reference DNA

Figure 7: Summary of methods utilised to obtain report on Fluorescence

- Laser capture microdissection of tissue sample
- DNA extraction
- Microarray
- DOP-PCR amplification
- DNA labeling
- Hybridisation
- Gene copy no. detection
- Image capture
- Image analysis
2.4 Evaluation of expression of Topoisomerase gene (amplified on CGH microarrays) by immunohistochemistry

Immunohistochemistry was used to evaluate the expression of one of the genes (Topoisomerase II alpha gene) in which amplifications were detected on CGH microarrays. Expression of this gene was evaluated in a larger cohort of prostate cancer and benign prostatic hyperplasia cases.

2.4.1 Patients and tumours used in immunohistochemical study

The immunohistochemical study included 100 patients with adenocarcinoma of the prostate and 42 patients with benign prostatic hyperplasia (BPH). The adenocarcinoma cases included 59 transurethral resection of prostate (TURP) specimens and 41 radical prostatectomy specimens. The 42 BPH cases were transurethral resection of prostate (TURP) specimens. These cases were diagnosed during the period 1999-2003 and were identified by performing a SNOMED code search of the Adelaide & Meath Hospital Incorporating the National Children's Hospital Cellular Pathology database. Formalin fixed blocks from these cases were retrieved from the files. Tissue microarrays were constructed as described previously.[111] 138 cases were present in triplicate and included representative areas of tumour or glandular tissue in cases of BPH. Sufficient tissue from one case of BPH was not represented on the tissue microarray and this case was excluded. 3 cases of adenocarcinoma were present in duplicate only as there was insufficient tissue from the third core present on the microarray. Histopathological
reports which included clinical details were reviewed for all adenocarcinoma cases and cases were classified into 4 categories benign prostatic hyperplasia (n=41), prostate carcinoma Gleason Score 6 (n=28), prostate carcinoma Gleason Score 7-8 (n=35), and prostate carcinoma Gleason Score 9-10 (n=37). We also compared prostate carcinomas Gleason Score 8-10 under the categories of hormone resistant (n=14) and hormone responsive (n=14). The 14 cases categorised as hormone resistant were recurrent prostate adenocarcinomas present in transurethral resection of prostate (TURP) specimens when clinical details indicated that the patient had received anti-androgen treatment. These cases were Gleason Score 8-10. 14 cases of matched Gleason Score prostate carcinomas who had not received anti-androgen treatment were compared with these cases using the U Mann Whitney test.

2.4.2 Immunohistochemical staining

Immunohistochemistry for Topoisomerase II-Alpha was performed using a monoclonal anti- Topoisomerase II-Alpha antibody with a dilution of 1:300. Sections of 5μm thickness were studied. Antigen retrieval was performed with dewaxed sections using a pressure cooker and the Trilogy system for 10 minutes. A standard avidin-biotin-peroxidase complex technique (DAKO) was used for visualisation with diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin and mounted. Tonsil samples from our routine files were used as positive controls for Topoisomerase II-Alpha.
2.4.3 Evaluation of Immunohistochemistry

Immunohistochemical staining was evaluated light microscopically using an X 40 objective and a Miller ocular. The Miller ocular eyepiece gave a square field, in the corner of which was a smaller ruled square, one-ninth the area of the total square. Nuclei with strong homogenous Topoisomerase II-Alpha staining were counted in the large square and the total number of nuclei, both positive and negative staining counted in the small square. The number of cells in the small square was multiplied by 9 to obtain the total number of cells in the large square. A mean of 240.5 epithelial cells were counted for the prostate cancer cases and a mean of 88 epithelial cells counted for the cases of benign prostatic hyperplasia. 5 non-overlapping square fields were counted for each tissue core, 3 in the y-axis and 2 in the x-axis. The percentage of positively staining nuclei within the 5 large squares was calculated for each core. In cases present in triplicate an average result was obtained from 3 cores and in cases present in duplicate an average was obtained from 2 cores. This result was the Topoisomerase II-Alpha index for the case. This method has been used in previous studies in the evaluation of immunohistochemical results in tissue microarrays.[112][113]

In addition each Topoisomerase II-Alpha index was obtained by image analysis using the Ariol system for tissue microarrays. An acceptable level of concordance was taken as within +/- 10% of TI index obtained using the Miller ocular.

Of 140 cases in total (138 present in triplicate and 2 present in duplicate) 14 cases (10%) showed discordance between Ariol image analyser and the Miller ocular TI indices. In all cases the Ariol image analyser overestimated TI indices compared to the Miller ocular method.
A second observer reviewed these discordant cases. This discordant group was composed of one Gleason score 7, two Gleason score 8, nine Gleason score 9, and two Gleason score 10 cases. These 14 cases tended to have higher Topoisomerase indices (mean TI of 13%) using the Miller ocular than the average Topoisomerase index for all 99 carcinoma cases analysed (mean TI of 3.69%).

Review of these discordant cases by a second observer using light microscopy and a Miller ocular correlated within 10% of the original Topoisomerase indices obtained by the first observer. The discrepancy between the Ariol image analysis results and the light microscopy results was due to a difficulty by the image analyser in distinguishing Topoisomerase negative tumour cells from stroma when there was infiltration of the stroma by less differentiated tumour. It therefore underestimated the number of total tumour cells within a field of analysis hence leading to an over-estimation of % cells positive for the Topoisomerase II alpha antibody.

Examples of immunohistochemical staining results obtained are presented in Figures 8 and 9.
Figure 8 A-F: Immunohistochemical Staining for TOPO II-Alpha in

(A) Low power view of TMA showing TOPO II-Alpha focal positivity in benign prostatic hyperplasia (BPH).

(B) High power view of a benign prostatic gland showing focal TOPO II-Alpha nuclear positivity.

(C) Low power view of TMA of a prostatic adenocarcinoma (Gleason score 3+3) showing focal cell positivity for TOPO II-Alpha.

(D) High power view of prostatic adenocarcinoma (Gleason score 3+3) showing focal discrete nuclear TOPO II-Alpha positivity in prostatic acini.

(E) Low power view of TMA of prostatic adenocarcinoma (Gleason score 9) showing multiple cells positive for TOPO II-Alpha.

(F) High power view of prostatic adenocarcinoma (Gleason score 9) showing discrete nuclear TOPO II-Alpha positivity in many tumour cells within the lesion.
Figure 9: High power view of androgen independent prostatic adenocarcinoma (Gleason score 9) showing discrete nuclear TOPO II-Alpha positivity in many tumour cells within the lesion.
2.5 Statistics:

Data were entered on to a computerised database and analysed by the SPSS statistical package using the U Mann Whitney test.
CHAPTER THREE

RESULTS OF CGH MICROARRAYS
Benign prostatic hyperplasia

Localised prostate cancer

CGH MICROARRAYS

Androgen independent prostate cancer

Metastatic prostatic cancer to bone marrow

Figure 10: Microarrays Showing fluorescence emissions
3.1 ARRAY CGH ANALYSIS

A report is generated for each array. This report tabulates among other variables the assigned target number, target clone name, cytogenetic location and the normalised test to reference intensity and a pooled correlation co-efficient. Normalised test to reference intensities more than the mean plus three standard deviations (~1.3) were considered as amplifications or gains of the indicated copy number.

One of these cases (Gleason Score 9) had been treated with anti-androgens and was known to be hormone resistant. The second case was a case of metastatic prostate cancer which had not been treated by anti-androgens. The third prostate sample was a
TURP surgical specimen with involvement of prostate chippings by Gleason Score 7 prostate adenocarcinoma. The fourth sample was a TURP surgical specimen with benign prostatic hyperplasia (BPH) and no evidence of prostatic carcinoma.

Figure 12: Example of report of fluorescence ratios generated by AmpliOnc GenoSensor software
Table 5:
Amplifications of the following genes were identified for the case of androgen independent advanced prostate cancer:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normalised test to reference intensity (fluorescence ratio)</th>
<th>Pooled correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET (tyrosine kinase, amplified in the transition between primary tumours and metastases)</td>
<td>4.27</td>
<td>0.91</td>
</tr>
<tr>
<td>HRAS (Plays role in normal growth and differentiation)</td>
<td>4.11</td>
<td>0.85</td>
</tr>
<tr>
<td>CBFA2 (core binding factor runt domain, involved in AML)</td>
<td>3.96</td>
<td>0.93</td>
</tr>
<tr>
<td>TOP2A (Involved in cell replication)</td>
<td>3.63</td>
<td>0.85</td>
</tr>
<tr>
<td>FES (feline sarcoma virus)</td>
<td>2.96</td>
<td>0.91</td>
</tr>
</tbody>
</table>
Table 6:
Amplifications of the following genes were identified for the case of metastatic prostate cancer:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normalised test to reference intensity (fluorescence ratio)</th>
<th>Pooled correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>FES (feline sarcoma virus,)</td>
<td>3.42</td>
<td>0.86</td>
</tr>
<tr>
<td>MYB (Alterations in MYB gene in more than 1/3 of human solid tumours)</td>
<td>2.19</td>
<td>0.87</td>
</tr>
<tr>
<td>BCL2 3’ (Blocks the apoptotic death of cells ie. lymphoctes)</td>
<td>1.85</td>
<td>0.91</td>
</tr>
<tr>
<td>TOP2A (Involved in cell replication)</td>
<td>1.74</td>
<td>0.91</td>
</tr>
<tr>
<td>AR 5’ (Can facilitate tumour cell growth in low androgen concentrations)</td>
<td>1.70</td>
<td>0.87</td>
</tr>
</tbody>
</table>
Table 7:
Amplifications of the following genes were identified for the case of Gleason score 7 localised prostate cancer:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normalised test to reference intensity (fluorescence ratio)</th>
<th>Pooled correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET (tyrosine kinase,)</td>
<td>5.36</td>
<td>0.82</td>
</tr>
<tr>
<td>SAS/CDK (Sarcoma amplified sequence,)</td>
<td>3.17</td>
<td>0.83</td>
</tr>
<tr>
<td>PGY1 (P-glycoprotein 1, also known as multi-drug resistance 1)</td>
<td>2.61</td>
<td>0.87</td>
</tr>
<tr>
<td>TOP2A (Involved in cell replication)</td>
<td>2.46</td>
<td>0.88</td>
</tr>
<tr>
<td>ERB2 (oncogene, most commonly amplified in breast carcinoma)</td>
<td>2.24</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Table 8:
Amplifications of the following genes were identified for the case of benign prostatic hyperplasia:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normalised test to reference intensity (fluorescence ratio)</th>
<th>Pooled correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>FES (feline sarcoma virus)</td>
<td>4.04</td>
<td>0.92</td>
</tr>
<tr>
<td>PIK3CA (Phosphatidylinositol, 3-kinase, sorts proteins to the vacuole)</td>
<td>3.56</td>
<td>0.88</td>
</tr>
<tr>
<td>GLI (Encodes a protein product with 5 zinc finger DNA binding motifs)</td>
<td>2.89</td>
<td>0.96</td>
</tr>
<tr>
<td>MOS (Moloney murine sarcoma viral oncogene homolog.)</td>
<td>2.06</td>
<td>0.94</td>
</tr>
<tr>
<td>TOP2A (Involved in cell replication)</td>
<td>1.54</td>
<td>0.97</td>
</tr>
</tbody>
</table>
As can be seen from the above tables amplification of MET gene was identified in the case of localised prostate cancer.

FES gene was amplified in androgen independent advanced prostate cancer, metastatic prostate cancer and benign prostatic hyperplasia.

However increased copy number of Topoisomerase II-Alpha (TOPO2A) was detected in all 4 prostate samples including the sample of BPH. Advanced hormone insensitive prostate carcinoma showed the most marked increase in copy number.

The results for the target of interest Topoisomerase II-Alpha are presented in Table 9

Table 9: Array CGH analysis for Topoisomerase II-Alpha gene

<table>
<thead>
<tr>
<th></th>
<th>Normalised test to reference intensity (fluorescence ratio)</th>
<th>Pooled correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced hormone insensitive prostate carcinoma</td>
<td>3.63</td>
<td>0.85</td>
</tr>
<tr>
<td>Metastatic prostate carcinoma</td>
<td>1.74</td>
<td>0.91</td>
</tr>
<tr>
<td>Localised Gleason Score 7 prostate carcinoma</td>
<td>2.46</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Topoisomerase II-Alpha is an essential cellular enzyme that functions in the segregation of newly replicated chromosome pairs, in chromosome condensation and in altering DNA superhelicity.

As Topoisomerase II-Alpha gene was significantly amplified in all our cases of prostate cancer and as it has been reported as a prognostic marker for tissues other than prostate this gene warranted further investigation as regards to it’s expression in prostate cancer. The fact that chemotherapeutic drugs that target the Topoisomerase II alpha gene are currently available increased the importance of investigating its’ expression in prostate tissue.
CHAPTER FOUR

RESULTS OF IMMUNOHISTOCHEMICAL STUDY ON
TOPOISOMERASE II ALPHA GENE EXPRESSION IN BENIGN
PROSTATIC HYPERPLASIA AND PROSTATE CANCER
4.1 Introduction

As Topoisomerase II-Alpha gene was significantly amplified in all our cases of prostate cancer and as it has been reported as a prognostic marker for tissues other than prostate this gene warranted further investigation as regards to it's expression in prostate cancer. The fact that chemotherapeutic drugs that target the Topoisomerase II alpha gene are currently available increased the importance of investigating its' expression in prostate tissue.

Examples of immunohistochemical staining for the Topoisomerase II-Alpha antibody are presented in Figures 13 and 14.
Figure 13 A-F: Immunohistochemical Staining for TOPO II-Alpha in

(A) Low power view of TMA showing TOPO II-Alpha focal positivity in benign prostatic hyperplasia (BPH).

(B) High power view of a benign prostatic gland showing focal TOPO II-Alpha nuclear positivity.

(C) Low power view of TMA of a prostatic adenocarcinoma (Gleason score 3+3) showing focal cell positivity for TOPO II-Alpha.

(D) High power view of prostatic adenocarcinoma (Gleason score 3+3) showing focal discrete nuclear TOPO II-Alpha positivity in prostatic acini.

(E) Low power view of TMA of prostatic adenocarcinoma (Gleason score 9) showing multiple cells positive for TOPO II-Alpha.

(F) High power view of prostatic adenocarcinoma (Gleason score 9) showing discrete nuclear TOPO II-Alpha positivity in many tumour cells within the lesion.
**Figure 14:** High power view of androgen independent prostatic adenocarcinoma (Gleason score 9) showing discrete nuclear TOPO II-Alpha positivity in many tumour cells within the lesion.
4.2 Statistics:

Data were entered on to a computerised database and analysed by the SPSS statistical package using the U Mann Whitney test.

Analysis of immunohistochemical results:

The U Mann Whitney test was used to determine whether there was a statistically significant difference between the Topoisomerase II-Alpha indices in each group. The median and range of Topoisomerase II alpha indices (TI) for each Gleason score are presented in Table 1 and median and range for the categories statistically analysed presented in Table 2 and 3.

There was no statistically significant difference between the groups benign prostatic hyperplasia and prostate carcinoma Gleason Score 6 (P=0.245). The difference between the groups prostate carcinoma Gleason Scores 6 and 7 was statistically significant (P<0.008). In addition there was a highly statistically significant difference between the groups prostate carcinoma Gleason Score 7 and Gleason scores 8,9 and 10 (not selected for hormonal status), (P=0.000).

When the category of Gleason score 8,9,10 was analysed as regard to hormone treatment and insensitivity (n=15) and cases with no hormone treatment (n=34) the difference in Topoisomerase indices approached statistical significance (P=0.081).

Results of the U Mann-Whitney test statistical analysis are presented in Table 10-13. Scatterplots were generated from the data. (Figures 15 & 16)
Table 10: Median and range of Topoisomerase indices according to Gleason score

<table>
<thead>
<tr>
<th></th>
<th>Gleason score 6</th>
<th>Gleason score 7</th>
<th>Gleason score 8</th>
<th>Gleason score 9</th>
<th>Gleason score 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>28</td>
<td>22</td>
<td>12</td>
<td>29</td>
<td>8</td>
</tr>
<tr>
<td>Median</td>
<td>0.3</td>
<td>0.9</td>
<td>1.65</td>
<td>2.6</td>
<td>12.55</td>
</tr>
<tr>
<td>Range</td>
<td>0-3.6</td>
<td>0-5.9</td>
<td>0.3-8.8</td>
<td>0-54.8</td>
<td>0.7-48.6</td>
</tr>
</tbody>
</table>

Table 11: Median and range of Topoisomerase indices according to categories analysed

<table>
<thead>
<tr>
<th></th>
<th>Benign prostatic hyperplasia</th>
<th>Gleason score 6</th>
<th>Gleason score 7</th>
<th>Gleason score 8,9,10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>41</td>
<td>28</td>
<td>22</td>
<td>49</td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>0.7</td>
<td>0.9</td>
<td>15.1</td>
</tr>
<tr>
<td>Range</td>
<td>0-3.3</td>
<td>0-3.6</td>
<td>0-5.9</td>
<td>0-48.6</td>
</tr>
<tr>
<td></td>
<td>Gleason score 8,9,10, hormone resistant, post hormone deprivation</td>
<td>Gleason score 8,9,10, no hormone treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------------------------------------------------------</td>
<td>------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of cases</td>
<td>15</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>5.5</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-48.6</td>
<td>0.3-36.3</td>
<td></td>
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</tr>
<tr>
<td>Categories analysed for statistical significance between Topoisomerase II alpha indices</td>
<td>Z test statistic</td>
<td>P value</td>
<td></td>
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<td>---</td>
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<tr>
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<tr>
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<tr>
<td>Hormone resistant Prostate Carcinoma Gleason Score 8-10 and Gleason Score 8-10 with no hormone treatment</td>
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<td>0.081</td>
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Figure 15: Scatterplot for Topoisomerase II-Alpha indices as determined by diagnostic category
Figure 16: Scatterplot for Topoisomerase II-Alpha indices comparing hormone resistant prostate cancer with group of prostate cancer with no hormone treatment.
CHAPTER FIVE

DISCUSSION
5.1 Introduction

Identification of features, which accurately predict the behaviour of prostate cancer occurring within a specific patient, is a major challenge. Gleason grading is based on morphological features and is a powerful prognostic indicator however there can be difficulties with interobserver reproducibility. In addition prostate carcinomas, which are morphologically indistinguishable and discovered incidentally, can behave in a clinically indolent fashion or aggressively. The identification of genes by the new microarray technology, which correlate with patient prognosis, is an exciting development with potential clinical application.

In this study increased Topoisomerase II-Alpha immunoexpression was associated with prostate cancer with the highest expression in hormone resistant prostate carcinomas. It also correlated with the known prognostic marker of Gleason Score.

Amplification of the Topoisomerase II-Alpha gene was identified in the four prostate samples investigated by CGH microarray indicating that increased expression of the gene is due to amplification at a DNA level.

5.2 Topoisomerase II-Alpha gene

Topoisomerase II-Alpha is an essential cellular enzyme that functions in the segregation of newly replicated chromosome pairs, in chromosome condensation and in altering DNA superhelicity. DNA topoisomerases, are involved in nearly all biological
processes governing DNA and untangle intertwined DNA strands prior to cell division by transiently breaking and then religating duplex strands of DNA. Chemotherapeutic drugs that target Topoisomerase II-Alpha such as etoposide, doxorubicin and mitoxantrone act by stabilizing a normally transient DNA-topoisomerase II complex, thus increasing the concentration of double-stranded DNA breaks. This phenomenon triggers mutagenic and cell death pathways.[114][115] Topoisomerase II-Alpha has been reported as a prognostic marker in a variety of tissues.[116][113][117][118][119][120][121][122][123][124][125][126][127]

To our knowledge there are only 2 papers in the literature which investigate Topoisomerase II-alpha expression in prostate cancer.[128][129] These studies do not investigate Topoisomerase II-Alpha expression in hormone resistant prostate carcinoma. This is of interest as Topoisomerase II-Alpha is the target of the drug etoposide that is an active agent in the combined chemotherapy of hormone-insensitive prostate carcinoma in clinical trials.[130][131]

In this study we examine Topoisomerase II-Alpha expression in benign prostatic hyperplasia and prostate cancer categorised by Gleason Score and by hormone sensitivity and investigate whether Topoisomerase II-Alpha expression is significantly increased in different diagnostic categories of prostate cancer.

There is little information regarding the expression of Topoisomerase II-alpha in prostate carcinoma or in particular in identifying whether its' expression is increased in comparison with benign prostatic hyperplasia. To our knowledge there is only one paper in the literature which attempts to compare Topoisomerase II-alpha expression in prostate cancer and benign prostatic hyperplasia.[129] This study reported little

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expression of Topoisomerase II-Alpha in benign prostatic hyperplasia however it only investigated 10 cases. In contrast to this study we identified Topoisomerase II-Alpha expression in benign prostatic hyperplasia (mean Topoisomerase index 0.9). In addition in our study there was no statistically significant difference in Topoisomerase II-Alpha expression between Gleason Score 6 prostate carcinoma and benign prostatic hyperplasia.

The previous two studies conclude that Topoisomerase II-Alpha expression correlates with Gleason Score. Our larger study also supports this finding. We also identified a statistically significant difference in Topoisomerase II-Alpha expression between prostate cancers classified as regards to Gleason Score.

Amplification of the Topoisomerase II-Alpha gene was identified in the four prostate samples investigated by CGH microarray indicating that increased expression of the gene is due to amplification at a DNA level. The degree of amplification was greatest in the hormone resistant prostate carcinoma however amplification of the gene was also identified in the case of benign prostatic hyperplasia. As Topoisomerase II-Alpha gene is a proliferation related gene and benign prostatic hyperplasia is a proliferative condition this is not surprising. This also correlates with the fact that we did not identify a statistically significant difference in Topoisomerase II-Alpha expression between Gleason Score 6 prostate carcinoma and benign prostatic hyperplasia.

Our study is the first study to our knowledge to examine Topoisomerase II-Alpha expression in hormone resistant prostate carcinoma. This is of particular interest as Topoisomerase II-Alpha is the target of the drug etoposide, which is an active agent in the combined chemotherapy of hormone-insensitive prostate carcinoma in clinical trials. We identified increased Topoisomerase II-Alpha expression in the hormone
resistant prostate carcinomas. This correlates with the fact that the clinical subgroup of hormone resistant prostate carcinomas has shown the best response to etoposide.\cite{130,131} A potential clinical application of the evaluation of Topoisomerase II-Alpha expression in prostate cancer tissue could be used to design rational combination therapies with Topoisomerase II-Alpha targeting drugs. High Topoisomerase II-Alpha immunoexpression was associated with hormone resistant prostate carcinomas. It also correlated with the known prognostic marker of Gleason Score. A number of studies have correlated the level of Topoisomerase II-Alpha expression with a response to anti-Topoisomerase II-Alpha drugs in cancer cell lines.\cite{132,133,134} In the future evaluation of Topoisomerase II-Alpha expression in prostate cancer tissue could be used to design rational combination therapies with Topoisomerase II-Alpha targeting drugs.

In the introductory chapter we discussed possible susceptibility genes for hereditary and sporadic prostate cancer. As we have demonstrated not only amplification but also increased expression of other genes Topoisomerase II-Alpha gene in advanced prostate cancer in this study we will now briefly discuss recent studies in which other genes have been found to be overexpressed in prostate cancer.

5.3 Other genes overexpressed in prostate cancer

Alpha-Methylacyl coenzyme A racemase
Alpha-Methylacyl coenzyme A racemase (AMACR) gene is known to be involved in the beta-oxidation of branched-chain fatty acids and fatty acid derivatives.[135] The enzyme encoded by the AMACR gene plays a critical role in peroxisomal beta oxidation of branched chain fatty acid molecules. AMACR positivity in prostate cancer could have important epidemiological and preventive implications, as the main sources of branched chain fatty acids are dairy products and beef, the consumption of which has been associated with an increased risk for prostate cancer in multiple studies.[136] Both untreated metastases and hormone refractory prostate cancers have been found to be strongly positive for AMACR.[137] AMACR expression has also been found to be a marker of tumour differentiation.[138] In diagnostic histopathology, AMACR marker demonstrates the ability to support a diagnosis of malignancy in prostate needle biopsies. Although it has limitations with respect to sensitivity and specificity AMACR will no doubt become a standard adjunctive stain used by pathologists seeking to reach a definitive diagnosis in prostate biopsies considered to be atypical but not diagnostic of malignancy on haematoxylin and eosin sections alone.[139]

Hepsin

The hepsin gene product is a membrane bound serine protease present in most tissues but at its' highest levels in liver tissue. This protein is thought to have an important role in cell growth. The hepsin gene product was found to be over-expressed in PIN and in prostate cancer using cDNA expression arrays. Hepsin expression levels molecularly distinguished prostate neoplasms using both micro arrays of complementary DNA and using tissue micro arrays of clinically stratified prostate cancer.[140] Expression of
hepsin protein in prostate cancer correlated inversely with patient prognosis.[141]

PIM1

PIM1 encodes a protein kinase upregulated in prostate cancer. The PIM1 gene product was also found to be over-expressed in PIN and in prostate cancer using cDNA expression arrays. PIM1 expression levels molecularly distinguished clinically stratified prostate neoplasms using micro arrays of complementary DNA and tissue micro arrays. A decreased expression of PIM1 kinase in prostate cancer correlated significantly with measures of poor patient outcome, to an even greater extend than hepsin.[141]

A remarkably similar co-transcriptional regulation or gene amplification of PIM1 and the oncogene c-myc (previously discussed) has been identified possibly mediating a synergistic oncogenic effect in prostate cancer.

MTA1

Expression of the metastasis-associated protein 1 (MTA1) has previously been found to be associated with progression to the metastatic state in various cancers. A recent study identified an association of MTA1 expression and prostate cancer progression. Metastatic prostate cancer demonstrated significantly higher mean MTA1 protein expression intensity compared with clinically localized prostate cancer or benign prostate tissue.[142]
EZH2

EZH2 is a developmental regulatory gene that is a transcriptional repressor and is found in higher concentrations in metastatic prostate cancers than in primary tumours.[143] Other genes such as CSEIL, ZNF217, MYBL2, and STK15 have also been found to be over expressed in prostate cancer.[144][145] Further research is required to determine whether the expression of some or all of these genes including the Topoisomerase II alpha gene in prostate cancers will be important prognostic factors for individual patients.

5.4 Recent theory on the role of inflammation in prostate cancer

Inflammation has a role in many cancers.[146] Symptomatic prostatitis occurs in at least 9% of men >40 years of age many suffering from multiple episodes.[147] The prevalence of asymptomatic prostatitis is not known.[148] Inflammatory cells produce numerous oxidants with potential to cause cellular or genomic damage in the prostate.[149] There is accumulating knowledge supporting the role of inflammation in prostate cancer including epidemiology studies to show there is a decreased risk of prostate cancer associated with the intake of anti-oxidants or non-steroidal anti-inflammatory drugs.[150][151]
In addition epidemiological studies have shown an increased risk of prostate cancer associated with sexually transmitted infections regardless of the particular infection suggesting that it is the associated inflammation rather than a particular causal infection that is involved in prostate cancer.[152][153]

Molecular pathology studies also support the hypothesis that inflammation is important in the aetiology of prostate cancer. Two of the prostate cancer susceptibility genes identified thus far and discussed in the introductory chapter, RNASEL and MSR1 encode proteins with critical functions in host responses to infections. In addition a polymorphism of TLR4 is associated with an increased risk of prostate cancer. TLR4 encodes a receptor, which is a central player in the signalling pathways of the innate immune response to infection by Gram-negative bacteria.[8] [15] [22]

Diagnostic histopathologists have also proposed that a prostatic lesion called proliferative inflammatory atrophy (PIA) is a precursor to prostatic intraepithelial neoplasia and prostate cancer. The term proliferative inflammatory atrophy applies to focal atrophic lesions associated with chronic inflammation and often adjacent to foci of prostatic intraepithelial neoplasia or prostate cancer. These lesions are thought to arise as a consequence of the regenerative proliferation of prostate epithelial cells in response to injury caused by inflammatory oxidants.[154] Somatic genomic abnormalities similar to those in prostatic intra-epithelial neoplasia and prostate cancer have been found in foci of proliferative inflammatory atrophy.[155] Epithelial cells in lesions of proliferative inflammatory atrophy also show many
molecular signs of stress, such as high levels of GSTP1, glutathione S-transferase A1 (GSTA1), and cyclooxygenase-2 (COX-2).[156] [157][158] Loss of GSTP1, probably as a result of hypermethylation of the CpG island sequences of GSTP1, may define the transition between proliferative inflammatory atrophy and prostatic intraepithelial neoplasia or prostate cancer.[159][160][161] Phenotypically intermediate cells between basal and secretory cells have been identified in normal prostate epithelium and these cells are increased in proliferative inflammatory atrophy lesions. The finding of a large number of highly proliferating intermediate cells in proliferative inflammatory atrophy indicates that these cells may serve as preferred target cells in prostate carcinogenesis.[162]

Growth Factors

Growth factors are important in the normal regulation of prostate development and growth. However the inappropriate expression of members of the growth factor families has been associated with prostate cancer progression.[163]

Interleukin-6

Interleukin-6 (IL-6) modulates cell growth and apoptosis. It is a multifunctional cytokine that activates the signalling pathways of Janus kinases-signal transducers and activators of transcription (STAT) and/or mitogen-activated protein kinases (MAPK). IL-6 levels are elevated in tissues and sera from prostate cancer patients and IL-6
receptor expression has been detected in prostate cancer cell lines and clinical specimens.

Chronic exposure of prostate cancer cells to IL-6 has been found to facilitate tumour growth in vivo by abolishment of the growth control by pRb and activation of the MAPK signalling pathway.[164] Interleukin-6 has also been shown to have a role in the interaction between epithelial and stromal cells in prostate cancer.[165]

Epidermal, Transforming, Vascular Endothelial and Insulin Growth Factors

Many of these growth factor receptors engage the Ras/mitogen-activated protein (MAP) kinase pathway as part of their signalling activities. These growth factors have been shown to be associated with invasion and metastasis of prostate cancer.[93] [166][167][168] Transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) can also cause prostate cancer progression by acting as angiogenic factors increasing micro vessel density around the cancer.[169] There is evidence that chronic activation of endogenous c-Ras by autocrine and paracrine growth factor stimulation sensitises the androgen receptor transcriptional complex to sub physiological levels of androgen. Progression to hormone refractory disease is often correlated with over expression of growth factors and receptors capable of establishing autocrine and/or paracrine growth-stimulatory loops.[93] Chemotherapy with the aim of interrupting these loops may be a possibility for the treatment of prostate cancer in the future.

Growth Factor Receptors
Growth factor receptors have been recognised as important oncogenes in many cancers, particularly the growth factor c-erb 2 (Her-2 Neu).

C-erb 2 (Her-2 neu)

There is some controversy over the role of c-erb 2 (Her-2 neu) in prostate cancer. C-erb 2 belongs to the epidermal growth factor receptor family. There have been FISH studies of primary prostate cancer specimens which have suggested that c-erb 2 gene amplification and neu overexpression are significantly correlated with DNA content, advanced grade and advanced stage.[170][171][172] However, large studies using FISH (339 cases) and CISH (126 cases) demonstrate that ERBB2 is not amplified in prostate cancer.[173][174]

Immunohistochemical studies have given rise to conflicting results due to different methodologies and different antibodies. Some studies report c-erb 2 (Her-2 Neu) overexpression in prostate cancer and some suggest that it increases in expression levels as prostate cancer progresses to androgen independence.[175][176] Other studies have not identified Her-2/neu amplification or over-expression in prostate cancer.[177][178][179] In summary, when evaluated scientifically the research to date demonstrates that c-erb 2 (Her-2 Neu) gene is not amplified in prostate cancer. Whether c-erb 2 (Her-2/neu) is over-expressed in prostate cancer remains controversial but studies with immunostaining as well as Q-RT-PCR have demonstrated that the expression of c-erb 2 is much lower than for example in breast carcinomas with c-erb 2 (Her-2 Neu) amplification and over expression.[180] Chemotherapy currently targeted towards c-erb 2 over expression in breast cancer is unlikely to have similar clinical application in prostate cancer.
Fas/Fas Ligand

Fas is a Type I membrane receptor of the tumour necrosis factor/nerve growth factor family. On binding to Fas ligand, a Type II transmembrane protein, the Fas/Fas ligand complex, induces apoptosis in target cells. Dysregulation of Fas and Fas ligand mediated apoptosis is thought to be involved in prostate tumorigenesis. Fas/Fas ligand has been found to be elevated in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. [181]

c-MET

Hepatocyte growth factor and its receptor, the c-met proto-oncogene product (c-MET), have been implicated in embryogenesis, tissue re-organization and tumour progression. C-met protein has been detected in a substantial number of prostate cancers and has been found more often in metastatic growths of prostate carcinoma and in androgen insensitive prostate cancer cell lines. There is also evidence that c-met (hepatocyte growth factor) enhances the invasive potential of prostate cancer cells. [182] High c-met receptor expression has also been identified in prostate cancer metastasis to bone. [183][184]

Topoisomerase II alpha gene is important in cell replication and proliferation and was found to be amplified on CGH microarray analysis in our study in the case of benign prostatic hyperplasia as well as the prostate cancer cases. This supports the theory that increased proliferation in the prostate gland increases the susceptibility to prostate cancer.
The evidence for a role of inflammation in prostate cancer is convincing with support from epidemiological, molecular and histopathological studies. In the near future it may be common practice for the prescription of anti-inflammatory drugs to decrease the risk of development of prostate cancer just as the prescription of aspirin is common practice now to decrease the risk of stroke.

5.5 Possible future advances in the diagnosis of prostate cancer – Proteomics

The system-wide study of proteins presents an exciting challenge in this information-rich age of whole-genome biology. Although traditional investigations have yielded abundant information about individual proteins, they have been less successful at providing us with an integrated understanding of biological systems. The promise of proteomics is that, by studying many components simultaneously, we will learn how proteins interact with each other, as well as with non-proteinaceous molecules, to control complex processes in cells, tissues and even whole organisms.

Proteomics presents a new horizon for biomarker discovery and uses protein-profiling technologies that can simultaneously resolve and analyse multiple proteins. Identification of proteomic patterns in serum has been used to distinguish neoplastic from non-neoplastic disease within the prostate.

Study cohorts of healthy controls, benign prostate neoplasia and prostate cancer could be separated based on the over expression or under expression of nine protein masses. This study required only the mass values of the proteins using a protein biochip mass spectrometry approach coupled with an artificial intelligence learning algorithm.
Knowing the protein identities was not required for the purposes of differential diagnosis.

Efforts are underway to identify and characterise these peptide/protein biomarkers as this knowledge will be important in understanding what biological role they have in prostate cancer oncogenesis. A protein known as Growth Differentiation Factor 15 has been identified as a proteomic alteration in a recent study using laser capture micro dissection and may be involved in early prostate carcinogenesis. Down-regulation of interferons has also been identified by proteomic analysis. Proteomics is a very exciting molecular tool and studies to date have shown a higher specificity for prostate cancer than PSA screening.

In the future it should be possible to detect Topoisomerase alpha II protein expression in prostate cancer using proteomics.

5.6 Conclusion

The importance of the increased expression of Topoisomerase II alpha is that there is a potential clinical application in the evaluation of Topoisomerase II-Alpha expression in prostate cancer tissue as it could be used to design rational combination therapies with Topoisomerase II-Alpha targeting drugs.

The molecular pathology of prostate cancer is complex, not only are multiple genes involved in its' pathogenesis but additional environmental factors such as inflammation are also involved. There has been exhaustive research into prostate cancer to date, which has demonstrated a complex interaction of multiple genes and environmental
factors, some of which may be more important in individual prostate cancer cases. This is an exciting era with the emergence of new investigative tools such as DNA microarray technology and the application of the field of proteomics to the study of human cancers. Knowledge of genetic changes underlying initiation, development and progression of prostate cancer is accumulating rapidly. With increasing knowledge it may be possible to distinguish indolent from aggressive prostate tumours by molecular fingerprinting.

In this study we have identified increased expression of Topoisomerase II alpha gene in advanced prostate cancers with high Gleason scores.
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This thesis almost didn’t get written as I have now moved on to pastures new in the John Radcliffe Hospital, Oxford. I have to thank my supervisor Prof. John O’Leary for his constant encouragement and enthusiasm throughout this work and the publications which arose from it and for stimulating my motivation to complete this thesis.

Finally I have to thank my husband John. He has been incredibly supportive throughout this work. He has been through the process of thesis submission himself and used his experience to help me particularly with the compilation of the references.
APPENDICES
The term proliferative inflammatory atrophy applies to focal atrophic lesions associated with chronic inflammation and often adjacent to foci of PIN or prostate cancer. These lesions are thought to arise as a consequence of the regenerative proliferation of prostate epithelial cells in response to injury caused by inflammatory oxidants. Somatic genomic abnormalities similar to those in PIN and prostate cancer have been found in foci of proliferative inflammatory atrophy. Epithelial cells in lesions of proliferative inflammatory atrophy also show many molecular signs of stress, such as high concentrations of GSTP1, glutathione S-transferase A1, and cyclooxygenase 2.

"The evidence for a role of inflammation in prostate cancer is convincing with support from epidemiological, molecular, and histopathological studies."

Loss of GSTP1, probably as a result of hypermethylation of the CpG island sequences of GSTP1, may define the transition between proliferative inflammatory atrophy and PIN or prostate cancer. Phenotypically intermediate cells between basal and secretory cells have been identified in normal prostate epithelium and these cells are increased in proliferative inflammatory atrophy lesions. The finding of a large number of highly proliferating intermediate cells in proliferative inflammatory atrophy indicates that these cells may serve as preferred targets in prostate carcinogenesis.

The evidence for a role of inflammation in prostate cancer is convincing with support from epidemiological, molecular, and histopathological studies. In the near future it may be common practice for the prescription of anti-inflammatory drugs to decrease the risk of development of prostate cancer, just as the prescription of aspirin is common practice now to decrease the risk of stroke.

CONCLUSIONS

Powerful new molecular pathology tools such as DNA microarrays are providing information that is already being incorporated into diagnostic pathology such as AMACR staining in prostate cancer cells. The molecular pathology of prostate cancer is complex; not only are multiple genes involved in its pathogenesis, but additional environmental factors such as diet and inflammation also play a role.

In other cancers such as colon cancer there are gatekeeper genes and multistep models of carcinogenesis. No such genes have been identified in prostate cancer despite exhaustive research. There is a complex interaction of multiple genes and environmental factors, some of which may be more important in individual patients with prostate cancer. This may explain why the molecular pathology findings in prostate cancer have not been useful in clinical practice to date; however, this looks likely to change.

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Topoisomerase II-α expression increases with increasing Gleason score and with hormone insensitivity in prostate carcinoma

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Aim: To investigate and compare topoisomerase II-α expression in benign prostatic hyperplasia (BPH), prostate cancer of varying Gleason scores and hormone-insensitive prostate cancer.

Methods: The immunohistochemical expression of topoisomerase II-α antibody in the above-mentioned diagnostic categories was investigated and compared.

Results: Increased expression of topoisomerase II-α was seen in the prostate cancers of Gleason scores 7 and 8–10 (p = 0.000) compared with prostate cancers of Gleason score 6 and BPH (p = 0.245). Statistically significant differences were found in the topoisomerase II-α gene expression between prostate cancers categorised by Gleason Score. Also, increased expression of topoisomerase II-α was seen in the known hormone-resistant prostate carcinomas compared with prostate cancers with no hormone treatment in the subgroup with Gleason scores 8–10, which approached statistical significance (p = 0.081). No statistically significant difference was observed in topoisomerase II-α expression between the groups with BPH and prostate carcinoma of Gleason score 6 (p = 0.245).

Conclusion: Topoisomerase II-α expression was found to increase with the known prognostic marker Gleason score and with hormone insensitivity. Objective evidence is provided for clinical trials with drugs targeting topoisomerase II-α to be targeted to patients with prostate cancers of Gleason Score >6 and, in particular, prostate cancers of Gleason Scores 8–10.

Abbreviation: BPH, benign prostatic hyperplasia
Immunohistochemical staining

Immunohistochemical staining for topoisomerase II-α was carried out by using a monoclonal anti-topoisomerase II-α antibody at a dilution of 1:300. Sections of 5 μm thickness were studied. Antigen retrieval was performed with dewaxed sections by using a pressure cooker and the Trilogy system for 10 min. A standard avidin–biotin–peroxidase complex technique (Dako Glostrup, Denmark) was used for visualisation with diaminobenzidine as a chromogen. Sections were counterstained with haematoxylin and mounted. Tonsil samples from our routine files were used as positive controls for topoisomerase II-α.

Evaluation of immunohistochemistry

Immunohistochemical staining was evaluated by light microscopy using a ×40 objective and a Miller ocular. The Miller ocular gave a square field, in the corner of which was a smaller ruled square, one ninth the area of the total square. Nuclei with strong homogeneous topoisomerase II-α staining were counted in the large square and the total number of nuclei with both positive and negative staining was counted in the small square. The number of cells in the small square was multiplied by nine to obtain the total number of cells in the large square. A mean of 240.5 epithelial cells was obtained in the prostate cancer cases and a mean of 68 epithelial cells in the cases of BPH. Five non-overlapping square fields were counted for each tissue core, three on the y axis and two on the x axis. The percentage of positively staining nuclei within the five large squares was calculated for each core. An average result was obtained from three cores in cases with tissue microarrays in triplicate and from two cores in cases with tissue microarrays in duplicate. This served as the topoisomerase II-α index for the case. This method was used in previous studies in the evaluation of immunohistochemical results in tissue microarrays.

In addition, each topoisomerase II-α index was obtained by image analysis that used the Ariol system for tissue microarrays. An acceptable level of concordance was taken as within +10% or −10% of the topoisomerase II-α index obtained with the Miller ocular.

Of the 140 cases in total (138 in triplicate and 2 in duplicate), 14 (10%) cases showed discordance between the topoisomerase II-α indices from the Ariol image analyser and the Miller ocular. In all cases the Ariol image analyser overestimated topoisomerase II-α indices compared with the Miller ocular method.

A second observer reviewed these discordant cases. This discordant group had one case of Gleason score 7, two cases of Gleason score 6, nine of Gleason score 8, nine of Gleason score 9 and two of Gleason score 10. These 14 cases tended to have higher topoisomerase indices (mean topoisomerase II-α index 13%) when using the Miller ocular than the average topoisomerase index for all 99 carcinoma cases analysed (mean topoisomerase II-α index 3.69%). A review of these discordant cases by a second observer, using light microscopy and a Miller ocular, correlated within 10% of the original topoisomerase indices obtained by the first observer. The discrepancy between the results from the Ariol image analyser and light microscopy was due to difficulty in distinguishing topoisomerase-negative tumour cells from stroma by using the image analyser when there was infiltration of the stroma by a less differentiated tumour. The image analyser therefore underestimated the total number of tumour cells within a field of analysis, leading to an overestimation of the percentage of cells positive for the topoisomerase II-α antibody.

Statistics

Data were entered on to a computerised database and analysed with the SPSS statistical package and the Mann–Whitney U test.

Analysis of immunohistochemical results

The Mann–Whitney U test was used to determine whether there was a significant difference between the topoisomerase II-α indices in each group.

RESULTS

Immunohistochemical staining

Figures 1 and 2 show the results of immunohistochemical staining.

Statistical analysis

Table 1 shows the median and range of topoisomerase II-α indices for each Gleason score. Tables 2 and 3 show median and range for the categories statistically analysed.

We found no significant difference between the groups with BPH and with prostate carcinoma of Gleason score 6 (p = 0.245). The difference between the groups with prostate

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Figure 1  Immunohistochemical staining for topoisomerase II-α (TOPO II-α). (A) Low-power view of the tissue microarray (TMA) showing TOPO II-α focal positivity in benign prostatic hyperplasia (BPH). (B) High-power view of a benign prostatic gland showing focal TOPO II-α nuclear positivity. (C) Low-power view of TMA of a prostatic adenocarcinoma (Gleason Score 3+3) showing focal cell positivity for TOPO II-α. (D) High-power view of prostatic adenocarcinoma (Gleason Score 3+3) showing focal discrete nuclear TOPO II-α positivity in prostatic acini. (E) Low-power view of TMA of prostatic adenocarcinoma (Gleason Score 9) showing multiple cells positive for TOPO II-α. (F) High-power view of prostatic adenocarcinoma (Gleason Score 9), showing discrete nuclear TOPO II-α positivity in many tumour cells within the lesion.

Figure 2  Immunohistochemical staining for topoisomerase II-α (TOPO II-α) in a high-power view of a hormone-insensitive prostatic adenocarcinoma (Gleason Score 9) showing discrete nuclear TOPO II-α positivity in many tumour cells within the lesion.
carcinoma of Gleason scores 6 and 7 was significant (p<0.008). In addition, the groups with prostate carcinoma of Gleason score 7 and Gleason scores 8-10 (not selected for hormonal status) differed significantly (p = 0).

When the category of cases with Gleason Scores 8-10 was analysed with regard to hormone treatment and insensitivity (n = 15) and cases with no hormone treatment (n = 34), the difference in topoisomerase indices approached significance (p = 0.081). Table 4 shows the results of analysis by the Mann-Whitney U test. Figures 3 and 4 show scatter plots generated from the data.

**DISCUSSION**

Little information is available on the expression of topoisomerase II-α in prostate carcinoma tissue or, in particular, in identifying whether its expression is increased in comparison with BPH. To our knowledge, only two papers in the literature investigated topoisomerase II-α expression in prostate carcinoma tissue and one paper attempted to compare topoisomerase II-α expression in prostate cancer and BPH. That study, which investigated only 10 cases, reported little expression of topoisomerase II-α in BPH. In contrast with this study, we identified topoisomerase II-α expression in BPH (range 0-3.3). In addition, our study showed no statistically significant difference in topoisomerase II-α expression between prostate carcinoma of Gleason Score 6 and BPH.

The previous two studies conclude that topoisomerase II-α expression correlates with Gleason score, which is supported also by our larger study. We also identified a statistically significant difference in topoisomerase II-α expression between prostate cancers classified with respect to Gleason score.

To our knowledge, our study is the first of its kind that examined topoisomerase II-α expression in hormone-resistant prostate carcinoma. This is of particular interest as topoisomerase II-α is the target of the drug etoposide, which is an active agent in the combined chemotherapy of hormone-insensitive prostate carcinoma in clinical trials. We identified increased topoisomerase II-α expression in the hormone-resistant prostate carcinomas compared with prostate carcinomas with no hormone treatment, which approached statistical significance. The number of cases of hormone-resistant prostate cancer of Gleason scores 8-10 was limited (n = 15) compared with that of prostate carcinoma of Gleason scores 8-10 with no hormone treatment (n = 34). Further studies with increased statistical power and increased numbers of hormone-resistant prostate carcinoma are required to show conclusively that this hormone-resistant subgroup of prostate carcinoma shows increased topoisomerase II-α expression compared with prostate carcinoma with no hormone treatment.

The importance of this finding would be in providing clinicopathological correlation for the fact that this clinical
subgroup of hormone-resistant prostate carcinomas has shown the best response to the drug etoposide that targets the topoisomerase II-α gene.16, 19

Our study showed with statistical significance that higher topoisomerase II-α immunoreactivity was associated with poorly differentiated prostate carcinomas (Gleason scores 8–10) compared with moderately differentiated prostate carcinoma (Gleason score 7), and between moderately differentiated prostate carcinoma (Gleason score 7) and well-differentiated prostate carcinoma (Gleason score 6).

Several studies have correlated the level of topoisomerase II-α expression with a response to anti-topoisomerase II-α drugs in cancer cell lines.20–24 As treatment options for advanced prostate carcinoma are limited, and advanced prostate carcinomas are more likely to be of higher Gleason score, it is important to attempt to identify potential targets for chemotherapy agents in this patient group.

In conclusion, we believe that the topoisomerase II-α gene is a potential target for chemotherapy in this group of patients, particularly in patients with prostate carcinomas of Gleason scores 8–10.

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