A retrospective histopathological study and selected molecular genetics of archival prostatic cancer tissue

Elizabeth Helen Lombard
MBChB(Pret), MMed (Haem Path)(US), FCPATH (SA) Anat Path

Thesis submitted in fulfilment of the requirements for part III of the Master’s Degree in Medicine, Faculty of Health Sciences, University of Cape Town

Supervisor: Professor P Hall
Division of Anatomical Pathology
Faculty of Health Sciences
University of Cape Town
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Declaration

I declare that this thesis is my own work. It has not been previously submitted for any diploma, degree or examination at another institution. This work was carried out in the Division of Anatomical Pathology, Health Sciences Faculty, and University of Cape Town. The opinions and conclusions drawn are not necessarily those of the University of Cape Town.

Elizabeth Helen Lombard

3/6/5

Date
Acknowledgements

I would like to thank:

- Professor Pauline Hall: For her support and guidance
- Professor Dhiren Govender for his assistance in the Gleason grading of some of the tumours and for the opportunity to complete the MMed degree in the division of Anatomical Pathology.
- Ms Nafiesa Allie, Division of Anatomical Pathology, University of Cape Town, for her patience and invaluable technical assistance in performing the immunohistochemical stains
- Dr Pascale Willem and Ms Sabera Kolia from the department of Molecular Medicine and Haematology, University of the Witwatersrand School of Pathology, for teaching me the FISH technique on paraffin tissue, and for their invaluable encouragement and support
- Dr Sedick Isaacs for his assistance with the statistical analysis
- Dr Andre van der Merwe and Dr Phillip Nakangombe from the division of Urology, University of Cape Town and Groote Schuur Hospital, for clinical information and advice
Abstract

Prostatic adenocarcinoma is the second most common carcinoma, and a major cause of death, in men, in Western populations. The risk of developing prostatic carcinoma in South Africa has been calculated as 1 in 14 for white men, 1 in 61 for black men, 1 in 50 for men of mixed race and 1 in 47 for Asian men\(^1\). The diagnosis is usually made by transrectal needle biopsy or transurethral resection of the prostate (TURP) and the tumours are graded according to the Gleason grading system\(^2\). Treatment is dependent on the stage of the disease and consists of surgery or hormonal therapy. The molecular mechanisms involved in the development and progression of prostatic carcinoma are complex\(^3,4\).

The aims of this study were to determine the age at presentation and the racial distribution of prostatic adenocarcinoma in the Western Cape region and to correlate this with histological grade; to correlate the expression of androgen receptor, bcl-2, p53 and Cox-2 with the Gleason grade of disease and patient demographic data and to establish a method to determine androgen receptor (AR) gene amplification in formalin fixed prostatic carcinoma tissue. Also, to assess the role of AR receptor amplification in the small cross-section of patients used to establish the method and to correlate the expression of androgen receptor, bcl-2, p53 and Cox-2 with the AR receptor amplification.

The demographic, clinical and histological data of newly diagnosed cases with prostatic carcinoma from January 1999 to December 2001 were reviewed. From the above group 61 cases with adequate archived histological material were selected for immunohistological studies determining AR surface, bcl-2, p53, Cox-2 and HPV product expression. Ten specimens from patients who showed progression of their disease on hormonal therapy were selected for the determination of androgen receptor gene amplification as well AR surface, bcl-2, p53 and Cox-2 expression.
The mean age of presentation in the mixed race group in the patients within our study was significantly younger than the white race group. In the group of 61 evaluated for Gleason score there was a significant increase in cases within the Gleason score 8 to 10 group (59%) and there was a positive correlation between Gleason score, AR expression, p53 expression and bcl-2 expression. A high percentage of cases expressed p53 (67%) and the mean p53 expression was significantly higher in the cases that expressed Cox-2 in stromal cells. This indicates that mutagens may play a significant role in prostatic carcinogenesis. A disproportionate number of specimens from black patients were positive for bcl-2 indicating a possible anti-apoptotic mechanism in the development of prostatic carcinoma within this group. One of the cases with progression of disease after hormonal therapy showed loss of Cox-2 and bcl-2 expression during evolution of the clone with gain of amplification of the gene, indicating a possible mechanism responsible for the clonal evolution.
Abbreviations

AAH  atypical adenomatous hyperplasia
AR   androgen receptor
CEP X X chromosome centromere probe
COX  cyclo-oxygenase
FISH fluorescent in situ hybridisation
GSH  Groote Schuur Hospital
HPV  human papilloma virus
IDC  interdigitating dendritic cell
PAF  protease activation factor
PIN  prostatic intra-epithelial neoplasia
PSA  prostate specific antigen
TURP transurethral resection of the prostate
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Chapter 1

Introduction and Literature review

1.1 Epidemiology

Prostatic adenocarcinoma is the second most common carcinoma, and a major cause of death, in men, in Western populations. The annual incidence of prostatic carcinoma in South Africa shows racial variation; in the white population the incidence is 43 per 100 000 and in the black population it is 13 per 100 000. These figures are in contrast to North American statistics where the incidence is 42 per 100 000 in the white population and 72 per 100 000 in the African American population. Several factors are proposed to play a role in the racial variation in North America and these include higher levels of sex hormone binding globulin in the tissue, that may activate the androgen receptor (AR) in African American patients.

The risk of developing prostatic carcinoma in South Africa has been calculated as 1 in 14 for white men, 1 in 61 for black men, 1 in 50 for men of mixed race and 1 in 47 for Asian men. Possible polymorphic variation in the gene that encodes vitamin D binding protein may play a role in the racial variation in South Africa.

The mean age of diagnosis for prostatic carcinoma quoted in the American literature, not broken down according to racial distribution, is 67 to 75 years.

1.2 Diagnosis and grading

Serum Prostate-Specific Antigen (PSA) is the most accurate and clinically useful biochemical screening marker in the detection of carcinoma of the prostate and is advocated for use in men older than 40 years who are at increased risk of carcinoma of the prostate and in all men over 50 years. The definitive histological diagnosis is usually
made by transrectal needle biopsy, using a spring-driven 18 gauge needle or by transurethral resection of the prostate (TURP).

The Gleason grading system is universally gaining popularity as the preferred grading system for prostatic adenocarcinoma. This system was developed by Dr Donald Gleason and the Veterans Administration Cooperative Urological Research Group. It assigns histological patterns from 1 to 5 based on the degree of architectural differentiation, with 1 being well differentiated and 5 undifferentiated. The primary grade is the most common or predominant grade and the secondary grade is the next most common but should comprise at least 5% of the tumour. The score (out of ten) is composed of the sum of the primary and secondary growth patterns (both out of five).

For statistical purposes the grades are usually grouped as Gleason score 2 to 4 (well differentiated), Gleason score 5 and 6 (moderately differentiated), Gleason score 7 (moderate to poorly differentiated) and Gleason score 8 to 10 (poorly differentiated).

Several nomograms are used to predict final pathological stage on radical prostatectomy. These nomograms use Gleason score on biopsy, clinical stage, serum PSA and the extent of cancer on biopsy to predict the risk of extraprostatic disease, seminal vesicle invasion and lymph node metastases. The treatment is based on these findings and therefore, an accurate Gleason grade is crucial.

1.3 Pathogenesis

Prostatic adenocarcinoma usually arises in the peripheral zone of the prostate (68%) but may also arise in the peripheral and transition zone. Two possible premalignant lesions of the prostate have been described: high grade prostatic intra-epithelial neoplasia (PIN) and atypical adenomatous hyperplasia (AAH). Of these two, a convincing argument has only been made for the premalignant nature of high grade PIN. High grade PIN is usually found in the peripheral zone of the prostate and has been described in up to 72% of cases of carcinoma and only 17% of cases without carcinoma. On occasion, carcinoma may arise directly from an area of high grade PIN.
Several factors have been identified that influence the development of prostatic carcinoma. These include a positive family history and dietary factors\textsuperscript{15}. Specifically, a diet rich in fructose and low in calcium may reduce the risk of developing prostatic carcinoma. Presumably this effect is due to increased hydroxylation of vitamin D\textsuperscript{16}, which has an antiproliferative effect in prostatic cancer cell lines\textsuperscript{17}.

Figure one summarises the factors potentially involved in prostate carcinoma development. The factors examined in this study will be discussed further in more detail.
Figure 1.1 The factors potentially involved in the development of prostatic carcinoma. (adapted from Ruitjter et al.)
1.3.1 Molecular mechanisms

The molecular mechanisms involved in the development of prostatic carcinoma are complex. Abnormalities in the androgen receptor (AR), p53 and bcl-2 genes or gene products have been described, amongst others, and will be discussed.

1.3.1.1 Androgen receptor

The actions of androgens, specifically testosterone and 5α-di-hydrotestosterone, are mediated by a specific receptor protein, the androgen receptor. This receptor is encoded by a single copy gene located on the X chromosome. This receptor protein is a member of the superfamily of steroid hormone receptors and modulates a range of processes during embryology and adulthood. During embryogenesis, normal AR function is critical to the development of the male phenotype18.

A number of studies have documented considerable heterogeneity of AR expression early in the development of prostatic cancer, but it is accepted that AR is expressed in advanced forms of prostate cancer18.

Palliative treatment for disseminated prostatic carcinoma aims to down regulate the concentration of circulating androgen. Treatment by orchidectomy decreases the circulating androgens by 95%, but adrenal androgens remain intact and prostatic cancer cells adapt to the low concentrations of androgens by enhancing production of dihydrotestosterone from the adrenal precursor androgens. This is usually achieved by AR gene amplification by the tumour cells19.

There is currently no effective treatment for recurrent carcinoma after androgen depletion or oestrogen therapy. An understanding of the underlying mechanisms in the development of resistance to hormonal therapy is important in the development of new therapeutic strategies.
Several mechanisms may contribute to AR activity in androgen independent prostate cancer apart from AR amplification, such as:

- AR mutation,
- altered expression of AR coactivator and corepressor proteins and
- activation of other pathways that can enhance AR function\(^{20}\).

C-myc has been found to act downstream of AR through multiple growth effectors. Thus c-myc is required for androgen dependent growth and following ectopic expression, can induce androgen--independent growth\(^{21}\).

### 1.3.1.1a Expression of surface protein

The expression of several genes involved in the regulation of the cell cycle and apoptosis, may be regulated via the androgen receptor in the prostate. Up regulation of bcl-2 is associated with androgen independent prostate cancer progression\(^{22}\). A significant association between AR and bcl-2 expression was found by Amirghofran *et al.*, however, no prognostic value was found for AR regarding its correlation with stage and grade\(^{23}\). In pre-treatment, transurethral specimens, of hormonally treated prostate cancer patients, Noordzij *et al.* found androgen receptor did not correlate with tumour stage and only marginally correlated with tumour grade, but when a combined bcl-2 /androgen receptor score was used, it was an independent prognostic marker to predict clinical progression\(^{24}\).

Cronauer *et al.* found that over expression of wild type p53 decreases androgen function, whereas p53 expressed at physiological levels stabilises AR signalling. Therefore, there is a balance of AR and p53 expression during the androgen-dependent growth of prostatic cancer, which is lost during the further progression of the disease\(^{25}\).

In studies comparing the development of AR gene amplification and AR protein expression, an increase in AR expression was seen with the development of AR amplification in paired tumours and the level of AR expression was significantly higher in hormone resistant tumours compared to hormone sensitive tumours\(^{26,27}\). A recent study has shown that high levels of androgen receptor expression is associated with aggressive
clinicopathological features and decreased biochemical recurrence-free survival in prostate cancer patients treated with radical prostatectomy.\textsuperscript{28}

1.3.1.1b Amplification of androgen receptor gene

In studies examining AR gene mRNA levels, expression was preferentially seen in patients with high grade, high stage tumours after combined androgen blockade treatment.\textsuperscript{29} Apart from AR gene amplification, an increased copy number of chromosome X have been found.\textsuperscript{30}

Although AR gene amplification has been found in significant numbers of tumours following resistance to androgen deprivation,\textsuperscript{26,27} the rate is still too low to be solely responsible for the development of resistant tumour.\textsuperscript{31}

Ford \textit{et al.} found that AR gene amplification increases AR expression on tumour cells but does not affect survival.\textsuperscript{27}

Controversial results are found in the literature regarding the presence of AR gene amplification in untreated tumours. Some studies have found no evidence of amplification in untreated tumours \textsuperscript{32-34} while other studies have found the AR gene amplification in up to 35\% of untreated tumours.\textsuperscript{31}

In the clinical setting AR gene amplification and point mutations are reported in patients with metastatic disease. Specifically AR gene amplification has been demonstrated by fluorescence in situ hybridisation (FISH) in bone metastases from hormone refractory prostate cancer patients.\textsuperscript{35}

1.3.1.2 bcl-2

Bcl-2 was the first negative regulator of programmed cell death to be identified.\textsuperscript{36} Bcl-2 suppresses apoptosis by direct action on mitochondria to prevent increased permeability and by mediating other proteins. For example, Bcl-2 may bind with the pro-apoptotic protease activating factor (PAF). PAF is the protein that activates cytochrome c, to
trigger the caspases and thereby set in motion the proteolytic events that kill the cell. The anti-apoptotic mechanisms of bcl-2 are illustrated in figure 1.2.

**Figure 1.2.** Schematic representation of the role of bcl-2 in apoptosis

A marked decrease in pro-apoptotic signals in prostates of aging rats has been described and may play a role in the human disease as well.

The expression of bcl-2 has been found to correlate with clinical grade and tumour progression in surgically resectable prostatic carcinomas. However, in a study by Noordzij *et al.* of pre-treatment transurethral resection specimens of hormonally treated (orchidectomy and anti-androgen therapy) prostate cancer, the bcl-2 scores did not
correlate with tumour grade or stage, but when a combined bcl-2 /androgen receptor score was used, it was an independent prognostic marker to predict clinical progression with Gleason grade and stage classification\textsuperscript{24}.

Bcl-2 has been found to be upregulated after androgen ablation of prostate carcinoma cell lines and in castrated rats\textsuperscript{42,43}. Over expression of the gene may confer resistance to androgen withdrawal and correlates with androgen resistance \textsuperscript{22,43,44}. It may have a synergistic effect with p53. Bcl-2 has been shown to correlate with biochemical recurrence after radiotherapy\textsuperscript{45,46}.

1.3.1.3 p53

p53 is a cell-cycle regulatory protein that inhibits progression of genetically damaged cells through the S-phase. Mutations in the p53 gene represent the most common genetic mutation in human malignancies. The wild type of p53 has a very short half-life and is usually not detected by immunohistochemistry, but mutated p53 has a longer half-life and may be detected. p53 mutation has been found to be a late event in tumour development in the prostate gland by Berner et al.\textsuperscript{47}, but other authors have described p53 mutations in the early stages of prostatic cancer\textsuperscript{48,49}.

In studies p53 positivity in prostatic adenocarcinoma cases has ranged from 6\%\textsuperscript{50} to 61\%\textsuperscript{51}.

Expression of p53 is associated with an androgen independent phenotype and androgen receptor gene amplification\textsuperscript{32}. There has also been positive correlation between p53 expression, Gleason grade, stage, disease progression and recurrence \textsuperscript{52}. A study by Brewster et al. has shown that p53 and Gleason score were independent predictors of biochemical relapse after radical prostatectomy\textsuperscript{53} and Theodorescu et al. have shown that in p53 immunohistochemically positive tumours the 15 year survival was 38\%, compared to 87\% for those with negative immunoreactivity\textsuperscript{54}.

The expression of p53, in cancer localised to the prostate gland, has been found (like bcl-2), to be associated with radiotherapy treatment failure\textsuperscript{55}.
It has been suggested that tumour regression in human prostate cancer is due to cell cycle arrest rather than to apoptosis and that emergence of androgen-independence is associated with a release from cell cycle arrest\(^5^6\). Amplification of the AR gene in hormone-refractory patients has been shown to be associated with an increase in p53 expression.

### 1.3.2 Other pathogenetic mechanisms

#### 1.3.2.1 COX-2

Cyclo-oxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandins. Prostaglandins inhibit apoptosis and stimulate angiogenesis and invasiveness\(^5^7\). During the conversion of arachidonic acid to prostaglandins, free radicals are generated and xenobiotics can be oxidised to form mutagens and in addition, a direct mutagen, malondialdehyde may be formed. Inhibition of COX enzymes leads to an increase in arachidonic acid, which can stimulate the conversion of sphingomyelin to ceramide, a mediator of apoptosis\(^5^8\). Two isoforms of COX have been identified. COX-1 is expressed constitutively in many tissues while COX-2 is inducible by cytokines, growth factors and tumour promoters \(^5^9\). Figure 1.3 illustrates the COX-2 catalytic pathways.
Non-steroidal anti-inflammatory drugs (NSAID’s) inhibit the activity of both COX-1 and COX 2. It has been observed that patients taking NSAID’s, had a decreased risk of colon cancer 60 and recent studies have shown that there was a trend towards reduced risk of advanced prostate cancer with regular use of non-steroidal anti-inflammatory drugs (NSAID’s)61,62. An increase in COX-2 expression has been found in prostatic carcinoma cells in contrast to no increased in expression in the cells of benign prostatic hypertrophy 63. The expression of COX-2 and therefore sensitivity to treatment by NSAID’s may present additional therapeutic options.

It has been found that Cox-2 inhibition down regulates bcl-264. This may contribute to mediation of apoptosis.
COX-2 is highly expressed in prostate cancer cell lines and found to be increased significantly in the prostates of aging rats. Numerous studies have demonstrated overexpression of COX-2 in prostatic adenocarcinoma tissue using immunohistochemistry. Madaan et al. found heterogeneous expression of COX-2 in prostate tumour cells and differences in localisation of staining between benign prostatic hypertrophy (BPH) glandular cells and prostatic adenocarcinoma cells. In the carcinoma cells the staining was cytoplasmic and moderate to strong, increasing with increasing grade, while in the BPH cases, the staining was membranous. In both the BPH and carcinoma cases there was no COX-2 staining of the stromal cells. Kirschenbaum et al. demonstrated that in benign prostate COX-2 expression was limited to smooth muscle cells and some basal epithelial cells while in high grade PIN increased COX-2 expression was found in 86% of PIN cells and their surrounding basal cells. They also found intense COX-2 staining in 87% of their prostatic adenocarcinoma samples without relation to the histological grade and COX-2 staining was demonstrated in the smooth muscle stromal cells of the adenocarcinoma cases. The expression of Cox-2 in the surrounding stroma may also be important in the paracrine regulation of tumour development.

Fujita et al. have demonstrated that COX-2 contributes to prostate cancer progression mediated in part by vascular endothelial growth factor.

Therefore, the upregulation of Cox-2 may promote tumour development by several mechanisms including; generation of free radicals, increase in cell proliferation, decrease in apoptosis and induction of angiogenesis.

1.4 Treatment

Several nomograms are used to predict final pathological stage on radical prostatectomy. These nomograms use Gleason score on biopsy, clinical stage, serum PSA and the extent of cancer on biopsy to predict the risk of extraprostatic disease, seminal vesicle invasion and lymph node metastases. Treatment is based on the stage of the disease and consists of surgery or hormonal therapy.
Palliative treatment for disseminated prostatic carcinoma aims to downregulate the concentration of circulating androgen by hormonal therapy. Hormonal therapy may involve orchidectomy, anti-androgenic agents, or a combination of both. Treatment by orchidectomy decreases the circulating androgens by 95%, but adrenal androgens remain intact and prostatic cancer cells adapt to the low concentrations of androgens by enhancing production of di-hydrotestosterone from the adrenal precursor androgens. This is usually achieved by AR gene amplification by the tumour cells\textsuperscript{19}.

About 70 to 80\% of prostatic carcinoma patients initially respond to hormonal therapy but often the disease progresses after a few months or years. The average survival of non-responders or patients with progression of the disease, is four to fifteen months\textsuperscript{69}. There is currently no effective treatment for recurrent hormone refractive carcinoma of the prostate and an understanding of the underlying mechanisms is important in the development of new therapeutic strategies.

1.5 Aims of the study

- To determine the age at presentation and the racial distribution of prostatic adenocarcinoma in the Western Cape region and to correlate this with histological grade.
- To correlate the expression of androgen receptor, bcl-2, p53 and Cox-2 with the Gleason grade of disease and patient demographic data.
- To establish a method to determine androgen receptor (AR) gene amplification in formalin fixed prostatic carcinoma tissue. This method can then be utilised in further studies investigating the role of AR gene amplification in the progression of prostatic carcinoma.
  - To assess the role of AR receptor amplification in the small cross-section of patients used to establish the method.
  - To correlate the expression of androgen receptor, bcl-2, p53 and Cox-2 with the AR receptor amplification in the small cross-section of patients used to establish the method.
Chapter 2

Materials and methods

2.1 Patient data

The demographic, clinical and histological data of newly diagnosed cases with prostatic carcinoma from January 1999 to December 2001 were reviewed.

The data was collected from histology records and the following were noted:

- Racial group
- Age at presentation

From the above group sixty-one consecutive cases, who had received TURP were selected for review of histological grade and immunohistochemical studies. Only TURP cases were selected to ensure adequate material for immunohistochemical studies.

2.2 Gleason grading

For histological grading of the tumours, the Gleason grading system was used\(^7\). This system takes into account the heterogeneity of prostatic adenocarcinoma by including the two most prominent growth patterns. The primary grade is the most common or predominant grade and the secondary grade is the next most common but should comprise at least 5% of the tumour.

The score (out of ten) is composed of the sum of the primary and secondary growth patterns or grades (both out of five). The patterns are shown in figures 2.1 to 2.5.
Figure 2.1  Gleason pattern 1: A circumscribed mass of simple, monotonously replicated round acini that are uniform in size, shape and spacing.

Figure 2.2  Gleason pattern 2: Similar to 1 except for lack of circumscription of the focus. Slight variation in the acinar size and shape is noted.
Figure 2.3 Gleason pattern 3: Prominent variation in the size, shape and spacing of the acini but the acini remain discrete and separate. Cribiform glands may be present as is illustrated here.

Figure 2.4 Gleason pattern 4: Fusion of the acini is present with ragged infiltrating cord and nests at the edges.
Figure 2.5 Gleason pattern 5: Fused sheets and masses of haphazardly arranged acini.

The sixty-one cases described in 2.1 were subjected to review of the Gleason score. A Gleason score was assigned without prior knowledge of the score attributed at diagnosis. Prof D Govender, Head of the Division of Anatomical Pathology, independently scored a random cross-section of cases.

For statistical purposes the grades are usually grouped as Gleason score 2 to 4 (well differentiated), Gleason score 5 and 6 (moderately differentiated), Gleason score 7 (moderate to poorly differentiated) and Gleason score 8 to 10 (poorly differentiated)\textsuperscript{8}.

2.3 Immunohistochemistry

The sixty-one consecutive cases were submitted for immunohistochemical studies. Archived blocks were used. All slides of each case were reviewed and the block containing an adequate amount of the highest grade tumour pattern, was selected.

**Antibodies**

The following protein products were determined on fresh section of paraffin fixed tissue:

- Androgen receptor
- P53 protein (DO7)
Anti-Human bcl-2 oncoprotein clone 124
Cox-2 mouse monoclonal antibody

Method (for full method see appendix A)
Paraffin-embedded sections were heat fixed overnight and dewaxed. Endogenous peroxidase activity was blocked using H₂O₂ in water and antigen retrieval was performed by pressure cooking slides in either citrate buffer or EDTA. Non-specific binding was blocked by treating slides with a 5% Goat Serum Solution (DAKO #X0907). Sections were incubated with primary antibody at room temperature at specified times and dilutions. Sections were incubated with DAKO Envision labelled Polymer HRP (DAKO #K4001). Positivity was developed by applying 3.3-diaminobenzidine (DAKO#K3466). Slides were immersed in 1%CuSO₄, counterstained in haematoxylin and blued in Scott's tap water.

Scoring of results
Cox-2 and bcl-2 were evaluated only for positive and negative staining. Expression of antibody in more than 10% of cells was considered positive.

AR and p53 nuclear staining were scored as 1, 2 or 3 according to intensity of staining and a hundred cells were evaluated. The final semiquantitative score out of 300 was determined in a similar way as the H-score used in the evaluation of oestrogen receptor expression in breast carcinoma⁷¹:
H-score = 3 (percentage of nuclei stained intensely) + 2 (percentage of nuclei stained moderately) + 1 (percentage of nuclei stained weakly).

2.4 Fluorescence in situ hybridisation (FISH) for androgen receptor gene amplification
Ten specimens from patients who showed progression of their disease on hormonal therapy (orchidectomy or oestrogen therapy) were selected for the determination of androgen receptor gene amplification. These cases were identified in the large, original group of patients by the presence of more than one TURP and were then clinically confirmed to have received hormonal treatment.
Concurrent androgen receptor protein, p53 protein expression, bcl-2 expression and COX-2 determination was done to determine correlation between immunohistochemical and molecular results.

Androgen gene amplification on the X-chromosome was determined using fluorescence in situ hybridisation dual labelling with probes for the X chromosome centromere (CEP X) chromosome spectrum green (Vysis) and LSI androgen receptor gene locus on X spectrum orange (Vysis). These studies were done on sections of paraffin fixed tissue.

**Method (for full method see appendix B)**

Paraffin sections were mounted on positively charged slides and baked overnight at 56-60°C prior to deparaffinisation. The slides were immersed in Xylene twice, dehydrated twice in 100% ethanol for 5 min at room temperature, and air dried on a slide warmer. For acid treatment the slides were immersed in 0.2 N HCl for 20 minutes at room temperature. For chaotrope treatment the slides were immersed in pre-treatment buffer at 80°C for 30 minutes, rinsed and immersed in 2x SCC. Then protease was added to prewarmed protease buffer and the slides were placed in protease solution at 37°C for 10 to 15 min, they were rinsed twice in 2x SCC and placed on a slide warmer to dry. The slides were immersed in 1% formaldehyde at room temperature for 10 min and rinsed twice in 2x SCC, then dried on slide warmer. The slides were placed in denaturation solution at 75°C for 5 min and dehydrated in 70-90-100% ice cold ethanol, 5 min each. The probe mixture was denatured at 75°C for 5 min and the probes were placed on ice for at least 3 min, and spun down briefly. The appropriate amount of probe was added to the slide, covered with a coverslip and sealed with rubber cement. The slides were placed in a FISH chamber in an incubator at 36°C overnight. The slides were washed, counterstained with DAPI and the coverslip fixed with Vectashield.

The AR gene amplification was scored and interpreted according to the method published by Miyoshi et al.\(^{33}\) using fluorescent microscopy. AR gene probe signal is orange and CEP X probe signal is green. To establish AR gene amplification an AR : CEP X ratio of \(\geq 10\) should be present.
2.5 Statistical methods

The data were analysed using the Pearson two tailed correlation test and the ANOVA regression model for multivariate prediction.

2.6 Limitations

The following limitations were identified in this study:

- The 61 specimens selected for immunohistochemical evaluation, were selected for specimen adequacy (opportunistic sample) and this may influence the results obtained.

- The number of cases included in the AR gene amplification study is not sufficient for statistical analysis. However, this group was selected to establish a method only and will be increased in further studies for full statistical analysis.
Chapter 3

Results

3.1 Patient demographic data
A total of 287 cases of prostatic adenocarcinoma were found from the beginning of January 1999 to the end of May 2002. Of these cases, 216 had enough demographic information regarding both race and age, to be included.

From the group of 216, 61 consecutive cases with specimens obtained by TURP were selected for immunohistochemical analysis. Of the 61 cases, 59 had enough demographic data regarding both age and race, to be included in the demographic study.

3.1.1 Race
Of the 216 cases: 45 (21%) were black, 126 (58%) were of mixed heritage and 45 (21%) were white.

Of the 59 cases used in the immunohistochemical analysis: 21 (36%) were black, 31 (53%) were of mixed heritage, and 7 (11%) were white.

3.1.2 Age
The age distribution of the 216 cases is shown in table 3.1 the mean age of the mixed race group is significantly younger than the white group (p=0.004, using Scheffe’s test for post hoc hypotheses). All other comparisons are not significant on the two-tailed test.

(For full results of statistical analysis see Appendix D)
Table 3.1 The age distribution of the 216 cases.

<table>
<thead>
<tr>
<th>Group</th>
<th>Range (years)</th>
<th>Mean (years)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>All n = 216</td>
<td>48 to 91</td>
<td>70.97</td>
<td>9.029</td>
</tr>
<tr>
<td>Black n = 45</td>
<td>52 to 88</td>
<td>71.84</td>
<td>9.415</td>
</tr>
<tr>
<td>Mixed n = 126</td>
<td>48 to 90</td>
<td>69.64</td>
<td>8.412</td>
</tr>
<tr>
<td>White n = 45</td>
<td>56 to 91</td>
<td>75.53</td>
<td>9.702</td>
</tr>
</tbody>
</table>

The age distribution of the 59 cases used in the immunohistochemical analysis is shown in table 3.2.

Table 3.2 The age distribution of the 59 cases used in the immunohistochemical study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Range (years)</th>
<th>Mean (years)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>All n = 59</td>
<td>50-87</td>
<td>70</td>
<td>8.82</td>
</tr>
<tr>
<td>Black n = 21</td>
<td>65-85</td>
<td>75</td>
<td>6.17</td>
</tr>
<tr>
<td>Mixed n = 31</td>
<td>50-87</td>
<td>69</td>
<td>9.10</td>
</tr>
<tr>
<td>White n = 7</td>
<td>56-78</td>
<td>66</td>
<td>10.41</td>
</tr>
</tbody>
</table>

3.2 Gleason score

Gleason score was determined in the 61 cases selected for immunohistochemistry. Of these six patients (10%) had Gleason score 4, five patients (8%) Gleason score 5, seven patients (11%) Gleason score 6, seven patients (11%) Gleason score 7, twelve patients (20%) Gleason score eight, eighteen patients (30%) Gleason score 9 and six patients (10%) Gleason score 10.
Within the four recognised “statistical” groups six patients (10%) were Gleason score 4 to 6 (group 1), twelve patients (20%) Gleason score 5 to 6 (group 2), seven patients (11%) Gleason score 7 (group 3) and thirty six patients (59%) Gleason score 8 to 10 (group 4).

The distribution according to race of the 59 patients with information available as to racial group is illustrated in table 3.

**Table 3.3** Distribution of race and Gleason score according to “statistical groups” 1-4.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mixed race</th>
<th>Black</th>
<th>White</th>
<th>Total</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>6 (10%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>12 (20%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>6 (10%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>13</td>
<td>4</td>
<td>35 (60%)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>31</strong></td>
<td><strong>21</strong></td>
<td><strong>7</strong></td>
<td><strong>59</strong></td>
<td></td>
</tr>
</tbody>
</table>

There was no statistically significant difference in the distribution of Gleason score related to race (see Appendix D for p values).

There was a significant positive correlation between Gleason score and p53 expression (p=0.034), Gleason score and AR surface expression (p=0.014) and Gleason score and expression of bel-2 (p=0.005) (see appendix D).

**3.3 Androgen receptor**

**3.3.1 Surface expression**

The mean expression of androgen receptor was 177/300, median 190/300 and the standard deviation was 57.56. Figure 3.1 illustrates androgen receptor expression.
Figure 3.1 Androgen receptor surface expression. 3+ positivity is indicated by the blue arrow, 2+ positivity by the red arrow and 1+ positivity by the green arrow.

There was a significant (p=0.014) positive correlation between androgen receptor expression and Gleason grade. There was no significant correlation between androgen receptor expression, age, p53 expression or bcl-2 expression. Statistical analysis yielding these results is in Appendix D.

3.3.2 Androgen receptor gene amplification

Within the original group of 216 patients, ten TURP specimens were obtained with a clinical history of anti-androgen therapy to the patient before the procedure. Ten specimens were submitted for androgen gene amplification detection. Of these, two specimens did not show any signal and were left out of further analysis. Of the remaining eight specimens, three specimens showed androgen gene amplification as is illustrated in figure 3.2, and five specimens revealed normal expression of the gene with no amplification.
Figure 3.2 AR gene amplification is present in the centre cell. X chromosome probe is green (one copy per cell) and AR gene probe is red (multiple copies). The cell to the right shows normal expression of androgen receptor gene (only one copy of each).

Within the group of eight specimens two patients were represented twice, once just after relapse post orchidectomy and again 12 months after the first debulking procedure. One of these patients exhibited AR gene amplification in both specimens (case 5 in table 3.4). The other patient showed no amplification in the first specimen but did have amplification of the AR gene in the second specimen (case 2 in table 3.4).

As illustrated in table 3.4 there was no association between p53 protein, bcl-2 expression, AR surface expression and AR gene amplification.
Table 3.4 AR protein, p53, bcl-2 and Cox-2 expression compared with AR gene amplification. AR protein, bcl-2 and p53 were present in the malignant cells while Cox-2 was present in the stromal cells.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2a</th>
<th>2b</th>
<th>3</th>
<th>4</th>
<th>5a</th>
<th>5b</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AR protein</strong></td>
<td>200</td>
<td>210</td>
<td>200</td>
<td>185</td>
<td>210</td>
<td>200</td>
<td>200</td>
<td>160</td>
</tr>
<tr>
<td><strong>p53</strong></td>
<td>3</td>
<td>170</td>
<td>170</td>
<td>0</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>bcl-2</strong></td>
<td>negative</td>
<td>positive</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td><strong>Cox-2</strong></td>
<td>negative</td>
<td>positive</td>
<td>negative</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td><strong>AR gene amplification</strong></td>
<td>negative</td>
<td>negative</td>
<td>positive</td>
<td>negative</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
<td>negative</td>
</tr>
</tbody>
</table>

3.4 p53

p53 expression ranged from 0 to 290 out of 300. The median expression was 40/300 with a standard deviation of 79.52. There was significant positive correlation between p53 staining and Gleason score \((p=0.034)\), see appendix D.

Twenty out of 61 cases (33%) were negative for p53 staining. There was no difference in the median Gleason score between the p53 negative and p53 positive group, see appendix D.

p53 expression is illustrated in figure 3.3.
Figure 3.3 p53 expression. 3+ positivity is indicated by the blue arrow, 2+ positivity by the red arrow and 1+ positivity by the green arrow.

3.5 bcl-2

Bcl-2 expression was determined in 61 specimens. Fifteen out of 61 specimens expressed bcl-2 (25%). Seven out of the 15 (47%) positive specimens were black patients, 7 (47%) of mixed descent and only one patient (6%), white. When compared to the demographics of the group; 36% black, 53% mixed race and 11% white, a disproportionate number of specimens from black patients were positive for bcl-2.

There was a highly significant correlation between bcl-2 expression and increased Gleason score ($p=0.005$, see appendix D). All but one of the patients that expressed bcl-2 were in statistical Gleason score group 4. The remaining case was in group 2.

There was no significant correlation between bcl-2 expression, AR expression, p53 expression or age (see appendix D).

Positive bcl-2 expression is illustrated in figure 3.4.
3.6 Cox-2

Cox-2 expression was determined in 61 cases. Cox-2 expression was present in the interdigitating cells (IDC’s) in all the cases examined (figure 3.5) and in the stromal cells of 51 out of 61 cases (84%) (figure 3.6). The positivity in stromal cells was present surrounding benign and malignant glandular tissue. The cells of origin (IDC’s or stromal cells) were identified on H&E stained slides.

The racial distribution of patients with specimens expressing Cox-2 in stromal cells, reflected the racial distribution of the patients selected.
Figure 3.5  Cox-2 expression in interdigitating dendritic cells surrounding high grade tumour.

Figure 3.6  Cox-2 expression in stromal cells.

Comparison of Gleason score and expression of androgen receptor and p53 in the Cox-2 positive and Cox-2 negative cases is illustrated in table 3.5. Comparison of bcl-2 and
Cox-2 expression is illustrated in table 3.6.

**Table 3.5** Comparison of Gleason score, expression of androgen receptor and p53 in Cox-2 positive and negative cases.

<table>
<thead>
<tr>
<th></th>
<th>Gleason grade Median (standard deviation)</th>
<th>Androgen receptor Median (standard deviation)</th>
<th>P53 Median (standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox-2 negative (n=10)</td>
<td>8 (2.01)</td>
<td>200 (46.3)</td>
<td>6.5 (35.5)</td>
</tr>
<tr>
<td>Cox-2 positive (n=51)</td>
<td>8 (1.8)</td>
<td>190 (59.9)</td>
<td>65 (81.8)</td>
</tr>
<tr>
<td>Significance</td>
<td>No significant difference</td>
<td>No significant difference</td>
<td>Highly significant difference (p&lt;0.0001)</td>
</tr>
</tbody>
</table>

**Table 3.6** Cox-2 and bcl-2 expression

<table>
<thead>
<tr>
<th></th>
<th>Cox-2 negative n=10</th>
<th>Cox-2 positive n=51</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl-2 positive n=15</td>
<td>5 (8%)</td>
<td>10 (17%)</td>
</tr>
<tr>
<td>bcl-2 negative n=46</td>
<td>5 (8%)</td>
<td>41 (67%)</td>
</tr>
</tbody>
</table>
Chapter 4

Discussion

The statistics for the racial distribution of patients with prostatic carcinoma may reflect the racial distribution of patients attending Groote Schuur Hospital (GSH). Due to changes in hospital policy, racial distribution of all patients presenting to GSH during the period of the study were not included in statistical reports and no conclusion could be reached for racial predominance. To accurately determine the racial distribution of prostatic carcinoma in the Western Cape, data from all cases presenting in private practice and the several hospitals within the Western Cape would need to be combined and then compared to known racial distribution figures within the province.

The mean age of presentation in the mixed race group was significantly younger than the white race group. This may indicate a different underlying genetic mechanism in the development of prostatic carcinoma. However, no studies investigating the presence of candidate genes within the mixed race group, have been identified in the literature to date.

No significant difference in the mean age of presentation was found between the black patients and the other two groups. Mohler et al. 5 found African American men with prostatic carcinoma to be significantly younger than American white men at presentation. These differences may be linked to different genetic or environmental mechanisms in the development of prostatic carcinoma in African American individuals 72,73 (originating from Central and West Africa) that is not present in the South African population in this study.

Gleason score of prostatic cancer is one of the strongest predictors of biologic behaviour and metastatic potential and specifically, foci of Gleason pattern 4 and 5 are predictive of adverse outcome74,75. In the group of 61 evaluated for Gleason score there was a
significant increase in cases within the Gleason score 8 to 10 group, 59%, when compared to published figures of 12-20% \(^7\). Therefore, more than half of the patients in this study are expected to have a poor prognosis.

The cause for a higher histological score may be linked to specific molecular mechanisms early in carcinogenesis as is reflected by the positive correlation between Gleason score, AR expression, p53 expression and bcl-2 expression.

There was no statistical difference in the Gleason score when related to race. This finding was in concordance with an American study by Mohler \textit{et al.}\(^5\) but contrary to another American study by Fowler \textit{et al.}\(^7\) who found that more than twice as many black American than white patients harboured cancer with a Gleason grade of 8 or more. The increase in high grade tumours in the white patients in our study may be linked to factors relating to the lower socio-economic status of the patients attending Groote Schuur Hospital. Once again a wider study incorporating patients of higher socio-economic status that will be treated in private practice is needed to determine the possible mechanisms responsible for the bias towards high histological grade patterns in our population.

There was a significant correlation between AR surface expression and Gleason grade in the group as a whole. A recent study has shown that high levels of androgen receptor expression is associated with aggressive clinicopathological features and decreased biochemical recurrence-free survival in prostate cancer patients treated with radical prostatectomy\(^28\). Since Gleason score is one of the most important factors in determining outcome\(^11\), it is expected that the level of AR expression will correlate with the Gleason grade.

No pattern could be detected for AR expression, Cox-2 expression or bcl-2 expression when compared to presence or absence of AR gene amplification. This is in contrast to Koivisto \textit{et al.} who found expression of p53 to be associated with an androgen independent phenotype and AR gene amplification.\(^19\). However, this was a small group
of cases primarily selected to establish methodology, and a larger study is indicated to determine accurate results.

Of interest is the evolution of the tumour in one case (figure 3.4, case 2). In the first specimen there was no evidence of AR gene amplification and expression of Cox-2 (in stromal cells) and bcl-2 expression were present. In the second specimen, taken 12 months later, AR gene amplification was present and bcl-2 expression and Cox-2 expression (in stromal cells) has been lost. This may indicate definite molecular mechanisms in the evolution of the clone. A larger study looking at cases of relapsed androgen independent tumours is indicated.

The high percentage of cases expressing p53 (67%) makes it likely that mechanisms causing mutation of p53 are responsible for carcinogenesis in our patients. The positive correlation between p53 expression and Gleason score is in keeping with findings in the literature. This may indicate that tumour regression and increased grade observed in this study is partly due to cell cycle arrest.

In accordance with Moul et al., Buhendorf et al. and Apakana et al. a positive correlation was found between bcl-2 expression and Gleason score. This finding may indicate a role of anti-apoptotic factors in the development of the disease.

The most important finding in the evaluation of bcl-2 expression was that a disproportionate number of cases from black patients were positive for bcl-2. This is in contrast to a study by Guo et al. who found bcl-2 at significantly higher levels in tumours from Caucasian than African-American patients. The trend to racial predominance in the expression of bcl-2 in our prostatic carcinoma cases may indicate a genetic or environmental factor linked to race that influence apoptotic mechanisms.

In contrast to Amirghofran et al. who found a significant association between AR and bcl-2 expression, there was no significant correlation between androgen receptor expression and bcl-2 expression in this study.
The constant expression of Cox-2 in the interdigitating dendritic cells served as a built in control for the method. Cox-2 is expressed in activated interdigitating dendritic cells and in the presence of malignancy, activated interdigitating dendritic cells are expected.

None of the cases showed Cox-2 expression within the tumour cells. This finding contrasted with studies by Madaan et al., Kirschenbaum et al. and Gupta et al. who found Cox-2 expression in prostatic carcinoma cells. The absence of Cox-2 in epithelial cells in our study may be due to differences in antibodies and methods used. The sources of the antibodies in the quoted studies are not apparent and small details in methodology may not have been mentioned in the papers. Correlation with other studies in our department (Dr H Holm, MMed thesis), have shown Cox-2 expression within epithelial tumours, using the same antibodies and method and performed by the same technologist.

Eighty three percent of cases showed Cox-2 expression within stromal cells. Kirschenbaum et al. demonstrated Cox-2 positivity in stromal cells of both the normal and cancerous prostate as was found in the specimens in this study. The expression of Cox-2 in the surrounding stroma may be important in the paracrine regulation of tumour development.

No significant difference in androgen receptor expression was detected between the specimens that were Cox-2 positive and negative in stromal cells. In the cases examined for androgen gene receptor amplification no segregation of positive or negative cases was found with amplification of the androgen receptor gene. No difference was seen in the Gleason grade of Cox-2 positive and negative cases. These findings may indicate that Cox-2 expression in stromal cells does not influence prognosis. However, a study incorporating patient survival data is indicated to determine definitively whether the presence of Cox-2 in stromal cells has prognostic value.

The mean p53 expression was significantly higher in the cases that expressed Cox-2 in stromal cells. The link between Cox-2 expression and mutation of p53 may be explained
by the fact that up regulation of Cox-2 increases the formation of mutagens and free radicals (figure 1.3).

When Cox-2 is induced there is a shift of the metabolic pathway to the production of prostaglandins as is illustrated in Figure 1.3. However, without Cox-2 induction there is an increase in apoptosis. This may be achieved by the down regulation of bcl-2. This means that in cases without Cox-2 expression in the stromal cells, no bcl-2 expression is expected. And conversely when Cox-2 is present it may be possible to detect upregulated bcl-2. Only 8% of the specimens were negative for both Cox-2 and bcl-2 and 17% of cases expressed both Cox-2 and bcl-2. This may be due to alternate pathways responsible for the increase in bcl-2 expression that is not linked to Cox-2 expression in some cases.
Conclusion and further studies

The mean age of presentation in the mixed race group of patients presenting to Groote Schuur hospital was significantly younger than the white race group. To accurately determine the racial distribution of prostatic carcinoma in the Western Cape, data from all cases presenting in private practice and the several hospitals within the Western Cape should be combined.

A significant number of the patients presenting at Groote Schuur hospital had a Gleason score of 8 to 10 group. The cause for a higher histological score may be linked to specific molecular mechanisms early in carcinogenesis as is reflected by the positive correlation between Gleason score, AR expression, p53 expression and bcl-2 expression. To establish whether the higher Gleason score found in over half our cases is in fact associated with a poorer clinical outcome a study incorporating patient survival data is indicated.

The high percentage of cases expressing p53 (67%) makes it likely that mechanisms causing mutation of p53 are responsible for carcinogenesis in our patients. The mean p53 expression was significantly higher in the cases that expressed Cox-2 in stromal cells and the link between Cox-2 expression and mutation of p53 may be explained by the fact that up regulation of Cox-2 increases the formation of mutagens and free radicals (figure 1.3).

The evolution of the tumour in one of the cases investigated for AR gene amplification, in which amplification of the gene is gained and Cox-2 (in stromal cells) and bcl-2 expression lost may indicate definite molecular mechanisms in the evolution of the clone. A larger study looking at cases of relapsed androgen independent tumours as well as the association between Cox-2 and bcl-2 expression is indicated. The patterns of Cox-2 expression and specifically the lack of Cox-2 expression in epithelium warrants further investigation.

A disproportionate number of specimens from black patients that were positive for bcl-2 and warrants further investigation with a larger group of patients matched for race.
Bibliography

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http://www.cansa.co.za/registry_prostate.asp.
17. Zhuang, S.H & Burnstein, K.L. Antiproliferative effect of 1alpha,25-
dihydroxyvitamin D3 in human prostate cancer cell line LNCaP involves


Appendix

A. Immunohistochemistry Method

- Paraffin-embedded sections were cut onto APES coated slides and heat fixed overnight at 60°C
- Sections were dewaxed
- Endogenous peroxidase activity was blocked by treating slides with a 1% H₂O₂ in water solution for 15min
- Slides were washed well in water
- Antigen retrieval was performed by pressure cooking slides in either citrate buffer or EDTA (androgen receptor only) for 2min at full pressure
- Slides were immediately immersed in water
- Slides were washed in phosphate buffered saline solution (PBS pH 7.6)
- Non-specific binding was blocked by treating slides with a 5% Goat Serum Solution (DAKO #X0907)
- Serum was drained off
- Sections were incubated with primary antibody at room temperature at specified times and dilutions

<table>
<thead>
<tr>
<th>Antibody dilution</th>
<th>bcl2</th>
<th>p53</th>
<th>Cox2</th>
<th>AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time</td>
<td>1/20</td>
<td>1/50</td>
<td>1/200</td>
<td>1/150</td>
</tr>
</tbody>
</table>

- Sections were washed well with PBS buffer
- Sections were incubated with DAKO Envision labelled Polymer HRP (DAKO #K4001) for 30 min at room temperature
- Sections were washed well with PBS buffer
- Positivity was developed by applying 3.3-diaminobenzidine (DAKO#K3466) for 5-10min
- Slides were washed in water
- Slides were immersed in 1% CuSO₄ solution for 5 min
- Slides were washed in water
• Slides were counterstained in haematoxylin, blued in Scott’s tap water
• Slides were washed in water, dehydrated, cleaned and coverslipped

B. Fluorescent in situ hybridisation method

• 3µm thick paraffin sections are mounted on positively charged slides
• Slides are baked overnight at 56-60°C prior to deparaffinisation
• Deparaffinisation
  o The slides are immersed in Xylene for 15 min at room temperature
  o Repeat twice using new Xylene each time
  o Dehydrated twice in 100% ethanol for 5 min at room temperature
  o The slides are air dried on a slide warmer at 45-50°C for 2-5 minutes
• Acid treatment
  o The slides are immersed in 0.2N HCl for 20 minutes at room temperature
  o Rinsed for 3 min in distilled water
  o Rinsed for 3 min in 2x SCC (wash buffer)
• Chaotrope treatment
  o The slides are immersed in 50ml pre-treatment buffer at 80°C for 30 minutes
  o They are rinsed in distilled water for 1 min at room temperature
  o They are immersed in 2x SCC for 5 min at room temperature, repeat
• Protease treatment
  o The protease is added to prewarmed protease buffer (37°C)
  o The slides are placed in protease solution at 37°C for 10 to 15 min
  o The slides are rinsed twice in 2x SCC for 5 min each at room temperature
  o The slides are placed on a slide warmer to dry at 45-50°C for 2-5 min
• Post fixation
  o The slides are immersed in coplin jar containing 50ml 1% formaldehyde at room temperature for 10 min
  o The slides are rinsed twice in 2x SCC for 5 min each at room temperature
  o The slides are dried on slide warmer at 45-50°C
• Denaturation
• The slides are placed in denaturation solution at 75°C for 5 min
• The slides are dehydrated in 70-90-100% ice cold ethanol, 5 min each
• The slides are placed on slide warmer
• While the slides are dehydrating (90%) the probe mixture is denatured at 75°C for 5 min
• The probes are placed on ice for at least 3 min, and spun down briefly

• Hybridisation
  • The appropriate amount of probe is is added to the slide, covered with a coverslip and sealed with rubber cement
  • The slides are placed on a slide warmer until the rubber cement dries
  • The slides are placed in a FISH chamber in an incubator at 36°C overnight

• Washing
  • The rubber cement is carefully removed
  • The slides are placed into wash buffer at room temperature and the coverslip is allowed to float off
  • The slides are placed in wash buffer at 75°C for 2 min

• Counterstain
  • The slides are placed in a coplin jar with 50ml DAPI solution for 15min in the dark
  • The slides are rinsed twice in 2xSCC and Tween for 2 min at room temperature
  • The excess liquid is removed and a coverslip is fixed with a drop of Vectashield
## C. RESULTS

Results of 6 patients used in immunohistochemical study

<table>
<thead>
<tr>
<th>gleason grade</th>
<th>Sum</th>
<th>Group</th>
<th>p53</th>
<th>bcl2</th>
<th>AR</th>
<th>age</th>
<th>col</th>
<th>black</th>
<th>white</th>
<th>cox</th>
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</thead>
<tbody>
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<td>0</td>
<td>2</td>
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<td>0</td>
<td>90</td>
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<td>0</td>
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<td>66</td>
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<td>+</td>
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<td>0</td>
</tr>
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<td>10</td>
<td>0</td>
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<td>75</td>
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## D. STATISTICAL ANALYSIS

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** Correlation is significant at the 0.01 level (2-tailed).
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<td>57</td>
<td>2.442</td>
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<td>Total</td>
<td></td>
<td>203.246</td>
<td>60</td>
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</tbody>
</table>

* a Predictors: (Constant), AR, P53, BCL2
* b Dependent Variable: Gleason grade

### Coefficients

<table>
<thead>
<tr>
<th>Model</th>
<th>B</th>
<th>Std. Error</th>
<th>Beta</th>
<th>t</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(Constant)</td>
<td>5.161</td>
<td>.672</td>
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<td>7.674</td>
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<tr>
<td>P53</td>
<td>8.141E-03</td>
<td>.003</td>
<td>.352</td>
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<td>3.112</td>
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<tr>
<td>BCL2</td>
<td>.982</td>
<td>.276</td>
<td>.405</td>
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<td>3.559</td>
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<tr>
<td>AR</td>
<td>7.722E-03</td>
<td>.004</td>
<td>.241</td>
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<td>2.176</td>
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* a Dependent Variable: Gleason grade
Age by race descriptive Statistics

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<th>race</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>N</th>
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<tbody>
<tr>
<td>1</td>
<td>69.64</td>
<td>8.412</td>
<td>139</td>
</tr>
<tr>
<td>2</td>
<td>71.84</td>
<td>9.415</td>
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<tr>
<td>3</td>
<td>75.53</td>
<td>9.702</td>
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<tr>
<td>Total</td>
<td>70.97</td>
<td>9.029</td>
<td>216</td>
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<table>
<thead>
<tr>
<th>race</th>
<th>Mean</th>
<th>Std. Error</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
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<tr>
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<td>68.165</td>
<td>71.115</td>
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<tr>
<td>2</td>
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<td>1.315</td>
<td>69.252</td>
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<tr>
<td>3</td>
<td>75.53</td>
<td>1.560</td>
<td>72.457</td>
<td>78.606</td>
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</table>

P=0.003
Race 1= mixed heritage
Race 2= black
Race 3= white

Race 1 differs significantly from 3 (p=0.004). All other comparisons are not significant on the two-tailed test.
Using Scheffe's test for post hoc hypotheses