THE EXPRESSION OF A MUTANT EPIDERMAL GROWTH FACTOR RECEPTOR IN PROSTATIC TUMOURS

By

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Declaration

The work on which this thesis is based is my own independent work except where acknowledged.

Ẹmiọla Oluwabunmi Ọlapade-Ọlaọpa
January 2000
Dedication

This project is dedicated to Olapade, Oluremi and Oluwaseun; though apart, we are one as always soli Deo gloria.

Emiola Oluwabunmi
Daodu,
January, 2000

Quotations

“Seek and you shall find”

Luke 11:9

“Gbajumọ ki iwà nkan tì”

A Yoruba proverb

“Very truly I say to you, —you will have pain, but your pain will turn to joy”

John 16:20
Synopsis

Prostate cancer is now the most commonly diagnosed cancer in men and is thus a significant cause of morbidity and mortality. The factors that govern the natural history of the disease however remain poorly understood, resulting in a stagnation of the principles of its management (especially advanced stage disease) for over a century. Research efforts are therefore currently focused on the molecular and cellular pathways that regulate normal and abnormal growth of the gland. Reports from these studies point to the acquisition of molecular mechanisms of autonomous proliferation as a pre-requisite for malignant transformation and its progression in the prostate and other solid organs.

Peptide growth factors and their receptors are cellular signaling molecules that are an integral part of the regulatory pathways/mechanisms in solid organ homeostasis. Of these the Epidermal growth factor receptor has been most commonly implicated in carcinogenesis. Studies on EGFR expression in prostatic tumours have produced conflicting results. Newer, and more specific, antibodies have however pointed to a progressive depletion of the receptor as prostatic tissues become increasingly malignant, but the reason for this remains unclear. This progressive loss of EGFR in prostatic tissues has remained unexplained, although several hypotheses have been postulated including the expression of a mutated receptor. The most common mutant EGFR found in human cancers is EGFRvIII (Type III), which is a constitutively active tyrosine kinase that is able to initiate cell division independent of ligand, and thus (potentially) independent of hormonal control. This aberrant EGFR has been implicated in the pathogenesis and progression of several cancers but has not been detected in prostate cancer.

This project tested my postulation that, in addition to the normal receptor, prostatic tumours also express an aberrant EGFR, and that the contradictory findings in previous studies are due to the detection of this mutant receptor by some but not all of the different techniques used. Tissues from normal, benign prostatic hyperplasia (BPH), carcinoma of the prostate (CaP) and metastatic tissues were scrutinised retrospectively for the presence of EGFRvIII and WT-EGFR using western blotting and immunohistochemical techniques. The levels of expression of both receptors within these neoplasms were then compared using statistical tests. The tests confirmed the presence of the EGFRvIII protein in prostatic neoplasms but not in normal glands. They also demonstrated an inverse relationship between the expression of this variant EGFR and the wild-type protein in these tissues. This study also reported for the first time on the expression of EGFRs (normal and variant) expression in prostate cancer metastases. We also assessed the clinical significance of EGFRvIII expression in prostate cancer by correlating its level of expression with levels of accepted prognostic indices of the disease.

The specificity of these results indicates that EGFRvIII is a potential histological marker for prostate cancer. This variant EGFR may also be of clinical significance in prostate cancer patients as its level of expression was predictive of an aggressive disease phenotype and the progression to hormone refraction. Taken together, these findings suggest that EGFRvIII could be a target for modern anti-cancer regimes including gene therapy.

Apart from the above, this study also identified areas that may benefit from future studies. These are:

1) Quantification of total EGFR (WT-EGFR and EGFRvIII) expressed in benign and malignant prostatic tissues.
3) Evaluation of the EGFRvIII as a possible target for anti-CaP therapy.
4) Evaluation of the presence of other mechanisms of hormone resistance in prostate cancer.
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ABBREVIATIONS

α-FGF  acidic fibroblast growth factor
AMPS  Ammonium persulfate
AR  Amphiregulin
bp  base pair
β-FGF  basic fibroblast growth factor
BPH  Benign prostatic hyperplasia
BSA  Bovine serum albumin
CaP  Carcinoma of the prostate
DNA  Deoxyribonucleic acid
DRE  Digital rectal examination
DU145  Human prostate cancer cell line
DXT  Deep x-ray therapy
EGF  Epidermal growth factor
EGFR  Epidermal growth factor receptor
EGFvIII  Type III mutant epidermal growth factor receptor
EP anti-PSA  Europath anti-PSA antibody
FGF  fibroblast growth factor
HAR  Human androgen receptor
Hb  Haemoglobin
HCG  Human chorionic gonadotrophin
HGPIN  High grade prostatic intra-epithelial neoplasia
Ig  Immunoglobulin
IGF  Insulin-like growth factor
IGFR  Insulin-like growth factor receptor
LHRH  Luteinising hormone-releasing hormone
LNCaP  Human prostate cancer cell line
mRNA  Messenger ribonucleic acid
NV anti-PSA  Novacastra anti-PSA antibody
PAGE  Polyacrylamide gel electrophoresis
PAP  Prostatic acid phosphatase
PC3  Human prostate cancer cell line
PDGF  Platelet derived growth factor
PSA  Prostate specific antigen
SDS  Sodium dodecyl sulfate
Tfm  Testicular feminising
TGF-α  Transforming growth factor alpha
TGF-β  Transforming growth factor beta
TRUS  Transrectal ultrasound scan
UGM  Urogenital mesenchyme
WT-EGFR  Wild-type epidermal growth factor receptor
PUBLICATIONS ARISING FROM THIS PROJECT

Published Papers
EO Olapade-Olaopa, DK Moscatello, EH MacKay, T Horsburgh, DPS Sandhu, TR Terry, AJ Wong, and FK Habib.
Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer.
British Journal of Cancer, 82:188-194, 2000

EO Olapade-Olaopa, EH MacKay and FK Habib.
Variability of immunohistochemical reactivity on stored paraffin slides (letter).
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Papers Submitted for Publication
EO Olapade-Olaopa, JO Ogunbiyi, EH MacKay, C Muronda, TO Alonge, A Danso, DK Moscatello, DPS Sandhu, OB Shittu, TR Terry, AJ Wong, and FK Habib.
The effect of tissue preservation and storage on immuno-reactivity in archival sections: implications for collaborative multi-centre immunohistochemical studies

Related Papers
EO Olapade-Olaopa, EH MacKay, NA Taub, DPS Sandhu, TR Terry and FK Habib.
Malignant transformation of human prostatic epithelium is associated with the loss of androgen receptor immuno-reactivity in the surrounding stroma.
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Expression of a constitutively active variant EGFR correlates with increased cell proliferation but not decreased cell death in primary prostate cancer.
British Journal of Urology, 83(Suppl 4): 15, 1999

EO Olapade-Olaopa, DK Moscatello, N Mistry, TR Terry, DPS Sandhu, AJ Wong, and FK Habib.
The expression of wild-type and variant EGF receptors in human prostate cancer cell lines.
Mechanisms for hormone resistance in prostate cancer in black and white men.

EO Olapade-Olaopa, N Mistry, DPS Sandhu, TR Terry, and FK Habib.
A constitutively active variant EGF receptor is expressed by human prostate cancer cell lines.

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High expression of a mutant epidermal growth factor receptor by epithelial cells is associated with a marked reduction of androgen receptor expression in stromal nuclei in prostate cancer.

EO Olapade-Olaopa, T Horsburgh, EH Mackay, DPS Sandhu, TR Terry, DK Moscatello, AJ Wong, and FK Habib
A mutant epidermal growth factor receptor is over-expressed in prostate cancer.

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Expression of a constitutively active variant EGFR correlates with increased cell proliferation but not decreased cell death in primary prostate cancer.
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EO Olapade-Olaopa, EH MacKay, DPS Sandhu, TR Terry, DK Moscatello, AJ Wong, and FK Habib.
Expression of a constitutively active variant EGFR correlates with increased cell proliferation but not decreased cell death in primary prostate cancer.
EO Olapade-Olaopa, DK Moscatello, N Mistry, TR Terry, DPS Sandhu, AJ Wong, and FK Habib.

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*Mechanisms for hormone resistance in prostate cancer in blacks and white men.*

**British Association of Urological Surgeons meeting - June 1998, Harrogate, United Kingdom.**

EO Olapade-Olaopa, N Mistry, DPS Sandhu, TR Terry, and FK Habib.

*A constitutively active variant EGF receptor is expressed by human prostatic cell lines.*

**British Association of Urological Surgeons meeting - June 1998, Harrogate, United Kingdom.**


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3rd Biennial Conference of the Pan-African Association of Urological Surgeons - August 1997; Kruger Gate, South Africa.

EO Olapade-Olaopa, T Horsburgh, EH Mackay, DPS Sandhu, TR Terry, DK Moscatello, AJ Wong, and FK Habib

High Expression of a mutant epidermal growth factor receptor by epithelial cells is associated with a marked reduction of androgen receptor expression in stromal nuclei in prostate cancer.
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Chapter 1 - Anatomy and Physiology of the Prostate

1.1 The Prostate Gland

The prostate is a cone shaped gland situated at the base of the male urinary bladder and surrounds the proximal urethra. The presence of the gland has long been recognised and the Greek word *prostatis* means “in front of” (the bladder).

1.2 Prostatic Embryology, Growth and Development

The prostate arises from the urogenital sinus at about the 12th week of intra-uterine life [1]. The default setting of the development of the urogenital tract produces the female phenotype. However, under the influence of testicular androgens, differentiation into the male genitalia occurs. The growth and maturation of the prostate occurs in four distinct phases during progression from embryogenesis to adulthood [2]. After the completion of differentiation, and until death, prostatic cells are in continuous cellular activity i.e. proliferation, apoptosis and trans-differentiation.

1.3 Anatomy of the prostate

1.3.1 Gross Anatomy

The adult prostate gland is shaped like a chestnut and is similar in size. Often described as an inverted cone to aid orientation, its cranial aspect is the base of the cone, whilst the apex of the cone (and the gland) is the caudal aspect. McNeal [3] divided the prostate into four definitive regions: peripheral, central, transitional, and periurethral [Figure 1]. The peripheral zone is by far the largest accounting for 75% of the prostatic volume, while the central zone makes up 20%. The transition zone constitutes 4% of the prostate and is made up of two areas of glandular tissue that surround the proximal segment of the prostatic urethra and the pre-prostatic sphincter. The periurethral zone accounts for only 1% of the prostate by volume.

**Figure 1**

Diagram of the prostate cut along oblique coronal plane, showing relationships between the transition zone (T) and the periurethral region (U) to sphincter (S) glandular prostate (P) and the verumontanum (V). The anterior fibromuscular stroma (F), the bladder (B).

*Adapted from McNeal JE 1970*
1.3.2 Prostatic Histology

The prostate gland is composed of stromal and epithelial compartments [Figure 2]. The epithelium is composed of three distinct cell types that share a common origin [4]. These are: 1) the basal layer, which is composed of cuboidal, (mainly) androgen-receptor and PSA negative, androgen-independent cells which are thought to contain the proliferative compartment [5]; 2) the secretory (columnar) luminal layer which represent the major phenotype of normal and abnormal epithelium [2]; and 3) endocrine-paracrine cells, a minor cell population of undetermined origin, function and significance.

Adult prostatic stroma carries the neuro-vascular bundles as well as the reticulo-endothelial cells, and is composed mainly of fibroblasts and smooth muscle cells [6]. The fibroblasts contribute fibrous tissue to the substance of the stroma and retain their ability to differentiate to other cell types. The smooth muscle cells are oriented around the prostatic ducts and acini, and are responsible for the contraction of the gland during ejaculation.

Figure 2

Micrograph showing the cytologic detail of prostatic gland lining epithelium. It is composed of tall, pale-staining columnar cells (C) and occasional small basal cells (B) with darker staining nuclei. Note the delicate supporting stroma of the papillary ingrowth (P) and the narrow periglandular layer of smooth muscle fibres (SM). The lumen contains an uncalcified, concentrically laminated corpus amyloaceae (CA).

1.4 Physiology of the Prostate

One of the main functions of the prostate is the production of secretions [1]. These fluids make up about 15 to 30% (0.5 to 1.0 ml) of the volume of semen. The pH of prostatic fluid is about 6.5 and its ionic constituents are: Anions (Na+, K+, Ca++, Mg++, Zn++); Cations (Cl-, Citrate, and Ascorbate) [7]. The concentration of both Ca++ and Zn++ are higher than in other body fluid, and both ions are absorbed by spermatozoa. Calcium, in particular, is required for spermatic motility [8]. The function of citrate is currently unknown. Prostatic fluid also contains serine proteases (PSA, PAP and tissue plasminogen activator) and enzymes that produce fructose (the major source of energy for sperms) from other sugars. Other prostatic fluid constituents are peptide hormones (e.g., thyrotropin releasing hormone, lipocortin, inhibin and calcitonin), which may have antibacterial functions [9], and the polyamines (spermine and spermidine) whose function is currently unknown.
The best known function of seminal proteins is seminal coagulation and coagulum dissolution. The serine proteases are actively involved in the dissolution of the coagulum, and the best known of these is PSA which is an important prostate cancer (CaP) marker [10].

1.5 Hormonal Control of the Prostate
1.5.1 Androgens and the Prostate.
The prostate is primarily an androgen target organ, and its growth and differentiation are closely regulated by circulating testosterone [1]. This is converted in the prostatic cells to its active metabolite dihydrotestosterone by the enzyme 5 alpha reductase [11]. These hormones are required for prostatic growth and development and also for the maintenance of the glandular structure and function of the adult organ [12]. They are also thought to be involved in the malignant transformation of prostatic epithelium as androgen deprivation is an effective (if temporary) treatment for CaP [13].

Testosterone
Testosterone is the most important androgen in the circulation, and is produced almost exclusively (95%) by the Leydig cells of the testis [14]. The remainder (5%) is converted in the periphery from androgens (androstenedione) produced by the adrenal glands. Both the testes and adrenal also produce other androgens, particularly dehydroepiandrosterone. Testosterone production depends on the stimulation of Leydig cells by luteinising hormone (LH) which is synthesized by the anterior pituitary [15]. The anterior pituitary, in turn, is stimulated by LHRH which is secreted by the hypothalamus. The production of testosterone is regulated by a negative feedback loop effect of androgens on LHRH and LH production [Figure 3].

Figure 3

Androgen production by the testes.

Adapted from Campbell's Urology
Dihydro-testosterone

Although testosterone is the main circulating androgen, on entering prostatic cells it is metabolised to its active metabolite 5 alpha-dihydrotestosterone (DHT) [16] [Figure 4]. DHT was first described by Farnworth and Brown [18], and is produced by the action of the enzyme 5 alpha-reductase on testosterone. Two isoenzymes of 5 alpha-reductase have been described in men (Types I and II) [17]. Type I is found mainly in non-androgen dependent tissues (e.g. liver), whilst Type II is the predominant form in the prostate. Both testosterone and DHT bind to the androgen receptor which is expressed by both stromal and epithelial compartments of the prostate [18].

**Figure 4**

Testosterone metabolism in the Prostate.

Key

- **T** = Testosterone
- **DHT** = Dihydro-testosterone

*Adapted from Campbell's Urology*

Other hormones

In addition to androgens, other hormones have an influence on prostatic function including oestrogen and prolactin. Oestrogens are thought to stimulate proliferation of the basal cells which are known to express oestrogen receptors. Prostatic cells also express prolactin receptors [19], and following hormonal ablation, prostatic volume is only restored in the rat by the joint administration of prolactin and testosterone [20].

1.6 A summary of the pathology of benign and malignant enlargements of the prostate

Although the prostate may be affected by many inflammatory or neoplastic disorders, enlargement of the gland in the older man is usually caused by benign and malignant diseases (BPH and CaP).
Benign prostatic hyperplasia
Benign nodular enlargement of the prostate arises exclusively from the peri-urethral glands in the transition zone. Microscopically, BPH tissue is distinguishable from the normal prostate by the presence of these nodules which are classified as either stromal or glandular, depending on the predominant tissue [21]. In mature BPH, whilst the volume of epithelial tissue is similar to that of normal prostate, the contribution of stromal tissue and glandular lumina is increased.

Malignant Enlargement of the Prostate
Most malignant growths of the prostate are adenocarcinomas. Other histologic types are small cell, transitional cell and primary sarcomas. Secondary deposits from other tumours to the prostate are a rarity, but local invasion from tumours of the colon and bladder may occur [22].
Chapter 2 - Carcinoma of the Prostate

2.1 Natural History of Prostate Cancer

The development of prostate cancer appears to be a multi-step progression from normal epithelium, through various stages of dysplasia, to intra-epithelial neoplasia, and to invasive disease [23]. This progressive de-differentiation of the glandular epithelium is associated with the acquisition of multiple genetic abnormalities, each of which may contribute to the eventual development of the malignant phenotype [Figure 5]. However, BPH is not considered as a pre-malignant condition despite its frequent finding in CaP glands [24].

Figure 5

Genetic changes associated with malignant transformation in the Prostate.

Adapted from Urology 48(5) 1996

Majority (75%) of prostatic adenocarcinomas arise in the peripheral zone of the gland [25]. CaP is usually multi-focal and is composed of malignant epithelial cells that infiltrate the surrounding stroma. In keeping with the slow growing nature of CaP, mitotic figures are fewer than seen in other cancers [26]. Well differentiated tumours retain the acinar pattern, similar to normal glands, but with a single layer of epithelium (the basal cell layer is absent). These acinar structures become progressively disorganised as the disease advances [27]. An interesting feature of CaP is that areas of differing differentiation are often adjacent to each other within the same tumour, and this characteristic has to be taken into consideration during histological grading of the cancer.
2.2 Epidemiology

Incidence and Mortality Worldwide

Prostate cancer is a major health problem in both developed and developing countries [28]. The disease is now the most commonly diagnosed cancer in men in the United States [29], Nigeria [30] and Jamaica [31], and the second leading cause of cancer related male deaths in the US [Figure 6]. It has been calculated that a newborn male has a 13% life-time risk of developing CaP, and that men older than 55 years have a 3% chance of dying from the disease [32]. Data from the US National Cancer Institute coordinated Surveillance Epidemiology and End Results (SEER) programme show that, between 1973 and 1992, the age-adjusted incidence rates for CaP in black and white American men has more than doubled [33] [Figure 7]. The highest rates of CaP, in all age-groups, has been recorded in blacks (age-adjusted relative risk black vs white 1.8) [34].

![Figure 6](image)

Worldwide incidence of Prostate Cancer.

Adapted from Cancer Incidence in Five Continents, Lyan International Agency for Research in Cancer, 1992 vol.6

However, in general, men die with the disease than from it [35]. Similar to its incidence, CaP mortality rates vary widely across the globe from 1.3 to 22.1/100,000 [36]. The disease also appears to be more aggressive in black men resulting in higher death rates in this racial group than in whites [37] [Figure 7]. In most western countries the disease is responsible for 1% of all deaths in males and about 13% of all cancer related deaths.
In the US, whilst mortality rates from all cancers have remained stable with an increased incidence of 3.1% (1975-79 vs 1987-91), that due to CaP appears to have increased by 14% with a 65% rise in incidence [38]. There are however recent reports that suggest that mortality from the disease may be decreasing [39].

Figure 7


Source - NCI SEER Program

Incidence, Prevalence and Mortality of CaP in the UK [40]

In the UK specifically, the incidence of CaP has risen steadily over the last 15 years, and the disease is now the fourth commonest cancer in men in the UK (after skin, lung and large bowel cancers). As regards mortality, CaP is the third commonest cause of cancer death after lung and bowel cancer. In 1990 13,481 new cases were registered in England and Wales, giving an incidence of 54.2 per 100,000 men [41]. In the same countries in 1993, the disease was responsible for 8689 deaths, a mortality rate of 33.8 per 100,000. Using the current figures, it has been projected that there will be approximately 19,800 new cases in the year 2011. Furthermore, it is estimated that the prevalence of this disease is 3.6 times the incidence. Using this formula, the prevalence of CaP in 1990 would be 195.1/100,000 (incidence of 13,481, total cases 48,531). This makes it obvious that the burden of prostate cancer on sectors of the health care systems in the UK is going to rise over the next decade.

2.3 Risk factors for CaP

Several risk factors have been identified for the development of prostate cancer and these include: 1) Age [42] (with constant exposure of the organ to a carcinogen; 2) Genetic factors (i.e. black race) [43], and familial susceptibility [44, 45]; 3) Dietary factors [46]; 4) Hormones (higher serum levels of androgens in black American men and (pregnant) black American women [47, 48]; 5) high grade prostatic intraepithelial neoplasia HGPIN (possible CaP precursor [49]).
2.4 Common prognostic factors

It has long been recognised that there is a wide variation in progression rates of CaP with many lesions remaining latent and undiagnosed [50]. Autopsy studies have now shown a discrepancy between the high incidence of the disease in post-mortem specimens (14 - 48%) [51, 52], and its relative rarity as a cause of death (3 - 22%) [38]. However, a minority of lesions advance rapidly, invading local tissue and metastasizing (to bone and lymph nodes) and presently, clinicians are unable to predict which cancers possess the aggressive phenotype. The identification of factors which accurately predict the biological behaviour of individual cases would therefore be invaluable in improving the management of this disease. The current major CaP prognostic factors are discussed briefly below.

2.4.1 Histopathological parameters

Histological grade
This is considered the most important prognostic factor in CaP. The commonest system of histological grading for prostate cancer is the Gleason score [27]. This system recognises five different types of glandular patterns of prostate cancer tissues with pattern 1 being most differentiated (good prognosis), and pattern five least differentiated (poor prognosis). The scores for the two most dominant patterns in any tumour are added together to give the Gleason score (2 - 10). For the purposes of prognosis Gleason scores are classed into 3 groups: 2-4, low grade; 5-7, medium grade; 8-10, high grade.

Others
Other prognostic histopathological parameters are pathological stage (which encompasses tumour volume, extracapsular extension and lymph node involvement) [53].

2.4.2 Clinical Parameters

The suggested clinical markers of poor prognosis in CaP are age, tumour volume, capsular penetration seminal vesicle invasion, and the presence of metastases [54, 55].

2.4.3 Biochemical parameters

Serum PSA
The biochemical index most commonly used as a CaP marker is serum PSA, which is a glycoprotein secreted almost exclusively by prostatic epithelium [56]. High concentrations of the enzyme are found in prostatic ducts and seminal fluid. Under normal circumstances only a small amount of PSA is absorbed into the blood stream, but this increases with damage to the basement membrane. As such high PSA levels are found in men with CaP, which increase as the disease advances. Although the highest levels of PSA are associated with malignant tumours, moderate elevation of the marker is
also found in prostatitis and BPH. PSA has recently become the most widely used serum marker for CaP and has been studied extensively in this role [10]. Another biochemical marker is serum PAP (which is useful indicator of skeletal metastatic disease [57].

2.5 CaP Detection
The aim of detection of CaP can be divided into: 1) screening of asymptomatic men; and 2) diagnosis of primary and metastatic disease in symptomatic men.

The methods currently used to detect CaP are:
1) *Digital rectal examination (DRE)* - The major limitation of this test in this role has been its poor sensitivity. However, the test appears to be more accurate at excluding CaP [58].
2) *Transrectal ultrasound (TRUS)* - This test is mostly used as a second line investigation in patients with a raised PSA or abnormal DRE due to its high cost, time consumption and discomfort [58].
3) *Measurement of Serum PSA* - Serum PSA measurement (free and total) is the most recent and simplest method for CaP screening. Although several test kits are available, with varying reference levels, a cut-off level of 4ng/mL (Hybritech's Tandem-R assay) is commonly used to define positivity requiring further evaluation. The specificity of PSA testing of asymptomatic men ranges from 87-97% [59].
4) *Others* - Renal function tests, plain and contrast radiological examinations and prostatic biopsies.

2.6 Clinical Features of Prostate Cancer
Men with CaP may present either with symptoms of localised disease (60-70%), or those of advanced disease (30-40%) [22]. The symptoms of localised CaP commonly include urinary outflow obstruction, detrusor irritability and haematuria. Clinical examination of the prostate may be unremarkable or may reveal a nodular hard gland. On the other hand, patients with advanced CaP (extracapsular disease and metastases) may present with both local symptoms and those attributable to the organs involved. Common symptoms of metastatic disease are those of bone secondaries (sudden-onset progressive low-back pain, paraplegia and pathological fractures.

2.7 Treatment of Prostate Cancer
Essentially treatment options can be broadly divided into those available to men with localised disease and those reserved for treating the inevitable progression to metastatic disease.

*Treatment of Localised Prostate Cancer* [60]
The treatment options in men with clinically localised CaP are:
1) Watchful waiting - this regime is generally recommended to men with well to moderately differentiated CaP with a life expectancy less than 10 years.

2) Radical prostatectomy - this is usually prescribed to younger men with moderately-poorly differentiated CaP.

3) Radical radiotherapy (including brachytherapy) - this is more likely to be offered to men with moderately-poorly differentiated localised disease who are less fit or who are older than those offered radical surgery.

4) Combination therapy - these include include adjuvant or neoadjuvant androgen deprivation in association with DXT and surgery.

Treatment of Advanced Prostate Cancer [22]
The aim of treatment of this stage of CaP is to slow down the progression of the disease as well as palliate symptoms. The main treatment methods used are:

1) Hormonal Manipulation (in hormone naïve glands) - this treatment is usually first line in the treatment of advanced CaP, and may be achieved by either surgical (orchidectomy) or medical castration (LHRH analogues and/or anti-androgens).

2) Palliative treatments for hormone-refractory disease - all CaP eventually escape hormonal control and require other palliative therapy which is usually surgical or radiation. Other palliative treatments are: radioactive isotopes such as Strontium-89 and Clodronate (for bone pain), oestrogens, and cytotoxic drugs).

2.8 Conclusion:
CaP is now the most commonly diagnosed cancer in men. Despite the prevalence of the disease however, the principles of its management (especially its advanced stages) have remained unchanged since the first descriptions of orchidectomy [61] and the use of hormones [62] as effective treatment for prostatic enlargement. Furthermore, it is now well recognised that despite the initial response of the majority of men with CaP to any of the current modalities of treatment (surgery, DXT or hormonal ablation), the malignancy eventually progresses to hormone refractory and metastatic disease which is eventually fatal [63]. In addition, all of the treatment regimens are attended by significant risk of complications that may be distressing for the patient. Indeed, severity of the unwanted sequelae of surgical castration led to the questioning of the wisdom of its prescription shortly after its description as treatment for prostatic enlargement [64]. Finally, there is also the problem of the small proportion of men who present, ab initio, with hormone refractory disease which tend to advance rapidly. This difficulty in the long term management of CaP patients has led to attempts to delineate the molecular pathways involved in the initiation and progression of this malignancy. These research efforts have now established the fact that androgenic influences on the normal and abnormal growth of the prostate are mediated via the regulation of the interaction between stromal and epithelial compartments of the gland.
Chapter 3 - Cellular regulation of prostatic growth and differentiation

3.1 Introduction

Like most other solid organs, the growth of the prostate is regulated by the interaction between several different pathways [1] [Figure 1]. The pathways are:

1) Endocrine pathway: This is via factors (hormones) produced by distant organs and arrive via the blood stream.

2) Paracrine pathways: This is via the action of polypeptide growth factors produced by cells in the vicinity of the target cells which possess the specific receptors for these proteins. This cellular interplay forms the basis of stromal-epithelial interaction.

3) Autocrine pathway: This is via proteins expressed into the extracellular matrix by target cells which then act on surface receptors of the same cells.

4) Intracrine pathway: This is via proteins that are produced within the cell and act on receptors in the same cell.

Figure 8

Mechanisms of regulation of solid organ growth.

Adapted from Campbell’s Urology

Although these mechanisms are inter-dependent, the endocrine pathway maintains overall control on prostatic structure and function by regulating activity in the other pathways. Normal and abnormal prostatic growth is then a result of a complex interaction between androgens, multiple growth factors and their receptors, as well as other co-regulatory molecules involved in cell-cell communications. The paracrine mechanisms of interactions between the stromal and epithelial compartments play a significant role in these inter-cellular communications.
3.2 Stromal-Epithelial Interaction in solid organs

Solid organs are made up of stromal and epithelial cells. There is now growing evidence that this chemical signaling (i.e. stromal-epithelial interaction) plays a crucial role in the developmental biology, cyto-differentiation and organo-genesis of solid organs [4, 65]. Stromal-epithelial interactions are also involved in the maintenance of the phenotypic expression of these tissues in the adult. This cell-cell communication is mediated by the action of polypeptide growth factors on their specific receptors.

Inter-cellular signaling was first described in the prostate [83], and this organ remains the best model for studying the nature of these complex communications between the two cell-types. The interactions between prostatic epithelium and the surrounding connective tissue are known to be critical, and influence the normal growth and androgen responsiveness of the organ [66] [Figure 9]. It is therefore possible that perturbations of these interactions play a crucial role in prostatic carcinogenesis, tumour progression and the development of hormone refractoriness. Furthermore, were definite alterations in stroma-epithelial interactions to be identified in prostatic tumours, these could present new targets for anti-cancer strategies.

Figure 9

Stromal-Epithelial interaction in normal Prostate.

Adapted from Campbell’s Urology
3.3 Stromal-Epithelial Interaction in the Prostate

3.3.1 Stromal-epithelial interaction in the normal prostate

Prostatic development is dependent on the interaction between mesenchymal and epithelial elements of the urogenital sinus in the first few weeks of life. The earliest expression of AR is seen in the urogenital mesenchyme (UGM) [67]. This observation led to the suggestion that the effects of androgens in inducing prostatic epithelial development is mediated via the adjacent mesenchyme. Other studies [68] have demonstrated that subsequently, under the influence of testicular androgens, UGM induces both the expression of AR in the adjacent epithelium and ductal morphogenesis and also regulates epithelial proliferation. The developing epithelium, in turn, induces the proliferation, differentiation and spatial patterning of the surrounding mesenchyme, ensuring the reciprocal nature of this relationship.

Following full differentiation of the various component cells of the prostate, the interaction between the stromal and epithelial compartments continue to play an important role in maintaining the structure and function of the organ under the influence of testicular androgens. Using elegant chimeric recombinant experiments, Cunha et al [69] demonstrated that stromal elements produce factors that have a proliferative influence on adult epithelium via a paracrine pathway. In these experiments, a positive effect was observed when wild-type UGM was combined with adult prostatic ducts. No such effect was seen when UGM from the Tfm variant was used. In addition, combining wild-type UGM with either wild-type or Tfm variant adult bladder epithelium produces adult prostatic epithelium. Interestingly, castration of the host animal in which the recombinants were grown led to prostatic atrophy, which was reversible by testosterone administration. Since the Tfm variant bladder epithelium does not express functional AR [70]), these experiments confirm the importance of AR positive wild-type stromal elements in mediating androgenic effects on both epithelial differentiation and regeneration via a paracrine mechanism.

3.3.2 Stromal-epithelial interaction in neoplastic prostatic tissues

Initiation and progression of malignant transformation

The normal intercellular communications between the stromal and epithelial compartments are commonly subverted in neoplastic prostatic growths. The possibility that stromal-epithelial interactions may play a part in the development of primary tumours of the prostate was first muted by Moore in 1943 [71], and reiterated by Franks in 1954 [72]. More recent studies have indicated that tumour-associated fibroblasts do not confer tumourigenicity on adjacent normal epithelium. However, tumourigenic stroma may induce immortalised but marginally transformed human prostatic epithelium to participate in carcinogenesis [68]. Also, in a rather ingenious
study, Cunha et al co-cultured normal, "benign" and tumour stroma with normal and BPH epithelium in various combinations. Their results demonstrated that stroma was the more important determinant of the rate of growth of prostatic tissue [G Cunha - Presentation at the Experimental Biology 1997 Conference]. In these experiments the highest rate of growth was seen when tumour stroma was combined with BPH epithelium [Figure 10]. It would therefore seem that stromal cells contribute to the initiation and maintenance of prostatic mutagenesis.

\[
\text{Figure 10}
\]

Effects of various stromal cells on growth of prostatic epithelium.

Alterations in stromal-epithelial interactions may also play a significant role in the local progression of cancers. In their studies, Goldenberg et al and Pathak et al found that inoculated mitotic human epithelium induced neoplastic changes in the adjacent stroma [73, 74]. For our part, we have shown in a separate but related study that malignant transformation of prostatic epithelium is associated with a loss of androgen receptor immunoreactivity in the surrounding stroma [75]. This in-vivo evidence is in support of previous suggestions from animal studies that local extension of cancers within solid organs may rely, at least in part, on the mutagenic field effect of the "altered" tumour stroma [68]. Another possible explanation for these changes is that transformed epithelial cells emit as yet undetermined "epigenetic signals" that alter the biological characteristics of the surrounding tumour associated stroma. Other investigations into the role of prostatic stroma in this process have also produced interesting results. Condon et al have shown from experiments using the (rodent) Dunning R3327H CaP and normal rat fibroblast that stromal cells critically influence the growth of malignant prostatic epithelium [76]. Their findings also indicated that the stromal effect may vary significantly in different tumours as well as in different stages of tumour progression.

**Progression to hormone resistance and metastatic disease**

Despite the initial response to androgen ablation in most patients, progression of CaP to hormone resistance and metastatic disease is almost inevitable [54]. Disruption of
the dependence of epithelial cells on (androgen-mediated) regulatory paracrine signals from the adjacent stroma is one of the pre-requisites for the acquisition of the potential for autonomous proliferation that characterises malignant transformation. In our earlier report quoted above [75], the absence of androgen receptor immuno-reactivity in stroma adjacent to malignant prostatic epithelium was independent of the hormone status of the glands. This observation suggests that a cessation of the androgen-stimulated chemical signals from the stroma to the epithelium occurs in the early stages of primary CaP irrespective of grade and progresses as the disease advances. The survival of malignant cells would then rely on their ability to switch to androgen-independent proliferation pathways sooner rather than later following transformation. Other evidence is offered by studies using in-vivo cell-cell recombination model of non-tumourigenic human CaP cell lines (LNCaP) and bone stromal cells [77, 78]. In these tests, by varying the hormonal environment, these researchers found that stromal cells are capable of inducing both the progression of CaP from androgen-dependence to androgen-independent status as well as the acquisition of metastatic potential.

Abnormalities of stromal-epithelial interactions are also of particular significance in the development of metastases. Once seeded, the metastatic cells interact with the stroma of the host tissues to establish a "secondary" inter-cellular communication. Applying this principle, Nemeth et al detected an intense stromal-epithelial reaction around tumourigenic prostatic cells (PC3) inoculated into SCID mice [79]. In addition, Gleave et al investigated the effect of co-inoculation of cell-conditioned media (CM) from a panel of non-tumourigenic fibroblasts on LNCaP cells [80]. They found that the proliferation of the CaP cells was stimulated only by bone and prostate-derived CM, whereas no such effect was seen with CM derived from lung, normal rat kidney or 3T3 fibroblast.

3.4 Conclusion
The interactions between stromal and epithelial cells are pivotal in many aspects of the biology of the prostate. The observations above illustrate that these cells modulate each other's differentiation and proliferation in normal and abnormal growth of the organ through paracrine mechanisms. Malignant transformation of the prostate may therefore involve cumulative abnormalities in the signaling between the epithelial and stromal compartments. Polypeptide growth factors and their receptors play a critical role in these complex cellular interactions. Changes in the cellular phenotype consequent on neoplastic transformation are therefore likely to be reflected in the expression and interplay of these molecules. As such, growth factors/growth factor receptor pathways are being continually studied to identify the specific alterations that characterise malignant transformation.
Chapter 4 - The Role of Growth factors and Growth factor receptors in Oncogenesis

4.1. Growth factors, growth factor receptors and normal growth

Growth factors are a diverse group of functionally related soluble proteins involved in several physiological processes including the (positive and negative) modulation of cell growth, cell differentiation and maturation, angiogenesis and activation of the immune system [81-84]. The definition of distinct intercellular pathways has led to the characterisation of several groups of these proteins. These comprise the epidermal growth factor receptor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin growth factor (IGF), transforming growth factor-beta (TGF-β) families and the haemopoetic growth factors [83] [85, 86]. Growth factors exert their regulatory effects by binding to specific receptors present on the cell surface of virtually all cells. This interaction, along with the action of cytokines and hormones on their receptors, triggers a cascade of intracellular biochemical signals that lead to the activation or repression of various subsets of genes.

The growth factor receptor is involved in both signal reception (as the recipient of extra-cellular stimuli) and signal transmission (as the primary transmitter of the information received to within the cell. The archetypal growth factor receptor is a transmembrane protein with three domains; an extracellular (ligand binding domain), a connecting transmembrane domain, and a cytoplasmic domain with intrinsic tyrosine kinase activity [86]. Ligand binding induces receptor dimerization and activation of the internal tyrosine kinase domain [Figure 11]. This autophosphorylation of the receptor and the adjacent proteins is initial step involved in signal transduction. The tyrosine kinase domain is the most conserved and is absolutely required for receptor signaling [87, 88]. Mutations in the external and transmembrane domains, on the other hand, may lead to constitutive activation of the receptor in the absence of ligand stimulation [89, 90]. On the basis of sequence homology, four classes of tyrosine kinase receptors have been described. These are: EGF receptor, insulin receptor, PDGF and FGF receptor families [86]. The presence of a variety of growth factor receptors on different cell types indicates that both cell development and phenotype are determined by a combination of interacting stimuli.
4.2 The Growth factors-Growth factor receptor axis and the cell cycle

Exposure of cells in the resting or quiescent phase (G0) to growth factors stimulates a series of events which results in their entry and progression through the cell cycle. This mitogenic response occurs in two parts; first the resting cell must be advanced to the G1 phase by “competence” factors (EGF, FGF, PDGF), traverse the G1 phase, and then become committed to DNA synthesis under the influence of “progression” factors (e.g. IGFs)[91] [Figure 12]. Transition through the G1 phase requires sustained growth factor stimulation for several hours, and should the stimulus be disrupted during this critical period, the cell reverts to the resting state [92]. There is also a critical period in G1 when the cell must be stimulated by both “competence” and “progression” factors in order to proceed through the cell cycle [93]. However after this restriction point only progression factors (e.g. IGF-1) are required. The actions of the individual growth factors appear to be specific as whilst some growth factors can induce both proliferation and differentiation of progenitor cells, others can only induce proliferative responses [94]. Interestingly, the removal of growth factors from a dependent cell does not always result in simple growth arrest as might be expected, but can in some cell types, cause a rapid onset of apoptosis [95]. This indicates that growth factors may regulate both the proliferation state and the short term survival in some cells.
Phases of the cell cycle.

- **G0**: Resting phase
- **G1**: Gap 1 phase (accumulation of nutrients and proteins)
- **S**: Synthesis (DNA phase)
- **G2**: Gap 2 phase (DNA repair)
- **M**: Mitosis phase (separation of chromosomes and cell division)

Despite the diversity of growth factors and their receptors, each growth-factor-receptor interaction utilises a common mechanism for initiating the proliferative response [86, 96]. Ligand-induced dimerisation and autophosphorylation stimulate binding of several cytoplasmic proteins to the catalytic domain of the receptor, and these are in turn phosphorylated by the receptor on tyrosine residues [Figure 11]. The connections between biochemical signals emanating from primary receptor substrates and the resulting changes in the nucleus also remain largely undefined. What is certain is that mitogenic signals affect the transcriptional activation of specific sets of genes and the inactivation of others [85]. However the number of steps involved in this process is currently not clear.

### 4.3 Growth factors-Growth factor receptor axis and Cancer

The coordination of cellular differentiation and proliferation is obligatory to the development and maintenance of the structure of solid organs. Malignant growths arise from a stepwise progression of genetic events that result in the uncoupling of these interdependent processes, leading to the transformation of epithelial cells. These events include the unregulated expression of growth factors and their receptors, or other components of their signaling pathways [83].

Adapted from Campbell’s Urology
The first indication that the growth factor-growth factor receptor axis may be involved in the processes that initiate malignant transformation came from the finding that oncogenes of animal retroviruses encode aberrant copies of growth factor receptor tyrosine kinases [97]. A number of these retroviral oncogene products are similar to the protein kinase encoded by the v-src product which specifically phosphorylates tyrosine residues [85]. Examples of these viral oncogenes include the v-erbB, which is a truncated form of the EGFR and has similarity to the v-src product, and v-sis oncogene which is similar to the PDGF B-chain [85, 98]. Other growth factor/growth factor receptor genes identified on the basis of viral oncogenes are the FGF-3, FGF-4 and FGF-5, as well as the M-CSF and SLF receptors [83].

Amongst the different mechanisms by which growth factors and their receptors may contribute to the progression of carcinogenesis, autocrine stimulation of cells is of particular interest to researchers [85]. Autocrinicity is the acquisition, by cells, of mechanisms that allows them to proliferate and survive independent of external stimuli and regulatory controls and may explain the progressive autonomy of transformed cells from their environment. Autocrine stimulation is achieved via two different mechanisms which are: 1) the expression of both the mitogenic growth factor and the relevant receptor by the same cells; and 2) the expression of constitutively active signalling proteins and growth factor receptors that can initiate proliferation independent of ligand binding.

The presence of the components of both autocrine proliferation pathways have been detected by several researchers in immortalised cancer cells [99-102], and is thought to contribute to their tumourigenicity. Further evidence of the importance of this pathway is provided by reports that inhibition of autocrine stimulation of cancer cells in vitro results in decreased cell growth [103]. Until recently, although these mechanisms had also been reported in native human tumours [104-107], the exact role of autocrinicity in tumour evolution was unclear. However, findings from current studies have indicated that factors that confer autocrinicity may confer an aggressive phenotype on human cancers [108-110].

Growth factors may also contribute to the malignant process by initiating the development of new vasculature that provides nourishment to the neoplastic growth [111]. In the absence of neo-angiogenesis, tumours can only grow to a few millimeters by relying on osmotic diffusion of nutrients [112]. To overcome this limitation, malignant growths express angiogenic growth factors (e.g. EGF and FGF) that are chemotactic for endothelial cells and induce their proliferation, resulting in the extension of nearby blood vessels towards the source of the factors [82]. This usually culminates in a rapid increase in tumour size, and also provides a route for metastatic spread.
There are extensive reviews on genetic alterations affecting growth factors or their receptors in human tumours including prostate cancer [83-86]. Sarcomas and glial tumours are known to exhibit persistent PDGFR activation due to autocrine stimulation of the receptor by ligands produced by the same cells [100]. Another autocrine loop detected in tumours involves EGFR stimulation by aberrantly expressed TGF-α, which is frequently seen in tumours expressing large amounts of EGFR [104, 113]. The expression of bFGF has also been detected in melanomas as opposed to normal melanocytes, which require the mitogen for proliferation [114]. In the case of IGFs, high levels of these growth factors and/or their receptors have been found in several tumours including those of the breast and kidney, as well as sarcomatous growths [115].

4.4.1 Growth factors in CaP
The role of growth factors and their receptors in CaP has been investigated using both tissue specimens and human prostate cancer cell lines which are able to grow and metastasize in nude mice. Alterations in growth factor signaling detected in these tissues indicate the presence of potential autocrine pathways of proliferation in this neoplasm. These include the TGF-α/EGFR [104], heregulin/erbB-3 [106], and IGF-II/IGF-IR [116] axes, and constitutive EGFR activity [103]. Several growth factor pathways have also been implicated in the progression of CaP. In particular, IGF has been shown to be capable of androgen receptor mediated transcription of metastatic prostate cancer cell lines in the absence of androgen [117]. This suggests that IGF may be involved in hormone-independent growth of CaP. Furthermore, TGF-α, and the TGF-β and FGF families are also considered as important factors in the development of metastatic prostatic disease [118].

4.5 Clinical Implications of Growth Factors and Growth factors Receptors in Cancer with emphasis of Prostate Cancer
Growth factor and growth factor receptors are now established in several protocols of cancer management in which they are used either as tumour markers, prognostic indicators or as targets for anti-cancer therapies [119].

Growth Factors and Growth factor receptors as Tumour Markers
The relative specificity and sensitivity of abnormalities in the expression of several growth factors in malignancies means they can be used as tumour makers. This property is currently being exploited for use in the diagnosis, staging and follow-up of treatment of various cancers in several centres. Amongst the malignancies in which such protocols have been established are B and T lymphomas and chronic
leukaemias [120], stomach cancer [121]. No growth factor or growth factor receptor has yet been identified as a suitable marker of prostatic neoplasia.

**Growth Factors and Growth factor receptors as prognostic Indicators**

Quantification of the expression of growth factors and/or their receptors is also being used in the evaluation of tumour aggressiveness. EGFR expression defines a poor prognosis in several cancers including those of the breast [122], bladder [123], stomach [124], oesophagus [125] and ovarian [126]. Over-expression of ErbB-2 also appears to indicate a more malignant phenotype in breast and ovarian cancer [123]. Interestingly, this study also showed that co-expression of both EGFR and ErbB-2 defines a particularly aggressive biology in breast cancers. In the prostate peptide growth factors and receptors are being evaluated as potential indicators of aggressive disease and include TGF-beta 1, TGF-beta I and beta II receptors [127] and bFGF [128].

**Growth Factors and Growth factor receptors as targets for anti-cancer therapy**

Growth factors and their receptors have been recognised as potential targets of anti-cancer therapies for a while [119]. Several anti-cancer treatment protocols now target growth factors and their receptors as adjuvant therapy to various chemotherapeutic protocols [129] [130]. However, the most attractive use of growth factor-related cancer treatment strategies is the possibility of specific tumour-cell destruction and established treatment schedules in this regard include cytokines such as Interferon-alpha [131]), Interferon-gamma and Interleukin-2 [132, 133]. Anti-tumour treatments can also be directed at different levels of the signaling pathway (growth factor, growth factor receptor and tyrosine kinases). Drugs and various monoclonal antibodies against growth factors and their receptors have been developed and are undergoing trials [134][135]. The EGFR, in particular, has been a popular target for these novel treatments [136]. Anti-cancer regimes directed at the EGFR have now been established in brain [137], lung, head and neck [138] and breast cancer [139]. Anti-EGFR antibodies may exert their inhibitory effects by initiating either an immunological response or terminal differentiation of the cells[140]. Other treatments being developed include Tyrophostins which are molecules that inhibit tyrosine phosphorylation [141], anti-sense oligonucleotides [142] and dimerisation inhibitors [143].

In CaP research, laboratory experiments have shown antibodies that bind to TGF-α, EGFR and IGF-IR inhibit the growth of metastatic prostatic cells [101, 116]. Drugs that mimic or block the action of growth factors and their receptors have also been designed. One such synthetic protein is JB1 which is an IGF-1 analogue which binds
competitively with IGF-IR thus preventing critical auto-phosphorylation of the receptor and interrupting the autocrine loop [144]. Monoclonal antibodies that inhibit constitutive EGFR phosphorylation [103], as well as drugs directed at the receptor signaling pathways [145] are also being tested in CaP cell lines. Efforts to develop effective gene therapy in CaP continue and several anti-sense regimes are currently undergoing trials with promising results [146].

4.6 Conclusion
A clear link has now been established between growth factors (and their receptors) and oncogenesis and its subsequent progression. Of these regulatory pathways the EGF-EGFR axis was the first to be identified and is the most researched. These investigations have frequently implicated EGFR abnormalities in the development and progression of several cancers.
Chapter 5 - The Epidermal Growth Factor Receptor

5.1 Introduction
The WT-EGFR belongs to the Type 1 group of growth factor receptors [147, 148]. This family of proteins share a variable sequence homology and includes the ErbB-2 [149], ErbB-3 [150] and the recently described ErbB-4 [151] proto-oncogenes. Over-expression of WT-EGFR, ErbB-2 and ErbB-3 is thought to be one of the causes of malignant transformation as high levels of these peptides have been found in many solid organ tumours, whilst the status of ErbB-4 in cancers is yet to be determined [152]. WT-EGFR was the first member of the family to be identified, and is by far the most commonly detected in malignancies [153]. As can be seen from the last chapter, this receptor and its ligands have a pride of place as a focus of research amongst the signaling molecules. Much of the biochemical characterisation of WT-EGFR has resulted from the isolation of the human epidermoid carcinoma cell line A431 [154] that was shown to over-express the receptor (approximately 2.6 x 10^6/cell).

The EGFR gene is located on the short arm of human chromosome 7 [155]. This codes for a 170 kDa transmembrane glycoprotein that plays an important role in the differentiation and proliferation of epithelial cells [156]. The native EGF receptor consists of a 621 amino acid extracellular “ligand” binding domain, attached by a 23 amino acid transmembrane region to a 542 amino acid cytoplasmic domain with tyrosine kinase activity [157]. Binding of the receptor by any of its ligands (see below) results in its activation, down-regulation of the ligand-receptor complex, and the initiation of a cascade of reactions that ultimately result in DNA replication and cell division [152, 158, 159]. WT-EGFR is found in a wide variety of normal human epithelial cells and the tumours arising from these tissues [160]. However, the level of the expression of the receptors in cancers is comparatively higher that those in the normal progenitor cells. Furthermore, WT-EGFR expression varies widely in normal tissues, and some cells (e.g. keratinocytes) express relatively high levels of the receptor (2 x 10^5/cell) whereas lymphoid cells express none or very low levels of the receptor [160].

5.2 EGFR Ligands
Presently several ligands (the EGF family) are known to bind to WT-EGFR. All members of the EGF family possess the “EGF unit”, which is a sequence of about 40-45 amino-acids with three disulphide bridges [137]. In addition, with the exception of cripto, all the other “EGF-like” molecules inhibit the binding of 125I-EGF to EGFR [161, 162].

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i) **EGF** - This was the first member of the group to be discovered (1962) [163]. The human EGF is found in the epithelia of almost every organ system in the body [164]. Its detection led to the search for and identification of its receptor (EGFR), and the detection of other members of the EGF family. It remains the best known mitogen for epithelial cells and their malignant counterparts.

ii) **Transforming growth factor –alpha (TGF-α)** - TGF-α is thought to play an important role in normal foetal development, tissue regeneration and angiogenesis [158]. Furthermore, as many neoplastic cell which over-express EGFR also express TGF-α, it may also be involved in autocrine stimulation of tumours [158, 165]. Although the TGF-α competes with EGF for binding with EGFR [165, 166], the two ligands interact with different sites on the receptor [167] and induce differential processing of the receptor [168].

iii) **Amphiregulin (AR)** - AR is a secreted heparin growth factor detected in 1988 [169]. Its biological activity differs from those of EGF and TGF-α although it is structurally and functionally related to them [170]. It inhibits the growth of tumours that proliferate in response to both of these mitogens, and it stimulates the proliferation of normal fibroblasts and keratinocytes. It however acts exclusively through the EGFR [159]. AR is found in many normal tissues and is comparatively over-expressed in the corresponding tumours [171]. Similar to EGF and TGF-α, this peptide has been implicated in autocrine stimulation of cancers [172].

iv) **Other members of the EGF family of ligands** - These ligands are structurally related to the EGF and include the heparin-binding growth factor (HBGF) and the vaccinia virus growth factor (VVGF) [147] which are virally encoded products.

5.3 **WT-EGFR as an Oncogene**

Among growth factor receptors, the most frequently implicated in human cancers has been WT-EGFR, and it is now clear that over-expression of this receptor plays a critical role in the initiation and progression of several human cancers [113, 126, 173-175]. The activated EGFR acts as a competence factor, and genetic alterations that activate the critical regulatory molecules within the “competence” signaling pathway are commonly associated with malignant transformation [91]. Findings from studies investigating the complementary roles of the “competence” and “progression” factor pathways in the proliferation of epithelial cells have provided evidence in support of the hypothesis that the signaling pathway of competence factors ordinarily limits growth in vivo [176]. Abnormalities of this signaling mechanism would then play a particularly important role in the excessive proliferation that is the *sine qua non* of carcinogenesis.
The first indication of the oncogenic potential of this receptor came from the finding that the cytoplasmic domain of EGFR has a striking 97% homology to the avian erythroblastosis virus oncogene product (v-erbB) [177]. This oncogene is a truncated protein that lacks the extracellular ligand-binding domain, and its constitutive activity is thought to be responsible for its ability to induce malignant transformation in chickens. Also of interest was the report that the expression of a truncated EGFR lacking the EGF binding domain (as with the v-erbB product) led to the transformation of immortalized rat fibroblasts and the activation of intrinsic tyrosine kinase [178].

WT-EGFR is thought to exert its oncogenic potential as a result of an increase in receptor tyrosine kinase activity [179]. This may be achieved through several mechanisms. These are:

1) Amplification of the EGFR gene and/or over-expression of the receptor, which leads to an increase in the absolute numbers of the receptors on the cell surface. In 1987, Velu et al reported that EGFR-related transforming potential is directly related to the number of receptors expressed [180]. In addition, cells which over-express EGFR were transformed to the malignant phenotype when grown in EGF-supplemented media [181].

2) Autocrine activation of the EGFR. As stated earlier (Section 4.4), TGF-α expression is seen in most squamous cell cancers that overexpress EGFR, thus providing the mechanism for a potential (persistent) autocrine stimulation of these cells [113]. This mechanism has also been detected in CaP [104, 182, 183].

3) Non-degradation of the activated EGFR within the cell cytoplasm. WR-EGFR induced proliferative signals are usually limited by degradation of the ligand-activated receptor in the cytoplasm [100]. The degradation domain is located in the cytoplasmic domain of the receptor and abnormalities of this domain can result in non-degradation of the activated EGFR with persistence of tyrosine kinase activity [184, 185].

4) Constitutive EGFR activation. This has been detected in established human CaP cells in-vitro [103]. Ligand-independent activation of EGFR could lead to persistent (and thus increased) tyrosine kinase activity. The current role played by this pathway in the biology of tumours is currently undetermined.

Although there is evidence to implicate all these mechanisms of increased tyrosine kinase activity (as detailed earlier in this section) in the malignant transformation of epithelial cells, over-expression of the receptor and/or amplification of the EGFR gene is the most commonly detected in human cancers [186, 187]. Interestingly,
EGFR amplification is more commonly found in glioblastomas which arise de novo in older patients with no previous clinical history of low-grade glial tumours (astrocytoma) [188]. In contrast, glioblastomas which develop (in younger patients) from the progressive de-differentiation of astrocytoma are more commonly associated with inactivating mutations of p53 (a tumour suppressor gene). Furthermore, EGFR amplification has been associated with rearrangement of the gene in tumours of the brain [187], breast [189], cervix and kidney [190], and squamous cells [191]. This has led to the scrutiny of these neoplasms for the expression of mutated forms of the receptor protein with some success (see below).

5.4 Mutant EGFRs in Cancer

The EGFR gene is often amplified and highly expressed in neural tumours, and this may be associated with increased receptor kinase activity [187, 192]. Investigation of this association led to the detection of EGFR mutant proteins in glioblastomas [193]. Since these first reports, mutations of EGFR have been found in several human tumours [89, 105] [194-197].

Most research directed at determining the biochemical and physiological properties of altered EGF receptors has involved laboratory studies on transfected glioblastoma cell lines. Although genetic changes have been detected in all three domains of the EGFR (cytoplasmic [198], transmembrane [199], and external [193]), alterations of the external domain are more common in human tumours [105, 109]. Furthermore, these structural abnormalities can either be due to insertion ([199]) or deletion [200]) mutations, both of which can result in constitutive activation of the receptor.

Mutant EGFRs resulting from deletion mutations of the external domain have been classified into three types on the basis of size and location of the mutation (Types I-III [89]) (Figure 13). Each of these antigens is thought to contribute to neoplastic transformation by conferring a significant growth advantage on epithelial cells in which they are over-expressed.
Wild type and mutant epidermal growth factor receptors.

Adapted from Biochem Biophys Res Comm 178; 1418; 1991

The Type I and Type III mutant EGFR genetic alterations result from deletions in the ligand-binding sub-domains of the extra-cellular domain of the native receptor [192, 200, 201]. Not surprisingly therefore, neither variant receptor binds the EGF or any other known EGFR ligand but are constitutively active and thus capable of autophosphorylation. This is in keeping with the report by Kwatra et al [202] that the EGFR ligand binding domain is not required for receptor dimerization. As such, both Type I and Type III mutant EGFR are structurally and biochemically similar to the retro-viral v-erbB gene product and are considered to be oncogenic. In contrast, the Type II mutants result from a ligand domain-sparing 83 amino-acid deletion in the same domain [89] and is thus able to bind EGF and TGF-alpha. Over-expression of this mutated form of EGFR is also capable of transducing EGF-stimulated mutagenic signals in culture.

5.4.1 The Type III mutant EGFR (EGFRvIII)

The Type III mutant epidermal growth factor receptor (EGFRvIII) is the most prevalent EGFR variant. This mutant EGFR has been detected in several tumours including: adult and paediatric glioblastomas (72% and 66% respectively), medulloblastomas (86%), breast cancers (78%), ovarian carcinomas (73%) [105], and non-small cell lung cancers (16%) [203]. EGFRvIII results from the deletion of exons 2-7, which leads to an 801-bp in-frame (267 amino acid) deletion of the
external domain of the normal receptor [200]. The rearrangement removes most of
the first two extracellular sub-domains of EGFR, but preserves the reading frame of
the receptor message. As such, this aberrant EGFR is not bound or down-regulated
by any known EGFR ligand [108, 194], but is a constitutively dimerized activated
tyrosine kinase which initiates mitosis independent of ligand-binding [204].
Currently, the initiating factor(s) of this alteration in the EGFR gene is unknown.
EGFRvIII has been detected in transformed cells only, and its expression is both
membranous and cytoplasmic [105, 204, 205].

Like the v-erbB avian oncogene (with which it has similarities), the EGFRvIII is
thought to play a significant role in the pathogenesis and progression of cancers
[108, 206], although both oncogenes may signal through different intracellular
pathways [204]. Evidence implicating this mutant receptor in neoplastic
transformation includes the ligand independent transformation of immortalised
rodent fibroblasts in culture and the development of tumours in athymic mice
inoculated with EGFRvIII [105, 207]. Similar to the WT-EGFR, over-expression
and non-degradation of EGFRvIII both result in increased tyrosine kinase activity
[204]. However, the oncogenic activity of the altered receptor may not be entirely
due to its intrinsic tyrosine kinase activity [207]. EGFRvIII peptide is thought to
confer an aggressive phenotype on malignant epithelial cells by increasing mitosis
and decreasing apoptosis [109, 208]. In addition, EGFRvIII transformation is
dependent on a high level expression of the antigen, such as has been found in
EGFRvIII-expressing glioblastomas [204].

The prevalence of the EGFRvIII in naturally occuring human tumours has led to the
prediction that it actively contribute to the malignant phenotype of these tissues [207].

5.5 Conclusion
The overall conclusion from the data presented above is that over-expression of the
EGFR and its mutants and/or the expression of an aberrant form of the receptor may
play a critical part in the development and progression of malignant transformation
of certain organs. Furthermore, the unique properties of the Type III mutant receptor
mean that its presence in human tumours may be exploited for the purposes of
developing new therapeutic approaches.
Chapter 6 - Controversy surrounding EGFR expression in human prostate cancer

6.1 Introduction

The overall conclusion from the data presented in Chapter 5 is that over-expression of the EGFR and its ligands and/or the expression of an aberrant form of the receptor may play a critical part in the development and progression of malignant transformation of many organs. Despite this abundance of information on EGFR expression in most malignancies, reports on the expression of the receptor in prostatic tumours are conflicting. The expression of EGFR mRNA is increased in these neoplasms [209-212]. Inspite of this, several studies have detected a decrease in the expression of the receptor protein with increasing malignant transformation of the epithelial cells (including two of the studies reporting an increase in the mRNA [211, 212]) (Table 1). However, other investigations have suggested that the levels of EGFR protein expression in benign prostatic hyperplasia (BPH) and carcinoma of the prostate (CaP) are similar. These inconsistent results have limited progress in the understanding of the role of this ubiquitous receptor in CaP, and the development of anti-EGFR strategies in CaP.

6.2 A review of previous studies

A critical review of previous investigations into the expression of EGFR in normal and abnormal prostatic tissues was a pre-requisite to carrying out this project. This perusal indicated that the confusion on the level of EGFR protein in prostatic tissues was probably due to the varied methodology used in the different studies. The different methods include:

1) The use of homogenised tissues - The early EGF-binding experiments used homogenised tissues [166, 210]. These test samples would have been contaminated by varying amounts of stromal cell which do not express EGFR, and this may account in part for the different results obtained. To avoid this problem, subsequent comparative immunostaining tests have mainly been done on tissue sections either frozen ([182, 211, 213-215]), or archival ([183, 212, 216-219]). However, formalin preservation is known to alter the structure of the cell membrane and thus mask antigenicity. As such the effectiveness of antibodies in detecting their relevant antigens may be reduced. However, various methods of antigen retrieval have been devised [220]. In addition some antibodies are able to react with archival material following de-waxing and deparaffinisation only. However, the use of tissue preserved in different ways in EGFR studies may also contribute to the variability of results reported.
2) **The use of different anti-EGFR antibodies** - The recognition of cellular proteins by indirect immunohistochemistry has in-built limitations. This is because antibodies react with specific peptide sequences during immunoreactions and different clones of antibodies directed at the same antigen may bind with different parts of the protein. As such, these reagents may recognise varied quantities of the antigen in similar material. Moreover, in addition to binding to the WT-EGFR, some of these antibodies may also react with some of the mutant EGFRs described in the literature further confusing the issue. Several anti-EGFR clones have been used to detect the receptor in EGFR studies, and these include: 1) anti-EGFR1 [214, 215]; 2) anti-EGFR (Ab-1) [104, 211, 213] [21]; 3) anti-EGFR (clone 31G7) [212, 217-219].

3) **Cells types compared during evaluation of EGFR expression** - Whilst it is generally accepted that EGFR immunostaining is strongest in the basal cells of normal and BPH glands, different methods have been used to assess EGFR expression. Most studies evaluating the expression of the receptor in different prostatic histotypes have compared the anti-EGFR immunostaining in the outermost cells of both malignant and benign (normal and BPH) glands [104, 211-215, 218]. However, in their study Glynne-Jones et al took the characteristic absence of basal cells in invasive CaP into consideration [183], and compared immuno-staining levels observed in the luminal cells of both BPH and CaP glands instead. This method of analysis may be responsible, in part, for the contradiction between their results and those reported in other studies [182, 211, 213-215, 218]. Their findings were however in keeping with those reported by Cohen et al [104].

4) **Differences in methods used to assess immunohistochemical staining.** Several methods of evaluating immunohistochemical staining have been applied to EGFR immunostaining in prostatic tissues thereby adding another variable to the controversy. These include: A) Cut-off points [104, 213, 216]; B) Visually graded scoring of the intensity of staining [30][182, 183, 211, 212, 214, 219]; C) Image analyser comparison of the intensity of EGFR immunostaining [215]; 4) The modified H-score [218, 219].

From the above it can be seen that the variability in IHC detection of EGFR protein in prostatic tissues may depend on the antibody used, tissue preparation and the method of analysis of the IHC result (Table 1). Inspite of these competing factors however, most of the studies investigating EGFR expression in prostatic tumours have reported a depletion of the receptor protein as the tissues de-differentiated [182, 212-214, 216, 217] [104, 211] [215, 218, 219].
Table 1
Table of Results of Previous Studies on Immunostaining Detection of EGFR Protein in Human Prostate Cancers

<table>
<thead>
<tr>
<th>No</th>
<th>Authors</th>
<th>Specimen Preparation</th>
<th>Anti-EGFR Antibody Clone</th>
<th>Method of Evaluation of IHC Result</th>
<th>CaP immuno-reactivity (no. positive/no studied) (cell type compared)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maygarden et al., 1992/</td>
<td>Frozen</td>
<td>Ab-1</td>
<td>COP Positivity (&gt;20%)</td>
<td>25% (5/20) (CaP &lt; Basal cells)</td>
</tr>
<tr>
<td>2</td>
<td>Mellon et al., 1992/</td>
<td>Frozen</td>
<td>EGFR1</td>
<td>VGIS</td>
<td>17% (5/29) (CaP &lt; BPH)</td>
</tr>
<tr>
<td>3</td>
<td>Fox et al., 1992/</td>
<td>Paraffin</td>
<td>12E</td>
<td>COP Positivity (10%)</td>
<td>40% (18/45) (CaP &lt; BPH)</td>
</tr>
<tr>
<td>4</td>
<td>Visakorpi et al., 1992/</td>
<td>Paraffin</td>
<td>31G7</td>
<td>VSIG</td>
<td>47% Uniform (69/147) (CaP &lt; Basal cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39% Partial (58/147) (CaP &lt; Basal cells)</td>
</tr>
<tr>
<td>5</td>
<td>Ibrahim et al., 1993/</td>
<td>Frozen</td>
<td>EGFR1</td>
<td>Image analyser GIS</td>
<td>Low/absent in 37 cases (CaP &lt; Basal cells)</td>
</tr>
<tr>
<td>6</td>
<td>Myers et al., 1993/</td>
<td>Frozen</td>
<td>Ab-1</td>
<td>VGIS</td>
<td>64% (7/11), 80% of cell positive (CaP &lt; Basal cells)</td>
</tr>
<tr>
<td>7</td>
<td>Montone et al., Paraffin</td>
<td>1. 31G7</td>
<td>VSIG</td>
<td>% cells positive</td>
<td>8% (1/13) (CaP &lt; BPH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. OM-11-951</td>
<td>VGIS</td>
<td></td>
<td>77% (10/13) (CaP &lt; BPH)</td>
</tr>
<tr>
<td>8</td>
<td>Cohen et al., 1994/</td>
<td>Frozen</td>
<td>Ab-1</td>
<td>COP Positivity (&gt;20%)</td>
<td>94% (94%) (CaP = BPH)</td>
</tr>
<tr>
<td>9</td>
<td>Tukeri et al., 1994/</td>
<td>Frozen</td>
<td>Ab-1</td>
<td>VGIS</td>
<td>53% (16/30) (CaP &lt; PIN &lt; BPH)</td>
</tr>
<tr>
<td>10</td>
<td>Maygarden et al., 1994/</td>
<td>Paraffin</td>
<td>31G7</td>
<td>Mod. H-score</td>
<td>40% (41/102) (Cancer &lt; Basal cells)</td>
</tr>
<tr>
<td>11</td>
<td>Moul et al, 1996/</td>
<td>Paraffin</td>
<td>31G7</td>
<td>Mod. H-score</td>
<td>47% (49/104) (CaP &lt; BPH)</td>
</tr>
<tr>
<td>12</td>
<td>Glynne-Jones et al.,</td>
<td>Paraffin</td>
<td>(anti-EGFR external domain ?clone)</td>
<td>VGIS</td>
<td>80% (44/55) (CaP &gt; BPH secretory cells)</td>
</tr>
</tbody>
</table>
6.3 Hypotheses proposed as potential explanations of the decreased EGFR expression in prostatic neoplasms

Several hypothesis have been proposed as possible explanations for decreased EGFR expression in (prostatic) tumours and these include: 1) down-regulation of the receptor expression in these tissues [211, 215]; 2) rapid turnover of the receptor [215]; 3) protease digestion of the receptor and a lack of recognition of the products of digestion by the antibodies [215]; 4) absence of EGFR gene expression [221]; 5) internalization of the receptor [222]; 6) expression of a truncated/mutated receptor [186, 218, 223] that is recognised by some but not of all the anti-EGFR antibodies. Hitherto, none of these postulations has been proved conclusively.

There are reports supporting these hypotheses of the mechanism of alteration in EGFR expression in other tissues. In their study Gamou et al detected a lack of expression of the receptor gene in small lung cell carcinomas that did not demonstrate EGF-binding activity [224], whilst McCune et al found that transient internalization of the EGFR prevented its detection in liver cells [225]. Furthermore, A431 carcinoma cells have been shown to regulate expression of the receptor via density-induced down-regulation [226].

However, the most attractive of the potential mechanisms explaining a decrease in EGFR protein expression in human neoplasms is the expression of a mutated receptor along with, or instead of, the WT-EGFR. This hypothesis has been the focus of concerted research efforts. As mentioned earlier, several types of mutant EGFRs have been found in solid organ tumours [89, 105, 194, 195], and these mutant EGFRs have also been implicated in carcinogenesis. The most prevalent of these variant receptors is the constitutively active tyrosine kinase Type III mutant EGFR. However, despite the detection of the EGFRvIII in several cancers [105], to our knowledge, the presence of this variant EGFR in prostatic tissues has not been investigated.

6.4 Conclusion

The EGFR is a ubiquitous protein, and is one of the most commonly implicated growth factors receptors in solid organ malignancy. However, results on its expression in prostatic tumours have been inconsistent and this has limited, not only the understanding of the role of this receptor in prostate cancer, but also its potential role as a target for new treatment regimes. Most reports have detected a decrease in EGFR protein expression in the more malignant prostatic neoplasms despite an increase in the receptor mRNA in these tissues. Several hypotheses have been proposed as explanations for this disparity including the expression of a truncated or mutant EGFR. Recently several mutations of the EGFR have been detected in solid tumours, and both the native and abnormal receptors have been implicated in carcinogenesis. However, until now, there are no reports confirming the expression of mutant EGFRs in prostatic tumours.
Chapter 7 - Scope of this Thesis

7.1 Summary - The magnitude of the problem.
Prostate cancer is a major burden on health-care resources world-wide as it is now the most commonly diagnosed cancer in men. It is thus a significant cause of morbidity and mortality. The disease represents a heterogeneous entity with varying degrees of biological behaviour. Unfortunately, despite the prevalence of CaP and the improvements in its detection in earlier stages, the factors that govern its natural history are still poorly understood. As a result, the principles of prostate cancer management have remained essentially unchanged for a century, especially when the disease becomes metastatic and/or hormone refractory. As a result of this, research efforts are increasingly focused on the molecular mechanisms that regulate normal and abnormal growth of the gland. Peptide growth factors and their receptors are an integral part of these pathways, and have been the target of many investigations.

It is accepted that alterations in stromal-epithelial interactions play a crucial role in the development and progression of CaP. Of particular interest are the current reports that point to the liberation of prostatic epithelium from (androgen mediated) stromal regulation in the malignant prostate. These findings suggest that the acquisition of molecular mechanisms of autonomous proliferation is a pre-requisite for prostatic mutagenesis. The Type III mutant EGFR (EGFRvIII) is a constitutively active tyrosine kinase which is capable of initiating mitosis independent of ligand-binding, and thus independent of hormonal control. This aberrant EGFR has been implicated in the pathogenesis and progression of several cancers but has not been detected in CaP.

7.2 Scope of this Thesis
Research into the inconsistency of reports on EGFR expression in prostatic tumours has waned over the years. Although the newer antibodies have indicated strongly that there is a progressive depletion of the receptor as the tissues become increasingly malignant, there are recent reports suggesting a comparative EGFR expression in benign and malignant tumours, thus continuing the debate.

This project sought to study this unexplained area of controversy in the molecular biology of prostatic neoplasms using newer (and more sensitive and specific) investigative techniques. We postulated that, in addition to the normal receptor, prostatic tumours also express a mutated EGFR, and that the contradictory findings in previous studies are due to the detection of this mutant receptor by some but not
all of the different techniques and/or antibodies used. We also hypothesized that the expression of such an aberrant EGFR could be of clinical relevance in CaP. As the most commonly detected mutant in solid organ tumours the EGFRvIII was considered to be a suitable candidate for investigation. The aim of this project was to scrutinize normal, BPH, CaP and metastatic tissues for the expression of EGFRvIII and WT-EGFR using immunohistochemical techniques, and to compare the expressions of both receptors in these neoplasms. We also aimed to assess the clinical significance of EGFRvIII expression in prostate cancer by correlating its level of expression with known prognostic indices of the disease. The detection of a variant type EGFR may also provide a new target for the anti-CaP strategies including gene therapy.
Chapter 8 - Materials

8.1 General Chemicals and Materials for Immuno-histochemistry and Western Blotting.

Most general chemicals and other material for immuno-histochemistry and Western blotting were purchased from Sigma Chemicals (Poole, UK), Aldrich Chemical Company Ltd (Gillingham, UK), Bio Rad Laboratories (Hemel Hempstead, UK), or BDH Laboratory Supplies (Poole, UK). Materials purchased from other companies are listed below.

8.1.1 Immuno-histochemistry

a) Hydrogen peroxide solution - Thorton and Ross Limited (Huddersfield, UK)
b) Tissue-Tek O.C.T compound embedding medium - R A Lamb (London, UK)
c) Cork discs - ICN Flow (Bucks, UK)
d) Superfrost®/Plus microscope slides - Fisher Scientific (Pittsburgh, USA).
e) Washed microscope slides - Gold Star (Warley, UK)
f) Cover-slips - Deckglasser (Germany)

8.1.2 Western blotting

a) Streptavidin-Peroxidase photoluscent conjugate and ECL western blotting detection reagent - Amersham International Plc (Bucks, UK).
b) Biomax scientific imaging film - Kodak (UK)

8.2 Solutions prepared by myself

8.2.1 Immuno-histochemistry (Appendix 1)

a) Tris buffered saline  b) Citric acid buffer

8.2.2 Western blotting (Appendix 2)

a) Homogenising buffer
b) Dialysis Buffer
c) 6% sodium dodecyl sulphate polyacrylamide gel (SDS- PAGE)
d) 6% Stacking gel

8.3 Other Equipment used

8.3.1 Immuno-histochemistry

a) Reichert-Jung 2800 Frigocut N - Leica (Milton Keynes, UK)
8.3.2 Western blotting

a) Curix 60® developer - AGFA-GEVAET Ltd, (Brentford UK).
b) Ultrasonic Processor - JENCONS Scientific Ltd (Leighton Buzzard, UK)

8.4 Antibodies

The antibodies used in this study were obtained from:

a) Mouse anti-human Cytokeratin Pan (Ck Pan) - Sigma Chemical Company (Poole, UK.)
b) Rabbit anti-human Prostatic Acid Phosphatase (PAP)-Sigma Chemical Company Poole, UK.
c) Mouse anti-PSA (clone 22MPA) - Euro-Path Ltd (Cornwall, UK.)
d) Rabbit anti-EGFRvIII (a gift) DK Moscatello and AJ Wong, Kimmel Cancer Institute (KCI), Thomas Jefferson Univ., Philadelphia USA
e) Mouse anti-EGFR (clone 31G7, Zymed) - Cambridge Bioscience, (Cambridge UK.)
f) Mouse anti-Ki67-MM1 - Novacastra Laboratories Ltd, (Newcastle UK.)

8.5 Immunohistochemical Detection Kits

a) Alkaline phosphatase anti-alkaline phosphatase detection system - Sigma Chemical Company (Poole, UK.)
b) Streptavidin-Biotinylated Horseradish Peroxidase System - DAKO (High Wycombe, UK.)

8.6 Fresh frozen Prostatic Tissues

Sections from fresh frozen prostatic tissues were used in the initial staining tests. Homogenised samples from these tissues were also used for the Western blots. Freshly cut prostate chips were obtained during transurethral resection of the gland at the LGH. The chips were transported to the laboratory in labelled pots containing “Transport medium” (RPMI + 10% fetal calf serum [FCS] + Penicillin and Streptomycin), and flash frozen to minus 170° C in liquid nitrogen within an hour of resection. Five-μm thick sections were cut from the chips at minus 20° C onto gelatinised microscope slides using the Frigocut® cryostat (Appendix 3). The remaining tissue was stored in liquid nitrogen dewars. Representative
sections of the tissue were sent for routine haematoxylin and eosin staining for histological evaluation.

8.7 Archival Tissue Sections
The bulk of this study performed using formalin-fixed paraffin-embedded archival tissues.

8.7.1 Sources of archival material

8.7.1.1 Archival skin sections
Four-µm sections of archival normal human skin were obtained from the Pathology Department of Leicester General Hospital, Leicester.

8.7.2.2 Archival prostatic sections
Sections (4-µm thick) from archival surgical specimens obtained in 1993 from 38 patients with CaP and 19 age-matched patients with BPH, were supplied by the Pathology Department of Leicester General Hospital [Figure 14]. Thirty-one of the malignant cases were newly diagnosed patients who had not been subjected to hormonal manipulation, and 7 glands were from patients with hormone-resistant disease. Tissue blocks for the study were selected by an experienced Consultant Pathologist (E. Hugh MacKay [EHM]), and sections from these blocks were cut onto silane coated (Sigma) and Superfrost®/Plus microscope slides and oven dried for at least 24hrs (range 24-72 hrs). Sections from 12 archival metastatic deposits (6 bone and 6 lymph node) were also obtained from the Department of Surgery, Western General Hospital Edinburgh for screening. All tissues had been fixed in 10% buffered formalin and embedded in paraffin. Histological evaluation was performed by routine haematoxylin and eosin staining of representative sections from each block of archival tissue. The primary malignant prostatic tumours were graded using the Gleason score [34], and classified as well differentiated (G1, [Gleason score 2-4]), moderately differentiated (G2, [Gleason score 5-7]) or poorly differentiated (G3, [Gleason score 8-10]). Glands with high grade prostatic in-situ (HGPIN) were seen in 14 CaP sections.
8.7.2.3 Archival KCI EGFRvIII mouse tumour

A specimen of archival mouse tumour derived from cells transfected with the human EGFRvIII [35] was kindly provided by Drs Moscatello and Wong. Sections (4-μm thick) were cut onto both silane coated and Superfrost microscope slides.
8.8 Clinical Data

Clinical information was available for retrieval in all 38 CaP patients, and was complete in 34 (90%). All data were included for analyses (Appendix 4). The minimum time interval between tissue retrieval and data review was 36 months. Clinical parameters recorded were:

1) Age  
2) Histological grade  
3) PSA at the time of tissue retrieval  
4) Hormone status  
5) Presence or absence of metastasis at diagnosis.  
6) Time to disease progression following hormonal therapy i.e. a rise in PSA level by greater than twice the nadir level, or the appearance of new metastases.  
7) Survival status.  
8) Follow-up period or length of survival. The minimum follow-up period was 36 months and the maximum was 52 months, with a median of 37 months (mean 39.2 months). Thirteen patients died before the review, and all but 1 death was from CaP related causes (as recorded in the death certificates).
Chapter 9 - Immunohistochemical detection of tissue antigens

9.1 Introduction

Immunohistochemistry was the main investigative tool used during this research project [227]. The basic principle of immunohistochemistry is the detection of a specific tissue antigen by an antibody. The technique was developed by Albert H. Coons et al. [228] who detected various antigens by using antibodies labeled with fluorescent dyes. The antigen to be detected may be intra-cellular or membranous, and if intra-cellular it may be either nuclear or cytoplasmic. The specificity of the antigen-antibody reaction makes it possible to identify tissue constituents with a high degree of certainty. Immunohistochemical techniques are being continuously improved with the development of more sensitive reagents and detection systems, and it is now possible to visualise immunocytochemical reactions at the ultrastructural level. By labeling antibodies with the relevant elements, immunoreactions can now be detected by auto-radiography and scanning electron microscopy, as well as the conventional light microscopy. A schematic representation of the principles of immunohistochemistry is shown in Figure 15.

Figure 15

Schematic representation of the principles of indirect immunohistochemical identification of cellular antigens using the streptavidin/biotin-HRP method.
Antibodies are usually gamma-globulins and are produced in the serum of animals (mice, rabbits, guinea pigs etc.) inoculated with antigen. To ensure the specificity of the antibodies raised, manufacturers use antigens that are as "pure" as possible. Despite this precautionary step, the antibodies produced are not directed specifically and solely to the injected antigens, and recognise various parts of the antigenic molecule and/or the carrier protein. The serum of the donor animal also contains several natural antibodies which may react with test tissue components. An immunoreaction that appears positive cannot, therefore, be assumed to be as result of the desired specific antigen-antibody reaction unless rigorous controls are maintained. This is done by including known positive and negative controls in each staining run.

In general "pure" or monoclonal antibodies are produced by the inoculation of mice with splenic lymphocytes (source of the antibody) which have been fused with mouse myeloma cells in culture. One cell produces only one antibody, and the cultured hybrid cells, with their ability to continually grow and divide in culture, are gradually cloned into cell-lines which produce one antibody only. Polyclonal antibodies are less specific, and are raised in a variety of animals e.g. rabbit and goat. However, although they react with more than one antigen, they do so with varying degrees of specificity and this characteristic allows for their use in immunohistochemical tests.

9.1.2 Requirements for Immunochemistry
In order to detect the desired antigen with immuno-histochemical techniques, there are some essential conditions that must be met. Briefly these are:

1. The antigen must be well preserved and must be available (exposed) for recognition by the relevant antibody
2. The detection system used must be able to achieve specific recognition of the antigen by antibody with minimal interference from other cellular proteins.
3. The antibody must be well characterised i.e. it must have a high affinity for the corresponding antigen. It should also bind the antigen avidly enough to avoid being washed off the section during the staining process.
4. The end product of the antigen-antibody reaction must be labeled in a manner as to be easily visible to the final detection system (light microscopy, radio-assay etc.). This preferably should also allow some degree of qualitative and quantitative comparison between sections.

9.1.3 Immunohistochemical detection systems
There are four main immuno-histochemical detection systems available. These are: the alkaline phosphatase-anti alkaline phosphatase system, the vectastain system, the
avidin-biotin system and the streptavidin-biotinylated/horse raddish peroxidase (HRP) system. The Streptavidin-Biotinylated/HRP system was recommended for use in this project to ensure uniformity with other workers in local research/pathology laboratories. After a few supervised sessions I was able to carry out my experiments independently.

9.1.3.1 Streptavidin-Biotinylated/HRP System
This system relies on the same principle as the avidin-biotin system which utilises the specificity of the high affinity of avidin (a glycoprotein derived from egg-white) for biotin, a member of the vitamin B complex [229]. Streptavidin is a protein that is chromatographically purified from *Streptomyces avidinii*. It shares similar properties with avidin including the ability to bind to biotin. In these reactions, several molecules of biotin bind to one avidin/streptavidin molecule, thereby amplifying the signal several times. In the course of several years, many basic avidin/streptavidin-biotin techniques have evolved. The most recent, and currently the most widely used technique, is the “ABC” (or “preformed complex”) [230]. With this system a preformed complex between avidin/streptavidin and a biotinylated enzyme is applied following incubation of the tissue with a biotinylated primary or secondary antibody.
9.2 Development of protocol for immuno-staining frozen prostatic sections with mouse and rabbit antibodies using the Streptavidin-biotin/HRP system.

9.2.1 Introduction
The antibodies to be tested for use in the detection of the relevant antigens in this study were of both mouse and rabbit origin. The experiments done to gain expertise in immuno-histochemistry were therefore designed to ensure familiarity with the peculiarities of staining with each type of antibody. Frozen prostatic sections were used at this stage of the project as immuno-histochemical reactions occur much more readily on fresh tissue (and without pretreatment). Immuno-positivity of test sections was only accepted as specific when immuno-staining was absent in the negative control serial sections included in the staining runs.

The protocol for immuno-staining of our frozen prostatic sections using the Streptavidin-biotinylated/HRP system was developed by titration of reagents at relevant steps of the manufacturer’s data sheet (Appendix 5) to ensure that optimal results were obtained. The pre-determined antibody dilutions (CK-pan = 1/100, PAP = 1/250) used in our laboratory were used during tests to determine optimal combination of the other variable factors of the Streptavidin-biotinylated/HRP system described below. Finally, the optimal antibody concentration for use with the standardised protocol was confirmed by titration.

9.3.2 Materials
- Tissue sections: Frozen prostatic (BPH) sections.
- Antibodies:
  1) Mouse anti-human Cytokeratin Pan
  2) Rabbit anti-human Prostatic Acid Phosphatase
- Negative Controls:
  1) BPH sections incubated with anti-rabbit Ig.
  2) Prostatic sections incubated with anti-mouse Ig.

9.2.3 Methods
9.2.3.1 Preparation of tissue sections (Appendix 5, Step 3)
Tissue sections were prepared for immuno-staining by thawing and rehydration in TBS. Following this intrinsic peroxidase activity was extinguished.
9.2.3.2 Extinguishing Intrinsic Peroxidase Activity (Appendix 5, Step 3)

a) Introduction - Human tissues possess intrinsic peroxidase activity. This results in a high background staining when using a peroxidase based immuno-reaction system leading to signal interference. This activity is quenched in frozen sections by immersion in a solution of hydrogen peroxide in methanol.

b) Method - Immersion of frozen prostatic sections in 0.6% hydrogen peroxidase in methanol for 5, 10, 20, 30 and 40 minutes. The staining process was completed as per the DAKO protocol.

c) Result - Background staining due to intrinsic peroxidase activity was reduced to a minimum when sections were immersed for 30 minutes or longer. The prescribed immersion of frozen sections in 0.6% hydrogen peroxidase in methanol for 5 minutes (DAKO protocol) was insufficient to extinguish peroxidase activity in our frozen prostatic sections probably because of the high amount peroxidase activity in prostatic tissues.

d) Conclusion - Frozen prostatic sections were to be immersed in 0.6% hydrogen peroxidase in methanol for 30 minutes to extinguish intrinsic peroxidase activity.

9.2.3.3 Elimination of Non-specific Background Staining (Appendix 5, Step 10)

a) Introduction - Proteins in the animal serum in which antibodies are raised can bind non-specifically to tissue epitopes during immuno-staining thereby masking the specific antigen-antibody reaction sought. To block such reactions, sections are incubated with normal serum for varying periods. Dilution of the antibodies in normal serum instead of a buffered solution also contributes to the reduction of non-specific interactions.

b) Method - Following the preparation and the extinguishing of peroxidase activity as above, the prostatic sections were incubated with normal (goat) serum for 5, 10 and 20 minutes. Comparison was also made between sections stained with antibodies diluted in buffered solution and those diluted in normal serum. The staining process was completed as per the DAKO protocol.

c) Result - Optimal results were seen when sections were incubated for periods longer than 10 minutes. Furthermore, staining of non-epithelial elements was further reduced in sections stained with primary antibodies diluted in normal serum.

d) Conclusion - Non-specific binding of epitopes in our sections was blocked by incubation with goat serum for at least 10 minutes, and by dilution of antibodies in normal serum.
9.2.3.4 Determination of Incubation Time for Optimal Antigen-Antibody Reactions
(Appendix 5, Step 13)

a) **Introduction** - The reaction of the antigen with the primary antibody is the most important step in immuno-histochemical experiments. Accurate determination of the time required for this reaction to occur is therefore critical in any project utilising this technique as its investigative tool.

b) **Method** - The earlier steps in the staining process were carried out as described above. The optimal incubation period time for antibody-antigen reaction was then determined for each of the antibodies by comparing the intensity of the substrate staining for 15 minutes, 30 minutes, 1hr, 2hrs and 3hrs at room temperature (RT), and overnight at 4°C. The staining process was completed as per the DAKO protocol.

c) **Result** - Poor staining results were obtained when sections were incubated with the primary antibody for 30 minutes or less at RT. Stronger reactions were seen with incubation for 1-2 hr, though somewhat better immuno-reactions were seen when sections were incubated overnight at 4°C. Staining for longer than 2hrs resulted in increased non-specific staining of tissue elements.

d) **Conclusion** - Frozen sections to be incubated with primary antibodies for 1-2 hours at RT or overnight at 4°C.

9.2.3.5 Determination of Optimal Antibody Dilutions

a) **Introduction** - Incubation of sections with an adequate concentration of antibody is necessary to enable reaction with an antigen. In addition, optimization of the antibody dilution ensures not only the sensitivity of the interaction, but also its specificity, by minimising interference from non-specific staining of tissue elements due to excess antibody in the solution.

b) **Method** - Optimal dilutions for each antibody were determined by titration using dilutions currently used in our laboratory as a guide. The anti-Cytokeratin Pan (anti-Ck Pan) dilutions used were: 1/50µl, 1/100µl and 1/150µl; whilst anti-PAP dilutions were: 1/300µl, 1/400µl and 1/500µl. The staining process was completed as per the DAKO protocol.

c) **Result** - The sharpest contrast of positive epithelial versus negative stromal staining with anti-Ck Pan was seen at 1/100µl dilution, whilst that of anti-PAP was seen at 1/400µl. Some stromal positivity was observed in sections stained with anti-PAP.

d) **Conclusion** - Optimal anti-Ck Pan and anti-PAP dilutions for frozen prostatic sections using the Streptavidin-biotinylated/HRP system were 1/100µl and 1/400µl respectively.
9.2.3.6 Completion of the Staining Process (Appendix 5, Steps 14 - 28)
The staining process was completed as prescribed in the DAKO protocol. The sections were incubated in succession with biotinylated secondary antibody (goat anti-mouse or anti-rabbit immunoglobulin as appropriate), then the streptABComplex/HRP solution. Finally a freshly prepared chromogen mix of buffered 3',3' diaminobenzidine tetrahydrochloride (DAB substrate) solution was added. The sections were counter-stained with Mayer's Haematoxylin, cleared and mounted with glycerol gelatin aqueous medium [Figure 15].

9.2.4 Discussion
After 17 independent staining runs, reproducible and satisfactorily positive results were obtained. Histologically confirmed (by EHM) prostatic epithelium within our sections stained consistently strongly for both Ck Pan and PAP. Stromal elements showed only minimal immuno-positivity for either antibody.

Cytokeratin Pan is expressed by epithelial cells in general, whilst Prostatic Acid Phosphatase is a prostatic epithelial cell marker. By comparing the results of these tests with histological evaluation of the tissues, the specificity of the antibodies was ascertained. In addition to confirming the successful completion of staining runs, immuno-positivity for these antigens identified which cells within our sections were of prostatic epithelial phenotype. The ability to obtain reproducible results when staining for these antigens confirmed my familiarity with immuno-histochemical techniques.

9.2.5 Conclusion
Frozen sections stained with either mouse anti-human CK Pan or rabbit anti-human PAP with the Streptavidin-biotinylated/HRP system were suitable for use as positive controls during the development of the protocol for staining of archival sections.
9.2.6 Confirmation of development of staining frozen tissue sections using the Streptavidin-biotinylated/HRP system

Figure 16

(A) Positive CK Pan staining in frozen section of a BPH gland (x 250)
(B) Negative control (section stained with anti-mouse IgG)
(C) Positive PAP staining in frozen section of a BPH gland (x 250)
(D) Negative control (section stained with anti-rabbit IgG)
9.3 Development of protocol for immuno-staining paraffin prostatic sections with mouse and rabbit antibodies using the Streptavidin-biotin/HRP system.

9.3.1 Introduction

The protocol for immuno-staining archival prostatic sections was developed in a manner similar to that of frozen sections. Briefly, titration tests were done at relevant points in the protocol recommended by the manufacturer (Appendix 5). The system was checked during each staining run by the inclusion of positive and negative controls. Immuno-positivity of test sections was only accepted as specific when specific staining was seen in positive controls included in the staining runs, whilst the negative controls were negative.

9.3.2 Materials

Tissue sections - 1) Test archival sections from BPH and CaP glands.
2) Control frozen BPH sections

Antibodies - 1) Mouse anti-human Cytokeratin - Pan (frozen sections)
2) Mouse anti-human PSA (frozen and archival sections)
3) Rabbit anti-human PAP (frozen and archival sections).

Controls- 1) Positive - Frozen prostatic sections of BPH stained with mouse anti-human Ck Pan or rabbit anti-human PAP.
2) Negative - Paraffin and frozen prostatic sections incubated with anti-rabbit or anti-mouse Ig.

Guide protocol - Manufacturer’s instructions

The monoclonal anti-Ck Pan used in the earlier tests was not effective on paraffin sections (per manufacturer’s data sheet). It was therefore replaced with a monoclonal anti-PSA known to react with archival material as the test mouse antibody. PSA is a more specific marker of prostatic epithelium than PAP. Having confirmed the specificity of the anti-PAP in recognising prostatic epithelium in our sections, the substitution of anti-Ck Pan with the anti-PSA antibody at this stage was necessary as this antigen was to be the marker for prostatic epithelial cells in future tests. Sections were immuno-stained with the rabbit anti-PAP both as a test rabbit antibody, and also as a positive control for the specificity and sensitivity of the monoclonal anti-PSA antibody.

None of these antibodies required pre-treatment prior to immuno-staining. Antibody dilutions suggested by the manufacturers for use on archival material were used in the initial titration runs. Optimal dilutions were later determined.
9.3.3 Methods

9.3.3.1 Immuno-staining of frozen tissue control sections
Frozen tissue control sections were stained as per the protocol described above in Section 9.2.3.1.

9.3.3.2 Preparation of archival tissue sections
The archival sections were prepared for immuno-staining by de-waxing and rehydration.

De-waxing and rehydration of Paraffin Sections (Appendix 5, Steps 1 - 4)
a) Introduction - All archival sections require the removal of paraffin wax and rehydration as a preliminary step prior to immuno-histochemical testing.
b) Method - Sections were placed in a 60°C oven for 5 minutes and then immersed sequentially in containers of xylene, 99%, 95% and 70% alcohol (two minutes in each). Following this the sections were rinsed first in tap water, then in distilled water, and equilibrated in TBS for 5 minutes.
c) Conclusion - Sections were ready for next steps in immuno-staining. If further steps for antigen-exposure were required, they were done at this point, otherwise the experiment proceeded to the intrinsic peroxidase activity quenching step (see below).

9.3.3.3 Extinguishing Intrinsic Peroxidase Activity (Appendix 5, Step 6)
a) Method - Immersion of archival prostatic sections in 3% and 6% hydrogen peroxide solution (in distilled water) for 5, 10, 20, 30 and 40 minutes. The staining process was completed as per the DAKO protocol.
b) Result - Immersion of sections in 3% hydrogen peroxide for up to 40 minutes insufficient to quench peroxidase activity. However enzyme activity extinguished after immersion in 6% solution for 20 minutes.
c) Conclusion - Archival prostatic sections were to be immersed in 6% hydrogen peroxidase solution for 20 minutes to extinguish intrinsic peroxidase activity.

9.3.3.4 Elimination of Non-specific Background Staining (Appendix 5, Step 10)
a) Method - Following preparation and quenching of peroxidase activity as above, the sections were incubated with normal (goat) serum for 10, 15, 20 and 30 minutes. All antibodies were diluted in normal serum. The rest of the staining process was completed as per the DAKO protocol.
b) **Result** - Optimal results were seen when sections were incubated for periods longer than 15 minutes.

c) **Conclusion** - Non-specific binding of epitopes was blocked by incubation of our sections with goat serum for at least 15 minutes. All antibodies were also to be diluted in normal serum.

### 9.3.3.5 Determination of Incubation Time for Optimal Antigen-Antibody Reactions

*(Appendix 5, Step 13)*

a) **Method** - The initial steps in the protocol were carried out as described above. The optimal incubation period time for antibody-antigen reaction was determined for each of the antibodies by comparing the intensity of the substrate staining for 30 minutes, 1hr, 2hrs and 3hrs at room temperature (RT), and overnight at 4°C. The rest of the staining process was completed as per the DAKO protocol.

b) **Result** - Weakly positive results were obtained when sections were incubated with the primary antibody for 30 minutes at RT. Stronger reactions were however seen with incubation for 1-2hrs. The best immuno-reactions were again seen when sections were incubated overnight at 4°C. Staining of archival sections for longer than 2hrs at RT resulted in increased non-specific staining of tissue elements.

c) **Conclusion** - Paraffin sections were to be incubated with primary antibodies for 1-2 hours at RT or overnight at 4°C.

### 9.3.3.6 Determination of Optimal Antibody Dilutions

a) **Method** - Initial steps in the immuno-staining process were carried out as described above. The paraffin sections were then incubated with the following antibody dilutions: mouse anti-PSA antibody - 1/5μl, 1/10μl and 1/20μl; and anti-PAP: 1/200μl, 1/250μl and 1/300μl. The rest of the staining process was completed as per the DAKO protocol.

b) **Result** - The sharpest contrast of positive epithelial versus negative stromal staining with mouse anti-PSA antibody was seen at 1/10 dilution, whilst that of anti-PAP was seen at 1/250. Some weak stromal positivity was again observed with anti-PAP.

c) **Conclusion** - Optimal dilutions for immuno-staining archival prostatic sections with the mouse anti-PSA antibody and anti-PAP dilutions were 1/10 and 1/250 respectively.
9.3.3.7 Completion of the Staining Process (Appendix 5, Steps 14 - 28)
As above, the remaining steps of the staining process were continued to the counter-
staining stage as described in section 9.2.3. Following counter-staining with Mayer's
Haematoxylin, archival sections were dehydrated and cleared by successive
immersion in 70%, 95% and 99% alcohol, and Xylene. The sections were mounted
with neutral mounting medium.

9.3.4 Conclusion
The sensitivity of the Streptavidin-biotinylated/HRP system in detecting antigens
with either mouse or rabbit antibodies has been confirmed. Prostatic sections stained
with either mouse anti-human PSA or rabbit anti-human PAP were considered
suitable for use as positive controls during the development of the protocols for other
antibodies to be used during the project.

9.3.5 Confirmation of development of staining archival tissue sections using the
Streptavidin-biotinylated/HRP system.

Figure 17

(A) Positive PSA staining in archival section of a BPH gland (x 250)
(B) Negative control (section stained with anti-mouse IgG)
(C) Positive PAP staining in archival section of a BPH gland (x 250)
(D) Negative control (section stained with anti-rabbit IgG)
9.4 Development of protocol for immuno-staining paraffin tissue sections with mouse anti-EGFR (clone 31G7).

9.4.1 Introduction

The EGFR protein is expressed by a variety of normal human tissues including skin and placenta. The mouse anti-EGFR antibody (clone 31G7, Zymed) used in this study was purchased commercially. This antibody is known to identify a 170kD band from A-431 cells [177], and to react with the peptide backbone of the extracellular domain of the EGFR protein. It competes with EGF for binding with the receptor, and is known to recognise the native form of the EGFR (WT-EGFR) only showing no cross-reactivity with other highly homologous members of the erb-B family of receptors [99]. Unlike several other antibodies which are able to detect this protein on frozen tissue sections only, this antibody is effective on prostatic archival material which has been pre-treated by enzyme (pronase) digestion [217-219].

During the development of this protocol immuno-positivity of test sections was only accepted as specific when positive controls included in the staining runs were positive, and the negative controls negative.

9.4.2 Materials

Tissue sections - 1) Test archival sections of normal skin.
2) Control archival BPH sections

Antibodies - 1) Mouse anti-human EGFR (clone 31G7)
2) Mouse anti-human Prostatic Specific Antigen

Controls - 1) Positive - Paraffin BPH sections stained with mouse anti-human PSA
2) Negative - Paraffin skin sections stained with anti-mouse Ig.

Immunohistochemical detection system - Streptavidin-biotinylated/HRP system (DAKO)

9.4.3 Methods

9.4.3.1 Preparation of archival material

The tissue sections were prepared for immuno-staining by de-waxing, rehydration and quenching of peroxidase activity as previously described.
9.4.3.2 Antigen retrieval by protease treatment of paraffin sections (Appendix 5, Step 5)

a) Introduction - Enzymatic degradation of formalin fixed tissue is another method used to retrieve antigenic epitopes [220, 231]. Pre-treatment of archival sections with protease enzyme XIV was the manufacturer's recommended method to expose antigenic sites in paraffin tissues to this anti-EGFR antibody. The suggested concentration to expose the antigen on archival skin sections using this method was immersion in 0.1% protease for 5 minutes at 37°C, or 20 minutes at RT. Using this as a guide, the protocol to be used in this project was determined by titration.

b) Method - Following routine preparation for staining, test sections were immersed in 0.1% protease for 5, 10 and 15 minutes at 37°C, and 15, 20 and 25 minutes at RT. Additionally, to test the possibility of reducing the period of immersion by increasing the concentration of the enzyme, sections were also immersed in 0.5% protease for 1, 2, and 5 minutes at 37°C, and 5, 10 and 15 minutes at RT. At the end of pretreatment sections were rinsed in distilled water and equilibrated in TBS.

c) Results - Antigenic sites were not exposed in sections incubated with the 0.1% concentration of the enzyme at RT, or at 37°C for 5 minutes. The epitopes were however unmasked adequately in sections immersed for 10 minutes at 37°C. Immersion of sections in 0.1% protease solution for longer than 10 minutes, or in 0.5% solution for any period resulted in over-treatment of the sections. This led to very high background staining due to intense non-specific staining of the tissue elements.

d) Conclusion - EGFR antigen in archival material was optimally unmasked for recognition by anti-EGFR antibody (clone 31G7) after pre-treatment with 0.1% protease for 10 minutes at 37°C.

9.4.3.3 Determination of Incubation Time for Optimal Antigen-Antibody Reactions

a) Method - Following routine preparation (including protease digestion) for staining, test skin sections were incubated with anti-EGFR antibody at 1/100μl dilution (Joy Ware, Virginia; personal communication) for 30 minutes, 1, 2, and 3hrs at room temperature (RT), and overnight at 4°C. The rest of the staining process was completed as per the standardised protocol for the immuno-staining archival material using the Streptavidin-biotinylated/HRP system.

b) Result - Sections incubated for 30 minutes at RT were only weakly immuno-positive whilst stronger reactions were seen in sections incubated for 1-2hrs. As with the other experiments, sections incubated overnight at 4°C had the best immuno-reactions. Significant non-specific staining was observed in sections incubated with antibody for longer than 2hrs at RT.
c) **Conclusion** - Paraffin sections were to be incubated with 31G7 anti-EGFR antibody for 1-2 hours at RT or overnight at 4°C.

### 9.4.3.4 Determination of Optimal Anti-EGFR Antibody Dilutions

**a) Method** - Preliminary steps in the staining process were carried out as previously described. Test sections were then incubated with anti-EGFR antibody dilutions using: 1/25µl, 1/50µl, 1/100µl and 1/120µl dilutions. The rest of the staining process was completed as per the standardised Streptavidin-biotinylated/HRP protocol.

**b) Result** - The best contrast of positive epithelial versus negative stromal staining with anti-EGFR antibody was seen at 1/100µl dilution.

**c) Conclusion** - The dilution of the mouse anti-EGFR antibody to be used for immuno-staining archival sections in this study was 1/100µl.

### 9.4.3.5 Completion of the EGFR Staining Process

As above, the remaining steps of the staining process were completed as previously described above in Section 9.4.3.

### 9.4.4 Conclusion

The protocol for the detection of the EGFR antigen on paraffin sections with the monoclonal anti-EGFR antibody (clone 31G7) using the Streptavidin-biotinylated/HRP system had been developed. Also, archival skin sections were suitable for use as positive controls during anti-EGFR immuno-staining experiments on prostatic sections.
9.4.5 Confirmation of development of protocol for immuno-detection of EGFR in archival tissue sections using the Streptavidin-biotinylated/HRP system

Figure 18

(A) Protease pre-treated archival skin immunostained with 1/100ml of anti-EGFR (clone 31G7).
(B) Negative control (section stained with anti-mouse IgG)
9.5 Development of protocol for immuno-staining paraffin tissue sections with rabbit anti-EGFRvIII.

9.5.1 Introduction
The rabbit anti-EGFRvIII antibody used in this study was developed by Drs Moscatello and Wong at the Kimmel Cancer Institute, Philadelphia USA (Appendix 6a). It had previously been shown to recognise the variant EGFR and not the WT-EGFR [105, 205]. This antibody is effective on archival material following antigen retrieval by microwave cooking of tissue sections in buffer. Drs Moscatello and Wong also kindly provided their immuno-staining protocol (Appendix 6b) as a guide, and this was modified to suit the Streptavidin-biotinylated/HRP detection system protocol I developed in Section 9.3.

Archival prostatic sections stained with rabbit anti-PAP were included as positive controls to check the system. Test results were only accepted as specific when positive controls included in the staining runs were positive, and the negative controls negative.

9.5.2 Materials

*Tissue sections* - Test archival sections of KCI mouse tumour.

*Antibodies* -
1) Rabbit anti-human EGFRvIII
2) Rabbit anti-human Prostatic Acid Phosphatase

*Controls* Positive Paraffin sections of BPH stained with rabbit anti-human PAP

Negative 1) KCI mouse tumour sections incubated with anti-rabbit Ig.
2) KCI mouse tumour sections incubated with goat serum.

*Immunohistochemical detection system* - Streptavidin-biotinylated/HRP system (DAKO)

9.5.3 Methods

9.5.3.1 Preparation of tissue sections
The archival tissue sections were prepared for immuno-staining by de-waxing and rehydration as previously described in Section 9.3.3.2 - 4.

9.5.3.2 Antigen retrieval by microwave cooking of paraffin sections

a) *Introduction* - Formalin fixation of tissues results in the masking of some antigenic epitopes by changing the cell wall configuration. Heat retrieval (by pressure or microwave cooking) is one of the commonest methods used to
overcome this problem [232]. Microwave cooking was the preferred method for this study because of its ease of use, and also because it was the method used in the Kimmel Institute protocol (which prescribed 9 minutes as the optimal cooking time). Furthermore a microwave cooker was already available in our laboratory.

b) Method - Test sections were microwaved for 5, 7, 10, and 15 minutes at high power (800W) in 300ml of citric acid buffer. The container was covered loosely to avoid pressure build up, and the buffer solution was topped up during cooking to prevent the sections from drying out. At the end of the cooking period, the sections were cooled by immersion in distilled water for 5 minutes to prevent over-cooking. The rest of the staining process was completed as per the modified Streptavidin-biotinylated/HRP system protocol using a 1/1000μl concentration of anti-EGFRvIII as suggested in the KCI protocol.

c) Results - Test tumour sections cut onto silane coated slides were used in the first set of experiments. There was a high tissue loss from these sections when buffer solution boiled during microwave cooking. Attempts were made to avoid this problem by increasing the quantity of the buffer solution to 1 litre, thus increasing the cooking time before boiling to approximately 6 minutes (instead of approximately 3 minutes). The total cooking time was unchanged (15 minutes maximum). Though the sections were retained on the slides microwaved for short periods (7 minutes) with this adjustment, the antigenic sites were not exposed. The loss of material due to boiling was however unabated in sections cooked for longer periods.

A change was therefore made to sections cut onto Fisher Scientific Superfrost Plus® slides for the subsequent microwaving runs. These slides have a positive charge on their surface that increases the adherence of tissue sections. Using these slides, sections withstood boiling of the buffer solution for prolonged periods without tissue loss (20 slides in 300mls buffer, boiling at approximately 3 minutes).

Microwaving the test sections for 5 and 7 minutes resulted in incomplete exposure of the antigens. However, strong intensity of the antigen-antibody reaction was seen after cooking for 10 minutes (5 minutes x 2, with addition of 20mls of distilled water after the first period of heating). There was no increase in the intensity of the immuno-reaction seen in sections cooked for longer periods.

d) Conclusion - EGFRvIII antigen in archival material optimally exposed after microwave cooking for 10 minutes.
9.5.3.3 Determination of Incubation Time for Optimal Antigen-Antibody Reactions

a) **Introduction** - The suggested incubation time (Appendix 6b) was 4 hrs at RT. This was considered too long, especially as significant background staining had been observed in sections stained for longer that 3 hours during earlier tests. Furthermore, the anti-EGFRvIII antibody had been improved since the development of the KCI protocol.

b) **Method** - The preceding steps in the staining process were carried out as previously described. The test mouse sections were then incubated with anti-EGFRvIII antibody at 1/1000μl dilution (KCI protocol) for 30 minutes, 1, 2, 3 and 4hrs at room temperature (RT), and overnight at 4°C. The rest of the staining process was completed as per the modified Streptavidin-biotinylated/HRP system protocol.

c) **Result** - Sections incubated for 30 minutes at RT stained poorly for EGFRvIII but stronger reactions were seen with incubation for 1-2hrs. As with previous tests, the best immuno-reactions were seen when sections were incubated overnight at 4°C. As mentioned above, staining of archival sections for longer than 3hrs at RT resulted in increased non-specific staining of tissue elements.

d) **Conclusion** - Paraffin sections to be incubated with primary antibodies for 1-2 hours at RT or overnight at 4°C.

9.5.3.4 Determination of Optimal Anti-EGFRvIII Antibody Dilutions

a) **Method** - Preliminary steps in the staining process were carried out as previously described. Test sections were then incubated with 1/1000μl - 2000μl anti-EGFRvIII antibody dilutions in increments of 200μl. The rest of the staining process was completed as per the modified Streptavidin-biotinylated/HRP system protocol.

b) **Result** - The sharpest contrast of positive epithelial versus negative stromal staining with anti-EGFRvIII antibody was seen at 1/1200μl dilution.

c) **Conclusion** - Rabbit anti-EGFRvIII to be used for immuno-staining archival sections in this study at 1/1200μl.

9.5.3.5 Completion of the EGFRvIII Staining Process

The staining process was completed as previously described in Section 9.4.3. The stained sections were then dehydrated cleared and mounted.
9.5.4 Conclusion
The protocol for the detection of the EGFRvIII antigen with the rabbit anti-EGFRvIII antibody using the Streptavidin-biotinylated/HRP system was confirmed. Archival sections of the EGFRvIII mouse tumour were therefore considered suitable for use as positive controls during immuno-staining experiments on prostatic sections.

9.5.5 Confirmation of development of protocol for immuno-detection of EGFRvIII in archival tissue sections using the Streptavidin-biotinylated/HRP system

Figure 19

(A) Microwave pre-treated archival mouse tumour immunostained with 1/1200 ml of anti-EGFRvIII.
(B) Negative control (section stained with anti-rabbit IgG)
9.6 Development of protocol for immuno-staining paraffin tissue sections with mouse anti-Ki67-MM1.

9.6.1 Introduction
The Ki67 antigen is found in granular components of the nucleolus of cells in the late G1, S, G2 and M phases of the cell cycle. The Ki67 antibody therefore identifies cells in the proliferative phase of the cell cycle [233]. A commercially available (Novacastra, UK) mouse anti-Ki67 antibody was used in this study. Similar to the anti-EGFRvIII, this antibody was effective on paraffin sections only after antigen exposure by microwave cooking. Staining due to abnormal antigen-antibody reactions was excluded by omitting the microwave step in a tissue section stained with the anti-Ki67.

Immuno-positivity of test sections was only accepted as specific when positive controls included in the staining runs were positive, and the negative controls negative.

9.6.2 Materials

**Tissue sections**
- 1) Test archival sections of a tonsil
- 2) Control archival sections of BPH
- 3) Control sections of archival KCI mouse tumour

**Antibodies**
- 1) Monoclonal anti-Ki67-MM1 (Novacastra, UK)
- 2) Mouse anti-human PSA (Europath, UK)
- 3) Rabbit anti-EGFRvIII (KCI, USA)

**Controls**
- Positive 1) Paraffin sections of BPH stained with mouse anti-human PSA
- 2) Archival KCI mouse tumour stained with anti-EGFRvIII
- Negative 1) Paraffin sections of a tonsil incubated with anti-mouse Ig.

**Immunohistochemical detection system** - Streptavidin-biotinylated/HRP system (DAKO)

9.6.3 Methods

9.6.3.1 Preparation of tissue sections
The archival tissue sections were prepared for immuno-staining by de-waxing and rehydration as previously described.

9.6.3.2 Antigen retrieval by microwave cooking of paraffin sections

a) **Method** - Although the Novacastra protocol prescribed pressure cooking for this purpose, microwave cooking was used in this project for reasons already discussed above. In a manner identical to that used when determining the cooking time required to expose the EGFRvIII antigen, test sections cut on Superfrost slides were microwaved for 5, 7, 10, 15, and 20 minutes at high power (800W) in 300ml of citric acid buffer.
b) Results - Antigenic sites were exposed only after a minimum cooking time of 7 minutes. However, maximal exposure of the antigen was seen after cooking for 15 minutes. There was no increase in the intensity of the immuno-reaction seen in sections cooked for longer periods.

c) Conclusion - Ki67 antigen in archival material were optimally exposed after microwaving cooking for 15 minutes.

9.6.3.3 Determination of Incubation Time for Optimal Antigen-Antibody Reactions

a) Method - The suggested incubation time (Novacastra protocol) was 30 minutes at RT. Preliminary steps in the staining process were carried out as previously described. Test sections were then incubated with anti-Ki67 antibody at 1/100μl dilution (Novacastra protocol) for 30 minutes, 1, 2, and 3 hours at room temperature (RT), and overnight at 4°C. The rest of the staining process was completed as per the standardised Streptavidin-biotinylated/HRP protocol.

b) Result - Stronger immuno-staining was observed in sections incubated for 1-2hrs as compared to those incubated for 30 minutes only, but the sections incubated overnight at 4°C gave the best results. Excessive background staining was seen in sections incubated with antibody for longer than 3hrs at RT.

c) Conclusion - Paraffin sections to be incubated with anti-Ki67 antibody for 1-2 hours at RT or overnight at 4°C.

9.6.3.4 Determination of Optimal Anti-Ki67 Antibody Dilutions

a) Method - The suggested range of dilutions was 1/100-200μl (Novacastra data sheet). Preliminary steps in the staining process were carried out as previously described. The sections were then incubated with anti-Ki67 antibody at: 1/80μl, 1/100μl, 1/150μl and 1/200μl dilutions. The rest of the staining process was completed as per the standardised Streptavidin-biotinylated/HRP protocol.

b) Result - The best contrast of positive epithelial versus negative stromal staining with anti-Ki67 antibody was seen at 1/150μl dilution.

c) Conclusion - The dilution of the mouse anti-Ki67 antibody to be used for immuno-staining archival sections in this study was confirmed at 1/150μl.

9.6.3.5 Completion of the Anti-Ki67 Staining Process (Appendix 5, Steps 14 - 28)

As above, the remaining steps of the staining process were completed as previously described in Section 9.4.3.
9.6.4 Conclusion
The protocol for the detection of the Ki67 antigen on paraffin sections with the monoclonal anti-Ki67 antibody using the Streptavidin-biotinylated/HRP system has been developed. Also archival sections of tonsils were confirmed as suitable positive controls during immuno-staining experiments for the antigen on prostatic sections.

9.6.5 Confirmation of development of protocol for immuno-detection of Ki-67 antigen in archival tissue sections using the Streptavidin-biotinylated/HRP system

Figure 20

(A) Microwave pre-treated sections of archival sections of lymph nodes immunostained with 1/150ml of anti-Ki67.

(B) Negative control (section stained with anti-mouse IgG)
9.7 Summary of protocols for immuno-staining archival prostatic sections with selected antibodies

Protocols for immuno-staining of human prostatic tissues with the antibodies to be used during this project had been standardised (Appendix 7i-iv). The antibodies were:

1. Anti-human PSA (a prostatic epithelial cell marker).
2. Anti-EGFR clone 31G7 (to identify prostatic cells expressing the wild-type EGF receptor).
3. Anti-EGFRvIII (to identify prostatic cells expressing the candidate variant EGFR).
9.8 Routine methods of analyses.

9.8.1 Evaluation of immuno-staining

The criteria to be assessed and the scoring system to be used were agreed with the Pathologist who had agreed to be an independent assessor (Dr EH MacKay) prior to embarking on the study. The criteria were: 1) the intensity of staining, and; 2) the number of cells positive for the antigens. Immuno-positivity was defined as staining that was present in test sections but absent in negative controls. Areas of inflammation, infarction and tissue autolysis were not assessed. Immuno-reactions to the anti-cytoplasmic/membranous antigens and the anti-nuclear antigens were evaluated separately. Accordingly, the immuno-staining pattern was assessed independently, and without prior knowledge of histological grading, by both myself and Dr Mackay.


Immuno-reactivity to anti-EGFRvIII, anti-PSA, and anti-EGFR antibodies was scored using a modification of the H-scoring system [218]. Briefly, the intensity of the reaction in homogeneous histological areas of the sections was scored on a previously agreed scale of 0 - 3 (0 = absent, 1 = weak, 2 = moderate, and 3 = strong), and multiplied by the percentage of the epithelial cells that had a positive immuno-reaction. The product of that calculation was expressed as a percentage of 300 (the highest mark possible). The final immuno-staining scores were graded as follows; Grade 0 = negative (0%), Grade 1 = weak (1-33%), Grade 2 = moderate (34-67%), Grade 3 = strong (>67%). Following my evaluation, the sections were reviewed and scored by EHM for confirmation.

9.8.1.1 Evaluation of anti-nuclear antigen antibodies

Immuno-positivity to the antinuclear antibody (anti-Ki67) was defined as percentage number of nuclei positive in 400 cells in epithelial and stromal cells at x 400 magnification [75].

9.8.2 Statistical Methods.

The final scores were reported as proportions and means (±SD) with 95% Confidence Intervals. Statistical comparison of the mean scores in individual histological groups was done using the paired and unpaired two-sample (adjusting for unequal standard deviations) t-tests as appropriate. Spearman's correlation coefficient was used to examine the univariate associations between EGFRvIII expression and the clinical indices. Multivariate analysis of the expression of EGFRvIII and the prognostic parameters was done using Cox's proportional hazards ratio. The impact of EGFRvIII and Ki-67 expression on survival was studied using
the Kaplan-Meir method. All tests were two-sided where appropriate and were performed at the two-sided 0.05 level of significance.
On completion of the analyses, independent review of the results was obtained from a Lecturer in Statistics at the University of Leicester (Dr Nick Taub).
PART 3 - RESULTS AND DISCUSSION

Chapter 10 - Investigation of the effect of storage on the immuno-reactivity of archival prostatic sections
Chapter 11 - Confirmation of prostatic epithelial phenotype in BPH, CaP and Metastatic sections to be used in this study.
Chapter 12 - Confirmation of anti-WT-EGFR and anti-EGFRvIII antibody specificity by western blotting
Chapter 13 - WT-EGFR Immunostaining in BPH, CaP and Metastatic sections.
Chapter 14 - EGFRvIII Immunostaining in BPH, CaP and Metastatic sections.
Chapter 15 - Ki67 Immunostaining in BPH, CaP and Metastatic sections.
Chapter 16 - Discussion
Chapter 10 - Investigation of the effect of storage on the immuno-reactivity of archival prostatic sections

10.1 Introduction

Ordinarily, paraffin sections used in immunohistochemistry are stained shortly after preparation. Recently there have been concerns that the storage of unstained paraffin slides results in alterations in immuno-reactivity with antibodies used to detect various antigens in these sections. A few studies have investigated these concerns and these have reported a wide range of changes in immuno-reactivity with increasing age of archival sections [234, 235]. In addition, the chemicals used in tissue-preservation (i.e. formalin and paraffin wax) may alter the factors that determine antigen-antibody reactions in these sections. Whilst some studies have evaluated the effect of formalin-fixation on immuno-histochemical results [236], there are no reports on the effect of the different tissue-preservation protocols used in different centres on the immuno-staining patterns observed in sections used in multi-centre studies.

As part of the initial investigations in this project, we have examined the effect of storage on unstained sections of archival prostatic tissues on immuno-reactivity with a panel of antibodies. As well as providing more information on the characteristics of antigen-antibody reactions, this preliminary study was necessary to ensure standardisation of the immuno-detection results obtained during subsequent tests.

10.2 General Materials and Methods

10.2.1 Antibodies

A panel of 8 antibodies was used in this study. In addition to the 6 antibodies previously used in this study 2 more antibodies were tested in these series of experiments. The anti-cellular

Table 2
Panel of Antibodies Tested

<table>
<thead>
<tr>
<th>No</th>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Type</th>
<th>Positive Control</th>
<th>Test Tissue</th>
<th>Pre-treatment</th>
<th>Cellular Compartment of Immuno-reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anti-EGFRvIII*</td>
<td>DKM/AJW</td>
<td>Polyclonal</td>
<td>KCI Mouse tumour</td>
<td>CaP</td>
<td>Microwave</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Anti-MIB 1</td>
<td>Dianova</td>
<td>Monoclonal</td>
<td>Tonsil</td>
<td>CaP</td>
<td>Microwave</td>
<td>Nuclear</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Anti-Ki67</td>
<td>NCL-Ki67-MMi</td>
<td>Monoclonal</td>
<td>Tonsil</td>
<td>CaP</td>
<td>Microwave</td>
<td>Nuclear</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Anti-PSA</td>
<td>NCL-PSA</td>
<td>Monoclonal</td>
<td>BPH</td>
<td>BPH</td>
<td>Microwave</td>
<td>Membranous</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Anti-PSA</td>
<td>Europath</td>
<td>Monoclonal</td>
<td>BPH</td>
<td>BPH</td>
<td>None</td>
<td>Membranous</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Anti-PAP</td>
<td>Sigma</td>
<td>Monoclonal</td>
<td>BPH</td>
<td>BPH</td>
<td>None</td>
<td>Membranous</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Anti-hAR</td>
<td>NCL-AR-2F12</td>
<td>Monoclonal</td>
<td>BPH</td>
<td>CaP</td>
<td>Microwave</td>
<td>Nuclear</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Anti-EGFR</td>
<td>31G7</td>
<td>Monoclonal</td>
<td>BPH</td>
<td>BPH</td>
<td>Protease</td>
<td>Membranous</td>
<td></td>
</tr>
</tbody>
</table>

Key
* A Type III mutant EGFR (ref)
CaP = Carcinoma of the Prostate
BPH = Benign Prostatic Hyperplasia
proliferation marker (anti-MIB 1 antibody) was included as control reagent for the anti-Ki67 antibody [237], whilst the anti-androgen receptor (anti-hAR) antibody was included to evaluate the effect of storage on the immunoreactivity of this steroid hormone receptor. The antibodies (and the dilutions used), the location of the immuno-reactions and the pre-staining treatments required are listed in Table 2.

10.2.2 Archival prostatic sections
Sections from archival specimens of transurethral and retropubic prostatectomies for BPH and CaP and bone metastatic CaP deposits were obtained from the LGH Pathology Department as previously described in Section 8.7.2.2. Four-um serial sections were prepared from selected blocks, and stored in slide racks at room temperature until stained.

10.2.3 Immunohistochemistry
Antigen-antibody binding were detected by streptavidin-biotin/HRP reactivity using the DAKO (Cambs, UK) immunodetection kit as described in Sections 9.3 - 9.6. The immuno-positivity of the sections was assessed using the criteria described in Section 9.8. [75, 218].

10.2.4 Selection of test tissues
Initial immuno-staining studies were done on freshly prepared prostatic sections (within 72hrs of preparation) to identify specimens that had the highest intensity of staining with each antibody. High PSA, PAP and EGFR expression was seen in BPH sections, whilst hAR, Ki67 and EGFRvIII immuno-reactions were strongest in sections from CaP glands. Ten cases of BPH and CaP each were randomly selected from the positive specimens as test tissues for the subsequent studies.

10.3 Specific Methods and Results
10.3.1. Investigation of age-related decrease in immuno-reactivity
Serial sections from the selected BPH and CaP blocks and the 6 bone metastasis were then stained with the panel of relevant antibodies on days 0-3, 10, 20, 30, 90, and 1 year of storage. During these tests unstained slides were stored in covered boxes. Freshly prepared sections from the same tissue blocks were included in each staining run for comparison.

Figure 21
Serial sections of archival CaP glands stained with anti-EGFvIII 1/1200ml (x250)
Very strong immuno-positivity is seen in the section stained with this antibody on day 2 (A), and this intensity is virtually unchanged in the section stained after 1 year of storage at room temperature (B).
Results

The strength of immuno-reactivity seen in sections incubated with 4 antibodies (anti-EGFRvIII, anti-PAP, anti-MIB1 and Novacastra (NV) anti-PSA) was virtually unchanged in serial sections stained at intervals of storage of up to a year. However, the strength of staining with the remaining 4 antibodies decreased progressively with the age of the sections (anti-Ki67, anti-hAR, anti-EGFR, Europath (EP) anti-PSA) [Figure 21].

The gradual loss of immuno-reactivity was most noticeable in anti-hAR staining as immuno-reactivity to this antibody reduced sharply in sections stained after 10 days storage at room temperature, and was completely lost after 3 weeks [Figure 22]. On the other hand, anti-EGFR and EP anti-PSA, and anti-Ki67 immuno-reactivity was stable for 20 days and 3 months respectively, but decreased thereafter. Both anti-EGFR and anti-Ki67 staining were lost after a year, but weak EP anti-PSA staining was seen in sections of similar age. However, immuno-staining to the anti-EGFRvIII decreased only slightly after a year of storage.

Figure 22

A) Strong immuno-positivity in section stained on day 3 (no counterstaining)
B) Complete loss of immuno-staining in section stained on day 20 (no counterstaining)
C) Negative control (no counterstaining)

Similar alterations in the patterns of immuno-staining was seen in stored archival primary prostatic and metastatic CaP sections using the panel of antibodies tested [Table 3].
Table 3
Effect of Age on Immunoreactivity of LGH Primary and Metastatic Prostatic Tissue Sections

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Test Tissue</th>
<th>Grade of Immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0-3</td>
<td>Day 10</td>
</tr>
<tr>
<td>NV anti-PSA</td>
<td>BPH</td>
<td>4</td>
</tr>
<tr>
<td>Anti-PAP</td>
<td>BPH</td>
<td>4</td>
</tr>
<tr>
<td>Anti-MIB-1</td>
<td>CaP</td>
<td>4</td>
</tr>
<tr>
<td>Anti-EGFRvIII</td>
<td>CaP</td>
<td>4</td>
</tr>
<tr>
<td>EP anti-PSA</td>
<td>BPH</td>
<td>4</td>
</tr>
<tr>
<td>Anti-Ki67</td>
<td>CaP</td>
<td>4</td>
</tr>
<tr>
<td>Anti-EGFR</td>
<td>BPH</td>
<td>4</td>
</tr>
<tr>
<td>Anti-hAR</td>
<td>CaP</td>
<td>4</td>
</tr>
</tbody>
</table>

Key: BPH = Benign Prostatic Hyperplasia
     CaP = Carcinoma of the Prostate

Table 3 Legend
Table showing variable effect of storage on immunoreactivity of panel of antibodies on archival LGH primary prostate tissue sections. The sections were incubated with x1 antibody concentrations i.e.: NV anti-PSA = 1/100ul; Anti-MIB 1 = 1/20ul; Anti-PAP = 1/250ul; Anti-EGFRvIII = 1/1200ul; EP Anti-PSA = 1/10ul; Anti-Ki67 = 1/150ul; Anti-EGFR = 1/100ul; Anti-hAR = 1/25ul

10.3.2. Investigation of age-induced antigen degeneration as a cause of reduction in immuno-reactivity

We postulated that the storage-related reduction in immuno-reactivity to the 4 antibodies recorded above (NV anti-Ki67, anti-hAR, anti-EGFR, EP anti-PSA) was as a result of age-determined decay, with the eventual loss of expression of the proteins. To test this hypothesis, serial sections of the relevant test tissues stored for the same periods as before (i.e. days 0-3, 10, 20, 30, 90, and 1 year) were incubated with half-dilutions (i.e. x 2 concentration) of the relevant antibodies. In addition, staining runs were done at intermediate time intervals (i.e. on days 15, 25, 60 and 180) to increase the sensitivity of the results.

Results
Immunostaining freshly prepared slides with double concentrations of the antibodies led to non-specific staining of the tissue elements. However, stronger immuno-reactivity was seen in stored slides stained within the time intervals that a reduction in immuno-reactivity had been seen with standard antibody dilutions (i.e. 10-15 days for anti-hAR; 90-180 days for anti-EGFR and anti-Ki67; and 1 year for EP anti-PSA) [Figure 23]. Interestingly, immuno-reactivity was not recovered in sections stained after time intervals when complete loss of immuno-reactivity had been observed earlier (20 days for anti-hAR, and 1 year for anti-EGFR and anti-Ki67).

73
Serial sections of archival BPH glands stained with anti-EGFR (x250)
Strong immuno-positivity is seen in a section stained with 1/100ml anti-EGFR on day 2 (A). But after 90 days of storage, only moderate immuno-reactivity is achieved in a section incubated with double the concentration of the antibody (1/50ml) (B). No immuno-reactivity is seen with the anti-EGFR after 180 days of storage at room temperature.

10.4 Discussion
Factors which affect immuno-reactivity in paraffin sections have significant implications for immunohistochemical studies. These are of particular importance in both research and confirmatory diagnostic tests as slides used for these purposes may have been in storage for a while, and are sometimes obtained from several centres. As a consequence preliminary investigations are necessary in these studies to evaluate the effects of age and tissue-preparation on the immuno-reactivity of various antibodies to be used in archival sections.

Time-elapsed variability in immuno-staining patterns may be antigen and/or antibody specific. This assertion is supported by our finding that the rate of decline in immuno-reactivity differed with each antigen and antibody tested. Our finding that the reduction in immuno-staining in aging sections may be reversed, prior to complete loss of expression, by increasing the concentration of antibody used provides further evidence that the decrease in immuno-staining in stored slides is due to a progressive decay of the antigens. Furthermore, the rate of the decline in expression, which overall was independent of the cellular location of the immuno-reaction, was highest in nuclear androgen receptors as compared to other antigens investigated. These results...
are similar to findings from an earlier study which reported that a loss of anti-oestrogen receptor immuno-staining occurred earliest amongst a panel of antibodies that showed a gradual decrease of immuno-reactivity with stored unstained slides [235]. Despite this similarity, we are unable to explain this higher sensitivity of nuclear steroid receptors to storage-dependent decay.

Interestingly, the strength of immuno-reactivity was maintained in 2/2 (100%) rabbit polyclonal antibodies used, as compared to 2/6 (33%) of the monoclonal reagents. This finding may be due in part to the decreased specificity of polyclonal antibodies which allows binding to a wider range of proteins. In addition, the loss of immuno-staining occurred to a similar degree with antibodies that required pre-treatment (microwave cooking and protease digestion) of archival sections as well as those reagents that did not require pre-treatment of the slides. However, the detection of PSA in year-old sections by an antibody that required antigen-retrieval and not by one that did not suggests that pre-treatment to expose epitopes may facilitate antibody binding to decaying antigens. Accordingly, antibodies (polyclonal or monoclonal) which require pre-treatment, or polyclonal antibodies that do not, may be preferable when staining paraffin sections retrieved from storage. Apart from this, better cell proliferation antigen results were obtained in year-old sections that were stained with MIB1 as compared with anti-Ki67. This is in keeping with previous studies which suggested that the antibodies against the MIB1 epitope are better suited for the detection of Ki67 protein in paraffin sections [237].

10.5 Conclusion
These results suggest that previous conflicting results from immunohistochemical studies on archival tissues could be due in part to differences in the age of stored sections used. Consequently, freshly prepared paraffin sections should be used preferentially when carrying out both diagnostic and research studies. However, immunohistochemical systems should be checked for aging induced antigen-degradation (by incubating with increased antibody concentrations) if the use of sections that have been in long-term storage be unavoidable. We have applied these protocols whilst carrying out subsequent tests on archival material in this study [75, 238, 239].
Chapter 11 - Confirmation of prostatic epithelial phenotype in BPH, CaP and Metastatic sections to be used in this study.

11.1 Introduction
PSA immuno-reactivity is the most commonly used marker to identify epithelial cells of prostatic origin [240]. As such it was the marker of choice in this study. Representative sections from primary prostatic (BPH and CaP) and metastatic cases were stained with anti-PSA antibodies using the protocol described in Appendix 7i. Storage induced antigen-degradation was excluded by staining all archival section within 3 weeks of preparation.

11.2 Evaluation of PSA Immuno-reactivity

11.2.1 Sections from BPH Glands [Figure 24a]
All sections from BPH glands were positive for PSA. PSA staining was strong in 16/19 sections (84%, C.I. 60 to 97%), moderate in 2/19 (11%, C.I. 1 to 33%), and weak in 1/19 (5%, C.I. 0 to 26%). Furthermore, moderate-strong PSA immuno-reactivity was also seen in the BPH glands within CaP sections.

11.2.2 Sections from CaP Glands [Figure 24b]
Anti-PSA reaction was strong in 12/38 sections (32%, C.I. 18 to 46%), moderate in 10/38 (26%, C.I. 13 to 43%), and weak in 13/38 (34%, C.I. 20 to 51%). In contrast to the universal PSA positivity seen in BPH glands, malignant glands in 3/38 CaP sections were negative for the marker (8%, C.I 2 to 20%). Overall, the intensity of PSA staining appeared to decrease with increasing de-differentiation of the epithelium, but there was no significant difference between adjacent histological grades. Of note in this regard was the fact that the 3 PSA-negative sections were from (anaplastic) G3 tumours.

11.2.3 Sections from Metastatic deposits [Figure 24c&d]
PSA immuno-reactivity was seen in metastatic prostatic cells in all metastatic deposits (bone and lymph) screened. The reaction was strong in 1/12(8%, C.I. 0 to 39%), moderate in 7/12 (58%, C.I. 28 to 85%), and weak in 4/12 (33%, C.I. 10 to 65%).
Figure 24: Sections from prostatic tissues incubated with 1/10ml (x250).

BPH glands (A) stained very strongly for PSA in the epithelium, whilst CaP (B) and bone (C) and lymph metastatic (D) tissues stain moderately and weakly with the antibody respectively. (E) Negative control (BPH section) incubated with anti-mouse IgG.

11.3 Discussion
As mentioned earlier, despite its limitations PSA is now the most commonly used marker for prostate cancer. It is also used commonly in the immunohistochemical identification of prostatic epithelial cells, especially within metastatic deposits. The prostatic phenotype of glandular tissue within all of BPH sections, and 35/38 of CaP sections was confirmed by immuno-positivity for this antigen. However, there were limitations to the use of PSA for this purpose, as the PSA-negativity of malignant glands in 3/38 of our CaP section shows. In these sections anti-PAP immuno-reactivity was used to determine that the mitotic cells were of prostatic epithelial origin.
As shown in the "results" sections above, in general malignant prostatic cells stain less intensely for PSA when compared to BPH glands. This observation is in keeping with reports from other studies, as is the finding that some CaP glands do not stain for PSA [240, 241]. Two mechanisms are thought to contribute to these findings, and these are: 1) It is estimated that on a cell-per-cell basis CaP glands secrete less PSA than BPH; and 2) The disorganisation associated with transformation to the invasive phenotype may result in the leakage of the antigen into the circulation. Although the mechanism by which PSA enters the circulation is currently unknown, the loss of basal cells in CaP may explain the higher serum levels and the weaker tissue expression of the marker in CaP.

11.4 Conclusion
Epithelial cells of prostatic origin in all BPH, CaP and metastatic sections to be used during subsequent tests in this project have been identified.
Chapter 12 - Confirmation of anti-WT-EGFR and anti-EGFRvIII antibody specificity by western blotting

12.1 Introduction
A multitude of antibodies is available for the detection of EGFR proteins each of which reacts with a different epitope of the antigens. There was therefore a potential risk of cross-reactivity between the reagents chosen to investigate the native and variant forms of EGFR in this project. To avoid this pitfall the sensitivity and specificity of the anti-WT-EGFR and anti-EGFRvIII antibodies used in this study was confirmed by SDS- PAGE gel electrophoresis as previously described by Laemmli [242].

12.2 Methods
12.2.1 Principles of western blotting
Western blot analysis of tissue samples aims to identify constituent proteins by dissociating them into individual polypeptide sub-units under conditions that minimise aggregation [243]. Most commonly SDS-polyacrylamide gels is used for this analysis. The strongly anionic detergent SDS binds to the denatured polypeptides which become negatively charged. Independent of the peptide sequence, the amount of SDS bound to each polypeptide is almost always equal to its molecular weight. The SDS-polypeptide complexes then migrate through the polyacrylamide gels and are separated according to their sizes. This is done by the sieving action of the gels and is dependent on the size of the “pores” in the gel, which in turn is a function of the absolute concentration of acrylamide and bisacrylamide used to cast the gel. By using known molecular weight markers, the size of the separated polypeptide chains in the lysates can then be estimated. SDS-PAGE gel electrophoresis is commonly done with a discontinuous buffer system in which the buffer is of a different pH and ionic strength from that used to cast the gel. The separated polypeptides are then transferred by direct electrophoresis from the gel to a nitrocellulose membrane [244]. Following electroblotting, the membranes are immuno-stained with the relevant antibodies. The respective proteins are then detected by the localisation of the antibody-antigen reaction in the relevant kD region using photoluscent reagents.

12.2.2 Preparation of Tissue Lysates for Western Blotting
Tissue lysates were prepared by homogenising frozen tissue samples from BPH and CaP glands known to express both WT-EGFR and EGFRvIII (by immuno-histochemistry), as well as endothelial cells (negative controls) in homogenising buffer. Histological diagnosis of the prostatic samples was confirmed by routine Haematoxylin and Eosin staining of representative sections of the specimens. The
prostatic chips were then macro-dissected to ensure near histological homogeneity (benign or malignant) of the tissue. The homogenate was centrifuged to remove debris, boiled and dialysed in dialysis buffer for 18h at 4°C.

12.2.3 SDS-PAGE electrophoresis
6% SDS-PAGE gels were poured with 5% stacking gels. The gels were mounted on the gel apparatus (Bio-Rad), which was then filled with Tris/glycine running buffer. Tissue lysates were suspended in reducing-SDS buffer, boiled in a water bath for 5mins and loaded onto the stacking gel using a pipette. High molecular weight standards were used in all gels. The apparatus was run at a constant current, 150mV, until the bromophenol blue dye front had reached the bottom of the gel.

12.2.4 Immunoblotting
The SDS-PAGE gels were taken from the apparatus and loaded into a Biorad Transblot blotting cassette. The cassette was loaded in layers and all components apart from the gel were pre-soaked in transfer buffer. First, one scotchbrite (3M USA) pads, then 1 layer of Whatman no. 1 filter paper, followed by the gel were stacked into the cassette which was covered by nitrocellulose membrane, 1 cm larger than the gel all round. The membrane was covered with filter paper and one more scotchbrite pad. This assembly was then placed in the Transblot cell (Bio-Rad), filled with transfer buffer and run for 1hour at 100 mA with the gel towards the cathode. The nitrocellulose membrane was then removed from the cassette, blocked with 5% BSA for 1h and then incubated with either polyclonal anti-EGFRvIII rabbit antibody, or anti-EGFR mouse monoclonal antibody for 1h. Secondary biotinylated antibodies were applied to the membranes for 1h followed by a photoluscent streptavidin/ABC complex. The blots were exposed to Kodak Biomax film for 30secs and developed using the Curix 60® developer.

12.3. Results
12.3.1 WT-EGFR Western Blotting
Western blot analysis of BPH and CaP lysates using the anti-WT-EGFR antibody detected a single band in the 170 kDa region, corresponding to the native EGFR protein in BPH and CaP [Figure 25a]. The sensitivity of the antibody was confirmed by the strong signal seen in the A431 lane (WT-EGFR positive control) [174], and its specificity by the lack of a signal in both the Jurkat Human T-lymphoma (EGFR-negative control [160]) and the HC2d/202 (EGFRvIII positive control[105]) lanes.

12.3.2. EGFRvIII Western Blotting
Anti-EGFRvIII immunoblotting detected a 148-kDa band in lysates from both BPH
and CaP, which corresponds to the variant EGFR [Figure 25b]. A weak EGFR\textsubscript{vIII} signal was also seen in the 120 kDa region in the CaP lysate. As previously described [105], very strong signal was also seen in the HC2d/202 lane, whilst there was no signal in either the A431 or the Jurkat Human T-lymphoma lanes.

12.4 Discussion

The size of proteins detected by the antibodies used is similar to earlier reports on the two types of EGFR protein. The 170kDa WT-EGFR has been previously reported in lysates of both normal and abnormal human tissues, including prostatic tumours [166, 175].

**Figure 25**: Expression of WT-EGFR and EGFR\textsubscript{vIII} in BPH and CaP analysed by western blotting.

Aliquots of lysates containing 250mg of BPH and CaP, 25mg of HC2 20d/2c tumour (EGFR\textsubscript{vIII} positive control), 50mg of A431 tumour (WT-EGFR positive control), and 50mg of Jurkat human T-cell lymphoma (EGFR negative control) were added to the wells. The lysates were run on 7.5% PAGE at 200mV, and then immunoblotted with antibodies to either the wild-type or variant EGF receptor.

A single band of proteins was recognised in the 170 kDa region in BPH and CaP by anti-WT-EGFR clone 31G7, whilst bands of protein were detected by the anti-EGFR\textsubscript{vIII} antibody in BPH and CaP lysates in the 140 kDa region. A 90kDa band was also seen in the HC2 20d/2c and CaP lanes of the EGFR\textsubscript{vIII} blot confirming the expression of multiple isoforms of the mutated protein by tissues which overexpress the EGFR\textsubscript{vIII} as previously reported (Ekstrand et al., 1992; Moscatello et al., 1996; Moscatello et al., 1995). Specificity of the antibodies used is confirmed by the strong anti-WT-EGFR signal seen at 170 kDa region in A431 lane and anti-EGFR\textsubscript{vIII} signal seen in the HC2 20d/2c lane in the absence of cross-reactions (signals) in these respective lanes in the immunoblots for each antigen. There were no signals in the negative control lanes in either blot.
Although this is the first description of the presence of EGFRvIII protein in prostatic neoplasm, isoforms of EGFRvIII have been detected in lysates from human brain tumours [105, 108, 194]. The different sizes of this aberrant receptor are thought to be due to multiple deletions in both the external and internal domains of WT-EGFR occurring in variable combinations [194]. However multiple isoforms of EGFRvIII are thought to be preferential to cancers which express high levels of the mutant antigen [204]. This assertion may explain the detection of several bands of EGFRvIII protein in the CaP lane.

12.5 Conclusion
These results indicated that both the WT-EGFR and EGFRvIII are expressed in prostatic tumours. In addition, these findings also confirmed the specificity of the antibodies used for the relevant antigens and thus their suitability for use in the subsequent immunohistochemical studies.
Chapter 13 - WT-EGFR Immunostaining in BPH, CaP and Metastatic sections.

13.1 Localisation of WT-EGFR Immuno-reactions
Following the western blotting results paraffin sections from BPH and CaP glands were immuno-stained with anti-EGFR (clone 31G7) to detect WT-EGFR. WT-EGFR expression was seen only in prostatic epithelial cells in our sections, and staining was mainly membranous [Figure 26a-d]. Immuno-reactivity was strongest in the basal/outermost cells of the glands giving the impression of an outer rim to the glandular structures. Due to the heterogeneity of BPH and CaP normal/atrophic glands and glands at varying stages of de-differentiation are adjacent to each other within tumour sections. WT-EGFR reactivity was found to be dependent on the histology of the individual glands within our sections, and similar immuno-reactions were seen in normal/atrophic and BPH glands in both BPH and CaP sections. The highest expression of the native EGFR was seen in normal/atrophic glands and the mean expression decreased as the epithelial cells de-differentiated.

13.1.1. BPH Sections
All normal/atrophic and BPH glands stained either strongly or moderately for WT-EGFR [Figure 26a & b]. Normal/atrophic glands in 17/19 [89%, C.I. 67 to 99%] and 13/19 [68%, C.I. 44 to 87%] of BPH glands expressed the normal receptor strongly, whilst moderate expression was seen in 2/19 [11%, C.I 1 to 26%] and 6/19 [32%, C.I. 13 to 57%] of the same groups of glands respectively.

13.1.2. CaP Sections
In the HGPIN glands within CaP sections, strong WT-EGFR immunoreactivity was seen in 1/16 (6%, C.I. 0 to 30%), whilst 7/16 (44%, C.I. 20 to 70%) and 8/16 (50%, C.I. 25 to 75%) stained moderately and weakly respectively [Figure 26c]. WT-EGFR expression was either weak or undetectable in 31/38 (81%) of malignant glands (26/38 [68%, C.I. 51 to 83%] and 5/38 [13%, C.I 4 to 28%] respectively) [Figure 26d]. Anti-WT-EGFR immuno-reactions were strongly positive in 3/38 (8%, C.I. 2 to 21%), and moderate in 4/38 (11%, C.I. 3 to 25%) of CaP sections. In addition, the mean expression of the normal receptor was similar in hormone resistant and hormone sensitive glands.

13.1.3. Metastatic Deposits
WT-EGFR was not detected in 10/12 metastatic sections (83%, C.I. 52 to 98%), whilst one deposit each (8%, C.I. 0 to 39% respectively) expressed moderate and weak amounts of the antigen [Figure 26e].
Figure 26: WT-EGFR immuno-staining in prostatic tumors (x 250). Sections were counterstained with haematoxylin.

(A) Normal/atrophic glands showing high WT-EGFR expression.
(B) BPH glands showing moderate expression of WT-EGFR.
(C) HG PIN glands showing minimal WT-EGFR staining of transformed cells whilst basal cells of adjacent benign gland showed strong immunoreactivity.
(D) Weak WT-EGFR expression in CaP glands.
(A-D) Membranous WT-EGFR immunoreaction was strongest in basal cells and appeared as an outer rim surrounding the glands.
(E) Lymph node metastasis showing no WT-EGFR staining in cells of prostatic or lymphoid origin.
(F) Mestatic prostatic deposit in bone also showing no WT-EGFR expression in either the invading or native cells of the tissue.
(G) WT-EGFR negative control (BPH section x 100)
13.2 Statistical analyses (Appendix 811c)
Statistical comparison of the mean WT-EGFR expression in the histological groups (normal/atrophic, BPH, PIN, CaP, and metastases) revealed highly significant differences. Normal/atrophic vs BPH \( p = 0.0001 \), PIN vs CaP \( p = 0.006 \), and all others \( p = <0.0001 \). The differences between the scores in adjacent grades of CaP were not significant. Furthermore, WT-EGFR expression was similar in the hormone sensitive and hormone resistant cases of CaP.

13.3 Clinical correlation
There was no significant univariate association between WT-EGFR expression and any of the clinical indices measured in CaP patients in our study (age; \( p = 0.33 \); serum PSA, \( p = 0.2 \); histological grade, \( p = 0.26 \); metastatic disease, \( p = 0.35 \); time to disease progression, \( p = 0.452 \); and death at 3 years, \( p = 0.759 \)). Similarly, multivariate analysis showed no direct relationship between these factors and WT-EGFR. As such, similar to earlier reports [216], WT-EGFR expression had no predictive value in CaP.

13.4 Discussion
As discussed in Chapter 6, since the first report of the presence of EGFR in BPH [166], there have been conflicting reports on the levels of the receptor protein found in prostatic tumours. A possible contributing factor to this controversy is the different methodology used in the different studies, in particular the large number of commercially available anti-EGFR antibodies (each of which may recognise different epitopes of the normal or variant EGFR or indeed both receptors). Further, there may also be other factors involved including: (1) the impact of storage on the loss of tissue antigens in archival material; (2) technical difficulties with EGFR antigen retrieval in archival prostatic tissues when compared to frozen sections. To overcome these obstacles we have used an antibody known to recognise the native receptor only. We also excluded the effects of antigen degradation on immunoreactivity by staining all sections within 3 weeks of preparation [245].

Taking the above into consideration, we have confirmed earlier reports (from studies using this clone of anti-EGFR) of the depletion of WT-EGFR in prostatic tumours [212, 217-219]. Furthermore, we report for the first time the lack of expression of this receptor in most metastatic deposits of prostate cancer. Several hypothesis have previously been proposed as possible explanations for this peculiarity of EGFR expression in prostatic tumours including the expression of an abnormal form of the receptor [213, 223].
13.5 Conclusion
At the end of this phase of this project, we postulated that the decreased expression of WT-EGFR in prostatic neoplasms is due to the expression of a mutated form of the receptor. Furthermore we hypothesised that the EGFRvIII was a suitable candidate for the elusive protein.
Chapter 14 - EGFRvIII Immunostaining in BPH, CaP and Metastatic sections.

14.1 Localisation of EGFRvIII Immuno-reaction
To detect EGFRvIII expression in prostatic tissues serial sections from BPH and CaP glands were stained with the anti-EGFRvIII. EGFRvIII was expressed by abnormal prostatic epithelial cells in our sections only, and the mean expression increased as the tumours became more malignant with poorly differentiated tumours and metastases staining the strongest [Figures 27a-e]. Similar to the observation with WT-EGFR expression, anti-EGFRvIII immuno-reactivity was dependent on the histology of the individual glands within the BPH and CaP sections. Although some membranous EGFRvIII staining was seen, the variant antigen was expressed mainly in the cytoplasm, and for the most part this appeared as a distinct peri-nuclear deposit. However, no nuclear staining was observed. Also, EGFRvIII was expressed mostly at the luminal surface of basal cells in BPH glands, and on the luminal surface of luminal cells in CaP.

14.1.1. BPH Sections
EGFRvIII was not expressed in normal/atrophic glands in BPH sections, but weak to moderate expression was seen in adjacent hyperplastic glands [Figure 27a & b]. The mutant receptor was expressed weakly by BPH glands in 17/19 sections (89%, C.I. 67 to 99%) and moderately in the remaining 2/19 (11%, C.I. 1 to 26%).

14.1.2. CaP Sections
In the HG PIN glands seen within CaP sections, EGFRvIII immunoreactivity was strong in 4/14 (29%, C.I. 8 to 58%), and moderate in 10/14 (71%, C.I. 42 to 92%) [Figure 27c]. EGFRvIII was detected in all CaP sections, strongly in 26/38 (68%, C.I. 51 to 83%), moderately in 10/38 (26%, C.I. 13 to 43%), and weakly in 2/38 (5%, C.I. 0 to 18%) [Figure 27d]. Furthermore, comparison of mean scores revealed that the expression of the mutant was significantly higher in hormone resistant CaP than in untreated glands [p = 0.012].

14.1.3 Metastatic Deposits
Eleven of twelve metastatic deposits (92%, C.I. 62 to 100%) stained strongly for EGFRvIII, while 1/12 (8%, 0 to 39%) stained moderately [Figure 27e]. In these deposits, EGFRvIII expression was seen in the metastasized prostatic cells only, and the adjacent osteoid or lymphoid cells were negative for the variant antigen.
(A) EGFRvIII was not expressed in normal/atrophic glands.
(B) Weak EGFRvIII immuno-reaction in BPH glands.
(C) HG PIN gland showing strong EGFRvIII staining in fully transformed cells whilst histologically benign cells within the gland and the adjacent BPH gland stained weakly.
(D) Strong expression of EGFRvIII in CaP glands.
(A-D) Cytoplasmic EGFRvIII staining was seen mainly as a perinuclear deposit on the luminal side of the tumour cells and gave an impression of an inner rim within fully formed glands.
(E) Metastatic prostatic cells in a lymph node showing strong EGFRvIII staining in the midst of negative lymphoid cells.
(F) CaP deposit in bone showing high EGFRvIII expression in the metastatic cells whilst surrounding osteocytes are negative.
(G) EGFRvIII negative control (CaP section x 100).
14.2 Statistical analyses (Appendix 8iiic)
Statistical evaluation of the mean scores revealed significant differences between EGFRvIII expression in benign and partially or fully transformed glands: BPH vs PIN or CaP \[p = <0.0001\], and also between CaP and metastases \[p = 0.004\]. EGFRvIII expression in G2 and G3 CaP was significantly higher than the expression in G1 tumours \[p = 0.006\].

14.3 Clinical correlation
Correlation of EGFRvIII expression with known prognostic parameters for CaP in our patients revealed a significant association with serum PSA \[p = 0.005\] \[Figure 28\], and the time to disease progression \[p = 0.05\] only. There was no direct relationship between expression of the variant receptor and histological grade, metastases, and age \[p = 0.063, 0.136, \text{and} 0.508 \text{respectively}\].

Figure 28
A scatter graph comparing EGFRvIII scores with serum PSA levels in individual CaP patients. This showed a significant association between EGFRvIII expression and serum PSA level at the time of tissue retrieval \[p = 0.005\].

Determination of the relationship between the prognostic factors and the survival of our patients by univariate analysis revealed that only the time to disease progression following hormone therapy \[p = 0.007\], and the presence of metastasis at diagnosis (i.e. advanced disease at presentation) \[p = 0.04\] had a significant impact on survival. At the time of data review, 12 patients had died from CaP related causes but the level of EGFRvIII expression did not have a significant influence on survival during the follow-up period \[p = 0.83\] \[Figure 29\]. Although EGFRvIII activity was not significant as an individual prognostic factor for CaP, its association with the time to disease progression indicates its over-expression may be predictive of a poor response to hormonal manipulation. Multivariate analysis using Cox's proportional hazards regression confirmed the importance of advanced clinical stage.
at presentation as an indicator of poor prognosis, as this was the only factor that had a significant influence on survival at 3 years \( p = 0.01 \). Neither the expression of EGFRvIII, nor any of the other known prognostic factors had a significant impact on survival during this period, i.e. EGFRvIII \( p = 0.31 \), age \( p = 0.11 \), and histology \( p = 0.22 \).

![Figure 29](image)

Kaplan-Meir overall survival estimates showing minimal effect of EGFRvIII over expression on the survival of CaP patients in this study.

14.4. Discussion

Although EGFRvIII has been detected in other cancers, to our knowledge this is the first study to report its presence in prostatic tumours. In keeping with reports from other studies, this variant EGFR was expressed by transformed prostatic cells only and in a cell-specific manner. The cell-specificity was best seen in PIN glands where EGFRvIII was over-expressed by the fully transformed cells only. In high-grade cancers EGFRvIII over-expression was seen in both single neoplastic cells and those arranged into glandular structures. In addition, metastatic CaP cells in both bone and lymph nodes stained strongly for the antigen whilst the surrounding osteoid and lymphoid cells were consistently negative. The specificity of EGFRvIII over-expression by malignant cells only in PIN, CaP and metastases, suggest that a high expression of this receptor may be required for the malignant transformation of prostatic cells and that it would be a useful marker when screening tissues for prostate cancer cells.

In previous studies of the clinical relevance of EGFRvIII expression in glial tumours,
high level expression was only found in the highest grade tumours, i.e. glioblastoma multiforme [200]. The association between mean high expression of EGFRvIII and prognostic indicators of CaP suggests that over-expression of this antigen may be predictive of aggressive growth in prostate cancer independent of histological grade. Earlier reports from in-vitro studies had concluded that EGFRvIII bestows a growth advantage on tumours by increasing the rate of proliferation, and by reducing apoptosis [109]. Furthermore, EGFRvIII increases the tumorigenicity, and the metastatic ability, of transformed cells [108]. Our finding of EGFRvIII over-expression in all metastatic deposits of prostate cancer screened implicates this aberrant receptor in the metastatic potential of malignant prostatic cells as well.

14.5. Conclusion

The differential expression of the EGFRvIII by transformed prostatic epithelial cells only may be a critical factor in the as yet unexplained depletion of the native receptor in tumours of this gland. Furthermore, the presence of EGFRvIII in all BPH and CaP glands scrutinised suggests that this mutant protein plays an important role in the transformation of prostatic epithelial cells.
Chapter 15 - Ki67 Immunostaining in BPH, CaP and Metastatic sections.

15.1 Introduction

The EGFRvIII studies above indicated that over-expression of this protein is an indicator of an aggressive phenotype in CaP. The highly significant association between the level of expression of the variant EGFR and serum PSA in our patients indicated that this biologic effect of the EGFRvIII was, at least in part, proliferative in nature.

It is now possible to measure the mitotic rate on sections from archival material using the anti-Ki67 antibody. The Ki67 antigen is found in granular components of the nucleolus of cells in the late G1, S, G2 and M phases of the cell cycle. The anti-Ki67 antibody therefore identifies cells in the proliferative phase of the cell cycle. Previous studies measuring the Ki67 labelling index have found it to be of prognostic value in several cancers including CaP [246-248]. In addition, using this (Ki67) index, Moch et al. [248] had suggested that over-expression of the EGFR was associated with a rapid proliferation of renal cancer cells.

I sought to confirm the role of the EGFRvIII in the autonomous proliferation of malignant prostatic cells by evaluating its association and the rate of cell proliferation by measuring the Ki67 index in serial sections from the CaP glands. Sections from 5 archival BPH specimens were included in the study as negative controls.

15.2 Localisation of anti-Ki67 immuno-reaction

Ki67 immuno-positivity was predominantly nuclear, with minimal cytoplasmic staining in some sections. Only epithelial cells with nuclear staining were included in the analysis. Sections from 5 BPH glands were used as controls, and the Ki67 index was low in 5/5 (100%, C.I 48 to 100%) [Figure 30a].

In CaP cases, a high Ki67 index (>3) was recorded in 21/37 (57%, C.I. 40 to 73%) of sections, whilst the remaining 16/37 (43%, C.I. 27 to 61%) had a low (0-2) proliferation rate [Figure 30b]. Similar to EGFRvIII expression, higher grade cancers had a significantly higher Ki67 index than G1 tumours [p = 0.0009]. All 6 (100%, C.I. 54 - 100%) metastatic deposits scrutinised had a high Ki67 index, but this was not statistically different from CaP scores [p = 0.06] [Figure 30c].
Figure 30: Ki-67 immuno-staining in prostatic tumors (x 250). Sections were counterstained with haematoxylin.

(A) BPH glands showing low (<3/100) Ki-67 index
(B) CaP glands showing high (>3/100) Ki-67 index.
(C) Lymph metastasis showing high (>3/100) Ki-67 index.
(D) Negative control (Tonsil section).

15.3 Statistical analyses.

15.3.1 Correlation between EGFRvIII expression and the proliferation rate in primary and metastatic prostate cancer

Statistical analysis revealed a significant association between the EGFRvIII score and the Ki67 index in serial sections from the primary and metastatic CaP cases investigated in this study \( p = 0.006 \) [Figure 31]. This direct relationship was seen in sections with high and low scores of the two indices \( p = 0.001 \) and <0.0001.

Correlation between Ki-67 index (cell proliferation) and EGFRvIII expression in CaP

Figure 31
15.3.2 Clinical Correlation.

Correlation of the Ki67 index with clinical data revealed a highly significant association with serum PSA \( p = 0.001 \), advanced disease at presentation and the time to disease progression \( p = <0.0001 \). Furthermore, there was a direct association between Ki67 index and death at three years in our patients \( p = 0.001 \). In contrast, the relationship between Ki67 and the Gleason grading of the tumours was not significant \( p = 0.11 \).

15.4 Discussion.

The use of the Ki67 proliferation index in determining the cell kinetics of benign and malignant prostatic tumours has been well described \[26, 247\]. In CaP it has been evaluated by several as a predictor of the aggressiveness of primary and recurrent prostate cancers. In this regard, the Ki67 index has previously been shown to correlate significantly with the disease recurrence following surgery and radiation \[249, 250\], survival \[251\], histology \[252\], and other indicators of cell proliferation \[247\].

However, questions have been raised concerning the usefulness of this index as an independent indicator of the biology of malignant prostatic growths. Of particular note are those studies in which measurement of the Ki67 index added little more information about the biology of CaP over that provided by the traditional prognostic indicators (Gleason score, pathological stage and ploidy) \[253, 254\]. In addition this index was unable to predict the time to tumour progression following hormonal treatment in patients with CaP \[255\].

In this present study, I found a significant correlation between EGFRvIII expression and Ki67 index in archival sections from CaP glands. This finding provides evidence to support the hypothesis that the effect of EGFRvIII expression on the biology of CaP is exerted in part through an increase in proliferation of the malignant cells. This assertion is in keeping with earlier reports from in-vitro studies had concluded that EGFRvIII bestows a growth advantage on tumours by increasing the rate of proliferation \[108, 109\]. This finding is of particular importance as it indicates that strategies targeted at the EGFRvIII may reduce or halt the progression of CaP tumours.

15.5 Conclusion

Despite the small numbers involved in this study, our results are validated by the similarities between our Ki67 findings and previous reports on the index. Furthermore, the demonstration of a direct association between the two indices (EGFRvIII expression and Ki67 index) and accepted CaP prognostic factors (PSA,
time to tumour progression and advanced disease) also lends credence to the accuracy of the results and the interpretation of the same. Interestingly, although patients with a high Ki67 index were more likely to be dead at the time of review, no such association was found between EGFRvIII expression and survival. This difference may indicate that the Ki67 is a better predictor of survival in patients with CaP than this variant EGF receptor. The EGFRvIII may however have additional effect on these tumours via its noted ability to increase the tumorigenicity, and thus the metastatic potential, of transformed cells.
CHAPTER 16: Discussion

16.1 Introduction

The processes of cellular proliferation and the acquisition of the different specialized tissue phenotypes involve highly co-ordinated mechanisms in multi-cellular organisms. Signaling between growth factors and their receptor is an integral aspect of these processes in solid organs [66, 83]. The malignant phenotype of a tumour relies on three characteristics of neoplastic cells; uncontrolled growth, local invasiveness, and metastatic ability. Abnormalities of the diverse interactions between these cellular proteins contribute to each of these characteristics of malignancies [85]. These pathways have been under intense scientific scrutiny, with the aim of identifying definite alterations in cell-cell communications in neoplasms that could present new targets for anti-cancer strategies.

The EGFR plays an important role in the initiation of cellular division of epithelial cells. Although an increased expression of this protein has been found in several cancers, findings from studies investigating its levels in prostatic tumours have been inconsistent. However, with the advent of more specific antibodies which recognise the native receptor only, recent studies have demonstrated a depletion of EGFR expression as prostatic tissues de-differentiate despite increased levels of receptor mRNA [182, 212-214, 216, 217] [104, 211] [215, 218, 219]. Until now the reasons for this unique finding have remained unclear and the aim of this study was to investigate the hypothesis that prostatic neoplasms express a variant type EGFR protein. The expression of such a protein could also be an important factor in the depletion of the wild-type EGFR in these tissues.

The EGFR family of receptors consists of several receptors which share sequence homology and have a tyrosine kinase residue at their cytoplasmic end. Currently, four distinct c-erbB receptors have been identified (c-erbB-1 – c-erbB-4), all of which are thought to play important parts in carcinogenesis. These receptors require ligand-binding for activation in the main, and several ligands have been identified [152], although none has been shown to bind c-erbB-2. Several deletion mutations of the external domain of the wild-type EGFR (c-erbB-1) have also been reported in the medical literature [89], and these have been found in abnormal epithelial cells only. The most common of these mutant receptors is the EGFRvIII which has been detected in several cancers [105]. This variant EGFR is a consequence of an 801-bp in-frame deletion of the external domain of the normal receptor [193]. This rearrangement removes the ligand-binding extracellular sub-domains of EGFR whilst preserving the reading frame of the receptor message. The EGFRvIII is thus a
constitutively active tyrosine kinase which is capable of initiating cell division independent of ligand. However, it has been suggested that the oncogenic potentials of this aberrant peptide are not entirely due to its intrinsic tyrosine kinase activity [207]. The prevalence of the EGFRvIII in naturally occurring human tumours has led to the prediction that it actively contributes to the malignant phenotype of these tissues [207].

From the fore-going a possible explanation for the initial conflicting results on the amount of EGFR expressed in normal and abnormal prostatic tissues was the lack of specificity of the methodology used in the different studies. This could have resulted in cross-reactivity with, and recognition of, one or more members/mutations of the EGFR family. As described in greater detail in Chapter 9, an important aspect of this current project therefore was the development of a methodology that ensured the specificity and sensitivity of the reagents used for the detection of the native and variant EGFR in our tissues. This was achieved by the use antibodies previously shown to possess these characteristics, and by carrying out initial confirmatory experiments on test tissues. Furthermore, tests were done to exclude time-induced antigen decay, thereby ensuring that maximal amounts of the proteins were available for detection. These preliminary experiments were crucial to the validity of the conclusions drawn from the subsequent qualitative and quantitative evaluations of the immuno-detection of the WT-EGFR and EGFRvIII.

16.2 Project Methodology

16.2.1 Methodology in general

16.2.1.1 Archival versus frozen tissue material for research projects

Despite the obvious advantage of the ease of detecting tissue antigens (by immunohistochemistry) on frozen sections, there is a preference for the use of archival material in research projects for the following reasons:

1). A vast bank of tissue is readily available in all pathology departments, and this makes large retrospective studies (especially those investigating the clinical significance of immunohistochemical findings) possible.

2). The histological diagnosis of the tissue blocks chosen for study may be predetermined, and this allows specific investigation of the different tissue histotypes. The problems of sampling error which plagues the use of frozen material (tissue obtained at the time of retrieval may not be representative of eventual histological diagnosis) is also avoided. This is of particular importance when using tissue obtained during prostatic resections as in this situation tissues for examination are randomly selected.

3). Formalin treatment preserves the cellular architecture of tissues. This
characteristic is of particular importance in indirect immunohistochemistry as the test allows the direct localisation of antigen-antibody reactions in the cellular anatomy. Our determination of the spatial relationship between the expression of the native and variant EGFR proteins (WT-EGFR – basal cells/membranous; EGFRvIII – luminal cells/perinuclear) was dependent on this property of paraffin prostatic sections. On the other hand, sectioning of frozen tissues often results in the loss of tissue architecture (especially the luminal cells in prostatic glands).

The use of archival and frozen tissue in EGFR studies
Most of the earlier studies on EGFR protein in prostatic neoplasms have used frozen sections ([182, 211, 213-215]. This is because of technical difficulties with the exposure of the EGFR epitopes in archival tissues to the most commonly used antibodies (EGFR1 and clone Ab-1). We also experienced similar difficulties during initial tests in this project. However, we were able to overcome these problems by the exhaustive search of the antibody library to find anti-EGFR and anti-EGFRvIII reagents that were known to react effectively with the relevant antigens on archival material [212, 217-219].

16.2.1.2 Detection of receptor proteins versus transcripts
In this project the detection of both WT-EGFR and EGFRvIII proteins was done in preference to mRNA or DNA studies. This is because the detection of the fully formed proteins was the preferred indicator of the expression of functional EGF receptors by prostatic epithelial cells. The detection of the mRNA/DNA transcripts, in contrast, would only confirm the presence of the receptor messages in the cell genome. The alterations that result in the depletion of EGFR expression in prostatic tumours have been recognised as most likely being at the translational level [211, 212, 215]. Furthermore, the EGFRvIII is a variant receptor that results from a deletion mutation that occurs at the mRNA level [200]. Taken together, these findings suggest that there could be a significant difference in the amount of mRNA of both EGFR present in prostatic tumours and the levels of the proteins expressed. Indeed in subsequent RT-PCR studies done on BPH and CaP tumours at the KCI Institute [239], the previously reported increased level of WT-EGFR mRNA in these neoplasms [209, 211] was confirmed, but smaller quantities of EGFRvIII mRNA were detected. This suggests that as prostatic tumours advance they increasingly express this variant protein in preference to the normal receptor.

16.2.1.3 The use of immunohistochemical techniques in research projects
1. Western blotting
Western blotting tests are commonly used to detect the presence of proteins in tissue lysates using previously characterised antibodies. The tests are more sensitive than indirect immunohistochemistry for this purpose as proteins prepared from fresh frozen tissue (by tissue lysing), and separated into their separate molecular weights (by gel electrophoresis) are more readily available for antigen-antibody reactions (immuno-blotting) than antigens in intact tissues sections (frozen or archival). Western blotting may also be used to evaluate the sensitivity and specificity of newly developed antibodies by carrying out experiments on appropriate positive and negative control tissues. In this study, western blotting tests were used for both purposes. The EGFRvIII has not been previously detected in the human prostate, and the test was used to confirm its expression in neoplastic prostatic tissues. Gel electrophoresis was also used to confirm the specificity and sensitivity of the anti-WT-EGFR and anti-EGFRvIII antibodies used in the subsequent indirect immunohistochemical tests, and to exclude cross-reactivity between the antibodies.

2. **Indirect immunohistochemistry**

Indirect immunohistochemistry is the most common method of detecting tissue antigens in research projects. The test is attractive to the research investigator for several reasons and, prominent among these is the wide range of commercial immunohistochemical detection kits available. This development has ensured that these tests can now be carried out in a standard and easily reproducible fashion. In addition, immunohistochemical techniques are used by most pathology departments for diagnostic purposes. As such, a novice Researcher can rely on the experience of the staff of the local pathology department as regards which tests to carry out and which kits to use.

Despite the advantages listed above, indirect immunohistochemistry has its limitations, and these have to be taken into account to ensure validity of the results obtained. These include:

1. **Sensitivity and specificity of immuno-reactions.**

These concerns centre around the sensitivity and specificity of the antibodies used in immuno-reactions. In general, sensitivity and specificity are inversely related, with polyclonal antibodies being more sensitive but less specific than the monoclonal antibodies. However, improvements have been made in the purification stage of the production of rabbit derived antibodies and this has gone some way in making these reagents more specific whilst retaining their specificity [105]. Moreover, the specificity and sensitivity of various antibodies can be evaluated by preliminary western blotting tests.

2. **Availability of several types of antibodies.**
Antibodies are directed against specific epitopes of the relevant antigen. As a result, different immunohistochemical studies using different antibodies may produce different, and at times conflicting results, from similar material. This problem may be responsible, in part, for the stated confusion over the expression of the EGFR in prostate malignancies due to the possibility of cross-recognition of antigens. Identification of the clones of antibodies used in individual studies now enables better comparison of results obtained from the use of similar reagents. This precaution was taken in this study.

3. **Quantification of immuno-staining results.**

Reproduced (and objective) measurement of the immuno-reactions is necessary to allow accurate evaluation of the results obtained. Over the years several methods of assessing EGFR staining results have been applied, and this has made it difficult to compare results from different immunohistochemical studies on the same tissues. Although attempts have been made to reach a consensus on the quantification of immuno-staining [256], several methods are still being used. These include: 1) cell count [75]; 2) grading of the average staining intensity [211]; 3) grading of scores [217]; 4) cut-off scores [216]; 5) image analysis [215]; 6) the H-score [257] and its modification [218]. The two indices chosen to quantify the immunohistochemical results in this study were: 1) the modified H-score (which takes both the percentage cell positivity and the average staining intensity into consideration) for the membranous antigens (PSA, WT-EGFR and EGFRvIII); and, 2) the proportion of target cells with positive staining for the nuclear antigen (Ki-67).

The use of the percentage of a stated number of cells staining positive is now generally acceptable, especially when evaluating immuno-reactions aimed at detecting nuclear antigens [26, 75, 255]. However, controversy has surrounded the intensity of staining as visual assessment of this index is considered difficult to reproduce, but this parameter has to be taken into consideration when evaluating immuno-staining of membranous antigens. The H-score and its modifications attempt to reduce this inherent subjectivity by assessing both the percentage cell positivity and staining intensity. This method of quantification has been used in earlier studies which evaluated EGFR expression in prostatic tissues [218, 219].

4. **Antigen masking in paraffin sections.**

Formalin-fixation induces changes in tissues that may make immunohistochemical detection of antigens difficult. This masking of tissue antigens by aldehyde-based fixatives is thought to be due to the formation of methylene bridges between reactive sites on different portions of the same molecule or of adjacent proteins.
Despite this, it has been shown that formalin treatment does not alter the secondary protein structure [258]. On the basis of these findings researchers now assume that tissue antigens are simply masked in archival tissues and not destroyed. Although partial restoration of antigenicity is achieved by rinsing tissue sections in water [259], or Tris buffered solutions [260], the reversal of formalin-induced masking of some antigen may require enzymatic or microwave pretreatment [220] as was done in this study.

5. The effect of storage on the detection of antigenic epitopes in archival sections.

Localisation of cellular proteins on tissue sections using immunohistochemical techniques is commonly done using paraffin sections of varying age. This may be either because the original tissue blocks are unavailable, or because of other procedural delays. The recent concerns about the possibility of storage having an effect on immunohistochemical results have been borne out by findings from preliminary investigations during this project. These storage-induced alterations are of particular relevance in collaborative tests where transportation delays may prescribe the use of sections that have been in storage for awhile. When taken along with previous reports [234, 235], my findings indicate that the effect of storage [245] on the detection of tissue antigens is variable.

The foregoing indicate that in addition to the differences in the clonal origin of antibodies used, the previous conflicting results from immunohistochemical studies on archival tissues could be also due in part to differences in the age and preparation of the sections. Consequently, freshly prepared (i.e. less than 10 days if kept at room temperature) paraffin sections should be used preferentially when carrying out both diagnostic and research studies. However, immunohistochemical systems should be checked for aging induced antigen-degradation if the use of sections that have been in long-term storage be unavoidable. Furthermore, in collaborative multi-centre studies, preliminary tests should be done to ascertain the effect of differences in tissue preparation techniques on the immuno-detection of the antigens under investigation so as to ensure standardisation of results obtained in sections from the various centres. These protocols were applied whilst carrying out subsequent immuno-staining tests on archival material in our laboratory [75, 238, 239].

16.2.2. Methodology of EGFR detection

16.2.2.1 Immuno-detection of EGFR proteins

As mentioned above (Section 7.4), the use of different types of antibodies in previous studies has been partly responsible for the controversy surrounding EGFR expression in prostatic tumours. This potential problem was excluded by using clones of
antibodies with proven sensitivity and specificity for the wild-type and variant EGFR proteins being investigated in this project (anti-WT-EGFR [clone 31G7], [177], and anti-EGFRvIII, [205]). These reagents have also been shown to be reactive with paraffin sections [105, 217]. The tissues used as positive controls for these antibodies in this project had also been previously identified as suitable in these studies (A431 for EGFR, [177]; and EGFRvIII transfected mouse tumour for EGFRvIII [105]). As mentioned above, these earlier reports were confirmed by carrying out preliminary western blotting on lysates of our test prostatic specimens, thus ascertaining that these reagents were effective for use, and not cross-reactive, in the subsequent indirect immuno-histochemical tests on our specimens. The western blot tests also confirm the expression of the EGFRvIII in neoplastic prostatic tissues. These preliminary qualitative tests were crucial to the validity of the results obtained in subsequent tests as the EGFRvIII had been previously undetected in prostatic tissues. In this regard, the use of an anti-EGFR known to react effectively with prostatic archival material with consistent results ([212, 217-219]) was particularly important as a control test of the methodology of the study.

Following the confirmation of two types of EGFR in prostatic tumours, quantification of the total amount of EGFR expressed by these tissues by indirect immunohistochemistry was a logical next step. This especially as an inverse relationship had been demonstrated between the expression of the variant and the wild-type receptors in these tissues [Figure 32]. As the EGFRvIII results from a deletion mutation in the external domain of the native receptor, antibodies directed at the internal (cytoplasmic) domain of the EGFR molecule would react with both EGFRs as would reagents which recognise a sequence of the external domain preserved by the EGFRvIII mutation. However considering the variable sequence homogeneity with the other members of the erbB family of receptors (erbB-2, erbB-3 and erbB-4), the specificity of the former group of antibodies was considered inadequate for this particular purpose because of the risk of cross-reactivity with these proteins. An anti-EGFR antibody (clone Ab-1) directed at the external domain that would detect both WT-EGFR and EGFRvIII positive cells [261] was therefore chosen. However, although good immuno-reactions were seen with this antibody in frozen sections, the results obtained on archival material were inconsistent. Further testing using other antibodies was deferred due to the constraints on time.
Graphical representation of WT-EGFR and EGFRvIII means scores (+/- S.D.) in the different histological grades of prostatic tissues demonstrating an inverse relationship between the levels of expression of the two types of EGFR. The difference between WT-EGFR and EGFRvIII means scores (+/− S.D.) was statistically significant in each tissue histotype (p < 0.0001).

16.3 EGFRvIII expression in prostatic tumours

16.3.1 Detection of WT-EGFR and EGFRvIII expression in prostatic neoplasms

A) EGFRvIII expression as a critical factor in WT-EGFR depletion in prostatic tumours.

As the evidence for the decreased expression of the native EGFR in prostatic tumours has increased, several hypothesis have been proposed as possible explanations for this observation as discussed in detail in Section 6.3. Among the various hypotheses, the postulation that tumours with a decreased level of EGFR protein express a truncated form of the receptor [186] has remained attractive to researchers [213, 223]. This especially as newer members of the erbB family of receptors have been detected in various human tissues [152, 262]). A further indication of the involvement of abnormal EGFR’s in tumours that express decreased levels of the receptor was provided by a recent study in breast cancer that showed that down-regulation of the WT-EGFR in breast malignancies was associated with an over-expression of c-erbB-2 [263]. Whilst this study suggested an inverse relationship between the expression of the two receptors in this (breast) neogrowth, no such association between EGFR and c-erbB-2 has been found in prostatic neoplasms [216, 217]. However, despite the concerted efforts of researchers, until now, there have been no reports of the detection of a truncated EGF receptor in prostatic neoplasms.
I also hypothesized that prostatic tumours express a variant (truncated) EGFR, and my tests have confirmed the presence of one such protein (EGFRIII) in these tissues. Although EGFRIII has been detected in many other cancers, to our knowledge, this is the first study to confirm its expression in prostatic neoplasms. As in the previous studies, EGFRIII was expressed by neoplastic prostatic cells only. This is also the first study to evaluate the association between the expression of the native and variant forms of the receptor in human neoplastic tissues. Despite the small numbers in our study, the inverse relationship between the level of immunoreactivity of these two antigens supports our hypothesis that the differential expression of the EGFRIII by transformed epithelial cells of this gland may be a critical factor in the unexplained depletion of the native receptor in these tumours [Figure 32].

The presence of the EGFRIII in neoplastic tumours has also been independently confirmed by the detection of the receptor transcript by RT-PCR tests done on our tissues (D Moscatello, KCI). Interestingly, as we have recently reported [264], these studies showed that whilst both WT-EGFR and EGFRIII mRNA were detected in both BPH and CaP, the expression of the variant receptor protein increased with dedifferentiation of prostatic epithelial cells with a concomitant decrease in WT-EGFR expression. This suggests that, similar to high grade gliomas [187], as prostatic tumours advance they increasingly express this constitutively active variant protein in preference to the normal receptor.

B) WT-EGFR and EGFRIII have different patterns of expression
The staining patterns observed in our sections show that although the expression of both WT-EGFR and EGFRIII is cell-specific, the patterns of expression of the two receptors differ. WT-EGFR is expressed mainly on the cell-membrane, whilst EGFRIII expression is mainly cytoplasmic/perinuclear. In addition, we have found that the prostatic cell-type which expresses the highest amount of EGFR, wild-type or mutant is dependent on the histology of individual glands (basal cells in normal/atrophic and BPH glands, and luminal cells of malignant glands). WT-EGFR expression therefore gives the impression of an outer rim surrounding the gland, whilst EGFRIII expression is like an inner rim [Figure 26a and 27b]. The presence of a perinuclear deposit in EGFRIII-positive prostatic cells is similar to earlier observations in glial tumours [108, 204] and may be due to the immuno-localisation of the internalised variant receptor (following ligand-independent dimerisation). This spatial relationship between the observed localisation of the immunoreactions involving the external domain of the EGFR and EGFRIII provides further evidence of the identification of two different types of EGFR.
proteins. However, the significance of this difference in the cellular distribution of the two types of EGFR is at present unclear.

16.3.2 Implications of EGFRvIII expression in benign and malignant prostatic tumours

A) The role of EGFRvIII in the initiation and progression of neoplastic transformation of prostatic cells

Although EGFRvIII has been detected in other cancers, this is the first study to investigate the expression of this variant protein in neoplastic tissues of varying grades of de-differentiation. Previous studies had shown that high EGFRvIII levels were more prevalent in glioblastoma multiforme [187]. These tumours usually develop de novo in older patients who have no prior clinical history of the less malignant form of neural tumour (astrocytoma) [188].

The pattern of EGFRvIII expression observed in this study suggests that this variant protein may be involved in the initiation and progression of neoplastic prostatic growths. This assertion is supported by the specificity of its expression by abnormal prostatic cells, and by the progressive increase in the expression of this variant protein as the tissues become increasingly malignant. Previous reports of the ability of EGFRvIII to induce malignant transformation in in-vivo and in-vitro studies provide additional evidence [105, 207].

The ability to proliferate autonomously is a characteristic of all cancers and expression of the EGFRvIII would confer this ability on its host cells. The specificity of EGFRvIII over-expression by only fully malignant cells in HGPIN, CaP and metastases, suggest that a high expression of this receptor may contribute to the malignant transformation of prostatic cells. However, the EGFRvIII is also expressed by BPH cells, but in reduced quantities. It is therefore possible that acquisition of this potential (for autonomous proliferation) is one of the early steps in the neoplastic pathway with progression to malignant status being triggered when the expression of this aberrant protein reaches a critical level. Another possible explanation for the observed over-expression of EGFRvIII in malignant prostatic cells is the preferential expression of the variant receptor by these cells following transformation. In addition, the universal expression of EGFRvIII in metastatic deposits screened is in keeping with earlier reports that this EGFR variant increases the metastatic potential of transformed cells [108].

B) EGFRvIII and hormone resistance in prostate cancer

Several molecular pathways have been proposed as leading to hormone resistance in
hormone responsive tumours [265, 266]. These are:

1). Alterations in steroid hormone receptor expression (decrease/loss or mutation) with the resultant utilisation of steroid receptor independent pathways. Evidence in support of this hypothesis has been detected in CaP [75].

2). Changes in ligand metabolism and availability/ligand-independent steroid hormone activation. These alterations may involve an increase or decrease in metabolism of the ligand and may lead to an increase or decrease in the amount of ligand available for binding with the steroid receptor. This may result either in a prolongation or reduction in steroid receptor activation which may eventually lead to a hormone refractory state.

3). Changes in post-steroid receptor events/steroid hormone receptor element. There are recent reports of the activation of the steroid–receptor pathways by growth factors in established human prostate cancer cell lines [267]. These pathways are considered to contribute to the autonomous (hormone-independent) proliferation of these cells and thus their ability to survive in laboratory conditions. Alteration of the androgen response element may also result in aberrant activation of the androgen receptor signaling pathways independent of stimulation by androgens.

4). Alterations in the growth factor/growth factor receptor signaling pathways (mutation/constitutive activation). Mutations of several growth factors have been detected in tumours including those of TGF-β [110], FGF [90], and EGFR [89, 152, 195-197]. Over-expression of some of these abnormal receptors has been associated with the development of hormone resistance [110, 268].

5). Loss of co-regulating proteins. Co-regulating proteins (co-activators and co-repressors) are responsible for regulating steroid hormone receptor pathways following activation by the relevant hormone [269]. Loss of these proteins would result in persistent signaling in these pathways once activated by ligand-binding, and thus contribute to hormone independence of the tumour.

Considering the above, our detection of the EGFRvIII in prostatic tumours confirms the presence of one of the possible pathways to hormone refraction in these neoplasms. Along with our report of an alteration in hAR expression in the stroma surrounding CaP glands [75], this current finding indicates that more than one of these potential pathways may be in operation in the development of hormone independence in hormone responsive cancers.

C. Correlation of EGFRvIII expression and the biology of prostate cancer

In our CaP specimens, this variant EGFR was over-expressed irrespective of
histological grade. Furthermore, EGFRvIII expression was directly related to the Ki67 (proliferation) index. This suggests that the oncogenic effect of EGFRvIII on CaP tumours could be mediated, at least in part, by an increase in mitosis as has been reported from in-vitro studies on other tumours [108] [109]. Thus the association between expression of EGFRvIII by cancerous prostatic cells and known clinical parameters of CaP (serum PSA and time to tumour progression) suggests that over-expression of this mutant is predictive of aggressive disease.

Considering the finding that a high EGFRvIII level is predictive of an aggressive CaP phenotype, expression of this protein may be involved in the observed racial differences in the biology of the disease. As such comparison of the levels of EGFRvIII expression in black and white men with CaP may improve our understanding of the malignancy. We therefore carried out initial studies to test this hypothesis by scrutinising archival BPH and CaP specimens from black African men and comparing the results with those observed in UK whites. Contrary to our expectations however, we found no significant difference between EGFRvIII expression in prostatic tissues obtained the two racial groups of men [p = 0.551] [238]. This suggests that the biology of this tumour is determined by the interaction of multiple genetic and environmental factors.

D) EGFRvIII expression provides further evidence of the graded molecular/cellular changes associated with transformation from benign to malignant prostatic disease

Although it is generally accepted that BPH does not progress to CaP, reports from molecular studies indicate that the levels of cellular indices of abnormal growth in these benign neoplasms is intermediate between that found in normal tissues and CaP. The graded alterations (increase and decrease) in the mean expressions of both WT-EGFR and EGFRvIII as prostatic tissues became increasingly transformed is supportive of the findings in studies investigating other factors such as C44 [270], PSMA [241], HK2 [271], and Telomerase [272]. Furthermore, there is increasing evidence from clinical and basic science research that PIN is a precursor of invasive prostate cancer [49]. High grade PIN lesions are usually found in close association with frank prostatic malignancy, and the levels of most molecular markers in these glands is in-between levels found in CaP and benign tissues (hAR - [75]; EGFR - [211]; HK2 - [271]; PSMA- [241]). As such, the observation that the mean expression of EGFRvIII (and WT-EGFR) is intermediate between the mean expressions in BPH and CaP could provide further evidence in support of the potentials for malignant growth of these glands.

E) EGFRvIII as a prostatic tumour marker
In sections from both BPH and CaP cases, EGFRvIII was expressed by benign hyperplastic and malignant neoplastic prostatic cells but not by normal cells. However, only fully malignant cells (in PIN, CaP and metastases) expressed high levels of the protein. Over-expression of the EGFRvIII may therefore be a useful histological marker when screening primary and metastatic tissues for malignant prostatic cells especially when PSA staining is weak or absent [241]. Recently, antibodies to tumour-specific antigens have been used to identify residual or recurrent malignant disease after radical prostatectomies [273]. However, this antibody reacts with the cytoplasmic domain of its target antigen (PSMA) and therefore requires a breach of the cell membrane for the antigen-antibody reaction to occur. Since \textit{in-vivo}, only damaged cells have defective cell surfaces, it is possible that the antibody detects only dead or dying cells. In contrast, the anti-EGFRvIII reacts with the external domain of the relevant protein. As the EGFRvIII is over-expressed in a cell specific manner in CaP, this antibody may therefore be more suitable for labeling minute quantities of viable neoplastic tissues inadvertently left behind following surgery. The cells so identified could then be targeted with either genetic, immunological or radiation therapies.

\section*{F) EGFRvIII as a potential target for anti-tumour strategies}

In light of the detection of EGFRvIII over-expression by cancerous prostate cells in this study, this mutant receptor may also be a suitable target for such therapies in CaP. There are recent reports on the results of trials in which the immunisation of immunocompetent laboratory animals with anti-EGFRvIII vaccines prior to inoculation with transformed cells have proved effective in inhibiting tumour formation [274]. Immunisation with the same antibody following inoculation also appeared effective in stimulating regression of established tumours, and both effects were due to the stimulation of an immune response against the EGFRvIII peptide sequence. Furthermore, EGFRvIII expressing cells were killed in preference to those expressing the native EGFR by an anti-EGF-receptor-specific toxin [275]. Whilst EGFRvIII is variably expressed by BPH and CaP alike, the specificity of the high levels of expression by carcinomatous cells means that treatments targeting this mutant receptor are likely to yield more rewarding results in the treatment of malignant prostatic disease.

Further, as mentioned earlier (Section 4.5.4), WT-EGFR has been the target of several anti-cancer treatments [138, 139, 276]. Similar to the controversy surrounding the expression of the receptor protein in prostatic tumours, assessment of the therapeutic effect of anti-EGFR antibodies in studies on established prostate cancer cells have produced conflicting results. Whilst some studies have reported
inhibition of the growth of tumour cells both in-vivo and in-vitro [277, 278], others have reported fluctuating growth responses on similar cell lines [279] and these discrepancies remain unsolved. It is however possible that these inconsistent therapeutic results are due to the variable recognition of the normal and mutant type EGFRs by the different antibodies used in these studies. The reports of EGF-independent EGFR activation in CaP cells in-vitro [103] provides independent evidence in support of this postulation. It would therefore be expected that our demonstration of the presence of multiple types of EGFR protein in CaP, which play an active role in the malignant process, would lead to improved characterisation of antibodies to be directed at the receptor in future studies.
PART 4 - CONCLUSION AND FUTURE STUDIES

Chapter 17 - Conclusion
Chapter 18 - Future studies
CHAPTER 17: Conclusion

The progressive loss of WT-EGFR as prostatic tissues de-differentiate remains unexplained. Although the expression of a mutated receptor had been considered, the techniques with which to detect such variant proteins have only been recently developed. The EGFRvIII is the most common mutant EGFR, but until now its presence in prostatic tumours had not been confirmed. This study has demonstrated the presence of the EGFRvIII protein in prostatic neoplasms but not in normal glands, and demonstrated an inverse relationship between the expression of this variant EGFR and the wild-type protein in these tissues. This is also the first report on the expression of EGFR (wild-type and mutant) expression in prostate cancer metastases. The specificity of my findings suggests that EGFRvIII is a potential histological marker for prostate cancer. The clinical significance of the presence of this constitutively active receptor in prostate cancer patients was also evaluated, and we found that its level of expression is predictive of an aggressive disease phenotype. Furthermore, EGFRvIII over-expression appears to contribute to the progression of CaP. The EGFRvIII is thus a potential target for modern anti-cancer regimes including gene therapy.
CHAPTER 18: Future studies

18.1 Quantification of total EGFR expressed in prostatic tumours
As discussed above, this investigation remains attractive. In view of the quantification of both WT-EGFR and EGFRvIII in prostate neoplasms, measuring the total amount of EGFR should further clarify whether the expression of this receptor increases or decreases in these tissues. However, it would be necessary to use antibodies that do not cross-react with any of the other members of the erb-B family to ensure accurate results.

18.2 Evaluation of the role of EGFRvIII in anti-apoptosis in primary and metastatic prostatic tumours
Carcinogenesis results in the disruption of the net balance between proliferation and apoptosis in solid organs, and there is evidence implicating the EGFRvIII in both cellular events in glial tumours [109]. Having confirmed the mitotic properties of this variant receptor in CaP, determination of its effect on programmed cell death in these tissues would improve our understanding of the molecular processes in these tumours. Initial tests in this direction have suggested that there is no direct relationship between EGFRvIII expression and anti-apoptosis in primary prostatic neoplasms [264]. However, the numbers incorporated in the study were small and a larger study is required to examine the relationship further.

18.3 Evaluation as a possible target for anti-CaP therapy
Presently there is no effective long lasting treatment of CaP, especially when it eventually progresses to hormone independence. The fact that EGFRvIII is overexpressed by both primary untreated and hormone resistant cancer indicates that similar positive therapeutic responses to anti-EGFRvIII treatment can be expected from both stages of CaP. The cell specificity of the protein should ensure that only abnormal cells are affected by such strategies. Gene and immune therapies directed against specific growth factors and/or their receptors involved in the various cancers are currently evolving in the laboratory, and several trials are already in place [280]. Considering the findings of this study, the EGFRvIII would be a suitable target for such studies in CaP.

18.4 Evaluation of the presence of other mechanisms of hormone resistance in prostate cancer
As mentioned above several molecular pathways have been proposed as leading to hormone resistance in cancers of hormone responsive organs. However, it is likely that more than one of these pathways is present in these diseases. I have investigated
these hypotheses in CaP and detected the presence of another potential pathway to hormone resistance in these tissues (the absence of the expression of androgen receptors in the stroma surrounding malignant prostatic epithelium) [75]. This significant finding indicates the loss of the mechanism of androgen regulation of (via the stroma [66]) epithelial proliferation in CaP and confirms the presence of multiple hormone resistance pathways in these tissues. Further studies are therefore required to investigate the presence of other pathways in prostatic tumours, and to determine their possible roles in the development of hormone resistant disease.
PART 5- APPENDICES AND BIBLIOGRAPHY
Appendix 1

Protocol for Preparation of Tris-Buffered Saline (TBS) x 10 Concentration

1). Dissolve - 87.6g of NaCl + 78.8g of Tris/HCl in 1L of distilled water using heated stirrer.
2). Adjust pH to 7.6 using 5M NaOH/HCl as appropriate
3). Store in 500ml flasks in fridge
4). Dilute 500ml to 5L for use as required.

Protocol for Preparation of Citric Acid Buffer (x 20 concentration)

1) Dissolve 82g of Citric acid monohydrate (MW = 210.14) in 500ml of distilled water.
2) Adjust pH to 6.0 using 5M NaOH (40g in 200ml dist H2O in fume cupboard)
3) Make solution up to 2L in volumetric flask
4) Store in 500ml flasks in fridge
5) Dilute 100ml to 2L for MW use as required.
Appendix 2

Protocol for Preparation of Tissue Lysates

Tissue Homogenisation

1. Weigh, then cut up tissue into very small pieces: add 1ml homogeneising buffer per 100mg tissue.
2. Homogenise tissue using the Ultra-tumax T25 homogeniser (rinsing machine between samples)
3. Pre-cool centrifuge by spinning it at a low speed for 5 minutes.
4. Centrifuge samples at 4°C for 1 hour, 11,000 rpm with no brake.
5. Boil segments of Visking® tubing (10,000 daltons) in distilled water for 2 minutes: press out water and clip one end.
6. Add sample, clip and label.
7. Dialyse, 150ml per sample for 18 hours at 4°C.

Preparation of Homogenising buffer

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Preparation of Dialysis Buffer (2 Litres)

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<td>PMSF (0.1 mmol/l)</td>
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Western blotting

Preparation of 6% Stacking Gel (20ml)

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<td>10% AMPS (1g in 10ml water)</td>
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Preparation of 6% Resolving Gel (20ml)

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<td>50 mmol/l Tric.cl (pH 6.8)</td>
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*** Note - The other solutions used for western blotting (Electrophoresis buffer, Blotting buffer, SDS sample buffer, Wash buffer, Blocking buffer, Incubation buffer) were purchased from Bio-Rad Laboratories UK.
Appendix 3
Cutting and Fixing Frozen Sections using Frigocut® Cryostat

a) Cabinet should be set at -20°C for cutting kidney.
b) Defrost timer should be set for 12 midnight,
c) Clearance angle of knife block should be set at 7.5° (the first mark represents 5°),
d) Cutting thickness should be set at 5μm.

Cutting sections
1. Transfer biopsy taken from liquid nitrogen tub to cryostat cabinet in small dewar flask (use protective equipment provided - mask and gloves). Danger of vial explosion due to rapid expansion of liquid nitrogen trapped in vial.
2. Place vial containing cork mounted biopsy in the cabinet and leave for 1 hour to warm to -20°C.
3. Mount cork on object block with OCT and place on freezing stage until OCT has hardened.
4. Place object block on cutting stage. Adjust cutting stage position as required. Trim biopsy until tissue is being cut, then collect sections on knife edge using anti-roll plate (position altered as required). Pick up sections on gelatinised microscope slides. Cut enough sections for 2 staining runs.
5. Leave sections to dry in air for at least one hour before fixing.

Fixing sections
1. Place slides with section in rack and into black dishes containing acetone at room temperature for 10 minutes. Allow to air dry.
2. Store half the slides in racks in labeled silica gel-containing sample bags in -20°C freezer (or overnight at room temperature for staining next day). Wrap other half with tape and dividers, place in labeled sample bags containing silica gel and store in archive boxes in minus 20°C room. Enter details in cut biopsy record book.

**Slides dipped in solution of 1.5 g gelatin in 250 ml distilled water + 0.1g chrome alum (added after gelatin is dissolved). Slides are then left to air dry and stored in large sandwich box.
Appendix 4

Clinical Data of Study Patients

BPH Patients

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## Appendix 4ii

- **Clinical Data of Study Patients continued**

### CaP Patients

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**Key:** Hist; histology; Mets, metastasis; NR, not recorded; NDM, no detectable metastases; Ac Pan, Acute Pancreatitis
Appendix 5

streptABComplex/HRP

STAINING PROCEDURE FOR CRYOSTAT SECTIONS AND PARAFFIN-EMBEDDED TISSUE SECTIONS

Sections are cut and placed on slides as for routine histological examination.

A Using a mouse primary antibody

<table>
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<th>Cryostat sections</th>
<th>Paraffin-embedded tissues</th>
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<tr>
<td>1) Fix in acetone for 10 minutes</td>
<td>1) Deparaffinize and rehydrate tissue sections</td>
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<tr>
<td>2) Incubate for 5 minutes with 0.6% hydrogen peroxide in methanol</td>
<td>2) Incubate for 5 minutes with 3% hydrogen peroxide in distilled water</td>
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The hydrogen peroxide will quench any peroxidase activity that may be present in the tissue.

3) Rinse with distilled water and place in Tris-buffered saline (TBS) for 5 minutes

4) Incubate for 20 minutes with normal rabbit serum (DAKO Code No. X 902) diluted 1:5 in TBS for blocking of non-specific background.

5) Tap off serum and wipe away the excess.

6) Incubate for 20 - 30 minutes with a mouse antibody diluted optimally in TBS.

7) Tap off liquid and place slide in TBS bath for 5 minutes.

8) Incubate for 20 - 30 minutes with biotinylated rabbit anti-mouse immunoglobulins (DAKO Code No. E 354 or E 413)** diluted 1:200 - 1:600 in TBS.

9) Same as step No. 7.

10) Incubate for 20 - 30 minutes with strepABCoomplex/HRP (DAKO Code No K 377) prepared according to instructions.

11) Same as step No. 7.

12) Incubate for 5 - 15 minutes with a peroxidase substrate solution***

13) Rinse with distilled water.

14) Counterstain and mount with coverslip.

B Using a rabbit primary antibody

Proceed as for the mouse antibody but replace steps 4, 6, and 8 with the following:

4) Incubate for 20 minutes with normal swine serum (DAKO Code No. X 901) diluted 1:5 in TBS.

6) Incubate for 20 - 30 minutes with a rabbit antibody diluted optimally in TBS.

8) Incubate for 20 - 30 minutes with biotinylated swine anti-rabbit immunoglobulins (DAKO Code No. E 353 or E 431)** diluted 1:300 - 1:800 in TBS.

*** Two common chromogens for peroxidase are 3, 3-diaminobenzidine tetrahydrochloride (DAB) and 3-amino-9-ethylcarbazole (AEC). DAB forms an end-product which is insoluble in organic and aqueous solvents. Thus, counterstains and mounting media containing organic or aqueous solvents can be used for slides stained with DAB. In contrast the AEC end-product is soluble in organic solvents and only an aqueous counterstain and an aqueous mounting medium should be used (for example Mayers hematoxylin and DAKO Glycergel Code No. C 563).

DAB substrate solution: Dissolve 6 mg of DAB in 10 mL of TBS, pH 7.6. Add 0.1 mL of 3% hydrogen peroxide. Filter, if precipitation occurs.

AEC substrate solution: Dissolve 4 mg of AEC in 1 mL of N,N-dimethylformamide. Add 14 mL of 0.1 M acetate buffer, pH 5.2. Add 0.15 mL of 3% hydrogen peroxide. Filter, if precipitation occurs.

The DAKO ""Handbook of Immunoenzymatic Staining Methods"" by Janice B. Wells is an excellent guide to immunohistochemical staining techniques and can be obtained from all DAKO distributors.

KP/25.02.91
Appendix 6a

Purification of Anti-EGFRvIII Antibody for Use in Immunohistochemistry.
Antibody against pepEGFRvIII (LEEKKGNYVVTEDHC) conjugated to Keyhole Limpet Hemocyanin (KLH) was prepared in New Zealand White rabbits as previously described (Humphrey). After the primary affinity purification, further purification was achieved by the removal of antibodies which cross-reacted with the normal EGF receptor. This was achieved by sequential adsorption onto Pierce Sulfo-Link beads (Rockford, IL) to which the flanking peptides LEEKKC- and NYVVTEDHC- had been conjugated (the C-terminal cysteine residue was included in all peptides for conjugation purposes). Western blotting confirmed that the antibody flow-through recognised only the EGFRvIII.
Appendix 6b

IMMUNOHISTOCHEMISTRY PROCEDURE FOR PARAFFIN SECTIONS USING AFFINITY-PURIFIED ANTI-EGFRvIII POLYCLONAL ANTIBODY


1. PREPARATION OF PARAFFIN SECTIONS:
   a) Tumors were paraffin embedded in an automated machine. Mouse tumors were fixed in paraformaldehyde 4% for 3-4 months at 4 C, or paraformaldehyde 4% for 24h and stored in PBS 3-4 months.
   b) 3-4 micron sections were cut, floated on warm water (55 C) and picked up on positively charged slides (LABCRAFT or FisherBrand Superfrost /Plus, catalog no. 12-550-15). **Do NOT use poly-lysine coated slides, as the sections will come off during the antigen retrieval procedure. Paraffin blocks must be put on ice for easy cutting, frequent immersion in cold water during cutting helps also.
   c) Dry the slides in an 37- 45 C oven 2-4 hours minimum, preferably overnight

2. DEPARAFFINATION PROCEDURE
   ** Deparaffinate only immediately before the immunostaining. Once deparaffinated you must go through the whole process and never let dry.
   In Coplan jar or in 50 ml Falcon tubes (2 slides/tube, back to back):
   1. Xylene (3 changes of 5 minutes each; for thick sections warm to 37 C)
   2. Absolute ethanol. (2 changes 2 minutes each)
   3. 95% Ethanol (2 changes 2 minute each)
   4. Wash in running water for five minutes.

3. BLOCKING ENDOGENOUS PEROXIDASE
   Incubate the slides in methanol containing 0.06% H2O2 for 20 minutes.

4. MODIFIED ANTIGEN RETRIEVAL PROCEDURE PURPOSE:
   Microwave Antigen retrieval produces improved staining for a wide range of monoclonal and polyclonal antibodies and reduces background staining. Essential for detection of EGFRvIII in paraffin sections.
   REAGENT: 10X H.I.E.R. Buffer (HIER101) (BioTek Solutions, Inc., 120 B Cremona Drive, Santa Barbara, CA 93117 USA); OR Vector Laboratories, Inc. #H-3300 Antigen Unmasking Solution, 100X (30 Ingold Road, Burlingame, CA 94010 USA; Phone (800) 227-6666; FAX (415) 697-0339.
   MICROWAVE: Whirlpool 750 Watts.
   MODIFIED ANTIGEN RETRIEVAL PROCEDURE:
   1. Wash paraffin section slides in distilled water.
2. Place slides in Tissue Tek Slide holder; always use 24 slides, use blank slides if necessary in order to maintain temperature.

3. Place in PBS into the holder 5 minutes (220 ml)

4. Fill the holder with 250 ml 1X working citrate buffer solution (HIER buffer)

5. Fit cap loosely, but secure with tape.

6. Position the slide holder in the center of the microwave; have paper towels to absorb liquid run over.

7. Microwave on HIGH for 5 minutes *

8. Open the microwave door. Add 15 - 25 ml distilled water and Microwave again on HIGH 4 minutes. (Check liquid level after 2 min; if necessary add more 1X unmasking buffer; sections must remain fully submerged at all times!).

9. Take out the slides immediately, open the lid. Allow slides to cool for 20 minutes in the microwave solution.

10. Rinse slides in three changes of distilled water 2 minutes each.

*Note: Time may need to be adjusted to compensate for differences between Microwave Brands and Models; water should be boiling within the first 3 minutes or so. Using the Vector Labs Antigen Unmasking Solution, we have found that addition of 15-25 ml dH2O plus 25 ml 1x unmasking solution is necessary after the initial 5 min. heating, and that the second 4 min. microwaving changed to 2 x 2 min., with an additional 25 ml unmasking solution added before the final 2 min. heating.

5. VECTASTAIN IMMUNOSTAINING

We use the Vectastain ABC Kit (Avidin DH/ biotinylated horseradish peroxidase H) from Vector Laboratories, 30 Ingold Road, Burlingame, CA 94010 USA; (415) 697-3600. Other such reagents probably work, but have not been tested.

1. Rinse the slides in distilled water 5 minutes.

2. Wash in 1X PBS for 20 minutes.

3. Incubate with 10% normal goat serum in PBS (serum from animal in which the secondary antibody was raised) for 20 minutes.

4. Blot the excess of serum and incubate in primary antibody (anti-EGFRvIII antibody at 1:2000 to 1:1000, i.e. 0.25 to 1µg/ml in 1X PBS, depending upon batch) for 2 hours (at lower conc. can be done overnight at 4 C) in a humid chamber.

5. Wash in 1X PBS very carefully. Use a wash bottle of PBS and a tray for collecting the PBS, let run PBS over the section several seconds; after that put the slides in the humid chamber, and incubate in PBS for 5 minutes 2 times.

6. Incubate the slides in secondary antibody (e.g. Vector Labs biotinylated goat anti-rabbit prepared according to the kit directions) for 30 minutes.

7. Repeat the PBS washes as above.

8. Incubate the sections in VECTASTAIN ABC solution for 30 minutes. It must be
prepared 30 minutes before use.

9. Repeat the PBS washes as above.

10. Incubate the sections 5 minutes in DAB substrate solution. (Diaminobenzidine and H2O2, prepared without modifications immediately before using). This results in a brown product.

11. Wash the slides in distilled water three times 2 minutes each.

6. COUNTERSTAIN

Prepare the following containers to go through:

1. Hematoxylin (40 seconds.)
2. Water.
3. Lithium carbonate (prepared 1% in water) 1 minute.
5. Ethanol 95 % (5X up and down)
6. Ethanol 95 % (5X up and down)
7. Ethanol absolute (5X up and down)
8. Ethanol absolute (5X up and down)
9. Xylol (5X up and down)
10. Xylol (5X up and down)

Let slides dry (20 minutes to overnight). Mount in Pro-Texx or similar medium.

NOTES: The antibody works very well with frozen sections, which should ideally be freshly cut, or stored sealed at -80 C before use (inconsistent results are obtained with sections stored for >24 hours). No antigen retrieval steps are needed, and staining steps are carried out in 1% BSA/PBS. One can also stain cultured cells grown directly on slides or coverslips and fixed with ice-cold methanol or with 2% formalin in PBS.
Appendix 7 - Final Immunostaining Protocols

Appendix 7i

Final Protocol for Streptavidin and Biotinylated Horseradish Peroxidase Staining

Technique for the detection of PSA in Paraffin-Embedded Prostatic Tissue Sections

Staining Kit: 1). DAKO streptABComplex/HRP
2). DAB chromogen

Paraffin-embedded tissue sections were supplied by the Pathology Department.

A. Using Anti-PSA dilution 1/10μl - Europath Laboratories

Paraffin-embedded sections

1) Remove excess wax by placing sections in 60 degree oven 5 min

2) Deparaffinize and rehydrate tissue sections

   Xylene 2 min
   99% alcohol 2 x 2 min
   95% alcohol 2 min

   Drain in-between each solvent

3) Rinse in first in running tap water and then in distilled water

4) Wash in TBS* pH 7.6 5 min

5) Immerse in 6% hydrogen peroxide in distilled water 30 min

6) Rinse in distilled water.

7) Equilibrate in Tris-buffered saline (TBS)* pH 7.6 5 min

8) Wipe excess buffer from slides and place slides in a humid chamber

   Do not allow the sections to dry.

9) Incubate with 100 ul of 20% normal goat serum in TBS to block non-specific staining. 20 min

10) Dilute mouse antibody optimally in 20% goat serum

11) Tap off excess liquid and wipe around sections.

12) Incubate with 100ul of diluted antibody in serum Overnight at 4°C

   (Leave normal goat serum on negative control sections)

13) Rinse sections twice with TBS 5 min

14) Wipe off excess buffer and replace slides into the humid chamber

15) Incubate with 120ul of secondary antibody diluted 1:400 in TBS Biotinylated Goat Anti-mouse Ig 30 min

16) Prepare streptABComplex/HRP immediately after applying secondary antibody:

   TBS 5ml
   Reagent A 1 drop(45ul) 1 drop(45ul) for 20 slides
   Reagent B Vortex

17) Rinse sections twice with TBS 5 min

18) Wipe off excess buffer and replace slides into the humid chamber
19) Incubate with 100ul of streptABCComplex/HRP solution **30 min**
20) Rinse sections twice with TBS **5 mins**
21) Prepare: **DAB Substrate**
   - Distilled water 5ml
   - Buffer stock 2 drops (vortex)
   - DAB 4 drops (vortex) **for 20 slides**
   - Hyd Peroxide 2 drops (vortex)
22) Wipe off excess buffer and replace slides into the humid chamber
23) Incubate with DAB substrate solution **30sec**
24) Rinse in distilled water
25) Counterstain with Mayer’s Haematoxylin **10 sec**
27) Dehydrate in graded alcohol, clear in xylene, and mount with XAM
Appendix 7 ii

Final Protocol for Streptavidin and Biotinylated Horseradish Peroxidase Staining Technique for the Detection of EGFR in Paraffin-Embedded Prostatic Tissue Sections

Staining Kit: 1). DAKO streptABComplex/HRP
               2). DAB chromogen

Paraffin-embedded tissue sections are supplied by the Pathology Department.

A. Using Anti-EGFR (clone 31G7) - Zymed laboratories mouse monoclonal antibody.

Paraffin-embedded sections

1) Remove excess wax by placing sections in 60 degree oven 5 min

2) Deparaffinize and rehydrate tissue sections:

   Xylene 2 min
   99% alcohol 2 x 2 min
   95% alcohol 2 min
   70% alcohol 2 min

   Drain in-between each solvent

3) Rinse in first in running tap water and then in distilled water

4) Wash in TBS* pH 7.6 5 min

5) Incubate with 0.1% pronase at 37°C 10 min

6) Incubate with 6% hydrogen peroxide in distilled water 30 min

Hydrogen Peroxide will block any peroxidase activity present in the tissue sections.

7) Rinse in distilled water.

8) Equilibrate in Tris-buffered saline (TBS)* pH 7.6 5 min

9) Wipe excess buffer from slides and place slides in a humid chamber

   Do not allow the sections to dry.

10) Incubate with 100 ul of 20% normal goat serum in TBS to block non-specific staining 20 min

11) Dilute mouse antibody optimally in TBS

12) Tap off excess liquid and wipe around sections.

13) Incubate with 100ul of 1/150ul dil anti-Ki67 in serum x 2h at room temperature or overnight at 4°C (Leave normal goat serum on negative control sections)

14) Rinse sections twice with TBS 5 min

15) Wipe off excess buffer and replace slides into the humid chamber

16) Incubate with 100ul of secondary antibody diluted 1:600 in TBS Biotinylated Goat Anti-Mouse Ig 30 min

17) Prepare streptABComplex/HRP immediately after applying secondary antibody:

   TBS 5ml
   Reagent A 1 drop(45ul) for 20 slides
   Reagent B 1 drop(45ul)
18) Rinse sections twice with TBS 5 min
19) Wipe off excess buffer and replace slides into the humid chamber
20) Incubate with 100ul of streptABComplex/HRP solution 30 min
22) Rinse sections twice with TBS 5 min
23) Prepare: **DAB Substrate**
   - Distilled water 5ml
   - Buffer stock 2 drops(vortex)
   - DAB 4 drops(vortex) } for 20 slides
   - Hyd Peroxide 2 drops(vortex)
   - Nickel 2 drops (vortex)
24) Wipe off excess buffer and replace slides into the humid chamber
25) Incubate with DAB substrate solution 30sec
26) Rinse in distilled water
27) Counterstain with Mayer’s Haematoxylin 10 sec
28) Mount with coverslip
Appendix 7iii

Final Protocol for Streptavidin and Biotinylated Horseradish Peroxidase Staining Technique for the detection of EGFRvIII in Paraffin-Embedded Prostatic Tissue Sections

Staining Kit: 1). DAKO streptABComplex/HRP
2). DAB chromogen

Paraffin-embedded tissue sections are supplied by the Pathology Department.

A. Using AntiEGFRvIII - Kimmel Cancer Institute rabbit polyvalent antibody

Paraffin-embedded sections

1) Remove excess wax by placing sections in 60 degree oven 5 min
2) Deparaffinize and rehydrate tissue sections
   Xylene 2 min
   99% alcohol 2 x 2 min
   95% alcohol 2 min
   Drain in-between each solvent
3) Rinse in first in running tap water and then in distilled water
4) Wash in TBS* pH 7.6 5 min
5) Microwave in citric acid pH 6.0 2 x 5 min
6) Incubate with 6% hydrogen peroxide in distilled water 30 min

Hydrogen Peroxide will block any peroxidase activity present in the tissue sections.
7) Rinse in distilled water.
8) Equilibrate in Tris-buffered saline (TBS)* pH 7.6 5 min
9) Wipe excess buffer from slides and place slides in a humid chamber
   **Do not allow the sections to dry.**
10) Incubate with 100 ul of 20% normal goat serum in TBS to block non-specific staining. 20 min
11) Dilute mouse antibody optimally in TBS
12) Tap off excess liquid and wipe around sections.
13) Incubate with 100ul of 1/150ul dil anti-Ki67 in serum x 2h at room temperature or overnight at 4°C (Leave normal goat serum on negative control sections)
14) Rinse sections twice with TBS 5 min
15) Wipe off excess buffer and replace slides into the humid chamber
16) Incubate with 100ul of secondary antibody diluted 1:600 in TBS Biotinylated Goat Anti-Mouse Ig 30 min
17) Prepare streptABComplex/HRP immediately after applying secondary antibody:
   TBS 5ml
   Reagent A 1 drop(45ul) for 20 slides
   Reagent B 1 drop(45ul)
   Vortex
18) Rinse sections twice with TBS 5 min
19) Wipe off excess buffer and replace slides into the humid chamber
21) Incubate with 100ul of streptABComplex/HRP solution 30 min
22) Rinse sections twice with TBS 5 min
23) Prepare: DAB Substrate
   
   Distilled water 5ml
   Buffer stock 2 drops(vortex)
   DAB 4 drops(vortex) for 20 slides
   Hyd Peroxide 2 drops(vortex)
   Nickel 2 drops (vortex)

25) Wipe off excess buffer and replace slides into the humid chamber
25) Incubate with DAB substrate solution 30sec
26) Rinse in distilled water
27) Counterstain with Mayer’s Haematoxylin 10 sec
28) Mount with coverslip
Appendix 7iv

Final Protocol for Streptavidin and Biotinylated Horseradish Peroxidase Staining

Technique for the detection of Ki-67 in Paraffin-Embedded Prostatic Tissue Sections

Staining Kit:
1). DAKO streptABComplex/HRP
2). DAB chromogen

Paraffin-embedded tissue sections are supplied by the Pathology Department.

A. Using Anti Ki-67 - Novacastra mouse monoclonal
   NCL-Ki67-MM1

Paraaffin-embedded sections

1) Remove excess wax by placing sections in 60 degree oven 5 min
2) Deparaffinize and rehydrate tissue sections

   Xylene 2 min
   99% alcohol 2 x 2 min
   95% alcohol 2 min
   70% alcohol 2 min

   Drain in-between each solvent

3) Rinse in first in running tap water and then in distilled water
4) Wash in TBS* pH 7.6 5 min
5) Microwave as per appendix 2 3 x 5 min
6) Incubate with 6% hydrogen peroxide in distilled water 30 min
   Hydrogen Peroxide will block any peroxidase activity present in the tissue sections.
7) Rinse in distilled water.
8) Equilibrate in Tris-buffered saline (TBS)* pH 7.6 5 min
9) Wipe excess buffer from slides and place slides in a humid chamber
   Do not allow the sections to dry.
10) Incubate with 100 ul of 20% normal goat serum in TBS to block non-specific staining 20 min
11) Dilute mouse antibody optimally in TBS
12) Tap off excess liquid and wipe around sections.
13) Incubate with 100ul of 1/150ul dil anti-Ki67 in serum x 2h at room temperature
    or overnight at 4°C (Leave normal goat serum on negative control sections)
14) Rinse sections twice with TBS 5 min
15) Wipe off excess buffer and replace slides into the humid chamber
16) Incubate with 100ul of secondary antibody diluted 1:600 in TBS Biotinylated Goat
    Anti-Mouse Ig 30 min
17) Prepare streptABComplex/HRP immediately after applying secondary antibody:

   TBS 5ml }
   Reagent A 1 drop(45ul) } for 20 slides
   Reagent B 1 drop(45ul) }

132
18) Rinse sections twice with TBS
19) Wipe off excess buffer and replace slides into the humid chamber
22) Incubate with 100ul of streptABComplex/HRP solution
22) Rinse sections twice with TBS
23) Prepare: DAB Substrate
   
   Distilled water 5ml } 
   Buffer stock 2 drops(vortex) } 
   DAB 4 drops(vortex) } for 20 slides 
   Hyd Peroxide 2 drops(vortex) } 
   Nickel 2 drops (vortex) } 
26) Wipe off excess buffer and replace slides into the humid chamber
25) Incubate with DAB substrate solution 30sec
26) Rinse in distilled water
27) Counterstain with Mayer’s Haematoxylin 10 sec
28) Mount with coverslip
Appendix 8
Results of Immunostaining experiments on prostatic tissues

Key to classification of scores:
1 = w (weak); 2 = m (moderate); 3 = s (strong).

A: PSA Staining of Prostatic Tissues

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w = 1(5%, 0-26%)
m = 2(11%, 1-33%)
s = 16(84%, 60-97%)
Appendix 8ib- PSA staining of CaP Glands

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\[w = 3\ (28\%, 6-61\%)
\[m = 3\ (28\%, 6-61\%)
\[s = 5\ (46\%, 17-77\%)

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\[w = 3\ (33\%, 10-65\%)
\[m = 3\ (33\%, 10-65\%)
\[s = 4\ (40\%, 8-81\%)

135
### Appendix 8ib contd

**G3 CaP**

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*a = 3 (18%, 2-51%)*

*w = 7 (41%, 14-73%)*

*m = 4 (24% 4-57%)*

*s = 3 (18%, 2-51%)*
## Appendix 8ib contd

### PSA Staining of Metastatic Tissues

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\[ w = 4 \ (33, 10-65) \]
\[ m = 7 \ (58, 28-85) \]
\[ s = 1 \ (8, 0-39) \]
Appendix 8iia

Key:

Classification of scores:
0 = a (absent); 1 - 33% = w (weak); 34 - 66% = m (moderate); >67% = s (strong).

HR = Hormone resistant disease

WT-EGFR Immunostaining Results in Prostatic Tissues

WT-EGFR Expression Normal/Atrophic Glands in BPH Sections

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Mean: 77.9m = 2 (11%, 1-40%)

SD: 8.6 s = 17 (89%, 60-9%)
### WT-EGFR Expression in Benign Hyperplastic Glands in BPH Sections

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Mean: 66.3 s = 13 (68%, 36 - 91%)

SD: 7.5 m = 6 (32%, 13-57%)
Appendix 8iib

*WT-EGFR Expression in HGPIN Glands in Primary CaP Sections*

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Mean 34m = 6 (43%, 18 - 71)
SD 15w = 8 (57%, 29 - 82)
### WT-EGFR Expression in Primary CaP Sections

#### G1 CaP

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**Mean**: 32.1  
**SD**: 25.8

- s = 2 (18.2%, 1-61%)
- m = 2 (18.2%, 1-61%)
- w = 5 (45.5%, 11-83%)
- a = 2 (18.2%, 1-61%)
Appendix 8iib contd

**WT-EGFR Expression in Primary CaP Sections**

**G2 CaP**

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**Mean** 19.3  
**SD** 23.6

- **m = 0** (0%, 0-41%)
- **w = 8** (80%, 35-99%)
- **a = 1** (10%, 0-51%)
Appendix 8iib contd

WT-EGFR Expression in Primary CaP Sections

G3 CaP

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Mean 17.8  s = 1 (6%, 0-36%)
SD 40.1  m = 1 (6%, 0-36%)

w = 13 (76%, 43-96%)
a = 2 (12%, 1-44%)
### WT-EGFR Expression in Metastatic CaP Sections

<table>
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<th>Intensity</th>
<th>% Cells</th>
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<th>Classification</th>
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</table>

Mean: 8  \( \text{m} = 1 \ (8\%, \ 0-39\%) \)

SD: 29  \( \text{w} = 1 \ (8\%, \ 0-39\%) \)

\( \text{a} = 10 \ (83\%, \ 52-98\%) \)
Appendix 8iiic

Cumulative Scores for WT-EGFR Expression in Prostatic Tissues

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<th>Intensity of immunoreaction</th>
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<td>(Total no)</td>
<td>Strong (%)</td>
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<td>77.9 (8.6)</td>
<td>17/19 (89)</td>
</tr>
<tr>
<td>BPH (19)</td>
<td>66.3 (7.5)</td>
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<tr>
<td>HG PIN (14)</td>
<td>34 (15)</td>
<td>0/14 (0)</td>
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<td>CaP (38)</td>
<td>22.4 (47.2)</td>
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<tr>
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<td>Lymph (6)</td>
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Key
- BPH = Benign prostatic hyperplasia
- HG PIN = High grade prostatic intra-epithelial neoplasia
- CaP = Primary prostatic cancer
## Appendix 8iiiA

*EGFRvIII Expression in Benign Hyperplastic Glands in BPH Sections*

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**Mean**

- s = 0 (0%, 0-24%)
- m = 2 (11%, 1-40%)
- w = 17 (89%, 60-100%)
- a = 0 (0%, 0-24%)

***Note: All Normal/atrophic glands were negative for EGFRvIII***
### Appendix 8iiiib

**EGFRvIII Expression in HGPIN Glands in Primary CaP Sections**

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Mean $s = 4$ (29%, 8 - 58)

SD $m = 10$ (71%, 42 - 91)

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### Appendix 8iiiib continued

*EGFRvIII Expression in Primary CaP Sections*

**G1 CaP**

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<td>3+</td>
<td>50</td>
<td>150</td>
<td>50</td>
<td>m</td>
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**Mean**  
\[56.7\]  
\[s = 4 (36\%, 7-77\%)\]

**SD**  
\[18.3\]  
\[m = 5 (45\%, 11-83\%)\]

\[w = 2 (18\% 1-51\%)]
### Appendix 8iiiib continued

*EGFRvIII Expression in Primary CaP Sections*

**G2 CaP**

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**Mean**

74.3 (s = 7 (70%, 26-96%))

**SD**

35.4 (m = 3 (30%, 4-74%))
**Appendix 8iiib continued**

*EGFRvIII Expression in Primary CaP Sections*

**G3 CaP**

<table>
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<tr>
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<td>280</td>
<td>70 s</td>
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Mean: 77.5 s = 15 (88%, 56-99%)

SD: 14.1 m = 2 (12%, 1-44%)
EGFRvIII Expression in Metastatic CaP Sections

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<th>No</th>
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<th>% Cells stained</th>
<th>Total</th>
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<th>Classification</th>
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<td>240</td>
<td>80</td>
<td>s</td>
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<td>2+</td>
<td>90</td>
<td>180</td>
<td>60</td>
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Mean: 83.5  s = 11 (92%, 62-100%)
SD: 8.9  m = 1 (8%, 0-39%)
### Appendix 8iiic

**Cumulative Scores for EGFRvIII Expression in Prostatic Tissues**

<table>
<thead>
<tr>
<th>Histology</th>
<th>Mean Score</th>
<th>Intensity of immunoreaction</th>
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<td></td>
<td>(Total no)</td>
<td>(SD)</td>
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<tr>
<td>Normal/Atrophic (19)</td>
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<tr>
<td>BPH (19)</td>
<td>17.9 (13.5)</td>
<td>0/19 (0)</td>
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<td>HGPIN (14)</td>
<td>55 (12)</td>
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<tr>
<td>CaP (38)</td>
<td>70.6 (16.5)</td>
<td>26/38 (68.4)</td>
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<td>G1 (11)</td>
<td>56.7 (18.3)</td>
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<td>G2 (10)</td>
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<td>G3 (17)</td>
<td>77.5 (14.1)</td>
<td>15/17 (88.2)</td>
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<tr>
<td>Lymph (6)</td>
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**Key**

BPH = Benign prostatic hyperplasia  
HGPIN = High grade prostatic intra-epithelial neoplasia  
CaP = Primary prostatic cancer
Appendix 8iva

*Ki-67 Expression in Prostatic Tissues*

**Classification of scores**

0-3 = 1 (low); >3 = h (high)

**Ki-67 Expression in control BPH Sections**

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<th>% Nuclei</th>
<th>Classification</th>
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**Mean**

<table>
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<tbody>
<tr>
<td>&lt;1</td>
<td>1 = 5 (100%, 35-100%)</td>
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**SD**

2
Appendix 8ivb

Ki-67 Expression in Primary CaP Sections

G1 CaP

<table>
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<th>Path No.</th>
<th>Histology</th>
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Mean: 3 low = 6 (67%, 22-96%)
SD: 4 high = 3 (33%, 4-78%)

G2 CaP

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Mean: 7 low = 4 (50%, 10-90%)
SD: 10 high = 4 (50%, 10-90%)

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Appendix 8ivb contd

Ki-67 Expression in Primary CaP Sections

G3 CaP

<table>
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Mean 12 low = 3 (18%, 2-51%)
SD 8 high = 14 (82%, 56-99%)
### Appendix 8ivb contd

**Ki-67 Expression in Metastatic CaP Sections**

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<th>Classification</th>
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<td>Low</td>
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**Mean**

9 low = 6 (100%, 41-100%)

**SD**

5
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