Prostate Specific Antigen Negative Prostate Cancer

A submission for the award of MD thesis by

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Abstract

Prostate specific antigen (PSA) has been used in the diagnosis and monitoring of prostate cancer for almost 20 years. Most men who present with metastatic prostate cancer have markedly elevated serum levels of PSA. However, approximately 1% of cases have serum PSA levels that are much lower than the tumour burden would suggest - so-called "PSA-Negative" tumours. Their diagnosis may be delayed, and management compromised. Little is known about this patient group. The aim of this study was to improve the understanding and management of "PSA-negative" prostate cancer.

The clinical history and tissue from 33 patients who presented with treatment-naïve metastatic prostate cancer and a serum PSA < 10 ng/ml were included in this study, the largest series so far reported. Clinical and immunohistochemical features were defined and alternative biomarkers investigated. Potential mechanisms underlying PSA-negativity were explored using prostate cancer cell lines and archival tissue.

From the clinical case notes review, patients presenting with low serum PSA and metastatic prostate cancer have a similar pattern of disease to men with high PSA prostate cancer. However, response duration to first line hormonal treatment and overall survival were shorter.

Immunohistochemistry performed on archival prostatic tissue has shown that the majority of the cancers are positive for PSA, despite low serum levels. The extent of PSA immunostaining is patchy and could be missed on biopsy. PSMA and AR are expressed, however, and represent alternative diagnostic aids. The study indicates that PSMA and PAP should be explored as potential serum biomarkers in this patient

group.

The androgen receptor (AR) remains expressed in over 90 % of these cases and therefore defects in this pathway are unlikely to explain the low serum PSA levels. Neither loss of heterozygosity nor gene methylation of AR or PSA appear to be mechanisms underlying low serum PSA levels.

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Papers

A.J.Birtle, A.Freeman, H.A.Payne, J.R.W Masters, S.J.Harland, & Contributors to the BAUS Section of Oncology Cancer Registry. "The clinical features of patients who present with metastatic prostate cancer and serum PSA < 10 ng/ml- the "PSA-negative" patients." *Cancer*, 2003;98(11):2362-2367

A.J.Birtle,A.Freeman, H.A.Payne, J.R.W.Masters & S.J.Harland. "Neuroendocrine differentiation in human prostate tissue: is it detectable and treatable ?" *British Journal of Urology International.*September 2003(92):490-492

A.J.Birtle, A.Freeman, H.A.Payne, J.R.W.Masters, S.J.Harland & Contributors to the BAUS Section of Oncology Cancer Registry. "The immunohistochemical characteristics of patients presenting with metastatic prostate cancer and a serum PSA of less than 10 ng/ml" In submission to the American Journal of Surgical Pathology.

A.J.Birtle, A.Freeman, H.A.Payne, J.R.W.Masters, S.J.Harland & Contributors to the BAUS Section of Oncology Cancer Registry. "Serum prostate specific antigen (PSA)-negative metastatic prostate cancer: histopathology and an alternative diagnostic marker." BCRM 2003 abstracts

Presentations

United States & Canada Academy of Pathology Meeting, Vancouver, March 2004. "Prostate specific membrane antigen (PSMA) may assist in the diagnosis of prostate cancer in patients presenting with low serum prostate specific antigen (PSA) relative to clinical stage."

British Association of Urological Surgeons Annual Meeting, Manchester, June 2003 "The clinical features of untreated metastatic PSA-negative prostate cancer"

British Cancer Research Meeting, Bournemouth, July, 2003. "Serum prostate specific antigen (PSA)-negative metastatic prostate cancer: histopathology and an alternative diagnostic marker"

Declaration

I hereby declare that this thesis is the result of my own work, and includes nothing which is the outcome of work done in collaboration, unless otherwise stated.

I further state that my thesis is not substantially the same as any I have submitted for a degree or diploma or any other qualification at any other University, and that no part has already been or is concurrently submitted for any such degree, diploma or other qualification.

Alison Birtle

August 2004.

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Chapter 1 General Introduction

1.1 Prostate Cancer Overview

1.1.1 Prostate Cancer Incidence

Prostate cancer is the second most frequent cause of male cancer deaths in the western world, accounting for 15.3 % of male cancers with 534,000 new cases worldwide (Greenlee 2001). In the UK, there are around 20,000 new cases and almost 10,000 deaths per annum (Parkin, et al. 2001). With an increasingly ageing population, the number of clinically significant cases will grow and it is likely that the incidence of prostate cancer will overtake that of lung cancer within the next 5 years (Office for National Statistics 1999).

1.1.2 Epidemiology of Prostate Cancer

1.1.2.1 Age

The mean age of men presenting with prostate cancer in the UK is 72.3 years with 80.5 % diagnosed over the age of 65 years (British Association of Urological Surgeons Section of Oncology 2002). From autopsy studies, it has been suggested that the majority of men aged 85 and above have histological evidence of prostate cancer (Breslow et al. 1977; Sakr et al. 1993), albeit small foci in many cases. For the majority of these men, prostate cancer was neither symptomatic nor a contributory factor in their deaths. It is important therefore, to identify those tumours that are more likely to cause a clinical problem by virtue of such prognostic factors as tumour size, histopathological grade and PSA.

1.1.2.2 Geography

There is a significant geographical and racial variation in prostate cancer incidence, with the highest incidence in Caribbean men, African-Americans in the USA (137 per 100,000 per year) and in Scandinavia, with the lowest incidence in Asia, particularly China (1.9 per 100,000 per annum). Whilst screening plays a part in the number of cases detected in the USA, it is clear that in the three main ethnic groups in the USA, there is a continuing disparity between incidence rates, with blacks having a 60% greater rate than Caucasians and 200 % higher than Orientals (JCNI Editor 1997). It is likely that multifactorial environmental and genetic factors are involved.

1.1.2.3 Genetic Predisposition to Prostate Cancer

A full description of this topic is beyond the scope of this thesis. It is, however, important to set the subject in context. 10-15 % of patients with prostate cancer have at least one relative who is also affected (Whittemore et al. 1995) and first-degree relatives of men with prostate cancer have a 2-3 times increased risk of developing prostatic malignancy. The risk is increased with the number of relatives affected and a younger age at diagnosis of the index case. Monroe (Monroe et al. 1995) suggested a recessive or X-linked pattern of inheritance, although an autosomal dominant inheritance of a high-risk gene has also been proposed (Carter et al. 1992a). Linkage analysis studies have mapped high risk loci to chromosomes 1, X, 20, 17 and 8 (Berry et al. 2000;Smith et al. 1996;Xu et al. 2001). Smith also showed evidence of a linkage to chromosome region 1q24-25 in North American and Swedish families, particularly in those with early onset disease and this locus was named HPC1. Polymorphisms of genes regulating androgen metabolism and apoptosis may also be involved in prostate cancer risk (Lange et al. 2000). The androgen receptor gene contains three polymorphic repeats. In

particular, the CAG repeat in exon 1, which codes for a polyglutamine track, has been studied. In vitro analysis has shown that the length of the repeat is inversely related to the transcriptional activity of the AR (Chamberlain, et al. 1994; Kazemi-Esfarjani et al. 1995). It has been suggested that there is heightened sensitivity to androgenic stimulation in prostatic epithelial cells with short CAG repeats (Elo & Visakorpi 2001) and thus increased oncogenic potential. The prevalence of short CAG repeats (< 22) is higher among African Americans than Caucasians (Irvine et al. 1995), with the longest repeats found among Chinese men (Hsing et al. 2000). This may partly explain the much higher risk of prostate cancer among African Americans, particularly when compared to Chinese men. Other polymorphisms that have been suggested to be associated with the risk of prostate cancer affect genes such as those for 5α-reductase type 2 (SRD5A2), cytochrome P450 family members (CYP3A4 and CYP17), and HPC2/ELAC2 (Elo & Visakorpi 2001). Mutations in BRCA1 and BRCA2, genes most usually associated with high-risk breast and/or ovarian malignancy, have been associated with a genetic predisposition for prostate cancer. Germline mutations in BRCA2 account for up to 5 % of cases of prostate cancer in familial cancer clusters. Edwards et al (2003) calculated an overall relative risk of 4.6 (3.5-6.2) for prostate cancer in 173 families identified as having BRCA2 gene mutations. In these families, germline mutations were present in 2% of men with prostate cancer who were aged 55 or younger (Edwards et al. 2003). The ongoing European IMPACT study (identification of men with a genetic predisposition to prostate cancer and their clinical treatment) should provide information on the role of both BRCA1 and BRCA2 in familial cases of prostate malignancy.

Chromosomal loss, in particular of 8p and 13q, is implicated in prostate cancer. It is

likely that inactivation of tumour suppressor genes at these loci is an early event in the development of prostate cancer (DeMarzo et al. 2003). In addition to mutation, an alternative mechanism to inactivate the remaining allele of a tumour suppressor gene is hypermethylation of the CpG islands of the promoter of the gene (see section 1.5.4). The most common genetic event in prostate cancer is probably hypermethylation of the π-class glutathione S –transferase (GSTP1) gene promoter (Lee et al. 1994). GSTP1 can detoxify environmental electrophilic carcinogens and oxidants, and may prevent oxidative DNA damage and hence mutational susceptibility (Nelson et al. 2001). Other genes known to be involved in the pathogenesis of the disease include metastasis suppressor genes such as CDH1 (E-cadherin) (Umbas et al. 1994), KA11 (Dong et al. 1995), CD44 (Gao et al. 1997) and PTEN (Liaw et al. 1997). Loss of tumour suppressor genes such as Rb (Bookstein et al. 1990) and p53 (Konishi et al. 1995) have also been implicated. Oncogenes identified as having a potential role in prostate carcinogenesis include ras, which is more common in Japanese rather than in western study populations of prostate cancer (Carter et al. 1990), c-myc (Buttyan et al. 1987) and c-erbB2 (Her-2/neu) (Zhau et al. 1996).

1.1.2.4 Environmental and Lifestyle Factors

The western diet, in particular high consumption of red meat, fat and dairy products (Chan et al. 2001;Hayes et al. 1999) has been linked to prostate cancer whilst diets high in phyto-oestrogens such as soya appear to have a protective effect (Strom et al. 1999). It may be the cooking process, in particular frying or charcoal grilling, which leads to the formation of potentially carcinogenic heterocyclic amines, rather than the meat intake itself, that is the contributory factor (Augustsson et al. 1999). Lycopene (an antioxidant found in tomato sauce), selenium and vitamin E have also been found to

have a preventative role (Giovannucci et al. 2002; Heinonen et al. 1998; Redman et al. 1998). Finally, insulin-like growth factor 1 (IGF-1), a growth factor involved in cancer cell metabolism, appears to confer a 1.7-4.3-fold increased risk of prostate cancer when present in high concentrations (Stattin et al. 2000).

1.1.2.5 Hormones

As prostate cancer does not occur in eunuchs, it is clear that there must be androgenic involvement in the development of the disease. Prostate cancer is known to be an androgen-responsive tumour and androgens are vital for the development of the normal prostate. Prostate cancer may be treated with medical or chemical castration, with response rates of around 80 % in the context of metastatic disease. In animal studies, both testosterone and its derivative dihydrotestosterone (DHT), which is produced within the prostate, have shown oncogenic ability (Gronberg 2003). It is attractive therefore, to link androgen concentrations to the pathogenesis of prostate cancer. Some authors have noted increased DHT levels in black men, lowest in Asian men, consistent with prostate cancer incidence in these racial groups (Parnes et al. 2005).

1.1.3 Diagnosis of Prostate Cancer

The majority of prostatic neoplasms produce a serum glycoprotein, prostate specific antigen (PSA) that may be used in both the diagnosis and monitoring of treatment of prostate cancer. Debate exists with regard to the usefulness of PSA-screening but it is at present the only serum marker validated for detection of prostate cancer in the asymptomatic patient. The combination of digital rectal examination (DRE), serum PSA level and transrectal ultrasonography (TRUS) form the basis of establishing a diagnosis of prostatic malignancy (Gerber & Chodak 1991). Histopathological diagnosis is

required to confirm the presence of carcinoma, and tissue is usually obtained by multiple core biopsies of the prostate performed under TRUS guidance. If a result using either DRE, TRUS or PSA is abnormal, the positive biopsy rate is 6-25 %; with two abnormalities this rises to 18-60 %, and if all three modalities are positive, the positive biopsy rate is 56-72 % (Gustafsson et al. 1992).

1.1.4 Histopathology of Prostate Cancer

The majority of prostate cancers (> 70 %) arise in the peripheral prostate zone with 5-15 % in the central region and the rest arising from the transition zone. Microscopic foci of latent prostate cancer are a common autopsy finding and approximately 30 % of men over the age of 40 have evidence of latent disease. Many of these microscopic tumours never become clinically evident due to the slow growth rate, but any tumour with a volume of greater than $0.5cm^3$ or one which is moderately or poorly differentiated is thought to be clinically significant (Partin et al. 1990). The histopathological pattern of prostate cancer correlates significantly with the biological behaviour. The Gleason grading system, which is based on the Veterans Administration Co-operative Urological Research Group (VACURG) study of more than 4000 patients between 1960 and 1975, is the most widely accepted method of histopathological grading. Although both intra-observer and inter-observer variability have been reported with the Gleason system (Cintra & Billis 1991;di Loreto et al. 1991), grade remains one of the strongest predictors of biological behaviour in prostate cancer, being strongly correlated with overall survival, disease free survival, and time to progression. The grade is used in nomograms to predict the likelihood of disease extension outside the prostate (Partin et al. 2001) and hence to determine treatment options. For example, a man with organ

confined disease but Gleason 8-10 will be offered different management strategies than if he had a well differentiated tumour. Poorly differentiated tumours have an improvement in survival and local control by the addition of long term hormonal therapy with an LHRH agonist to radical radiotherapy, (Bolla et al. 1997;Bolla et al. 2002).

Absence of the basal cell layer is critical in the diagnosis of adenocarcinoma of the prostate. Basal cells may be mimicked by compressed stromal fibroblasts but are usually only seen focally at the periphery of the glands. An intact basal cell layer is present at the periphery of benign glands, whereas in carcinoma, the basal cell layer is absent. Occasionally, diagnosis is hampered by observing small adenocarcinoma foci clustering around larger benign glands which have an intact basal cell layer, and confirmatory monoclonal antibody immunohistochemistry with such antibodies as cytokeratin 7 and 20, 5 and 14 (Bassily et al. 2000) or 34βE12 (Varma et al. 2003) is required to evaluate the basal cell layer. Perineural invasion and microvascular invasion, although not significantly associated with tumour grade, tend to be associated with more aggressive biological behaviour of the tumour (Epstein et al. 2000).

The percentage of biopsy cores involved with tumour has also been found to be predictive of the likelihood of recurrent or aggressive tumour, particularly in screen-detected T1c tumours where the probability of recurrence of disease strongly dictates management options (Gretzer et al. 2002). The percentage of cores positive for cancer has also been found to be a significant predictor of pathological stage after radical prostatectomy (Epstein et al. 2000;Gancarczyk et al. 2003).

1.1.5 Presentation of Prostate Cancer

Patients may present with asymptomatic prostate cancer detected at routine PSA evaluation, as an incidental finding in approximately 10% of men undergoing transurethral resection of the prostate or with symptoms attributable to the underlying neoplasm. The presenting symptoms of prostate cancer fall into two main groups: symptoms of urinary outflow obstruction (60-70%) and symptoms of advanced disease (30-40%), including haematuria, dysuria, incontinence, perineal pain, loin pain, rectal bleeding or obstruction, impotence and haematospermia. The classical pattern of metastatic disease is of lymph node metastases, bone metastases, and less commonly lung or liver (Small 1998). Bone metastases occur predominantly within the axial skeleton (85%) (Whitmore, Jr. 1984) pelvis and long bones, and may present with bone pain, spinal cord compression or be asymptomatic.

1.1.6 Staging of Prostate Cancer

The most frequently used staging system is the 2002 TNM (Tumour Node Metastasis) (Sobin & Wittekind 2002). The assessment of local tumour stage, in particular the distinction between intracapsular (T1 –T2) and locally advanced (T3-T4) disease has the greatest impact on treatment decisions. Clinical examination using DRE may underestimate the tumour extension and some authors have documented a positive correlation between DRE and pathological tumour stage in less than 50 % of cases (Spigelman et al. 1986). Both computed tomography (CT) and magnetic resonance imaging (MRI) may be utilised for the staging of local disease and pelvic lymph nodes with a reported accuracy of 66-83 % in distinguishing between organ-confined and extracapsular disease (Schiebler et al. 1993).

Elevated serum alkaline phosphatase levels may indicate the presence of bone metastases in 70 % of affected patients (Wolff et al. 1996). Bone scintigraphy using technetium diphosphonate is the most sensitive method of assessing bone metastases, being superior to clinical evaluation, bone radiographs, or serum levels of alkaline phosphatase or prostate specific acid phosphatase (McGregor et al. 1978).

Serum PSA levels increase with increasing age and age-related thresholds above which the likelihood of malignancy is greater have been established (Oesterling et al.1995). However, if PSA level alone is used in an individual patient, it seems to have a limited ability to predict the final pathological stage accurately. As PSA is produced by both benign and malignant prostatic tissue, there is a complex relationship between serum PSA concentration and clinical and pathological tumour stage. In the great majority of patients with metastatic disease, serum PSA is markedly elevated (Daver et al. 1988; Pantelides et al. 1992). However, when considering patients with apparently localised disease for potentially curative treatment options such as radical prostatectomy, radical external beam radiotherapy or Iodine^{125} brachytherapy, a combination of serum PSA level, Gleason score on prostate biopsy and clinical T stage have been used to construct nomograms to predict the likelihood of organ confined disease, seminal vesicle involvement and lymph node spread for particular PSA levels (Partin et al. 1997;Partin et al. 2001).

In the staging of newly diagnosed cases, various studies have recommended a threshold below which imaging investigations are unlikely to reveal metastases and hence argued against the need for imaging in certain circumstances because of the low cost:benefit ratio. In a study by Levran of 861 men with a PSA below 20 ng / ml, eight (0.9%) had

positive bone scans and 13 (1.5%) had CT- defined nodal disease (Levran et al. 1995). In reviewing 167 cases of M1 prostate cancer presenting over a 11 year period, eight cases with a serum PSA of less than 10 ng / ml were identified (Yamamoto et al. 2001a). Although the risk of having a positive bone scan increases with advancing tumour stage and grade, the positive predictive value remains low even in higher risk poorly differentiated tumours. Up to 5% of T1b or T1c have a positive bone scan (Gleave et al. 1996) and it can be argued that only in well differentiated, low stage with PSA <10 ng/ml tumours could a staging bone scan be potentially omitted.

1.1.7 Treatment of Prostate Cancer

The treatment of early prostate cancer can be divided into four therapies: radical prostatectomy, radical radiotherapy using external beam or brachytherapy implant, hormonal therapy alone or active surveillance. Decision-making is based upon the prognostic factors as described above and the patient's preferences. The current UK ProTecT (Prostate testing for cancer and treatment) study aims to randomise 100,000 men, counselled before PSA testing with regards to the implications of a raised PSA, and to randomise patients with a histologically proven tumour, to either active monitoring, radical prostatectomy or radical radiotherapy, with primary endpoints of 10 and 15 year survival. In addition, disease progression, complications, health economics and quality of life assessments will form the secondary endpoints (Donovan et al. 2003).

For locally advanced disease, combined modality treatment with external beam radiotherapy and hormonal therapy is usually pursued. In the context of metastatic disease, hormonal treatment remains the mainstay, with palliative radiotherapy reserved for areas of symptomatic discomfort. The median response duration to first line

hormonal therapy in metastatic disease, usually with an LHRH analogue, is around 18 months (Eisenberger et al. 1998). On initiation of second-line hormonal therapy, usually with the addition of an anti-androgen or oestrogen such as diethylstilboestrol, responses are seen in 35-45 % of patients, (Small & Vogelzang 1997) with a median survival time of 9-12 months after commencing second –line treatment (Hussain et al. 1994). Median overall survival in treated metastatic prostate cancer is around 27 months from the time of diagnosis. (Eisenberger et al. 1998).

1.2 PSA-Negative Prostate Cancer

1.2.1 Definition

In general terms, a "PSA-negative" prostate cancer produces much lower serum levels of PSA than the tumour burden would suggest. A PSA value of less than 10 ng/ml is often used as the value for determining suitability for radical prostatectomy. For the purposes of this thesis, therefore, I have defined PSA-negative disease as patients with serum PSA levels of less than 10 ng/ml despite the presence of widespread progressive metastatic disease.

1.2.2 Clinical and Pathological Aspects of PSA-Negative Prostate Cancer

PSA-negative prostate cancer may be divided into two groups:

- Treatment-naïve patients in whom the serum PSA is never raised : Primary PSA-negative

- Patients relapsing after previous treatment, usually for clinically localised prostate cancer, whose initial tumour was PSA-positive but now fails to produce raised levels of serum PSA: PSA-negative at recurrence

1.2.2.1 Primary PSA-Negative

At initial presentation serum PSA levels are much lower than expected given the volume of disease present. These may be locally advanced or metastatic tumours. The level of serum PSA in the great majority of patients presenting with metastatic disease is markedly increased (Daver et al. 1988). Given that 10 ng/ml is the usual cut-off for consideration of radical surgery (Peschel & Colberg 2003), a serum level below this value in a patient with clinically apparent metastatic disease is remarkable (Oommen et al. 1994;Wolff et al. 1998;Yamamoto et al. 2001a). PSA-negative disease has been estimated at occurring in 0.5-1% of prostate cancer patients with PSA levels below 10 ng/ml. (Oesterling et al. 1993b). There is a paucity of information on these tumours and the majority of the literature is based on small series and case reports. The literature is summarised in Table 1.1. Advanced prostate cancer associated with low PSA has generally been linked to highly undifferentiated tumours (Gleason 8-10) (Partin et al. 1990). This is in contrast to the majority of poorly differentiated prostate cancers which tend to be associated with higher levels of serum PSA because they are more advanced (Partin et al. 1990). In 7/8 cases presenting with serum PSA< 10 ng/ml and widespread metastatic disease over an 11 year period, 3 year survival was less than 33 % (Yamamoto et al 2001). The median survival for M1 prostate cancer overall has been reported as 24-27 months (Hussain et al 1994). The small series of PSA-negative prostate cancer reported in the literature have suggested a sub-optimal response to primary hormonal manipulation, and this has been postulated by Gaffney (Gaffney et

al. 1992) as being associated with a so-called "functional dedifferentiation" and more aggressive biological behaviour. An alternative model is that the tumours more closely resemble prostate stem cells and thus lack the cytological markers of terminal differentiation. A high rate of visceral metastases (up to 55 %) has also been suggested (Sella et al. 2000) and a non-typical pattern of bone metastases observed, with lytic rather than the characteristic osteloblastic and sclerotic bone lesions usually seen in prostate cancer. A significant link ($p<0.0001$) between low PSA (< 20 ng/ml) metastatic prostate cancer and osteolytic bone metastases has also been noted (Doherty et al. 1999).

Some histological subtypes of prostate cancer, including ductal adenocarcinoma, small cell carcinoma or those with neuroendocrine differentiation are associated with lower levels of PSA. These subtypes may occur in the treatment naïve patient or in the setting of disease recurrence after primary therapy.

1.2.2.2 PSA-Negative at Recurrence

This group of patients initially present with raised levels of PSA appropriate to their stage of disease as previously outlined. Following initial treatment with either radical prostatectomy, radical radiotherapy or androgen deprivation, they are deemed to have responded to treatment and continue on follow-up. They subsequently develop a local recurrence or distant metastases, but now fail to produce appropriate levels of PSA. It is possible that prostate tumours may dedifferentiate on recurrence and this can be associated with a secondary failure to produce PSA. A repeat biopsy in this setting often shows a large percentage of cells with neuroendocrine features, particularly in the context of previous hormonal treatment. Mai and colleagues (Mai et al., 1996) reported

two cases of hormone treated organ confined prostate cancer, which at subsequent metastatic relapse, had normal levels of serum PSA and complete absence of immunostaining at tissue level for PSA or PAP. Sciarra recently suggested that neuroendocrine markers such as Chromogranin A and neuron specific enolase (NSE), might complement the PSA assay in selected cases of poorly differentiated tumours (Sciarra et al. 2003). Further, that an increase in Chromogranin A expression, despite low PSA levels, may indicate progression and a poor prognosis. Tissue neuroendocrine markers including Chromogranin A and NSE were identified in a series of 18 patients with clinically progressive androgen-independent prostate cancer and low serum PSA, (Sella 2000) leading to the belief that the majority of low serum PSA metastatic prostate cancers were neuroendocrine in origin. It has been suggested that hormonal therapy enhances neuroendocrine differentiation, although this remains controversial (Van de Voorde et al. 1994). It is postulated that the hormonal treatment suppresses the non-neuroendocrine cells while having minimal effect on the neuroendocrine population, which themselves lack androgen receptors (Bonkhoff, Stein et al. 1993). Thus several authors have argued that both treatment naïve metastatic prostate cancer and those cases of clinically progressive hormone refractory prostate cancer which have extensive and multifocal neuroendocrine features are similar and that this is the mechanism for the low serum levels of PSA (Abrahamsson 1996;Bonkhoff 1998).

1.3 PSA as a Tumour Marker

1.3.1 Definition of Tumour Marker

A substance detectable in body fluids that is produced by, or in association with, tumours (Malkin 1992). Tumour associated antigens are those associated with tumours

arising from specific organs, but antigens with absolute specificity have not been isolated for any type of cancer.

Some uncertainty exists as to the first identification of PSA, thought to be by Ablin (1970), although the protein purification is generally attributed to Wang in 1979 (Wang et al. 2002) and PSA was first isolated from the serum of patients with prostate cancer in 1980 (Papsidero et al. 1980). The landmark description of PSA as a tumour marker was some years later (Stamey et al. 1987) and PSA was pioneered as a screening test for prostate cancer by Cooner (1988) and Catalona (1991) in the 1980's. Early biopsy studies by Cooner (1988) in patients with abnormalities detected on ultrasound, showed a strong correlation between biopsy results and sensitivity, specificity, and both positive and negative predictive values for PSA above a threshold of 4.0 ng/ml for ultrasound detected abnormality. Together with other studies, this led to the threshold of 4 ng/ml being used by many clinicians as an indication for biopsy. Between one third and one half of men with a PSA level above 4.0 ng/ml will have cancer on sextant biopsy of the prostate (Partin et al. 1990). The difficulty lies in determining which of these tumours would have become clinically significant.

1.3.2 Sensitivity and Specificity of PSA

1.3.2.1 Definition

Sensitivity describes the accuracy in detecting individuals who have malignancy whereas specificity is the ability to exclude non-malignancy.

1.3.2.2 PSA Screening

Although PSA has shown 58-90% sensitivity for the detection of prostate cancer, when examining large series of men with a PSA of > 4.0 ng/ml, particularly when compared

to its predecessor prostatic acid phosphatase (PAP) (Myrtle et al 1986), its specificity of around 60 % is not sufficient to allow its use as a sole agent in mass screening. Using a single value for men of all ages leads to the exclusion of a large number of patients with clinically significant early-stage disease. This is because 20-50% of such tumours occur in men with serum total PSA (tPSA) levels of <4 ng/ml (Schroder et al. 2000;Stenman et al. 1994). As serum PSA usually increases with age, partly due to BPH, age-related cut-off values have been suggested to increase the sensitivity in young patients and the specificity in older populations (Oesterling et al. 1993a). However, there remains debate about the use of these age-specific ranges as some authors argue that they result in missing an unacceptable number of clinically significant tumours in older men (Catalona et al. 2000).

With the advent of PSA screening in the USA in the 1990's, there has been a stage migration in prostate cancer at presentation, with a substantial increase in the numbers of early prostate cancers detected (Han et al. 2001). In the USA, T1c tumours are currently the most commonly diagnosed prostate tumour (> 60%) (Partin et al. 1990). In the U.K. this trend has also been noted to a lesser extent, with T1c cases representing 17.4 % of new prostate cancer diagnoses on the BAUS Cancer Registry 2001 database (British Association of Urological Surgeons Section of Oncology 2002). In total, 57.9 % of patients presented with organ confined disease (compared with 51.8 % in 1999), 30.2 % with locally advanced and 11.9 % with metastatic tumours. In addition, more men are presenting with well to moderately differentiated tumours (Gleason 4-6) and PSA levels of less than 10 ng/ml, often used as a cut-off for considering radical prostatectomy (Peschel & Colberg 2003). Although the incidence of prostate cancer in the USA has begun to fall (Hankey et al. 1999), it would appear that the cases had

begun to plateau before the advent of PSA screening, and that screening alone has not been responsible for the decline (Paquette et al. 2002). In other countries declines in prostate cancer mortality were observed within a few years of the introduction of wide-scale screening (Bartsch et al. 2001; Chirpaz et al. 2002),and so any screening effect must be due to early deaths avoided. This is perplexing, as the average mean lead time for PSA screening (the time screening brings forward the diagnosis in time) is at least 5 years, (Etzioni et al. 1998) while median survival of localised prostate cancer is > 10 years compared to around 30 months for advanced disease (Beemsterboer et al. 1999). Hence no effect would be expected for at least 5 years from starting a screening programme, other than that due to the earlier detection and management of advanced disease. Improved survival among cases diagnosed before screening commenced, due to more aggressive treatment options could well have contributed to the fall in deaths, especially since a similar decrease in prostate cancer mortality has been observed in the UK, despite the relative lack of PSA screening in the UK (Oliver et al. 2000). Conflicting advice exists in the USA with regard to prostate screening however. Current guidelines from the American Cancer Society and the American Urological Association are for annual PSA and DRE in men who are over the age of 50 and have a life expectancy of 10 years (Cookson 2001). In contrast, the Guide to Clinical Preventive Services from the US Preventive Services Task Force is not in favour of screening (diGuiseppi 1996). Crawford and colleagues recommended stratifying patients with PSA of less than 4 ng/ml, and that 5 yearly PSA testing was adequate for men with initial readings of <1, and 2 yearly for those with a PSA of 1-1.9. This lead to an earlier potentially positive test being missed in only 2.6 % of men (Crawford et al. 1999). In the UK, no formal guidelines currently exist for PSA testing, other than in men with

symptoms of bladder outflow obstruction, although the introduction of information packs outlining the pros and cons of PSA testing for men enquiring about PSA checks has begun.

At present two large-scale multi-centre randomised controlled trials are in progress: the European Randomised Study of Screening in Prostate Cancer (ERSPC) and the Prostate, Lung, Colon and Ovary Trial (PLCO) in the US, with more than 200,000 men randomised since the trials began in 1994 and 1995 respectively (de Koning et al. 2002). Together they have sufficient statistical power to detect a mortality reduction of 20 % or more after about 10 years of follow-up. At present in the ERSPC trial, over a third of prostate cancers identified were in the group of men with PSA concentrations lower than 4 ng/ml. (Schroder et al. 2000). Because of this, a protocol amendment has meant that prostatic biopsies are now performed on all men with PSA of 3 ng/ml and greater in the ERSPC study (Schroder & Wildhagen 2001). The first trial analyses, expected around 2008, should provide valuable evidence with regard to the benefit or not of population-based PSA screening for prostate cancer.

1.3.3 Improving PSA specificity

1.3.3.1 Use of Free PSA / Total PSA Ratio

In normal prostate, most free PSA (fPSA) consists of the mature protein that has been inactivated by internal proteolytic cleavage. In prostate cancer, the cleaved fraction is relatively decreased, resulting in lower fPSA to tPSA ratios (PSA index). In studies of men with tPSA between 4 and 10 ng/ml, a PSA index greater than 25 % indicates a risk of prostate cancer of less than 8 %, whilst the corresponding risk with a PSA index of less than 10% is much greater, more than 56 % (Catalona et al. 1997;Van Cangh et al.

1996). There appears to be an inverse relationship between fPSA percentage and increased histopathological grade and hence more aggressive tumours (Li et al. 1999). It may be of use in detecting unfavourable pathological findings in the presence of inconspicuous values of tPSA. The PSA index may also increase the sensitivity and specificity of PSA testing in men with < 4ng/ml tPSA, potentially reducing unnecessary biopsies (Djavan et al. 1999;Vashi et al. 1997).

1.3.3.2 PSA Density

To compensate for larger prostates which contain more PSA-producing epithelium, and the contribution of BPH to serum PSA levels, serum PSA is normalised to the prostate volume by dividing the PSA value by a TRUS derived volume, giving the PSA density. Densities greater than 0.15 have been thought more suggestive of prostate cancer (Benson et al. 1992). This method is limited, however, by variations in prostate shape, and also the ratio of epithelium to stroma. Catalona (1994) in a study comparing PSA and PSA density in detecting early prostate cancers, found that nearly half the cancers would be missed if the 0.15 density level was used as the determinant for a biopsy. It is likely that using free PSA and PSA density together may improve diagnostic accuracy.

1.3.3.3 PSA Velocity

PSA velocity is the slope of increase in PSA levels over time (Carter et al. 1992b) with values greater than 0.75ng/ml/year being thought predictive of clinical prostate cancer. However, this test is limited by the need for previous values being recorded, the uncertainty about the optimum time between PSA measurements and biological variations in PSA levels from non-malignant causes. The latter is particularly relevant as biological variability may account for a 25-30% difference between two PSA

measurements (Nixon et al. 1997).

1.4 The Biochemistry of PSA

1.4.1 General Overview

PSA is a serine protease, with a molecular weight of 28,430 daltons, and is a member of the tissue kallikrein family of proteases (Yousef & Diamandis 2001). It is produced principally by prostatic ductal and acinar epithelium. Small amounts are also found in paraurethral and perianal glands, apocrine sweat glands, breast tissue and thyroid, although these make no appreciable contribution to measurable serum levels. It has also been recorded at low levels in breast cancer (Partin et al. 1990) but the expression as determined immunohistochemically tends to decrease with increasing grade (Abrahamsson et al. 1988). Normal prostatic tissue contributes on average 0.2 ng/ml per gram of tissue to the serum concentration of PSA. The corresponding value for BPH tissue is 0.6 ng/ml and for cancerous tissue it is 2.0 ng/ml (Stamey et al. 1987). The normal prostate contains PSA levels about one million-fold higher than in serum with seminal fluid levels in the order of 0.2-5 mg/ml (Rittenhouse et al. 1998).

1.4.2 PSA as a Tissue Kallikrein

Kallikreins were initially described as serine proteases which digest particular high molecular weight proteins to reduced kinins or bioactive peptides. Two subdivisions exist: tissue and plasma kallikreins. 15 tissue kallikrein genes have been identified, (KLK 1 to 15), located on chromosome 19Q13.3-q13.4 (Yousef & Diamandis 2001). The genes cluster in a 60kb region and KLK1 is closest to the centromere (Figure 1.1). There is 40-80% sequence homology between kallikreins at both the DNA and protein level (Yousef et al. 1999), particularly between KLK1, 2 and 3 which are known as the

classical kallikreins, 4-15 being termed "new" kallikreins. KLK3 encodes for hK3 (human kallikrein 3) better known as PSA. The major difference between classical and new kallikreins is that the new ones contain extra exons (either 5' or 3') (Yousef & Diamandis 2001). The "kallikrein loop" is a 9-11 amino acid peptide sequence found only in classical kallikreins and precedes the aspartic acid in the active site (Yousef & Diamandis 2001). KLK3 (PSA) has been found to have splice variants in addition to the main 1.6kb transcript and the gene contains 4 introns and 5 exons, in common with the other classical kallikreins (Riegman et al. 1989). At least 12 of the kallikrein genes are steroid hormone regulated (Yousef & Diamandis 2001) and are expressed in multiple tissues. In the prostate, KLK 2, 4, 10, 11, 13 and 15 are all expressed and may share regulatory factors that are organ specific. KLK2 and its product hK2 (prostate specific glandular kallikrein) in particular shares 80 % sequence homology with PSA and is thus the subject of interest as a tumour marker (Stephan et al. 2000). Co-ordinated levels of expression of hK2 and PSA (Herrala et al. 2001) in both abnormal and normal prostatic tissue have been demonstrated and hK2 can activate the conversion of inactive (proPSA) to active PSA (see section 1.5.7) (Kumar et al. 1997). Herrala et al (2001) also demonstrated almost double the expression of mRNA for hK2 in prostate carcinoma tissues compared with PSA and the use of hK2 may enhance prostate cancer detection. The KLK 15 gene is also up regulated at the mRNA level in prostate cancer in comparison to normal prostatic tissue and has been associated with more aggressive tumours (Yousef et al. 2001).

1.4.3 Role of Kallikreins

The exact role of the tissue kallikreins in malignancy is not clear. Serine proteases are

involved in tumour progression, such as invasion, proliferation and tumour metastasis (Carroll & Binder 1999; DeClerck et al. 1997; Tryggvason et al. 1987). However, conflicting data suggests that they also act as tumour suppressors (Goyal et al. 1998), as anti-angiogenic factors (Fortier et al. 1999b), as apoptotic molecules (Balbay et al. 1999) or negatively regulate cell growth (Lai et al. 1996). KLK 10 expression is down-regulated in aggressive forms of prostate cancer (Luo & Diamandis 2000) and has been shown in animal models to act as a tumour suppressor gene in breast cancer (Liu et al. 1996).

1.4.4 Specific Function of PSA

Villoutreix and colleagues (Villoutreix et al. 1994) have shown that in comparison with other serine proteases, aspartate 189 is replaced with serine in the substrate binding pocket of PSA giving it a chymotrypsin-like activity and is hence responsible for the liquefaction of semen by the cleavage of semenogelin I and II in the seminal fluid (Lilja et al. 1987). The proteolytic action of PSA may cleave key signalling molecules such as insulin, interleukin-2, fibrinogen, PTHrP (Cramer et al. 1996) and TGFβ (Killian et al.1993). For example, insulin-like growth factor binding protein-3 (IGFBP-3), which is the major serum binding protein for insulin-like growth factor-1 (IGF-1) can be cleaved by PSA (Cohen et al. 1992). IGF-1 appears to have a mitogenic effect on prostate cancer and increased serum levels of this factor have been identified as a risk factor for prostate cancer (Chan et al. 1998). Cohen et al (1994) have demonstrated that cleavage of IGFBP-3 by PSA in vitro reduces binding of IGF-1 and can increase proliferation in response to added IGF-1. However, the significance of this PSA-induced cleavage in vivo is uncertain, although after definitive local treatment of prostate cancer by

prostatectomy, serum levels of IGFBP-3 have been found to be raised, suggesting an anti-tumour effect of intact IGFBP-3 (Bubley et al. 2002). PSA may play a role in bone metastases and osteoblastic responses. Potent mitogenic effects on osteoblasts in vitro by PSA have been shown, mediated either through TGFβ, or by alterations in cell surface receptors of the osteoblasts (Killian et al. 1993). In contrast, PSA's action of cleavage on PTHrP reduces the mitogenic action. Given that osteoblastic bone metastases are characteristic of prostate cancer, it is likely that PSA plays a key but as yet ill-understood role in the development of bone metastases.

It has been suggested that PSA may affect proteins involved in cell migration and invasion, facilitating tumour progression. It is involved in the cleavage of components of the extracellular matrix, and neutralising antibodies to PSA have been shown to prevent micro invasion of LNCaP cells, a prostate cancer cell line derived from a lymph node metastasis, in vitro (Webber et al. 1995). PSA, like hK2, can activate urokinase-type plasminogen activator by cleavage, which may promote invasion (Yoshida et al. 1995). However, PSA may have a tumour inhibiting rather than a tumour promoting capacity. It has been shown to inactivate fibroblast growth factor 2 (FGFR-2) (Heidtmann et al. 1999) and vascular endothelial growth factor (VEGF) (Fortier et al. 1999a), both factors being angiogenic in their normal state. This has been supported by in-vivo work examining paraffin–embedded prostate cancer sections, in which an inverse relationship was seen between PSA expression and microvessel density, this microvessel densitiy being used as an index of tumour angiogenesis (Papadopoulos et al. 2001). It is clear that the role of PSA as a tumour promoter or tumour suppressor is complex and has yet to be established. It can be argued that if PSA contributed significantly to the invasive potential of prostate cancer, one would expect the disease to

behave in a much more aggressive fashion than it actually appears to do.

1.4.5 PSA in Monitoring Treatment Response

After definitive primary treatment, PSA levels can be used to monitor the patient. Any detectable PSA subsequent to radical prostatectomy is interpreted as a sign of disease recurrence. PSA levels after radical radiotherapy are more complex to interpret: PSA levels usually remain low but still detectable after treatment, particularly after seed brachytherapy where a PSA "bounce" of between 0.2-0.3 ng/ml, is seen during the first 3 years after the implant (Cavanagh et al. 2000). The ASTRO definition of 3 successive rises after radiation therapy is in common use, although nadir PSA levels are also helpful in predicting relapse (Critz et al. 1996).

1.4.5.1 PSA in Recurrent Prostate Cancer

Killian et al. (1985) described how PSA elevation was seen prior to clinical relapse in 92 % of patients after primary local therapy. However, there is wide variation in the time from PSA relapse to clinical relapse, one study showing an 8-year interval in patients after radical prostatectomy (Pound et al. 1999). PSA doubling time was found to be of help, particularly when doubling occurred in less than 10 months (Partin et al. 1994).

1.4.5.2 PSA in Metastatic Prostate Cancer

In patients with metastatic prostate cancer serum PSA falls significantly after medical castration with LHRH analogues, and is accompanied by improvements in clinical symptoms. A PSA nadir of < 4 ng/ml has been identified as an indicator of the likely duration of remission (Miller et al. 1992). Debate exists as to whether PSA is an acceptable surrogate marker of response in metastatic disease. Its use in this setting is

attractive, given that it is often difficult to measure improvement of index lesions on bone scans accurately, due to the comparatively slow improvement in scan appearances. However, PSA responses may not reflect tumour responses, particularly in androgen-independent disease. In one study of orchidectomy and flutamide versus orchidectomy alone (Eisenberger et al. 1998), a PSA nadir of < 4 ng/ml was achieved by significantly more patients in the combination arm, but there was no association with increased overall survival. In contrast, retrospective series, (Kelly et al. 1993; Scher et al. 1999) suggest that a greater than 50 % fall in PSA after treatment is predictive of improved survival and thus PSA remains an important, albeit flawed, marker of response in the metastatic setting.

1.5 Hypotheses for PSA Negativity

Three main hypotheses may be considered, each of which will be discussed in greater detail:

a) Changes at the gene regulatory level

b) Alterations in the protein degradation pathways within prostatic tissue.

c) An alteration in protein processing and secretion

Before looking at the potential factors involved in the regulation of the PSA gene, an overview of the key processes is required.

The expression of a protein-encoding gene involves several steps: transcription of the gene; extensive processing of the initial product, the "precursor" messenger RNA; and export of the correctly processed "mature" mRNA from the nucleus to the body of the cell.

1.5.1 Gene Transcription

1.5.1.1 Definition

The process whereby one DNA strand is used as a template to synthesise a

complementary RNA sequence.

1.5.1.2 General Control of Transcription

DNA is transcribed into RNA by DNA-dependent RNA polymerases (RNA pol). The polymerases are part of the transcription complex, a larger aggregate of proteins that moves in a 5' to 3' direction along the DNA from a point of initiation to a point of termination (Lewin B 1994b). There are three RNA polymerases, RNA pol I, II and III, transcribing ribosomal RNA, messenger RNA and transfer RNA respectively. The activity of RNA polymerase II, and hence the rate of transcription and expression, is tightly controlled. The control involves two particular components - promoter or enhancer sequences upstream or downstream of the gene and transcription factors.

1.5.1.3 Promoters

Each promoter contains specific sets of short conserved sequences, recognised by the transcription factors and / or RNA pol. A promoter consists of a number of short conserved motifs or nucleotide sequences, the vast majority of which lie upstream of the start point. Although RNA pol I and III recognise a comparatively restricted group of promoters, those used by RNA pol II exhibit a greater sequence variation. The same DNA motif may be shared by multiple promoter sequences. One such is the consensus sequence TATA, the "TATA box". This sequence is usually located 25 base pairs upstream of the transcription start site. The factors that can act together with RNA pol II include basal factors, upstream factors and inducible factors. Basal factors interact with

RNA pol to form a complex around the start point and hence are involved in initiating RNA synthesis at all promoters. Upstream factors are DNA-binding proteins, which recognise specific short consensus elements upstream of the start point. These factors are themselves ubiquitous, and may act upon any promoter with the correct DNA binding site. These upstream factors improve initiation efficiency. Inducible factors act in a regulatory capacity and have a similar mode of action to the upstream factors. They are, however, activated at particular times, or are tissue specific. They bind key areas in genes known as response elements.

1.5.1.4 Enhancers

The activity of a promoter may be increased by an enhancer, a conserved sequence that may be positioned at a point varying from a few hundred to many thousand kilo bases from the transcriptional start site (Muller et al. 1988). The position of the enhancer relative to the promoter is not fixed and may be either upstream or downstream of a gene. Indeed, it can also function with the nucleotide sequence in either orientation. The essential role of an enhancer may be to increase the transcription factor concentration in the local environment of the promoter. Figure 1.2 illustrates some of the gene regulatory elements of transcription.

Initiation by RNA pol II may occur in a variety of ways; however the key factor is the binding of upstream or inducible factors to the specific sequence elements upstream of the start point. This binding leads to the formation of the transcription complex.

1.5.1.5 Transcription Factors.

These are proteins that bind to specific DNA sequences within promoters and enhancers, but are not themselves part of RNA polymerase. Some transcription factors

bind to multiple promoter regions and are essential in the transcription process. In contrast, some ensure tissue-specific expression of a gene, by binding only to promoters and enhancers associated with particular genes. The transcription factors often share short conserved amino acid sequences specific for DNA binding. These include the leucine zipper, which contains a leucine amino acid every seven residues along the polypeptide chain of the transcription factor. It is likely that these residues allow dimerisation of the transcription factors such as AP1 prior to DNA binding. In AP1, the oncogenic proteins Jun and Fos form heterodimers necessary for AP1 activation.

1.5.1.6 Basal Transcription Apparatus

RNA pol II, together with transcription factors, forms the basal transcription apparatus, required for gene transcription (Nikolov & Burley 1997). Initiation requires the transcription factors to act in a specific order to assemble the complex and as each factor is added, an increasing length of DNA is covered. Most promoters contain a sequence located 25 basepairs upstream of the start point found in all eukaryotes, the TATA box. The initial step in transcription complex formation is binding of a particular factor to a region upstream of the TATA box. This factor contains TBP (TATA-binding protein) and smaller subunits known as TBP- associated factors or TAF's. TBP is a small protein of approximately 30 kD, which binds in the DNA minor groove, allowing other DNA –binding proteins to act in the major groove. Subsequently, other basal transcription factors add to the complex, the binding of transcription factor IIF bringing RNA polymerase II to the complex. Once the RNA polymerase has initiated transcription, it moves along the template until it reaches a terminator sequence, at which point the completed RNA product is released and RNA pol dissociates from the DNA template (Hirose & Manley 2000).

The initial gene transcript pre-mRNA is subsequently spliced to remove introns and to form messenger RNA (Sharp et al. 1987). The junctions between the introns and exons contain short consensus signal sequences, which act as recognition signals for proteins that bind to these sites and allow mRNA cutting and splicing. The reactions are carried out by spliceosomes, which are built from five RNA molecules (known as small nuclear RNA's, snRNA's) and more than 50 proteins. Alternative splicing leads to the formation of multiple mRNA's and hence potential splice variants of the protein product. Splicing enhances the export of mRNA and certain proteins, including *Aly*, are required not only for mRNA export but are also involved in transcription and are thus multifunctional (Keys & Green 2001).

1.5.2 Gene Translation

1.5.2.1 Definition

In translation, transfer RNA (tRNA) molecules enable each codon, a particular triplet of nucleotides in mRNA, to be translated into the sequence of amino acids in the protein. Each tRNA is specific for a particular amino acid and a specific codon.

The growing mRNA is translated by ribosomes moving in a proximal to distal direction. A protein chain is released from the ribosome when any one of three stop codons is reached.

1.5.2.2 Control of Translation

The expression of most genes is controlled at the transcription level as detailed above. The control of transcription and translation is tightly linked. Some genes, however, are transcribed at a constant level and switched on and off by post-transcriptional regulatory processes. These processes include:

- Premature termination of the RNA transcript.
- RNA interference- small RNA molecules bind to the complementary portion of an mRNA (Lewin B 1994a). This may either prevent it from being translated by ribosomes or trigger its destruction.
- Alternative RNA-splice site selection
- Failure of a poly-A tail being added to 3' end which is necessary for RNA export.
- Control of transport from the nucleus to the cytosol
- RNA editing of RNA transcript sequences changing the meaning of the message.
- Control of the initiation of translation by specific factors such as e1F-2, which is phosphorylated by specific protein kinases to decrease the overall rate of protein synthesis.
- Regulation of RNA degradation.

The majority of these control processes require specific sequences in the RNA molecules being regulated, to be recognised by either a regulatory protein or a regulatory RNA molecule.

1.5.3 Control of PSA Transcription

Before considering PSA specifically, it is necessary to briefly outline normal prostatic androgen action (Figure 1.3). Testosterone, which is the main circulating androgen, circulates in the blood primarily bound to sex hormone binding globulin (SHBG) and albumin. On entering prostate cancer cells, free testosterone is converted by the enzymes 5α reductase, into dihydrotestosterone (DHT). These factors have a five-fold

greater affinity for the androgen receptor (AR) than testosterone.

1.5.3.1 The role of the AR

The AR is a member of the steroid-thyroid-retinoid nuclear receptor super family (Quigley et al. 1995) that act by binding DNA and inducing transcription. It is a 110-kDa protein composed of an N-terminus ligand–independent transcriptional activation domain, (AF1) spanning amino acids 141-338, a central domain containing 2 zinc fingers which are involved in DNA binding and a C terminal ligand binding domain (LBD) (Chang et al. 1995). The LBD contains a ligand dependent transactivation domain AF2. In the basal state, the AR is bound to heat shock proteins (HSP), as a heteromeric complex which prevents DNA binding (Smith & Toft 1993). Binding of androgens induces a conformational change in the AR such that HSP's are released and the receptor is phosphorylated, partly mediated by protein kinase A (Nazareth & Weigel 1996). Within the nucleus AR homodimer complexes are formed, which can bind androgen response elements (ARE) in the promoter region of PSA and other target genes. The steroid receptor-DNA complex then recruits co-regulatory proteins, co-activators or co-repressors to the AR complex. The binding of the ligand causes a change in the position of helix 12 of the receptor, forming a surface to which the co-activators can bind. The co-activators allow the AR complex to interact with the general transcription apparatus (GTA) causing stimulation or inhibition of transcription of PSA (McKenna et al. 1999). Many co-activators contain one or multiple copies of an α helical LXXLL motif (where L =leucine and X = any amino acid residue); this signature motif mediates ligand-dependent co-activator-nuclear receptor interactions (Heery et al. 1997).

1.5.3.2 Androgen Response Elements (ARE)

ARE's are found in the promoters of genes expressed in different tissues, including prostate, brain, kidney, liver and testis. The androgen regulation of PSA expression is mediated by three ARE within the 5.8 kb pair PSA promoter (Cleutjens et al. 1997) (see figure 1.4). Two of the ARE's are located within the proximal region of the promoter, together with the TATA box, at –170 (ARE I) and –395 (ARE II) (Cleutjens et al. 1996). ARE II functions as an ARE only in the presence of ARE I, and is termed an *androgen responsive region* (Cleutjens et al. 1996). The third (ARE III) is much more distal, located 4.2 kb upstream of the PSA promoter (Cleutjens et al. 1997) within a 440 bp PSA distal enhancer. Five additional low affinity ARE's, close to ARE III have also been located within the enhancer. They appear to participate in co-operative binding of multiple AR molecules to the PSA enhancer (Reid et al. 2001).

Multiple co-regulators and suppressors of the AR have been identified (Figure 1.5) Co-regulator complexes modulate AR-mediated PSA activation by interaction with general transcription factors or by remodelling chromatin. Most co-regulators appear to function as co-activators of the AR. Amplified levels of co-activators could lead to activation of the AR by less potent or by lower levels of androgens (Gregory et al. 2001). Certainly over expression of two co-activators, TIF2 and SRC1 are associated with increased AR activation, even at physiological levels of androgen (Gregory et al. 2001).

It is known that mutations of the AR are often present in androgen independent prostate cancer (AIPC) (Taplin et al. 1995). These may alter the ligand specificity of the AR, leading to its activation by non-androgens. In addition, amplification of the AR has been found in the context of advanced or metastatic disease, and put forward as a

potential mechanism underlying androgen independence (Visakorpi et al. 1995).

1.5.3.3 Growth Factors

Alternative pathways have been described whereby the AR may be activated or bypassed, allowing PSA transcription to occur (Feldman & Feldman 2001). A ligand independent activation of the AR: an "outlaw receptor pathway" (Culig et al. 1994) allows induction of PSA in the absence of androgen. Both growth factor-activated and receptor tyrosine kinase-activated pathways have been implicated. Insulin-like growth factor-1 (IGF-1) causes a five-fold rise in PSA secretion in LNCaP cells (Culig, Hobisch et al. 1994). Keratinocyte growth factor (KGF) and epidermal growth factor (EGF) are both ligands for receptor tyrosine kinases and initiate complex signalling cascades. Her-2/neu (c-erb-b2) is a member of the EGF-receptor family of receptor tyrosine kinases and has been shown to activate oestrogen receptor in the absence of the oestrogen ligand (Pietras et al. 1995). Over-expression of Her-2/neu can activate AR dependent genes, including PSA, in the absence of androgen, but not in the absence of the AR (Craft et al. 1999). This "outlaw" AR created by Her-2/neu over expression cannot be blocked by bicalutamide, an anti-androgen, showing that the pathway is separate to the AR ligand binding domain (Craft et al. 1999). Her-2/neu may also activate the AR through a mitogen-activated protein kinase (MAPK) pathway (Yeh et al. 1999). Another alternative pathway for Her-2/neu is by activation of the phosphatidylinositol/protein kinase B pathway (Zhou et al. 2000). The 3-phosphorylated inositols are second messengers that activate protein kinase B, which can phosphorylate the AR at two separate serine residues (Wen et al. 2000), turning it into an androgen-independent outlaw receptor.

Transforming growth factor β (TGFβ) has been found to be significantly elevated in patients with clinically evident metastases and correlated with increasing serum PSA levels (Ivanovic et al. 1995). It can enhance interaction between the AR and Smad3, a downstream mediator of TGFβ in the prostate cancer cell lines LNCaP and DU145 (Kang et al. 2001). The addition of Smad3 also increased PSA expression.

Interleukin 6 (IL-6) can activate the androgen-responsive promoters in LNCaP cells in the absence of androgen and increase PSA mRNA levels. Bicalutamide, an anti-androgen, abolished the IL-6 effect. In addition, pre-treatment with a MAPK inhibitor blocked the PSA rise secondary to IL-6, showing there is cytokine-mediated induction of the AR promoter (Lin et al. 2001).

The AR may be bypassed completely, facilitating PSA production and proliferation even in the absence of androgens and the AR. The anti-apoptotic gene Bcl-2 has been suggested as working this way in prostate cancer and has been found to be up-regulated in AIPC in both human and murine models (Colombel et al. 1993).

In summary, it is clear that the AR plays a vital role in PSA transcription and it may represent the key factor in the failure of PSA production in PSA-negative tumours.

1.5.3.4 Transcription Factors

It is likely that multiple transcription factors, some prostate specific, are involved in the regulation of the PSA gene.

A novel family of **P**rotein **I**nhibitor of **A**ctivated **STAT** proteins (where STAT is a signal transducer and activator of transcription) have been found to both positively and negatively affect AR-mediated transcriptional activity of PSA. Whilst the members

PIAS 1 and 3 enhance this transcription, PIASy is a potent inhibitor in prostate cancer cells (Gross et al. 2001).

GATA transcription factors, which are known to have a role in general mammalian development (Simon 1995) have been implicated in the development of metastatic AIPC in a transgenic mouse model (Perez-Stable et al. 1997). Six GATA transcription sites which flank an ARE in the far upstream PSA enhancer (Schuur et al. 1996) are required for optimum PSA expression and stimulation by androgen. In particular, the GATA-binding proteins 2 and 3 appear to modulate androgen induction of the PSA promoter (Perez-Stable et al. 2000).

Other putative consensus sites for transcription factors have been identified on the PSA promoter that could potentially activate PSA expression by other signal transduction pathways. These include an AP1 site, which partially overlaps with the ARE II. If the AP-1 family proteins (which include the jun and fos oncogenes) are elevated, AP-1 may bind to its DNA-binding site and block the binding of the androgen receptor (Sadar et al. 1999). Another potential site of PSA regulation is the cAMP response element (CRE), located at –3196 of the PSA enhancer, because transcriptional factors responsive to cAMP, including activating transcription factor (ATF) and CREB can bind at this site (Sadar et al. 1999).

1.5.3.5 Non-Androgen Dependent Transcription Factors

A novel prostate derived Ets transcription factor (PDEF) has been shown to interact with the AR and play a role in PSA gene regulation. Ets factors play a crucial role in haematopoiesis, angiogenesis and organogenesis, and several distinct chromosomal translocations involving Ets factors have been discovered in human cancers (Graves &

Petersen 1998). There are high affinity sites in the PSA promoter for PDEF and PDEF causes an 11-fold enhancement of PSA promoter activity in LNCaP cells, (a PSA and AR expressing prostate cancer cell line) in the absence of androgen. A 57- fold activation of the promoter activity was seen in the presence of both androgen and PDEF, showing that PDEF mediated PSA promoter transcription has both an androgen dependent and independent component (Oettgen et al. 2000).

P53, a known tumour suppressor gene, has been found to negatively regulate PSA expression, by blocking N-C interaction of the AR, thought to be responsible for homodimerisation of the receptor (Shenk et al. 2001). Gurova (2002) found that p53 suppression in LNCaP cells caused a 4-8 fold increase in PSA protein. The inhibitory effect of wild-type p53 was reduced by trychostatin A, a potent inhibitor of histone deacteylases (Murphy et al. 1999), (see section 1.5.4) suggesting the involvement of histone deacetylation in negative regulation of PSA promoter activity.

A 45 base pair cell specific transcription factor (**p45**) has been found to bind to a specific 150 base pair region within the PSA distal enhancer. In the absence of androgen, and independent of the androgen receptor, this can cause activation of the PSA promoter (Yeung et al. 2000).

To summarise, multiple factors are involved in the control of PSA transcription. One possible explanation for PSA-negativity is an alteration in the AR itself, either structural or functional, or in the PSA promoter or enhancer regions.

1.5.3.6 Prostate Specific Autocrine Factors

In a similar fashion to their response to paracrine growth factors, there may be an

autocrine mechanism involved, with interaction with co-activators of the AR and the receptor itself. This results in a highly transcriptionally active ARE III in the absence of androgen and other growth factors. One such protein has been termed NF-1 (nuclear factor 1) but remains to be fully characterised (Yeung et al. 2003).

1.5.4 Methylation and Deacetylation

In the normal mammalian genome, methylation occurs only at the 5' of a cytosine base of a Cp (phosphate) G dinucleotide. Many CpG dinucleotides have been lost by spontaneous deamination, and the remaining CpG areas have a high frequency of methylation (Jones & Laird 1999). Methylation promotes structural chromatin changes which can block transcription and thus inactivate a gene. CpG islands are GC and CpG-rich areas of approximately 1 kb, comprising about 1% of the genome, and usually located in the promoters of so-called housekeeping genes, namely those responsible for cellular function (Larsen et al. 1992). They are typically unmethylated. DNA is packaged into chromatin, which is composed of histone proteins around which the DNA is coiled. The HDAC and HAT proteins act together to modulate chromatin structure and transcription, via histone acetylation (Roth et al. 2001). Deacetylation of histone proteins appears to be the initial step in the recruitment of methyltransferase to the CpG islands, leading to hypermethylation (Patra et al. 2001). DNA methyltransferase and HDAC1 have been found to be up-regulated in prostate cancer, implying a role in inhibition of transcription via methylation (Patra et al. 2001) and hypermethylation of the PSA gene or AR gene may be responsible for failure of PSA production. This will be explored in Chapter 6.

1.5.5 LOH

1.5.5.1 Definition

Loss of one allele at a locus often followed by duplication of information from the remaining locus.

1.5.5.2 LOH in Prostate Cancer

In early prostate cancer, the loss of genetic material is five times more common than DNA amplification, with loss at 8p and 13q seen most frequently (Elo & Visakorpi 2001). LOH is an important factor in gene activity as it affects both number of gene copies and function. An illustration of LOH is shown in figure 1.6. LOH is a potential factor in loss of PSA expression and has never been previously studied in this context.

1.5.6 PSA Protein Degradation Pathways

Important degradation pathways for proteins exist in the cytosol, endoplasmic reticulum (ER) and in lysosomes. In the cytosol, degradation occurs on proteasomes, large protein complexes dispersed throughout the cell. Proteasomes act on proteins that have been specifically marked for destruction by the covalent attachment of a small protein known as ubiquitin. It is possible that a conformational change in the PSA protein makes it more vulnerable to ubiquitin-targeted proteolysis, and hence contributes to low serum PSA levels by increased protein destruction.

Newly synthesised proteins enter the biosynthetic-secretory pathway in the endoplasmic reticulum (ER). They are subsequently mobilised from the ER to the Golgi apparatus and hence to the cell surface or other compartment by transport vesicles. A short four-amino acid sorting signal, KDEL, mediates the retention of soluble resident proteins

within the ER. If this signal is transferred to proteins that are normally secreted, they will be retained within the ER and hence their systemic levels reduced. Protein modification, including phosphorylation and glycosylation occurs within the Golgi complex and can confer functional advantages to the protein product. Although protein degradation occurs within the ER, lysosomes remain the main site of intracellular digestion. Multiple pathways are involved, including endocytosis. However, some proteins contain specific sequences on their surface that direct them selectively to the lysosomes for degradation. An alteration in the normal surface sequence of PSA, by mutation, may cause enhanced lysosomal proteolysis and hence a reduced serum level of the protein product.

1.5.7 PSA Protein Processing and Secretion

Like many other secretory proteins, PSA is synthesised as an inactive protein precursor from which active molecules have to be liberated by proteolysis. PSA is synthesised as pre pro PSA, a 261 amino acid preprotein, with a 17 amino acid signal sequence at one end. This sequence is cleaved forming pro PSA, an inactive precursor protein of 244 amino acids (Lundwall & Lilja 1987) (Figure 1.7). A 7 amino acid activation sequence at the N terminal is subsequently removed by KLK2, cleavage usually occurring between arginine at position 7 and isoleucine at position 8, to produce the active 237 amino acid form of PSA. This has 5 intrachain disulphide bonds and a single asparagine-linked oligosaccharide. PSA may also be activated by other prostate kallikreins including hK4 or prostase (Lundwall & Lilja 1987). The cleavages occur in the *trans* Golgi network and in the secretory vesicles. As described later, differential processing may occur resulting in varying isoforms. Once an extracellular signal is

received, the secretory vesicles fuse with the plasma membrane to release their contents. Prostate secretory granules (PSG) act as reservoirs of PSA and prostatic acid phosphatase (PAP). These PSG's are almost absent in malignant cells (Cohen et al. 2000) and alternative pathways exist for PSA secretion (section 1.5.6)

In seminal plasma, about 30 % of PSA is the active enzyme, with a further 5 % in a complexed form with protein C inhibitor (PCI) (Mikolajczyk et al. 2000). The remainder of the PSA is composed of inactive forms, collectively known as benign PSA (bPSA) because of their anatomical location within the prostate transition zone and their relatively increased levels in BPH rather than in tumour (Mikolajczyk et al. 2000). They are created by internal cleavages between residues 85-86, 145-146, or 182-183.

In serum, the majority (70-90%) of PSA circulates as a complex with the serine protease inhibitor α1-antichymotrypsin (ACT) (Lilja et al. 1991). Trace amounts are also bound to API (previously known as α1-antitrypsin), ITI (interαtrypsin inhibitor) or PCI. Another fraction of the PSA is bound to the 25-fold larger α2-macroglobulin (A2M) molecule. Because of its larger size, this completely encapsulates the PSA molecule, leaving no free epitope sites of PSA for detection and the PSA-A2M can only be measured by assay after the complex has been opened (Zhang et al. 1998). Free PSA (fPSA) represents 10-30 % of total PSA (tPSA). This is largely composed of the catalytically inactive forms (bPSA) produced by the internal cleavages as described above. As the level of these components is greater in benign tissue, and the serum level consequently higher in patients with BPH rather than tumour, the ratio of free to total PSA has been used as a discriminator between BPH and prostate cancer with lower ratios present in prostate cancer patients (Catalona et al. 1998). The molecular forms of

PSA in prostate tissue, seminal fluid and plasma are summarised in table 1.2.

1.5.7.1 Mechanism of the Differential Expression of PSA in Cancer and Benign Disease

In prostate cancer, due to loss of the basal cell layer and disruption of the normal glandular architecture, there is a decrease in the luminal processing of both proPSA and of complexed PSA (ACT-PSA), and they are able to reach the circulation directly causing increased levels of both components in serum (Figure 1.8). In contrast, in normal tissue and in BPH, PSA reaches the circulation much more slowly by leaking "backwards" into the extracellular space where it is susceptible to proteolytic degradation (Stenman 1997). Chen et al (1997) predicted the existence of a protease substance in BPH nodules that was subsequently isolated from serum, and is responsible for the inactivation or "nicking" of PSA in BPH tissue. Such nicked PSA reacts poorly with ACT in vitro (Leinonen et al 1996). A higher level of nicked PSA in BPH may contribute to the reduced ability to form complexes with ACT. The reaction rates of PSA with inhibitors, together with the metabolic clearance rates of the different forms, determine the concentration of each form in circulation. PSA-A2M complexes are removed from circulation with a half –life of 2-5 minutes (Imber & Pizzo 1981). This is thus likely to be the major metabolic pathway for PSA reaching the circulation. Free PSA has a 1-2 hour half-life whereas PSA-ACT has a much longer half-life of 2-3 days (Bjork et al. 1998;Richardson et al. 1996).

The fact that PSA reaches the circulation by different routes in benign and malignant tissue may explain the comparatively higher levels of PSA in circulation in prostate cancer. It has been postulated that higher local ACT expression in prostate cancer could

facilitate formation of PSA-ACT complexes (Bjork et al. 1994). However, the proportion of complexed PSA in serum is low in men without prostatic disease, although the expression of ACT is similar in normal and malignant prostatic tissue. The PSA reaching the circulation through the extracellular space is more extensively "nicked" and can form complexes less easily than that secreted directly into the blood stream.

Other potential explanations include increased production of active PSA from proPSA in cancerous tissue compared to BPH (Mikolajczyk et al. 2000). From proteomic studies, levels of ACT may be reduced in some prostate cancers (Meehan et al. 2002) which would affect the level of serum PSA detected by most assays. With these factors in mind, it is clear that any alterations in the processing of PSA, in the formation of complexes (due to changes in the levels of the co-complexors or because of structural mutations in the PSA protein), or in secretion itself may affect serum PSA levels. An alteration in the half life of PSA due to increased systemic proteolysis may also occur.

1.5.7.2 Relationship between Serum and Tissue Levels of PSA

The serum PSA level for an individual with prostate cancer is proportional to the size of the prostate gland (Partin et al. 1990), volume of BPH (Cadeddu 1995), and volume and differentiation of the tumour (Abrahamsson et al. 1988). An inverse relationship between Gleason grade of the cancer and levels of PSA in serum and tissue has been described (Aihara 1994). A further study by Weir (2000) corrected the serum PSA in radical prostatectomy cases for gland size and tumour volume and examined tissue PSA staining. They confirmed the relationship between serum total PSA and volume of prostate carcinoma and that an inverse correlation exists between corrected serum PSA

density and Gleason score. They noted, however, that despite less serum PSA produced per gram of tumour, more poorly differentiated tumours of higher grades are associated with greater tumour volume, advanced stage and higher serum PSA (Partin et al. 1990). The positive relationship in these cases between serum PSA and Gleason score is thus mainly a function of tumour volume rather than tissue capacity for PSA production. This cannot explain the low PSA-negative tumours, where from the volume of metastatic disease one would expect serum PSA levels to be at least 100 ng/ml. Lack of correlation between serum and tissue PSA may relate to differences between the amount of PSA produced by prostate cancers and the amount secreted. In high volume disease, there is usually gross disruption of the architecture of the prostate gland, causing variations in the amount of PSA secreted into the systemic circulation. There may be variations between individual neoplastic cells in terms of their secretory regulation. In addition, there is often marked heterogeneity in tumours in terms of histological differentiation and the dominant clone may be primarily PSA negative at either tissue or serum PSA level, or indeed both. In the context of metastatic disease, the dominant clone may be in the primary tumour or in the metastasis.

1.5.7.3 PSA Isoforms

Truncated forms of proPSA, particularly those cleaved between leucine 5 and serine 6, termed [-2] pPSA are biologically inactive and circulate as part of the free PSA fraction in serum (Mikolajczyk 1997). They are assumed to result from post-translational proteolytic cleavage of the 7 amino acid leader sequence in proPSA. The same author found higher levels of this isoform in the fPSA fraction in biopsy positive patients with tPSA in the 6 to 24ng/ml range and these isoforms may be a useful adjunct to PSA testing. Monoclonal antibodies to [-7] pPSA, [-4] pPSA and [-2] pPSA have been used

to examine cancer serum in patients with total PSA levels around 10 ng/ml. (Mikolajczyk 2001) confirming five-fold greater levels of [-2] pPSA in biopsy positive patients compared to biopsy negative patients. Similar results were shown for the other pPSA isoforms and they remain the source of ongoing studies. When used in combination with levels of bPSA, which is being evaluated as a potential marker for BPH, they may provide a more specific serum test for prostate cancer, particularly in the context of rising total PSA after definitive local treatment (Mikolajczyk 2002).

1.5.7.4 Confounding Factors Affecting Serum PSA Levels

Biological

In an individual patient, serum concentrations of PSA vary with time (Prestigiacomo & Stamey 1996), with fPSA variation being greater than tPSA. The effect of ejaculation is small despite causing transitory rises in tPSA for up to a day (Herschman et al. 1997). Significant manipulation of the prostate by biopsy, cystoscopy and TRUS can cause rises in serum PSA although the effect of DRE is debated (Collins 1997).

Sample Handling

Storage of serum unfrozen may cause loss of PSA within a few days (Leinonen 1993) and this PSA decline has also been noted in samples stored for years at -20°C (Stenman et al. 1994). The initial changes in unfrozen sera are due to loss of free PSA and subsequently complexed PSA is also reduced, probably due to cleavage of ACT and the release of active PSA which then binds to A2M and becomes undetectable (Stenman et al. 1999).

Treatment of Benign or Malignant Prostate Disease

As PSA is an androgen-dependent gene (see section 1.5.3), serum levels of PSA fall

significantly following medical or surgical castration and also decline subsequent to TURP. The use of finasteride in BPH causes a 50 % or so reduction in PSA after 6 months of treatment by blockage of 5α-reductase II and PSA levels in patients receiving finasteride should be multiplied by two (Andriole 1998).

PSA Assays

Sensitive immunoassays are required due to the much lower levels of PSA in serum than in seminal fluid. The complexed forms of PSA tend to be underestimated especially by assays using polyclonal antibodies (Stenman 1991). This is because some of the antigenic sites on PSA are eliminated when it binds to ACT (Pettersson 1995).

PSA-ACT can be determined by using a sandwich assay with a catcher antibody to PSA and a detection antibody to ACT. However, the method is plagued by high levels of non-specific background staining (Leinonen et al. 1993). Similar sandwich assays have been developed for PSA-API (Zhang et al. 1996) and an immunoadsorption assay can be utilised for PSA-A2M (Zhang et al. 1998).

Assays however, demonstrate a huge variability in terms of the technique employed, and type of antibody used, which is further complicated by the number of commercially available assays. It is thus important for assay calibration standards to be performed, using such protocols as a standard composed of 90% PSA bound to ACT and 10 % free PSA. Other authors have suggested using a 100 % free PSA assay as a standard (Brawer et al. 1997). Difficulties arise when comparing sequential PSA results from the same patient performed using different assays.

1.6 Alternative Markers for PSA-Negative Prostate Cancer

Given the absence of raised serum PSA levels, and the possible lack of tissue immunostaining, other serum and/or tissue markers may aid diagnosis and management.

1.6.1 PAP

Before PSA evaluation was available, serum PAP levels were routinely used to monitor prostate cancer (Gutman et al. 1936). However, in a study of 74 patients, serum PSA never lagged behind PAP in identifying recurrence/progression, and it preceded PAP rises in 90% of patients. Due to these findings, PAP has been replaced by PSA as the tumour marker of choice. It has, however, been postulated that PAP may have a role in bone metabolism, by acting on bone remodelling cells to facilitate the formation of blastic lesions (Bok & Small 2002). Within the osteoblast, phosphatase and calcium act on the mineral apatite to induce crystal growth and bone formation, with the subsequent release of PAP into the bloodstream. PAP and PSA levels are usually concordant in prostate cancer, but cases of advanced disease have been noted wherein serum PSA remained undetectable whilst PAP levels were elevated (Goldrath & Messing 1989; Leibman et al. 1995). Discordance of results at tissue level has also occasionally been described (Aihara et al. 1994). Immunostaining with PAP and PSA often shows marked heterogeneity within tumour populations, with scattered or groups of cells showing negative reactivity for either one or both markers (Keillor & Aterman 1987). There is no information on PAP levels either in serum or at tissue level in PSA-negative prostate cancer, and it may prove a useful diagnostic or response to treatment marker in either capacity.

1.6.2 AR

The role of the AR in PSA transcription has been previously outlined (section 1.5.3.) Androgen receptor gene expression is amplified in AIPC (Linja 2001) with increased PSA-immunostaining seen in AR-amplified primary tumours (Koivisto & Helin 1999). One potential mechanism for the loss of PSA secretion in PSA negative tumours is the deletion, mutation or lack of function of the androgen receptor as described above. If this is indeed true, then immunostaining for AR will be absent. In addition, neuroendocrine cells lack AR (Bonkhoff et al. 1993) and hence AR loss may reflect this tumour subtype.

1.6.3 Kallikreins

The kallikrein family have been previously described in this chapter (Section 1.4.2) HK2 in particular, shows promise as an alternative tumour marker as it is both prostate-localised and tumour associated, although it has been shown to be expressed in breast tumours (Black. 2000). Nevertheless, there is 21% greater amplification at the HK2 gene level in prostatic tumours (Herrala et al. 2001) and HK2 serum levels have already been shown to add significant information in the detection of prostate cancer particularly at low serum PSA levels (Kwiatkowski. 1998; Recker 1998). However, difficulties exist with regard to immunoassays for different molecular forms of HK2 which has hampered large scale trials (Stephan et al. 2000).

1.6.4 PSMA

Prostate specific membrane antigen (PSMA) is a 100 kD type II integral membrane glycoprotein, expressed in all forms of prostate tissue, and over-expressed in prostate cancer (Israeli 1994). The gene, which is located on chromsome 11p encodes for a

protein consisting of a 19 amino acid internal portion, a 24 amino acid transmembrane portion and a 707 amino acid external portion (Leek 1995).

It appears to be up-regulated after androgen ablation (Wright, Jr. 1996) and is then correlated with androgen-independent progression (Murphy 1995). After examining bone marrow and serum from 50 patients with androgen-independent prostate cancer, Kim (2000) concluded that PSMA was a useful serum marker in AIPC with extensive metastatic disease but his work has not been duplicated as yet, primarily due to problems with antibody consistency. Alternative assay methods such as RT-PCR have been attempted which, despite improving sensitivity, are not sufficient to put into widespread clinical use (Murphy 1998) It is, however, a useful diagnostic tool when using a PSMA antibody complexed to $Indium^{111}$ as a radiological marker, particularly in the context of possible recurrent disease after definitive primary treatment (Elgamal et al. 1998). As a tissue marker, however, it has been shown, albeit in small series, not only to consistently immunoreact with prostate cancer specimens but to be positive in cases where PSA staining was negative (Chang 2001a; Wright, Jr., Grob et al. 1996). Increased PSMA staining has also been documented in poorly differentiated adenocarcinomas rather than in well-differentiated tumours (Kawakami & Nakayama 1997) and intensity of staining has been shown to correlate with the Gleason tumour score (Bostwick 1998 ;Burger et al. 2002). This latter author also found that prostate epithelial cells in most BPH did not express PSMA at a level detectable by immunohistochemistry. It is possible, therefore, that PSMA, may provide different diagnostic information in PSA-negative tumours.

1.6.5 AMACR

α-methylacyl-CoA racemase (AMACR) is a tissue marker that is significantly up-

regulated in prostate cancer at both the RNA and protein level, showing 97% sensitivity and 100 % specificity for prostate cancer detection (Rubin 2002). No studies exploring serum levels are yet in place.

1.7 Rationale for Thesis

Although men with treatment naïve PSA-negative metastatic prostate cancer represent a small number of cases, they pose a significant management dilemma. Diagnosis is often difficult and treatment may thus be delayed. There is little information outlining their presentation and response to treatment and prognosis. They have been assumed to be a different entity to high PSA prostate cancer. The absence of a useful biochemical marker makes monitoring response to treatment extremely difficult. Their histological characteristics have not been established and the mechanism for PSA-negativity has never been established.

The main aims of the work presented in this thesis are:

1) To collect a tissue bank and clinical database of patients presenting with treatment naïve metastatic prostate cancer and serum PSA < 10 ng/ml.
2) To describe the clinical presentation, response to treatment and prognosis of primary PSA negative metastatic prostate cancer.
3) To assess the expression of PSA and potential alternative tissue markers using immunohistochemistry.
4) To investigate the mechanism causing some prostate cancers to be PSA negative.

Alterations in any one of three pathways is plausible:

- Synthesis of the protein
- Degradation and metabolism within the tissue
- Secretion of PSA into the systemic circulation

Due to constraints on time and access to tissue blocks, this work principally examines the control of PSA protein synthesis although all hypotheses are equally valid. The key factors in synthesis that are presented are:

- The methylation status of the androgen receptor and PSA
- LOH of PSA gene

Table 1.1 Previous Studies of PSA-Negative Prostate Cancer (Serum PSA < 10ng/ml)

Study	No.	Previous Management	Pattern of metastases	Histology of primary tumour	Tissue PSA
Kageyama 1996	**3**	**Treatment naive**	Bone 2 Liver 1	**Poorly differentiated**	**All negative**
Gaffney 1992	**10**	**Treatment naive**	**Metastatic**	**Undifferentiated**	**All negative**
Yamamoto 2001	**8**	**Treatment naive**	**Bone 7** **Liver 1**	**Poorly differentiated 4** **Undifferentiated 2** **Heterogenous 1** **Moderately differentiated 1**	**Negative 3** **Positive 2** **No Tissue 3**
Cohen 1995	**8**	**Treatment naive**	**Locally advanced**	**Moderately differentiated**	**All negative**[a]
Feiner 1986	**7**	**Treatment naive**	**Metastatic**	**Undifferentiated**	**Negative 6**
Beado 2001	**1**	**Radical Prostatectomy**	**Bone**	**Poorly differentiated**	**Negative**
Leibman 1995	**2**	**Radical Prostatectomy**	**Lung 1** **Brain 1**	**Poorly differentiated**	**Positive 1** **Negative 1**
Sella 2000	**18**	**Androgen independent**	**Liver 10** **Bone 11**[b] **Lung 6**	**Small Cell 6** **Poorly differentiated 2** **Both 10**	**Positive 11**[c] Negative 4[c] No Tissue 3[c]

[a] when monoclonal PSA antibody used. 7/8 were positive with PSA polyclonal antibody

[b] lytic rather than osteoblastic lesions

[c] all positive for neuroendocrine marker

Molecular forms	Prostatic tissue		Seminal plasma	Serum	
	Carcinoma	Normal/BPH		PCa patients	BPH patients
TPSA	7.25 µg/mg protein	20.2 µg/mg protein	0.2–5 g/liter, 30–50% enzymatically active	>2 µg/liter	1–20 µg/liter
FPSA	5.58 µg/mg protein	15.1 µg/mg protein	>95% of tPSA	5–30% of tPSA	10–50% of tPSA
ACT-PSA	0.019 µg/mg protein	0.008 µg/mg protein	Not detectable	70–95% of tPSA	50–90% of tPSA
CPSA	0.068 µg/mg protein	0.099 µg/mg protein		70–95% of tPSA	50–90% of tPSA
API-PSA				3.2% of tPSA	4.1% of tPSA
ITI-PSA				<1% of tPSA	<1% of tPSA
A2M-PSA			Small amounts	12% of tPSA	17%
PCI-PSA			<5% of tPSA	Not detectable	
Nicked PSA	Smaller proportion	Higher proportion	30% of tPSA	Small amounts	
ProPSA	3.0% of tPSA	Small amounts	Not detectable	25% of fPSA	
BPSA	4.33% of tPSA	11.4% of tPSA 2.0 µg/mg protein			

Table 1.2 Molecular Forms of PSA (from Lilja et al 1991)

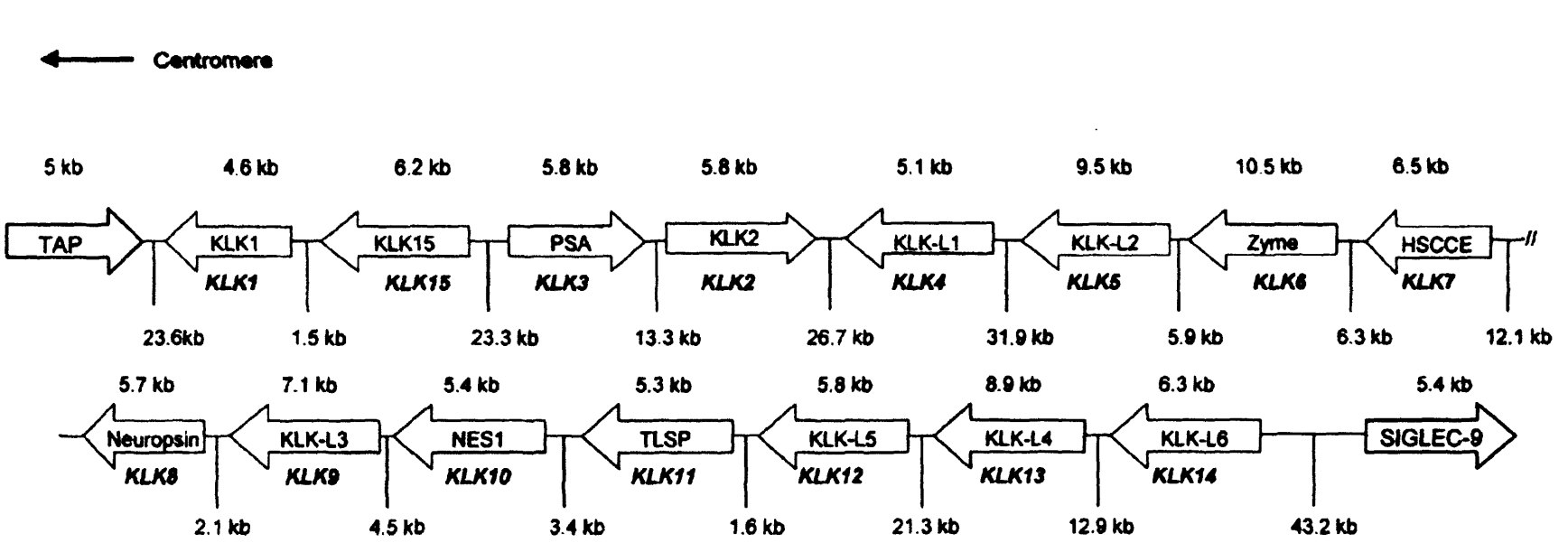


Figure 1.1 Gene Locus of the New Kallikrein Family Around Chromosome 19q13.4 at a 300kb Region

Direction of transcription indicated by arrows. Boxes represent the former gene names. The new kallikrein name is shown below the boxes. The genomic length of each gene is shown above the relative boxes. Distances between genes are shown between boxes. The kallikrein gene is framed by the Siglec and TAP genes

(After Yousef et al 2001 and Stephan et al 2000)

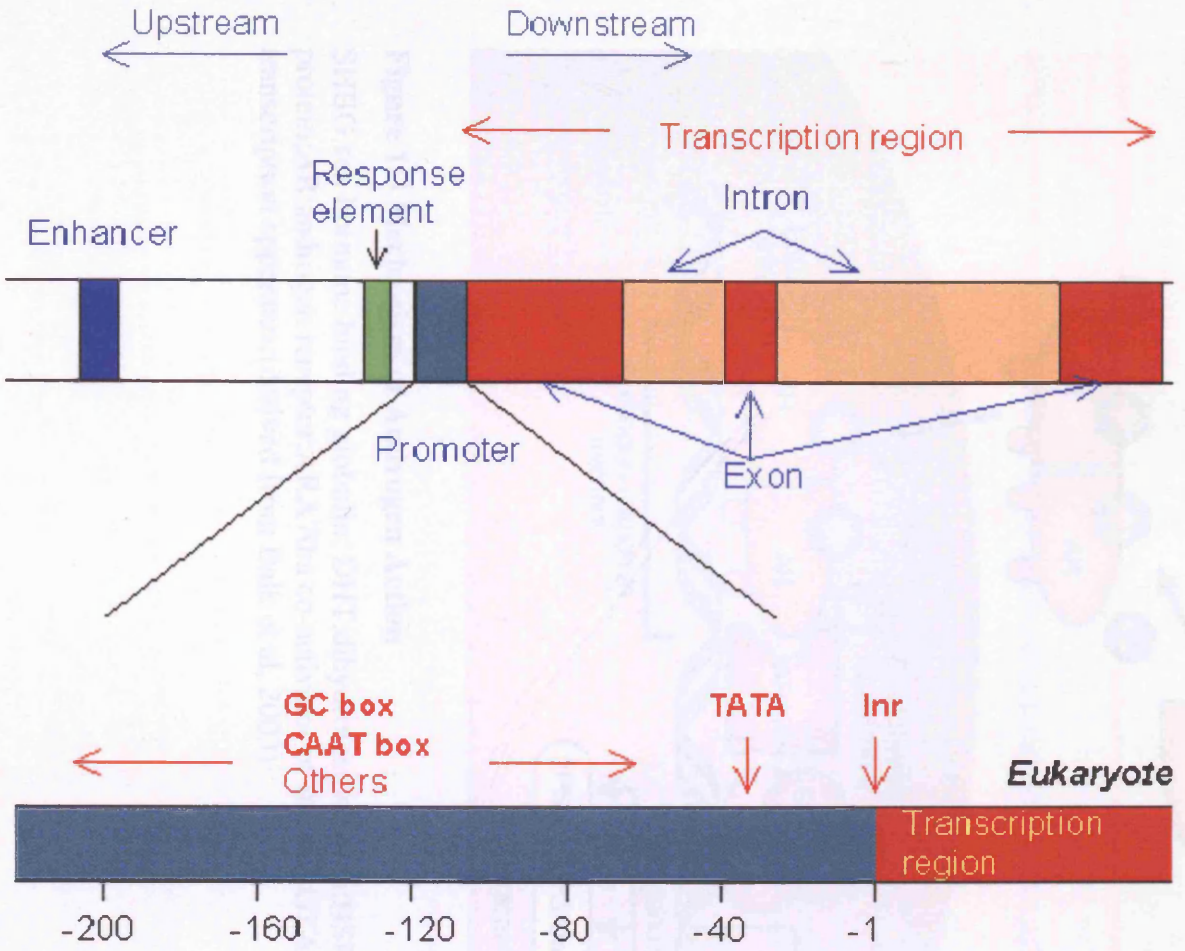


Figure 1.2 Gene Regulatory Elements in Transcription

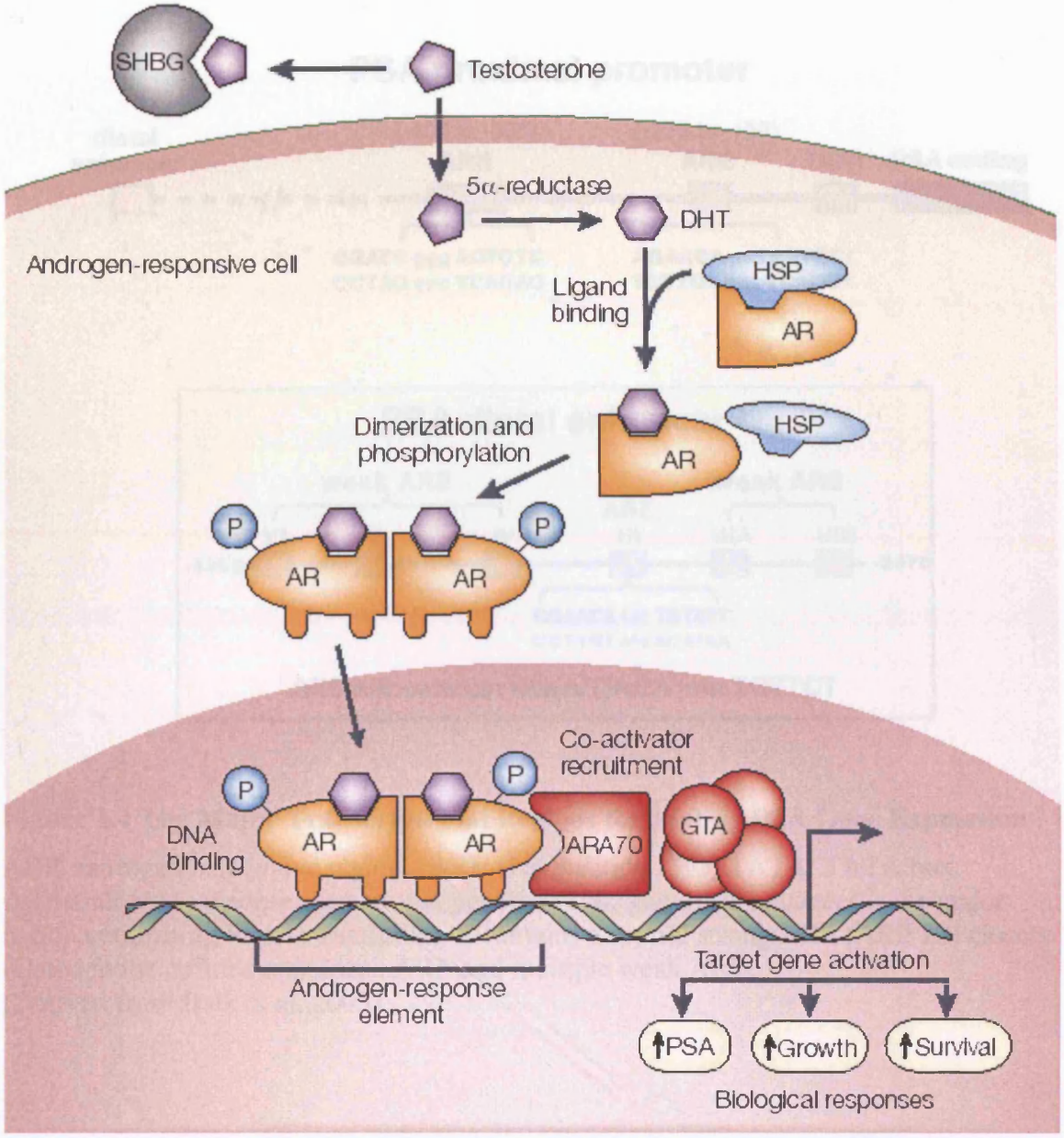


Figure 1.3 Mechanism of Androgen Action

SHBG:sex hormone binding globulin; DHT:dihydrotestosterone;HSP:heat shock protein;AR:androgen receptor;ARA70:a co-activator of the AR;GTA:general transcription apparatus;(derived from Balk et al, 2003)

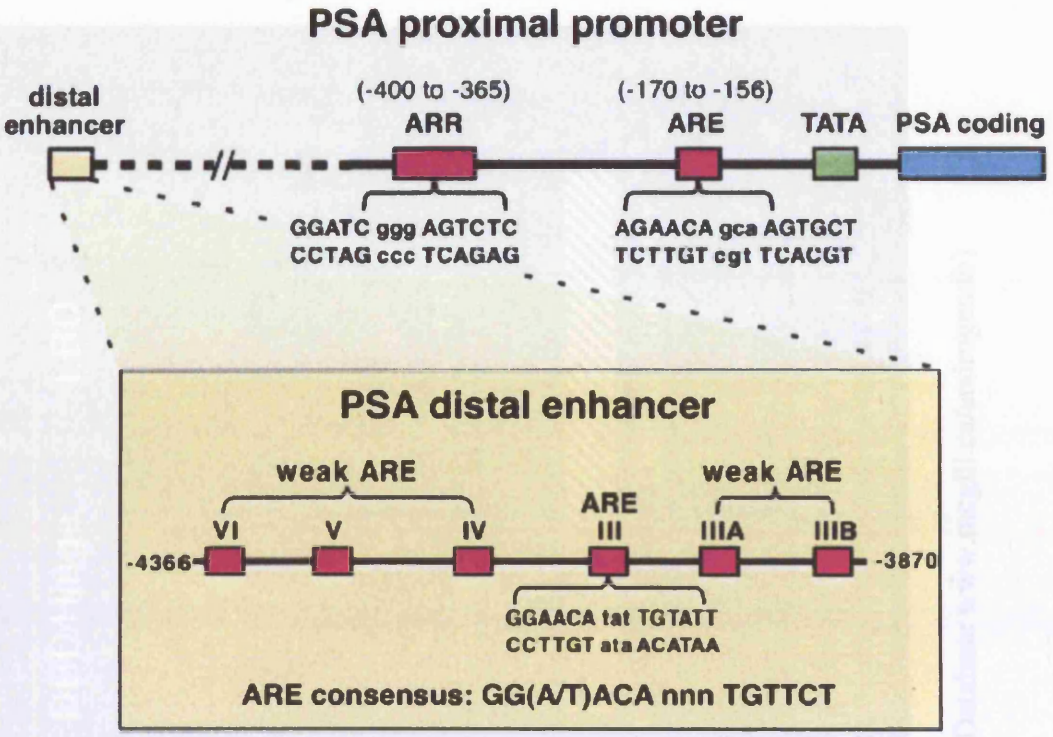


Figure 1.4 The Major Transcriptional Regions Regulating PSA Gene Expression

ARR:androgen responsive region. (ARE II at position -395) TATA: TATA box. ARE:androgen response element I at position -170. The distal enhancer is the major region controlling PSA transcription. It contains a central strong ARE (ARE III) closely related to the defined consensus ARE and multiple weak ARE.
(Derived from Balk et al, 2003)

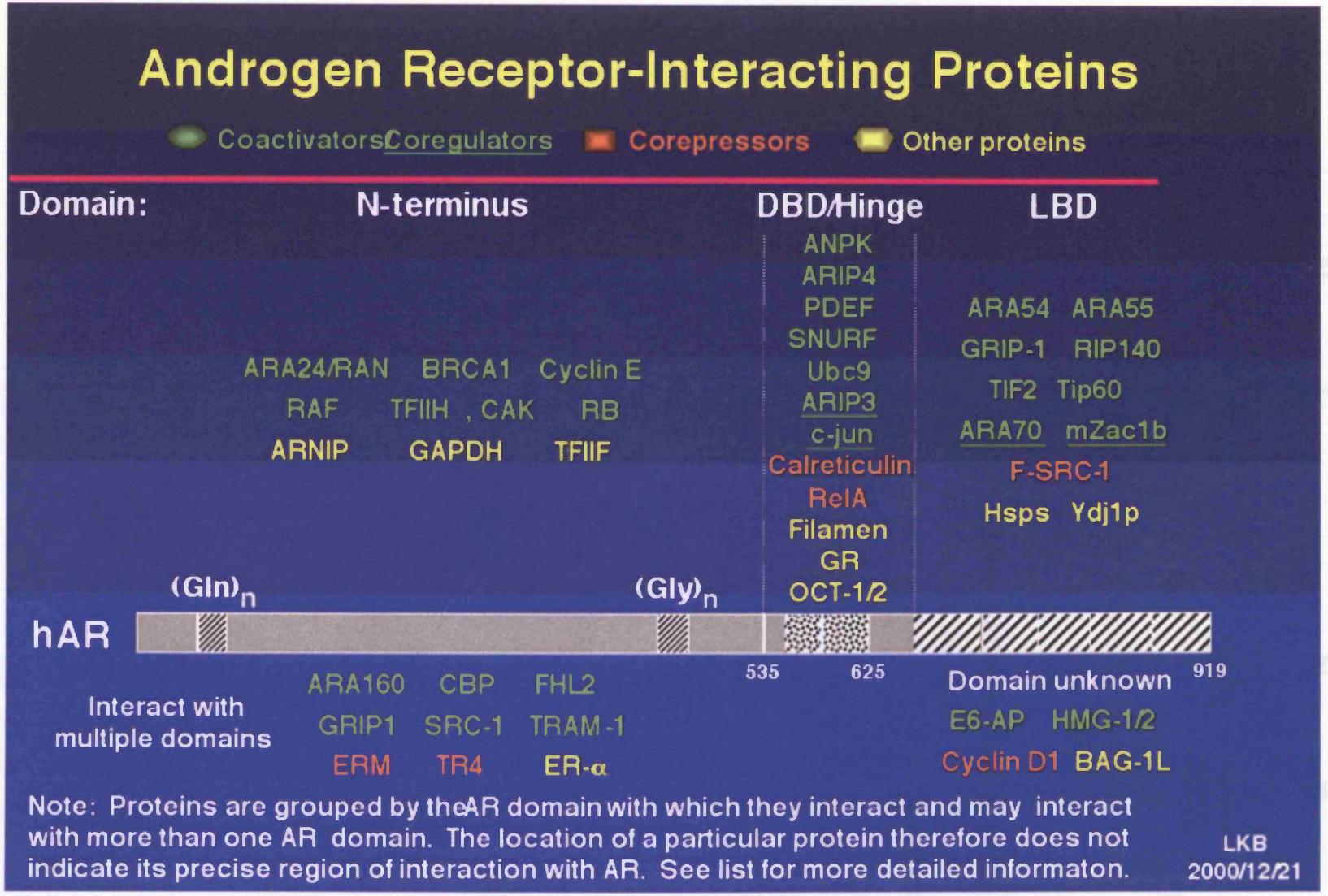


Figure 1.5 Proteins Interacting with the AR (from Androgen Receptor Database www.mcgill.ca/androgendb)

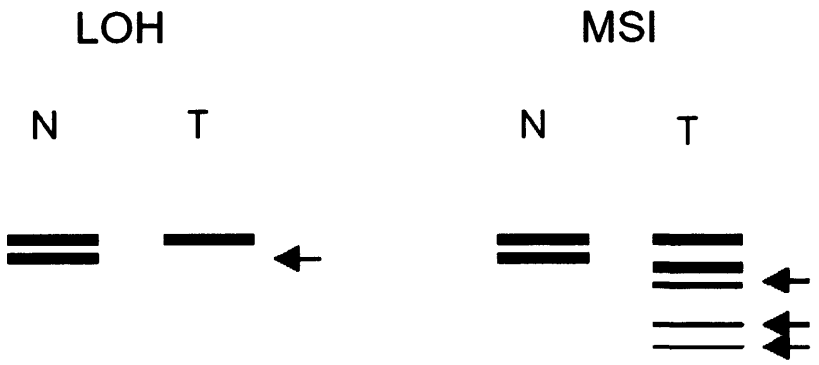


Figure 1.6 Schematic Illustration of LOH and Microsatellite Instability

LOH is seen as lack of one of the allelic bands in the tumour (T) (arrow) as compared to the heterozygous normal tissue (N), and microsatellite instability appears as new allelic bands in the tumour tissue (arrows).

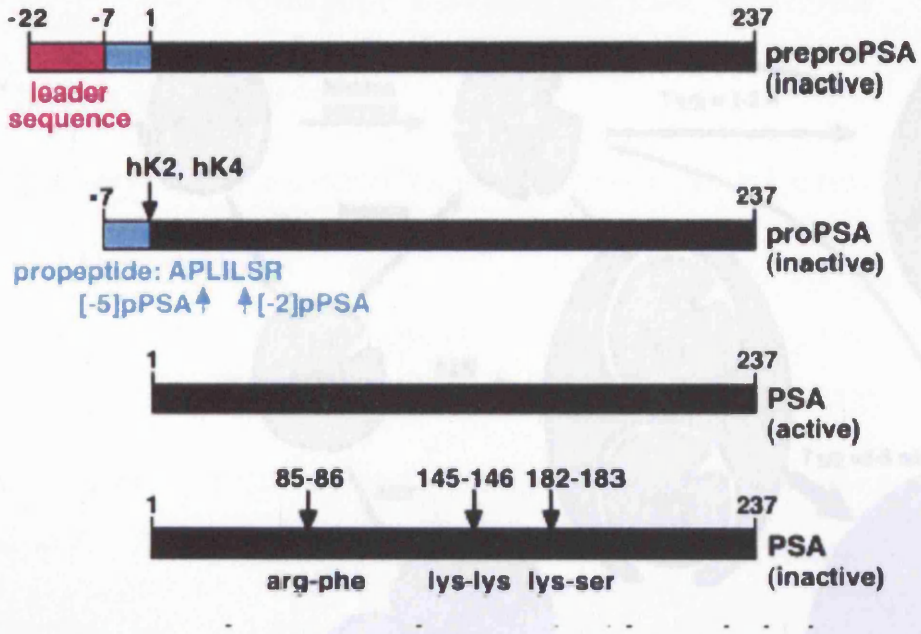


Figure 1.7 Structure of PSA Isoforms

Leader sequence on preproPSA is cleaved to generate proPSA, which is inactive. HK2 cleaves the propeptide in the lumen, generating active mature PSA. Truncated forms of proPSA can also be generated by alternate cleavage of the propeptide, producing [-2]pPSA and [-5]pPSA, which are inactive. Active PSA in the lumen of prostate ducts can be further cleaved at the indicated positions to generate inactive PSA. Active PSA in the bloodstream rapidly binds to protease inhibitor.
(After Balk, 2003)

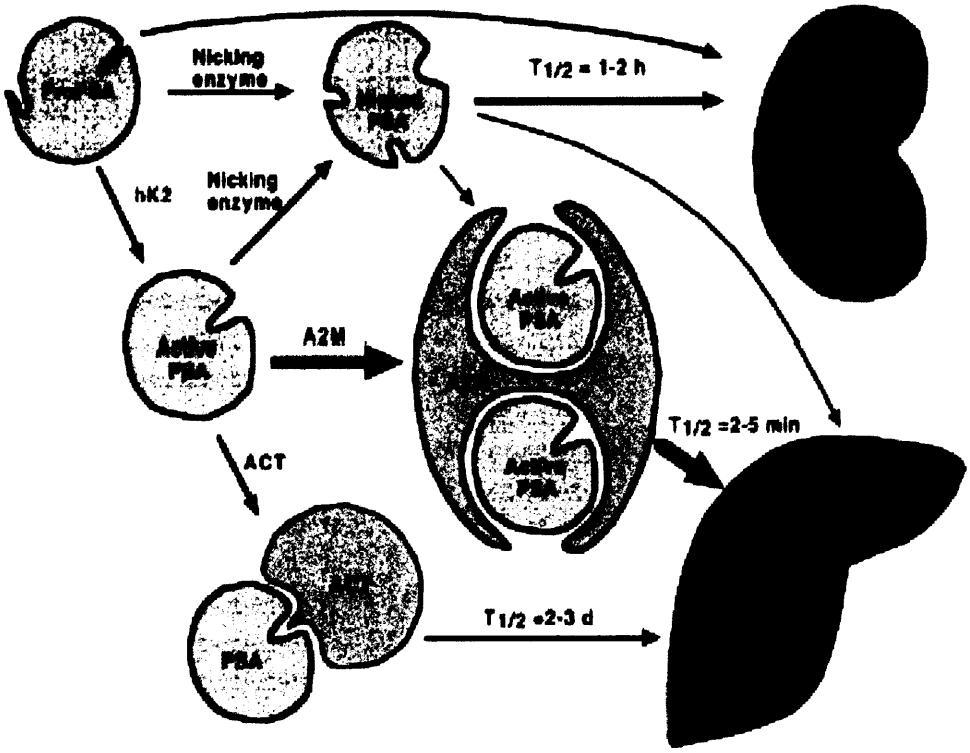


Figure 1.8 Model of the Metabolism of PSA in Extracellular Fluid and Circulation

PSA in normal and hyperplastic prostate leaks passively into extracellular fluid and the circulation. In cancer, it can be actively secreted due to disruption of normal architecture. "Nicked" PSA forms from further degradation of the active PSA and reacts slowly with A2M but not with ACT. Due to its rapid metabolism, formation of PSA-A2M appears to be the major catabolic pathway of PSA.
(After Stenman et al 1999)

Chapter 2 Materials and Methods

2.1 Principal Techniques

2.1.1 Immunohistochemistry on Cells Grown on Coverslips

2.1.1.1 Cell Fixation.

After cells were grown in the appropriate conditions for the experiment using a 24 well plate, (section 6.3.1) the medium was aspirated from each well and the coverslips were rinsed twice in 1ml of 1 x PBS. One ml of ice-cold 1:1 acetone:methanol was then added to each well, and the plate left on ice for 10 mins to allow cell fixation. The coverslips were then rinsed twice with 1ml of 1 x PBS and if not used immediately, stored at 4°C in 1ml 0.1 % sodium azide in 1 x PBS to prevent microbial growth. Cells were stable for up to 1 month under these conditions.

2.1.1.2 Immunostaining

The Vectastain Elite® (Vector PK-6100) ABC kit was used for immunostaining. The system is based on the biotin/avidin system, using unlabelled primary antibody, biotinylated secondary antibody and an **A**vidin and **B**iotinylated horseradish peroxidase macromolecular **C**omplex is formed, detected by diaminobenzidine tetrahydrochloride (DAB) as the chromagen. A fresh solution of 0.3% peroxide in 70 % methanol was prepared and 1ml added to each well to saturate any endogenous peroxidase to thus prevent background staining. The 24 well plates were incubated at room temperature for 30 mins, then each coverslip washed twice with 1 x PBS for 5 mins. The coverslips were then incubated with normal horse serum (NHS) (Blocking serum, Vector S-2000) 50µl in 2.5 mls of 1 x PBS at room temperature for 20 mins. After aspiration of the blocking serum and sequential washes with PBS, the coverslips were incubated with the

primary antibody (mouse anti human) at the appropriate dilutions (section 4.3.2) for 1-2 hours at room temperature. After sequential washes in 1 x PBS, the coverslips were incubated for 30 minutes at room temperature with biotinylated secondary antibody (horse anti mouse IgG), (Vector BA-1400) diluted 10µl in 1 ml of NHS. After sequential washes in PBS, the samples were incubated with VECTASTAIN Elite® reagent, a mixture of avidin DH and biotinylated horseradish peroxidase H for 30 mins at room temperature. After further washes with PBS, one drop of DAB solution, (DAB peroxidase substrate kit, Vector SK-4100) -a pre-prepared mixture of DAB, hydrogen peroxide and buffer- was added per coverslip and left until the desired stain intensity was obtained. This was achieved after ~ 3 mins. The DAB was aspirated, the coverslips washed with water and counterstained with Harris's haematoxylin for 10 seconds then rinsed with water. The coverslips were then dehydrated by passage through sequential alcohols, washed in xylene to remove the alcohol and mounted on a glass slide using DEPEX (Gurr) mounting medium.

2.1.2 Immunohistochemistry on Formalin Fixed Paraffin Embedded Sections

2.1.2.1 VectaBond™ Slides for Immunohistochemistry

Clean glass slides were placed in a rack and immersed in acetone for 5 minutes. 7ml of VECTABOND™ (Vector) reagent treatment were added to 350 ml of acetone and the slide racks were submerged in this solution for 5 mins, then gently dipped into distilled water and left to air dry.

2.1.2.2 H&E Staining

Samples from prostatic resection or diagnostic biopsy were obtained as described in

chapter 3. 3μm sections were cut onto non-bonded slides and stored at room temperature until required. The tissue sections were heated at 60°C in an oven for 30 minutes, then de-waxed for 3 minutes in xylene twice, and taken through a series of graded alcohols to dehydrate. After rinsing in running tap water, they were then stained with the regressive stain Harris' haematoxylin for 5 minutes, rinsed in tap water for 3 minutes and placed in 1 % acid alcohol for 6 seconds to differentiate. They were then left in Scott's tap water for 5 minutes, rinsed in tap water, counterstained with 1 % eosin for 5 minutes, rinsed in running tap-water for 60 seconds and then taken through a series of graded alcohols and xylene to dehydrate. Coverslips were then applied using DEPEX mounting medium.

2.1.2.3 Antigen Retrieval

3μm sections were cut onto VECTBOND-coated slides. For each antibody, a specific method of antigen retrieval was used (table 4.1). After dewaxing in xylene, the sections were taken through a series of graded alcohols to water. For PSMA, TIMP2, Bcl-2, MMP-2, FGF8 and FGFR, the optimum method of antigen retrieval was determined by using both of the microwave methods with each of the antigen retrieval solutions in turn, and then the pressure cooker method, to elicit the strongest degree of staining using positive controls. For the microwave method, the slides were placed in a rack in a large plastic container containing 600 mls of buffer (citrate, Dako pH 6.0 (S1699), Dako pH 9.9 (S3007) or Tris-EDTA buffer pH 9.0, (section 2.2.2) and microwaved at full power for 20 to 25 minutes, then the container was transferred to a sink of cold running water and allowed to stand for 10 minutes. For the pressure cook method, 3 L of citrate buffer pH 6.0 was placed in the pressure cooker, brought to boiling point and racks containing slides placed within and cooked at full pressure for 2 minutes. The

slides were then cooled with running tap water.

2.1.2.4 Antibodies

After antigen retrieval, endogenous peroxidase activity was quenched by incubation with peroxidase blocking solution (DAKO S2023) and the slide rinsed with washing buffer (DAKO K5006). 200 µl of each primary antibody was incubated at the required concentration in antibody diluent (DAKO S2022) for 60 minutes at room temperature. The EnVision system (DAKO), which uses a one-step system for the secondary antibody and streptavidin-biotin complex, and reduces background staining from endogenous biotin, (Vyberg & Nielsen 1998) was employed for all immunohistochemical staining with the exception of PSMA. For the EnVision technique, the EnVision HRP rabbit/mouse reagent (DAKO K5007), containing dextran coupled to peroxidase and goat secondary antibody molecules against rabbit and mouse was incubated at room temperature for 60 minutes. The immunoperoxidase antigen-antibody reaction products were visualised by incubation in diaminobenzidine (DAB) for 10 minutes at room temperature. The tissue was counterstained with Harris' haematoxylin for 2 minutes, dehydrated in alcohols and cleared in xylene.

2.1.2.5 Immunohistochemical Staining for Her-2/Neu

The Dako Hercept kit was used (A0485). After dewaxing in xylene, the sections were taken through a series of graded alcohols to water. 400mls of epitope retrieval solution (ERS) (Dako S1699) at a 1:10 dilution was heated for 12 mins at high power in an 800W microwave, placed in a water bath and maintained at 98°C. Sections were briefly pre-warmed to 98°C in the water bath, before placing in ERS for 40 mins. The ERS container was removed from the water bath, left to stand for 20 mins and the slides

rinsed in water, then in TBS/Tween (1 x TBS containing 0.1 % TWEEN) before incubation with peroxidase blocking solution for 5 mins. After rinsing in TBS/TWEEN, the pre-diluted 1/2000 Her-2 antibody was applied for 30 minutes. After further washes, the visualisation reagent was applied for 30 mins at room temperature, and a 10 minute incubation with DAB used to visualise the product as previously described. Sections were lightly counterstained with haematoxylin, washed in tap water and differentiated in 1 % acid alcohol, then dehydrated in the ethanols and cleared in xylene. Coverslips were applied using DEPEX mounting medium.

2.1.3 Protocols for Cell Line Experiments

Unless otherwise stated, the constituents of all solutions are detailed in section 2.2.4

2.1.3.1 Cell Culture

Three immortalised prostate cancer cell lines were used: LNCaP - originally derived from a supraclavicular fossa node, which expresses PSA and a mutated form of the androgen receptor (Horoszewicz et al. 1983); DU145 - derived from a brain metastasis, which expresses neither AR or PSA (Stone et al. 1978) and PC3-derived from a bone metastasis, which again expresses neither PSA or AR (Kaighn et al. 1979). All cell lines were obtained from Prof J Masters at the Prostate Cancer Research Centre. Cells were maintained in 5 mls RPMI complete medium in a T25 flask or in 15 mls of RPMI complete medium in a T80 flask. The medium was changed at 3 day intervals and the cells split using trysin-versene when 90% confluence was seen. Cells were maintained at 37°C with 4 % CO_2.

2.1.3.2 Cell Counting

Cell culture was performed as outlined above. The medium was aspirated from each

T80 flask and 3 ml of trypsin-versene added to detach cells. Trypsin-versene consists of 0.5g/L of trypsin at a ratio of 1:250 of 0.2g/L of versene in buffered saline solution. 7 mls of RPMI medium complete was added to terminate the reaction and the flask contents transferred to a 15 ml Falcon tube giving a cell sample suspended in 10 ml total. 100µl of cells in suspension was then added to 100µl 0.1 % trypan blue viability stain in a micro-centrifuge tube. The mixture was micro-pipetted onto the edge of a coverslip placed on a Neubauer® haemocytometer, allowing the suspension to be drawn under by capillary pressure, the cells counted and the number of cells per ml of solution calculated.

2.1.3.3 RNA Extraction Protocol

RNA extraction was performed using the RNeasy® mini kit (QIAGEN #74104)

The medium was aspirated from each T80 flask and 3ml of trypsin-versene added to detach the cells. 7 mls of RPMI complete medium were added to neutralise the trypsin and the suspension transferred to a 15 ml sterilin tube, and centrifuged at 1000 rpm at 4°C for 10 minutes and the supernatant removed. The pelleted cells were then disrupted by adding 350µl of Buffer RLT (#79216), which had been previously made stable by the addition of 10µl of β-Mercaptoethanol per 1ml of buffer RLT. Buffer RLT inactivates RNases to ensure isolation of intact RNA. Samples were vortexed and pipetted onto QIAshredder columns (#79654) and centrifuged for 2 mins at maximum speed to homogenise the cell lysate. 350µl of 70 % ethanol was added to the lysate to provide appropriate binding conditions and the sample applied to an RNA mini spin column in a 2 ml collection tube, and centrifuged for 15 seconds at 10,000 rpm. 700µl of Buffer RW1 (provided in kit) which acts as a DNA-ase, was added onto the RNeasy

column, centrifuged for 15 secs at 10,000 rpm to wash and the flow-through discarded. After transferring the column to a new collection tube, 500µl Buffer RPE (provided in kit) was added, and the column centrifuged for 15 secs at 10,000 rpm to wash and the flow-through discarded. 500µl Buffer RPE was added, and the column centrifuged at 10,000 rpm speed for 2 mins to dry the membrane and ensure no residual ethanol was present. The column was transferred into a new collection tube, 30µl of RNase-free water added, and centrifuged for 1 min at 10,000 rpm to elute. Samples were stored at -70°C.

2.1.3.4 RT-PCR

Reverse Transcriptase to Synthesise First Strand cDNA.

A 12 µl RNA / water mixture was made in a microcentrifuge tube with the following constituents:

1µg extracted RNA stored as Xµl volume**

1µl oligo (dT) 0.5µg/ml (Invitrogen Y01212)

12µl – (1+Xµl) of RNase free water (section 2.2.2)

**The RNA concentration was calculated using the OD obtained using the spectrophotometer. This provided a µg per µl concentration, giving a value of Xµl containing 1µl of RNA.

The RNA was heated for 10 mins at 70°C in a thermocycler then maintained on ice.

The following components were added to the mixture:

4 µl 5 x first strand buffer (Invitrogen Y00146)

2µl 0.1 M DTT (Invitrogen Y00147)

1µl 10mM dNTP (nucleotide triphosphates) mix (section 2.2.2)

The mixture was incubated at 42°C for 2 minutes, and 1 µl of Superscript II RNaseH reverse transcriptase (Invitrogen 18064-014, 200U/µl) added. The mixture was then incubated at 42°C for 50 minutes, heated at 70°C for 15 minutes to inactivate the reactions and the resulting cDNA stored at -20°C.

For all experiments an additional control tube was set up containing no RNA to ensure validity of the method.

Polymerase Chain Reaction (PCR)

A 50 µl master mix for each primer was made with the following components maintained on ice throughout:

3µl cDNA

5 µl 10 x Buffer IV (AB-0289)

3 µl $MgCl_2$ (25mM)

5 µl dNTP mix (section 2.2.2)

2µl Primer 1 (sense)

2µl Primer 2 (antisense)

29.5µl RNase free water

0.5µl Red Hot ® DNA polymerase (*Taq*) thermus icelandicus (AB-0406/B)

To the above reaction mixture, one drop of mineral oil was carefully layered over to prevent evaporation of PCR products.

For all PCR experiments, a negative control containing reagents only was set up to ensure no contamination of the technique had occurred.

PCR conditions were optimised for each experiment. The general conditions used were:

DNA denaturation/*Taq* activation	2 min	95°C	
DNA denaturation	45 secs	95°C	X 36 cycles
Primer annealing	45 secs	60°C	
Extension	45 secs	74°C	
Final extension	10 min	72°C	

2.1.3.5 Gel electrophoresis

Gels of the following composition were used:

200ml 1.5 % agarose gel for 28 well gel

3 g agarose (Cambrex BioScience 44366) was dissolved in 200 mls 1 x TAE (Section 2.2.2). 5µl per 100ml Ethidium bromide (10 mg/ml solution) was added (to allow bands of DNA to be visualised against a background of unbound dye). After the addition of appropriate amounts of PCR-product and loading buffer, (section 6.3), the gel was run

in an electrophoresis tank using 1 X TAE as running buffer to optimise product separation.

100 ml 1.5 % agarose gel

1.5 g agarose dissolved in 100 mls 0.5 x TBE with ethidium bromide added as before and 0.5 x TBE used as the running buffer.

2.1.3.6 Western Blotting

Unless otherwise stated, the constituents of all solutions are detailed in section 2.2.4 & 5.

Protein Extraction

Cells were trypsinised, washed twice with 1 x PBS, once in complete RPMI medium, spun and the pellet collected and kept on ice. 400µl of RIPA (radioimmunoprecipitation assay) lysis buffer was added to each tube, vortexed and left on ice for 15 minutes. Samples were vortexed, spun at 10,000 rpm in a microcentrifuge for 10 mins at 4°C and the supernatant protein extract kept on ice or stored at -70°C. Protein concentrations were quantitated using the Sigma Diagnostics Lowry method protein assay kit (P5656). A standard protein concentration curve was constructed using the protein standard solutions supplied, and the protein concentration of each sample measured with a spectrophotometer set at 500 nM wavelength read from the standard curve.

The lysates were separated using a 13 % gel or 8 % gel depending on the protein size. The stacking gel was poured onto the resolving gel, the comb inserted and left to set. 50µg of protein were loaded per well, made up to 20µl with RIPA buffer and 10µl 1 x SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) loading

buffer. SDS is an anionic detergent which denatures proteins. Samples were heated for 6 mins at 96°C and loaded onto the assembled gels in the electrophoresis tank filled with SDS-PAGE running buffer. Samples were run for 90 mins at 100 V. 10µl of a Rainbow ™ protein size marker (Amersham) was run alongside the samples. The proteins were transferred from the gel onto a PVDF (polyvinylidine difluoride) membrane in a blotting apparatus filled with 1 x transfer buffer overnight at 40V at 4°C.

The following day, the blot was incubated with blocking solution at room temperature for 60 mins on an orbital shaker to block non-specific binding. It was then incubated with primary antibody, (anti-AR, anti-PSA, or anti-actin) at the appropriate dilution (Section 6.3) in 4ml of 1 % milk protein:PBS, for 60 mins at room temperature on an orbital shaker. After washing with PBS-TWEEN , the membrane was incubated in a secondary antibody solution consisting of 1/5000 dilution of HRP-conjugated anti-mouse IgG in 1% milk protein, for 60 minutes at room temperature on an orbital shaker. After further washes with PBS-TWEEN and PBS alone, the blots were developed using the Supersignal® WestPico chemiluminescent substrate kit (Pierce 34080). If no signal was seen after developing the film after 5 or 30 mins exposure time, the blots were exposed overnight at 4°C.

2.1.4 LOH Protocols

2.1.4.1 DNA Extraction of Microdissected Specimens

Samples from prostatic resection or diagnostic biopsy were obtained as described in section 4.3 and Laser Capture Microdissection (LCM) performed as detailed in section 5.3.2. Two methods of DNA extraction were employed.

DNeasy® (QIAGEN #69504) Tissue Kit Method

The kit allows extraction of purified DNA free from contaminants and enzyme inhibitors. 180µl of Buffer ATL (tissue lysis buffer #19076) and 20 µl of proteinase K (20mg/ml #19131) to cause direct cell lysis were added to a clean 0.5µl Eppendorf® for each microdissected sample. The LCM cap was transferred to the eppendorf, firmly inserted and the eppendorf vortexed and inverted to ensure maximum contact of the microdissected cells with the tissue lysis solution. The samples were placed in a humidified oven at 55°C overnight. The buffering conditions subsequently optimise selective DNA binding to the DNeasy membrane. The following day, the samples were vortexed for 15 seconds and 200µl of buffer AL (#19075) added to each tube, vortexed and incubated at 70°C for 10 minutes. 200µl of 100 % ethanol were added, the samples vortexed and pipetted onto a DNeasy mini column sitting in a 2 ml collection tube, centrifuged at 8000 rpm for 1 minute and the flow-through discarded. 500µl of wash Buffer AW1 (#19081) was added, the samples centrifuged at 8000 rpm for 1 minute and the flow-through discarded. 500µl of wash buffer AW2 (#19072) was added, centrifuged for 3 minutes at 10,000 to dry the membrane and the flow-through discarded. The column was placed in a new microcentrifuge tube, and 100µl of Buffer AE (#19077) added to the membrane to elute. Samples were then incubated at room temperature for 1 minute, centrifuged at 8000 rpm for 1 minute and the elutate stored at -20°C.

In-House Protocol for DNA Extraction

Protocol obtained from Breakthrough Breast Cancer Laboratories, Institute of Cancer Research.

A sterile solution of proteinase K extraction buffer was made, using 50mM KCL; 10mM Tris-HCL, pH 8.0; 2.5 mM $MgCl_2$; 0.1 mg/ml gelatin; 0.45% NP40; 0.45% Tween 20. Proteinase K (PK) (Invitrogen # 25530049, 20 mg/ml) was diluted 1:40 in proteinase K extraction buffer and 20µl of the PK/ buffer solution added to a clean 0.5ml eppendorf. The LCM cap was then transferred to the eppendorf, pushed in tightly and inverted to ensure contact of the microdissected cells with the extraction solution. Samples were incubated overnight at 55°C in a humidified chamber. The following morning, the tubes were microcentrifuged for 30 seconds at maximum speed to spin down the solution, and placed in a thermocycler at 95°C for 10 minutes to inactivate the PK. The samples were microcentrifuged for 1 minute at maximum speed, the eluted DNA pipetted into a fresh tube and stored at -20°C.

2.1.4.2 PCR using LOH PSA Primer

A 15µl reaction mix was made with the following constituents:

1.5µl 10 x Reaction buffer (contains 750 mM Tris-HCL, pH 8.8)

6.1µl Distilled water

1.5µl dNTP 1:10 dilution

1.5µl $MgCl_2$

1.6µl PSA Primer sense (forward microsatellite marker) 5µM concentration

1.6µl PSA Primer antisense (reverse microsatellite marker) 5µM concentration

0.2µl AmpliTaq Gold (ABI 4311806)

1.0µl DNA

AmpliTaq Gold is a heat stable DNA polymerase obtained from the *Thermus Aquaticus* bacterium. AmpliTaq Gold® DNA Polymeraseis a chemically modified form of AmpliTaq® DNA Polymerase.When the chemical moiety is attached to the enzyme, the enzyme is inactive. During set-up and the first ramp of thermal cycling (when the reaction is going through non-optimal annealing temperatures), the enzyme is inactive. The result is that mis-primed primers are not extended. When the reaction reaches optimal annealing temperatures, the chemical moiety is cleaved during a heat activation step, releasing active AmpliTaq® DNA enzyme. The yield of specific product increases because reactants are not wasted in the formation of unintended products. AmpliTaq Gold® DNA enzyme's chemical hot-start capability can release active enzyme in a "time-release" manner. With or without a limited up-front heat activation step, active enzyme is released slowly during thermal cycling to match template concentration and increase specificity.

To the above reaction mix, one drop of mineral oil was added to each eppendorf to reduce evaporation of the PCR products. Optimisation of the PCR conditions is described in section 6.3.2.

The final conditions used were as follows:

DNA denaturation/*Taq* activation	15 mins	95°C	
DNA denaturation	45 secs	95°C	X 50 cycles
Primer annealing	45 secs	58°C	

Extension	45 secs	74°C
Final extension	10 min	72°C

The sequences for the PSA LOH primers were as follows:

Forward primer GGAGCTCTGACTCAGTTCTACTCTATC;

Reverse primer GACTGAGACATCCTCTCCCACCAT. The forward primer was labeled with FAM (6-carboxyfluorescein) to give a blue identification line on analysis.

Two controls were used throughout: LNCaP DNA as a positive control and reagents only as a negative control to ensure there was no contamination of the reaction.

2.2 Reagents & Buffers

2.2.1 Reagents for Immunohistochemistry

2.2.1.1 Harris Haematoxylin

10g	Haematoxylin powder
40ml	Acetic acid
50ml	Ethanol

Made up to 1L with distilled water.

2.2.1.2 Scott's Tap water

7g	Sodium bicarbonate
40g	Magnesium sulphate
2L	Tap water

2.2.1.3 Acid Alcohol 1 %

99ml	70% alcohol
1ml	Concentrated HCl
1%	Eosin (1g Eosin, 100ml Distilled water)

2.2.2 Buffers for Immunohistochemistry

2.2.2.1 Tris Buffered Saline (TBS) pH7.4

80g	Sodium chloride
6.05g	Tris (tris hydroxymethylamine)(Sigma)
44ml	1M HCl
10L	Distilled water

pH to 7.4 with 1M HCl.

2.2.2.2 Citrate Buffer pH 6.0

29.4g	Sodium citrate (BDH)
54ml	1M HCl
10L	Distilled water

pH to 6.0 with 1M HCl.

2.2.2.3 EDTA Buffer pH 8.0

18.61g	EDTA
52ml	1M NaOH
5L	Distilled water

pH to 8.0 with 1M NaOH.

2.2.2.4 Tris-EDTA Buffer (X10 concentrate)

12g	Tris
1g	EDTA
10ml	1M HCl
500ml	Distilled water

Keep refrigerated. Dilute 1/10 with distilled water and pH to 9.9 before use.

2.2.3 Reagents for Tissue Culture and Molecular Biology

2.2.3.1 RPMI Complete Medium

500ml	RPMI- 640 medium without L-glutamine (Invitrogen 31870-025)
5ml	L-Glutamine (stock 200 mM concentration)
40ml	Foetal calf serum (stock)

2.2.3.2 RNase-Free Water.

1ml DEPC (diethyl pyrocarbonate) (Sigma) to 100ml distilled water

Shake vigorously, incubate for 12 hours at 37°C then autoclave for 15 minutes to remove DEPC traces.

2.2.3.3 dNTP for RT

10µl of 100mM stock solution of each dNTP (Invitrogen) (dATP, dCTP, dGTP, dTTP) added to 60µl RNase free water

2.2.3.4 dNTP for PCR

10µl of 100mM stock solution of each dNTP (dATP,dCTP,dGTP,dTTP) added to 460µl RNase free water , vortexed.

2.2.4 Buffers for Tissue Culture and Molecular Biology (Electrophoresis)

2.2.4.1 0.5x TBE (Tris-Borate EDTA)

Diluted from 10x TBE stock (Promega V4251)

2.2.4.2 50x TAE

242g	TRIS Base (Sigma)
57.1ml	Glacial acetic acid
100ml	0.5M EDTA pH 8.0 (Merck)

Diluted to 1x

2.2.5 Reagents for Western blotting

2.2.5.1 5x PBS (Phoshate Buffered Saline)

42.5g	Sodium chloride
32.4g	Disodium hydrogen phosphate

0.78g Sodium phosphate

Made up to 1L with ultrapure water, pH 7.3 (de-ionised, RNA-ase free water)

2.2.5.2 20x TBS (Tris Buffered Saline)

60.6g Trizma hydrochloride (Sigma)
13.9g Trizma base (Sigma)
87.6g Sodium chloride (Sigma)
500ml Ultrapure water

2.2.5.3 RIPA Lysis Buffer

50mM Tris-HCl pH 8.0
150mM NaCl
5mM EDTA
1% (volume/volume) NP 40 (USB)
0.5% (weight/volume) sodium deoxycholate
0.1% (weight/volume) SDS (USB)
10% (volume/volume) glycerol

Immediately before using, the following protease inhibitors were added to the RIPA solution:

Phenylmethylsulphonyl chloride (PMSF) 1mM, aprotinin (10µg/ml), leupeptin (10µg/ml), and pepstatin (10µg/ml),

2.2.5.4 SDS-PAGE Gel Loading Buffer

4ml Distilled water
1ml 0.5M Tris pH 6.8
0.8ml Glycerol
1.6ml 10% SDS
0.4ml 2-mercaptoethanol
0.2ml 0.2% bromophenol blue

2.2.5.5 5x SDS-PAGE Running Buffer

7.5g Tris base
36g Glycine
2.5g SDS

2.2.5.6 10x Transfer Buffer for Electroblotting

15g Tris base (Trishydroxymethylmethylamine-BDH)
72g Glycine
10% Methanol

2.2.5.7 Blocking Solution

5% Milk protein (Marvel)
made up in:
500ml 1x PBS
0.5 ml TWEEN-20 (polyoxyethylene (20) sorbitanmonolaurate-BDH Lab Supplies)

2.2.5.8 Stacking Gel

3ml Water
0.67ml 30% acrylamide mix
0.25ml 1M Tris, pH 6.8
40µl 10% SDS
40µl 10% ammonium persulphate (APS)
6µl TEMED

2.2.5.9 Resolving Gels

	15%	**8%**
Water	2.5ml	4.6ml
30% acrylamide	5mls	2.7ml
1.5mM Tris buffer pH 8.8	2.5ml	2.5ml

To the above, 100µl 10% SDS, 100µl ammonium persulphate and 6µl of TEMED (Sigma-tetramethylethylenediamine, to catalyse acrylamide polymerization) were added, and 3.5 ml thin gel poured into the assembled gel plates, which were left for 30 minutes to polymerize.

2.3 Chemicals & Reagents Suppliers

Applied Biosystems (AB) Foster City, California, USA

Amersham Biosciences, Piscataway, New Jersey, USA.

Biocarta US, San Diego, California, USA

BDH (VWR-International Ltd), Poole, Dorset, UK

Boehringer-Mannheim, Germany

Cambrex Bio Science Verviers, S.P.R.L., Verviers, Belgium.

DakoCytomation, Glostrup, Denmark

Invitrogen Life Technologies, Paisley, UK.

Merck & Co Inc, New Jersey, USA

Monosan, Uden, The Netherlands.

Pierce Biotechnology Inc, Rockford, IL, USA

Qiagen Ltd, Crawley, West Sussex, UK

Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA

Sigma Diagnostics, St Louis, Missouri, USA.

Vector Laboratories, Burlingame, California, USA

Chapter 3 The Clinical Features of Patients Presenting with Treatment-Naïve Metastatic Prostate Cancer and Serum Prostate Specific Antigen (PSA) < 10 ng / ml - the "PSA-Negative" Patients

3.1 Introduction

As previously outlined, the level of serum PSA usually correlates with the volume of tumour present, hence in the great majority of patients with metastatic disease, PSA is markedly increased (Daver et al. 1988). Typically less than 1 %, (Oesterling 1993) present with abnormally low serum levels of PSA, below 10 ng/ml. ("PSA-negative" disease) (Oommen et al. 1994;Wolff, Zimnyet et al. 1998;Yamamoto et al. 2001b). Information on this patient group is sparse, though an association with high tumour grade (Gleason 8-10) has been described (Pantelides et al.1992;Partin et al. 1990).

Overall survival is anecdotally thought to be poorer than for patients with PSA-positive metastatic prostate cancer (Yamamoto et al. 2001a). The disease is thought to represent a different clinical entity, with a pattern of metastatic disease unlike that of high PSA tumours, showing an increased number of visceral and soft tissue metastases (seen in up to 55 % of patients) and non-typical pattern of bone disease (Sella et al. 2000). However, this data is obtained from patients with androgen-independent clinically progressive disease, rather than from patients presenting as treatment naive metastatic prostate cancer with low serum PSA.

3.2 Aims

The aim of this study was to describe the clinical features of patients presenting with

metastatic prostate cancer and serum PSA levels of less than 10 ng/ml, in the largest group thus far characterised.

3.3 Materials and Methods.

Patients presenting with histologically proven untreated metastatic prostate cancer and a serum PSA of < 10 ng / ml were identified from the British Association of Urological Surgeons (BAUS) Cancer Registry. Data was collected from patients who presented in 2000 and 2001. All database information was anonymised, with the patient identifiable by hospital ID and date of birth, referring consultant and referring centre. Details on stage, grade of tumour, presenting PSA and treatment decision were extracted. There was no policy of exclusion of cases with neuroendocrine histology. Referring consultants were contacted by post, and asked to forward consent forms and study information sheets on to patients. Copies of the patient information sheet, consent form and letters to the urologists, patients and pathologists can be seen in Appendix 1. Agreement had been previously obtained from the Multi Centre Research Ethics Committee that consent was not needed from next of kin where patients had died. Patients were also identified from local referrals to uro-oncologists at the Meyerstein Institute of Oncology at the Middlesex Hospital. Approval for this study was given by the Joint UCL / UCLH Committees on the Ethics of Human Research (Study number 01/0215) and the South East Multi-Centre Research Ethics Committee (MREC 02/01/16).

One investigator (AJB) then reviewed the patient's notes at centres nationwide. Presentation, pattern of metastases, initial PSA, histology, treatment and treatment response times, and overall survival were reviewed. Hall's definition of clinical

progression as "new symptoms or signs, or worsening of those already existing, clearly attributable to prostate cancer" was used (Hall 1997) together with the time to first subjective progression, evaluated using similar criteria for response as in the EORTC Phase III 30853 study of hormonal therapy in metastatic prostate cancer (Denis, Carnelho de Moura et al. 1993). In this study, subjective progression was defined as a progression of urological symptoms with the appearance of severe symptoms requiring surgical relief or catheterisation, weight loss of more than 10% within one year, or deterioration of WHO performance status. An increase in pain score as reflected by a step up in the WHO analgesia classification and ladder (Ventafridda 1985) was also considered to reflect progression. If sequential radiological assessment was performed, progression was defined as the appearance of new lesions on bone scan or new lesions on X-ray film, or a greater than 25% increase in index lesions measured as the sum of products of the largest perpendicular diameters. PSA response, which is often used as a surrogate marker for treatment response, was inappropriate for this study. Survival curves were plotted using the Kaplan-Meier method. Archival prostate blocks were obtained for a pathological and morphological review at the department of Histopathology, University College London Hospital.

3.4 Results

3.4.1 Cases Collected

60 cases were identified from the BAUS Cancer Registry, from 2708 cases of metastatic prostate in total diagnosed during the 2000 and 2001 database collection period, the PSA-negative group hence representing 2.21% of the total number of registered cases. 41 replies were elicited for these 60 cases. Of these, 14 cases were excluded either because review of the clinical notes revealed that the serum PSA was greater than 10 ng

/ ml (range 22.1-121) at presentation, lack of evidence of metastatic disease, equivocal diagnosis of prostate cancer, or refusal by the consultant to approach the patient for consent (due to such factors as terminal illness). Six other cases were collected from referrals to the local cancer centre at the Meyerstein Institute of Oncology. 33 sets of clinical case notes were reviewed. Three additional cases registered as having neuroendocrine differentiation were omitted as clinical case notes were missing and / or no pathology blocks were available.

3.4.2 Patient Demographics

The median age was 67 years (range 48-81 years) with median serum PSA at presentation of 3.5 ng/ml (range 0.1-9.8) (Table 3.1). The median age of the group was slightly lower than that of prostate cancer overall (72.3 years) (BAUS Section of Oncology 2002). No patient had a prior history of pelvic radiotherapy. The presenting features, pattern of metastases and extent of local disease are summarised in Table 3.1. The pattern of bone disease was typical of prostate cancer, predominantly affecting the axial skeleton, pelvis and long bones.

3.4.3 Histopathology

Histopathological confirmation of disease was obtained in all patients; 57 % of patients were diagnosed on needle biopsy, while the others had transurethral resection of the prostate for symptoms and diagnosis (Table 3.2). Referral pathology reports suggested that the Gleason scores for the PSA negative group were only slightly higher than those for all metastatic prostate cancer patients on the BAUS cancer registry (Figure 3.1). On review by a specified urological histopathologist (AF), 25 cases (76%) had high grade cancers with mean Gleason score of 9 (Table 3.2). None showed neuroendocrine

differentiation.

3.4.4 Treatment

All patients received hormonal manipulation as primary therapy (Table 3.3) with a subjective response rate of 73 %. Benefit in responding patients was symptomatic (80%), with either a reduction in the amount of analgesia required, or improvement of performance status. One patient received initial hormones as part of an intermittent hormonal study, which were then discontinued until rising PSA. 23/33 (69%) patients received palliative radiotherapy for symptomatic bone pain or spinal cord compression, with 3 patients receiving palliative prostatic radiotherapy to improve haematuria and perineal pain.

3.4.5 Responses to Treatment

Symptomatic response durations to primary treatment were 0-14 months with a median time to progression of 7 months (Figure 3.2). Subsequent treatment was most frequently (36%) the combination of an LHRH agonist and non-steroidal anti-androgen (maximal androgen blockade, MAB). Third line hormone therapy was commenced in 12 % of patients. 5/33 (15%) patients received chemotherapy (Table 3.3).

No evidence of symptomatic benefit was seen in 83 % of patients placed on maximal androgen blockade or in any of those commenced on third line hormones. Three symptomatic and radiological responses were seen in the five patients treated with chemotherapy (1 with single agent mitozantrone, 2 with epirubicin, carboplatin & 5-fluorouracil), but relapse occurred shortly after completing treatment (3-7 weeks). The number of chemotherapy cycles ranged from 3 to 6.

The frequency of serum PSA estimation subsequent to the initiation of treatment varied considerably. PSA levels fell from the baseline values in 6 cases, but an association between fall in PSA (i.e. within the 0-10 range) and improvement in clinical symptoms was seen in only 2 patients. In only 2 patients did PSA values rise above 10 ng / ml (to 27.8 and 43.1 respectively) at any time during the course of their disease. Hypercalcaemia was seen in 3 cases (9%), either at initial presentation or during disease progression.

3.4.6 Survival

Median overall survival was 12 months with a range of 1 –36 months (Figure 3.3). All 22 deceased patients died of their disease. With a median follow-up of 19 months, 11 / 33 (33%) patients remain alive, 7 with progressive disease and 4 (12%) alive and well.

3.5 Discussion

Metastatic prostate cancer that is "PSA-negative", presenting with abnormally low levels of PSA accounts for only a small proportion of patients, but presents a significant management dilemma. This study has described the clinical features from the largest series so far documented. The median age of the group was slightly lower than that of prostate cancer overall (67 years versus 72 years) (BAUS Section of Oncology 2002). The presenting symptoms were typical of metastatic prostate cancer in general, and most patients presented with symptoms of advanced local disease or symptomatic bone metastases. Despite reported rates of soft tissue metastases of up to 55% (Sella et al. 2000) there was a relatively low rate (30%) of non-bone disease, either visceral metastases or non-regional lymph nodes metastases in this study.

In the staging of newly diagnosed cases, various studies have recommended a threshold

below which imaging investigations are unlikely to reveal metastases. In a study of 861 men with a PSA below 20 ng / ml, eight (0.9%) had positive bone scans and 13 (1.5%) had CT- defined nodal disease (Levran et al. 1995).

It has been proposed that an undifferentiated component correlates with non-responsiveness to hormonal therapy (Gaffney et al. 1992) and other series have been composed almost exclusively of poorly differentiated and undifferentiated tumours (Yamamoto et al. 2001a). In 3 cases of metastatic prostate cancer with serum PSA < 10 ng / ml, all tumours were of high Gleason grade and the author postulated that the tumours had lost the characteristics of the original prostate tissue itself, and this affected PSA release into the blood stream (Kageyama. 1996). Other authors have also described the association between advanced prostate cancer stage with a low PSA and poorly differentiated tumours (Partin et al. 1990). However, although our series included a high proportion of poorly differentiated Gleason 8-10 tumours (76% on histology review), the remainder were moderately differentiated. It is possible that in these last cases the bulk of the tumour had a different morphology from that sampled. None of the 33 cases contained morphological evidence of neuroendocrine differentiation. In 3 further cases identified from the BAUS Cancer Registry that were not studied, either due to lack of case notes or no tissue remaining on the pathology blocks, there were features of neuroendocrine differentiation reported by the referring centre pathologist, but it was not possible to verify this.

Response to treatment in this group of patients is difficult to measure. PSA is of limited value given that baseline values were < 10 ng / ml. There was a fall from baseline PSA levels in 6 patients, but this correlated with improvement in clinical symptoms or signs

in only 2. In these two cases, it is likely that the dominant clone produced small amounts of PSA, contrasting with the other 4 patients who demonstrated a fall in PSA levels from baseline but had clinically progressive disease. In all but two patients, PSA values never rose above 10 ng/ml, a remarkable observation in the context of widespread symptomatic progressive disease. In the two patients demonstrating a PSA rise, this may reflect the presence of a mixed PSA-negative and PSA-positive clonal population. The total PSA rather than free/total PSA ratio was the only data available. It was not possible to stratify patients into the prognostic groups defined by Fossa (Fossa et al. 1992) based upon serum creatinine, haemoglobin and alkaline phosphatase, due to incomplete data recording at the referring centre.

Hypercalcaemia is uncommon in prostate cancer. Recent data from a bisphosphonate trial suggest <1% incidence of hypercalcaemia in the placebo arm in hormone refractory metastatic prostate cancer (Saad 1995). When reviewed, the rare cases of hypercalcaemia in prostate cancer have been largely associated with neuroendocrine carcinomas (Smith et al. 1992). The high rate (9%) of hypercalcaemia in our study would reinforce the differing biological nature of the PSA- negative tumour despite the absence of neuroendocrine differentiation.

The median duration of response to first line therapy in our study was 7 months, in marked contrast to the figure of 12 to 18 months seen in randomised studies of previously untreated metastatic prostate cancer (Eisenberger et al. 1998;Ventafridda et al. 1985). Our response rate is similar to that seen in a smaller case series of PSA-negative tumours, where response to 1st line hormonal therapy was seen in 2 / 4 patients (Yamamoto et al. 2001a). Nonetheless, the majority of patients did have a symptomatic

response to androgen deprivation and this remains the recommended initial treatment.

For patients with metastatic prostate cancer in relapse, further responses to subsequent endocrine treatments are seen in 35-45 % (Labrie 1988;Small & Vogelzang 1997) with a median survival time of 9 months after second line treatment is initiated (Hussain et al. 1994). In the current study, no responses were seen in 83% of patients in whom a non-steroidal anti androgen was added to their initial LHRH agonist or in any of those commenced on third line hormonal therapy.

Of the 5 patients receiving chemotherapy, 3 responses were seen, maintained throughout the chemotherapy course but not durable once treatment was completed. Response rates of 10-15% to chemotherapy have been quoted in the context of metastatic prostate cancer (Brausi 1995) with responses maintained for up to 9 months. More recently, Phase II studies have proved encouraging with responses of over 40% in selected hormone refractory patients for combinations such as 5-fluoruracil, epirubicin and cisplatin (Chao et al. 1997) or various taxane containing regimes (Petrylak 2002). Given the poor response to second or third line hormonal therapy in the PSA-negative group, early chemotherapy may well be worth considering in patients with good performance status.

The median overall survival time of 12 months for the patients studied is much shorter than that seen in treated metastatic prostate cancer overall, where a figure of 27 months (Eisenberger et al. 1998) or one year after the first progression is described (Denis et al. 1993).

This study has described the clinical features in the largest series thus far reported of patients presenting with metastatic prostate cancer and a serum PSA of less than 10 ng /

ml, the PSA-negative metastatic group. Although the presentation and pattern of metastases are similar to those of high PSA metastatic disease, response to hormonal therapy is poor, with short response durations to first line treatment and minimal response to subsequent management other than chemotherapy. Hypercalcaemia appears to be a prominent feature. Overall survival rates are much less than those of high PSA disease and alternative strategies for management, including early chemotherapy in appropriate patients should be considered.

Table 3.1 Patient Demographics

Age (years)	48 – 81 (median 67)
PSA at diagnosis (ng/ml)	0.1 – 9.8 (median 3.5)
Previous TURP for BPH	5 (15%)
Previous Urinary Investigations	6 (18 %)
Presenting symptoms	
Urinary symptoms/pelvic pain	17 (51%)
Acute retention	6 (18%)
Cachexia / malaise	3 (10 %)
Bone pain	7 (21 %)
Bone metastases	27 (81 %)
Axial skeleton	16 (48 %)
Pelvis	9 (27 %)
Femur / Humerus	8 (24%)
Soft tissue[a]	4(12%)
Lung	2
Liver	1
Glans penis (cutaneous)	1
T Stage	
T2	1
T3a / b	12
T4	20
Abdominal nodal metastases	6 (18 %)
Bone marrow infiltration	2 (6 %)

[a] In addition to bone metastases

Table 3.2 Histopathology

Source of material	
TURP	13 (39 %)
Prostatic biopsy	19 (57 %)
TUR bladder neck	1
Histology at local centre	
Gleason 8-10	20 (60%)
Gleason 6-7	13 (40%)
Histology review	
Gleason 8-10	25 (76 %)
Gleason 6-7	8 (24 %)

TURP transurethral resection of prostate

Table 3.3 Treatment and Response

Treatment	Number of patients	Responses
First line therapy	33	24
LHRH agonists[a]	27	21
NSAA	3	1
Orchidectomy	3	2
Second line therapy		
MAB	12	2
Zoladex restarted [a]	1	1
Third line therapy		
Diethylstilboestrol	4	0
Chemotherapy:	5	3
single agent	3	1
combination	2	2

MAB Maximal androgen blockade; [a] 1 patient treated with intermittent hormone therapy; NSAA non-steroidal anti-androgen monotherapy

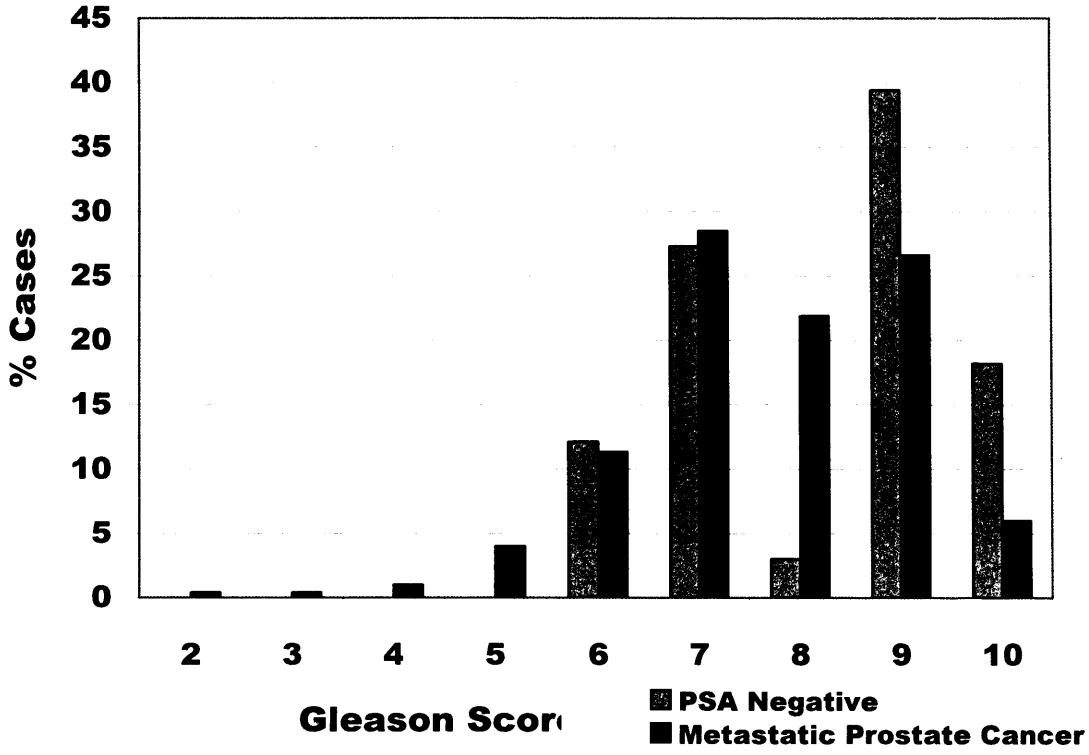


Figure 3.1 Histopathological Grading of PSA-Negative Metastatic Prostate Cancer and Metastatic Prostate Cancer Overall (data from local centres)

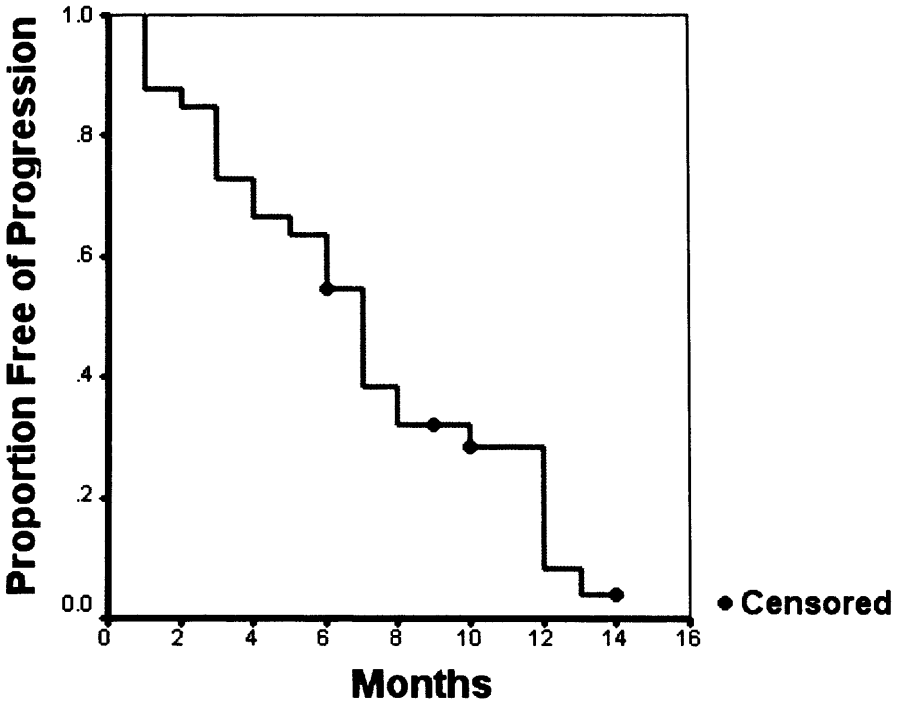


Figure 3.2 Progression Free Survival in Patients with PSA-Negative Metastatic Prostate Cancer

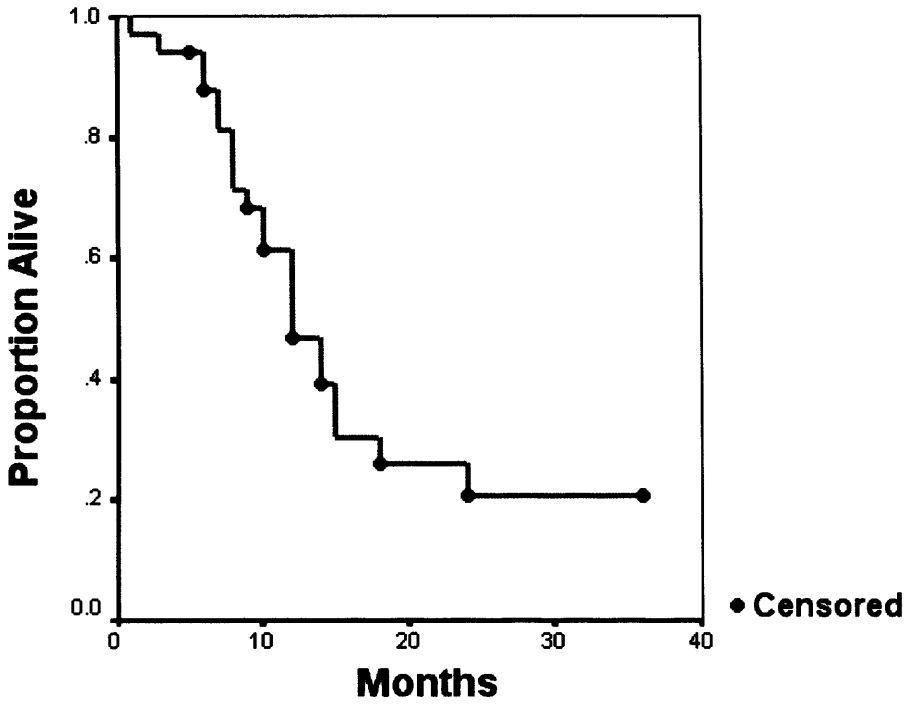


Figure 3.3 Overall Survival of Patients with PSA-Negative Metastatic Prostate Cancer

Chapter 4 The Immunohistochemical Features of Treatment-Naïve Metastatic Prostate Cancer Presenting with a Serum PSA < 10 ng/ml

4.1 Introduction

The management of prostate cancer has been transformed by the measurement of serum levels of PSA as already discussed (Stamey et al. 1987) with markedly elevated levels of PSA reflecting the tumour burden (Daver et al.1988). In this thesis, the low PSA group is defined as men presenting with untreated metastatic disease with serum PSA levels of less than 10ng/ml (Birtle et al. 2003). As outlined in chapters 1 and 3, the small percentage of men who present with previously undiagnosed metastatic prostate cancer and low serum PSA levels tend to have a worse prognosis. This is evidenced by their poor response to hormonal manipulation and the difficulty in monitoring, due to the lack of a reliable serum marker (Birtle et al, 2003).

The identification of alternative tissue markers could allow investigation of corresponding serum markers to facilitate diagnosis and monitoring in PSA-negative disease.

The two primary candidate alternative prostate cancer markers are prostatic acid phosphatase (PAP) and prostate specific membrane antigen (PSMA) neither of which has been evaluated in the context of low serum levels of PSA. PAP was in widespread clinical use to monitor prostate cancer prior to the advent of PSA, but is less sensitive and specific (Bogdanowicz 1991). Prostate specific membrane antigen (PSMA) was discovered after PSA, and has been shown to be present in larger amounts in malignant

than in benign prostatic tissue (Chang. 2001b). However, it has not found a routine role in clinical practice.

4.2 Aims

The aims of this study were:

- To examine the histopathological features of men presenting with untreated metastatic prostate cancer and serum PSA less than 10 ng/ml
- To investigate the extent of staining for tissue PSA
- To determine whether alternative tissue markers might be useful in this patient group to aid diagnosis and histopathological assessment.

4.3 Materials and Methods

4.3.1 Cases Studied

Patients presenting with untreated histopathologically proven metastatic prostate cancer and a serum PSA of less than 10 ng/ml were identified as outlined in Chapter 3. Paraffin embedded formalin-fixed archival prostatic tissue specimens were obtained. H & E slides of each case were cut and reviewed by a pathologist with a special interest in urological pathology (AF) to confirm the diagnosis and grade of carcinoma. The Local Ethics Committee of UCLH NHS Trust and the South East Multi-Centre Research Ethics Committee approved the study.

4.3.2 Specific Immunohistochemical Staining (see Section 2.1.2 for Immunohistochemistry General Technique)

3μm serial tissue sections from each case were mounted onto Vectabond-coated slides and immunostained using commercially available antibodies to PSA, androgen receptor

(AR), PSMA, PAP, Chromogranin A, and CD56. In addition, cytokeratins (CK) 7 and 20 and 34βE12 antibodies were used to exclude transitional cell carcinoma (Varma et al. 2003). Appropriate positive and negative controls were included for all immunohistochemistry using standard methods of antigen retrieval and previously validated antibody concentrations for all antibodies other than PSMA. The optimal dilution of and preferred method of antigen retrieval for PSMA was determined by titrating the antibody using a high-grade radical prostatectomy sample as a positive control. The tissue sections were dewaxed in xylene and taken through a series of graded alcohols to water. The method of antigen retrieval, primary antibody concentration and method of staining are summarised in Table 4.1. The general technique for immunohistochemistry using the EnVision method was followed as described previously (section 2.1.2).

4.3.3 PSMA Immunostaining

PSMA was visualised using a goat peroxidase anti-peroxidase system (Sternberger 1970). After antigen retrieval and washing as described above, slides were incubated with normal rabbit serum 1:10 for 10 minutes at room temperature. Primary antibody (goat) was then added for 60 minutes followed by a 1:200 dilution of rabbit anti-goat secondary antibody in excess. 200 µl of 1:100 goat-peroxidase-anti-peroxidase was then added for 60 minutes at room temperature with DAB detection as before.

Immunohistochemical staining was assessed independently by two observers, including a pathologist with a special interest in urological pathology (AF). A semi-quantitative assessment was performed taking into account the intensity (0: negative; 1+: low intensity, just above level of background staining; 2+: moderate intensity; 3+: high

intensity) and extent (focal/diffuse) of staining. For all immunohistochemistry, the score and extent were recorded in areas of carcinoma. Very focal areas (less than 10 cells) showing low intensity staining (1+) staining were classified as negative. Given the subjective nature of assessment, the 2+ and 3+ grades were reported together, in keeping with other authors.

4.3.4 Potential Diagnostic Markers as Therapeutic Targets

10 of the 33 cases were selected at random (5 biopsy specimens and 5 from transurethral resection) to perform an additional immunohistochemistry panel of 6 antibodies including TIMP2, MMP2, Bcl-2, Her-2, FGF8 and FGFR2. Each of these represented a potential molecular therapeutic target. The details of this small study are given in Appendix 2.

4.4 Results

4.4.1 Clinical and Histopathological Features

In 19 cases (57 %) archival tissue was obtained from ultrasound-guided prostatic biopsy specimens, with a further 13 specimens derived from transurethral resection of the prostate and one from a bladder neck resection. 27 / 33 cases (81%) had bone metastases, with four patients presenting with soft tissue metastases. All patients received primary hormonal manipulation (for details, see Birtle et al, 2003).

25 cases (76 %) had a combined Gleason score of 8-10, with the remaining 8 cases (24%) having a combined Gleason score of 6-7. No cases showed morphological features of neuroendocrine differentiation. One case classified as a prostatic carcinoma with a combined Gleason score of 10 was subsequently re-classified as a urothelial carcinoma because of the immunohistochemical expression of urothelial markers (see

below).

4.4.2 PSA Immunostaining

9/ 33 cases (27 %) showed negative staining for PSA with a further 3 classified as focal 1+ (Table 4.2). 21 of the 33 cases (63 %) demonstrated 2-3+ immunoreactivity which was focal in 19/33 cases. The association of PSA staining with AR, PSMA, PAP, chromogranin A and Gleason score is summarised in Table 4.2.

4.4.3 AR Immunostaining

A strong diffuse nuclear staining for AR was observed in 30/33 cases. Moderate or strong staining for AR was seen in 9/12 cases that were negative or focally 1+ for PSA (Table 4.2).

In these 3 latter cases, the AR staining was in an area that was negative for tissue PSA. Of the 3 cases that were completely negative for PSA/AR, one was later regarded as a urothelial tumour by virtue of positive CK 7 and 34βE12 staining.

4.4.4 PAP Immunostaining

24/33 cases (84 %) were positive for PAP, although the pattern of staining was mainly focal (79 %). There was also strong positive co-staining of PSA and PAP staining in 60% of cases, with 16 cases showing moderate or strong staining for both PSA and PAP and 3 cases being negative for both antibodies.

4.4.5 PSMA Immunostaining

30 / 33 cases (90 %) demonstrated PSMA staining, which was diffuse in 70% of the positive cases. In the 12 cases that were PSA negative or focal 1+, PSMA was diffusely

positive in 9 cases. In those tumours showing a mixture of PSA positive and negative areas, there was no association seen between the staining pattern for PSA and that for PSMA.

The pattern of immunostaining seen with respect to PSA, AR, PSMA and PAP is illustrated in Figure 4.1, where images A-E represent one patient with negative tissue staining for PSA, and images F-J represent a second case with positive immunostaining for PSA.

4.4.6 Chromogranin A Immunostaining

Chromogranin A immunostaining was negative in 24/33 cases (72%), with 8 of those cases also tissue PSA negative (Table 4.2). In 6 cases (18%) 2+/3+ intensity of immunostaining was observed, but the pattern of staining was focal. One case displayed focal 3+ intensity of staining, but this was present in only 2 cells, and in keeping with other authors' publications (Deftos et al. 1996;Sciarra et al. 2003), was not considered sufficient to reflect neuroendocrine differentiation.

4.4.7 Urothelial Markers

3 cases were examined with urothelial markers, as a result of uncertainty over the diagnosis of prostate cancer morphologically. 2 cases were CK 7 positive and CK 20 negative, the other CK 7 negative / CK 20 positive. 34 βE12 was positive in one of the 3 cases, which was also CK 7 positive and was thought more likely to be a urothelial tumour than a prostate carcinoma (data not shown).

4.5 Discussion

This study has investigated the immunohistochemical profile of 33 primary tumours in

men presenting with treatment-naive metastatic prostate cancer and serum levels of PSA less than 10 ng/ml. Although three quarters of cases were Gleason 8-10, the remainder were moderately differentiated. All 12 cases where tissue PSA immunostaining was classified as negative or focal 1 + were high grade tumours. Other authors have found the same association, and have also noted a similar relationship with serum PSA levels (Aihara et al. 1994). It has been suggested that such cases undergo "de-differentiation", losing their original prostatic tissue characteristics and hence altering the relationship between serum PSA levels and tumour volume (Kageyama et al. 1996). This de-differentiation has been associated with a more aggressive phenotype, and agrees with the finding that first line hormonal responses and overall survival are much shorter in this group of patients (Birtle et al. 2003) (Chapter 3).

Interestingly, given the low serum PSA levels, positive immunostaining for tissue PSA was seen in two thirds of the cases, although in many of these the staining was focal and could potentially be missed on prostatic biopsy sampling. Some authors have argued that both monoclonal and polyclonal antibodies to PSA should be used (Gray 1996) because prostate carcinomas showing negative immunostaining for PSA monoclonal antibody may have an epitope that cannot be recognised by monoclonal antibody. The PSA antibody used in this study was monoclonal but similar work by Feiner (Feiner & Gonzalez 1986) showed negative immunostaining for both monoclonal and polyclonal PSA antibody in high grade prostate cancers and monoclonal antibody work remains the mainstay of the diagnostic laboratory. Hence the current study did not repeat the staining using a polyclonal antibody.

Although PSA and PAP are usually concordant in advanced prostate cancer (Stamey et

al. 1987) occasional discordance in immunohistochemical studies has been described (Mettlin. 1993). Concordant results for PSA and PAP were seen in 28/33 cases (84%), although in only 6 cases (18%) was PAP immunostaining diffuse. In a similar fashion to PSA, PAP staining could be subject to a geographical miss on prostate biopsy and thus is likely to confer no additional diagnostic benefit.

In contrast, PSMA staining was seen in 30/33 (90%) cases and was diffuse in the majority, in agreement with the observations of Bostwick (Bostwick et al. 1998). This observation may be important in cases where tissue PSA immunostaining is negative or focal 1+. Our results are also similar to those obtained by Chang (Chang et al. 2001a) who found positive immunoreactivity for PSMA in prostate cancers where tissue PSA was negative. In tumours where both PSA negative and positive areas were seen, PSMA staining occurred in both regions, suggesting there is no association between the pattern of PSA and PSMA expression. As areas which are focally positive for PSA are easy to miss on biopsy, these results indicate that PSMA staining may be of value in this patient group to aid diagnosis and confirm prostatic origin despite the low serum PSA.

Alterations in the androgen receptor by mutation, deletion or over-expression have all been implicated in the development of hormone refractory prostate cancer (Feldman & Feldman 2001). Given that PSA is an androgen-dependent gene (Balk et al. 2003) androgen receptor deletion is a potential mechanism to explain the low levels of serum PSA in treatment-naïve metastatic prostate cancer. However, strong predominantly diffuse nuclear AR staining was observed in most of the tumours and was seen in all but three of the cases that were negative or weakly positive for PSA. A mechanism in which the AR is present but bypassed by a ligand-independent pathway, leading to an increase

in proliferation and a decrease in apoptosis has been suggested (Feldman & Feldman 2001) and this may be a more plausible explanation. From the results in the current study, it appears that the mechanism of low serum PSA cannot be explained by AR loss resulting in altered transcription of PSA. In a similar manner to the observations with PSMA, the diffuse nature of the AR staining could reduce the likelihood of misdiagnosis on prostate biopsy.

It has been previously been postulated that neuroendocrine markers might complement the PSA assay in selected cases of poorly differentiated tumors (Sciarra et al. 2003). In the same study, tissue neuroendocrine markers including chromogranin A and neuron-specific enolase were identified in 18 patients with clinically progressive androgen-independent prostate cancer and low serum PSA, leading to the belief that the majority of low serum PSA metastatic prostate cancers were neuroendocrine in origin. In contrast, in our group of hormone-naïve metastatic prostate cancers, no tumour had a predominantly neuroendocrine phenotype. CD56, which has also been previously useful in detecting neuroendocrine differentiation (Kaufmann et al. 1997) was unhelpful, being focally positive in only 3/33 (9%) cases. Neuroendocrine markers do not appear to provide additional information in this group of cases. Thus whilst neuroendocrine differentiation may be one mechanism by which prostate cancers develop resistance to androgen ablation, it does not explain the primary presentation of prostate cancer with low serum PSA levels in this group of patients.

In conclusion, men presenting with treatment-naïve metastatic prostate cancer and inappropriately low levels of serum PSA (less than 10 ng/ml) are difficult to manage and have a poor prognosis. Both tissue PSMA and AR immunostaining are often

diffusely positive in these tumours, particularly in cases where the PSA is negative or weak and focal, and may thus aid the histopathological diagnosis of prostate cancer in these patients, allowing treatment to be commenced more promptly. The potential value of PSMA as a serum biomarker of prostate carcinoma also merits further investigation.

Table 4.1 Summary of Primary Antibody and Conditions

Primary antibody	Concentration	Species/ Catalogue	Antigen Retrieval*
AR	1/100	Mouse/Dako M3562	9.9
PSA	1/20	Mouse/ Dako M0750	NT
PSMA	1/100	Goat/Santa Cruz sc-101271	9.9
PAP	1/1000	Mouse/Dako M0792	NT
Chr A	1/10	Mouse/Dako M0869	NT
CD56	1/10	Mouse/Monosan MON9006-1	PC
CK7	1/100	Mouse/Dako N1626	TE
CK 20	1/100	Mouse/Dako N1627	TE
34βE12	1/50	Mouse/Dako M0630	MWD

***9.9** : 25 minutes microwaving at full power, stand 10 mins, in Dako high pH retrieval solution (S3007)
NT : no antigen retrieval used
TE: 20 min microwave at full power in Tris-EDTA buffer, pH 9.0
MWD : 25 minute microwave at full power, 10 minutes stand, in Dako Retreival Solution (S1699)
PC : 2 minutes pressure cooking at full pressure in citrate buffer pH 6.0

Table 4.2 Summary of Immunohistochemistry Results in 33 Cases

PSA	0	F 1+	F 2+ / 3+ (Diffuse staining in 2 cases only)
AR			
0	3*	0	0
F 1+	0	0	1
Diffuse 2+ / 3+	6	3	20
PSMA			
0	1*	0	2
F 1+	1	0	8
Diffuse 2+ / 3+	7	3	11
PAP			
0	3	0	3
F 1+	1	0	2
2+ / 3+	5	3	16
No diffuse staining seen			
Chr A			
0	8	4	12
F 1+	1	0	2
F 2+ / 3+	0	1	5**
No diffuse staining seen			
Gleason score			
6	0	0	1
7	0	1	6
8	0	0	3
9	5	1	7
10	4	1	4

F: Focal
D: Diffuse
* One case which was AR/PSA/PSMA negative was positive for urothelial markers
** Very scattered staining

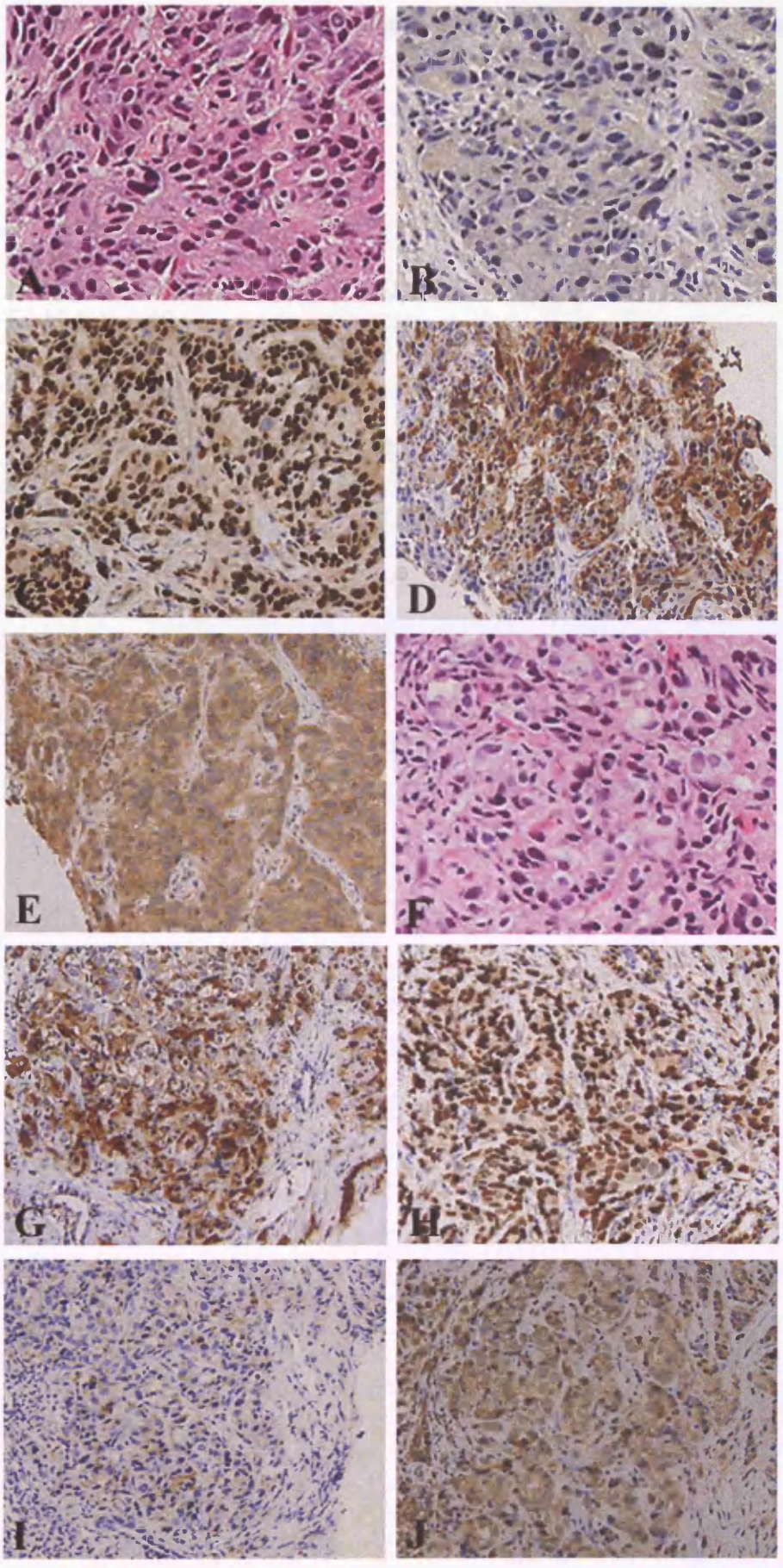

A
B
D
E
F
G
H
I
J

Figure 4.1 Immunohistochemical Features Illustrated by 2 Cases

Case 1 (A-E),
Case 2 (F-J)
Key:
A: H & E x 40
B: negative PSA staining x 40
C: 3+ nuclear diffuse AR staining x 40
D: 3+ focal PAP staining x 20
E: 3+ diffuse PSMA staining x 20
F: H & E x 40
G: 3+ focal PSA staining x 20
H: 3+ diffuse AR staining x 40
I: 1+ focal PAP staining x 20
J: 2+ diffuse PSMA staining x 40

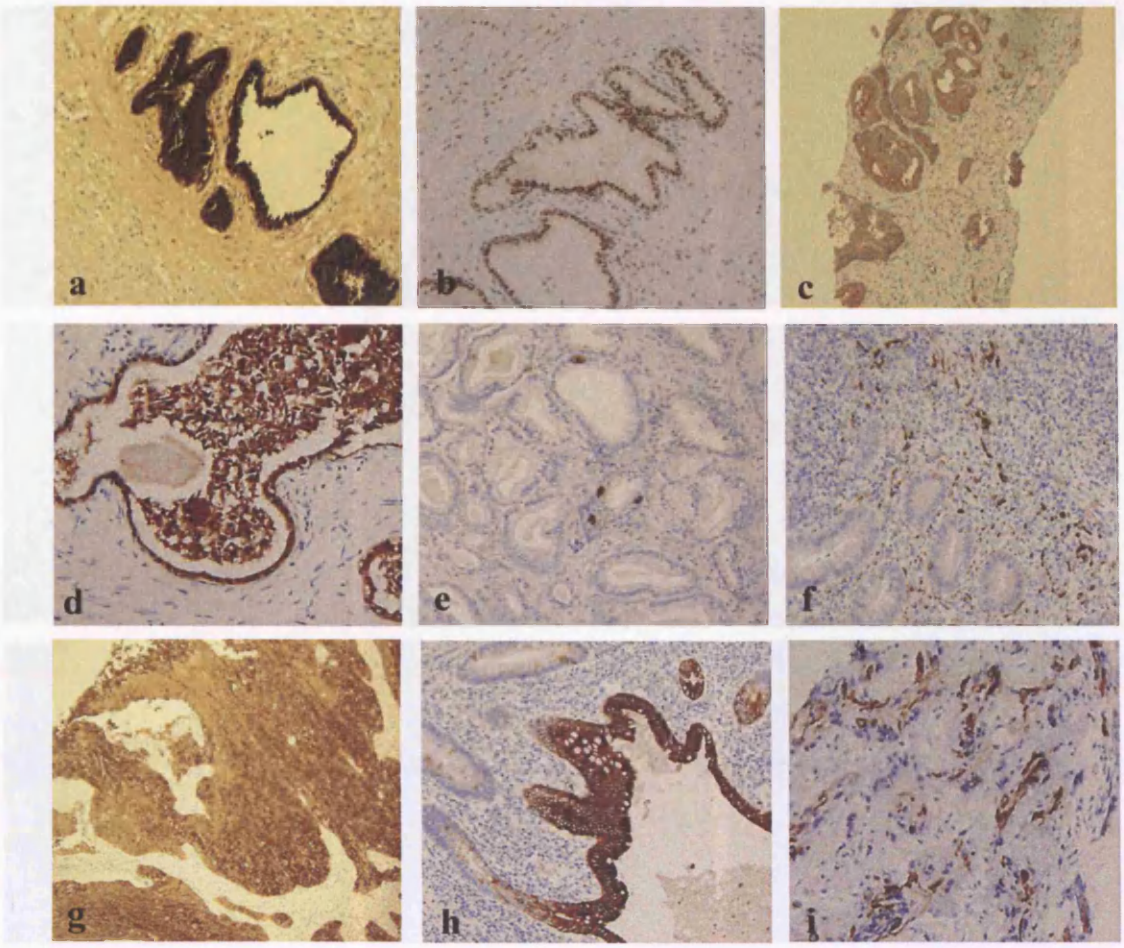
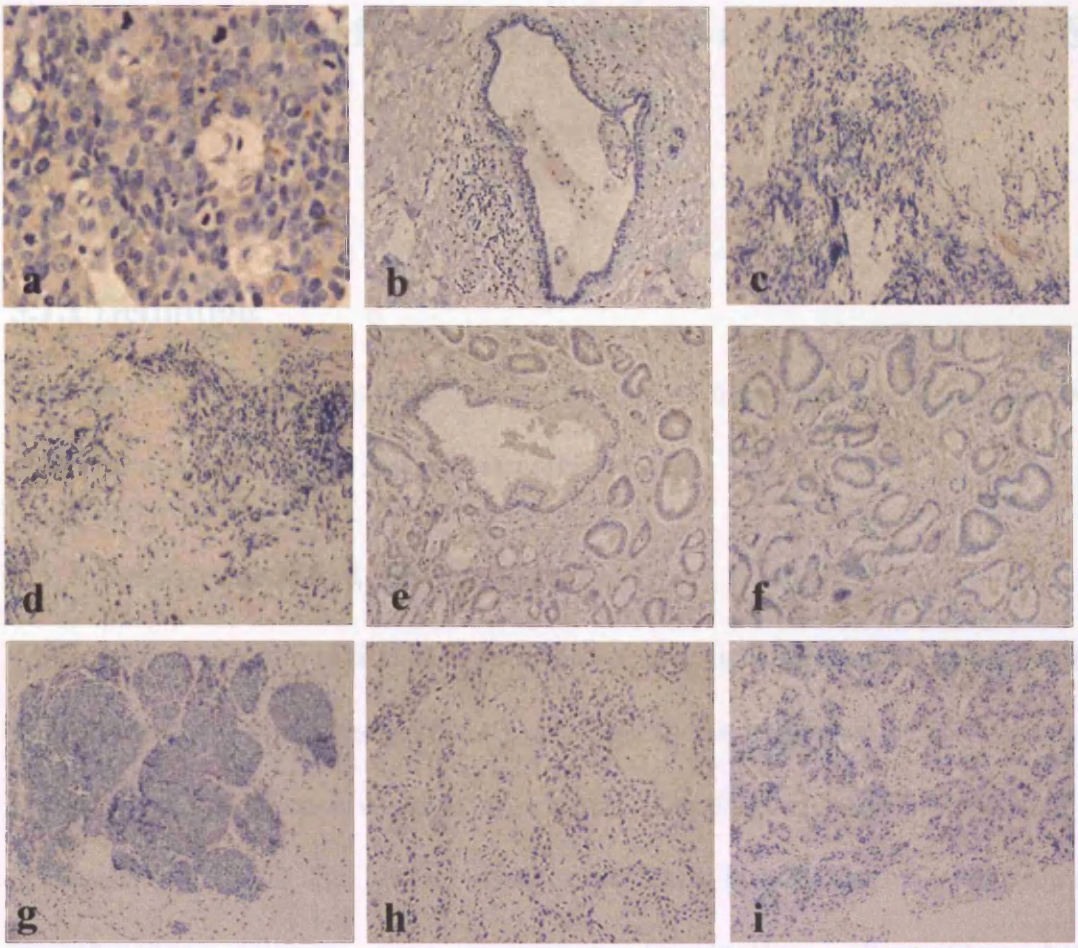


Figure 4.2 Positive Controls

a: normal prostate showing strong luminal staining for PSA (x 20)
b: normal prostate showing strong nuclear staining for AR (x 20)
c: prostate carcinoma showing strong diffuse staining for PSMA (x 10)
d: normal prostate showing strong luminal staining for PAP (x 40)
e: nerves showing strong focal staining in neuroendocrine cells for chromogranin A (x 40)
f: nerves showing strong focal staining for CD 56 (x 20)
g: invasive bladder carcinoma showing strong diffuse staining for 34βE12 (x 20)
h: invasive breast tumour showing positive staining for CK 20 (x 20)
I: invasive bladder tumour showing positive staining for CK 7 (x 40)



a: normal tonsil shows negative PSA staining (x 40)
b: normal breast, shows negative AR staining (x 20)
c: normal bladder shows negative PSMA staining (x 20)
d: normal bladder shows negative PAP staining (x 20)
e: normal breast shows negative chromogranin A staining (x 20)
f: normal breast shows negative CD 56 staining (x 20)
g: prostate carcinoma shows negative 34βE12 staining (x 20)
h: bone marrow showing negative CK 7 staining (x 20)
i:bone marrow showing negative CK 20 staining (x 20)

Chapter 5 Loss of Heterozygosity as a Mechanism for PSA-Negativity

5.1 Introduction

5.1.1 Definition

Loss of heterozygosity may be defined as loss of one allele at a locus followed by duplication of information from the remaining locus.

5.1.2 Background to LOH Detection

Allelic loss at a certain region of a chromosome at a high frequency is known to indicate the presence of a tumour suppressor gene. Due to the high level of specificity, loss of heterozygosity is often invaluable as a marker for diagnosis and prognosis of cancer (Thiagalingam 2002). The genetic changes that result in LOH known to date are mitotic recombination, deletion, gene conversion, translocation, chromosome breakage, chromosomal fusion or telomeric end-to-end fusions, or loss of a whole chromosome (Aaltonen. 1993).

In the non-coding regions of the genome, so-called tandem repeats exist which are areas of repeated DNA sequence eg $(CA)_n$. A microsatellite or short tandem repeat is one in which 1-5 base pairs are involved. For PSA, this is a dincleotide CA repeat. The number of the repeats is variable and they thus differ between the paternal and maternal alleles. This results in alleles of differing sizes and thus heterozygosity can be detected by high resolution electrophoretic techniques.

5.1.3 Clinical Relevance of LOH

In colorectal cancer, LOH has been associated with poorer outcomes in untreated

patients. Retention of heterozygosity appears to indicate improved chemotherapy responses (Barratt. 2002) and is being investigated as a potential marker to stratify patients into appropriate treatment groups. LOH may be of use in diagnosis and determining prognosis of cancer. Allelic loss is a common finding in prostatic adenocarcinomas, present in more than 50 % of cases on chromosomes 8, 10q and 16 q and allelic loss appears to be more common in high grade tumours (Stamey et al. 1987).

5.1.4 Microsatellite Instability (MSI)

Microsatellite instability describes the emergence of new microsatellite alleles within a tumour, of lengths that differ from the host's two parent alleles for that microsatellite. It was first observed in hereditary non-polyposis colon cancer (Aaltonen et al. 1993). Microsatellite DNA is thought to arise from strand misalignment during replication, recombination or repair and is attributable to mutations in mismatch repair genes. When DNA containing a microsatellite repeat is amplified by PCR, the resulting products will be of different lengths, even if by only a few base pairs. It has been identified in other tumour types such as colorectal and lung carcinomas and may be implicated in prostate cancer.

5.1.5 Assessment of LOH

To assess LOH, the sample must have two alleles of different lengths in the normal tissue and is termed *informative*. However, at about 30 % of loci, the alleles will be homozygous, having the identical number of microsatellite repeats within the amplified segment, and alleles of equal size. The sample is then deemed *non-informative* as LOH cannot be distinguished from the homozygous pattern in the normal tissue. Mutation may also result in a change in the number of repeats in the tumour tissue.

5.2 Aims

The main aim of this study was to investigate LOH at the PSA locus in treatment naïve metastatic prostate cancer with low serum PSA levels. If it is present, it is possible that LOH is the mechanism for the lack of PSA production in these patients. It may also be a useful prognostic molecular marker.

5.3 Materials and Methods

5.3.1 Clinical Specimens

10 formalin-fixed paraffin embedded prostate blocks were chosen from the bank of archival tissue collected as described in Chapter 3. All cases were men presenting with previously untreated metastatic prostate cancer and a serum PSA of less than 10 ng/ml. A mixture of diagnostic core biopsy and resection specimens were used and the blocks chosen were from tumours that had shown low immunoreactivity for PSA (Chapter 4). 3μm sections were taken and mounted onto non-coated slides. H & E staining was performed as outlined in Section 2.1.2, although only one slide for each patient was cover-slipped for the purposes of outlining areas of tumour and normal tissue. All other slides were left uncovered to facilitate laser capture microdissection (LCM). Areas of tumour and normal tissue were identified and outlined on each cover-slipped slide by one histopathologist with a site-specialised interest in urological tumours, Dr Alex Freeman in the Department of Histopathology at UCL.

5.3.2 LCM

This work was carried out with assistance from Pete Simpson and Chris Jones at the Breakthrough Breast Cancer Laboratories, The Institute of Cancer Research, Fulham Road, London. The PIX CELL II ARCTURUS system was used. The LCM technique

was developed by the NIH in the USA and has been commercially developed since 1997 with Arcturus. In principle, a specific transfer film is bonded to the underside of a transparent cap. A side arm positions the cap onto the area of interest on a section and a laser beam focally activates the transfer film. The targeted cells bond to the film and are held within the polymer. This is shown in figure 5.1. The diameter of the beam may be adjusted so that individual cells can be microdissected and beam diameters of 7.5, 15 and 30μm are available. The cap is then transferred to a 1.5ml Eppendorf and stored at room temperature until DNA extraction is performed. Multiple areas of tumour and benign tissue were microdissected for each case, where tissue availability permitted. To facilitate microdissection, sections were dehydrated in 70 %, 90% and 100 % alcohol for 2 minutes, then in xylene for 3-5 minutes and air-dried prior to microdissection. An initial laser energy of 85mW was applied, increasing to 100mW if microdissection was difficult. For samples that proved difficult to lift off, the alcohol and xylene step was repeated. Examples of LCM are shown in figure 5.2.

5.3.3 DNA Extraction

The methods of DNA extraction have been detailed in Section 2.1.4.

The DNeasy® (QIAGEN) kit was initially used as this provided purer DNA extracts by virtue of the mini column system. However, because of the small amount of microdissected tissue held on each cap, the samples obtained were extremely dilute and hence an in-house DNA extraction method, as practiced by the Breakthrough Breast Cancer Laboratories was employed (section 2.1.4.1). DNA was extracted from paired microdissected samples from tumour and benign areas for each of the 10 cases.

5.3.4 PCR Optimisation

A standard 15µl reaction volume was used for each PCR, as per the protocol in section 2.1.4. Optimisation of the PCR conditions in terms of number of cycles and annealing temperature was performed, using DNA previously extracted from LNCaP cells as a positive control. To optimize the conditions for the microdissected tissue DNA, LOH primer B1991, a primer known to provide validated results, was kindly provided by Charlotte Foley from The Institute of Urology Prostate Cancer Research Centre and the PCR repeated with the number of cycles changed from 40 to 50 and 60. The optimum results were found using 50 cycles.

5.3.5 Annealing Temperature

The melting and hence the annealing temperature for the microsatellite markers is affected by the number and proportion of base pairs, ie. the guanine-cytosine and thymidine-adenine bonds formed. The calculated melting point for each primer was obtained using the following equation:

$T_{melting} = 81.5 + [\ 0.41 \times (\%GC) - (675 / \text{number of base pairs})]$

For the forward primer this was 76.2°C, for the reverse primer 75.5°C, giving an average calculated melting temperature of 75.9°C. The annealing temperature was calculated from the following:

$T_{anneal} = Tm - (2 \text{ to } 6\ °)$

However, a range of annealing temperatures were tried, via a temperature gradient plate on the Hybaid thermocycler®, using temperatures from 48-60 °C with optimum results seen at a T anneal of 58°C.

Once the optimal conditions had been established, the PCR was performed as described in Section 2.1.4. PCR products were run on a 1.5% agarose gel to check that product of the correct size had been generated.

5.3.6 Construction of Plate for Genotyping

The spatial calibration for the ABI Prism 3700® DNA Analyser is carried out using Hi-Di Formamide (Applied Biosystems 43114320). Hence all samples run through the machine must be suspended in Hi-Di Formamide to ensure accurate sequencing. In a 1.5 ml eppendorf, 1ml of Hi-Di Formamide was added to 15µl of the size standard Genescan® 400High Density (ROX) (ABI PRISM 402985) and mixed. ROX consists of 21 single stranded fragments of 50-400 bp range. Each fragment is labelled by a single fluorophore which results in a single peak when run on a polyacrylamide gel in the ABI A3700. GeneScan™ Size Standards are sets of fluorescent-labelled DNA fragments of known sizes used for determining the size of unknown DNA fragments run on ABI PRISM® DNA sequencers. The standards are run in the same lane or capillary injection as the samples, which contain fragments of unknown sizes labelled with different fluorophores. GeneScan® Analysis Software automatically calculates the size of the unknown DNA sample fragments by generating a calibration or sizing curve based upon the migration times of the known fragments in the standard. The unknown fragments are mapped onto the curve and the sample data is converted from migration times to fragment sizes. 8µl of the HiDi/ROX mix was micropipetted into each well on a 96 well reaction plate (MicroAmp Optical)

Serial dilutions of the PCR products were made, giving 1:20, 1:40 and 1:80 concentrations. 2µl of the appropriate concentration was added to each well on the plate

giving a total volume of 10µl per well. One well contained water only as a negative control. The plate was then sealed, vortexed and the DNA denatured by heating in a PCR machine for 5 minutes at 95°C. This also allowed incorporation of the HiDi into the DNA. The plate was then placed in ABI PRISM 3700® DNA analyser for 4 hours and run. The ABI 3700® software allows each of the 96 lanes to be labelled with a sample name. The PCR products were therefore arranged in the 96 well plate so that the fluorescent electropherogram generated compared tumour with normal DNA for each of the 10 samples.

The ABI 3700® is a high voltage electrophoresis circuit. The machine loads the Hi/Di, ROX and PCR product mix into a well containing buffer and the cathode. Over a period of 50 seconds this reaction mix is injected into a capillary containing polyacrylamide gel. After the samples have been injected into the capillaries the injection wells are washed and re-filled with buffer. A 1000V potential difference is applied between a loading bar, the cathode, and an electrode, the anode, at the back of the cuvette assembly (Figure 5.3). PCR products migrate within the capillary to the other end, smaller products moving faster. As DNA fragments leave the other end of the capillary, they are carried through a transparent cuvette by a moving polymer, passing through the beam of an argon laser. As the fluorescently labelled fragments pass through the laser they emit a blue (FAM) or red (ROX) light that is detected by a camera. The amount of light detected is proportional to the quantity of product and so the amplitude of the curve on the fluorescent electropherogram.

5.3.7 Data Analysis

The data from the 3700 was imported for analysis into the Genescan® software. The

allelic peaks are preceded by so-called stutter peaks, which represent slippage of the *Taq* polymerase during DNA amplification (Bright et al. 1997). The allelic peak is the highest peak after the stutter peaks. The first allelic peak is usually higher than the second as it is made up of its own product as well as a stutter peak of the second, larger allele. The software was set to mark the size in base pairs of each peak within the predicted size range of 176-192, and the peak height to quantify each different length of DNA product.

The definition of loss of heterozygosity is based on the relative ratio difference between the two alleles from the normal tissue sample and the two alleles from the cancer. This can be calculated as the ratio of the smaller peak to the larger peak for the tumour DNA compared to the same ratio in the normal DNA from the same sample (Narla 2001) ie. X_{LOH}= ([T1/T2]/[N1/N2]). The value for LOH can hence range from zero (complete loss) to 1 (no allele loss).

The definition of a significant difference also varies throughout the literature and ranges from 0.3 to 0.75 (Barratt et al. 2002; Narla et al. 2001). For heterozygous alleles where one allele has been lost by the cancer, there should in theory be only one allele present in the cancer sample compared to two in the benign sample. In practice, however, it is difficult to avoid contamination with normal tissue. Thus the two alleles contained in these contaminants are detected and contribute to a smaller peak in the cancer sample.

As previously mentioned, if the sample in the normal tissue is homozygous, the pattern is deemed non-informative. This is because a homozygous result in the tumour sample could either be due to true LOH or to be simply a reflection of the homozygous pattern in the normal tissue.

5.4 Results

If sections had been dried for too long or at too high a temperature during the initial sectioning stage, microdissection proved extremely difficult. PCR products from the extracted tumour DNA were visualised on a 1.5 % agarose gel (Figure 5.4). Products were seen for all 10 tumour samples and the LOH protocol was thus then carried out. 8 of the 10 tumour /normal tissue pairs yielded peaks within the predicted range of 176-192 base pairs. Two of the tumour/normal pairs did not show a signal and it is likely that despite using a range of DNA concentrations, the DNA for these samples was too dilute. For all other samples, identical results were seen at all levels of DNA concentration (ie at 1:20, 1:40 and 1:80).

2/8 samples displayed a homozygous pattern in both tumour and normal tissue and were classed non-informative. (Fig 5.5)

2/8 samples displayed a heterozygous pattern, with a ratio for LOH of 1.0, showing that there was no allelic loss. (Fig 5.6)

4/8 samples showed microsatellite instability, namely that microsatellite alleles of different lengths from the normal tissue's two parent alleles were seen. (Fig 5.7)

None of the 8 matched tumour/normal tissue samples exhibited LOH.

5.5 Discussion

Although 10 matched tumour/normal tissue samples from each patient were analysed, two of the pairs did not produce a detectable signal. This was despite three different concentrations of DNA being loaded for the LOH analysis. For the other eight samples, identical results were seen at 1:20, 1:40 and 1:80 concentrations. When the results were

compared with the images and data recorded during the microdissection process, it was apparent that these two cases had been difficult to microdissect, even after an additional alcohol/xylene step. This probably reflected overdrying or overheating in the initial sectioning stage. It is likely that there were in fact very few cells lifted from these sections, and thus minimal amounts of DNA extracted.

There is debate as to whether samples showing microsatellite instability can be termed informative. Barratt et al noted that MSI may in fact obscure relative allele abundance in the tumour (Barratt et al. 2002). If the MSI samples are considered informative in this study, then 6/8 samples were informative in total. If there was only a 10% rate of LOH at the PSA locus, this would not be picked up with such a small sample size.

Although the samples were microdissected, there may have been some contamination of the tumour with benign tissue. In this case, if too many cycles were used during the PCR process, it is possible that the normal DNA acting as a contaminant of a supposed tumour sample was amplified, and any LOH in the tumour masked.

In addition, different cancer foci within the same tumour area may display varying characteristics, namely that some may have LOH and others not. In such circumstances, those foci without loss may mask those with LOH.

The high rate of MSI (50 %) seen in this study is unusual. The tissue specificity of microsatellite instability in other tumour types, such as hereditary non-polyposis colon cancer (Aaltonen et al. 1993) has been speculated to be an indication of tissue specific mutagens or the existence of tissue specific genes which are targets of spontaneous mutations, providing a growth advantage to that particular cell type (Eshleman & Markowitz 1995). As already described elsewhere in this thesis (section 3), patients

presenting with treatment naïve metastatic prostate cancer and a serum PSA of less than 10ng/ml have a much poorer response to hormonal treatment and significantly shorter overall survival than patients with metastatic prostate cancer and appropriately high serum PSA levels. It may be that MSI contributes to the aggressive phenotype of the PSA-negative metastatic tumour by conferring a selective growth or survival advantage.

An alternative explanation is that for microdissected samples, the amount of template DNA may vary considerably and interfere with optimum PCR amplification. Sieben (Sieben 2000) found that in particular, the DNA microdissected from paraffin-embedded sections had a higher rate of LOH artefact than that obtained from frozen sections. They recommended a minimum of 10 ng DNA for reliable PCR analysis from paraffin embedded material when using a di, tri or tetranucleotide marker, to reduce LOH errors. PCR artefacts may also occur due to fixation induced change of DNA and conditions in the amplification of the repetitive sequence, (especially for CG rich sequences) favouring mis-annealing and hairpin formation (Diaz-Cano 2001). The DNA extracted in this study was extremely dilute, and optical density reading could not be obtained on the samples extracted using the DNeasy method. The in-house DNA extraction protocol had been developed to improve the concentration of extracted DNA but it is possible that the amount of DNA present was still less than 10 ng and that the MSI seen was artefactual rather than a true result.

In summary, loss of heterozygosity for PSA was not found in these cases of treatment – naïve metastatic prostate cancer with low serum PSA levels. An element of caution is required however, as it is possible that a low rate of LOH could not be detected given the small number of cases examined. Microsatellite instability would appear to be a

frequent occurrence and may be a factor in the aggressive behaviour of these tumours, although technical difficulties may be implicated.

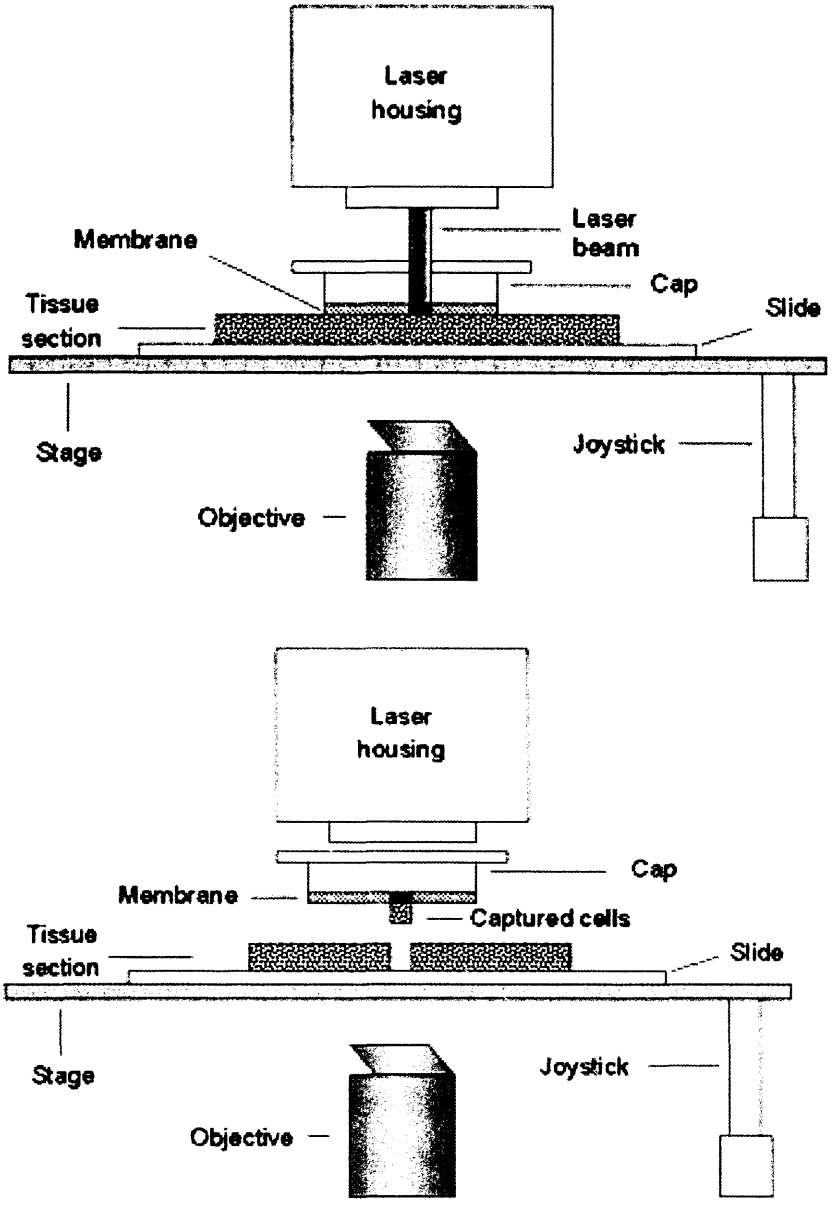


Figure 5.1 Showing Tissue Acquisition (top) and Tissue Transfer (bottom) During LCM

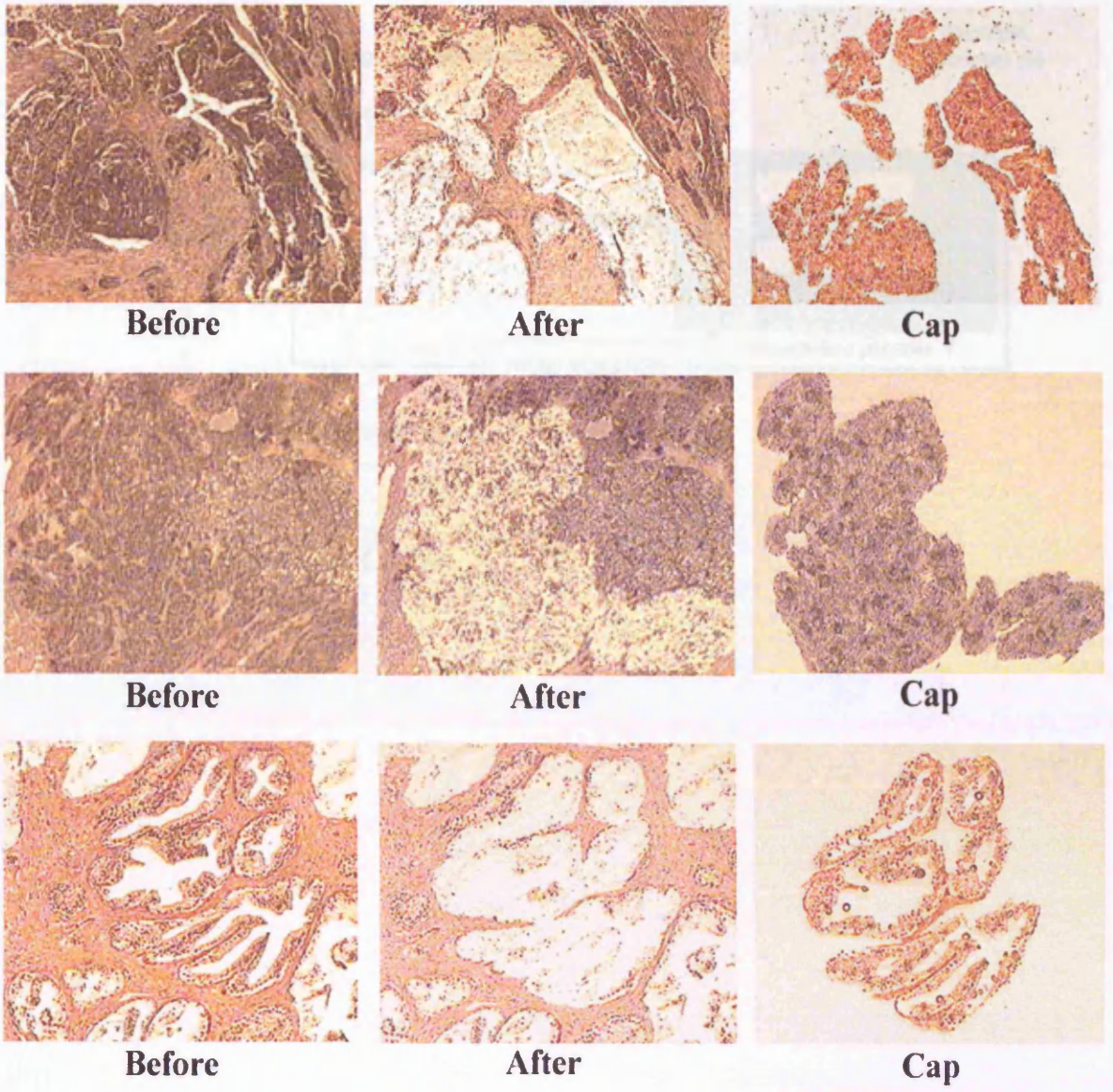


Figure 5.2 Examples of Microdissected Prostate Tissue

Lines 1 and 2 show adenocarcinoma microdissection
Line 3 shows normal prostatic tissue microdissection

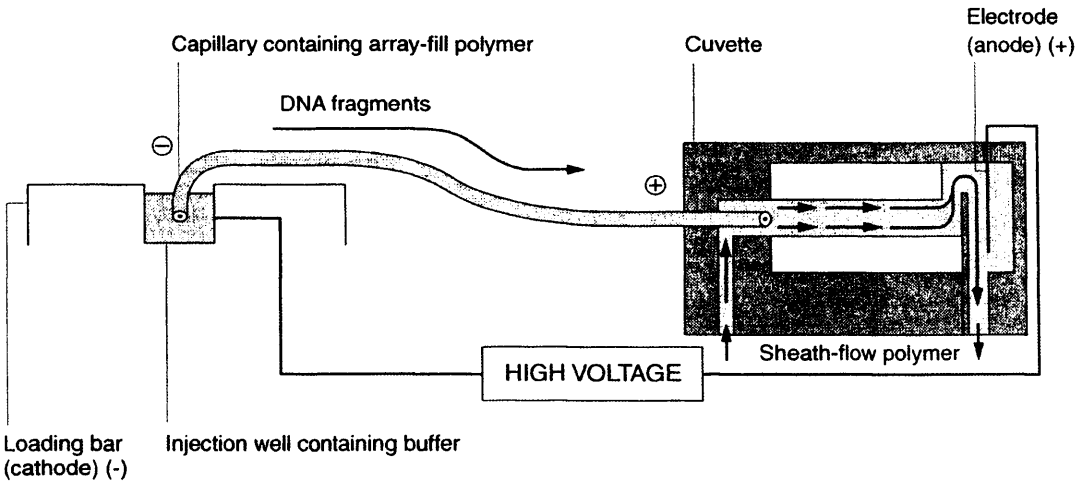


Figure 5.3 Electrophoresis Circuit

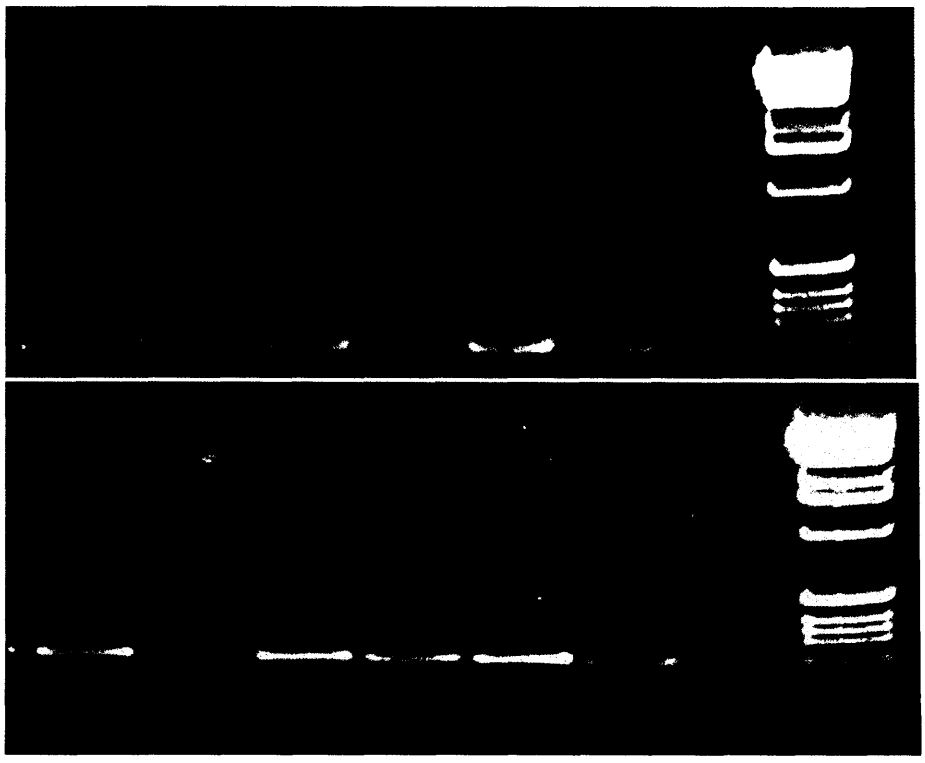


Figure 5.4 PCR Products for PSA Primer using Microdissected DNA

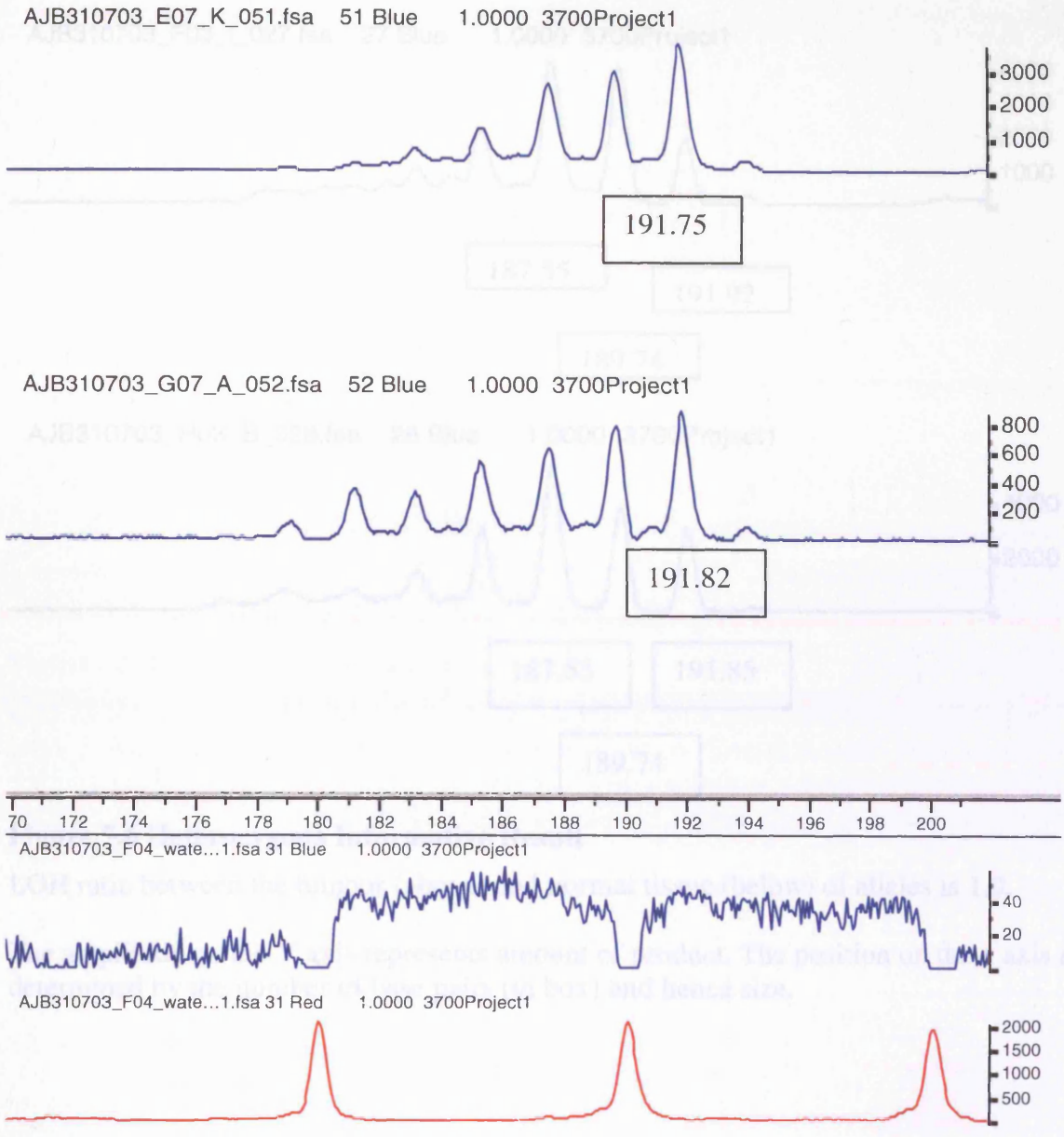


Figure 5.5 Example of Non-Informative Homozygous Sample

The polymorphic region in between the markers is the same length in the maternal and paternal alleles and so one allelic peak is seen in both tumour (top line) and normal tissue from the same patient (second line). The negative control (water) and the red size standard are also shown (bottom two lines). The amplitude on the Y axis represents amount of product. The position on the x axis is determined by the number of base pairs (in box) and hence size.

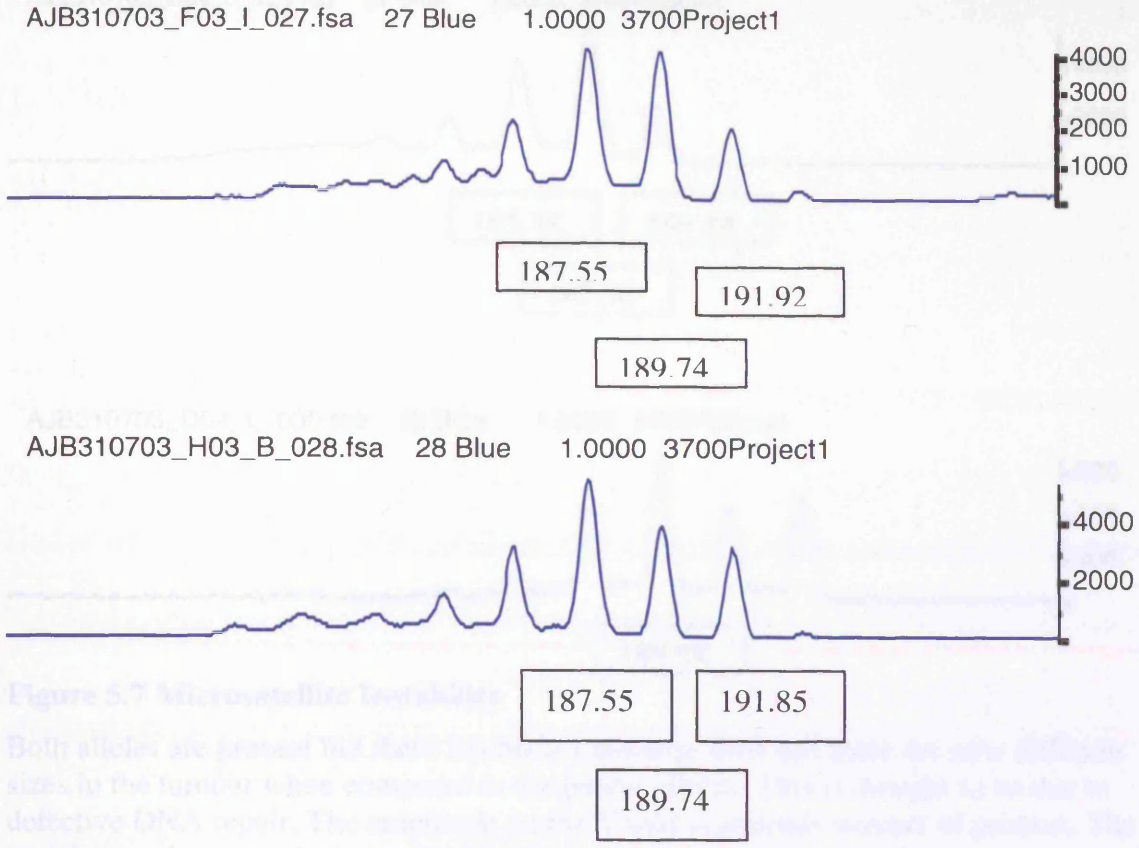


Figure 5.6 Heterozygous Informative Result

LOH ratio between the tumour (above) and normal tissue (below) of alleles is 1.0.

The amplitude on the Y axis represents amount of product. The position on the x axis is determined by the number of base pairs (in box) and hence size.

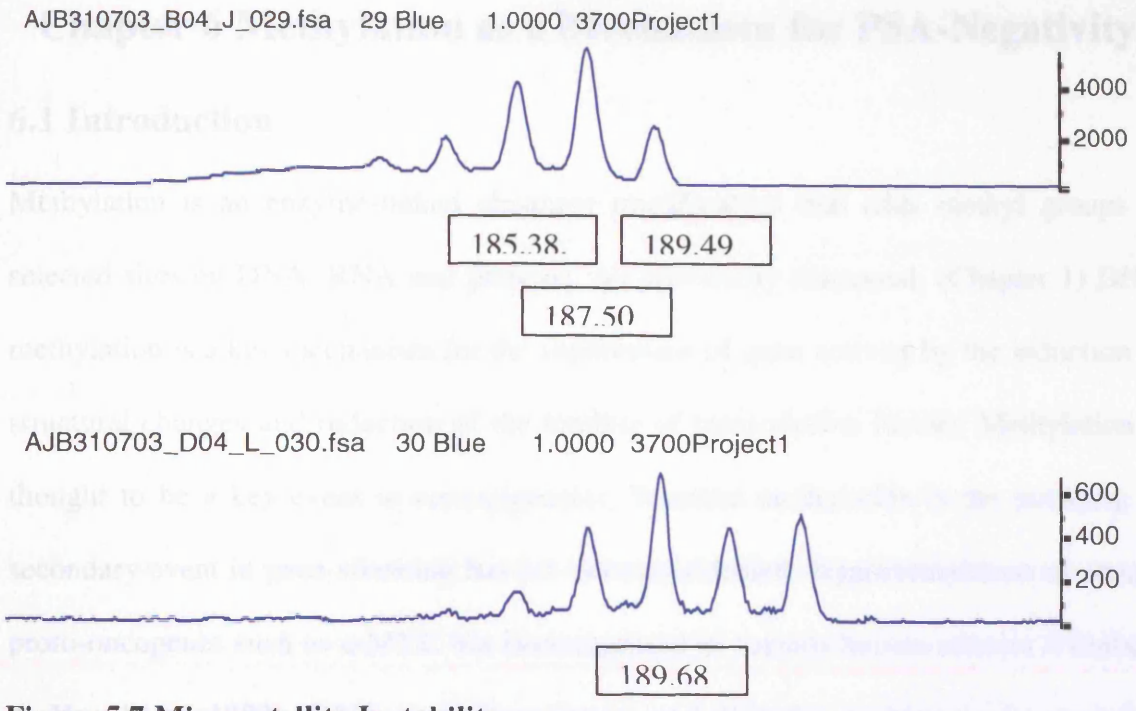


Figure 5.7 Microsatellite Instability

Both alleles are present but there has been a position shift and there are now different sizes in the tumour when compared to the parent alleles. This is thought to be due to defective DNA repair. The amplitude on the Y axis represents amount of product. The position on the x axis is determined by the number of base pairs (in box) and hence size.

Chapter 6 Methylation as a Mechanism for PSA-Negativity

6.1 Introduction

Methylation is an enzyme-linked chemical modification that adds methyl groups at selected sites on DNA, RNA and proteins. As previously discussed, (Chapter 1) DNA methylation is a key mechanism for the suppression of gene activity by the induction of structural changes and reduction of the binding of transcription factors. Methylation is thought to be a key event in carcinogenesis. Whether methylation is the initiating or secondary event in gene silencing has not been established. Hypomethylation of certain proto-oncogenes such as c-MYC has been reported in various human cancers (Feinberg & Vogelstein 1983). DNA methyltransferase and HDAC1 (a histone deacteylation enzyme) have been found to be up-regulated in prostate cancer, implying a role in inhibition of transcription via methylation (Patra et al. 2001).

Many tumour suppressor genes are found to be hypermethylated in malignancy (Jones & Laird 1999). In prostate cancer, GSTP1 promoter hypermethylation is frequently seen in prostate cancer and is present in potential precursor lesions (Jeronimo 2001). Aberrant methylation of the AR has been described (Chlenski 2001) and this may be a factor in functional silencing of the AR leading to lack of PSA production. There may also be methylation of the PSA promoter or enhancer.

DNA methylation has been considered as a therapeutic target, using demethylating agents such as 5-aza-2'-deoxycytidine (5-aza-2'CdR) which inhibits DNA methyltransferases and causes global hypomethylation. Methylation of the AR may be considered analogous to work in breast cancer where hypermethylation of the oestrogen receptor (ER) has been proposed as a mechanism for initiating or maintaining

oestrogen-receptor negative status in breast cancers (Lapidus et al. 1998). ERα has been found to be reactivated in prostate cancer cell lines after treatment with agents to induce demethylation (Li et al. 2000). These drugs cause terminal differentiation of cells by removing excess methyl groups. Difficulties with demethylation agents have arisen due to the non-specific nature of this class of drugs, but some success has been obtained in haematopoetic disorders such as acute promyelocytic leukaemia (Lo et al. 2002).

As PSA is an androgen-responsive gene, it is possible that methylation of the AR leads to functional silencing of both the AR and PSA. If this is the underlying mechanism of PSA-negativity, it may offer a potential therapeutic target.

6.2 Aims

The aim of this study was to investigate whether DNA methylation of the AR and of PSA itself was a significant feature in specific prostate cancer cell lines, which do not express PSA or the AR in their normal state. This will be done by:

- Investigating PSA and AR expression in prostate cancer cell lines by RT-PCR, after treatment with an agent known to cause demethylation
- Investigation of PSA and AR expression in prostate cancer cell lines by immunohistochemical staining after demethylation treatment.
- Investigation of protein expression of PSA and AR in prostate cancer cell lines by western blotting after demethylation treatment.

6.3 Materials and Methods

6.3.1 Cell Lines

The prostate cancer cell lines DU 145, PC3 and LNCaP were used (section 2.1.3) with

conditions maintained as previously described.

6.3.2 Demethylation Optimisation.

The most commonly used agent for demethylation is 5-aza-2'-deoxycytidine (5-aza) (Jones 1985). A variety of schedules have been used by other workers in demethylation studies with 5-aza concentrations ranging from 10nM to 10μM, with treatment times varying from 1 to 14 days and a similar range of days before harvesting cells. Initial optimisation of conditions was performed in conjunction with Mr Simon Bott in the Prostate Cancer Research Centre. Prostate cancer cell lines LNCaP and PC3 were treated with 3μM concentration of 5-aza (Sigma A3656) for one week and then harvested. A parallel protocol using 500nM concentration of 5-Aza and a treatment time of 24 hours, followed by a 5 day period before harvesting was also used, with GSTP1 methylation status assessed. The GSTP1 was used as a control gene known to be methylated in prostate cancer cell lines and in tissue (Singal et al. 2001). The 3μM concentration schedule was found to cause significant and excessive cytotoxicity affecting all cell lines. Re-expression of GSTP1 was noted with the 500nM schuedule (Bott 2002). This was therefore utilised as the basis of subsequent methylation protocols.

6.3.3 Demethylation of Prostate Cancer Cell Lines by Treatment with 5-Aza.

2 x T80 flasks of LNCaP, DU145 and PC3 cells, plated at 500,000 cells per flask and maintained as previously described were left to attach overnight. The following day at ~ 40% confluence, a 500nM solution of 5-Aza made up in fresh medium (RPMI complete) was added to one flask per cell type. The second of each pair was media

changed, and used as a negative control. After 24 hours, the medium was aspirated from all flasks and replaced with 15 ml of drug-free medium. Cells were harvested after 5 days and cell numbers per flask counted as previously outlined (section 2.1.3.2). The cell preparation obtained was used for western blotting or RT-PCR as detailed below.

6.3.4 RT-PCR using Primers for AR and PSA

RNA extraction, RT-PCR and the use of negative controls to ensure the validity of the method was performed as previously described. (section 2.1.3). The primers used were for PSA, AR, PSMA, PAP and GAPDH (glyceraldehyde 3 phosphate dehydrogenase, a housekeeping gene) as a positive control for the PCR process. The primer sequences were:

PSA (forward) 5'- ACTGCATCAGGAACAAAAGCGTGA-3'

PSA (reverse) 5'-CGCACACACGTCATTGGAAATAAC-3'

AR (forward) 5'-CGAAATGGGCCCCTGGATTTATAG-3'

AR (reverse) 5'-AGTCGGGCTGGTTGTTGTCGTGTC-3'

PSMA (forward) 5'-GGAATCTCCTTCACGAAACCG-3'

PSMA (reverse) 5-CACTGAAAGGTGGTACAATATCCGA-3'

GAPDH (forward) 5'-CCACCCATGGCAAATTCCATGGCA-3'

GAPDH (reverse) 5'-TCTAGACGGCAGGTCAGGTCCAC-3'

PAP (forward) 5'-TTGTGACTTTGGTGTTTCGGC-3'

PAP (reverse) 5'-TGTCATAGCACTCATCAAAGTCCG-3'

The conditions used for PCR were as previously outlined (section 2.1.3). All PCR products were separated by size after gel electrophoresis, the product size for PSA being 362 base pairs, AR 524 bp, and GAPDH 597 bp. 10 µl of PCR product together with 5µl of loading buffer was loaded into each well on a 1.5 % agarose gel, and the gel run at 100V for 40-60 minutes. Prior to electrophoresis, 3µl of a 1kb DNA ladder was loaded into an empty well on the gel, allowing comparison of PCR products size against DNA fragments of known size. Products were visualised under ultraviolet light. All experiments were performed in triplicate.

6.3.5 Demethylation of Prostate Cancer Cell Lines and Assessment of PSA and AR Status by Immunohistochemistry

6.3.5.1 Demethylation

DU145, LNCaP and PC3 prostate cell lines were used as before. Trypsinisation and cell counting were performed as described in section 2.1.3 and 12,500 cells of each cell type in 1 ml of RPMI complete medium were added to each well in a 24 well plate, each of which contained a cover-slip inserted under aseptic conditions. 4 wells were set up for each cell type and the cells were left to attach overnight. The following morning, the medium was aspirated and 1 ml of a 500 nM solution of 5'aza added to each well. This was removed after 24 hours, replaced with 1ml of fresh medium, and the plate left for 7 days. The cells were then fixed as described previously.

6.3.5.2 Immunohistochemistry of Cells Grown on Coverslips

The protocol for immunohistochemistry was preformed as detailed in section 2.1.1. The primary antibodies used were anti-PSA diluted 1:20 (mouse monoclonal BioGenex

AM01405M) and anti-AR (mouse monoclonal Biogenex AM256-2M) at 1:100 made up in normal horse serum (NHS). A negative control was set up using one cover-slip for LNCaP cells with secondary antibody and NHS only. LNCaP cells, which express PSA and a mutated form of the AR acted as the positive control. The experiments were repeated in triplicate.

6.3.6 Demethylation of Prostate Cancer Cell Lines and Assessment of PSA and AR Protein Expression by Western Blotting

Western blotting was performed following the protocol as described in section 2.1.3.6. Initially 25µg of protein was loaded for all three cell lines, with an additional well loaded with 2.5µg of the LNCaP protein extract. This was because of the concern of over-saturation of PSA and AR from the high expression found in LNCaP cells. A 15 % resolving gel (section 2.2.2) was used for AR and for actin, with a 6 % gel required for PSA due to the much smaller size product. The primary antibodies used were at the following initial concentrations:

AR 1:1000 Mouse monoclonal Biogenex (AM256-2M)

PSA 1:250 Mouse monoclonal Biogenex (AM01405M)

Actin 1:20,000 Mouse monoclonal (Santa Cruz sc-8432)

Due to poor results with the PSA antibody, this was repeated at a 1:100 concentration and the blot left to develop overnight. As no results were seen with the LNCaP positive control for PSA (see below) the experiment was subsequently repeated using a 1:100 concentration of a different primary antibody for PSA (Santa Cruz sc-7316 mouse monoclonal) which was again allowed to develop overnight. Protein expression was

normalised to actin.

6.4 Results

6.4.1 RT-PCR using AR and PSA Primers

6.4.1.1 Cell Numbers

(See table 6.1) After treatment with 5-Aza, all cell lines showed either a reduction in total cell numbers or only a small increase from baseline. The relative numbers of cells in the untreated flask were 3-20 fold greater than the treated group, illustrating the potential cytotoxic and cytostatic effect of the demethylating agent.

6.4.1.2 PCR Results

(See Figures 6.1 & 6.2)

AR

No re-expression of AR was seen in either DU 145 or in PC3 cells treated with 5'Aza. AR expression was seen in both the treated and untreated LNCaP cells, which are known to express AR in their normal state.

PSA

A product of the appropriate size for PSA (362 base pairs) was seen in the treated DU 145 cells. No product was seen in the untreated DU 145 cells which do not normally express PSA or in either treated or untreated PC3 cells. PSA expression was seen in both LNCaP control cells.

PAP and PSMA

PAP and PSMA expression was detected in untreated and treated LNCaP cells, with no

expression seen for either treated or untreated DU 145 or PC3 cells.

GAPDH

GAPDH expression was seen in all appropriate experimental lanes, with no band seen in the 2 control lanes, containing no RNA or no DNA respectively, confirming validity of the experimental method.

6.4.2 Immunohistochemical Staining for PSA and AR after Demethylation of Prostate Cancer Cell Lines

(See Figure 6.3) Strong staining for PSA and nuclear staining for AR was observed in both the control and demethylated LNCaP cells. PSA staining was observed in the treated DU 145 cells (demethylated) but was not seen in the untreated DU 145 cells, or the treated or untreated PC-3 cells. There was negative staining for AR in all untreated / treated DU 145 and PC-3 cells.

6.4.3 PSA and AR Expression after Demethylation using Western Blotting

(See Figure 6.4)

PSA protein expression

Initially, no PSA expression was observed with the experimental cell lines or with the positive LNCaP control, despite leaving the immunoblot in contact with the film overnight to develop. The experiment was hence repeated using a different PSA antibody (Santa Cruz) as described above and PSA protein expression was observed for the LNCaP controls only. No PSA protein expression was observed in either the

treated/untreated DU 145 cells, or the treated/untreated PC-3 cells.

AR protein expression

AR expression was seen in the positive control LNCaP cells only. No re-expression of the AR was observed in either the treated / untreated DU 145 cells or the treated / untreated PC-3 cells.

Actin protein expression

Actin expression was observed for all treated / untreated cell lines and for the LNCaP controls, confirming consistent protein loading for the experiment.

6.5 Discussion

A potential mechanism for PSA-negativity is functional silencing of the AR by hypermethylation. CpG islands are small areas where the frequency of CpG is much higher than usual and are found in over 60 % of human promoters. The AR contains a ~ 1.5 kb region extending across the transcriptional start site that is 65 % GC with an expected/ observed CpG ratio of 0.62 (Jarrard et al. 1998). This fulfils established criteria for identifying a CpG island. (Gardiner-Garden & Frommer 1987). Jarrard et al (Jarrard et al. 1998) found aberrant methylation in the AR expression negative prostate cancer cell lines DU 145, DuPro, TSU-PR1 and PPC1 and noted that re-expression of AR after demethylation was associated with re-expression of PSA. However, the results of treatment with a demethylation agent such as 5 aza-2'deoxycytidine (5-aza) have been conflicting. Nakayama et al (2000) found weak re-expression of mRNA AR in DU 145 cells after 5-aza treatment but no-re-expression in PC-3 cell lines.

In contrast, Chlenski et al (2001) described weak re-expression in PC3 cells with no re-

expression in DU145. There is thus no consistency between reported results, and a variety of demethylation protocols have been used, with differing concentrations of 5-aza, different treatment times, and varying times for the cells left to grow after removal of the drug. In our study, a schedule for demethylation was developed.

Methylation status has also been examined in prostate tumours. AR methylation was evident in 50 % of advanced AR-negative and metastatic prostate cancer, but not in primary localised prostate cancer or normal tissue (Kinoshita 2000). The methylation status of PSA itself has not been investigated, primarily because it is not thought to contain CpG islands. However, its expression may be affected by functional silencing of the AR.

PSMA has been identified as a potential alternative tissue and/or serum marker in PSA-negative prostate cancer (section 4.4.5). Its expression varies among prostate cell lines and methylation of the PSMA promoter represses PSMA transcription in LNCaP cells. However, work in the current study is in agreement with other authors who noted that demethylation did not reactivate PSMA transcription and subsequent protein expression in DU 145 cells (Noss, Singal et al. 2002). Similar results were observed for PAP which was not re-expressed following demethylation. This study indicates that for PSMA and PAP, methods other than methylation are involved in their expression.

In this study, PSA re-expression after demethylation treatment was observed in DU 145 cells, a cell line that does not normally express either PSA or the AR. This was clearly demonstrated by RT-PCR and to a lesser extent, by the immunohistochemistry. The fact that no protein was detected on immunoblot is likely due to the greater sensitivity of RT-PCR in detecting low levels of expression compared to western blotting.

It is interesting that no re-expression of the AR was seen after demethylation. This would imply that there is a pathway, other than that via the AR, which is methylated in prostate cancer cells, and directly affects expression of PSA. The identity of this remains unidentified; nevertheless, it would appear that methylation plays a role in PSA expression and may be a factor in PSA-negativity. Further work should include the investigation of methylation status of PSA and AR in prostate cancer tissue where serum PSA is inappropriately low. This could be achieved by methylation specific PCR.

Table 6.1 Cell Line Numbers with/without Demethylation

0.5×10^5 cells were originally inserted per flask.

Treated flasks were incubated for 24 hours with 500nM 5'Aza.

Cells harvested after 5 days.

Flask contents	Number of cells
DU 145 treated	3.15×10^5
DU 145 untreated	$58.8. \times 10^5$
LNCaP treated	6.15×10^5
LNCaP untreated	19.3×10^5
PC3 treated	8.46×10^5
PC3 untreated	27.9×10^5

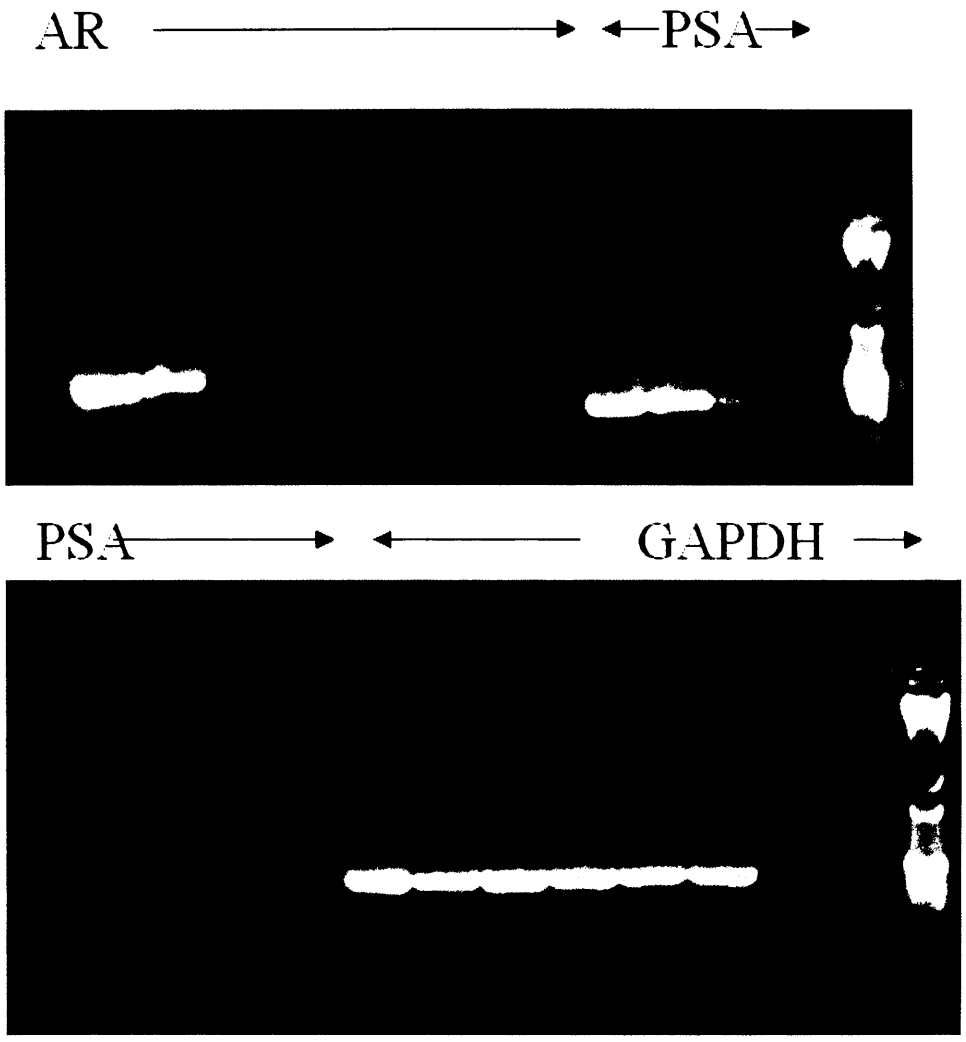
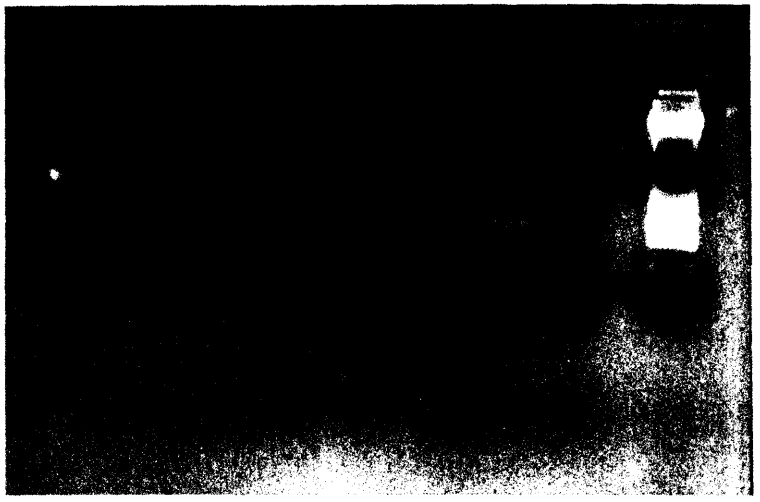


Figure 6.1 RT-PCR Results using PSA, AR and GAPDH Primers after Demethylation Treatment of Prostate Cancer Cell Lines with 5-Aza

Lane 1 LNCaP cells treated with 5 Aza
Lane 2 LNCaP cells untreated
Lane 3 DU 145 cells treated with 5 Aza
Lane 4 DU 145 cells untreated
Lane 5 PC-3 cells treated with 5 Aza
Lane 6 PC-3 cells untreated
Lane 7 No RNA
Lane 8 Reagents only



PSMA ⟶ ⟵ PAP

PAP ⟶ ⟵ GAPDH

Figure 6.2 PCR Results using PSMA and PAP Primers after Demethylation of Prostate Cancer Cell Lines with 5-Aza

Lane 1 LNCaP cells treated with 5 Aza
Lane 2 LNCaP cells untreated
Lane 3 DU 145 cells treated with 5 Aza
Lane 4 DU 145 cells untreated
Lane 5 PC-3 cells treated with 5 Aza
Lane 6 PC-3 cells untreated
Lane 7 No RNA
Lane 8 Reagents only

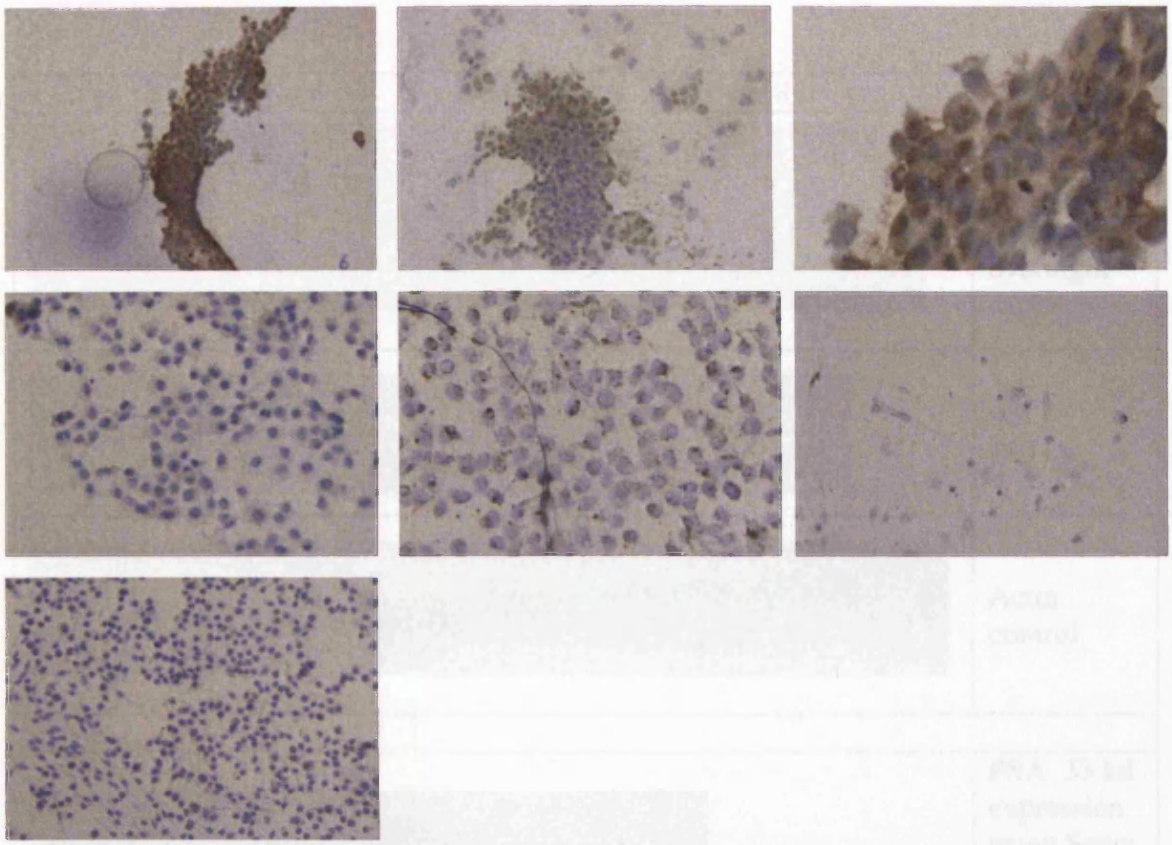


Figure 6.3 Immunohistochemical Staining of Prostate Cancer Cell Lines Grown on Coverslips

Row 1: Control LNCaP cells staining for PSA
Control LNCaP cells showing nuclear staining for AR
Treated DU 145 cells showing staining for PSA
Row 2: Untreated DU 145 cells showing negative staining for PSA
Untreated DU 145 cells showing negative staining for AR
Treated PC-3 cells showing negative staining for PSA
Row 3: Control LNCaP cells with secondary antibody only

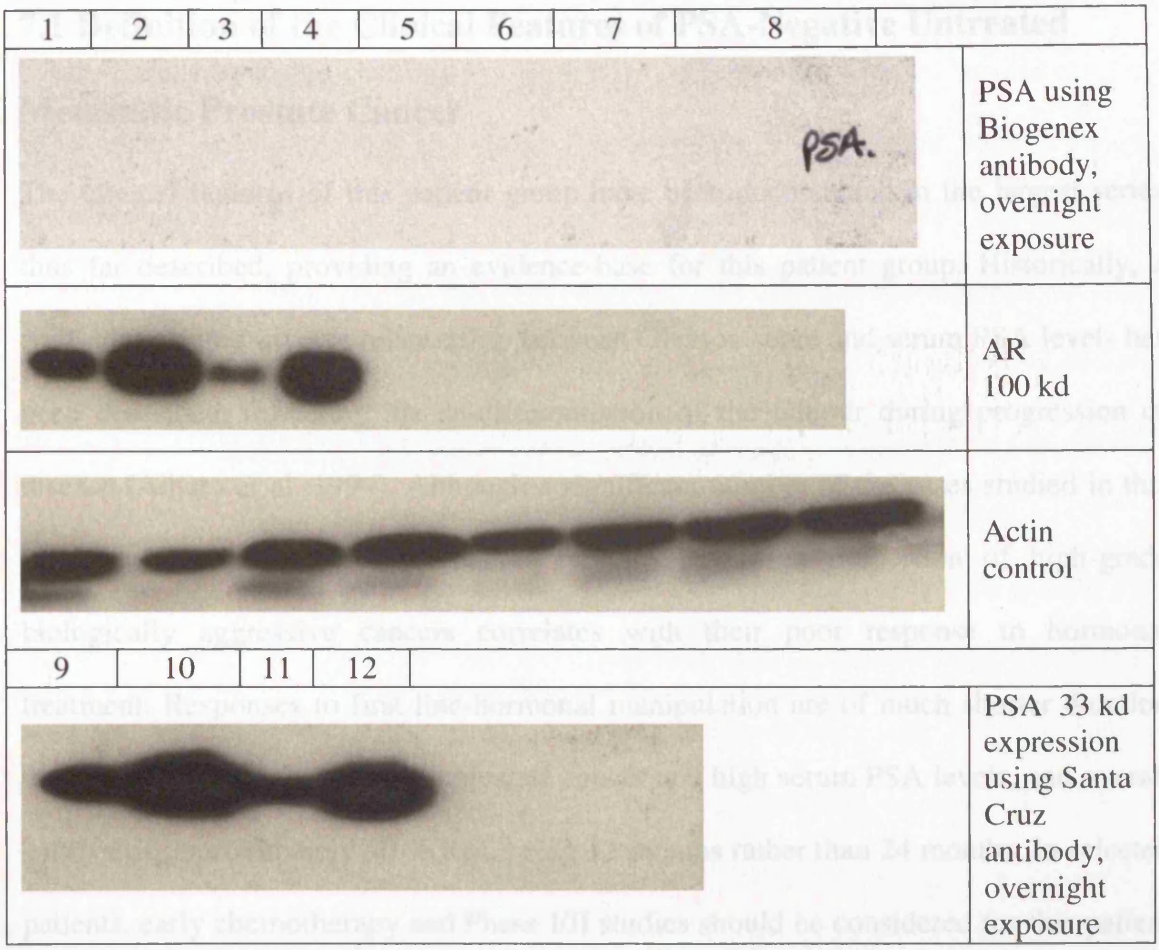


Figure 6.4 Western Blotting of Prostate Cancer Cell Lines Treated with 5-Aza to Investigate Re-Expression of PSA and AR after Demethylation

Lane 1 25.0 µg protein loaded, LNCaP cells treated with 5-Aza
Lane 2 25.0 µg protein loaded, LNCaP cells untreated
Lane 3 2.5 µg protein loaded, LNCaP cells treated with 5-Aza
Lane 4 2.5 µg protein loaded, LNCaP cells untreated
Lane 5 25.0 µg protein loaded, DU 145 cells treated with 5-Aza
Lane 6 25.0 µg protein loaded, DU 145 cells untreated
Lane 7 25.0 µg protein loaded, PC3 cells treated with 5-Aza
Lane 8 25.0 µg protein loaded, PC3 cells untreated
Lane 9 5.0 µg protein loaded, LNCaP cells treated with 5-Aza
Lane 10 5.0 µg protein loaded, LNCaP cells untreated
Lane 11 50.0 µg protein loaded, LNCaP cells treated with 5-Aza
Lane 12 50.0 µg protein loaded, LNCaP cells untreated.

Chapter 7 General Discussion

7.1 Definition of the Clinical Features of PSA-Negative Untreated Metastatic Prostate Cancer

The clinical features of this patient group have been documented in the largest series thus far described, providing an evidence-base for this patient group. Historically, a well-documented inverse relationship between Gleason score and serum PSA levels has been described, reflecting the de-differentiation of the tumour during progression of disease (Aihara et al. 1994). Although a significant number of the cases studied in this thesis were moderately differentiated tumours, the high proportion of high-grade biologically aggressive cancers correlates with their poor response to hormonal treatment. Responses to first line-hormonal manipulation are of much shorter duration than for patients with metastatic prostate cancer and high serum PSA levels, and overall survival is approximately 50 % less, being 12 months rather than 24 months. In selected patients, early chemotherapy and Phase I/II studies should be considered for this patient group. The entry of these patients into clinical studies will allow data to be collected prospectively, and a database of tissue and sera to be collected.

7.2 The Immunohistochemical Characteristics of Serum PSA-Negative Prostate Cancer

The initial premise in the current study was that as levels of serum PSA and tissue PSA are generally concordant, tissue staining for PSA might be absent in the group of patients studied in this thesis and that some alteration in protein synthesis, most likely due to a change in the AR was the culprit. Other authors have observed reduced tissue levels of PSA in high grade prostate carcinomas, by both PSA mRNA measurement

(Qiu 1990) and immunohistochemistry (Aihara et al. 1994). However, this work has clearly shown that in the majority of men with inappropriately low levels of serum PSA, there is positive tissue staining for both PSA and the AR. This makes silencing or deletion of the AR unlikely and shows that PSA synthesis at tissue level is present, although the extent of synthesis may be reduced. It should be born in mind that a functional assay of the AR was not performed and it is possible that although the AR is present, it is not functional. The archival prostate tissue used in this work was obtained from biopsy or resection of the primary carcinoma. It is well known that marked heterogeneity occurs within prostate cancer and there may be areas displaying differential amounts of tissue positivity for both PSA and AR within each tumour (van der Kwast 1991). It is possible that had tissue from the metastasis been available for each patient, the pattern of tissue PSA and AR immunostaining may have significantly differed from that observed in the primary tumours, and that a tissue PSA-negative metastatic tumour represented the dominant clone in this group of patients. It would be necessary to collect paired tissue from both primary and metastatic tumours to evaluate this hypothesis.

7.3 Alternative Diagnostic Tissue Markers

Positive tissue immunostaining for PSA was observed in two thirds of the cases, but was in general focal in nature and could potentially be missed on a diagnostic prostatic biopsy. The more diffuse pattern of immunostaining seen with PSMA in 90% of cases therefore reduces the chances of a geographical miss on diagnostic biopsy and should augment the histopathologist's panel of the antibodies used when serum PSA is low. It was not possible to analyse the tissue for hK2 status, which shares 70% sequence homology with PSA, due to difficulties obtaining a specific validated antibody.

7.4 Potential Serum Markers

Attempts have been made to utilise circulating serum PSMA levels as part of the screening of prostate cancer. Murphy and colleagues (1995) reported that serum PSMA levels are elevated in prostate cancer patients, and the elevation remains, even when PSA levels are low. Although Kim (2000) suggested that serum PSMA may be of use in androgen-independent prostate cancer, this has not been a universal finding. Attempts to improve detection have been made using RT-PCR assays; although RT-PCR of serum PSMA was more sensitive than that for serum PSA, it was not sufficiently sensitive to be used as a basis for clinical therapy (Murphy 1998). However, given the marked tissue immunostaining for PSMA in our group of patients, serum PSMA levels may well be of use in establishing early diagnosis in those cases presenting with metastatic disease and low serum PSA. In men with low serum PSA, serum PSMA should be explored as a potential tumour marker, but requires a reliable serum assay technique.

Before the advent of PSA as a serum tumour marker, serum PAP was extensively used in the diagnosis and management of prostate cancer (Gutman et al. 1936), but fell out of favour due to lack of tissue specificity and diurnal variations in serum levels. There have been occasional reports in advanced disease where PAP levels were elevated while the serum PSA remained undetectable (Goldrath & Messing 1989;Leibman et al. 1995). Perhaps it is time to re-vist PAP as a serum marker in the PSA-negative metastatic group.

7.5 Alternative Therapeutic Targets (Appendix 2)

The numbers examined for alternative molecular targets is too small to be reported with confidence. Nevertheless, the attraction of Her-2 as a molecular target is powerful.

These patients do behave as though they are hormone –refractory from the outset, given their extremely short duration of response to first-line hormonal management. As Her-2 has been shown to be over-expressed in hormone refractory prostate cancer, further exploration of Her-2 staining in these PSA-negative patients should be pursued as a potential therapeutic option.

7.6 The Mechanism of Low Serum PSA Levels –"PSA-Negativity"

7.6.1 Protein Synthesis

One aim of the work reported in this thesis was to investigate the potential mechanisms underlying PSA-negativity, specifically in relation to PSA protein synthesis. As described above, PSA is certainly expressed at tissue level, albeit in an often focal and patchy fashion, as demonstrated by the immunohistochistry results. The cell line work demonstrated that methylation of the AR causing functional silencing of PSA transcription was not a factor identified in this thesis. Some other factor affecting PSA production appeared to be methylated and PSA expression re-activated by appropriate demethylating agents. This implies that some mechanism, which bypasses the AR, appears to be methylated in prostate cancer cell lines and thus negatively affects PSA expression. One way of further examining this would have been to perform methylation specific PCR for PSA and AR using the prostate archival tissue after bisulphite treatment, but insufficient tissue remained to examine this in an adequate number of cases.

Loss of heterozygosity was not a factor in the samples studied. The method using archival laser-captured tissue was problematic, and analysis of the remainder of the samples would be optimised by macroscopically scraping slides where the volume of

tissue remaining permitted this to be done.

7.6.2 PSA Degradation

Protein synthesis was only one of three potential mechanisms involved in PSA-negativity, the others being PSA secretion and protein degradation. Free PSA and PSA bound to certain protease inhibitors may only be present temporarily in serum, due to prompt degradation. Use of immunohistochemical antibodies to ubiquitin would help determine whether rapid PSA protein degradation and metabolism occurs, which would markedly reduce serum levels of PSA. Electron microscopy could then determine in which regions of the cell ubiquitin was present, thus demonstrating where a change in the degradation pathway was taking place.

7.6.3 PSA Secretion

PSA secretion into the systemic circulation may be the limiting factor. It is clear that PSA within the systemic circulation exists in several forms. For this study, absolute serum PSA levels were documented and it was therefore not possible to correlate serum free:total PSA ratios. Neutralising antibodies to PSA have been reported in up to 5% of all sexually active women but have not, as yet, been identified in patients with prostate cancer. PSA is normally secreted from prostatic luminal epithelium as pro-PSA, from which the 7 amino acid leader sequence is cleaved. Differential cleavage in the post-translational modification of PSA results in splice variants such as [-2] pPSA and [-7] pPSA. Current serum PSA assays are unable to detect these splice variants, which have been recently reported as being present at up to five-fold greater values in prostate biopsy-positive patients when compared to biopsy-negative ones (Mikolajczyk et al. 2000;Mikolajczyk et al. 2001). It is feasible that men with inappropriately low serum

levels of PSA produce one such splice variant, which with the development of commercial assays, may be able to be routinely tested and used as a diagnostic and monitoring marker.

7.7 Further Work.

This work needs to be taken forward with further immunohistochemistry to examine hK2 status in these tumours and to assess this as an alternative diagnostic marker. As many of the patients examined in this work have already died, serum samples were not available but a pilot study is in place to collect sera prospectively from any patient presenting in this patient group to the Cancer Centre, and examine PSMA levels as a serum biomarker. Currently, serum PAP levels are also being routinely collected for a similar reason. Her-2 status should be examined in the remaining two-thirds of the samples.

In conclusion, diagnosis and monitoring of men presenting with untreated metastatic prostate cancer and low serum PSA levels may be augmented by the use of alternative tissue and serum marker such as PSMA. Given that reliable assays remain elusive, any such patient should have tissue and sera prospectively stored to enable retrospective analysis to be carried out when technical advances permit.

Appendix 1 Documentation For Clinical Features Study

- Letter to pathologist
- Letter to urologist
- Letter to patient from urologist
- Patient information sheet
- MREC consent form

CONFIDENTIAL

Consultant Histopathologist,
Hereford Hospitals NHS Trust,
The County Hospital,
Union Walk,
Hereford.
HR1 2ER.

19th July, 2002.

Dear Dr.,

Re: PSA-Negative Prostate Cancers.

I am currently investigating PSA negative prostate cancers as part of a clinical and laboratory-based study. The BAUS Section of Oncology has agreed to support this project by allowing access to its minimum dataset for urological cancer to identify those patients who are PSA-negative and to approach the Consultants involved with each case. I plan to study the patients' case notes to establish the clinical presentation, natural history and prognosis of this group of patients. The laboratory aspect of the project is to investigate the reason why these tumours are PSA negative. I hope to characterise the differentiation patterns of the tumours, confirm PSA negativity and check androgen receptor expression using immunohistochemistry. We also aim to examine mRNA expression and measure the methylation status of the gene. We hope to look for alternative tumour markers in addition.

The database has identified two patients of Mr ....., Hospital ID...., DOB.....(who has consented to the project and whose consent form is enclosed) and patient hospital no....., d.o.b..... who is deceased. Approval has been given by MREC not to seek consent from next of kin where patients have already died.

Mr ..... has received a copy of the MREC approval to forward on to the local LREC committee for their information. I have contacted Mr ..... who is happy to provide access to the patient's notes and I would hope to be able to collect the specimens in person on the same visit. If it is not possible to have a block from the prostate, I would be very grateful for as many 3μm slides as possible, as I am hoping to use 5-7 uncoated slides for the molecular biology work and 8 vecta bonded coated slides for the immunohistochemistry.

I look forward to hearing from you and please do not hesitate to contact me if I can provide you with any further information,

Yours sincerely,

Dr. Alison Birtle MRCP.FRCR.
Clinical Research Fellow, Pathology letter. Version1.9.01

CONFIDENTIAL

Dear Mr.....

Re: PSA-Negative Prostate Cancer

Patient :

I am currently investigating PSA negative prostate cancers as part of a clinical and laboratory-based study.

The BAUS Section of Oncology has agreed to support this project by allowing access to its mimimum dataset for urological cancers to identify those patients who are PSA-negative and to approach the Consultants involved with each case. I plan to study the case notes of patients with locally advanced or metastatic disease to establish the clinical presentation, natural history and prognosis of this group of patients. The laboratory aspect of the project is to investigate the reason why these tumours are PSA negative. I hope to characterise the differentiation patterns of the tumours, confirm PSA negativity and check androgen receptor expression using immunohistochemistry. We also aim to examine mRNA expression and measure the methylation status of the gene. We hope to look for alternative tumour markers in addition.

I would be very grateful if you could write to your patient, Hospital no. ....., D.O.B. .....to ask for consent for me to have permission to access the medical records and to obtain blocks of the prostate to perform the requisite investigations. I enclose a copy of the patient information sheet and consent form. I would be happy to meet with you, if you wished, to discuss the project and plan to review the medical records and to collect the samples in person.

If you are in agreement, please sign below, and retain a copy for your records.

I look forward to hearing from you and please do not hesitate to contact me if I can provide you with any further information,

Yours sincerely,

Dr.Alison Birtle MRCP.FRCR
Clinical Research Fellow

Please delete as appropriate:

1. I will send on the letter and information sheet to the patient, and when I have the patient's consent I will give you access to the notes. I will ask my secretary then to pull the notes.
2. I would like to meet with you to discuss the project

Signed .....
Date.....
Address.....

Urologist letter .Version 2. 10.01

CONFIDENTIAL

PSA – NEGATIVE PROSTATE CANCER STUDY

Dear

On behalf of my colleague, Dr Birtle, I would like to ask you to take part in a study into prostate cancer, which is being run from the Institute of Urology in London, and is a national project. It would involve extra tests being done on the prostate tissue that was removed at your surgery but would not mean any extra hospital visits or tests on yourself. It is hoped that this study will help in the diagnosis and treatment of prostate cancer.

I enclose the information sheet and consent form for the study and the contact details of the doctor involved. Please contact her if you have any questions, and forward the consent form back to her at the contact address.

Thank you for considering this proposal.

Yours sincerely,

CONSULTANT UROLOGIST.

Patient letter. Version 02.10.01

CONFIDENTIAL
PATIENT INFORMATION SHEET
PSA – NEGATIVE PROSTATE CANCER STUDY

Most prostate cancers produce a protein called PSA (prostate specific antigen) which circulates in the blood stream. This can be used to detect prostate cancer and to see how well treatment is working. However, some cancers can either stop producing PSA or never produce it from the start.

We are interested in men who have prostate cancer, and have much lower measurements of PSA in the blood than expected. We would do specific laboratory tests on the prostate tissue that was removed at surgery. No tests other than those relevant to this particular project would be done and at the end of the project, the samples would be returned to your original hospital. We would also see if there were any other substances produced by the cancer that could be used instead of PSA to diagnose prostate cancer and to see how treatment was working. Our results will not benefit you directly but in time we hope this will help earlier diagnosis and better treatment for men whose prostate cancer do not produce PSA.

We also would like to look at your medical records, to see if there was anything in common with other men with similar cancers. This information would be confidential and only used for this project. There would be no extra tests done on you physically, only on the cancer that has already been removed. There would be no extra visits to the hospital.

You do not have to take part in this study if you do not want to. If you decide to take part you may withdraw at any time without having to give a reason. Your decision whether to take part or not will not affect your care and management in any way.

If you have any questions about the study, please contact Dr. Alison Birtle, Research Registrar, The Institute of Urology,
. Telephone
All proposals for research using human subjects are reviewed by an ethics committee before they can proceed. This proposal was reviewed by the Joint UCL/UCLH Committees on the Ethics of Human Research.

Info sheet Version 03.03.02

Centre Number: :
Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **PSA – NEGATIVE PROSTATE CANCER**

Name of Researcher: Dr Alison Birtle, Clinical Research Fellow.
The Institute of Urology & Nephrology,

1. I confirm that I have read and understand the information sheet dated 3.02 (version 3) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the Institute of Urology or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study.

Name of Patient	Signature	Date
Name of Person taking consent (if different from researcher)	Signature	Date
Researcher	Signature	Date

Consent form Version 2.3.02. 1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 2 Potential Diagnostic Markers As Therapeutic Targets For Serum PSA-Negative Prostate Cancer

A2.1 Introduction

The poor prognosis of men presenting with untreated metastatic prostate cancer and low serum PSA has been previously described (Birtle et al 2003). Different management strategies are therefore needed and molecular targets represent an attractive option. Over-expression of particular gene products at tissue level may allow the development of targeted antibodies. Likely targets include Her-2/neu, matrix metalloproteinase 2 (MMP 2), bcl-2, and fibroblast growth factors.

A2.1.1 Her-2/neu (C-erb-B2)

Her-2 encodes the 185-kDa transmembrane tyrosine kinase receptor, first identified as a homologue of the epidermal growth factor receptor gene (Yamamoto et al 1986) and found to be over-expressed in 20-30 % of breast and ovarian cancers (Slamon et al 1989). In particular, over-expression of Her-2/neu (c-erb-B2) in prostate cancer models appears to be associated with androgen-independent growth, and may activate the AR in a ligand-independent manner (Craft et al 1999). In some studies, it has been associated with shortened survival (Sadasivan et al 1993). If Her-2/neu is involved in prostate cancer progression and the transition to androgen independence, it may be up-regulated in PSA-negative tumours, which themselves appear to behave in an androgen-independent manner from the outset. At present, analysis of prostate cancer specimens for Her-2 expression is not standard, but may be of value in identifying those patients likely to benefit from targeted therapy with such agents as Herceptin ™, a monoclonal antibody licensed for breast cancers over-expressing the protein, (Agus et al 1999), or

PKI 166 (Novartis Pharmaecuticals), a dual EGFR/Her-2 tyrosine kinase inhibitor found to be active in hormone refractory prostate cancer (Mellinghoff et al. 2002).

A2.1.2 Matrix Metalloproteinase 2 (MMP2) and its tissue inhibitor (TIMP2)

Matrix metalloproteinases (MMP1-20) are proteolytic enzymes capable of degrading the basement membrane and extracellular matrix promoting neoplastic cell invasion and metastases. Their activity is modulated by their tissue inhibitors (TIMP1-4). Increased expression of MMP's has been associated with poor prognosis and reduced survival in patients with a variety of malignancies, including carcinoma of the colon, stomach, oesophagus and breast (Stetler-Stevenson 1990). Several studies have described an association in prostate cancer between a high MMP-2 :TIMP-2 ratio and increased tumour stage or grade (Still et al 2000). In a similar fashion, several studies have found a correlation between MMP-2 and advanced or progressive prostate cancer. Co-expression of MMP-2 and its tissue inhibitor TIMP2, has a negative prognostic significance in patients with prostate cancer (Ross et al 2003). Pre-clinical studies of MMP inhibitors have shown anti-tumour effects in vivo (Lokeshwar 1999) and clinical studies of second-generation MMP inhibitors have shown clinical responses in pancreatic, lung and ovarian tumours (Brown & Giavazzi 1995).

A2.1.3 Fibroblast Growth Factors (FGF)

Fibroblast growth factors are multifunctional heparin binding polypeptides, which share structural similarity. Their signalling is mediated by transmembrane tyrosine kinase receptors, the FGFR's (Yamasaki et al 1996) and both FGF and FGFR are involved in development, wound healing, angiogenesis and tumorigenesis (Basilico & Moscatelli

1992). FGF8 or androgen induced growth factor has been shown to have a positive relationship with tumour grade (Leung et al 1996) although other workers have found the reverse (Wang et al 1999). Nevertheless, it would seem that FGF8 expression is linked with development of prostate cancer. Reduced expression of its receptor, FGFR2, has been suggested as a marker for the development of AIPC (Naimi et al 2002).

A2.1.4 Bcl-2

The anti-apoptotic factor Bcl-2 is frequently reported as being up-regulated in advanced prostate cancer (McDonnell 1992). Bcl-2 over-expression has been shown in AIPC and it has been suggested that this may be bypassing the apoptotic signal initiated by hormonal therapy (Feldman & Feldman 2001).

The aim of this pilot study was:

To investigate the expression of potential therapeutic targets for patients with untreated metastatic prostate cancer and low serum PSA.

A2.2 Materials and Methods

A2.2.1 General immunostaining

10 of the 33 cases collected as in chapter 4, were selected at random (5 biopsy specimens and 5 from transurethral resection) to perform an additional immunohistochemistry panel of 6 antibodies. Commercially available primary antibodies to TIMP2, MMP2, Bcl-2, Her-2, FGF8 and FGFR2 were used. Appropriate positive and negative controls were included for all immunohistochemistry. The optimal dilution of and preferred method of antigen retrieval was determined by titrating the antibody using a high grade radical prostatectomy sample as a positive control for Bcl-2 and an archival hyperproliferative keloid scar for TIMP2, MMP2, FGF8 and FGFR

(Table A2.1). The standard cell line positive and negative controls as supplied by the manufacturer were used for Her-2. In brief, the tissue sections were dewaxed in xylene and taken through a series of graded alcohols to water. The method of antigen retrieval, primary antibody concentration and method of staining is summarised in Table A2.1. An identical method of immunostaining was performed as previously outlined in chapter 4, using the EnVision sysytem.

A2.2.2 HER-2/neu immunostaining

The Dako Herceptest Immunohistochemical kit for immunostaining with HER-2/neu (Dako Corp., Carpenteria, CA) was used as described previously (Edorh et al 1995). Assessment of the degree of staining was performed as outlined below.

Immunohistochemical staining was assessed by one pathologist who had a site-specialised interest in urological tumours (AF) and semi-quantitative staining was performed taking in to account the intensity (0, +, ++, +++) and extent (focal/diffuse) of staining. Membrane staining for HER-2/neu protein was categorised, ranging from zero to +3, as defined by the developers of the commercial kit and compared with the supplied positive and negative cell line controls. Score zero was defined as per the manufacturer's instructions as undetectable staining or membrane staining in < 10% of the tumour cells. Score +1 was defined as faint membrane staining detected in > 10% of the tumour cells. Score +2 was considered as weak to moderate complete membrane staining observed in > 10% of the tumour cells and score +3 defined as moderate to strong complete membrane staining observed in > 10% of the tumour cells.

A2.3 Results

(See Table A2.2) 2/10 cases were positive for Her 2-neu, with 2/10 positive for Bcl-2.

(Figures A2.1 and A2.2) Weak immunoreactivity for FGF8 was observed in 5/10 cases. All 10 cases demonstrated some staining for TIMP-2 with 9/10 cases also showing MMP-2 immunoreactivity (data not shown). All cases were negative for FGFR2 (data not shown).

A2.4 Discussion

The mechanism of low serum levels of PSA cannot be explained by AR loss leading to altered PSA transcription, as strong nuclear AR staining was seen in the majority of patients (Chapter 4). It has been suggested that up-regulation of Bcl-2, found in cases of androgen independent prostate cancer could bypass the apoptotic signal provided by hormonal therapy (Feldman & Feldman 2001). We found positive staining for Bcl-2 in 2 of the 10 (20%) cases selected for the additional panel of antibodies, both cases being poorly differentiated tumours which were tissue PSA and AR negative. Overexpression of Her-2/neu has been observed in certain tumour types, including breast, ovary and lung carcinomas (Naber 1990;Slamon 1989), and associated with tumour progression. Recent studies in prostate cancer have suggested significant overexpression of this protein in primary metastatic prostate cancer, particularly after androgen-deprivation therapy (Osman 2001) and that Her-2/neu expression appears to increase with progression to androgen independence (Signoretti 2000). Given the short median duration of response to first-line hormonal therapy of 7 months in our group of patients with low serum PSA (Birtle et al 2003), it can be argued that those prostate tumours presenting with metastatic disease and low serum levels of PSA are hormone-refractory from the outset. It is an attractive option to consider targeted therapy with monoclonal antibody to Her-2/neu for these patients. However, Her-2/neu immunostaining was only performed in 10 patients with 1/10 showing 2+ staining and 1/10 1+ staining.

Fibroblast Growth Factor 8 (FGF 8) or androgen induced growth factor expression was studied as conflicting results for this factor in prostatic tissue have been documented in the literature. Leung has shown a positive relation between FGF8 and tumour grade in prostate cancer (Leung 1996). In contrast, other studies have shown an inverse relationship between these two parameters and a positive correlation with the AR (Wang 1999). We found only weak staining for FGF8 in 5/10 cases studied with no demonstrable association between either tumour grade or AR expression. In addition, FGF receptor 2 (FGFR 2) staining was negative in 10/10 cases. This agrees with work (Naimi 2002) where FGFR2 expression was downregulated in 18/30 prostate samples, particularly in androgen-independent ones, and it has been suggested that its inactivation may have a role in disease progression. Alternative methods such as real-time quantitative polymerase chain reaction would be needed to look at this potential downregulation of FGFR2.

Unregulated secretion of matrix metalloproteinases (MMPs) or their endogenous protein inhibitors (tissue inhibitor of metalloproteinases TIMPS) have been implicated in tumour invasion and metastases (Liotta 1986). MMP-2 expression has been associated with more aggressive biologic behaviour in colorectal, breast and stomach cancer (Stetler-Stevenson WG 1990). More recently, Still (2000) has suggested a tight association between MMP-2 and TIMP-2 expression with an increase in the ratio of MMP-2:TIMP-2 in high grade or high stage tumours. The current study has shown an inverse relationship between MMP-2 and TIMP-2 in 2 cases both of which were Gleason 9 tumours.

Although the additional immunohistochemical markers were examined in one third of

the cases only, over-expression of Her-2/neu may represent a potential therapeutic target. In addition, the down-regulation of FGFR and TIMP-2 may play a part in the particular aggressive pattern of disease in patients with treatment-naïve metastatic prostate cancer and low serum PSA. Further work is needed to increase the number of cases examined.

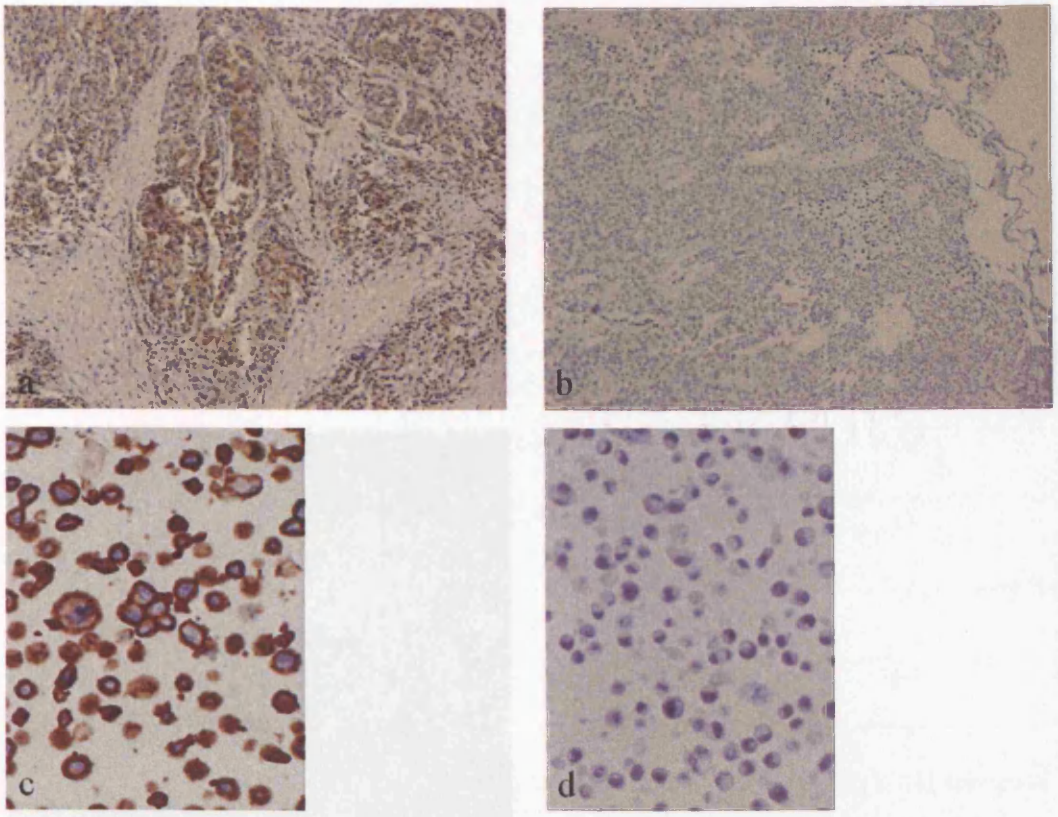


Figure A2.1 Her-2/Neu Immunostaining

A Serum PSA-negative prostate carcinoma showing 3+ Her-2/neu staining (x 20)
B Serum PSA-negative prostate carcinoma showing negative Her-2/neu staining (x 20)
C Positive cell line control for Her-2/neu supplied in Hercept test ® (x 40)
D Negative cell line control for Her-2/neu supplied in Hercept test® (x 40)

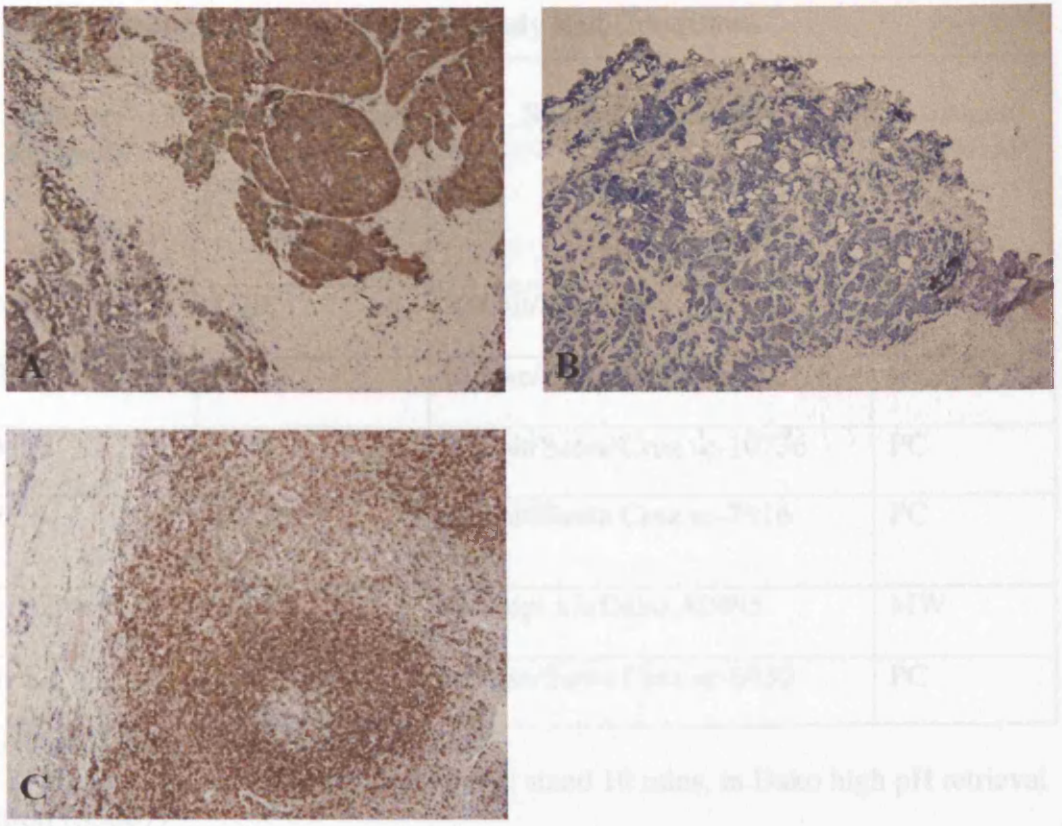


Figure A2.2 Bcl-2 Immunostaining

A Serum PSA-negative prostate carcinoma showing Bcl-2 3+ staining (x 20)
B Serum PSA-negative prostate carcinoma showing negative Bcl-2 staining (x 20)
C Lymph node as positive control for Bcl-2 (x 20)

Table A2.1 Summary of Primary Antibody and Conditions

Primary antibody	Concentration	Species/ Catalogue	Antigen Retrieval*
TIMP2	1/20	Rabbit/Santa Cruz	9.9
Bcl-2	1/10	Mouse/Dako M0887	PC
MMP2	1/100	Rabbit/Santa Cruz sc-10736	PC
FGF 8	1/200	Rabbit/Santa Cruz sc-7916	PC
HER-2/neu	1/2000	Hercept kit/Dako A0485	MW
FGFR2	1/100	Mouse/Santa Cruz sc-6930	PC

* Method of antigen retrieval

9.9: 25 minutes microwaving at full power, stand 10 mins, in Dako high pH retrieval solution (S3007)

MW: stand in Epitope Retrieval Solution (Dako S1699) at 98 °C in waterbath for 40 minutes (as per manufacturer's instructions)

PC = 2 minutes pressure cooking at full pressure in citrate buffer pH 6.0

Table A2.2 Immunohistochemistry Results of Additional Markers Panel in 10 Selected Cases

		Extent of Tumour Positivity						
Patient	**Gleason Score**	**PSA**	**TIMP2**	**MMP2**	**FGF8**	**FGFR2**	**Her-2/neu**	**Bcl-2**
1	10	0	F 2+	F 2+	F 1+	0	0	0
2	9	F 2+	F 1+	F 3+	D 1+	0	0	0
3	10	0	F 2+	F 1+	F 1+	0	0	D 3+
4	10	F 2+	F 2+	F 3+	0	0	0	0
5	9	F 2+	F 1+	F 2+	F 1+	0	2 +	0
6	10	0	F 2+	F 3+	0	0	0	0
7	8	D 3 +	F 2+	F 2+	0	0	0	0
8	9	0	F 3+	F 3+	0	0	0	F 1+
9	9	0	F 1+	0	F 1+	0	F 1+	0
10	9	F 2 +	D 2+	D 2+	0	0	0	0

F: Focal Staining
D: Diffuse Staining

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