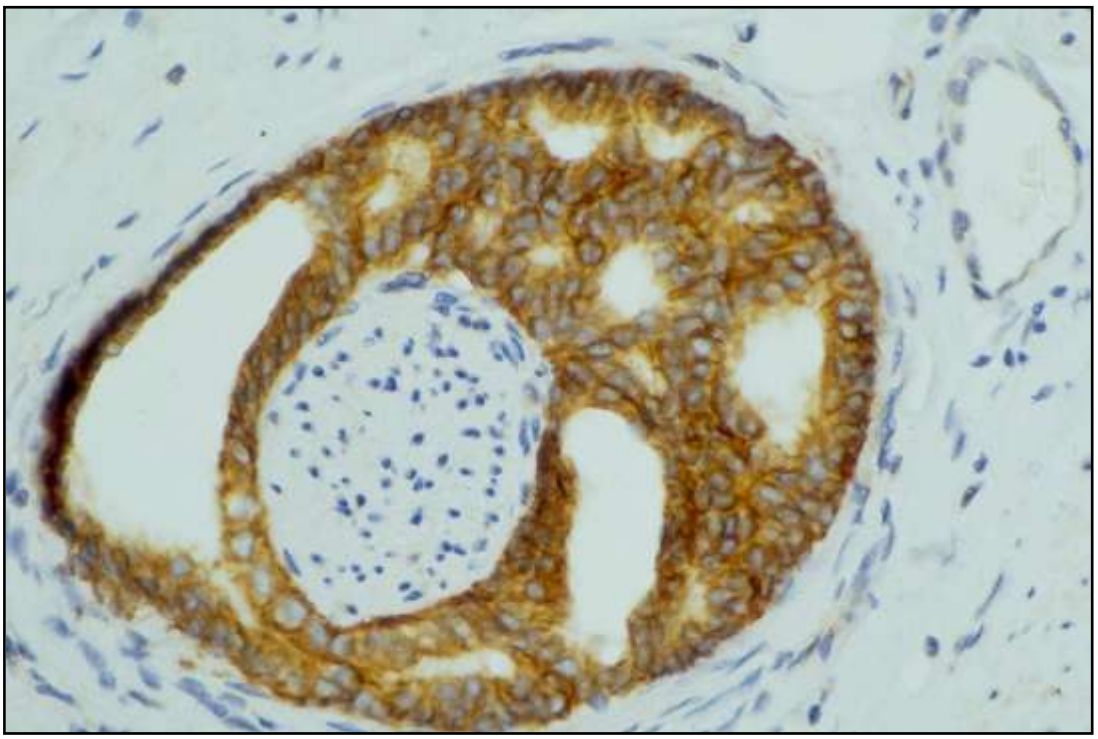
Prognostic indicators in prostate cancer



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MD thesis
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September 2000

Abstract

The incidence of prostate cancer is increasing in men under the age of 60 in the United Kingdom. This thesis examines clinicopathological variables and tumour biomarkers as prognostic indicators.

Ductal carcinoma of the prostate is a rare histological variant. This study shows that it has a prognosis similar to a high Gleason score microacinar carcinoma and treatment should be no different.

Radical prostatectomy aims to cure clinically localised prostatic adenocarcinoma but in a cohort of 217 men treated at the Bristol Urological Institute, 28% will have a biochemical recurrence in the first five postoperative years. In this study the Gleason score in 75 preoperative biopsies was found to be a significant indicator of biochemical recurrence after radical prostatectomy: the Gleason score of the tumour in the radical prostatectomy specimen was significant only univariately. In a larger series of 129 patients the presence of capsular penetration by tumour, and of positive surgical margins were both significant predictors of recurrence on multivariate analysis.

The tumour biomarkers p53, bcl-2, CD44 and E-cadherin were assessed by immunohistochemistry to determine whether any could predict biochemical recurrence after radical prostatectomy. Only p53 proved multivariately significant in the preoperative biopsies. p53 was only univariately significant in radical prostatectomies. bcl-2 appeared significant multivariately in a small series of radical prostatectomy specimens but in a larger series this was found significant only univariately. CD44 and E-cadherin did not prove to be useful predictors of recurrence.

The amplification of Her-2/neu oncogene has been shown to be an indicator of poor prognosis in patients with breast cancer. Using in situ hybridisation techniques increased copy numbers of Her-2/neu were found in only two patients with prostate cancer, but these were related to polysomy for chromosome 17 rather than true amplification.

In conclusion, tumour markers are useful in our understanding of tumour progression and clinicopathological data provide reliable prognostic information.

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[†] All photomicrograph magnifications stated are the original magnifications (om).

Abbreviations

AGM – Dr Angus MacIver, (Consultant Pathologist, Southmead Hospital).

AR – Androgen receptor

CT - Computer tomography

DRE – Digital rectal examination.

ER – Oestrogen receptor.

FISH – Fluorescent in situ hybridisation.

JDO – Dr Jon Oxley

Ki67 – An immunohistochemical marker which is positive in proliferating cells.

MRI - Magnetic resonance imaging

NPH – Nodular prostatic hypertrophy.

OM – Original magnification.

ONS – Office for National Statistics.

PIN – Prostatic intraepithelial neoplasia.

ppv – Positive predictive value.

PrAP – Prostatic acid phosphatase.

PSA – Prostate specific antigen.

SSC - Sodium chloride/sodium citrate buffer.

TRUS – Transrectal ultrasound.

TURP – Transurethral resection of the prostate.

Introduction

Prostatic adenocarcinoma is the second commonest cause of cancer death after lung cancer in European men. There is increasing knowledge of the molecular abnormalities in prostate cancer but no single marker has proved to be a useful predictor of outcome. This thesis aims to study the histological features of prostatic adenocarcinomas and tumour biomarkers to examine their predictive and prognostic attributes.

The anatomy of the prostate gland and the origins of tumours

The prostate gland lies beneath the urinary bladder and surrounds the neck of the bladder and the prostatic urethra. The normal prostate weighs approximately 20 grams but it can become greatly enlarged by nodular prostatic hypertrophy, so that the gland in a 65 year old will be on average three times larger than that of a 25 year old. The prostate can be divided into several zones: peripheral, central, transitional, and periurethral. These zones are important as 70% of the prostatic adenocarcinomas arise in the peripheral zone. The prostate gland itself is comprised of tubuloalveolar glands embedded in a fibromuscular stroma. These glands have an internal columnar epithelium surrounded by a cuboidal basal epithelium, which is itself surrounded by a basement membrane layer. Prostatic adenocarcinomas are derived from the internal columnar layer and the malignant glands lack the normal basal layer epithelium. The carcinoma cells often have large nuclei with prominent nucleoli. Glands that have these nuclear characteristics but have the presence of the basal layer are thought to be the precursor of adenocarcinoma and the term high grade prostatic intraepithelial neoplasia (PIN) has been used to describe these glands. When PIN was first described it was divided into three categories, PIN 1, 2 and 3 in

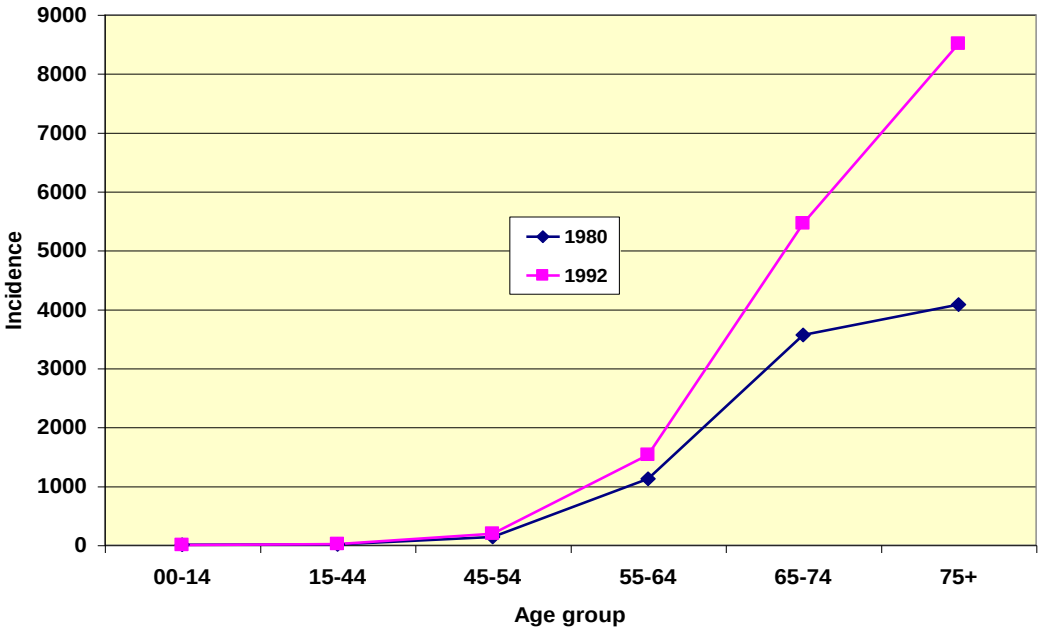
a similar way that cervical intraepithelial neoplasia has been divided. Nowadays PIN is classified as either high or low grade. Low grade PIN is characterized by the proliferation of the columnar cells within the ducts and large acini. The nuclei show a wide range in size from normal to enlarged and have open nuclei but at most small inconspicuous nucleoli. This may be difficult to impossible to distinguish from proliferation within nodular hyperplasia and as a result there is poor reproducibility. The significance of low grade PIN is also questioned and it is rarely diagnosed. High grade PIN depicts cytologically malignant cells lining the glandular units of the prostate and its presence in a needle core biopsy is an indication for further biopsies as up to 70% of patients will have a carcinoma diagnosed in a subsequent biopsy. This is high percentage is probably due to having missed the tumour with the first set of biopsies rather than evolution of tumours from the PIN in the time span between the two sets of biopsies.

Epidemiology and aetiology of prostate cancer

In England and Wales nearly 9,000 men died of prostate cancer in 1992 compared with 4313 in 1974 [Office for National Statistics (ONS), 1999]. This increasing trend probably reflects an ageing population in whom there is a high prevalence of the disease (see Figure 1). Although this may be the case, there were still 600 deaths in 1992 in men under the age of 65 years.

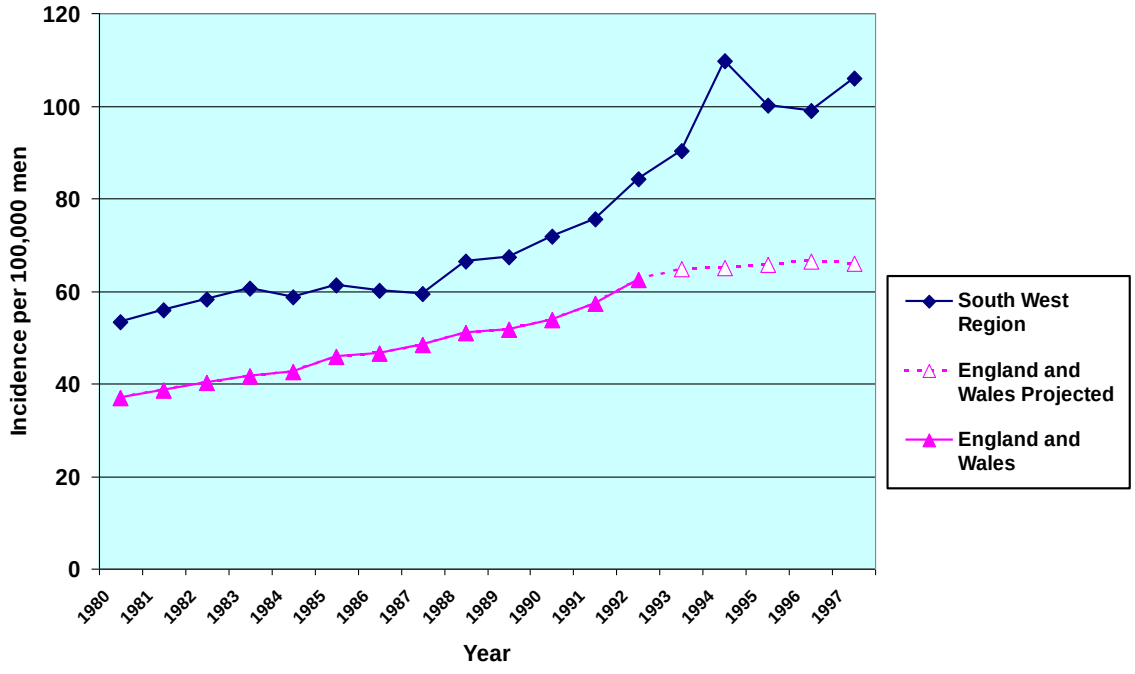
Data in the UK are incomplete from 1992 onwards but the South West region has complete data up to 1997. There has been a rapid rise in incidence of prostate cancer since the late eighties and there appears to be a plateau from 1994 onwards (Figure 2)[ONS, 1998]. There is a marked difference in the incidence rates in the South

Figure 1: Age group incidence for prostate cancer in England & Wales for 1980 & 1992.



West compared with the whole of England and Wales: this may be partly due to this region having 2% more men over 70 years of age than England and Wales as a whole [personal communication from Alistair Harvey].

Figure 2: Incidence rates for prostate cancer (1980-1997).



This rapid increase in incidence is related to increased detection of the disease resulting from the widespread availability of prostate specific antigen (PSA) tests on serum combined with transrectal ultrasound (TRUS) and needle core biopsy. There has also been increasing use of transurethral resection of the prostate (TURP) in which there is an incidental finding of tumour in approximately 10% of cases [Post *et al*, 1998 & 1999]. As a result more men are diagnosed with prostate cancer at an earlier stage and this leads to an apparent improved survival (lead-time bias). A European study looking at survival between 1985-1989 showed that England does not perform well in comparison with its neighbours having a 45% 5 year relative survival compared with 72% in Switzerland [Post *et al* 1998]. These authors point out that the role of early diagnosis, diagnosis of insignificant tumours and the variation in treatment is difficult to disentangle but they conclude that there had been only a modest improvement in survival since 1978. Although this is a recent study, the data period is before widespread use of PSA testing and radical prostatectomy in England. Unfortunately the lack of complete data from 1993 onwards makes future analysis impossible.

The aetiology of prostate cancer is uncertain but it likely to be multifactorial involving environmental and genetic factors. Studies from the United States have shown that the incidence and mortality rates in African-American men are twice those of Caucasian men, and African-American men present earlier in life with higher grade cancer [Selly *et al*, 1996]. Other risk factors shown to be important include family history of prostate cancer, occupational exposure (eg cadmium) and high dietary fat intake. The sex hormones are linked to prostate cancer, though the evidence remains inconclusive. Castration and high levels of oestrogen may be

protective factors. There have been conflicting results when other factors such as sexually transmitted diseases, viruses, and vasectomy, have been examined. Overall there appears to be a complex interplay between age, endogenous hormones, genetic factors and environmental influences [Selly *et al*, 1996].

Diagnosis of prostate cancer

The diagnosis of prostate cancer relies on sequential use of digital rectal examination (DRE) and PSA testing with suspicious results leading on to TRUS and needle core biopsy.

DRE is a rapid test with no significant complications. Reported sensitivity of DRE alone ranges from 44% to 97% whilst specificity ranges from 22% to 96%, and the positive predictive value (ppv) ranges from 13% to 69%. The variation is probably as a result of variations in case selection and diagnostic criteria [Selly *et al*,1996]. The combination of PSA levels and DRE increases the sensitivity by approximately 25% when the PSA is greater than 4 ng/ml.

PSA is a 34kD serine protease produced almost exclusively by prostatic epithelium and is thought to liquefy seminal coagulum that is formed at ejaculation. There are numerous assays available and the normal range is between 0 and 4 ng/ml. There are men with values above 4 ng/ml who do not have prostate cancer and the converse is true [Selly *et al*, 1996]. Various factors are known to elevate serum PSA: these include nodular prostatic hypertrophy, prostatitis and diagnostic examinations. PSA sensitivity ranges from 57-99%, with a specificity of 59-97%; again the variations in the studies are probably due to the factors identified for DRE above. Gillatt *et al* [1995] assessed the capacity of PSA to discriminate between patients with localised

prostate cancer and controls. They found that with a cut-off of 4 ng/ml the sensitivity was 99% with a specificity of 87%. Increasing the cut-off to 8 ng/ml improved the specificity to 97% but the sensitivity was reduced to 94%.

Several methods have been developed to try to improve the specificity of PSA, though most remain experimental. These include age-specific reference ranges, PSA density, PSA velocity and the measurement of free and complex PSA. Age-specific reference ranges are based on the theory that as men age their prostates enlarge and may be subject to low-grade chronic prostatitis, both led to an increasing PSA level. By correcting the PSA cut-off for age this can be corrected for but unfortunately studies have reported conflicting results. PSA density (or PSA index) relies on malignant tissue producing more PSA per gram than benign tissue. The PSA density is calculated by dividing the serum PSA by the gland volume in millilitres. The volume of the prostate is calculated using TRUS. Again there has been conflicting evidence on its usefulness due to inaccuracy of TRUS at measuring the true volume of the prostate. PSA velocity is determined from measurement of PSA at two consecutive points, normally one year apart. Increasing rates of 0.75 ng/ml or greater per year have been used as a cut-off and in one study there was a specificity of 90% compared with 60% for PSA alone [Carter *et al*, 1992]. A major drawback is the requirement for at least two PSA tests before the velocity can be measured but it may prove to be a useful improvement. PSA is known to occur in the serum in two different molecular forms • free and bound to $alpha_1$-antichymotrypsin. The bound form is the predominant form and the proportion bound is increased in prostate cancer. It is hoped that raised PSA due to nodular hyperplasia (a normal ratio) can

be separated from cancer (increased bound:free ratio). Assay techniques are still being developed but the results appear promising.

Transrectal ultrasound is used for a variety of functions including estimating the size of the prostate, diagnosing prostate cancer, guiding needle biopsies, staging the cancers detected and monitoring disease before and after therapy. 95% of prostate cancers are hypoechoic but up to half of the hypoechoic areas can be benign. There are also a proportion of tumours that are isoechoic or hyperechoic making accurate detection impossible. The sensitivities, specificities and ppvs for TRUS alone vary from 38% to 98%, 30% to 94%, and 9 to 59% respectively [Selly *et al*,1996]. The ppv increases when it is combined with DRE and PSA.

Needle core biopsy still remains the diagnostic 'gold standard'. The implementation of spring loaded automatic biopsy guns using 18 gauge needles has largely superseded aspiration cytology of the prostate. The biopsy can be guided digitally or using TRUS. Studies have shown that TRUS is superior to digital guidance but the limitations of TRUS, as discussed above, mean that in a prostate without hypoechoic areas random or systematic biopsies are needed. Systematic biopsies are taken from particular regions of the prostate and have been shown to detect smaller volume cancers which may be missed by TRUS guided biopsy. The numbers of cores and the parts of the prostate that are sampled are also important in detecting tumour. Ravery *et al* [2000] used an extensive biopsy protocol in which at least 10 cores were taken and found an overall 6.6% improvement in detection of prostate cancer in comparison to a standard sextant biopsy protocol. Peller *et al* [1995] showed a correlation among the number of positive sextant biopsies with preoperative PSA,

tumour volume, pathological stage, Gleason score, seminal vesicle involvement and capsular penetration.

Methods for clinical staging of prostate cancer

When the diagnosis of prostate cancer has been made the extent of the tumour spread needs to be assessed, as surgery will only cure localized disease. DRE is the most commonly used method but it can only reach the posterior aspect of the prostate. TRUS is better than DRE at identifying capsular breach and seminal vesicle involvement. Also the volume of tumour on TRUS has been shown to be related to the presence of metastatic disease but, as discussed earlier, the accuracy of volume measurements is questionable.

Magnetic resonance imaging (MRI) can look at the prostate in 3 dimensions and early studies were promising but more recent reports were conflicting. One study reported a sensitivity of 72%, specificity of 84% and accuracy of 78% in differentiating localized from extracapsular disease [Bezzi *et al*, 1988]. As this technology progresses there is promise for the future but in Bristol it is not used for routine staging.

Computer tomography (CT) is used to delineate the extent of tumour spread and identify local pelvic metastases. It can accurately display the gland shape and size, this information is essential for radiotherapy if that treatment is chosen. CT has a relatively high false positive rate at predicting lymph node metastasis and upstaging has been estimated to occur in approximately 40 to 50% of cases [Selly *et al*, 1996]. The majority of patients in Bristol will have a CT scan before either surgery or

radiotherapy is planned especially those who have high serum PSA or a high Gleason score tumour.

The bone is the commonest site for distant metastases in prostate cancer and radionuclide bone scans are the primary method for checking for these. This technique is accurate but expensive and Oesterling *et al* [1993] suggested that for patients with a serum PSA below 20ng/ml and no skeletal symptoms, staging by radionucleotide bone scans does not appear necessary as only 0.6% were found to have metastases. In patients with a serum PSA below 10ng/ml a staging bone scan does not appear necessary and this policy has been adopted in Bristol since the mid 1990's.

The evaluation of pelvic lymph nodes for metastasis prior to surgery is important as those with positive nodes have a markedly worse prognosis compared with those with negative nodes. The risks and costs of this procedure mean that it is rarely undertaken routinely in Bristol, but frozen section analysis of these nodes prior to radical prostatectomy is becoming common practice. Laproscopic lymph node dissection is now an option and this is being increasingly used in Bristol.

Treatment of prostate cancer

Once a diagnosis of localised prostate cancer has been made, the three main treatments are radical prostatectomy, radiotherapy and conservative management. Hormonal therapy is used when there is advanced or metastatic disease. Neoadjuvant hormonal therapy or radiotherapy have been used with radical prostatectomy but they have not shown any survival advantage over surgery alone in

localised prostate cancer [Banerjee *et al*, 2000]. Whether radical prostatectomy or radiotherapy or watchful waiting is the best treatment is unknown due to the lack of randomised controlled trials. Most studies have been observational in design and there has been bias, for example in patient selection (patients considered for surgery would have less co-morbidities than those receiving radiotherapy or conservative treatment). Other problems include the ability of radical prostatectomy to allow pathological staging of the disease whereas the other treatments rely on clinical staging, which may be very inaccurate. These problems are being addressed with the start of the ProtecT study in the UK. This study aims to compare the three therapies in a randomised way in a non-symptomatic population screened with PSA tests. The data from this study and others may provide the answer to which therapy is ideal, though the results from the ProtecT trial will not be known for many years.

The improvement in surgical techniques has led to an increase in the number of radical prostatectomies performed. There are two major approaches • retropubic and perineal. There are also nerve-sparing techniques which aim to preserve potency. The postoperative mortality rate ranges from 0.2 to 1.2%. The commonest long-term complications are incontinence (34% mild, 32% requiring pads) and loss of sexual function (89%) [Selly *et al*, 1996].

Radical prostatectomy aims to cure organ-confined prostate cancer and in the majority of cases it appears to achieve this [Walsh *et al*, 1994]. Disease relapse during the first ten postoperative years is observed in 30-56% of men with clinically-localised prostate cancer who have undergone radical prostatectomy, most within 36 months of surgery [Partin *et al,* 1993, Trapasso *et al,* 1994, Dillioglugil *et al*, 1997].

This treatment failure may be defined biochemically as a detectable serum PSA or as local or distant clinical disease recurrence. Biochemical relapse is considered to precede clinical failure by up to 48 months [Frazier *et al*, 1993]. Such disease relapse may be a result of incomplete local cancer excision or development of occult micrometastases. Partin *et al* [1993] studied 955 men undergoing radical prostatectomy, noting a rising PSA alone in 58% of those who relapsed during follow-up; the remainder had clinically detectable recurrences. Patients who relapse are treated with radiotherapy or hormonal therapy [Fichtner, 2000 and Moul, 2000].

There are no strict selection criteria of patients undergoing radical prostatectomy in Bristol and each patient is assessed as an individual case. The patient is counselled as to the complications and chances of recurrence, with the final decision being left to the patient. In the late 1980's and early 1990's patients with clinically localised disease and a life expectancy of at least 10 years would be offered a radical prostatectomy. Following studies in the mid 1990's the preoperative serum PSA level and Gleason score were also considered, as patients with either high Gleason scores or PSA levels above 20 ng/ml had been shown to have a high recurrence rate [Partin *et* al, 1993]. In Bristol these groups of patients would be carefully staged using bone scans and either CT scans or MRI to confirm that the disease was localised and advised of the high risk of recurrence with the final decision being the patient's choice.

Radiotherapy is being increasingly used in localised disease as well as more extensive disease. The commonest technique uses external beam radiation. Complications are related to field size and acute complications include cystitis,

diarrhoea, and proctitis, whilst long-term complications include urethral stricture and impotence [Selly *et al*, 1996]. Radiotherapy has the best curative rate in patients with localised, small volume, low-grade tumours (as in radical prostatectomy).

There is no convincing evidence that hormonal therapy produces a survival advantage in localised prostate cancer. In symptomatic disease there is an 80% chance of reduction of either bone pain or PSA or both with some form of hormonal manipulation. The options are either surgical castration, or luteinising hormone releasing hormone analogues (e.g. goserelin), or oestrogen therapy (e.g. stilboestrol), or anti-androgens (e.g. cyproterone acetate) [reviewed by Moffat, 1999]. Further studies of the use of these therapies in localised disease are needed.

Conservative management (watchful waiting) has been traditionally the treatment option in asymptomatic patients. It is not possible to predict which tumours will progress from localised to extensive disease. Some patients with localised disease would benefit from radical therapy, whilst others would not as their disease would not have progressed. The capacity to predict which tumours will behave badly is the 'Holy Grail' of prostate cancer pathology and has led to a large number of studies examining both clinical and pathological biomarkers.

Predictors of outcome

Clinicopathological markers

Stage, grade and serum PSA.

In order to compare studies the stage of prostate cancer has been classified. The system widely used is the TNM system, which was revised in 1992 (Figure 3), though some studies use the American system (modified Whitmore-Jewett). The TNM system was further revised in 1997 with slight changes to the categories (Table 1).

Figure 3: Prostate cancer staging using the TNM system, 1992 revision. (Source: Bostwick *et al*, 1997)

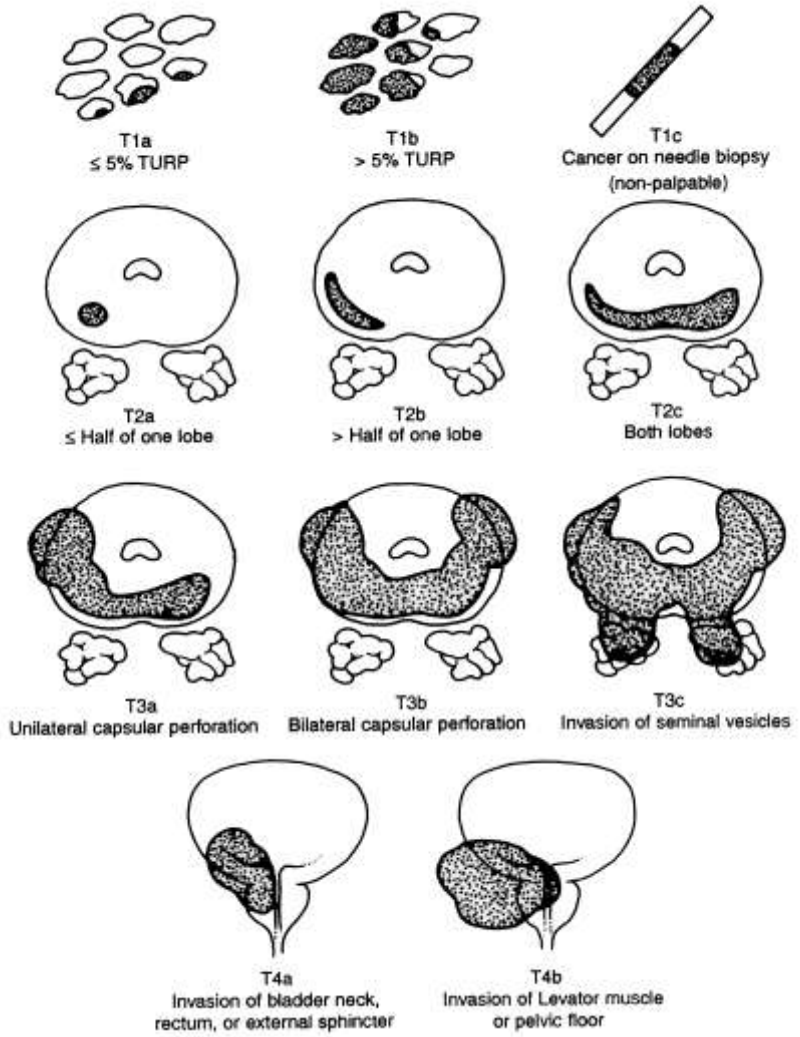


Table 1: Prostate cancer staging using the TNM system, 1997 revision. [Sobin and Wittekind, 1997]

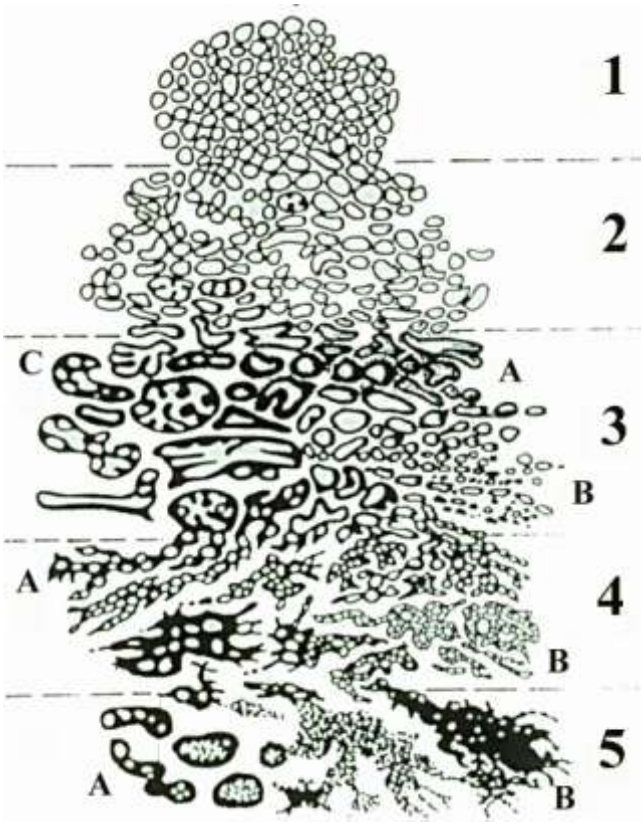
T1		Not palpable or visible
	T1a	≤ 5%
	T1b	> 5%
	T1c	Needle biopsy
T2		Confined within prostate
	T2a	One lobe
	T2b	Both lobes
T3		Through prostatic capsule
	T3a	Extracapsular
	T3b	Seminal vesicle(s)
T4		Fixed or invades adjacent structures: bladder neck, external sphincter, rectum, levator muscles, pelvic wall.
N1		Regional lymph nodes
M1a		Non-regional lymph nodes
M1b		Bone(s)
M1c		Other site(s)

The tumours are staged clinically based on the TRUS, DRE and any evidence of metastatic disease. The tumours are staged pathologically when the radical prostatectomy specimen is examined. The clinical stage is prefixed by a 'c' whilst the pathological stage is prefixed by a 'p'. The widespread use of PSA has led to an increase in cT1c tumours detected, and as there is no pathological equivalent to this these tumours are artificially 'upstaged' to T2 or even T3 after radical prostatectomy. The capacity to predict prostatic capsular invasion clinically is poor as a result a high percentage of tumours will be pT3 when the prostatectomy specimen is examined when the tumour was thought to be cT2.

Prostatic adenocarcinomas have been graded using a variety of systems. The most commonly used system is the Gleason grading system which is based on data from a prospective study of nearly 5000 patients between 1960 and 1975 [Gleason *et al*

1973, and Gleason 1992]. Nine histological patterns were identified and survival data were correlated to each pattern. Some of the patterns showed a similar biological phenotype and were grouped together so that there were three sets of related patterns and two distinct patterns arranged into five grades. These five grades were numbered in increasing biological malignancy. Gleason found that nearly half of the tumours had more than one grade. Patients with two different grades had a mortality rate between those two groups of patients who had pure tumours of those two grades rather than the mortality rate of the worst grade. This led to the combination of two grades to give a score [Gleason 1992]. The score is expressed as the commonest grade + the second commonest grade = score (e.g. 3+4 = 7). If there is only one pattern then the grade is doubled, i.e. 3+3 = 6. A standardised drawing for the grading system allowed pathologists to use the system independently (Figure 4) and also decreased inter- and intra-observer errors.

Figure 4: Prostatic adenocarcinoma – standardised drawing for the Gleason grading system [adapted from Gleason, 1992].



The Gleason grading system makes no allowance for nuclear cytological grade whereas the World Health Organization or Mostofi system has four histological growth patterns (rated 1 to 4) and three degrees of nuclear anaplasia (rated 1 to 3). The rating of each of these categories are added and scores 2-3 correspond to grade I, 4-5 grade II, and 6-7 grade III [Mostofi 1975]. A study of interobserver reproducibility of five different grading systems, including the Gleason system and the Mostofi system, found that none had a high degree of reproducibility (kappa coefficient >0.7). The worst agreement was in the Mostofi system as assessment of nuclear grade proved difficult especially when the tissues were suboptimally fixed [ten Kate *et al*, 1986]. Despite these problems in reproducibility the collective experience supports the clinical use of grading prostatic adenocarcinoma [Bostwick and Dundore, 1997].

As with the clinical and pathological stage there is also variation between the Gleason score of the needle core biopsy and the tumour in the radical prostatectomy specimen. Mills *et al* [1986] examined this in 53 biopsies and found identical Gleason scores in 51% of cases; in 4% the biopsy score was higher and in 45% the radical prostatectomy score was higher. Most of the disagreement occurred in low score tumours and when there was little tumour in the biopsy. Mills *et al* recommended a repeat biopsy in cases of small tumour volume if the clinical management was dependent on an accurate grade. Bostwick *et al* [1994] recommended Gleason scoring of all needle core biopsies and most UK centres have now adopted this.

The pretreatment PSA, Gleason score and clinical stage have all been used to predict the pathological stage as well as biochemical recurrence after radical prostatectomy. The ability to predict the pathological stage is important as approximately 60% of men newly diagnosed with prostate cancer are believed to have organ-confined disease but fewer than half of these men actually have organ confined disease when the radical prostatectomy is examined histologically [Partin *et al*,1993]. A multi-institutional study of 4133 men with clinically localised cancer led to the calculation of a nomogram which predicts the pathological stage based on the biopsy Gleason score, the clinical stage and the pretreatment PSA [Partin *et al*,1997]. Partin *et al* [1995] and Bauer *et al* [1996a] developed biostatistical model equations to give relative risk of recurrence based on PSA, postoperative Gleason score, organ confinement and, in Bauer's model, race [Moul, 2000]. The major drawback of these equations is their reliance on postoperative data, which restricts their usefulness in determining which patients may benefit from early adjuvant therapy.

Morphological variants – ductal carcinoma

Most prostatic adenocarcinomas have a microacinar pattern but there are many rarer variants which have specific histological appearances. The recognition of these variants permits some degree of prognostication. Variants include ductal carcinoma, small cell carcinoma, sarcomatoid carcinoma, mucinous adenocarcinoma, signet ring cell carcinoma, and lymphoepithelioma-like carcinoma [Randolph *et al*, 1997].

Since the description of ductal carcinoma of the prostate over thirty years ago by Melicow and Pachter [1967] its exact histiogenesis and behaviour has been debated. Owing to the tumour's immunoreactivity with antibodies to prostatic acid

phosphatase (PrAP) and prostate specific antigen (PSA) its origin is now widely accepted as being prostatic rather than from Müllerian remnants as originally described by Melicow. There is also electron microscopical evidence of prostatic origin as the tumour cell has a prominent Golgi apparatus [Young *et al*, 1973]. The original terminology of endometrial carcinoma of the prostate, which reflected both the histological appearance and its believed origin, has been felt to be inappropriate by many authors and has been replaced by ductal carcinoma with endometrioid features [Young *et al*, 1973]. Histologically there are two patterns, type A (papillary) or type B (cribriform) [Randolph *et al*, 1997]. The papillary pattern is characterised by papillary fronds lined by high grade tumour cells, ranging from a single to stratified, tall columnar epithelium. This type is seen in suburethral/urethral locations, in which there is relatively more room to grow than in the parenchyma. Type B is more heterogeneous and is characterised by intraductal papillary, solid or complex glandular growth. The two patterns often coexist and merge. Differentiation between cribriform microacinar carcinoma and cribriform ductal carcinoma is difficult and for study 1 only cribriform tumours which had at least 50% papillary areas, were considered as ductal tumours (see Figure 5).

Not only are the terminology and origin of this tumour controversial but so is the best form of treatment. In the early 1970s oestrogen therapy was felt to be inappropriate owing to the proposed origin from Müllerian remnants and its resemblance to endometrial carcinoma of the uterus. Only after a patient with ductal carcinoma underwent orchiectomy and showed tumour regression was treatment with oestrogen accepted [Young *et al*, 1973]. The reported prognosis of this tumour

is varied: in some reports the long-term survival was good whilst in others it was poor [Young *et al*, 1973, Vale *et al*, 1992, Millar *et al*, 1996].

Nodular prostatic hyperplasia (NPH) and microacinar carcinoma of the prostate are known to express androgen receptors (AR) which can be detected immunohistochemically [Miyamoto *et al*, 1993]. Oestrogen receptors (ER) have been shown to be present rarely in epithelial cells of hyperplastic prostates but not in prostatic microacinar carcinoma [Brolin *et al*, 1992]. Ductal carcinoma has been found similarly to lack oestrogen receptors [Lee, 1994] but the presence of AR has not been studied immunohistochemically.

Tumour biomarkers

p53

p53 is a tumour suppressor gene located on chromosome 17p13.1. The gene encodes a phosphoprotein made up of 393 amino acids which was discovered because it binds the virally-encoded large 'T' antigen which is responsible for the transformation of cells by simian virus 40. Antibodies to this large 'T' protein precipitated a 53kD protein which was originally thought to be the product of an oncogene. Further work showed that the transformed cells contained p53 mutations and normal p53 acted to control cellular proliferation [Steele *et al*, 1998].

p53 is now accepted as central to the cell response to stress due to hypoxia, DNA damage or heat. Activated p53 leads to cell cycle arrest through the activation of p21 products, a cyclin dependent kinase inhibitor, as well as DNA repair via the GADD45 (growth arrest and DNA damage). If the repair is successful then p53 activates a gene called mdm2, which binds to p53 and inactivates it. If repair is

unsuccessful then p53 activates the apoptosis-inducing gene bax and the cell dies [Cotran *et al*, 1999].

As p53 acts as a transcription factor most of the significant mutations in tumours occur in the DNA binding domain. The result is that the mutant p53 allows damaged cells to survive so they can continue to have further mutations in other oncogenes. Normal (wild type) p53 protein has a short half-life of between 5 and 45 minutes but p53 protein is seen to accumulate in many tumours. This may be due to two reasons: that the mutant protein is more stable and that the inability of mutant p53 to activate mdm-2 to bring about its own destruction.

Immunohistochemistry with the monoclonal antibody DO7 detects the p53 protein but cannot differentiate between wild type and mutant p53. It is now accepted that positive immunohistochemical staining provides indirect evidence of p53 mutation, though protein accumulation can occur in the absence of p53 mutation (for example when there are mutations in the mdm-2 gene) [Burton *et al*, 2000].

In prostate cancer there have been widely ranging reports of incidence of p53 expression immunohistochemically ranging from 4% to 79% [Voeller *et al*, 1994, Van Veldhuizen *et al*, 1993]. Most of the variations are attributed to methodological differences in tissue sampling, scoring and the antibody clone used. Although not unanimous, most reports have shown a correlation between p53 immunohistochemistry staining and disease progression [Theodorescu *et al*, 1997, Bauer *et al*, 1996, Byrne *et al*, 1997, Matsushima *et al*, 1997 and Stackhouse *et al*, 1999]. Cheng *et al*[1999] studied p53 expression in lymph node positive prostate

cancers and found concordance between p53 expression in the primary tumours and the metastases. On univariate analysis, expression of p53 in these metastases was associated with disease free survival but it was not significant on multivariate analysis. p53 immunohistochemistry on needle core biopsies has been shown to predict patient prognosis and this was further improved when it was combined with bcl-2 protein expression [Matsushima *et al*, 1997]. Stackhouse *et al* [1999] studied needle core and subsequent radical prostatectomies and found that p53 in the radical prostatectomies was a significant predictor of recurrence but this was not the case when the preoperative biopsy was used. This study concluded that the differences were attributable to the limited amount of tumour in the needle core biopsies which were not representative of the tumour in the radical prostatectomies.

bcl-2

bcl-2 was the first anti-apoptotic gene identified and is a member of a large family of homodimerising and heterodimerising proteins responsible for either inhibiting (as in the case of bcl-2 and bcl-xL) or promoting apoptosis (as in the case of bax, bad and bcl-xS). The bcl-2 gene is located on chromosome 18q21 and is involved in a translocation in 65% of follicular B cell lymphomas. The bcl-2 family of proteins regulate a group of proteolytic enzymes called the caspases which are involved in apotosis (Cotran *et al*, 1999). Overexpression of bcl-2 leads to a cell avoiding apoptosis, which in turn can result in the survival of mutated cell.

bcl-2 overexpression has been shown to be present in 20-26% of prostate cancers [Matsushima *et al*, 1997, Bubendorf *et al*, 1996, Bauer *et al*, 1996, Stackhouse *et al*, 1999]. bcl-2 expression has also been shown to occur in high grade prostatic intraepithelial neoplasia (PIN) suggesting that it may be associated with early

prostate tumorigenesis [Baltaci *et al*, 2000]. Matsushima *et al* [1997] studied 146 patients by needle core biopsy and found that expression of bcl-2 correlated with stage and was an independent prognostic marker. Bubendorf *et al* [1996] and Bauer *et al* [1996] on radical prostatectomy specimens showed that lack of bcl-2 expression correlated with a longer disease-free survival after radical prostatectomy. Stackhouse *et al* [1999] used radical prostatectomies and preoperative needle biopsies and found that though bcl-2 was a significant marker in the radical prostatectomy specimens this was not the case for the needle cores. This study concluded that the sampling of tumours by Needle core biopsies was insufficient to detect particular clones that were positive for bcl-2.

CD44

The CD44 gene is located on chromosome 11p13 which has been shown to produce a variety of related integral membrane glycoproteins by a process of mRNA splicing [Gao *et al*, 1997]. One of its roles is as an adhesion molecule expressed on T lymphocytes, which use CD44 to bind to a hyaluronate on high endothelial venules in lymphoid tissues. The standard form of CD44 (CD44s) is expressed on most cell types and is the shortest CD44 protein. CD44s is derived from the splicing of exons 1-5 of the CD44 gene to exons 16-20. Exons 6-15 are termed variable exons, v1-v10, and are absent in the standard form. Alternate splicing of these 10 exons leads to CD44 variants (CD44v). These exons are in the extracellular region of the molecule [Verkaik *et al*, 1999]. Post-translational modifications can further alter these products leading to a polymorphic family of trans-membrane proteins [Tarin, 1997].

Tumour cells metastasize by circulating in the blood and adhering to endothelium at a distant site. Gunthert *et al* [1991] used DNA splicing to overexpress a particular part of the CD44 gene and this led to a rat tumour clone expressing CD44v6 and gaining metastatic potential. Studies of the CD44 family in neoplasia have shown that it is not necessarily a defective expression of a particular exon but an overall disorganised pattern of expression. Overexpression of CD44s has been linked to increased aggressiveness in many tumours including lymphoma and colon and rectal adenocarcinoma: in contrast, decreased expression of CD44 has been found in bladder carcinoma, endometrial carcinoma and adenocarcinoma of the lung [De Marzo *et al*, 1998].

In prostate cancer, most studies have shown a decreased expression of CD44s [Noordzij *et al*, 1997, De Marzo *et al*, 1998]. Lokeshwar *et al* [1995] studied CD44 expression in two prostate carcinoma cell lines and showed a high level of CD44 expression: normal controls had a low level of expression. Nagabhushan *et al* [1996] looked at CD44s expression in whole prostates and in lymph node metastases and found 60% of primary prostatic tumour expressed CD44s moderately to strongly whilst none of the metastases did. They also found a correlation with histological grade, as have other authors [Kallukury *et al*, 1996]. Noordzij *et al* [1997] found it to be unrelated to grade and stage. These studies suggest that loss of CD44 expression may be an indicator of poor prognosis in prostatic carcinoma but there have been no studies examining CD44s expression in preoperative biopsies.

E-cadherin

The CDH1 gene lies on chromosome 16q22.1 and encodes the 120kDa E-cadherin protein. E-cadherin acts as a calcium dependent intercellular adhesion molecule present at the zonulae adherentes of epithelial cells. It has an important role in establishing and maintaining intercellular connections and morphogenesis. The normal function of E-cadherin is dependent on its linkage to a group of molecules called catenins. E-cadherin binds directly to either β-catenin or γ-catenin, whereas α-catenin links the bound E-cadherin complex to the actin cytoskeleton [Richmond *et al*, 1997]. Downregulation of E-cadherin has been shown to be inversely correlated with differentiation and advanced stage in many tumours including bladder, breast, cervical, colorectal, endometrial and lung tumours [Jian *et al*, 1997]. In some tumours E-cadherin is structurally normal but its expression is reduced owing to abnormal catenins.

In prostate cancer, studies using immunohistochemistry on frozen sections showed a decreased expression was correlated with an increasing Gleason score, advanced clinical stage and poor clinical outcome [Umbas *et al*, 1994]. A further study by Cheng *et al* [1996] used paraffin wax embedded tissue and found decreased expression in metastatic deposits in comparison to the primary tumours. They also criticised the previous paper by Umbas *et al* [1994] on several points including the exclusion of 32% of the patients on the basis of heterogeneous staining pattern in the prostates. Cheng *et al* felt that heterogeneous staining was part of the spectrum between normal and abnormal staining as well as due to the multifocal nature of prostate cancer. Umbas *et al* had showed a significant survival difference between

patients with a normal and a decreased E-cadherin expression but as Cheng *et al* point out this was based on only 3 years follow-up. A subsequent study examining TURP specimens showed a significantly lower survival rate in patients with abnormal E-cadherin expression on univariate analysis but on multivariate analysis it was independent of Gleason score but not of tumour metastasis [Richmond *et al*, 1997].

De Marzo *et al* [1999] studied E-cadherin in radical prostatectomies using paraffin wax embedded tissue and found that it was an independent predictor of high stage disease but in the 10 patients with metastases in lymph nodes these deposits appeared to express E-cadherin at least moderately. This study concluded that it must be only a transient loss of E-cadherin in the primary tumours, which would mean that it was not due to a genetic mutation. Ruijter *et al* [1997 & 1998] addressed the findings of heterogeneous staining for E-cadherin and found that fixation of radical prostatectomy specimens could affect E-cadherin staining but there was also heterogeneity within the tumours even when fixation was optimal. Because of this heterogeneity and the fact that needle core biopsies were small and subject to sampling effects, Ruijter *et al* [1998] felt that E-cadherin in core biopsies would not be useful.

Her-2/neu

Her-2/neu oncogene is located at chromosome 17q21 and encodes one of the epithelial growth factor receptors (HER 2) on the cell membrane. Interest in this comes as a result of its overexpression in 25-30% of breast cancers, which when present is associated with a poor prognosis in node positive cases [Wang *et al*, 2000].

Early studies in prostatic adenocarcinoma using immunohistochemistry showed a varying degree of overexpression of Her-2/neu gene product from 29% by Ross *et al* [1993] to 80% by Zhau *et al* [1992]. Another study found it expressed in 100% of tumours and in 20% of prostatic hyperplasias [Gu *et al*, 1996]. The advent of fluorescent in situ hybridisation (FISH) and commercially available DNA probes has enabled the direct visualisation of amplified oncogenes in archival material. In breast cancer 90% of the cases of overexpression of the protein have been attributed to gene amplification, though a more recent study suggested a lower incidence [Wang *et al*, 2000]. Assessment of gene amplification using differential polymerase chain reaction analysis in 53 cases of prostatic adenocarcinoma showed no amplification [Kuhn *et al*, 1993], but using FISH Ross *et al* showed that 41% have amplification of the gene and this also correlated with tumour recurrence [Ross *et al*, 1997a]. A subsequent study using a different commercial probe (Vysis) has shown a lower incidence of amplification of 9.3% [Mark *et al*, 1999]. The largest study of prostate cancers and Her-2/neu amplification used a combination of FISH using the Vysis probe and microarrays [Bubendorf *et al*, 1999]. This technique permitted 262 separate tumours to be successfully assessed by taking small samples (0.6 mm in diameter) and mounting them on a single slide, which was then used for FISH. Microarrays have a great advantage in that they can screen large numbers of tumours for gene amplifications but there can be sampling error, as most tumours are heterogeneous. Bubendorf *et al* addressed this by using tumours from different stages in the disease from localised to metastatic. They found Her-2/neu was not amplified at any stage of the disease.

The advent of a monoclonal antibody to the Her-2/neu protein, Herceptin (trastuzumab), and its use as an adjuvant therapeutic agent has brought even greater interest in Her-2/neu oncogene. Recent studies in human prostate xenograft models showed a response to Herceptin in androgen dependent tumours [Agus *et al*, 1999] but not androgen independent tumours.

AIMS

Study 1: ductal carcinomas of the prostate.

The aim of this study is to investigate the expression of PSA, PrAP, AR and ER in a group of ductal carcinomas of the prostate. Expression of Ki67 and p53 would also give an insight into the prognosis of these tumours.

Study 2: preoperative p53, bcl-2, E-cadherin and CD44 immunohistochemistry as predictors of biochemical recurrence following radical prostatectomy.

The aims of this study are to examine clinicopathological parameters (age, serum PSA, Gleason score, clinical and pathological stage) and tumour biomarkers in both preoperative biopsies and radical prostatectomies to determine if any are significant predictors of biochemical recurrence after radical prostatectomy.

Study 3: postoperative p53 and bcl-2 as predictors of biochemical recurrence following radical prostatectomy.

This study is an extension of study 2 with a larger series of radical prostatectomy specimens. The number of clinicopathological parameters examined was expanded (age, serum PSA, Gleason score, seminal vesicle invasion, margin status, and pathological stage).

Study 4: amplification of Her-2/neu in prostate cancer.

The aim of this study is to examine the amplification of Her-2/neu in the largest series to date, using a modification of the FISH method in which enzymatic techniques rather than fluorescent detection are used.

Materials and methods

Clinical data

Ductal carcinoma of the prostate

Twelve cases of ductal carcinoma of the prostate were identified from the pathology files of Southmead Hospital, Bristol between 1982 and 1996. Clinical information was obtained from the clinical case notes and death certificates. Eleven of the specimens were from transurethral resections of the prostate (TURP) and one was from a retropubic prostatectomy. A representative area of tumour was selected for immunohistochemistry.

Radical prostatectomies

Radical prostatectomy was performed on 320 patients at Southmead Hospital or nearby Weston General Hospital between 1987 and 1999 by a single urological surgeon (Mr D Gillatt). 269 patients had follow-up of greater than 12 months. 75 of these patients underwent either TURP or needle core biopsy at Southmead and subsequent radical prostatectomy were studied in study 2. There was insufficient tumour in four of the radical prostatectomy specimens in study 2 for p53 and bcl-2 immunohistochemistry. The remaining 71 patients and a further 58 patients were included in study 3 (total number 129). The 58 patients were selected on the basis of available tissue blocks containing adequate tumour volumes for immunohistochemistry. Study 4 included 117 patients of these 129 and they were selected on the basis of having sufficient tumour remaining in the tissue blocks after study 3.

Biochemical recurrence was defined as at least two consecutive serum PSA elevations above 0.2 ng/ml with the second elevation measured a minimum of 6 months after the first.

All the needle cores and the radical prostatectomies were reviewed by a histopathologist (JDO) and the Gleason score and the pathological stage were recorded. The entire radical prostatectomy specimen was embedded in whole mounts from 1996 onwards and prior to 1996 standard cassettes were used. The apex and base margins were examined using a shave technique. Any breach of the capsule, regardless of the volume of tumour breaching the capsule, was recorded as positive capsular breach (stage pT3). The tumour was considered to have reached the surgical margin if the apex or base shave or the circumferential margin (whether it be intra or extraprostatic) contained tumour. If tumour reached an intraprostatic surgical margin then the tumour was considered as pT2 unless there was capsular breach elsewhere. An area with the highest Gleason grade was selected for studies 2, 3 and 4.

Immunohistochemistry

Tissues were fixed in 10% unbuffered formal saline, paraffin wax-embedded and processed routinely. Serial sections 3 μm thick were cut onto Vectabond treated slides (SP1800, Vector Laboratories Ltd., Peterborough, Cambridgeshire, U.K). For study 2 paired tissue sections from the preoperative biopsy and the radical prostatectomy were placed on the same microslide. The primary antibodies used in each study are shown in Table 2. A three-step immunoperoxidase method was used. Briefly, the sections were dewaxed and hydrated. Sections for PSA and PrAP were digested with trypsin for 10 minutes (Difco 1:250, 1% trypsin in 1% calcium chloride, pH 7.8 at 37^{o}C). Sections for the other markers were pressure-cooked for

105 secs in 1500 mls of 0.01 M sodium citrate buffer (pH 6.0). The slides were transferred to an automated immunostainer (Optimax, BioGenex, Menarini Diagnostics, Pentos House, Falcon Business Park, Ivanhoe Road, Finchhampstead, Reading, UK) for the following steps. After incubating with 3% aqueous hydrogen peroxide to block endogenous peroxidase, the sections were covered with normal goat serum (prediluted, HK112-9K, BioGenex) for 13 minutes to block non-specific binding sites. The slides were then incubated with the appropriate primary antibody (Table 2). In study 1, detection was carried out using the Super Sensitive immunodetection system (BioGenex) at 1/50 dilution. In the remaining studies detection was carried out with the Duet immunodetection system (K0492, Dako UK Ltd., High Wycombe, Berkshire, UK) at a dilution of 1/100 for an incubation time of 30 minutes. The enzyme activity was developed using liquid diaminobenezedine (HK153-5K, Biogenex) as per instructions, for 9 minutes. The sections were counterstained with haematoxylin, dehydrated and mounted. Negative controls for each case were performed by replacing the primary antibody with Optimax wash buffer (HK583-5K, BioGenex). In study 1 two medium and two high grade microacinar prostatic adenocarcinomas as well as ER positive breast carcinoma were used as positive controls. A high-grade prostatic carcinoma was used as a positive control for p53. Human tonsillar tissue was used as a positive control for bcl-2 and CD44. Normal gastric mucosa was used as a positive control for E-cadherin.

Table 2: Primary antibodies.

study	1° antibody	source	pretreatment	dilution	incubation (min)
1	p53	Dako (DO7)	pressure cooked	1/20	30
1	ER	Dako (M7047)	pressure cooked	1/25	60
1	AR	BioGenex (AM256/2M)	pressure cooked	1/30	30
1	Ki67	Dako (A047)	pressure cooked	1/150	30
1	PSA	Dako (M750)	trypsin	1/1000	30
1	PrAP	Dako (A027)	trypsin	1/400	30
2	CD44	Dako (DF1485)	pressure cooked	1/40	75
2	E-cadherin	R+D Systems (HECD-1)	pressure cooked	1/100	60
2,3	p53	Dako (DO7)	pressure cooked	1/60	30
2,3	bcl-2	Dako (124)	pressure cooked	1/40	120

Scoring immunohistochemistry

Study 1

The results of the immunostaining were evaluated by two histopathologists (JDO and AGM). PSA and PrAP were scored on intensity, 1+ weak, 2+ moderate, 3+ strong. p53, AR, Ki67, ER staining were scored by percentage of positive nuclei in 100 cells in an area of greatest staining intensity, 1-25% was considered as 1+, 26-50% as 2+, 51-75% as 3+, and 76-100% as 4+ . The mitoses per mm^2 of both the ductal and microacinar areas were assessed on a haematoxylin and eosin (H&E) slide. The microacinar carcinomas were graded using a Gleason sum score. The

histological pattern of the ductal component of the tumours was recorded as cribriform or papillary.

Studies 2 and 3.

Immunohistochemical staining was assessed by a single pathologist, blinded to treatment outcomes. One hundred consecutive malignant cells in the area of highest staining were counted. Cytoplasmic bcl-2 staining in malignant glands was considered positive. The same technique was used for scoring p53 but only nuclear staining was counted as positive. p53 and bcl-2 expression were considered positive regardless of the percentage of cells staining, as previously suggested [Bauer *et al*, 1996b]; absence of staining was considered negative. CD44 expression was scored in the areas of highest Gleason score by using a semi-quantitative method: negative = none of the malignant cells were staining; focal = less than 10% of cells staining; regional = 11 - 50% cells staining; diffuse = greater than 50% cells staining. CD44 staining was considered abnormal when negative or focally positive membranous expression was observed, or when stain was confined to cytoplasm, as previously suggested [Kallakury *et al*, 1996]. Membranous E-cadherin expression was scored in the areas of highest Gleason score as uniformly present, heterogeneous or uniformly absent. Staining was considered abnormal if the membranous expression was heterogeneous, uniformly absent or when stain was confined to cytoplasm, as previously suggested [Umbas *et al*, 1994].

In situ hybridisation

5 μm sections from the paraffin tissue blocks were mounted onto sialinised slides. The sections were dewaxed in xylene followed by two washes in 100% ethanol

before being air-dried. After this they were placed in the pretreatment solution of 30% sodium bisulphite (S9000, Sigma Aldrich Co. Ltd., Fancy Road, Poole, Dorset, UK) for 45 minutes at 45^{o}C. After this the slides were washed in 2x sodium chloride/sodium citrate (SSC) (0.3M sodium chloride (S-3014, Sigma Aldrich), 0.03M sodium citrate (S-4641, Sigma Aldrich) at pH 7.0). Protein digestion was then carried out by immersing the slides in proteinase K (P-2308, Sigma Aldrich) at 100mg/l for between 30 and 50 minutes at 45^{o}C. The digestion time was calculated based on the degree of propidium iodide uptake as per the manufacturer's instructions (propidium iodide antifade, S1370-6, Quantum Appligene Lifescreen, Salamander Quay West, Park Lane, Harefield, Middlesex, UK). Following digestion the slides were dehydrated via 70%, 90% and 100% ethanol and then air-dried. 8 μl of Her-2/neu digoxigenin labeled probe/hybridisation mix was added to the tissue (P5111-DG.5, Quantum Appligene Lifescreen). A 22 mm^{2} coverslip was then applied and rubber solution used to seal the coverslip. The slides were placed on a hotplate at 70^{o}C for 5 minutes in order to denature the probe and the tissue DNA. The slides were then incubated in a humidified chamber at 37^{o}C overnight. The following day the rubber solution was pealed off and the slides were soaked in 2x SSC so that the coverslip fell off. This was followed by a stringency wash in 2x SSC at pH 7.0 at 73^{o}C for 5 minutes. The slides were washed in Optimax wash buffer (HK583-5K, Biogenex) and 25μl of 1/20 anti-digoxigenin peroxidase (1207733, Roche Diagnostic Ltd, Bell Lane, Lewes, East Sussex, UK) was applied with a plastic coverslip and incubated for 4 hours at 37^{o}C in a humidified chamber. After two further washes in Optimax buffer the enzyme activity was developed using liquid diaminobenzidine (HK153-5K, Biogenex) and counterstained with haematoxylin. Stromal nuclei and non-neoplastic glands acted as internal controls.

Her-2/neu was considered to have a significant increase in the copy number if there were 5 or more signals per nucleus in greater than 20% of the nuclei [Ross *et al*,1999a]. A minimum of 100 nuclei were examined per case using a 100x objective and constant adjustment of the microscope focus. Any tumours that showed increased copy number of Her-2/neu underwent in situ hybridisation using the same technique on a serial section with a chromosome 17 alpha satellite probe (CP5040-DG.5, Quantum Appligene Lifescreen, mixed 1/20 with Hybridsol VI, S1370-30, Quantum Appligene Lifescreen). Scoring was the same as Her-2/neu, and a ratio of the percentage showing Her-2/neu amplification to the percentage showing chromosome 17 amplification was calculated. If this ratio was greater than 2 then the case was considered to be true amplification.

Statistical analysis

Studies 2 and 3

For study 2 complete data were available for the variables: age, PSA, clinical stage, pre-operative and post-operative Gleason scores and post-operative pathological stage. In study 2 the serum PSA was considered as a continuous variable during the statistical analysis. In study 3 the serum PSA was grouped - <11, 11-20 and >20. As further patients and fewer biomarkers were examined in study 3 so more clinicopathological data was included (margin status and seminal vesicle involvement). The preoperative serum PSA was included in both the preoperative and postoperative analyses, as previous authors had used it to predict biochemical recurrence after radical prostatectomy [Partin *et al*, 1995 and Bauer *et al*, 1996a].

To allow for patients with insufficient tumour in the needle core or radical prostatectomy to be included in the analyses, it was necessary to include indicators

for missing data in the regression models fitted. Thus all analyses included an adjustment for missing data (unless indicated otherwise). The outcome of interest was biochemical recurrence. Patients not experiencing a recurrence were censored at death or last follow-up. Cox proportional hazards regression was used to identify factors associated with biochemical recurrence. The models were built in stages using the modelling scheme suggested by Collett [1994]. Factors significant at the 10% level or less were retained [Collett, 1994]. Interactions among variables were examined, but none were found in study 2. Survival curves for the factors found to be associated with the outcome were produced using the Kaplan-Meier method. The proportional hazards assumption was assessed using the method described by Grambsch and Therneau [1994] and implemented in the statistical package Stata (Stata Statistical Software version 6. Stata Corporation, College Station. Texas).

Results

Study 1: ductal carcinomas of the prostate.

Clinical data

Twelve patients with ductal carcinoma were identified • six of the tumours had a pure papillary pattern whilst six had a mixed ductal and microacinar pattern. The patients' age range was 67 • 84 years at presentation. Eight of the patients presented with obstructive symptoms and four of these had haematuria (Table 3). Three of the patients had had previous TURP, two had prostatic hyperplasia and one microacinar carcinoma.

At cystoscopy, the tumour had a fronded appearance in four patients and in another four the urethra was narrowed by tumour. The serum prostatic acid phosphatase or prostate specific antigen data was available in 8 patients and it was elevated in all except one of these patients.

Microscopic

The ductal component of the tumour had two distinguishable sub-types: cribriform, in which the tumour appeared solid but had papillary areas at the edges amounting to at least half of the tumour (Figure 5a), and papillary, in which the tumour was uniformly papillary. Of the twelve tumours, seven were cribriform, the remainder were papillary. The nuclei of the ductal areas had prominent nucleoli (Figure 5b). The Gleason sum score of the microacinar areas in the mixed group ranged from 4 to 9. High mitotic counts (greater than 4 per mm^2) were present in the ductal component of eight patients. Only patient 11 had a high mitotic count in a microacinar area.

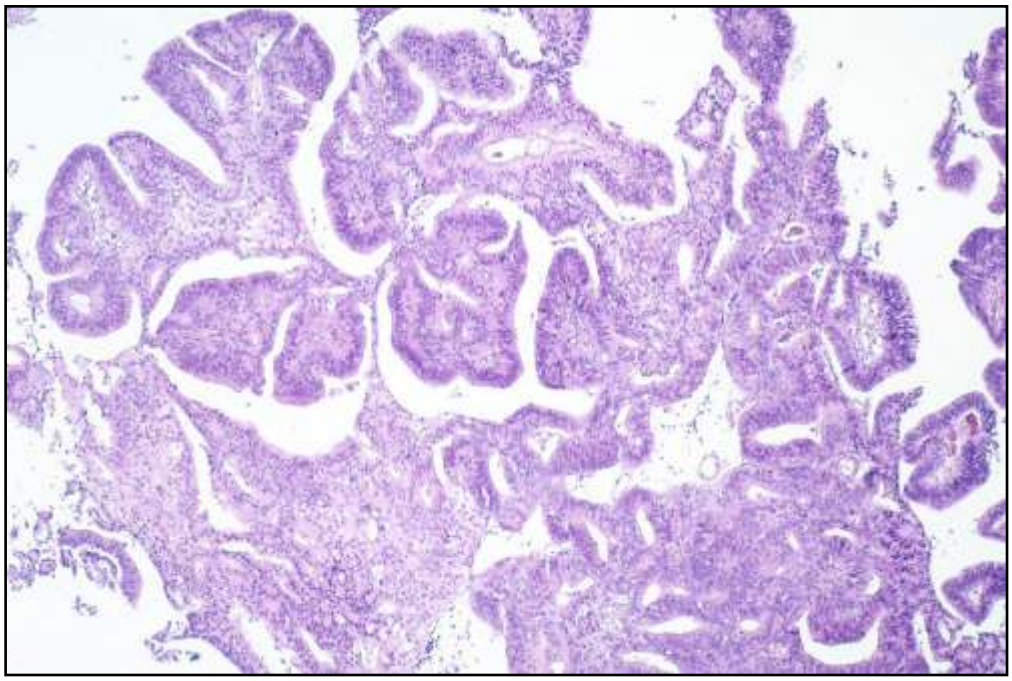
Table 3: ductal carcinoma of the prostate; clinical data.

Case	Age	Presenting symptoms	Urethral appearance	Serum PSA [a]	PrAP	Previous TURP	Therapy [b]	Skeletal at presentation	Metastasis at death	Outcome [c]
1	78	obstruction	fronded		180	no	O	yes	yes	DOD 4yr
2	75	obstruction	necrotic		86	benign	S, DXT	yes	yes	DOD 2yr
3	78	n/k [d]	n/k		n/k	n/k	n/k	n/k	n/k	n/k
4	73	obstruction &haematuria	narrowed		3	no	RP	no	n/k	A 13yr
5	73	n/k	n/k		n/k	n/k	n/k	n/k	yes	DOD 4yr
6	77	obstruction &haematuria	fronded	65		no	n/k	n/k	n/k	A 6mo
7	67	obstruction	narrowed		47	benign	O, C	yes	yes	DOD 2yr
8	80	n/k	friable		n/k	n/k	n/k	no	n/k	DWD 6yr
9	70	obstruction	narrowed		17	malig.	O, S	no	yes	DWD 8yr
10	74	obstruction &haematuria	fronded		14	no	C, DXT	no	yes	DOD 3yr
11	77	n/k	n/k		n/k	n/k	O	n/k	yes	DOD 1yr
12	84	obstruction &haematuria	fronded		102	no	C	yes	yes	DOD 1yr

[a] Normal values: PSA less than 4 ng/ml, PrAP between 2 and 10 U/L.
[b] C = Cyproterone acetate, O = orchiectomy, S = Stilboestrol, RP = retropubic prostatectomy.
[c] A = alive with disease, DOD = died of disease, DWD = died with disease.
[d] n/k = not known.

Figure 5: Prostatic ductal carcinoma showing

(a) cribriform and papillary areas (Haematoxylin and eosin, om x40)



(b) cribriform areas with prominent nucleoli, apoptosis and mitosis. (Haematoxylin and eosin, om x400)

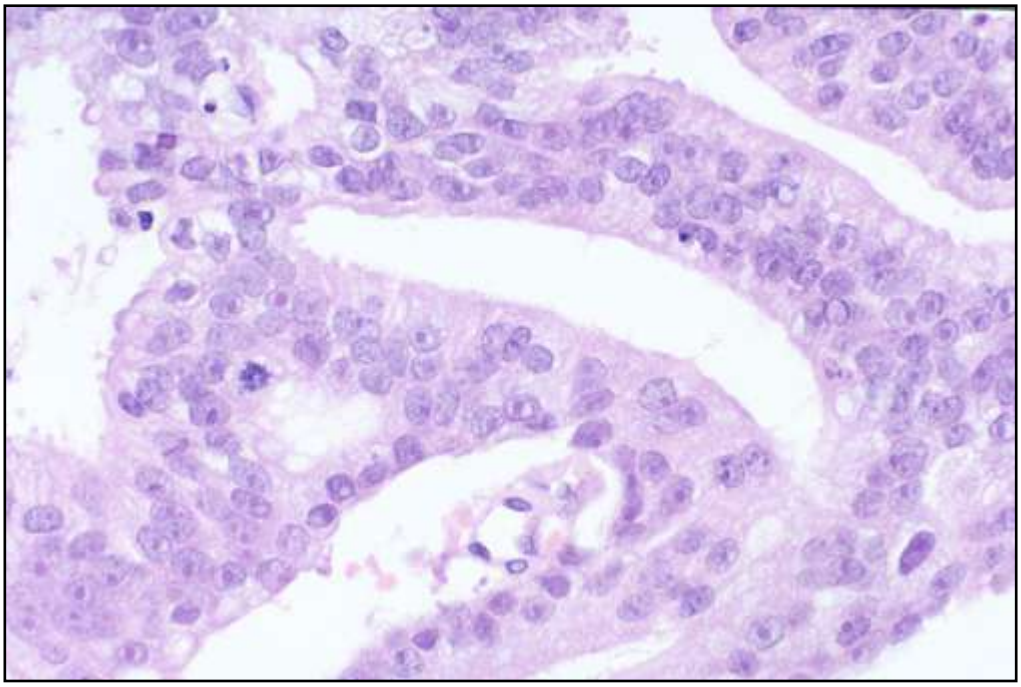
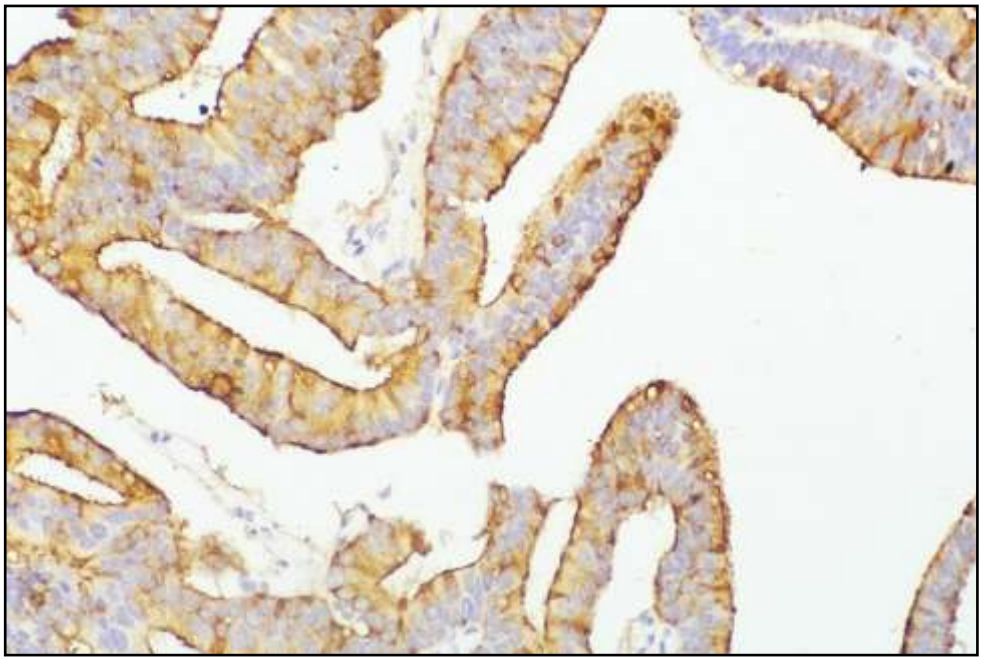
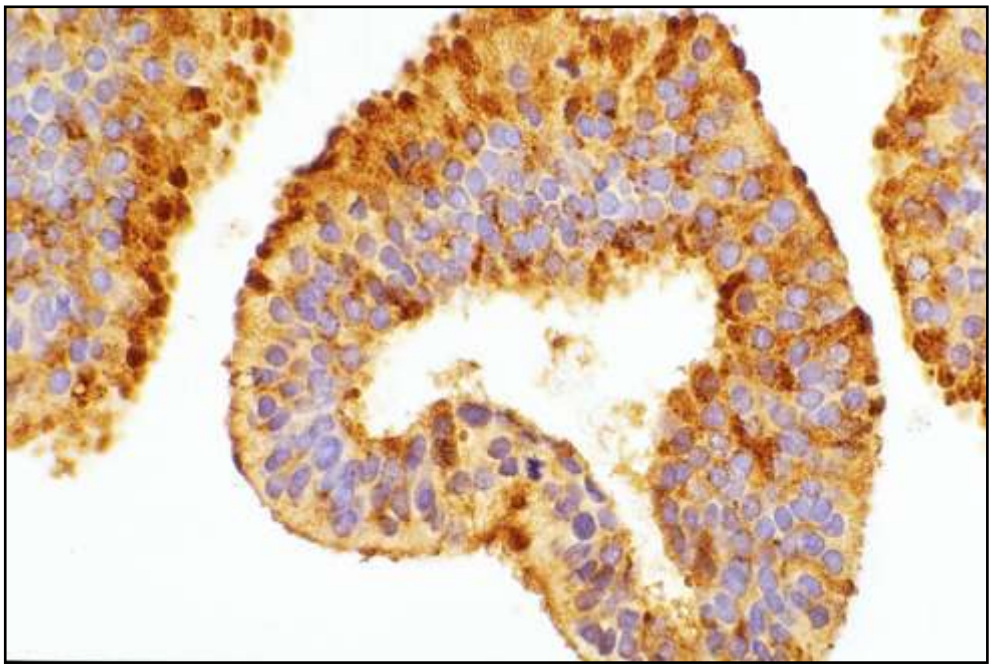


Figure 6: Ductal carcinoma of the prostate.
a) PSA immunoreactivity, showing strong luminal marking. (Immunoperoxidase, om x200)



b) PrAP immunoreactivity, showing strong luminal staining. (Immunoperoxidase, om x400)



Immunohistochemistry

The PSA and PrAP were positive in all areas of the tumours with intensity scores that were greater than or equal to 1+ (Figure 6a and b). There was no epithelial nuclear or cytoplasmic staining for ER nor was there any significant stromal staining. Three of the ductal tumours had 2+ nuclear staining with Ki67, whilst none of the microacinar areas had greater than 1+ (Tables 4 and 5). Two patients (3 and 11) had very strong nuclear staining with p53 (4+) in the ductal areas and four others had much weaker positivity (Figure 7). Ten of the ductal tumours had strong (4+) nuclear staining with AR, whilst patients 2 and 7 had 1+ and 2+ respectively (Figure 8a). The majority of the nucleoli of the cells were negative for AR (Figure 8b).

Table 4: Pure ductal carcinomas of the prostate, immunohistochemistry.

Patients	Ductal pattern	Mitotic count per mm^2	Ki67 [a]	p53 [a]	Androgen receptor [a]
1	**cribriform**	**0.5**	**1+**	**none**	**4+**
2	**cribriform**	**4.5**	**1+**	**none**	**1+**
3	**cribriform**	**4.5**	**1+**	**4+**	**4+**
4	**papillary**	**5**	**1+**	**none**	**4+**
5	**cribriform**	**4.5**	**2+**	**1+**	**4+**
6	**papillary**	**4.5**	**1+**	**1+**	**4+**

[a] Nuclear scoring: 1+ = 1 to 25% staining,
2+ = 26 to 50% staining,
3+ = 51 to 75% staining,
4+ = 76 to 100% staining.

Table 5: Mixed ductal and microacinar carcinomas of the prostate, immunohistochemistry.

Patient	Component	Gleason score	Ductal pattern	Mitotic count per mm^2	Ki67 [a]	p53 [a]	AR [a]
7	ductal		cribriform	5.5	1+	none	2+
	microacinar	2 + 2		0	none	none	1+
8	ductal		papillary	1	1+	none	4+
	microacinar	3 + 4		1	1+	1+	4+
9	ductal		papillary	5.5	1+	none	4+
	microacinar	4 + 3		0	1+	none	4+
10	ductal		papillary	1	2+	1+	4+
	microacinar	4 + 4		0	1+	1+	4+
11	ductal		cribriform	1	2+	4+	4+
	microacinar	5 + 4		4.5	1+	1+	none
12	ductal		cribriform	5	1+	none	4+
	microacinar	5 + 4		0.5	1+	none	4+

[a] Nuclear scoring: 1+ = 1 to 25% staining,
2+ = 26 to 50% staining,
3+ = 51 to 75% staining,
4+ = 76 to 100% staining.

Figure 7: p53 immunoreactivity in ductal carcinomas. (Immunoperoxidase, om x400)

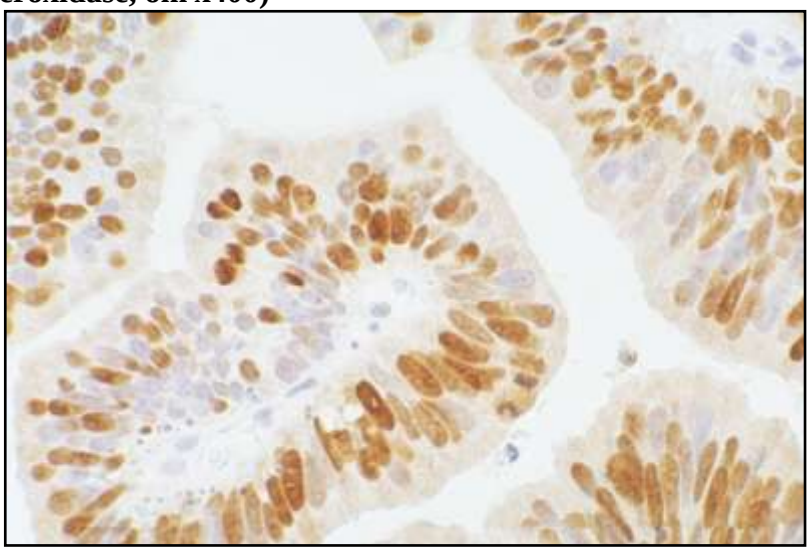
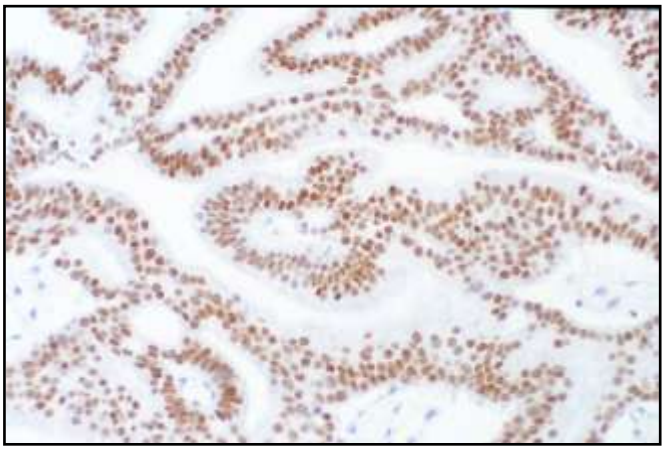
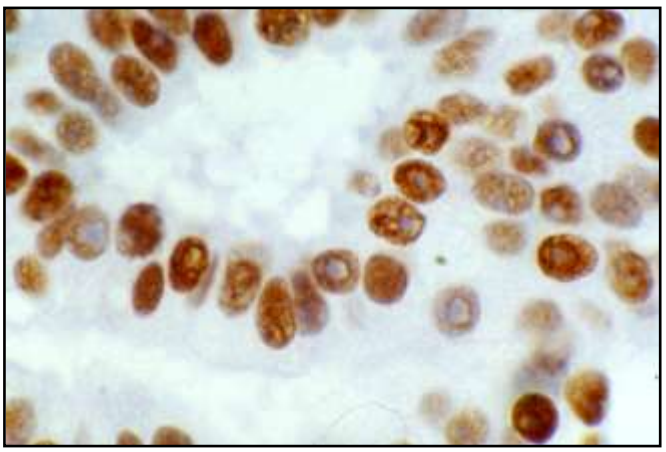


Figure 8: AR immunoreactivity in ductal carcinomas of the prostate. The nucleoli appear negative for AR. (a) medium (om x200) (b) high magnification (om x1000). (Immunoperoxidase)

(a)



(b)



Treatment and follow-up:

Therapy was known in 8 patients and 7 of these received hormonal manipulation; four had orchiectomies, one received stilboestrol and radiotherapy, one had a combination of stilboestrol and orchidectomy and three were treated with cyproterone acetate alone or with radiotherapy or orchidectomy (Table 3). Patient 4 had a ductal carcinoma diagnosed incidentally when he underwent a retropubic prostatectomy for obstructive symptoms. He had no evidence of metastasis at presentation and is still alive 13 years after his original surgery. Patient 6 has recently been diagnosed and is the only other patient still alive. The remaining patients with follow-up died between 1 and 8 years after diagnosis and all these had evidence of metastatic disease, and of these, seven died as a result of the metastatic disease.

Radical prostatectomies.

Between 1987 and 1999, 320 men have undergone radical prostatectomy performed by Mr Gillatt. Full data from 217 patients are available when those that have less than 12 months follow-up, neoadjuvant therapy and those lost to follow-up are excluded. The Kaplan Meier estimated overall rate of biochemical recurrence and survival are shown in Table 6 [personal communication from Mr M Winkler].

Table 6: Biochemical recurrence and survival data for whole series (n=217)

Time	% Recurrence free survival	95% CI	% Survival	95% CI
1 year	**95**	**90 – 98**	**99**	**96 - 100**
5 years	**72**	**62 – 81**	**89**	**80 - 95**
10 years	**40**	**18 – 61**	**69**	**44 – 84**

Study 2: Preoperative p53, bcl-2, E-cadherin and CD44 immunohistochemistry as predictors of biochemical recurrence following radical prostatectomy.

Clinical data

Data on all 75 patients were available with a mean follow-up of 65 months (median 60, range 7-152 months). The median age for the group at the time of surgery was 66 years (range 51-74). The median preoperative PSA was 11 (range 1-37). The clinical stage was cT1 in 27 (36%), cT2 in 48 (64%), whilst the pathological stage

was pT2 in 36 (48%) and pT3 in 39 (52%). Preoperative and postoperative Gleason scores ranged from 4 to 10. The Gleason scores were grouped into three groups, scores 2-4, scores 5 and 6, and scores >7. When the grouped Gleason scores are compared between the biopsy specimen and the radical prostatectomies then 49 (65%) agree, the biopsy specimen score was lower in 23 (31%) and higher in 3 (4%). The radical prostatectomy specimen had positive margins in 34 (45%).

To date, 31/75 (41%) patients have shown biochemical relapse, median time to recurrence 16 months (range 1-62 months), 18 relapsed within 24 months and 30 within 60 months. Data showing clinicopathological data and numbers who have had a recurrence is shown in Table 7.

Table 7: Clinicopathological variables and clinical outcome after radical prostatectomy (total number = 75, median follow-up = 60 months).

Variable	Number biochemical relapse	Number with no evidence of disease.
Margin		
negative	**8**	**33**
positive	**23**	**11**
Pathological stage		
pT2	**6**	**30**
pT3	**25**	**14**
PSA (ng/ml)		
less than 10 ng/ml	**9**	**19**
10 or greater	**22**	**25**
Biopsy Gleason Score		
less than 7	**22**	**43**
7-10	**9**	**1**

Preoperative and postoperative biomarker expression

There was insufficient tumour in the core biopsies for immunohistochemistry for p53 in 13/75 (17%), for bcl-2 in 11/75 (15%), for CD44 in 18/75 (24%) and for E-

cadherin in 20/75 (27%). Two of the radical prostatectomy specimens had no identifiable tumour within them, though they had been examined at multiple levels and review of the needle cores confirmed the presence of tumour. A further 2 radical prostatectomy specimens had insufficient tumour for immunohistochemistry for p53 and CD44. As further sections were cut there was insufficient tumour for bcl-2 in a total of 5 patients and for E-cadherin in 7 patients.

p53 expression data were available for 62 biopsies and 71 radical prostatectomy specimens. Positive expression was observed in 40 out of 62 (64%) biopsies (Figure 9) and in 39 out of 71 (55%) radical prostatectomy specimens (Table 8). Preoperative Gleason scores were <7 in 65 patients (85%), of whom biopsy p53 data are available for 53; 19 of these 53 have relapsed, 14 (74%) of whom expressed p53 while 5 (26%) expressed no p53. However from the group of Gleason scores <7, 19 patients with positive p53 and 15 with normal p53 have no evidence of disease recurrence. Data on bcl-2 immunohistochemistry were available for 64 biopsies and 70 radical prostatectomy specimens. Positive staining was observed in 7 (11%) preoperative biopsies (Figure 10) and in 14 (20%) radical prostatectomy specimens (Table 8).

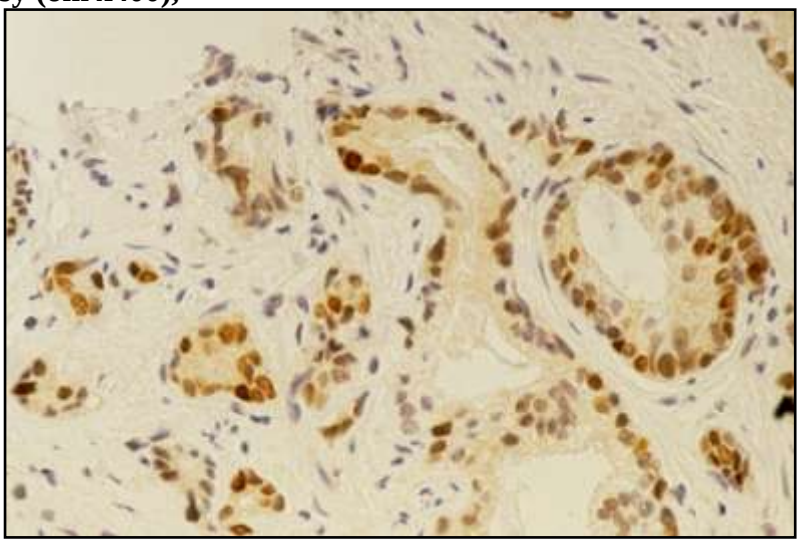
CD44 data were available on 57 biopsies and 71 radical prostatectomy specimens. Of the biopsies, 11 out of 57 (19%) exhibited normal diffuse or regional membranous immunoreactivity, while 46 (81%) stained focally or staining was absent (both regarded as abnormal, Figure 11). 26 radical prostatectomy specimens (37%) exhibited normal diffuse or regional membranous immunoreactivity, while 45 (63%) stained focally or staining was absent (Table 8). E-cadherin data are available

for 55 biopsies and 68 radical prostatectomy specimens. 49 biopsies (89%) were uniformly immunoreactive and 6 (11%) expressed heterogeneously (regarded as abnormal, Figure 12). No biopsy was uniformly negative. 36 radical prostatectomy specimens (53%) were uniformly immunoreactive while 32 (47%) exhibited heterogeneous immunoreactivity (Table 8). No radical prostatectomy was uniformly negative for E-cadherin.

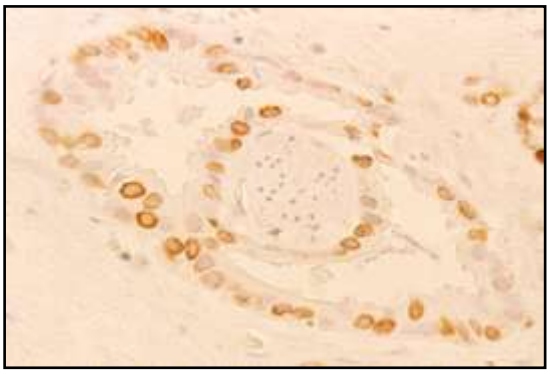
Table 8: Immunohistochemical expression of p53, bcl-2, CD44 and E-cadherin, and clinical outcome after radical prostatectomy.

Variable (n= number with available result)		Number treatment failure (% of available result)	Number with no evidence of disease (% of available result)
Biopsy			
p53 (n=62)	**negative**	**7 (11%)**	**15 (24%)**
	positive	**21 (34%)**	**19 (31%)**
bcl-2 (n=64)	**negative**	**23 (36%)**	**34 (53%)**
	positive	**5 (8%)**	**2 (3%)**
CD44 (n=57)	**normal**	**4 (7%)**	**7 (12%)**
	abnormal	**24 (42%)**	**22 (39%)**
E-cadherin (n=55)	**normal**	**23 (42%)**	**26 (47%)**
	abnormal	**4 (7%)**	**2 (4%)**
Radical prostatectomy			
p53 (n=71)	**negative**	**12 (17%)**	**20 (28%)**
	positive	**19 (27%)**	**20 (28%)**
bcl-2 (n=70)	**negative**	**23 (33%)**	**33 (47%)**
	positive	**8 (11%)**	**6 (9%)**
CD44 (n=71)	**normal**	**9 (13%)**	**17 (24%)**
	abnormal	**22 (31%)**	**23 (32%)**
E-cadherin (n=68)	**normal**	**16 (24%)**	**20 (29%)**
	abnormal	**13 (19%)**	**19 (28%)**

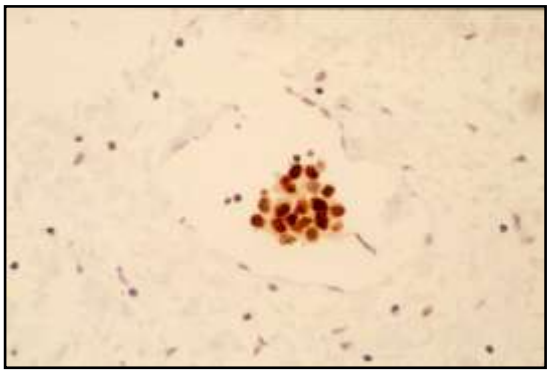
Figure 9: positive p53 immunostaining in prostate cancer.
(a) core biopsy (om x400),



(b) perineural invasion (om x400),



(c) intravascular invasion (om x400),



(d) showing variation between high and low Gleason grade in a heterogeneous tumour (om x640).

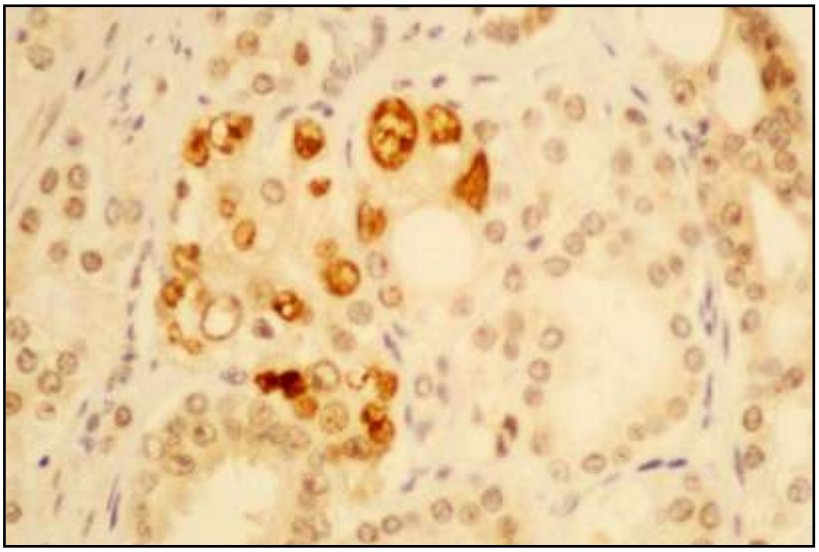


Figure 10: Positive cytoplasmic bcl-2 staining in prostatic adenocarcinoma (om x1000).

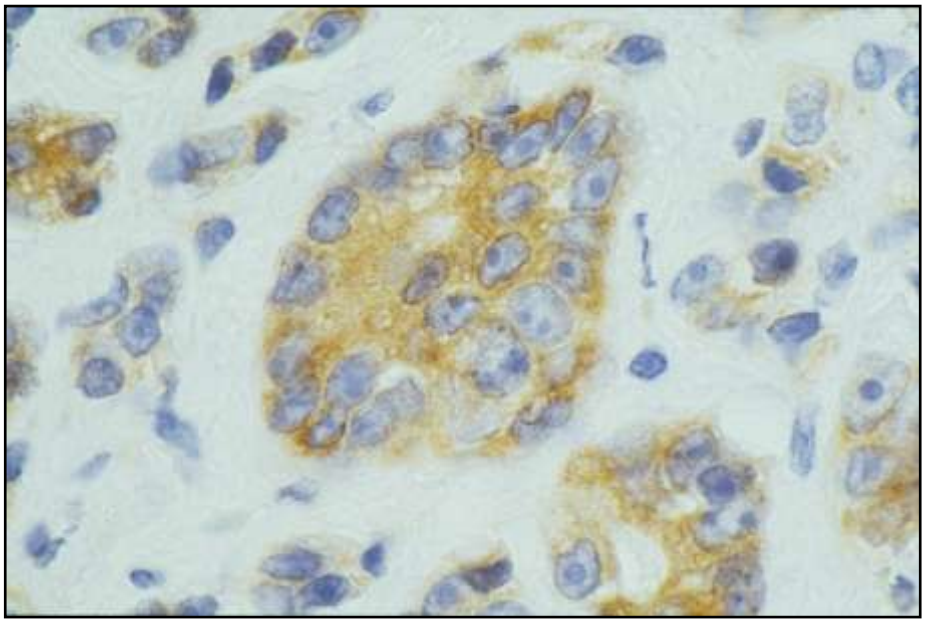


Figure 11: Membranous CD44 immunostaining in prostatic adenocarcinoma (om x1000).

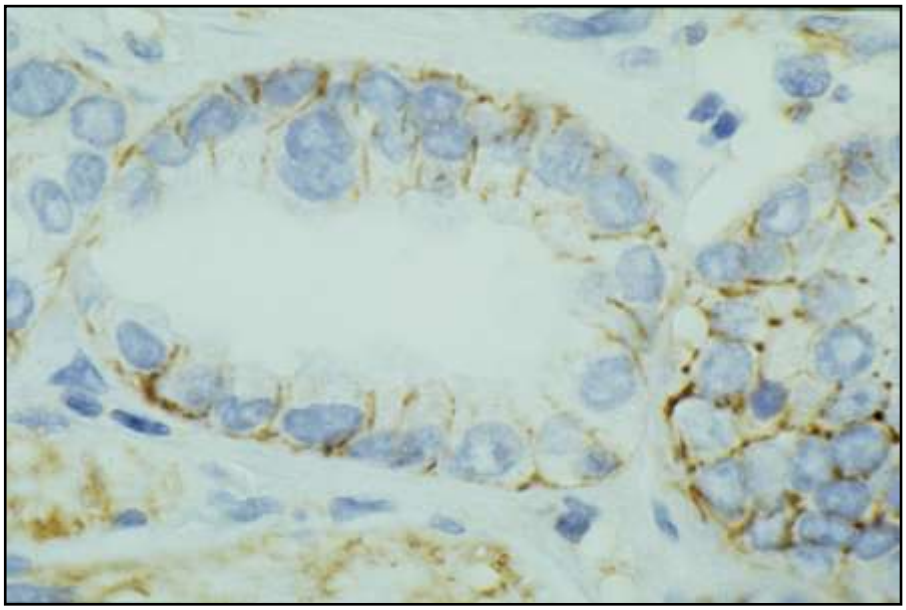
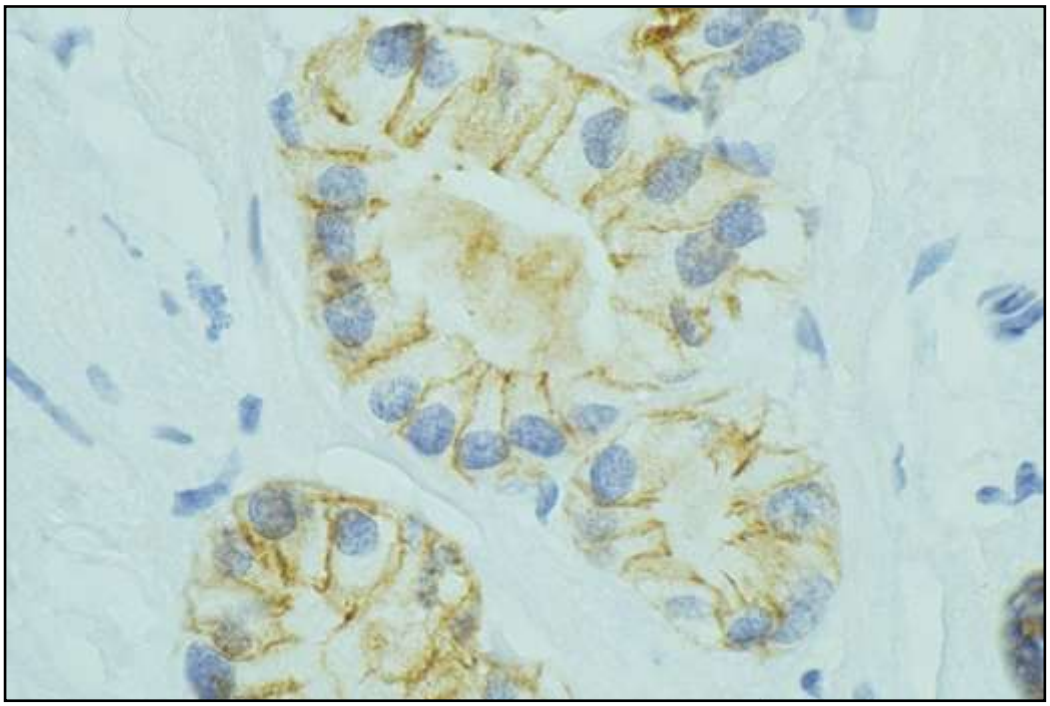
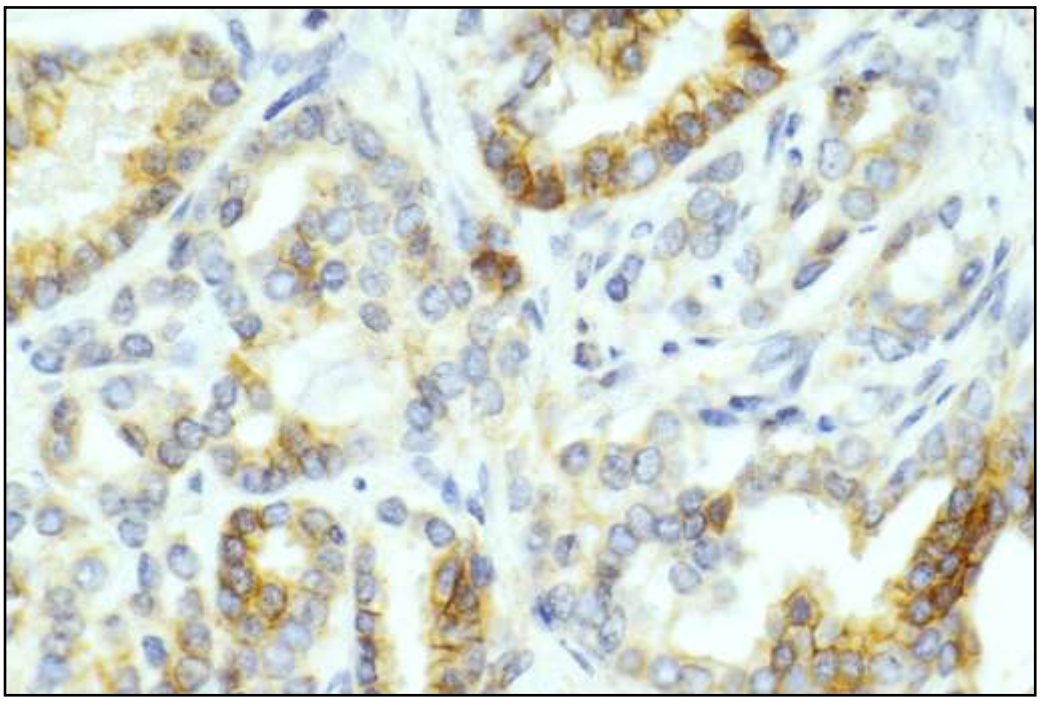


Figure 12: E-cadherin immunostaining in prostatic adenocarcinoma

(a) normal (om x1000),



(b) heterogeneous (om x400).



Statistical analysis

Preoperative indicators

Seven of the eight factors considered for univariate analysis were individually significant predictors of biochemical recurrence at the 10% level and were considered in the multivariate model: PSA (p=0.03), clinical stage (p=0.05), Gleason score (p=0.001), p53 (p=0.03), bcl-2 (p=0.09), E-cadherin (p=0.06) and CD44 (p=0.01). Age was not a significant factor for recurrence. However, due to the high level of missing data, with many patients having missing data for several variables (e.g. all 11 patients with missing bcl-2 also had missing CD44, with a further 7 having missing CD44 but recorded bcl-2) a multivariate model proved inestimable. When the data for CD44 were removed from the model then multivariate analysis was possible and this showed that only Gleason score, PSA and p53 were significant. The relative risks for recurrence are shown in Table 9. The likelihood of recurrence is similar in patients with a Gleason score of 2 to 4, and 5 or 6, but with a score of between 7 and 10 they are up to 6 times more likely to have a recurrence. For every unit increase in the PSA the relative risk of recurrence increases by 1.04. For an increase of 5 in PSA the relative risk increases to 1.22. The Kaplan-Meier survival estimates are shown in Figure 13.

Table 9: Relative risks of significant preoperative factors.

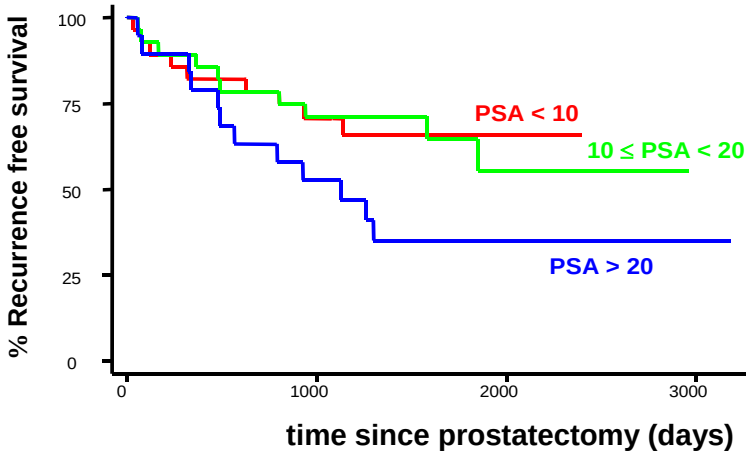
Variable	Relative* risk	95% Confidence limit
p53	**1.88**	**0.70-5.04**
Gleason score (7 to 10)	**6.34**	**2.01-19.97**
PSA (per unit increase)	**1.04**	**1.00-1.09**

*** Adjusted for missing data**

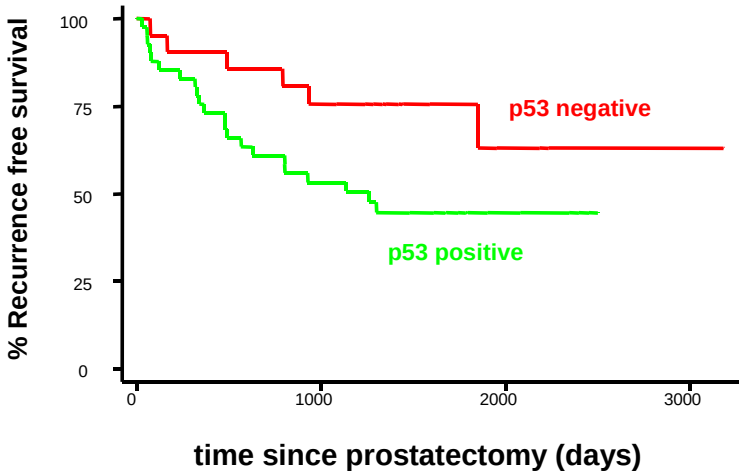
Figure 13: Kaplan-Meier survival estimates for significant pre-operative indicators.

a) Gleason score.

b) PSA



c) p53



Postoperative indicators

Seven factors were considered for inclusion in the model – PSA, pathological stage, Gleason score, p53, bcl-2, E-cadherin and CD44. The two patients with no tumour present in the radical prostatectomy specimen were excluded (neither experienced a recurrence). Similarly as only 4 of the 73 patients studied had a post-operative Gleason score of 2 to 4 these were considered with those who had a score of 5 or 6. Thus for Gleason score the comparison was between those with a score of less than 7 and those with a score of 7-10. Also, as only 2/73 patients had missing data for p53, bcl-2 and CD44 missing data indicators were not included for these variables, the more common response (bcl-2 = negative, p53 = positive and CD44 = abnormal) was assumed.

From initial univariate analysis four of these seven factors were individually significant predictors of biochemical recurrence at the 10% level – PSA ($p=0.03$), pathological stage ($p=0.0001$), Gleason score ($p=0.08$) and bcl-2 ($p=0.02$). The remaining three factors were not univariately significant (at the 10% level) – E-cadherin ($p=0.99$), p53 ($p=0.21$) and CD44 ($p=0.29$). Of these four factors significant on univariate analysis three, bcl-2, pathological stage and PSA, were found to be associated with biochemical recurrence in a multivariate model ($p<0.05$). The three factors that were not significant univariately were also not significant in the multivariate model ($p>0.3$ in all cases). The relative risks for recurrence for these three factors are shown in Table 10. A patient with pathological stage 3 is more than 5 times as likely to experience a recurrence compared to a patient with pathological stage 2. Similarly, a patient with positive bcl-2 is almost $2^1/_2$ times as likely to experience a recurrence compared to someone without bcl-2

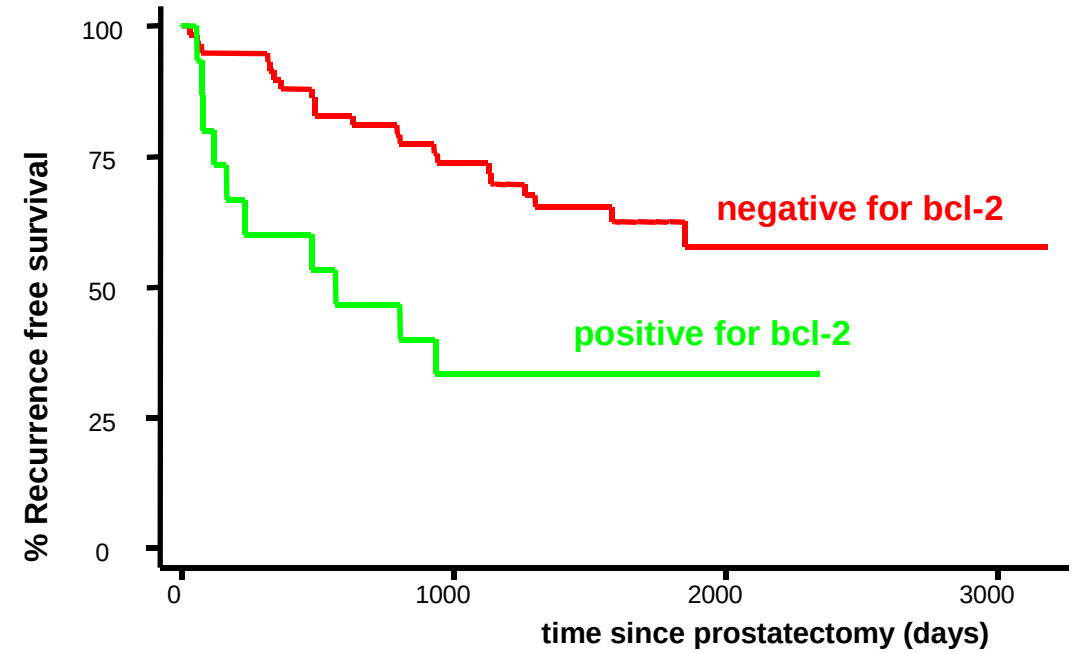
immunopositivity. In this model a unit increase in PSA increases the relative risk of recurrence by 6%. For an increase of 5 in PSA the relative risk increases to $(1.06)^5$ = 1.34 (34%). The Kaplan-Meier survival curves are shown in Figure 14.

Table 10: Relative risks for significant postoperative variables.

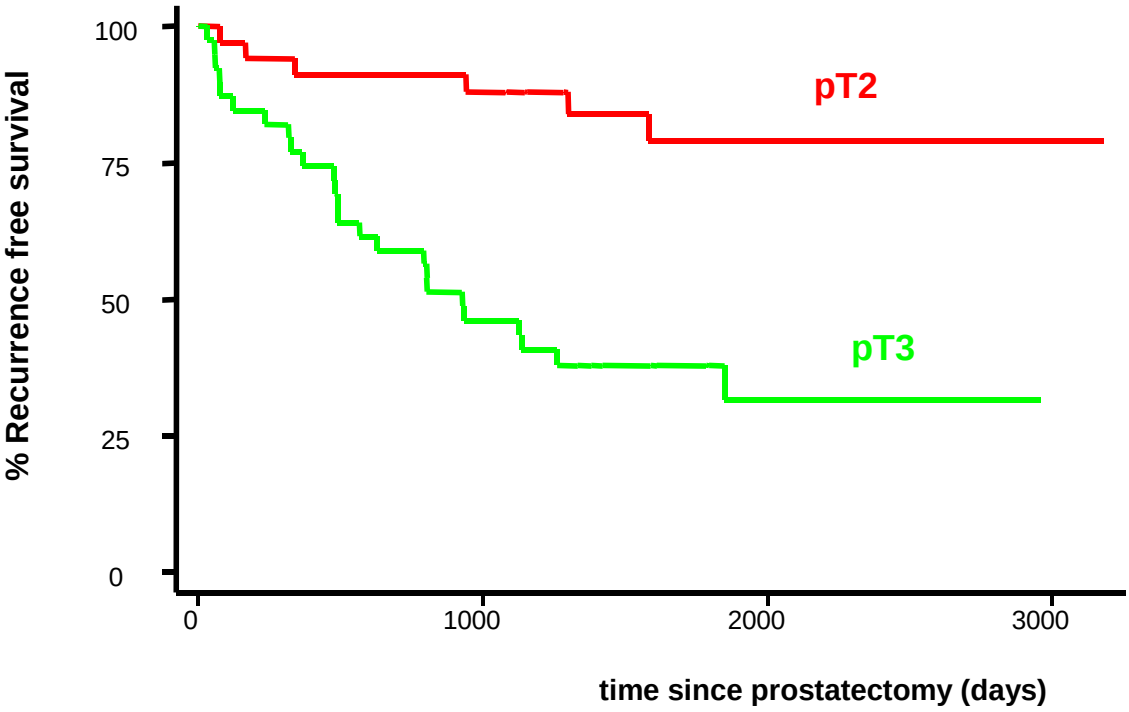
Factor	Relative risk	95% confidence interval
PSA	**1.06**	**1.01 – 1.10**
Pathological stage		
pT2	**1.00**	**-**
pT3	**5.68**	**2.22 – 14.50**
bcl-2		
negative	**1.00**	**-**
positive	**2.46**	**1.12 – 5.36**

Figure 14: Kaplan-Meier survival estimates for post-operative indicators.

a) bcl-2



b) Pathological stage



Study 3: Postoperative p53 and bcl-2 as predictors of biochemical recurrence following radical prostatectomy.

Clinical follow-up was available on all 129 patients in this series, follow-up ranged from 0.2 to 152 months (note that one patient died postoperatively, mean of 53 months and a median of 50 months). Of the 129 patients 50 (39%) had had a biochemical recurrence. Eleven patients have died, two of whom did not have evidence of biochemical recurrence at the time of death. Tumour involved the surgical margins in 49 (38%) patients and the seminal vesicles in 16 (12%). There was capsular penetration (pT3) in 67 (52%) of the patients. The PSA was less than 11 ng/ml in 51 (39%) patients. The Gleason score was less than 7 in 87 (67%) patients. p53 immunohistochemistry was greater than 0% nuclear staining in 92 (71%) patients, but 20 of these had less than 10% nuclear staining. bcl-2 showed >0% cytoplasmic staining in 22 (17%) patients, and only one patient had a score of between 1 and 10%. Eleven patients received neo-adjuvant hormonal therapy, these patients had been excluded from study 2 but were included in this study. The clinicopathological data and the biomarker results are shown in Table 11.

Table 11: Clinicopathological variables, p53 and bcl-2, and clinical outcome after radical prostatectomy (total number =129).

Variable	Number biochemical relapse (total number = 50)	Number with no evidence of disease (total number = 79)
Margin		
negative	18	62
positive	32	17
Pathological stage		
pT2	10	52
pT3	40	27
Seminal vesicle involvement		
negative	38	75
positive	12	4
PSA		
less than 11ng/ml	17	34
11 – 20ng/ml	13	34
>20ng/ml	20	11
Gleason Score (radical prostatectomy)		
less than 7	26	61
7-10	24	18
p53		
0%	12	25
1-9%	6	14
≥10%	32	40
bcl-2		
0%	37	70
1-9%	0	1
≥10%	13	8

Statistical analysis

All seven factors were univariately significant (Table 12).

Table 12: Univariate analysis showing all seven factors were significant (n=129).

Variable	p-value
PSA	**0.01**
Gleason score (<7 versus7-10)	**0.003**
Pathological stage (pT2 versus pT3)	**<0.0001**
Seminal vesicle involvement	**0.0008**
Margins	**<0.0001**
p53	**0.04 (0.01)***
bcl-2	**0.04 (0.04)***

***using a cut-off of ≥10% staining**

The final multivariate model contained the variables PSA, capsular penetration, positive margins and an interaction between PSA and margins (Table 13). Defining the cut-off for positive staining as >0% or ≥10% made no difference to the variables identified. Patients without capsular penetration had better survival than those with capsular penetration (Figure 15). Patients with negative margins had better survival than those with positive margins, provided their PSA was less than or equal to 20. Those patients with negative margins had slightly lower risk if their PSA was between 11 and 20 rather than less than 10 but the confidence interval crosses 1 and so this is not a significant difference. For those with PSA greater than 20, there was

no difference in the survival of patients with positive and negative margins (Figure 16).

Table 13: Significant variables on multivariate analysis with the relative risks (n = 129).

Variable	Numbers	Relative risk	95% Confidence interval
PSA ≤ 10, margins –ve	32	1.00	-
PSA ≤ 10, margins +ve	17	5.78	1.97 - 16.96
PSA 11-20, margins –ve	31	0.78	0.18 - 3.28
PSA 11-20, margins +ve	18	4.08	1.38 - 12.00
PSA >20, margins –ve	17	5.94	1.99 - 17.72
PSA >20, margins +ve	14	6.49	2.17 - 19.31
Organ confined	62	1.00	-
Capsular penetration	67	4.19	1.99 - 8.79

Figure 15: Kaplan-Meier curve for pathological stage (n=129).

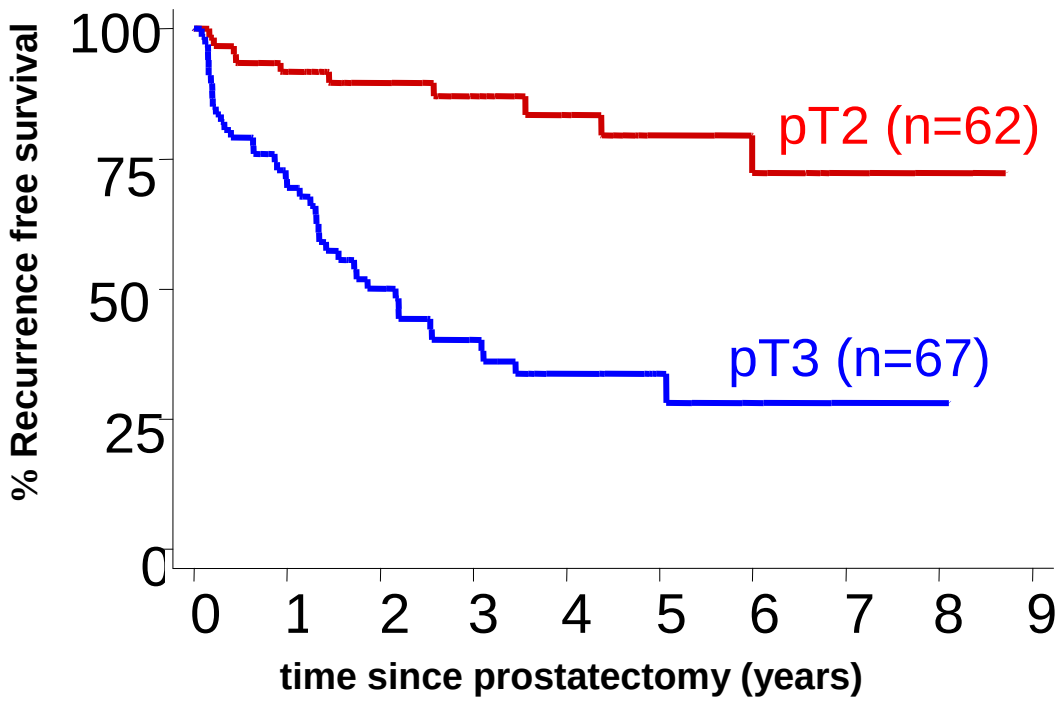
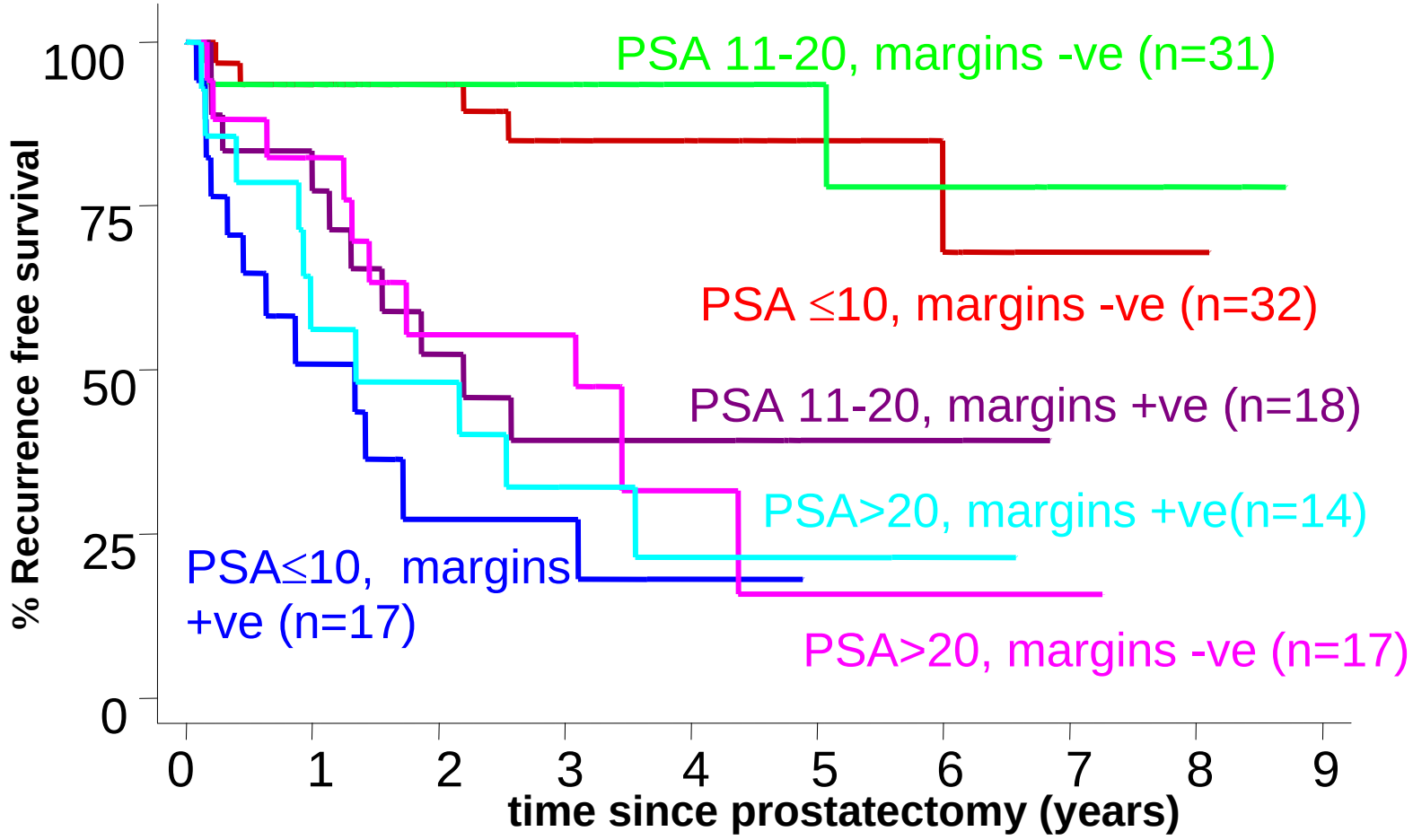


Figure 16: Kaplan-Meier curve showing interaction between PSA and margin involvement (n=129).



Statistical analysis after exclusion of patients treated with neo-adjuvant therapy.

A second model was developed excluding all patients who received neo-adjuvant therapy (n=11). The second model was therefore based on 118 patients, with a median follow-up of 53 months (range 0.2 to 152.4 months). The same prognostic factors were considered, however there were too few patients in the Gleason scores 2-4 category so they were grouped with Gleason scores 5 and 6. Univariate analysis showed similar results to the whole group (Table 14).

Table 14: Univariate models, excluding those who had received neo-adjuvant therapy (n=118)

Variable	p-value
PSA	**0.01**
Gleason score (<7 versus7-10)	**0.003**
Pathological stage (pT2 versus pT3)	**<0.0001**
Seminal vesicle involvement	**0.002**
Margins	**<0.0001**
p53	**0.04 (0.01)***
bcl-2	**0.05 (0.04)***

***using a cut-off of ≥10% staining**

The final multivariate model contained exactly the same factors as the first model, namely PSA, pathological stage, positive margins and an interaction between PSA and margins (Table 15). Again, it made no difference using a cut-off for positive p53 or bcl-2 staining of either >0% or ≥10% staining. Patients without capsular penetration (pT2) had better survival than those with capsular penetration (pT3)

(Figure 17). Again, patients with negative margins had better survival than those with positive margins, provided their PSA was less than or equal to 20. For those with PSA greater than 20, there was no difference in the survival of patients with positive and negative margins (Figure 18).

Table 15: Significant variables on multivariate analysis with the relative risks, after excluding the patients who had received neoadjuvant therapy (n=118).

Variable	Numbers	Relative risk	95% Confidence interval
PSA ≤ 10, margins –ve	31	1.00	-
PSA ≤ 10, margins +ve	17	5.86	1.99 - 17.25
PSA 11-20, margins –ve	29	0.85	0.20 - 3.59
PSA 11-20, margins +ve	17	3.83	1.27 - 11.49
PSA >20, margins –ve	11	5.95	1.91 - 18.42
PSA >20, margins +ve	13	6.18	2.03 - 18.72
Organ confined	55	1.00	-
Capsular penetration	63	3.78	1.73 - 8.22

Figure 17 : Kaplan-Meier curve for pathological stage in patients not receiving neo-adjuvant therapy (n=118).

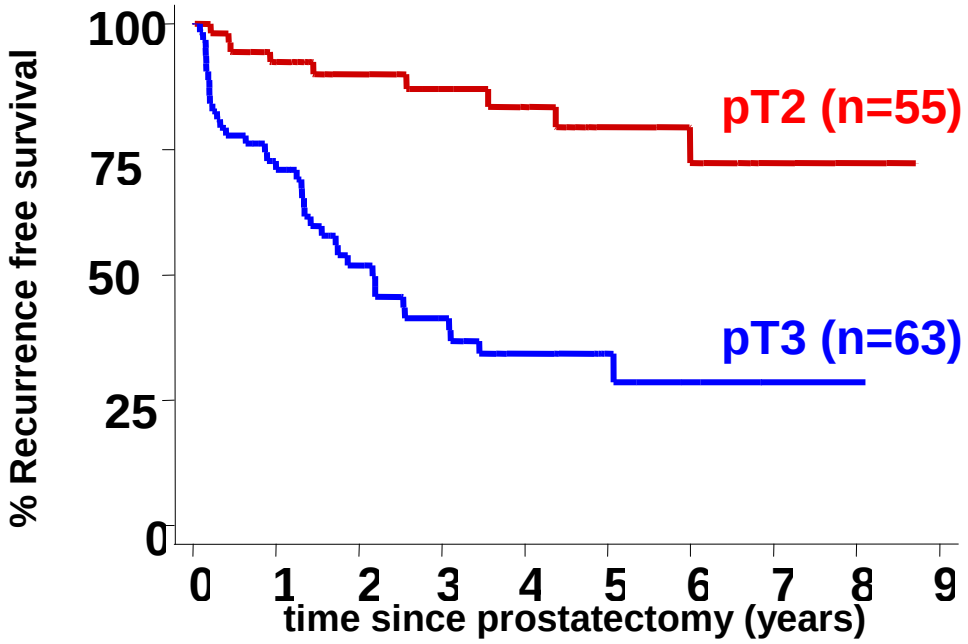
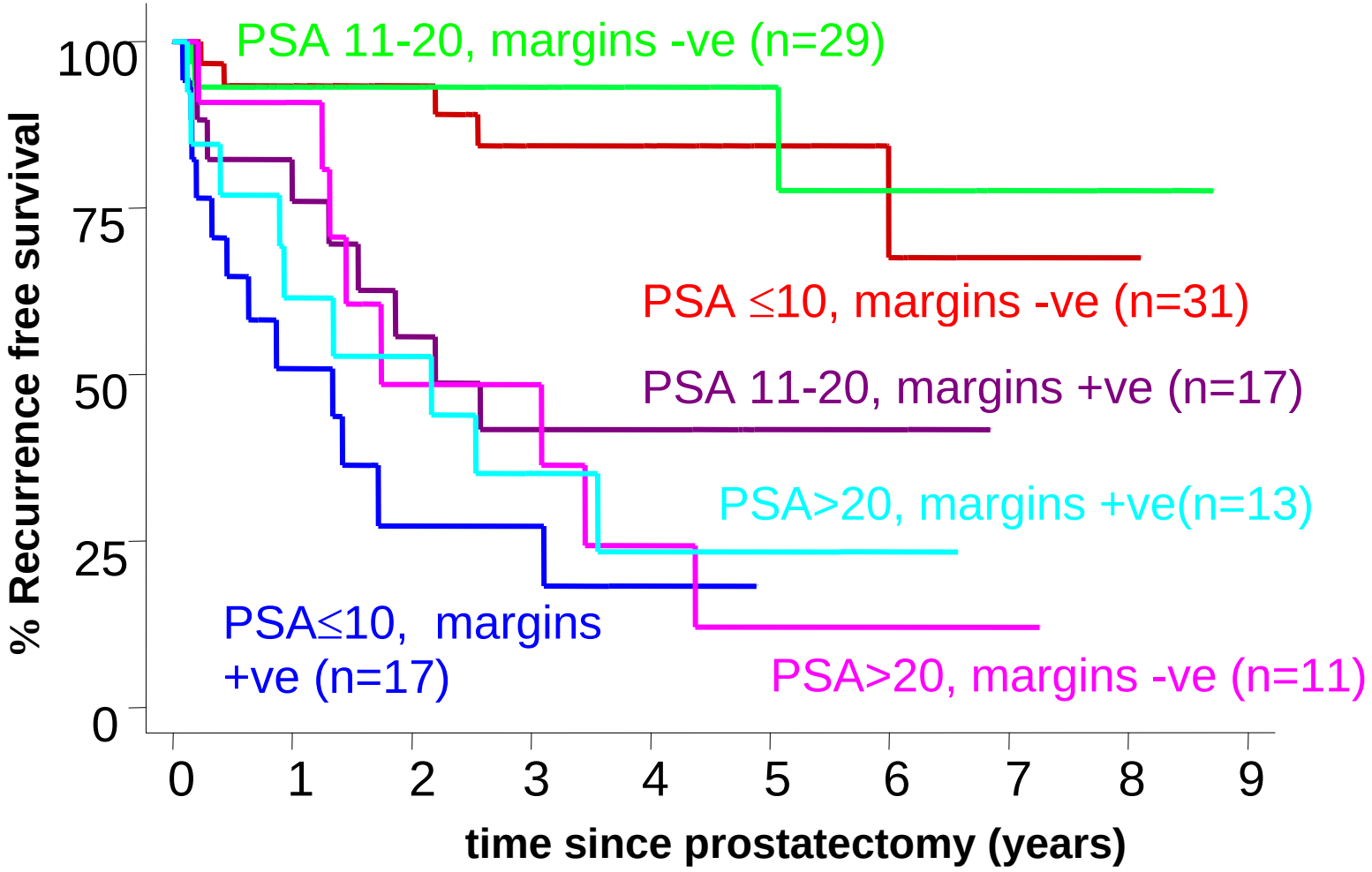


Figure 18: Kaplan-Meier curve showing interaction between PSA and margin involvement in patients not receiving neo-adjuvant therapy (n=118).

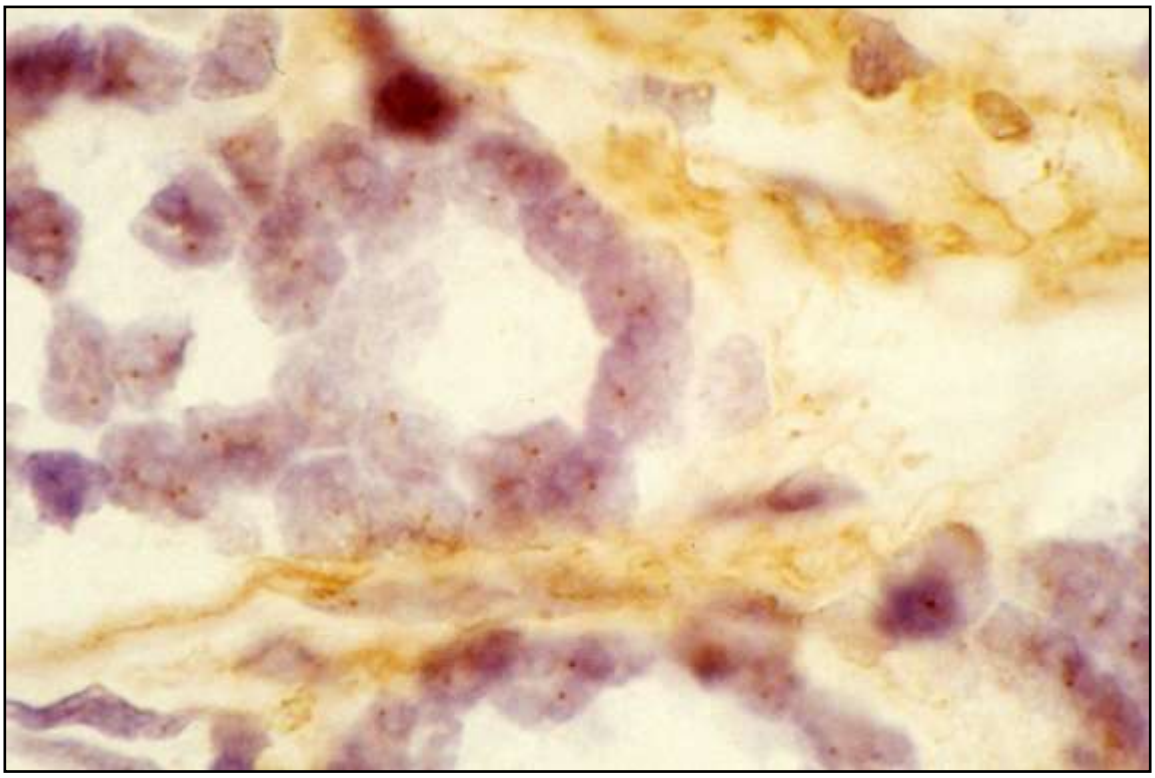


Study 4: Amplification of Her-2/neu in prostate cancer.

Follow-up of the 117 patients ranged from 14 to 152 months (median 53 months). The median pretreatment serum PSA was 13ng/l, (range 1 to 43ng/ml). 56 (48%) of these patients were classified as stage pT2, and 61 (52%) were classified as stage pT3/pT4. Three (2.5%) patients had positive lymph nodes at the time of radical prostatectomy (N1). Biochemical recurrence occurred in 50 (43%) patients (defined as a serum PSA of greater than 0.2 ng/l in at least two consecutive measurements). The median time to recurrence was 12 months (range 1 to 66 months). 24 (21%) had definite clinical recurrence and 12 (10%) have died. Nine (8%) had a Gleason score of less than 5, 72 (61%) either 5 or 6 and 36 (31%) had a Gleason score of 7 or greater.

In situ hybridisation was successful in 114 of the 117 patients. The signal from the probe was highly dependent on the degree of digestion and suboptimal results were repeated. There was a tendency for the signal to be located at the nucleolar membrane (Figure 19a). Significant increase in copy number of Her-2/neu was present in only 2 (1.75%) of the cases and this was at a low level (23% and 38%) (Figure 19b). The ratios to chromosome 17 were both less than 2 (0.8 and 1.6) (Figure 20a) and b)). These two cases both had a Gleason score of 7; one was stage pT3a and clinically recurred with distant metastasis after 19 months. The other was stage pT3b and clinically recurred after 2 months with distant metastasis.

Figure 19: In situ hybridisation for Her-2/neu in prostate cancer (peroxidase detection, om x1000)
(a) normal copy number,



(b) increased copy number.

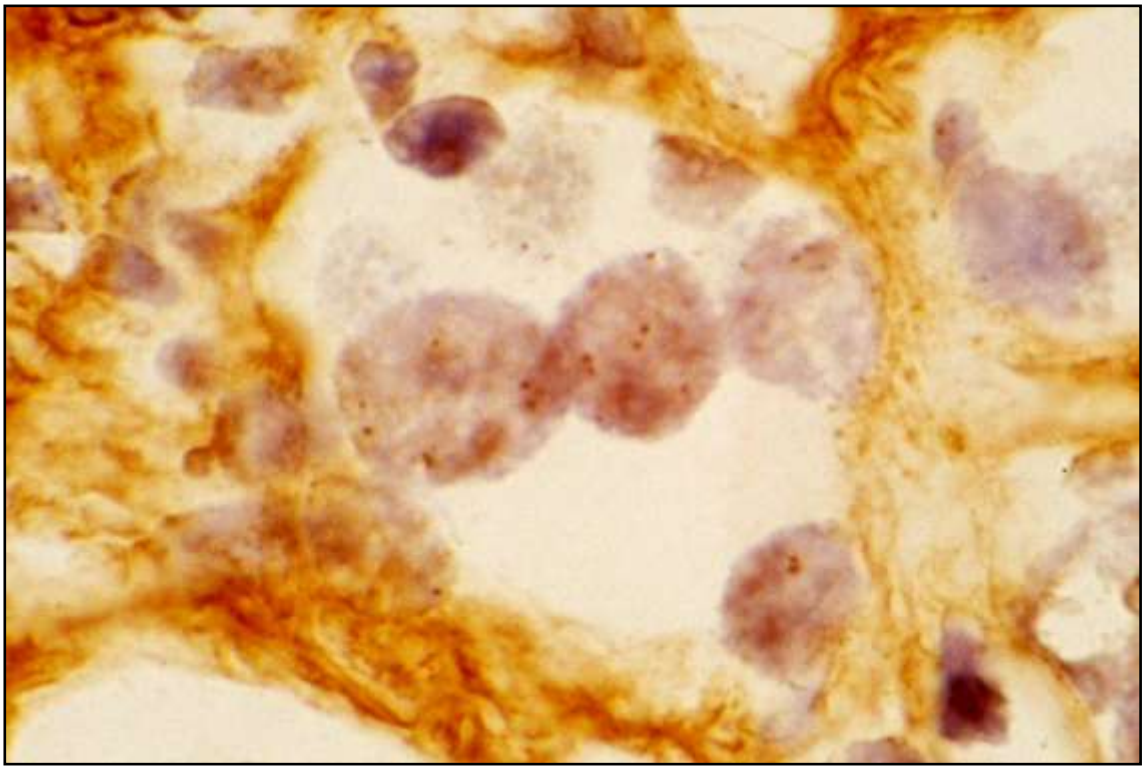
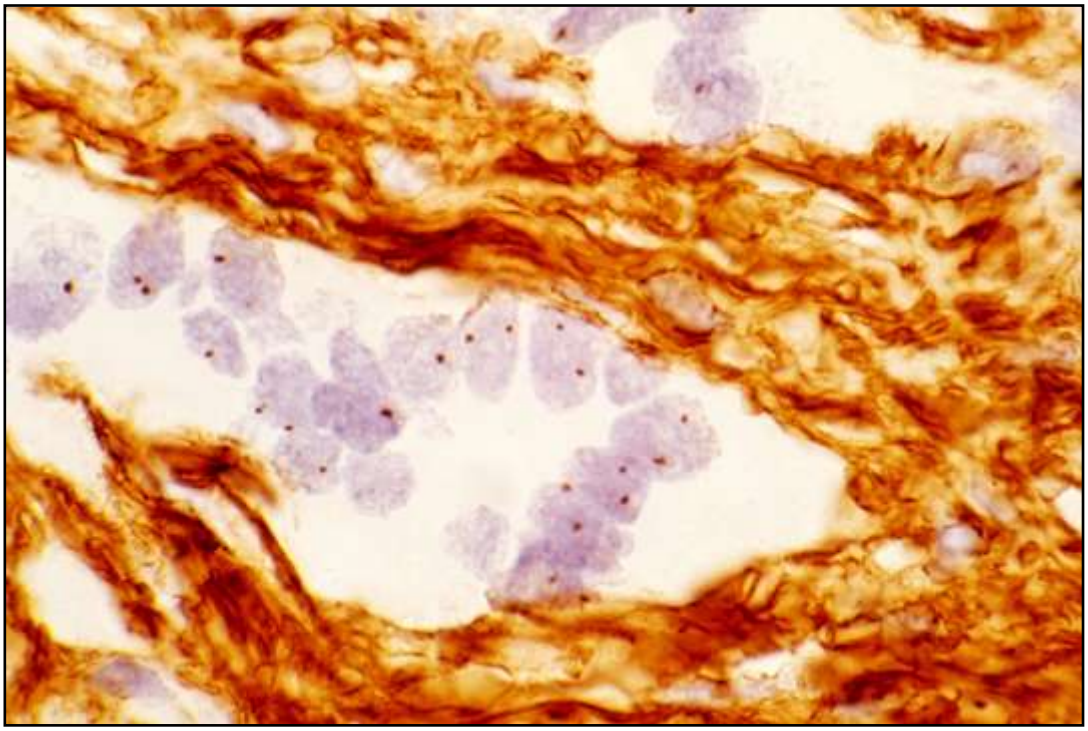
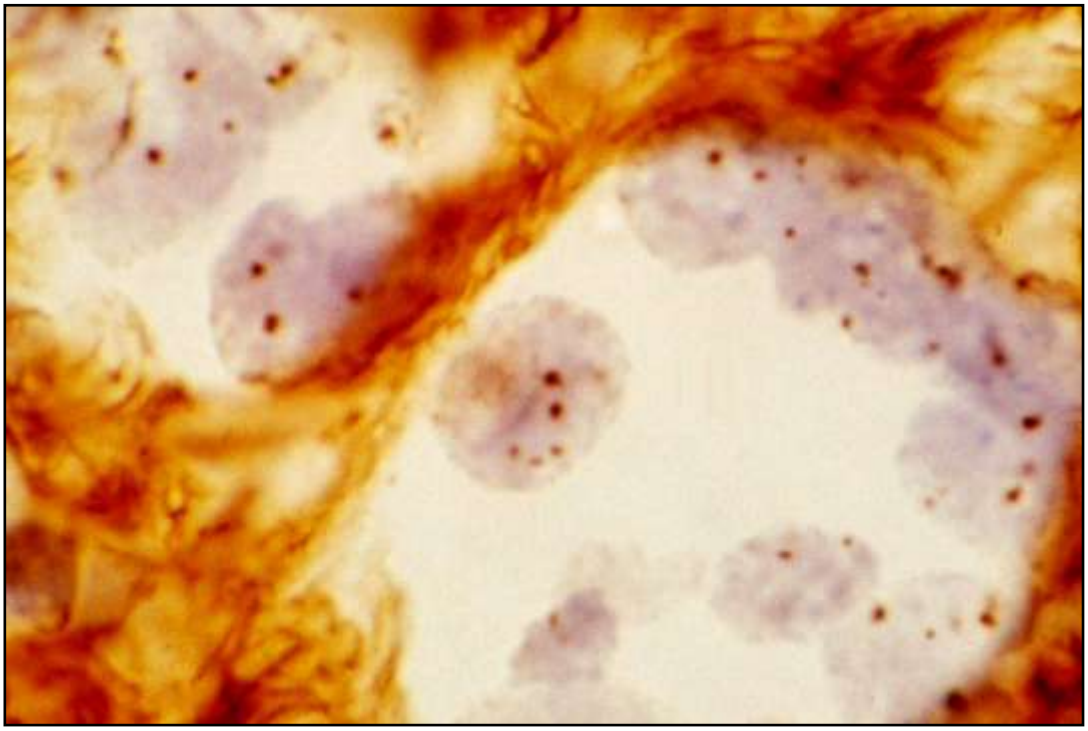


Figure 20: In situ hybridisation for chromosome 17 alpha satellite probe in prostate cancer. The signal is clearer than for Her-2/neu, (peroxidase detection, om x1000)

(a) normal copy number



(b) increased copy number



Discussion

Ductal carcinoma of the prostate

Pure ductal carcinoma of the prostate occurs almost exclusively in elderly men and its incidence is reported to be 0.2% - 1.3% of prostatic carcinomas [Tannenbaum 1975, Dube *et al*, 1973]. In the series studied by Dube *et al* [1973] 6% of all prostate carcinomas had a ductal as well as a microacinar component. Ductal carcinomas are thought to arise in the primary ducts of the prostate. The patients often have obstructive symptoms but may also present with haematuria. At cystoscopy the prostatic urethra can have a fronded or a papillary appearance. The serum PrAP or PSA are low in localised disease but are elevated in metastatic disease.

Immunohistochemical markers for PSA and PrAP as well as better antigen retrieval methods have answered the question of origin of this tumour. In all but two reports [Gillatt *et al*, 1986, Stavropoulos *et al*, 1993] the tumours have been positive for PSA and PrAP. In study 1 all the ductal areas were positive for both the antigens confirming the earlier reports and giving weight to the belief that the tumour has a prostatic histiogenesis.

The expression of oestrogen receptors (ER) has been examined in benign and malignant prostates. Brolin *et al* [1992] looked at ER in prostates and found that stromal nuclei were positive only in microacinar carcinomas. Epithelial cell nuclei were found to be rarely positive in hyperplastic and normal prostate [Miyamoto *et al*, 1993]. Lee [1994] found a diffuse weak positive staining for the oestrogen-regulated proteins PS2 and ER-D5 in ductal carcinomas of the prostate equivalent to

that of microacinar carcinomas and NPH. He concluded that as there was no difference between the two, then oestrogen therapy was indicated. Nuclear marking for ER is considered to be of the most significance and in none of the patients in this study was this found, nor was there any significant stromal positivity. These results refute the idea that the tumour has endometrial similarities and that oestrogen therapy should not be used [Gillatt *et al*, 1986].

Archive material can now be studied for androgen receptors after the introduction of reliable immunohistochemical markers. Androgen receptors have been reported by Miyamoto *et al* [1993] to be lost as prostate tumours become more anaplastic. In study 1, all the patients' ductal tumours had nuclear positivity for AR. All four of the control pure microacinar carcinomas, which were of low and high grade, were positive for AR (data not shown). These findings do not correlate with Miyamoto *et al* [1993] but this probably reflects differences in either antigen retrieval or antibody. This not only adds weight to a prostatic origin of ductal tumours but also shows that anti-androgen therapy is likely to have an effect.

Mitotic counts are of no use in grading microacinar carcinoma as they are often low even in aggressive disease. Many grading systems exist but the most widely accepted is the Gleason score, which is based on architectural patterns. Some authors consider ductal carcinomas as Gleason grade 3 and if they have necrosis as grade 5 [Bostwick, 1997]. Ductal tumours often have a cribriform appearance and this would fall into the grade 3 category whilst others have a pure papillary pattern which does not have an assigned grade. In the series reported by Bostwick *et al* [1985] 11 of the 13 cases had mitotic counts greater than 4 per 10 high power fields

and the overall prognosis was poor. Millar *et al* [1996] found that some cases had a high mitotic count but in general they were low and the long-term survival in this group was better than in Bostwick's series. The mitotic count of most of the ductal tumours in study 1 was high and this was reflected in the amount of Ki67 marking. The prognosis was worse in our patients when compared to Millar's but similar to Bostwick's.

The Ki67 antigen represents a nuclear cell proliferation-associated protein expressed in G1, S, G2 and M phases of the cell cycle, but not in non-proliferative G0 cells. The proliferation index in non-neoplastic prostatic acini has been found to be 0.19-4.0% whilst in malignant acini it ranges from 1.6-16% [Bubendorf *et al*, 1996a, Feneley *et al*, 1996]. The data for microacinar carcinoma components in study 1 correlate with the published results as none had greater than 1+ (1-25%) immunoreactivity. All the ductal tumours had at least 1+ reactivity and three had 2+ (26-50%) suggesting that Ki67 expression is greater in this variety of prostate cancer. The patients with high Ki67 expression died of their disease at 1, 3 and 4 years after diagnosis. Although not significantly different from the others, a point to note is that patient 11 had 2+ Ki67 and 4+ p53 expression and died of his disease after only 1 year.

As tumours become more aggressive so the expression of p53 is thought to increase. Kallukury *et al* [1994] showed that 20% of high-grade prostatic microacinar carcinomas were p53 positive whilst only 7% of low-grade tumours were positive. Two (17%) of the ductal tumours in study 1 were strongly positive for the p53 antigen whilst none of the microacinar components were. One of these patients died

within one year whilst the other was lost to follow-up. Although no strong conclusion can be drawn from this, the result suggests that the expression of p53 may be an indicator of aggressive disease in ductal tumours as well as microacinar carcinomas.

Prognostic indicators are difficult to assess due to small numbers in this study, the age of the patients and the presence of microacinar carcinomas in half of them. Localised disease and prostatectomy, as seen in one patient with a low serum PAP who had the tumour diagnosed incidentally, appear to be the best indicators as this patient is still alive 13 years after his original surgery. Skeletal metastasis at presentation, a co-existent high-grade microacinar carcinoma, high mitotic counts and high serum PAP or PSA all appear to be adverse prognostic indicators.

The main treatment modalities for microacinar carcinoma of the prostate consist of surgery or radiotherapy or anti-androgen therapy (either in the form of orchiectomy or by pharmacological methods). Ductal carcinomas are known to respond to orchiectomy [Young *et al*, 1973]. Cueva *et al* [1988] described a patient who failed to respond to oestrogen therapy but had a remission with estramustine phosphate. Radiotherapy was noted to be effective in at least one of the patients in this study. The expression of AR seen in the tumours in this study would confirm the theoretical use of anti-androgen techniques. Most of the group had extensive metastatic disease at presentation and it is difficult to assess treatment response. The greater age of patients with ductal tumours means that radical prostatectomies are often not considered but with the greater use of serum PSA and transrectal ultrasound earlier diagnosis may become more common.

This data and that from previous studies would suggest that patients with ductal carcinomas of the prostate should be treated no differently from those with microacinar carcinomas. In cases of ductal carcinoma diagnosed by TURP a coexistent microacinar carcinoma should be excluded.

Clinicopathological factors as predictors of outcome following radical prostatectomy.

In studies 2 and 3 clinicopathological variables were examined to see if they could predict biochemical recurrence after radical prostatectomy. Patients for these studies came from a cohort of 320 men who had undergone radical prostatectomy performed by a single surgeon between 1987 and 1999.

For study 2 patients were selected on the basis of availability of preoperative biopsies and having not received neo-adjuvant therapy. Statistical analysis had previously been performed after a follow-up period of 6 - 114 months (mean 38 months) and the results have been published [Brewster *et al*, 1999]. For this thesis a further period of follow-up was studied, ranging from 7 to 152 months (mean 65 months, median of 60 months) and a slightly different definition of biochemical recurrence was applied. Brewster *et al* [1999] used as a definition of biochemical recurrence a postoperative serum PSA of at least 0.2 ng/ml, increasing on at least one subsequent estimation, and this was based on the definition used by Bauer *et al* [1996b]. In this thesis the definition was a post-operative PSA of > 0.2ng/ml on two consecutive measurements at least 6 months apart. The definition of biochemical recurrence has been very variable; in previous studies some authors have defined it as a PSA greater than 0.4 ng/ml and increasing on at least one subsequent occasion

[Dillioglugil *et al*, 1997], 0.5 ng/ml or greater [Umbas *et al*, 1994], and greater than 1.0 ng/ml on two consecutive measurements [Noordzij *et al*, 1997].

In the first analysis, only 23 (30%) patients had experienced a recurrence whilst in this thesis this increased to 31 (41%) patients. The same preoperative and postoperative clinicopathological factors were examined. In the first analysis only the Gleason score (pre and post operative) and the postoperative margin status were significant clinicopathological factors, whilst in this study the Gleason score (only preoperatively), the serum PSA and the pathological stage were significant (margins were not included in the analysis). In study 2 the Gleason score postoperatively was significant univariately but not multivariately, which is not what was found by Brewster *et al* [1999]. The statistical analysis in study 2 used a continuous range of PSA, rather than a cut-off of 10 ng/ml used by Brewster *et al* [1999], which may have resulted in this factor becoming significant. The presence or absence of capsular penetration (ie pathological stage pT2 or pT3) appears significant in the later analysis with a relative risk of 5.68 between the two. The stage was not significant in the earlier analysis and this may have been due to either the longer follow-up or a different definition for the PSA recurrence in this thesis.

In study 3, 129 patients from the cohort were studied. These patients were selected on a basis of available follow-up. Neo-adjuvant therapy was given to 11 patients prior to surgery and the two statistical analyses with and without these patients showed similar results. More clinicopathological variables were included in study 3 than in study 2 as there were more patients and fewer tumour biomarkers examined. 16 patients had seminal vesicle involvement and 49 patients had involvement of the

surgical margins. When the prostate gland is removed the surgeon can cut into the prostate leading to tumour at a surgical margin (termed intraprostatic surgical margin), but if the tumour does not extend through the capsule in another part of the prostate then the tumour can be margin positive but capsular invasion negative. Of the 129 patients 37 had positive margins but were pT2 whilst 12 had positive margins but were pT3 (data not shown). This study did not differentiate between the intra or extraprostatic circumferential margins and the apex or bladder base margins. Tumour present at any of these margins was considered as a positive margin. All the clinicopathological factors were significant on univariate analysis at the 10% level but on multivariate analysis only the PSA, pathological stage and margins were significant. These results were similar to study 2, as one would have expected considering that over half of the patients were the same.

Epstein studied a group of 721 men who had undergone radical prostatectomy with a mean follow-up of 6.5 years [Epstein, 1998]. He used a definition of progression as a serum PSA of >0.2 ng/ml and none of the patients had received neo-adjuvant therapy. He found that 68% and 52% were free from progression at 5 and 10 years respectively, which compares with the data from 217 patients of this cohort of 72% and 40% (Table 6). He found that the Gleason score, seminal vesicle involvement, capsular penetration and margin status all significant univariately and on multivariate analysis on 617 men found the Gleason score, capsular penetration and surgical margins were significant [Epstein *et al*, 1996].

Banerjee *et al* [2000] studied a group of 485 men defining a biochemical recurrence as ≥0.4 ng/ml and that race, preoperative PSA, pathological stage and Gleason score

(postoperatively) were all significant multivariately. In this study they separated the Gleason scores into three groups, namely 6 and less, 7 and greater than 7. The aim of their study was to develop prognostic groups based on clinicopathological data and using a technique called recursive partitioning or tree-structured survival analyses. This technique uses a yes/no tree to separate various prognostic groups and the authors felt this would enable clinical trials to separate patient groups [Banerjee *et al*, 2000]. Both these large studies compare favourably with the results in study 3 though Gleason score was not significant multivariately in this series this may have been due to differences in the grouping.

p53, bcl-2, CD44, and E-cadherin in preoperative biopsies and in radical prostatectomies as predictors of biochemical recurrence following radical prostatectomy.

There has been increasing interest in the use of immunohistochemical markers as predictors of outcome following radical prostatectomy. Studies 2 and 3 aimed to examine the usefulness of these markers.

In study 2 preoperative biopsies and radical prostatectomy specimens were examined. The single major problem was the amount of tumour within the needle cores. As further markers were examined there became insufficient tumour for analysis. By the time CD44 staining was carried out only 57 patients could be assessed, this made the multivariate analysis impossible and required the exclusion of CD44 from the preoperative dataset. Two of the radical prostatectomy specimens showed no evidence of tumour, the so-called ‘vanishing cancer phenomenon’ [Goldstein *et al*, 1995]. There were also further problems with small volume

tumours being cutout of the radical prostatectomy specimens, but this was less common than in the preoperative biopsies.

Study 2 studied the adhesion molecules CD44s and E-cadherin as both have been shown to predict recurrence after radical prostatectomy [Umbas *et al*, 1994 and Kallakury *et al*, 1996]. Abnormal CD44 immunostaining varied greatly between the biopsies (81%) and the radical prostatectomies (63%). This discrepancy was even more marked for E-cadherin with 11% abnormal for the biopsies and 47% for the radical prostatectomies. Ruijter *et al* [1997 and 1998] showed that tissue fixation affected E-cadherin immunohistochemistry. When they compared how well biopsies correctly identified radical prostatectomies with abnormal E-cadherin staining they found a very low sensitivity of 15%. They felt this was due to the heterogeneity of tumour staining within the prostates. Study 2 appears to confirm the inaccuracy of biopsy E-cadherin staining and this may also explain the differences found with CD44.

Neither CD44 nor E-cadherin immunostaining in the radical prostatectomy specimens were significant univariately in predicting biochemical recurrence following radical prostatectomy. The study by Richmond *et al* [1997] looked at TURP specimens and found a correlation between abnormal E-cadherin expression and patient survival but 39% of their group already had bony metastases at presentation. Umbas *et al* [1994] studied a group of patients treated by various modalities and included 42 patients who underwent radical prostatectomy (three of whom had also received neo-adjuvant therapy). They used frozen tissue samples and found abnormal E-cadherin expression was significantly related to progression.

Cheng *et al* [1996] correlated E-cadherin expression with the Gleason score using paraffin wax sections but to this date no study, apart from study 2, has examined E-cadherin in paraffin wax sections as a predictor of recurrence after radical prostatectomy.

Noordzij *et al* [1997] studied CD44s immunohistochemistry in 97 radical prostatectomies and found an inverse correlation with Gleason score and pathological stage. There was a significant relation between CD44s and clinical progression in this group and this was also true for PSA progression though they only had PSA data on 29 of the patients. This study has a longer follow-up (mean of 84 months) in comparison to study 2 (mean of 65 months) but has a total mortality of 35% (13% of tumour related disease, 22% of unrelated causes) whilst the total mortality in study 2 is 11%. The differences in mortality rates and the small number of patients with PSA data in this study make direct comparison impossible. Study 2 shows that CD44s and E-cadherin are not useful as predictors of recurrence.

In the earlier analysis of this data by Brewster *et al* [1999] p53 was the only significant preoperative tumour biomarker on multivariate analysis, though bcl-2 was significant univariately. With further follow-up in this thesis the preoperative p53 still remains significant multivariately but the other markers are all univariately significant. Previously p53 was also a significant postoperative marker [Brewster *et al*, 1999] but this was not found in this later analysis, and it was not even univariately significant. In study 2 the postoperative bcl-2 was confirmed to be significant multivariately after a longer follow-up period. E-cadherin and CD44 were not significant univariately either in this analysis or in Brewster *et al* [1999].

The earlier analysis of the data in study 2 was the first time that all four of these biomarkers had been examined in pre- and postoperative biopsies with correlation with the outcome after radical prostatectomy [Brewster *et al*, 1999]. Since publishing the data a subsequent study by Stackhouse *et al* [1999] looked at p53 in 129, and bcl-2 in 103, preoperative biopsies and matched postoperative radical prostatectomies. These markers had previously been studied in the same radical prostatectomy specimens by Bauer *et al* [1996b]. Stackhouse *et al* [1999] found bcl-2 positive in 17%, and p53 positive in 50% of the biopsies, whilst in this thesis bcl-2 was positive in 11% and p53 in 64%. They did not find either significant univariately at predicting biochemical relapse (defined as a PSA of 0.5 ng/ml or greater or two serial measurements of 0.2 or greater). This paper also updated the series of 199 patients reported by Bauer *et al* [1996b] and confirmed that both p53 and bcl-2 were significant postoperative predictors on multivariate analysis. They concluded that the differences were probably as a result of some of the biopsies sampling foci of tumour which were unreactive for p53 or bcl-2, whilst the radical prostatectomy specimens contained multifocal tumour some of which were positive. This theory was supported by the fact that they found p53 positive in 50% of the biopsies but 68% of the radical prostatectomies, and bcl-2 positive in 17% of the biopsies and 33% of the corresponding radical prostatectomies [Stackhouse *et al*, 1999].

Both study 2 and the study by Stackhouse *et al* [1999] cover a group of patients who no longer reflect the patients undergoing biopsy and radical prostatectomy today. A significant proportion of patients in these two series were identified as palpable

tumours, but with the advent of PSA testing in non-symptomatic patients and increased use of sextant biopsy more tumours are identified at an earlier stage (stage T1c). These populations of patients still have limited follow-up but future data may show different results. Also with the use of systematic rather than directed biopsy, more tissue cores are taken so there is an increased likelihood that representatives of all the focal tumours would be sampled and this may allow greater accuracy for immunostaining in the future.

In study 3 the number of patients was increased to 129 and the biomarkers p53 and bcl-2 were examined in the radical prostatectomies. The incidence of positive p53 immunostaining was 71%, which was considerably higher than in study 2 (55%). This could be accounted for by the increase in low level staining, ie staining between 1 and 10%. The bcl-2 staining was similar between the two studies, at 20% and 17% respectively. Although some authors use a cut-off of >0% [Bauer *et al*, 1996b], others have used a cut-off of 10% as a level for positive staining [Bubendorf *et al*, 1996b, Theodorescu *et al*, 1997, and Matsushima *et al*, 1997] [reviewed by, de la Taille *et al*, 1998]. Statistical analysis was carried out using both these cut-offs and this did improve the level of significance univariately but not multivariately. The affect of neoadjuvant therapy is not well understood and as a result most studies exclude this subset of patients. In study 3 the statistical analysis showed that there was no difference in the results if these patients were included or excluded.

bcl-2 and p53 proved to be significant predictors of recurrence in univariate analysis but they were less significant than all the clinicopathological variables studied. On multivariate analysis neither bcl-2 nor p53 were significant in study 3, though bcl-2

had been significant in the smaller group studied in study 2. In study 3 margin status was also included and this was highly significant univariately, this would have replaced bcl-2 in the multivariate analysis. These results are conflicting with other authors as well as the earlier analysis of the data in study 2 [Brewster *et al*, 1999], but Bubendorf *et al* [1996] did not find p53 significant but did find bcl-2 significant. Even in those studies that found p53 and bcl-2 significant the sensitivity and specificity was too poor for them to advocate using either as a diagnostic test [Stackhouse *et al*, 1999].

Amplification of Her-2/neu in prostate cancer

Prostate cancer appears to lag behind the developments made in breast cancer, for example immunohistochemical assessment of oestrogen receptor status is now undertaken in every breast tumour in the UK. In the United States of America there is now Food and Drug Administration (FDA) approval for two FISH assays of Her-2/neu amplification in certain clinical applications in breast cancer [Wang *et al*, 2000]. One of these FISH assays – INFORM™ uses the Quantum Appligene probe (used in this study) (Ventana Medical Systems, Tuscon, Arizona), whilst the other PathVysion™ uses the Vysis probe (Vysis, Downers Grove, IL). The availability of these probes and the therapeutic agent Herceptin has led to increased studies of Her-2/neu in all tumours.

Early studies in prostatic carcinomas using immunohistochemistry to detect Her-2/neu gene products showed varied results. This was probably due to different antibodies, antigen retrieval, and small patient numbers. Similar variations have

been found in breast cancers but with the introduction of a standardised technique (HercepTest ^{TM}) these problems should be resolved. To date, no study of prostate cancer using this technique has been published.

FISH has shown there to be a high percentage of gene amplification in those cases of breast cancer showing protein overexpression. In prostate cancer, one group has found amplification of Her-2/neu oncogene in up to 44% of cases and this was associated with advanced pathological stage and higher Gleason score [Ross *et al*, 1997a and b, Kallakury *et al*, 1998]. Recent work by this group has shown a lower amplification rate of 10 to 25% (personal communications, Prof Ross). Mark *et al* [1999] using the Vysis probe found an amplification rate of 9%. The Vysis Her-2/neu probe has the great advantage over the Quantum Appligene probe in that it also contains an internal control of a chromosome 17 alpha satellite probe. As a result two colour FISH can be used and this allows a ratio of number of chromosome 17s to Her-2/neu to be calculated. The major drawback of two colour FISH is its reliance on computer assisted analysis, which may restrict the application of this technology to larger centres.

The largest study of prostate cancers and Her-2/neu amplification used a combination of FISH using the Vysis probe and microarrays [Bubendorf *et al*, 1999]. This technique allowed 262 separate tumours to be successfully assessed by taking small samples (0.6 mm in diameter) and mounting them on a single slide, which is then used for FISH. Microarrays have a great advantage in that they can screen large numbers of tumours for gene amplifications but there can be sampling error, as most tumours are heterogeneous. Bubendorf *et al* addressed this by using

tumours from different stages in the disease from localised to metastatic. They found Her-2/neu was not amplified at any stage of the disease.

The differences in amplification rates in these studies appear to be due to the definition of amplified (Table 16). Ross *et al* [1997a and b] made no control for polysomy whilst Mark *et al* [1999] used a ratio of Her-2/neu : chromosome 17 signals of 1.5 and Bubendorf *et al* [1999] used a ratio of 3. Although Bubendorf *et al* found none amplified there was a single case in the study by Mark *et al* with a ratio of 3.

Table 16: Previously reported criteria and amplification rates of Her-2/neu in prostate cancer

Author	Patient numbers	Probe	Criteria for amplification	% Amplified
Ross *et al* [1997a,b]	**113**	**Quantum Appligene**	**≥5 signals in ≥20%**	**41%**
Mark *et al* [1999]	**86**	**Vysis**	**ratio ≥1.5**	**9%**
Bubendorf *et al* [1999]	**262 tumour microarrays**	**Vysis**	**ratio ≥3**	**0%**
Study 4	**114**	**Quantum Appligene**	**≥5 signals in ≥20%**	**1.75%**
			ratio ≥2	**0%**

Study 4 is the largest series to date using complete sections of prostatic tumours as opposed to microarrays and is the first using enzymatic detection. The major

advantages of enzymatic detection are that it provides a permanent record and does not require fluorescent microscopy for scoring. Using the criteria for amplification as discussed by Ross *et al* [1997a] we found two cases of Her-2/neu amplification but if chromosome 17 polysomy is allowed for then none of these should be considered amplified. The low amplification rate found in study 4 when compared to Ross *et al* may have been due to the use of enzymatic rather than fluorescent detection, but it probably reflects the findings of the other groups (Table 16). A recent paper comparing the two probes in breast cancers found that there was concordance in 98% [Wang *et al*, 2000] but they used a cut-off ratio of 2 for the Vysis probe.

Defining a subset of clinically localised prostatic carcinomas that will have a recurrence post-prostatectomy has led to several biological markers being examined. Her-2/neu oncogene does not appear to be commonly amplified in early stage disease and as a result will not prove useful. Increased copy number does appear to happen in advanced stage and in hormone unresponsive cases. Intriguingly early studies in xenografts of the new therapeutic agent Herceptin have not been shown to be useful in hormone unresponsive tumours.

Conclusion

There is an increasing incidence of prostate cancer in men under the age of 60 years in this country and this seems unrelated to the increased detection of subclinical cases [Post *et al*, 1999]. This thesis examined clinicopathological variables and tumour biomarkers in order to see whether any would be useful prognostic indicators.

Ductal carcinoma of the prostate is an easily identifiable histological variant of adenocarcinoma of the prostate. This study confirmed its prostatic origin and the presence of androgen receptors. It often occurs in the presence of high-grade microacinar tumour and its treatment and prognosis are no different.

Although radical prostatectomy aims to cure clinically localised prostatic adenocarcinoma at least 60% will have evidence of biochemical recurrence after 10 years. This thesis has shown that the clinical stage and margin status are both significant predictors of recurrence. These data were derived from the postoperative specimen and so the search for preoperative markers led to the examination of tumour biomarkers in preoperative biopsies. Study 2 showed that biopsy p53 was a significant marker of recurrence as were the Gleason score and the serum PSA. When p53 immunohistochemistry is used on the radical prostatectomy specimens it appears to be only univariately significant. bcl-2 was not significant in the biopsy but showed multivariate significance in a limited series of radical prostatectomies. CD44 and E-cadherin did not appear to have any significant predictive ability.

Study 4 showed that gene amplifications can be studied in archival material using an enzymatic in situ hybridisation technique. Her-2/neu does not appear to be amplified in clinically localised prostate cancer. The results confirm the findings of other groups using standard FISH techniques.

Overall this thesis found:

1. A patient with a preoperative Gleason score of 7 or above is 6 times more likely than a patient with a Gleason score below 7 to have a recurrence after radical prostatectomy.
2. Patients with a preoperative PSA above 20 ng/ml are 6 times more likely to recur after radical prostatectomy than patients with a serum PSA below 20 ng/ml.
3. Patients with capsular breach are 4 times more likely to recur after radical prostatectomy than patients with organ confined disease.
4. The presence of positive surgical margins is a poor prognostic indicator.

In conclusion, the low sensitivities and specificities of the tumour biomarkers studied at predicting recurrence will mean that these will still remain a research tool rather than a diagnostic tool. PSA and Gleason score enable us to define a high risk group before they have surgery but we need to define a group of patients using other preoperative markers as one in five patients with either a Gleason score below 7 or a PSA below 20 ng/ml can also recur. This need is even more pressing as more men will be diagnosed with early, clinically localised prostate cancer as a result of increased detection.

Acknowledgements

Clinical data

Mr David Gillatt performed all the radical prostatectomies and allowed me to study the series. Mr Simon Brewster and Mr Mathius Winkler were both research registrars at the Bristol Urological Unit and provided me with detailed follow up on the patients.

Epidemiological data

Alistair Harvey and Gareth Davies at the South West Cancer Intelligence Unit gave advice and data.

Funding

Funding for this work came from the Showering Fund and the Southmead Research Foundation.

Immunohistochemistry

The majority was performed by Clive Abbott, with help from Karen Sibley, Hanna Lord, and Haydn Kendall at Southmead Hospital.

In situ hybridisation

Dr Adrian Charles gave me advice and Stewart Swann cut the tissue sections.

Statistics

All statistics were performed by Dr Chris Rogers and Kate Parry at the R & D Unit, Southmead.

Supervision

Dr Angus MacIver inspired my interest in prostatic pathology, but sadly he died before any of these projects could be published. Dr Dani Peat and Prof David Lowe gave useful advice and guidance on writing this thesis.

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