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INVESTIGATION OF A TUMOUR SUPPRESSOR GENE AT CHROMOSOME 10q23.3 IN PROSTATE CARCINOMA.

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ABSTRACT

Using various molecular genetic techniques, attempts have been made to identify a tumour suppressor gene (TSG) in prostate carcinoma. This gene will act as a genetic marker to identify patients at risk of disease progression from prostate cancer. The region at chromosome 10q23-24 is postulated to contain a TSG that plays an important role in the development and progression of tumours as it is deleted in a number of cancers including glioblastomas, endometrial and prostate cancer. In glioblastomas, chromosome 10 is partially or entirely deleted in approximately 90% of tumours.

The TSG called *Pten* has been identified on chromosome 10 in the region 10q23.3. The significance of loss of the *Pten* gene in prostate cancer is unknown. In this thesis, the clinical significance of single nucleotide polymorphisms (SNP'S) in the chromosomal region 10q23-24 was evaluated. Comparisons in the distribution of polymorphisms between Ninety-five prostate cancer patients and forty-three non-prostate cancer patients were made. Three intronic polymorphism markers within the *Pten* gene were used: a single-base A→G substitution in intron A, 96 bp upstream of exon 2. A 5-bp ATCTT insertion / deletion in intron D, 110 bp downstream of exon 4 and a single-base T→G substitution in intron H, 32 bp downstream of exon 8. This study did not isolate

any particular trend in polymorphism distribution in the *Pten* gene when comparisons were made between the two study groups.

The significance of loss of *Pten* gene in thirty-four prostate cancer patients was evaluated using four highly informative microsatellite markers: D10S541, D10S2492, D10S1765 and AFMa086wg9 located within and around the *Pten* locus. DNA was extracted from prostate cancer cells following microdissection of archival paraffin embedded radical prostatectomy specimens. Loss of heterozygousity (LOH) studies was performed on matching blood/tissue DNA using these four microsatellite markers.

For these case specimens, frequency of allele loss of 48% was found at the D10S541 locus; 39% at D10S1765; 32% at D10S2492 and 22% at the AFMa086wg9 locus. At all four microsatellite, the mean (range) LOH was 35.25% (22%-48%). Of the 34 case specimens 17(50%) showed LOH in at least one of the informative marker sites.

Correlating the significance of this region of LOH with pathological staging and known prognostic indicators in prostate cancer showed that the loss of the *Pten* gene was associated with late stage disease and likely to be involved in disease progression in prostatic adenocarcinoma.

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ABBREVIATIONS

A	Adenine nucleotide
AKT-1	serine/threonine kinase enzyme
APS	ammonium persulphate
AR	Androgene receptor
Bp	Base pairs
BPH	Benign prostatic hyperplasia.
C	Cytosine nucleotide
CA	Cancer of the prostate
C-onc	Cellular oncogene
CT	Computer Tomography.
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
dNTPs	deoxy-nucleotides triphosphate.
DRE	Digital rectal examination
EDTA	Ethylenediaminetetracetic acid
FSH	Follicle-stimulating hormone
G	Guanine nucleotide
H & E	Hematoxylin and Eosin stain
HETERO	Heterozygous
HOMO	Homozygous
ICRF	The Imperial Cancer Research Fund.
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LOH	Loss of heterozygousity

Mb	Megabases
MMAC1	Mutated in advanced cancers 1
MRI	Magnetic Resonance Imaging
NA	Not applicable.
PCR	Polymerase chain reaction
PIC	Polymorphic information content
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate specific antigen
Pten	Phosphatase and tensin homologue deleted from chromosome 10
PTKs	Protein tyrosine kinases
PTPs	Protein tyrosine phosphatases
RFLP	Restriction Fragment Length Polymorphism
RRP	Retropubic radical prostatectomy
SNP's	Single nucleotide polymorphisms.
SSLPs	Simple sequence length polymorphisms
STRs	Short tandem repeat polymorphisms
T	Thymine nucleotide
TEMED	N, N, N′, N′ – tetramethylethylenediamine
TEP1	Transforming growth factor β regulated and epithelial cell enriched phosphatase 1
TRUS	Transrectal ultrasound
TSG	Tumour suppressor gene
TURP	Transurethral resection of the prostate.
v-onc	Viral oncogenes

2.1 Materials and Reagents

Reagents:	Company/Origin
Agarose (2%)	GibcoBrl Invitrogen life technology Tel: 0800 269 210 Email:ukorders@invitrogen.com
AmpliTaq Gold DNA polymerase.	Applied Biosystems 7 Kingsland Grange Woolston Warrington Cheshire, WA17SR Tel: 01925 825650
dRhodamine dye terminators	Applied Biosystems 7 Kingsland Grange Woolston Warrington Cheshire, WA17SR Tel: 01925 825650
Ethidium bromide	Sigma-Aldrich Company Ltd. The Old Brickyard New Road Gillingham Dorset. SP8 4XT Tel: 0800717181
Orange G	Sigma-Aldrich Company Ltd. The Old Brickyard New Road Gillingham Dorset. SP8 4XT Tel: 0800717181
PCR Buffer	Applied Biosystems 7 Kingsland Grange Woolston, Warrington Cheshire, WA17SR Tel: 01925 825650

PCR buffer (15mM $MgCl_2$)	Boehringer Ingelheim Ltd Ellesfield Avenue Bracknell, Berkshire. RG12 8YS Tel: 01344 424600
Proteinase K	Sigma-Aldrich Company Ltd. The Old Brickyard New Road, Gillingham Dorset. SP8 4XT Tel: 0800717181
Puregene™ DNA isolation kit.	FLOWGEN –Gentra Findel House, Excelsior Road Ashby Park, Leicestershire LE65 1NG .Tel 0845 120 4519
QIAquick PCR Purification tubes	QIAGEN HOUSE FlemingWay Crawley,WestSussex RH109NQ. Tel: 01293-422911
ΦX174 DNA/*Hae* III Marker	Promega UK Ltd Delta House Chilworth Science Park Southampton SO16 7NS Tel:0800378994
TAMRA 500bp ladder	Perkin-Elmer. Warrington, chershire Tel: 01925 825650
TBE (Tris, Borate, EDTA)	Imperial Cancer Research Fund Clare Hall Labouratories South Mimms Potters Bar, Herts, EN6 3LD

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I would like to dedicate this thesis to the memories of my late father Dr Richard Adejumo Ajayi and my late brother Mr Adekola Adeniji Ajayi. May their souls rest in perfect peace.

I carried out the experiements reported in this thesis at the Imperial Cancer Research Technology, Dominion House, Barbican. The histopathologist at The Royal Free hospital, Dr Mike Jarmulowicz, performed the mapping of high-density prostate adenocarcinoma cells on H & E stained slides. I manually performed the microdissections of the prostate tissue on the slides.

PUBLICATIONS ARISING

The Expression profile and genomic organisation of the tumour suppressor gene PTEN. Hamilton JA, Stewart LMD, Ajayi LA, Roberts KG, Kaisary AV, Snary D Br J Cancer. May 2000.82(10) 1671-6.

My contribution to this paper involved collection of 138 blood samples, DNA extraction and analysis of three polymorphism sites.

POSTERS AND PRESENTATIONS ARISING

Single nucleotide polymorphism analysis in the *Pten* gene on patients with Prostate Cancer. Ajayi LA, Stewart LMD,Simmonds N, Snary D, Kaisary AV. The 6th Mediterranean Congress of Urology, Cairo, Egypt, September 1999.

DNA extraction technique using Microsatellite markers in paraffin embedded radical prostatectomy specimens. Ajayi LA, Roberts KG, Stewart LMD, Snary D, Kaisary AV. Urology research society. Royal college of Surgeons, January 2000.

Use of microsatellite markers as genetic markers to identify risks of tumour progression in prostate cancer. Ajayi LA, Stewart LMD, Roberts KG, Snary D, KaisaryAV. British association of urological surgeons. Birmingham 2000.

LOH studies on PTEN gene in prostate cancer. Ajayi LA, Stewart LMD, Roberts KG, Snary D, KaisaryAV. British Prostate Group meeting. Newport. November 2000.

The significance of the *pten* gene in prostate cancer. Ajayi LA, Stewart LMD, Roberts KG, Snary D, KaisaryAV. Societe Internationale d'urologie, section of oncology, Sharm-el-sheik, Eygpt. October 2003.

CHAPTER ONE

INTRODUCTION

1.1 Prostate Cancer

1.1.1 Demographics

Adenocarinoma of the prostate is the most common non-skin cancer in men. In 2003, an estimated 220,900 men will be diagnosed with this disease and 28,900 will die from prostate cancer in the USA (Jemal A et al, 2003). Public health records document that men in USA have a one in six lifetime risk of developing prostate cancer (Jemal A et al, 2003). Overall death rate from prostate cancer in the USA in the year 2000 was 30.6 per 100 000 (Stewart SL et al, 2004).

In the European Union, there are approximately 95,000 new cases registered and 35,000 men dying from the disease every year (Jensen OM et al 1990) and represents 12% of all new cancers diagnosed (Black RJ et al, 1997).

In England and Wales, it is a significant cause of morbidity with 23,109 new cases diagnosed in 2000 with an annual death rate of 8,973 for that same year (ONS, 2001). In the British association of urologogical surgeon's registry for 2001, there was 12,892 new prostate cancers registered, representing 53% of all new cancers and 23 new cases per Consultant for that year (BAUS Cancer Registry, 2001)

The uses of serum prostate specific antigen (PSA), transrectal ultrasound (TRUS) and biopsy technology as well as interests in detecting this disease (increase public awareness, media interest and screening) are major influences in the increase in the incidence of prostate cancer (Brawley OW and Kramer BS 1996).

The mortality from prostate cancer in the USA has showed a decline in trend at an average rate of 1% per year since 1992 (Mettlin CJ et al, 2000), Figure 1.1. This was felt to be due to the successful impact of PSA screening in the USA population. However, In the UK where the population screening for prostate cancer is not common practice, the reduction in mortality rate has been found to mirror that of the USA (Oliver SE et al, 2000). This contrasting observation questions the validitity of PSA testing as a screening tool. It has also focused the attention of researchers working in prostate cancer field on other factors including genetic, dietry and environmental factors as contributory factors responsible for the reduction in mortality rate in prostate cancer.

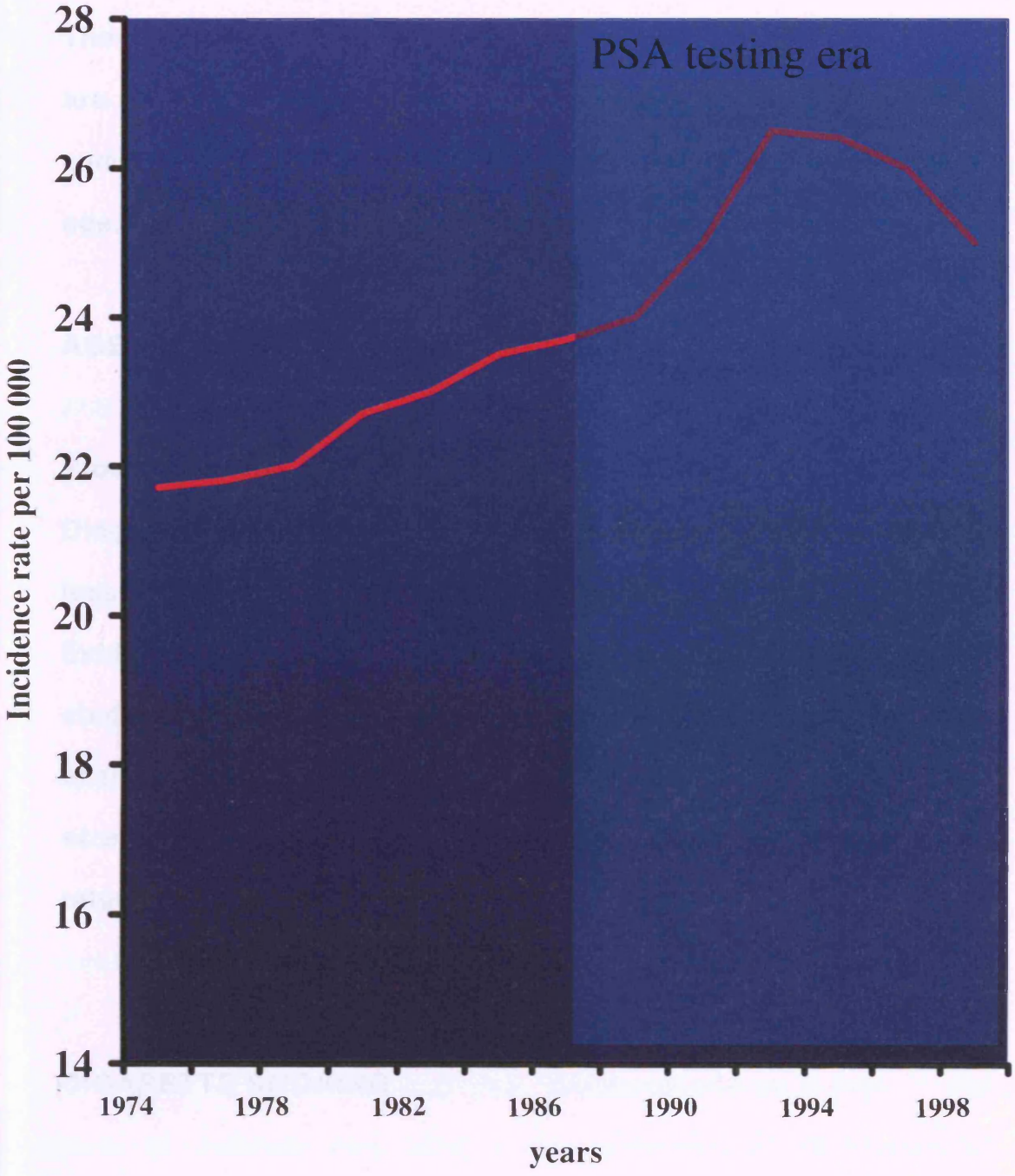

Figure 1.1: **Age-adjusted mortality rate in the Pre-& Post-PSA era from the SEER program in USA** Adapted from, Mettlin et al. *Microsc Res Tech* 2000;51:415-8

1.1.2 Aetiology

The mechanisms involved in the pathogenesis of prostate cancer are unknown. The disease is thought to occur as a result of a complex and yet unclear interaction between the environment, age, race, hormones, dietary fat, and genetic factors.

AGE

Prostate cancer has been known as a disease of elderly men. Diagnosis of prostate cancer is rare before the age of 50 with less than 1% of cases being diagnosed under the age of 40. Evidence of prostate cancer has been found in post-mortem studies on young males aged between 30 –39 years old who died of trauma and had no clinical history of prostate cancer (Sakr WA *et al* 1993). After the age of fourty, the incidence and mortality rates both increase exponentially.

CIGARETTE SMOKING

The effect of cigarette smoking on the epidemiology of prostate cancer is inconclusive and difficult to interpret. Cigarette smoking has been well established as a cause of human malignancies such as lung cancer and has been associated with the

development of certain urological malignancies especially bladder cancer. Plaskon, reported on a population-based case-control study on middle aged men and found a dose-response relationship between the number of pack-years smoked and prostate cancer risk (Plaskon LA, 2003). They also reported that cessation of smoking resulted in a decline in risk.

DIET

Dietary patterns have long been implicated in the development of different types of malignancies. Consumed food components may be metabolised to carcinogens, may alter hormonal balance, or may contain vitamins, minerals, and nutrients that could protect against the development of certain cancers. Dietary differences among racial groups, socio-economic classes, and geographic sites may explain some of the differences noted in prostate cancer epidemiology and behaviour.

There are studies showing that increased energy intake in the form of calories may lead to increase risk of carcinoma of prostate. A study by Grönberg H (1996) showed that men who considered they eat "somewhat more" or "much more" than the general population had odds ratios of developing prostate cancer of 2.22 and 3.89 respectively. The study also showed Body Mass

Index >26kg/m² had significantly increased risk of developing prostate cancer. This study implied that excess of calories or deficiency in other nutrient factors may influence carcinogenesis.

Soya is a dietary compound widely consumed in Japan: a nation with one of the lowest incidences of prostate cancer in the world. Soyabean products, whole grain cereals, seeds, nuts and berries are believed to have a protective effect against the development of prostate cancer. Phytoestrogens, including lignans and isoflavonoids such as genistein and daidzein contained in soyabeans and cereals may confer protection against prostate cancer (Landstrom M *et al* 1998). A typical western diet contains practically no soy products and a minute amount of phytoestrogens.

It is hypothesized that intestinal bacteria convert these substances into hormone-like compounds with antioxidative activities as well as other activities such as, inhibition of 5-α-reductase and 17β-hydoxysteriod dehydrogenase, inhibition of angiogenesis and endothelial cell proliferation (Adlercreutz H, 1995). These effects produce a profound inhibitory influence on malignant transformation, cell proliferation and angiogenesis.
Vitamin A or retinoic acid is essential for normal differentiation of epithelial cells. Vitamin A deficiency has been linked to a variety

of malignancies. It has been shown to have a chemoprotective role in head and neck malignancies (Kakizoe T, 2003). Retinoic acid is the metabolically active form of Vitamin A. It is fat-soluble and intake from vegetable sources is inversely proportional to prostate cancer incidence (Clinton SK *et al*, 1996). The benefit of dietry Vitamin A remains uncertain.

Lycopene is a carotenoid that cannot be converted to Vitamin A. Dietary lycopene can be found in fruits such as tomatoes, guava and watermelon. Lycopene is a potent antioxidant and the most significant free radical scavenger among the carotenoid family. In a large Seventh-day Adventist cohort study, Giovannucci reported that tomato consumption was strongly associated with reduced prostate cancer risk (Giovannucci E, *et al* 1998). This unique association suggests that lycopene supplementation may be beneficial in preventing progression of prostate cancer.

Vitamin E, is a major intracellular antioxidant in cell membranes, inhibits lipidperoxidation and has been demonstrated to have a wide range of anti-cancer properties. These properties include both protection against carcinogenesis and inhibition of tumour progression. The precise pathways of vitamin E's beneficial benefits are largely unknown.

Although Vitamin D is called a vitamin – it is in fact a steroid hormone. Vitamin D is naturally synthesised under the influence of ultraviolet (U-V) light with conversion into biologically active compounds by hydroxylation in the liver and kidneys. Although vitamin D is primarily involved in bone and calcium metabolism, there is increasing evidence that vitamin D may be important in preventing prostate cancer. The first suggestion that Vitamin D might be related to prostate cancer came from an epidemiological study, which showed that individuals with the highest calcium intake (which naturally suppresses vitamin D) have a 3-fold higher risk of prostate cancer (Schwartz GG and Hulka BS. 1990). A prospective evaluation of men in the Health professional follow-up study showed that men with high consumption of calcium had a relative risk of 2.97 of developing advance prostate cancer (Chan JM *et al*, 1998).

Rotruck and colleagues elucidated the biochemical function of selenium by demonstrating that glutathione peroxidase; an enzyme that protects the cell from oxidative damage is selenium dependent (Rotruck *et al*, 1972). A randomised control study of 58,279 men in the Netherlands showed an inverse correlation with selenium and risk of prostate cancer (Van den Brandt *et al*, 2003).

Although the precise mechanism by which dietary factors contribute to the prevention of prostate cancer is unknown, increasing evidence suggests that it may exert its effects by inhibiting oxidative damage within the prostate epithelium. Oxygen free radicals and oxidative damage to biomolecules is a major focus of recent aetiological cancer research (Sikka SC, 2003). There is strong evidence that oxygen free radicals generated both endogenously and from external sources are associated with carcinogenesis and cancer progression. Oxygen free radicals are generated endogenously as by-products of normal metabolic processes and cause oxidative damage to important biomolecules such as lipids, proteins and DNA.

In conclusion, the dietry factors discussed above may be instrumental in preventing or slowing the growth of prostate cancer. The difference between latent and clinical prostate cancer suggest that diet may be influential in the progression of the disease. However, definitive studies are lacking.

HEAVY METAL EXPOSURE

The prostate gland contains the highest level of Zinc of all organs in the body. Zinc is an essential ingredient of proper enzyme function and is required for DNA and RNA replication and repair. Cancerous prostate glands contain less zinc than normal glands (Platz EA and Helzlsouer KJ, 2001). No correlation has been found between serum levels of zinc and prostate cancer, but one study suggests that men with zinc intake of up to 100mg/day had a relative risk of advance prostate cancer of 2.29 (Leitzmann MF *et al*, 2003).

Cadmium is a trace element known to act as an inhibitor of zinc metabolism. Overexposure to this agent also has been implicated in prostate cancer pathogenesis (Achanzar WE *et al,* 2001). High levels of cadmium may result from industrial exposure in those who work with batteries, paint and metal weldering. Environmental sources of cadmium include cigarette smoke, water and soil. Prostate cancer mortality in workers with long term exposure to high levels of cadmium has been disproportionately high, and cadmium workers often have more aggressive tumours (Elghany NA *et al*, 1990). Cadmium exposure may contribute to prostate cancer risk directly, or the risk may result from the effect of cadmium on zinc availability.

HORMONES

The prostate gland consists of stromal and epithelial elements under androgenic influence. The hypothalamus produces luteinizing hormone-releasing hormone (LHRH), which stimulates the anterior pituitary to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates leydig cells of the testicles to produce testosterone. Testosterone enters the prostate and is converted by the enzyme 5-α -reductase type 2 into dihydrotestosterone (DHT), the active metabolite influencing prostatic development. Figure 1.2 shows endocrine control. Other hormones such as oestrogen and prolactin may also influence the growth of the prostate gland, either directly or indirectly by negative feedback inhibition.

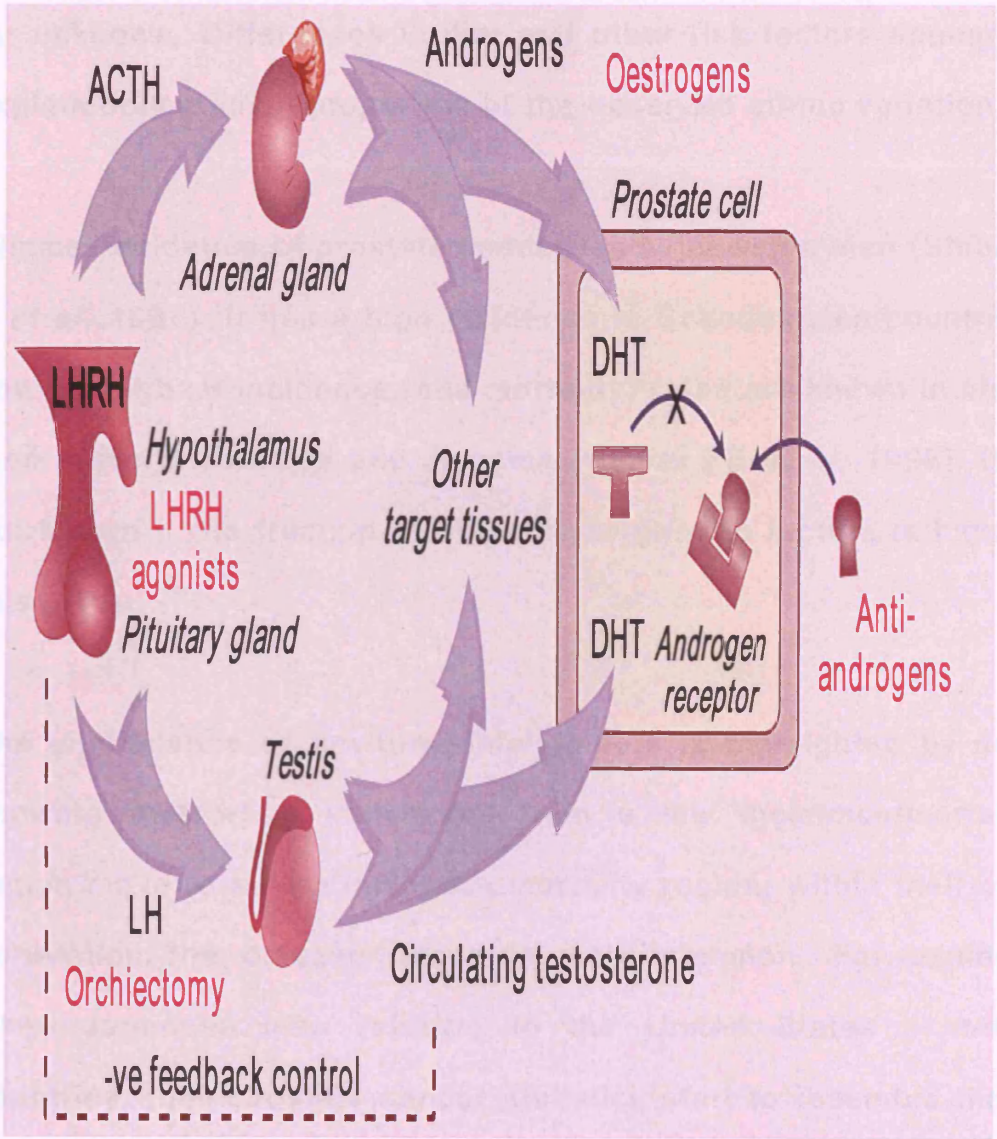

Figure 1.2: **Sites of action of hormonal therapy**
The hormones produced by the organs responsible for prostate cell function.

RACE

Prostate cancer incidence rates vary significantly between ethnic groups (Hsieh K and Albertsen PC, 2003). This variation is probably due to a combination of genetic and environmental factors, although the relative contribution of each of these is so far unknown. Differences in diet and other risk factors appear to explain only a small proportion of the observed ethnic variation.

Clinical incidence of prostate cancer is low in Asian men (Shibata A *et al,* 1997). It has a high incidence in Scandinavian countries, and the highest incidence (and mortality) rates are known in black men in North America and Jamaica (Glover FE *et al,* 1998). It is not known if the fraction attributable to genetic factors is high in this group.

The significance of environmental factors is highlighted by data showing that when individuals from a low incidence/mortality region move to a high incidence/mortality region, within their own generation the disease becomes more common. For example when Japanese men relocate to the United States or other countries, their prostate cancer statistics start to resemble those of the local population (Shimizu H *et al,* 1991). Younger age at the time of relocation and the length of time living in the new environment appears to correlate with the increase incidence.

In contrast, African –American men have a 60% higher incidence and a 2.3 times higher mortality rate than white men living in the same geographic location (Crawford ED 2003). Thus, although black men have more advanced disease upon diagnosis (40% are more likely to present with metastases), they have a higher mortality rate even when compared with white men presenting with identical disease stages (Crawford ED 2003).

To understand the genetic, environmental, or social basis of the epidemiological differences noted between black and white Americans, it is important to understand the biology of prostate cancer in Africa. These data are sparse and have started to accumulate only recently. Because of political instability, economic difficulties, shorter life expectancy, and competing morbidities, comprehensive clinical and histopathological data are yet to be produced. Nevertheless, new evidence exist that prostate cancer is a surprisingly common disease in black Africans (Kehinde EO. 1995). When black males in the USA are compared to blacks in Nigeria, they were found to have similar prevalence of prostate cancer, however the incidence of clinical prostate cancer was found to be six times greater in American blacks. The study also showed that the prostate cancer in Nigerian males was less invasive. In conclusion, race appears to be an important factor. However serious technical and conceptual

limitations hamper the ability of racial comparisons to illuminate the causative pathways. Factors concerning impact of socio-economic status influences, dietary and environmental influences must always be considered.

VASECTOMY

A history of vasectomy has been associated with an increased risk for prostate cancer (Giovannucci E *et al,* 1993), although an equal amount of studies provide evidence to the contrary (Bernal-Delgado E *et al* 1998, Lesko SM *et al,* 1999). The directive from the World Health organisation states that any causal relationship between vasectomy and the risk of prostate cancer is unlikely.

PROSTATIC INTRAEPITHELIAL NEOPLASIA

The search for the precursor of prostatic adenocarcinoma has in recent years focused on the spectrum of histopathological changes referred to as Prostatic Intraepithelial Neoplasia (PIN) (Bostwick DG. 1995). PIN is characterised by cellular proliferation within preexisting ducts and acini with cytological changes that mimic cancer, including nuclear and nucleolar enlargement.

The term PIN, was introduced in 1987 and was endorsed by consensus at an international conference in 1989 to replace other terminology used in the literature for the same lesion, including intraductal dysplasia, hyperplasia with malignant changes, marked atypia and duct- acinar dysplasia. The consensus group also proposed that PIN be divided into two grades (low and high) to replace the previous three-grade system (PIN 1, 2 and 3) (Bostwick DG and Brawer MK 1987).

The clinical importance of recognising PIN is based on its strong association with prostate cancer. It tends to be multifocal and occurs in the peripheral zone, as does the prostate cancer. It co-exists with cancer in more than 85% of cases (Bostwick DG and Brawer MK 1987). This finding allows PIN to have a high predictive value as a marker for adenocarcinoma. Its identification in biopsy specimens warrants further search for concurrent invasive carcinoma. If high grade PIN is identified; close surveillance and follow-up biopsy are indicated.

1.1.3 GENETIC EPIDEMILOGY

FAMILY HISTORY

Credit for the awareness of prostate cancer as a familial disease must go to the Mormons in Salt Lake City (Woolf CM. 1960). Their meticulous genealogical archives are a very valuable source of information for research on family aggregations of various diseases. In 1960, Woolf CM reported the familial clustering of prostate cancer, indicating a threefold higher risk that a relative of a prostate cancer sibling would acquire the disease (Woolf CM. 1960). Modern studies began in 1982, when Bishop DT *et al* reported that brothers of a sibling had a high risk of acquiring prostate cancer (a two-to-four fold increase over that expected) as compared with their brothers-in-law, who supposedly lived under similar environmental conditions (Bishop DT *et al,* 1982).

Further support that the disease may be truly hereditary and not due to a similar lifestyle was provided by the studies performed by Grönberg *et al* in 1997. They used the well-established Swedish "Twin Registry" and investigated the frequency of prostate cancer in twin brothers. The concordance rate amongst heterozygous twins was 0.043(compared to familial cancer), whereas the concordance rate in monozygotic twins was 0.192, almost five times higher. This value is markedly higher than for

breast cancer (0.125). Lifestyle was not particularly studied in this report, which may have influenced the results, but probably only marginally.

There are no prostate cancer associated genes yet identified, so the diagnosis of hereditary cancer cannot be confirmed by a laboratory test. A potential susceptibility gene, *HPC1*, has been mapped to chromosome 1q24-25(Eeles RA *et al*, 1997).

BREAST CANCER

Breast cancer has many features in common with prostate cancer, in incidence, pathology and hormone responsiveness. Breast cancer also occurs in a familial form, but not as marked as prostate cancer. In breast cancer two candidate tumour suppressor genes have been identified, supposedly linked to familial Breast cancer, named *BRCA1* on chromosome 17q (Hall JM *et al* 1990) and *BRCA2* on chromosome 13q (Tulinius *et al* 1992). Tulinius *et al* also reported that prostate cancer was the second most common malignancy related to familial breast cancer, and that the prostate cancers were invariably highly aggressive.

Gao *et al* supported the theory of a link between breast and prostate cancer by finding a high frequency of loss of *BRCA 1* in men with prostate cancer, but this was not based on linkage analyses (Gao X *et al,* 1995). Several reports have favoured a link between breast and prostate cancer, indicating an increased risk for sons of mothers with breast cancer to develop prostate cancer, as well as an increased risk for daughters of fathers with prostate cancer to develop breast cancer (Sellers TA *et al,* 1994). However, there are studies, which show a poor relationship between familial breast and prostate cancer (Isaacs SD *et al*, 1995). Therefore in summary, the real link between breast and prostate cancer is in ununknown and needs further proof.

1.2 Pathology and treatment

1.2.1 The natural history of cancer

Tumour progression and Heterogeneity

The natural history of cancer begins with transformation of a normal cell or cells, followed by tumour progression, accompanied by the development of tumour-cell heterogeneity (Weinstein IB. 1988). Genetic and non-genetic mechanisms have been implicated in the development of cancer. Various chemical, radioactive, and viral agents act at the genetic level while environmental factors are believed to act mainly through non-genitic mechanisms (Bishop JM 1991). Most cancers contain genetic damage, which may be, associated with activation or alteration of proto-oncogenes or tumour suppressor genes. Such genetic changes have been identified in many human cancers including cancers of breast, lung, colon, retinoblastoma, leukaemia and lymphomas. Genetic and karyotypic changes in prostate cancer have also been well documented.

The rate of growth of a particular tumour depends upon the size of the growth fraction and the balance between the rate of cell production and loss (Templeton DJ and Weinberg RA 1991).

The growth of a tumour is exponential during early stages of development but as it enlarges, growth becomes slower and at a clinically detectable size the majority of tumour growth is no longer exponential (Laster WR. 1969). Malignant transformation is also associated with acquisition of genetic instability, which makes the cells susceptible to a high rate of spontaneous genetic mutation (Nowell PC. 1976). Consequently, tumour cells develop increasing phenotypic diversity during progression. By the time a tumour is diagnosed, it consists of a heterogenous population of cells, which vary, in Karyotype, invasiveness, growth rate, hormonal responsiveness, metastatic abilities and susceptibility to cytotoxic drugs (Cotran, Kumar & Robbins 1989). Only those cell which survive host defence and ischaemic conditions, grow further. As a result, any emerging tumour will be enriched with those subclones that are most capable of survival, growth, invasion, metastasis and resistance to cytotoxic drugs.

Metastasis

Death from cancer usually occurs due to effects of metastatic disease (Liotta 1991). Treatment modalities currently available to treat metastatic disease have severe limitations. Drug and hormone resistance has emerged as major obstacle in the successful treatment of metastatic cancer. Radiotherapy and surgery play a role only in selective cases.

Metastatic cells are less cohesive and are characterized by the ability to traverse tissue boundaries. Tumour cells detach from a primary tumour and sequentially invade the epithelial basement membrane, extracellular matrix, and endothelial basement membrane before entering the lymphatics or vascular circulation.

Once within a vessel, those tumour cells, which survive immune defences, are carried as emboli to sites where they are arrested in the capillary bed. These tumour cells may then extravasate into the local target organ interstitium and parenchyma in a manner that is the reserve of that employed to invade from primary sites.

There are various theories regarding factors, which determine the site of metastasis. The "anatomical hypothesis" suggests that the eventual metastatic site is determined by the anatomic site of a primary tumour (Evans CW. 1991); The "Seed and Soil" hypothesis suggests that provision of a fertile environment in which the compatible tumour cells could grow is the principal determinant of metastatic sites and the "paracrine/autocrine hypothesis" suggests that seeding could be determined either by release of paracrine factors by target organs or autocrine factors by tumour cells in response to organ derived signals (Hart L, 1988).

1.2.2 Pathology

The prostate is an accessory sex gland which surrounds the urethra at the base of the bladder and functions by contributing secretory proteins to the seminal fluid. Found exclusively in mammals, the prostate is not required for viability or even basal levels of fertility: therefore, its primary significance stems from its relevance for human disease. In deed, it is intriguing to note that malignant prostatic tumours are among the most common neoplasia in men, whereas other ductal organs of male urogenital system, such as the seminal vesicles and bulbourethral (Cowper's) gland are virtually immune to neoplasia.

In the adult male, the prostate gland is a small acorn-shaped tissue composed of small glandular acini of varying shape and sizes embedded in fibromuscular stroma – a mixture of smooth muscle and connective tissue containing neurovascular and lymphatic tissue.

Adenocarcinoma arising from the prostatic epithelium accounts for 95% of prostatic malignancies and is usually composed of small glandular acini that infiltrate in an irregular, haphazard manner (Ellis WJ and Lange PH 1994). The critical feature in prostate adenocarcinoma is absence of the basal cell layer, which

may be detected immunohistochemically by using monoclonal antibodies against high molecular weight cytokeratin.

Prostatic adenocarcinoma originates in the peripheral zone in approximately 75% of cases, with the rest occurring in the transitional zone (McNeal JE *et al*, 1998). Figure1. 3 illustrate the view of the three zones of the Prostate. The tumours arising from these separate zones have different pathological features and clinical behaviour. Transitional zone tumours arise in or near foci of benign prostatic hyperplasia and are usually smaller and better differentiated. Peripheral zone cancers are often less well differentiated, larger in volume than transition zone tumours, and are frequently associated with greater stromal fibrosis, extracapsular extension, seminal vesicle invasion and lymph node metastases.

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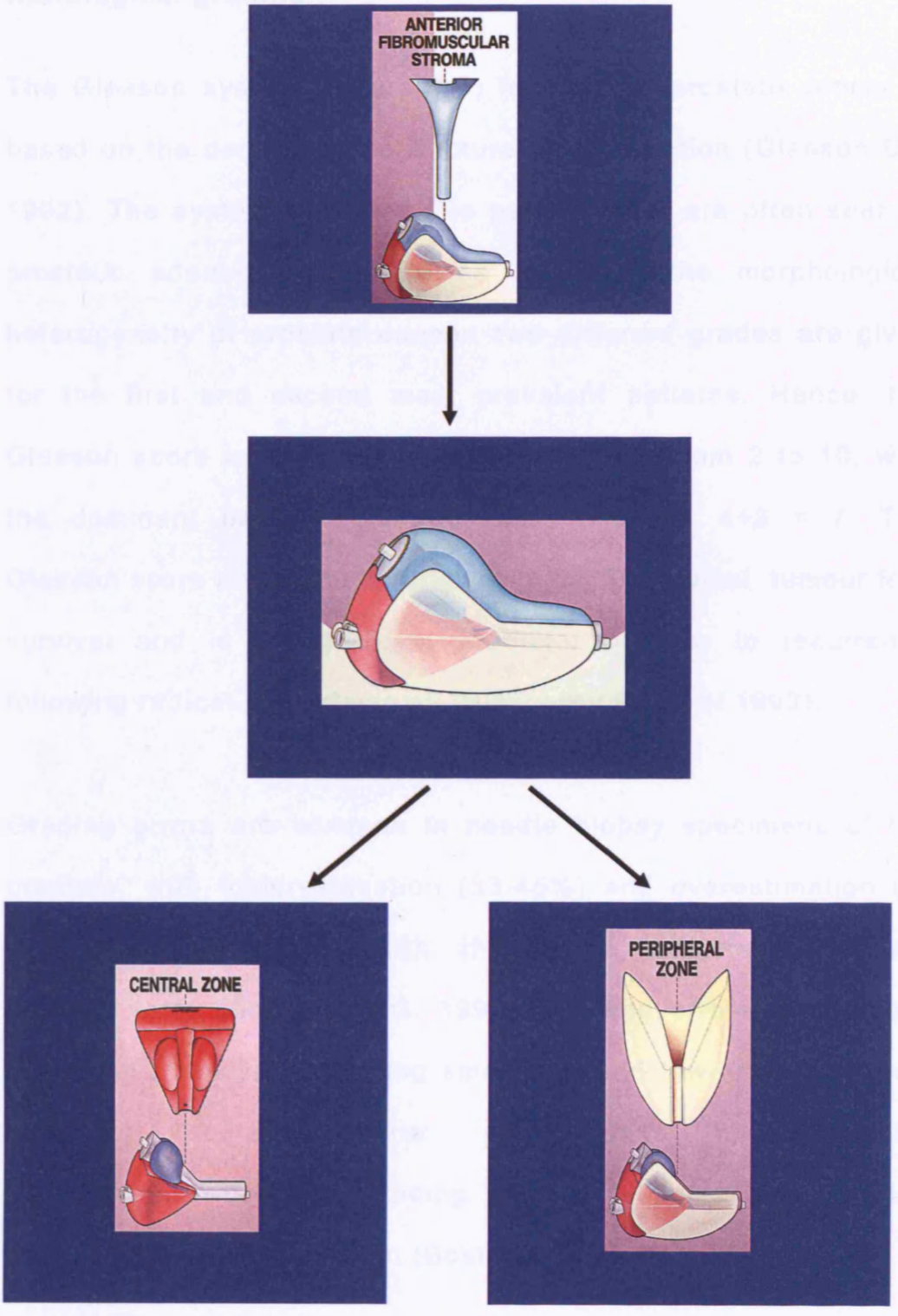


Figure 1.3: The zones of the prostate
Illustration of three zones of the prostate

Histological grading

The Gleason system (Figure 1.4) for grading prostate cancer is based on the degree of architectural differentiation (Gleason DF. 1992). The system identifies five patterns that are often seen in prostatic adenocarcinoma. As a result of the morphological heterogeneity of prostate cancer, two different grades are given for the first and second most prevalent patterns. Hence, the Gleason score is given as their sum, ranging from 2 to 10, with the dominant pattern recorded first, example, 4+3 = 7. The Gleason score correlates strongly with crude survival, tumour free survival and is a significant predictor of time to recurrence following radical prostatectomy (Humphrey PA *et al* 1993).

Grading errors are common in needle biopsy specimens of the prostate, with underestimation (33-45%) and overestimation (4-32%) when compared with the whole specimen following prostatectomy (Bostwick DG. 1994). Grading errors occur more readily in biopsies containing small foci and low-grade cancers, reflecting sampling error and tumour heterogeneity. Nevertheless, Gleason grading needle biopsies can provide useful predictive information (Bostwick DG. 1994).

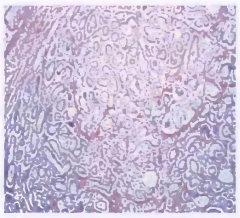
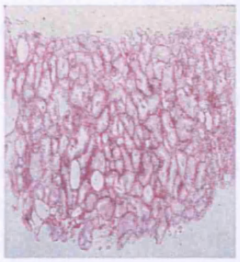
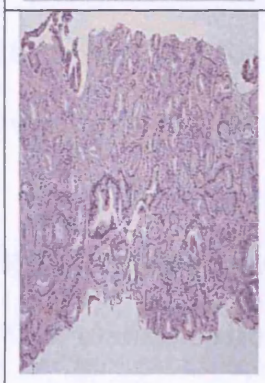
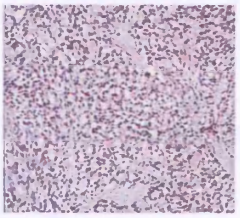
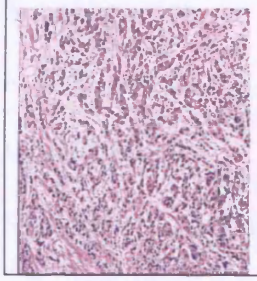
Prostatic Adenocarcinoma (Histological Patterns)	Pattern	Margins of Tumour areas	Gland pattern	Gland Size	Gland Distribution
	1	Well defined	Single, Separate round	Medium	Closely packed
	2	Less defined	Single, separate More irregular	Medium	Spaced up to one gland diameter apart, on average
	3	Poorly defined	Single, separate More irregular	Small, medium or large medium or Large	Spaced ≥ one gland diameter apart, rarely packed. Round masses with smooth edges
	4	Ragged infiltrating	Fused glandular masses	Small	Fused in ragged masses
	5	Ragged infiltrating or Poorly defined	Almost absent, few tiny glands or signet ring cells	Small	Ragged anaplastic masses of epithelium

Figure 1.4: Gleason pattern for adenocarcinoma of the Prostate

TUMOUR STAGING

The TNM (tumour, node, and metastasis) system is the most common classification used worldwide (Sobin LH & Wittekind CH. 1997). The classification of prostate cancer published in 1997(Table 1.1) was used in the staging of the patient's prostatic adenocarcinoma in this thesis as that was the classification in use at the time of experiements reported in this thesis. The classification has been revised in 2002 with a modification made to pT2 stage in which pT2b is defined as tumour involving more than half of one lobe but not both lobes and pT2c is defined as tumour invading both prostatic lobes.

RULES FOR CLASSIFICATION OF PROSTATE CANCER

The classification applies only to adenocarcinoma. There should be histological confirmation of the disease.

The following are the procedures for assessing T, N, and M categories:

T *categories*	Physical examination, imaging, endoscopy, biopsy, and biochemical tests.
N *categories*	Physical examination and imaging.
M *categories*	Physical examination, imaging, skeletal studies, and biochemical tests.

Regional Lymph Nodes are the nodes of true pelvis, which essentially are pelvic nodes below the bifurcation of the common iliac arteries. Laterality does not affect the N classification.

T- Primary Tumour	
Tx	Primary tumour cannot be assessed
To	No evidence of primary tumour
T1	Tumour clinically inapparent, not palpable nor visible by imaging
T1a	Incidental finding following TURP <5% of issue
T1b	Incidental finding following TURP >5% of tissue
T1c	Tumour identified by needle biopsy (e.g, because of elevated PSA).
T2	Tumour confined to the prostate, palpable or visible by imaging
T2a	Tumour involves half of one lobe
T2b	Tumour involves both lobes
T3	Tumour extends through the prostatic capsule.
T3a	Extracapsular extension (Unilateral or Bilateral)
T3b	Seminal vesicle invasion
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles; i.e bladder neck, rectum or external sphincter, levator muscles and/ or fixed to pelvic wall.
N – Regional Lymph Nodes	
NX	Regional lymph nodes cannot be assessed
No	No regional lymph nodes metastasis
N1	Regional lymph node metastasis
M – Distant Metastasis	
Mx	Distant metastasis cannot be assessed.
Mo	No distant metastasis
M1	Distant metastasis present
M1a	Non-regional lymph nodes
M1b	Skeletal metastases
M1c	Other sites

Table 1.1: The 1997 TNM Classification of Prostate cancer

In this thesis the above classification for prostatic adenocarcinoma was used to stage the patient's disease.

Unique Features of Prostate carcinoma

Several features tend to distinguish prostate adenocarcinoma from other common cancers. This list is not exhaustive but serves to highlight important questions in prostate cancer biology.

1. *Extreme age dependency of incidence*. Although the most common malignancy in men, this disease does not appear (at least in the detectable form) at a significant rates until the sixth decade of life (Sakr WA *et al*, 1993).

2. *Slow growth rate*. Doubling times measured in years are not uncommon.

3. *Sensitivity to androgens.* Most cancers respond to androgen therapy, virtually all become insensitive (Crawford ED. 1990).

4. *Multifocality.* The prostate of a man diagnosed with cancer contains an average of five independent lesions (Bastacky SI *et al,* 1995). These lesions are genetically heterogeneous, both inter and intratumourally. This multifocality is independent of family history of prostate cancer (Bastacky SI *et al,* 1995).

5. *Lack of ability to establish cell lines from clinical specimens of prostate cancer.* After hundreds of attempts by numerous investigators only a handful of cell lines exist (Limon J. 1990).

1.2.3 Prostate cancer Presentation and Detection

Historically, prostate cancer was diagnosed with the development of symptoms, either due to local disease resulting in voiding dysfunction, or disseminated disease commonly resulting in general lethargy, bone pain, pathological fractures and weight loss. This situation has changed dramatically with the PSA testing as an available tool which, when combined with the digital rectal examination (DRE) and transrectal ultrasound (TRUS), results in much greater ability to detect prostate cancer while still confined to the gland. The use of these methods is primarily responsible for the approximately threefold increase in incidence rates observed since 1988 (Brawley OW and Kramer BS 1996).

Prostate Specific Antigen (PSA)

PSA is a serine protease from the kallekrein family of glycoproteins with a molecular weight of 34kiloDaltons. Chromosome 19 contains the gene, which encodes for PSA. It is produced within the ductal cells of the prostate. PSA is secreted into the seminal plasma, where it is responsible for proteolysis of the sperm entrapping seminal coagulant formed in the ejaculate (Lilja H and Abrahamsson PA. 1988). It was first characterised following a forensic investigation in 1985 (Graves HC. 1985).

The antigen circulates in the blood mainly bound to protease inhibitors, including α-1-antichymotrypsin and α-2-macroglobulin; only a small fraction of the total PSA exists in a free state. Whereas α-2-macroglobulin encapsulates all the epitopes of the PSA protein, α-1-antichymotrypsin leaves some exposed; therefore, immunoassay techniques have been developed to assess free PSA bound to α-1-antichymotrypsin but not to α-2–macroglobulin (Christensson A *et al*, 1993). The use of free: total PSA ratios is used clinically to improve the postivity predictive value for transrectal ultrasound and biopsy in the diagnosis of prostate cancer. (Woodrum DL *et al*, 1998).

Serum concentrations of PSA are normally of the order below 4mg/mL, although this varies with age (Dalkin BL *et al*, 1993). With prostate pathology these levels can increase, in particularly dramatic fashion in the case of carcinoma. PSA is now well established as a useful investigation in the diagnosis and follow-up of patients with prostate cancer. A focus of intense research effort is on the ability to accurately interpret slightly elevated PSA levels that can be indicative of either benign or malignant disease (Oesterling JE *et al*, 1991). Serum PSA detection after prostatectomy or other treatment for prostate cancer is a reliable indication of disease progression (Oesterling JE *et al*, 1988).

Serum PSA has become the mainstay of screening and detection in some countries (Farkas A *et al*, 1998). PSA has the strongest positive predictive value for prostate cancer. In population selection and biopsy studies, the chance of finding cancer on biopsy are approximately 22% with a PSA value of 4.0ng/ml to 10ng/ml, rising to over 63% in those with a PSA greater than 10ng/ml (Catalona WJ *et al*, 1991). However in a multicentre study, it was shown that 18% of cancers would be missed if PSA alone was utilised as a screening tool (Catalona WJ *et al*, 1994). The combination of DRE and PSA testing improves the rate of cancer detection in the selection of patients for biopsy (Catalona WJ *et al*, 1994).

The greatest limitation of PSA is its lack of specificity. Various conditions can cause "false positive" results such as BPH and prostatitis. However, PSA concentrations are the best overall predictor of bone scan findings and can be used as a screening test for prostate cancer. (Catalona WJ *et al*, 1994).

Transrectal ultrasound (TRUS) guided biopsy

Prostate cancer may appear as a hypoechoic lesion on ultrasound. The degree of echogenicity is variable and dependent upon the amount of solid tumour per unit area. Only macroscopic cancer can be detected on TRUS. The sensitive 7MHz probe facilitates biopsy and the 18-gauge Biopty needle has improved

the accuracy of the biopsy needle placement. TRUS–guided biopsies of hypoechoic areas have increased the detection rate of prostate cancer in screening populations (Cooner WH 1990).

The protocol for TRUS–guided prostate biopsies vary between hospitals. During this research project the antibiotic prophylaxis protocol used in the prostate laboratory at The Royal Free Hospital was Gentamicin 120mg administered intravenously prior to the procedure followed by three day course of Ciprofloxacin 500mg twice daily. The use of periprostatic infiltration of local anaesthetic prior to biopsy is currently advocated; however this technique was not used, at the time of this study. Six (Sextant) biopsies were taken routinely from the base, mid-zone and apex of the prostate bilaterally. Additional biopsies were taken if suspicious looking areas were identified separate from the region of initial biopsy.

The radionuclear bone scans.

Prostate cancers most commonly metastasise to bone. The radionuclear bone scan using γ-camera images following an intravenous injection of a technetium salt can identify a potential bone metastasis long before it becomes apparent on plain X-ray. Simple technetium salts do not localise in the skeleton, but complexes or chelates of technetium with a number of phosphorous containing compounds do concentrate in bone.

Abnormalities on a skeletal technetium–99 labelled bone scan appear as areas of increased uptake which can represent areas of pathological process such as neoplasia or infection and is not a direct indicator of the pathological process itself. This is an important concept because it explains why bone scan itself cannot be used to accurately monitor progress of disease. However bone scan remains the most sensitive diagnostic tool that we have to detect small bone metastases in untreated patients. Recent trauma, infection, inflammatory joint disease, and Paget's disease can produce false positive results. These can usually be excluded on clinically together with plain X-ray.

Studies have shown that it is unnecessary to perform bone scans if a patient with prostate cancer has a PSA of below 10ng/ml (Chybowski FM *et al*, 1991). This is only true for an untreated patient because therapy, especially hormone treatment, may often lower the serum PSA to near normal levels, reflecting a decrease in PSA secretion by cancer cells, but leaves the metastasis still evident.

Computer Tomography (CT).

Cross-sectional imaging with CT is used in an attempt to detect local extension of prostate cancer and/or the presence of lymph node metastases. However, it is not routinely useful because of low sensitivity (Wolf JS Jr *et al*, 1995). Pelvic imaging for lymph

node assessment may be warranted in men at higher risk for metastases suspected by locally advanced on DRE, marked PSA elevation (PSA ≥ 20µg/l), or the presence of poorly differentiated cancer on needle biopsy.

Magnetic Resonance Imaging (MRI)

The role of MRI is to improve the accuracy of clinical staging by identifying patients, whose prostate disease has perforated the capsule. This prevents inappropriate therapy with risk of additional morbidity. Gross violation of the capsule by carcinoma with tumour invading the periprostatic fat is readily detectable (Chelsky MJ *et al* 1993). MRI is the only modality that can provide good images of the seminal vesicles. On T2 weighted images the seminal vesicles have a honeycomb appearance, the fluid having high intensity and the stroma low intensity. Work by Hricak and colleagues showed that MRI could have a direct influence on current decision making in the clinical management of prostate carcinoma (Hricak H 1994). The negative predictive value of extracapsular (92%) and seminal vesicle (98%) extension (i.e., that the capsule and seminal vesicles are not involved) is reasonable high and should reliably predict which patients may benefit from prostatectomy. MRI also has the advantage over CT scan in that the staging for extracapsular extension can be undertaking at the same time as looking for lymph node involvement.

1.2.4 Treatment of Early Prostate Cancer.

The treatment of early prostate cancer remains controversial, as there are no prospective randomised clinical trials comparing all treatment modalities to ascertain which treatment is most effective. As mentioned PSA has become the mainstay of detection and is used as a screening tool in many countries. However, the effect of PSA as a screening tool is itself controversial. A number of studies have indicated the potential of PSA in allowing early detection and treatment of prostate cancer, which may lead to a survival benefit (Lu-Yao GL & Yao SL 1997), (Labrie F *et al* 1999).

Currently the main treatment choices for early-localised prostate cancer involve radical therapy in the form of surgery or radiotherapy (external beam or brachytherapy), or a conservative approach using active surveillance and deferred treatment if necessary. The management decision is difficult due to the fact that the difference between the outcomes of the modalities is unknown and the natural history of the disease itself is not clearly understood.

Studies assessing conservative management have shown that a large percentage of men with prostate cancer will not die of their disease over 15 year follow-up period. Based on Gleason score of

histopathological samples from biopsies or TURP specimens, 767 men with localised cancer were followed-up 10-20 years after their initial diagnosis. Some men had undergone deferred hormonal therapy during this period. In those men with well-differentiated (Gleason score<4) carcinoma 4-7% were at risk of cancer specific death at 15 years. A Gleason score of 5 raised the risk to 6-11%, score of 6 to 18-30%, score of 7 to 42-70% and a poorly differentiated cancer gave 60-87% risk of death over 15 years. In the older man with a cancer that has a Gleason score of 6 or less, a conservative approach is a very real choice with a generally favourable outcome (Albertsen PC *et al,* 1998). Chodak *et al* had previously shown that in men with low grade prostatic carcinomas, 10 years disease specific survival was 87%, but only 34% in those with high grade lesion (Gleason score>7). In those men who were free of metastases at 10 years, 81% had prostate cancer with Gleason score<4 cancer, 58% with Gleason score 4-6 and 26% with Gleason score>7 (Chodak GW *et al,* 1994).

In a Swedish prospective population study, the disease specific survival was 85% at 10 years and 81% at 15 years (Johansson JE *et al*, 1994). These patients had undergone a programme of surveillance and deferred treatment (exogenous oestrogens or orchidectomy) if symptomatic, with excellent outcomes (Johansson JE *et al*, 1994).

Due to lack of prospective randomised controlled trials, various groups have tried to compare results retrospectively using pooled data analysis or population studies to gain information concerning the best radical treatment, surgery or radiotherapy, compared to conservative or deferred treatment. Adolfsson J *et al* (1993). Examined the literature from 1980 with respect to radical prostatectomy, external radiotherapy and deferred treatment. They tried to calculate a weighted mean disease specific survival at 10 years by using the number of patients per group as the weights. A 10 years the disease specific survival was 93% for surgery, 74% for radiotherapy and 83% for deferred treatment. This study suffers from bias in that prognostic factors were unevenly distributed. There were more poorly differentiated tumours in the surgery and radiotherapy arms compared to the conservative arm and although the stages were evenly spread, the group undergoing surgery had smaller tumours than in the other 2 groups. Those men undergoing radical prostatectomy had pelvic lymph node sampling and only in absence of disease did surgery proceed, thereby biasing patient selection.

The American Urological Association also attempted a similar literature search, the results of which were similar to those above but the main conclusion was that the data was unreliable and could not be used to compare the different treatment modalities (Middleton RG *et al,* 1995). Analysis of the Surveillance,

Epidemiology and End Results (SEER) program of nearly 60,000 patients with localised prostate cancer showed that for well or moderately differentiated tumours there was not a large difference between the three common treatment options. Only in those patients with a poorly differentiated lesion did radical prostatectomy have a survival benefit at 10 years of 67% versus 53% for radiotherapy and 45% for deferred treatment. This study is not without its problems of patient selection but the very considerable number of men in each group (15,000-25,000) makes it an important study (Lu-Yao GL, Yao SL. 1997).

The discussions concerning radical treatment against deferred treatment are in part due to the lack of concrete evidence that radical treatment is better in terms of risk-benefit analysis. That there maybe a moderate survival benefit over 10-15 years with radical treatment in some patients has to be set against the complications of radical pelvic surgery of radiotherapy. Radical prostatectomy is associated with a 0.3% mortality rate, incontinence rates of between 5-30% depending on whether the surgeon or patients is directly questioned and erectile dysfunction rates of between 25-75%. This also has to be seen in the context of an overall positive margin rate of 28% (Wieder JA and Soloway MS. 1998), thereby theoretically not curing the patient. However, this does not always translate into disease progression or directly impact upon survival (Pound CR 1999). The PIVOT trial, which

was set up in 1994, comparing Intervention against expectant management in patients with early prostate cancer, would hopefully demostrate which management approach will provide superior survival and quality of life in men (Wilt Tj & Brawer Mk 1994).

Radical radiotherapy is not without its side effects. It has been associated with a mortality rate of 0.2%, incontinence rate 0.9% and erectile dysfunction rates of between 40-70%(Shipley WU *et al,* 1999) however Intensity modulated radiation therapy (IMRT) reduces side effects to adjacent structures allowing for dose escalation to the prostate (Teh BS et *al,* 2002).

New therapies such as brachytherapy are gaining popularity but are yet not substantiated with long-term data. The 5-year biochemical free survival for brachytherapy is quoted as 71% (Brachman DG *et al*, 2000).

1.2.5 Treatment of advanced Prostate Cancer

Testosterone and its more potent metabolite Dihydrotestosterone are essential for normal prostate growth and also play a role in the growth of prostate adenocarcinoma. Since 1940, bilateral orchidectomy has been the mainstay of treatment of metastatic prostate carcinoma (Huggins C and Hodges CV. 1941). Subsequently, medical methods of anti-androgen therapy and medical castration have been employed. Lutenizing hormone releasing hormone (LHRH) agonist act by stimulating the pituitary gland to produce gonadotrophins which then inhibit further production due to a negative feedback mechanism. Testosterone levels rise initially but reach castration levels after 2-3 weeks. These agents are usually well tolerated by patients and may be administered every 3 months as depot injections.

The time to commencement of hormonal manipulation has been the subject of a number of investigations. After the commencement of hormonal therapy, relapse can occur and it has been thought that delaying treatment would stave off hormone relapse. Alternatively early treatment to prevent the onset of symptoms has been anecdotally been thought to aid survival. This question was addressed by MRC study of immediate versus deferred treatment, which showed a decrease in morbidity from local extension and also a survival benefit in the group receiving

immediate hormonal therapy (Kirk D, 1997). The use of radiotherapy in locally advanced disease has been shown to improve survival in patients who did not have lymph node involvement. Radiotherapy with adjuvant androgen suppression has shown improvements in both local control of disease and conferred survival benefit in those with poorly differentiated disease (Bolla M *et al* 1997, Pilepich MV *et al* 1997). These studies also imply a benefit may be gained from early use of androgen suppression. However, these benefits need to be seen in the context of side effects such as decreased libido, erectile dysfunction, lethargy, depression and osteoporosis.

The adrenal glands are also responsible for the production of a small concentration of androgens. There are classes of drugs, which have been developed to decrease production of androgens, such as ketoconazole, or to inhibit androgen receptor, such as bicalutamide (Casodex). These receptor antagonists have several benefits in that they have few side effects and preserve libido.

No matter which method of androgen ablation is used for locally advanced or metastatic carcinoma of the prostate, most patients show disease progression at a year or eighteen months. No therapy has been shown to prolong survival once the disease is hormone resistant and the survival is typically 2-3 years from the time of diagnosis of metastatic disease (Crawford ED 1990).

1.3 GENETICS

1.3.1 Cytogenetics of prostate cancer.

There is much evidence that cancer is the result of a primary genetic event. As the chromosome carries the genetic material, it is natural to study them to see if chromosomal changes lead to cancer.

The primary informational molecule of the cell is deoxyribonucleic acid (DNA). It is damage to this DNA that is considered to cause cancerous growths. DNA consists of two polynucleotide chains that coil around one another to form a double helix. The two chains are held together by hydrogen bonds between the nitrogen bases. For DNA there are four possible bases: adenine (A), guanine (G), cytosine (C) and thymine (T). It is the complementary base pair sequence (A=T and C=G) that contains the information necessary for cell growth, proliferation and differentiation. DNA is transcribed into ribonucleic acid (RNA), which acts to direct the formation of proteins. A gene is a segment of DNA involved in the manufacture of a single protein. Within the human nucleus, the DNA is tightly coiled in association with proteins to form chromatin. During cell metaphase, chromatin condenses for cell division and only then are characteristic chromosomes seen. Each chromosome is seen to be consisting of

two chromatids joined at the centromeres. Each chromosome has a short arm labelled p and a long arm labelled q.

Humans possess 46 chromosomes as 23 homologous pairs. There are 22 autosomes and 2 sex chromosomes. Genes located at the same point on each of pair homologous chromosomes are called alleles. One allele is inherited from each parent. If both alleles are identical, an individual is homozygous for that gene. If the alleles are different, the individual is heterozygous for that gene. Each species has a complement of chromosomes that is characteristic in number and structure and is referred to as the species Karyotype.

Somatic cells contain 46 chromosomes and are called diploid (2n). Germ cells contain 23 chromosomes and are called haploid (n). Prostate cancer cell may contain more than the normal number of chromosomes. If their chromosome complement is an exact multiple of the haploid number, then they are polyploid (e.g. triploid, 3n). If they contain an excess that is not an exact multiple of the haploid number they are termed aneuploid. Numerical abnormalities occur at the time of cell division.

Apart from numerical chromosomal abnormalities, there may be structural anomalies. These result from chromosomal breakages, which occur commonly in tumours. In normal cells chromosome

breakage occurs, but repair mechanisms join areas of breakage without delay.

Structural chromosomal abnormalities may be of different types. A translocation is the transfer of chromosomal material between two chromosomes. An insertion translocation occurs when there is a break in one chromosome that is filled by material from another chromosome. Loss of material from one chromosome is called a deletion. Duplication is the presence of two copies of a chromosome fragment. Inversion occurs when there are two breaks in a chromosome and the inverting fragments rotate 180°. An isochromosome occurs when one arm of a chromosome is deleted and the other is duplicated. Loss of heterozygousity (LOH) is the loss of an allelic band in tumour constitutional DNA and is recognized as indicative of putative tumour suppressor genes.

Several studies have attempted to address the relationship between prostate cancer karyotype, and tumour grade, stage and prognosis. Unfortunately because there are no consistent abnormal (marker) chromosomes, this has proved to be very difficult.

Clinicians are still unable to predict which tumour is likely to progress and which is will remain quiescent. It remains unclear if, how or when a latent tumour undergoes malignant transformation. The molecular mechanisms involved in the progression of prostate cancer remain relatively unknown in comparison with other common cancers. Since Nowell PC *et al* (1976) first described a genetically based model of clonal development and expansion (Nowell PC, 1976), accumulating evidence has implicated activation of oncogenes and inactivation of tumour suppressor genes within a cell lineage as the generally accepted model of carcinogenesis (Fearon ER and Vogelstein B. 1990). Such genetic malfunctions can occur spontaneously or they may be inherited. There has been no identification as yet of a conclusive, consistent 'marker' gene or genes for prostate cancer.

DNA transfection assays were heavily used in the early 80s to identify active oncogenes. Frequent allelic losses observed at specific chromosomal loci in several types of human carcinomas imply that putative tumour suppressor genes have been inactivated in the regions where deletions are detected. Although in prostate cancers the roles of most allelic losses have not been clarified, such losses appear to be frequent on chromosome arms 7q, 8p, 10q, 16q and 17p in prostate cancer tissue (Isaacs WB. 1995, Carter BS *et al*, 1990).

1.3.2 Oncogenes

The Conversion of normal cells to malignant cells appears to be a multistage process, and it appears that several mutational events are required for the development of carcinomas (Klein G and Klein E. 1985). The development of cancer at the molecular level appears to be determined by the complex interactions by two major groups of genes, the oncogenes and the tumour suppressors genes.

Cellular oncogenes

Some retroviruses induce neoplastic transformation in normal cells in culture and susceptible animals. This occurs when certain genes, viral oncogenes (*v-onc*), are integrated into the host cell's genome. In 1976 it was discovered that DNA homologous to viral oncogenes was present in all vertebrates (Stehelin D *et al*, 1976). These cellular homologues of viral oncogenes are called cellular or *c-onc* oncogenes. They appear to be critically involved in the regulation of normal cell growth and differentiation, since they are highly conserved and expressed in normal cells at various stages of the cell cycle and in the process of cell maturation. When activated, the oncogenes have the ability to transform cells to a malignant form, either by expression of an altered form of a normal cellular protein, or by over expression of an otherwise

normal gene. One mechanism of oncogenes activation is chromosomal translocation. Several proto-oncogenes are located near chromosomal breakpoints involved in translocations specific to certain malignancies (Mitelman F and Heim S. 1988).

Examples of oncogenes include c-*MYC* and *ras* gene. These oncogenes has been thoroughly investigated for their possible involvement in prostate tumourigenesis, but much of the data have strongly indicated that they do not play a significant role in prostate cancer (Carter BS *et al*, 1990) (Evans GI 1992). The *bcl-2* gene, an inhibitor of apoptosis located on chromosome 18q21 is thought to be associated with the transition prostate cancer to androgen resistant state (Colombel M *et al* 1993).

Aberrant expression of the oncogenes *Bcl-2, Ras* and *MYC* have been detected in prostate cancer cells, compared to expression in normal tissue. However, none of these genes have been shown to be consistently activated in prostate cancer and the overexpression mechanisms remain largely undefined (Isaacs WB 1995).

1.3.3 Tumour suppressor genes

Evidence for the existence of tumour suppressor genes came from an analysis of the inheritance of rare hereditary childhood cancers. Based on retinoblastoma, Knudson AG *et al* (1985), proposed a "two-hit" inactivation process in which both copies of a critical gene had to be inactivated for the disease to manifest. Work on retinoblastoma and Wilms tumours has shown that individuals affected inherit inactivation or deletion of one of the tumour suppressor genes. It is when the gene develops a mutation or deletion of the remaining allele that the tumour develops. A number of studies have investigated tumour suppressor genes in prostate cancer.

RB gene.

The *RB* gene was the first tumour suppressor gene found in human malignancies. This gene is the second most mutated tumour suppressor gene in human cancers. It is located on chromosome 13p and codes 110kDa nuclear phosphoprotein, which has been implicated in the regulation of cell cycle progression (Lee WH 1988). Loss of the *RB* gene is thought to occur in at least 50% of prostate tumours (Cooney KA *et al*,

1996). Although further studies are required to full assess the role of RB alterations in prostate carcinogenesis, studies carried out thus far, suggest that it is infrequently mutated in prostate cancer (Konishi N *et al* 1996) and that abnormal *Rb* expression has little prognostic significance in prostate cancer (Vesalainen S and Lipponen P 1995).

p53 gene

The *p53* gene is located on the short arm of chromosome 17 and encodes a 53 kDaltons nuclear phosphoprotein. Often referred to as the guardian of the genome, *p53* causes a G1-S cell cycle arrest in cells exposed to DNA damaging agents, which presumably allows the cell sufficient time to repair or restore its DNA damage before replication in S phase, or, depending on the degree of damage, induce apoptosis (Bellamy CO *et al*, 1997). The mechanisms by which *p53* induces apoptosis are still poorly understood. When *p53* is inactivated, damaged DNA can accumulate leading to the progression of the cells into a malignant transformation. In addition, inactivating mutations of *p53* have been shown to confer resistance to apoptosis induced by radiation and multiple chemotherapeutic agents (Mc Donnell TJ *et al*, 1997).

Genetic abnormalities of the *p53* tumour suppressor gene are associated with several human malignancies including bladder cancer (Sarkis AS et al 1993) and breast cancer (Borresen-Dale AL. 2003). Although *p53* mutations in early invasive prostate cancers tissues are relatively infrequent (Henke RP *et al* 1994), they occur more frequently in metastatic disease (Stapleton AM et al, 1997). In a study by Bookstein, almost all of the *p53* mutations were associated with high grade, metastatic, or hormone-refractory prostate cancer (Bookstein R *et al*, 1993). Therefore, *p53* mutations are not only late events in prostate tumour development, but are also less frequent (10-20%) than in other types of cancer (Bookstein R et al, 1993).

E-Cadherin **gene**

The concept of the tumour suppressor gene has been extended to genes, which are subjected to frequent downregulation in cancer, suggestive of an important tumour-suppressing activity despite the lack of evidence for mutation. The *E-Cadherin* gene is such an example, located at chromosomes 16q22.1 and encodes a cell adhesion molecule protein, which plays an important role in tissue development and epithelial cell differentiation (Umbas R *et al*, 1992). When down regulated, this cell adhesion molecule may be involved in oncogenic processes through inactivation of cell

adhesion-mediated growth control pathways. *E-Cadherin* protein is frequently reduced or absent in high Gleason grade prostate cancers, and this finding has prognostic significance (Umbas R *et al*, 1992). Although the predominant mechanism for down regulation of this gene has not been determined, studies have shown that inactivation via promoter methylation of E-cadherin gene is a common finding in prostate cancer cell lines, and at low but detectable rate in clinical specimens of prostate cancer (Graf JR *et al,* 1995).

1.4 *Pten* GENE.

1.4.1 Introduction

The region at chromosome 10q23-25 was postulated to contain a prostate cancer tumour suppressor gene. Enthusiasm for this hypothesis stems from work performed by the Imperial Cancer Research Fund, Clare Hall Laboratories (Gray IC *et al*, 1995). Using microsatellite dinucleotide triphosphate cytosine: Adenine (CA) repeat markers, the group constructed a detailed deletion map spanning chromosome 10q23-25 that incorporated *Mxil,* a candidate tumour suppressor gene which encodes a negative regulator of the Myc oncoprotein. Sixty-two percent (23 of 37) of the prostate tumour analysed exhibited some degree of loss in the region 10q23-25. Closer inspection of their data suggested the presence of a prostate tumour suppressor gene(s) near

the10q23-24 boundary, which was deleted in the overwhelming majority (22 of 23) of tumours showing loss. This area of loss was narrowed down more specifically, to between markers AFMa124wd9 and D10S583, a region spanning approximately 9cM. The study failed to show specific loss of *Mxil* gene.

Following this report by the Imperial Cancer Research Fund group, several groups isolated the putative tumour suppressor gene at chromosome 10q23 using different approaches.

One group (Li J *et al*, 1997) called it *Pten* because the gene product shares homology with a protein tyrosine phosphatase domain and extensive homology to tensin, a protein that interacts with actin filaments at focal adhesion complexes.

Another group (Steck PA *et al,* 1997) called it *MMAC1* (Mutated in advanced cancers 1) as alterations to the 10q22-25 region has been reported in prostate, renal, small cell lung and endometrial carcinomas, melanoma and meningiomas, suggesting the existence of one or more tumour-suppressive loci affecting several cancers in this chromosomal region.

A third group called the gene *TEP1* (Transforming growth factor β regulated and Epithelial cell enriched Phosphatase 1) (Li D-M and Sun H. 1997).

1.4.2 *Pten / MMAC 1 /TEP 1* a candidate tumour suppressor gene.

A number of studies have investigated the frequency of *Pten* mutations in various cancers. Such studies reported that *Pten* was mutated/deleted in 44% (Wang SI *et al,* 1997) of glioblastomas; 43% (Cairns P *et al,* 1997) or 50% (mostly by loss of expression) (Whang YE *et al,* 1998) of prostate cancers; and 50% in endometrial carcinomas (Tashiro H *et al,* 1997). *Pten* is also mutated in 80% of the autosomal dominant cancer predisposition disorders, Cowden disease (Liaw D. 1997, Marsh DJ 1998), Lhermitte-Duclos disease (Eng C. 1994) and Bannayan-Zonana syndrome (Bannayan G.A. 1971, Zonana J *et al*). Cowden disease is characterised by hamartomas of multiple organs including the lower gastrointestinal tract, breast, thyroid, central nervous system and hyperkeratosis of the skin. Lhermitte-Duclos disease is characterised by similar features, but also shows dysplastic gangliocytoma of the cerebellum that leads to altered gait, seizures and mental retardation. Bannayan-Zonana syndrome is characterised by hamartomatous polyps and multiple lipomas.

The tumour suppressor activity of *Pten* was demonstrated using adenoviral transfer of the Pten gene in glioma cells. This resulted in decreased proliferation, loss of anchorage-independent growth

in soft agar, and loss of tumourigenicity in mice in which the *pten* gene had been removed from both aleles (Cheney IW *et al,* 1998).

The generation of *Pten*-null (neither alleles present) mice has given further evidence to support the role of *Pten* as a tumour suppressor. Di Cristofano *et al* reported that *Pten* null mice (-/-) were embryonic-lethal and that chimeric mice (*Pten* +/-) tended to develop a number of neoplasia spontaneously (Cristofano A *et al,* 1998).

The study of *Pten* +/- mice revealed neoplastic growth in multiple organs, including endometrium, liver, prostate, gastrointestinal tract, thyroid, and thymus (Podyspanina K *et al* 1999). These results suggest a possible, causative role for *Pten* mutations in tumourigenesis, and are reminiscent of the results obtained from other known tumour suppressors, such as retinoblastoma gene product or *p53*, where mice with loss of these genes exhibit a marked increase in tumour susceptibility.

Patients with Cowden disease provided another line of genetic evidence to support the tumour suppressor role of Pten, where increased cancer susceptibility is correlated with germ line mutations of *Pten* (Liaw D 1997).

1.4.3 Structure and function of Pten.

The Pten protein

The *pten* cDNA encodes 403-amino-acid peptides with a relative molecular mass of 47 KDaltons. Two major RNA transcripts of approximately 2 and 5kb were detected in a wide variety of cell lines (Li J et al, 1997),(Li DM and Sun H 1997). PTEN is a cytoplasmic protein as observed by immunoflurescence (Li DM and Sun H 1997).

The structure was investigated for common motifs, which would indicate possible activities of the protein. PTEN contain a motif C(x5) R that defines the protein tyrosine phosphatase (PTPase) supergene family. PTEN exhibits dual specificity protein phosphatase activity in vitro (Myers MP *et al*, 1997). PTEN has been shown to dephosphorylate the inositol lipid second messenger phosphatidylinositol-3-4-5-triphosphate (PIP3). Generation of PIP3 is an upstream event in signalling through the serine/threonine kinase AKT-1, a mediator of cell survival and anti-apoptopic signaling.

Phosphoinositide is a component of signalling pathways that control critical cellular functions. Regulation of phosphoinositide levels is complex, requiring kinases, phosphatases and lipases to control biosynthesis and degradation of inositol lipids.

High levels of PIP3 are indirectly required to activate AKT-1 (Figure 1.4), resulting in inhibition of apoptosis and allowing cellular proliferation (Wu X *et al*, 1998). PTEN dephosphorylates PIP3 to PIP2. The levels of PIP3 decrease, AKT-1 is not activated and proliferation is inhibited. When pten gene is inactivated or mutated, the levels of PTEN protein is reduced; PIP3 can build up and send out the signals for inhibition of apotosis, cellular proliferation resulting in tumourogenesis.

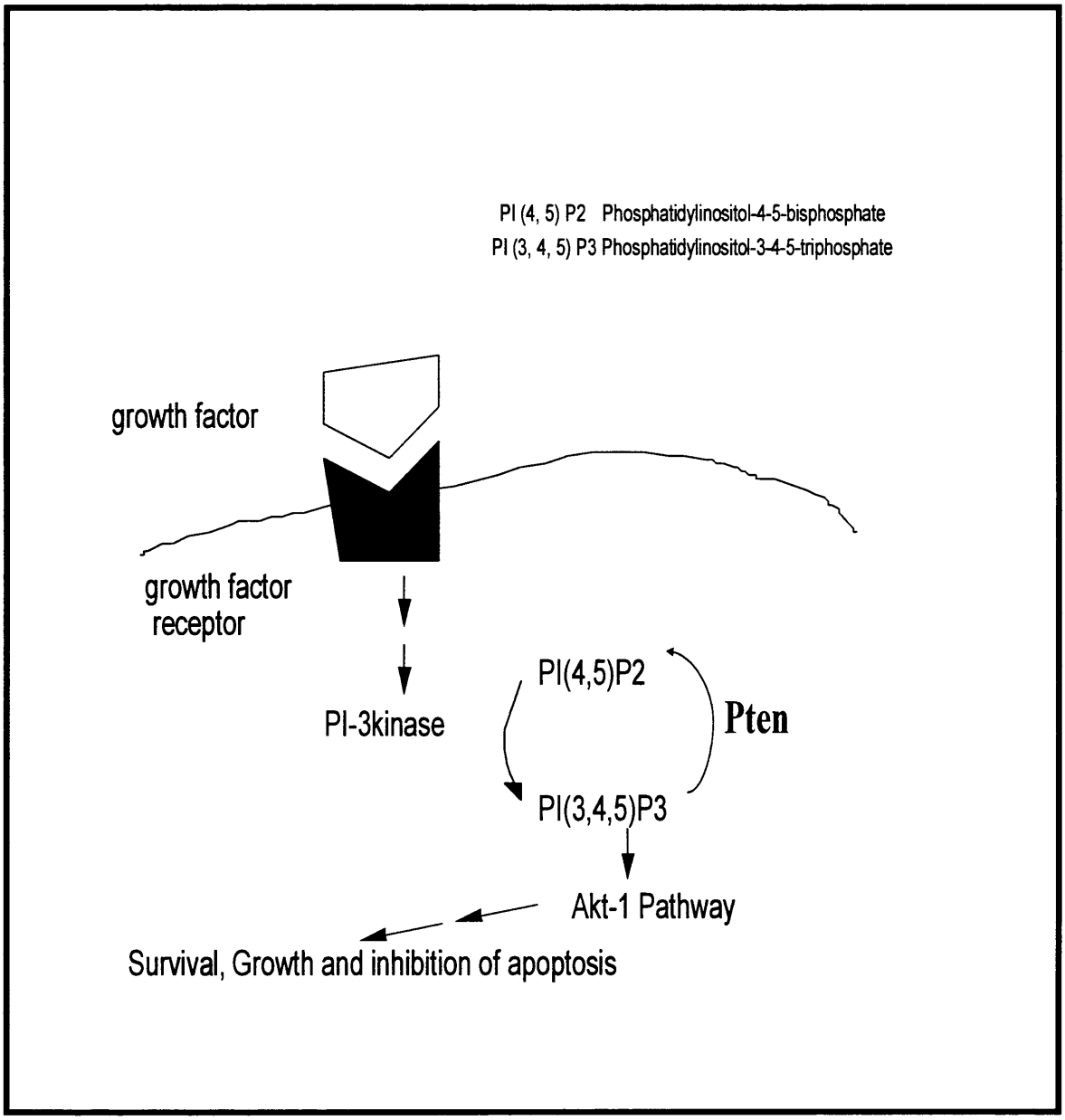


Figure 1.5 **Substrate specificity of Pten.**
Pten prevents the development of high intracellular concentrations of PIP3, which is required for the activation of AKT-1 pathway.

1.4.4 Microsatellite markers

Microsatellites are tandem arrays of short stretches of nucleotide sequences, which were identified, by Litt M and Luty JA in 1989. They are usually repeated between 15 and 30 times in a gene (Braaten DC *et al*, 1988). They are also called simple sequence length polymorphisms (SSLPs) (Tautz D. 1989) and short tandem repeat polymorphisms (STRs) (Edwards A *et al* 1991). They belong to the family of repetitive non-coding DNA sequences, which can be classified as follows:

1. Satellite sequences: arrays with repeat sizes ranging from 5 to 100 basepairs (bp) characteristically organised in a cluster up to 100 megabases (Mb). These are located in the heterochromatin near chromosomal centromeres and telemeres and are not as variable in size within populations as the other members of this family (Tyler-Smith C and Willard HF. 1993).
2. Minisatellites sequences: arrays with repeat sizes of 15 - 70 bp, which range in size from 0.5 to 30 kilobases (kb). These are found in euchromatic regions of the gene and are highly variable in repeat size within populations (Armour JA and Jeffreys AJ, 1992).

3. Microsatellite sequences: arrays with a repeat size of 2 – 6 bp. These are highly variable in size but ranging around a mean

of 100bp and are found in the euchromatin (Tautz D and Renz M 1984).

Microsatellites were initially identified in eukaryotic genomes as stretches of Thymine nucleotide = Guanine nucleotide (T=G) alternating sequence with varying length (Miestfield R *et al*, 1981). Subsequent reports demonstrated the PCR typability of T=G, C=A dinucleotide repeat microsatellites and showed the Mendelian co-dominant inheritance of the size polymorphisms (Litt M and Luty JA 1989). In order of decreasing abundance, A, C=A, A=A=A=N, A=A=N and A=G nucleotide repeats were identified as the most frequent sequence motifs in human microsatellites (Beckman JS and Weber JL. 1992). Disregarding (A) *n* multimers, whose size polymorphisms are difficult to type by PCR, (C=A) *n* microsatellites are estimated to number between 35 000 and 100 000 copies in the human genome, a marker density of approximately one microsatellite every 100 000bp, even in the most conservative of estimates (Weber JL. 1990) (Beckman JS and Weber JL. 1992).

It must be noted that although widely distributed. Microsatellites are not uniformly spaced along chromosomes, being under represented in subtelomeric regions (Weissenbach J 1993).

1.4.4.1 Origin and function of Microsatellite Sequences.

The origin and function of repetitive sequences is not clear at present. The initial occurrence of short repeat sequences could be due to chance (in a random sequence the probability of a (C=A)*3* repeat is 1/256), or they could have arisen as mutations from the poly (A)*n* sequences (Beckman JS and Weber JL. 1992).

The selective prevalence of (C=A)n repeats may be explained by the methylation of Cytosine nucleotide residues at 5′G=C3′ sequences normally present in the genome. Methylated Cytosine nucleotide residues can be deaminated, producing a transition of Cytosine to Thymine. This process leads to an abundance of 5′G=T3′ motifs and their complementary 5′C=A3′ motifs (Levinson G and Gutman GA 1987). Subsequently, the expansion of the repeat may occur due to strand slippage during DNA replication, creating length polymorphisms differing by a few repeats at a time. Additional sequence motifs may then arise by mutation of expanding C=A repeats (Schlotterer C *et al*, 1992).

It was originally thought that repeat sequences possessed a functional role in the genome, either directly via a role in gene regulation (Hamada H *et al*, 1984) or indirectly as hot spots for recombination (Slighton JL *et al*, 1980). To date no definite function can be ascribed to microsatellite sequences.

1.4.4.2 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

Single nucleotide polymorphisms (SNPs) are sites on the DNA sequences in which two or in some cases more than two bases can occur in different individuals. SNPs comprising some 80% of all known polymorphisms, and their density in the human genome are estimated to be on average 1 per 1,000 base pairs, however, their distribution is not uniform (Kwok PY *et al* 1996). SNPs have a low mutation rate per generation, making them the marker of choice in association studies, a commonly used technique for identifying polymorphisms with a role in a disease. In this design, the frequencies of the different alleles of a given polymorphism are compared in a control population and in prostate cancer group being investigated. If a significant difference in frequency is found then it is said that a positive association exists.

These SNPs are major contributors to genetic variation in the human genome (Kwok PY *et al*, 1996). These variations in SNPs may or may not be of functional significance, i.e. lead to a change in the structure or action of the protein coded for by that gene. This in turn may not have clinical significance. However, genes interact with other genes and also with their products, thus a polymorphism that on its own is of no significance may produce

phenotypic changes in conjunction with other genes or environmental factors.

If a SNP is found within a small, unique segment of DNA, it serves both as a physical landmark and as a genetic marker whose transmission can be followed from parent to child. This property makes SNPs useful markers in population genetics and evolutionary studies (Hacia JG *et al*, 1999). Most SNPs are located in non-coding regions of the genome, and have no direct known impact on the phenotype of an individual.

There is an enormous interest in SNPs because it is hoped that they could be used as markers to identify genes that predispose individuals to common, multifactorial disorders by using linkage disequilibrium mapping (Risch N and Merikangas K 1996).

2.5 Principles of polymerase chain reaction.

The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. The double-stranded DNA sample to be amplified, referred to as the template, is denatured by incubation at high temperature. The two strands, now dissociated, remain free in solution until the temperature is lowered sufficiently to allow them to reanneal. The primers are defined as the pair of synthetic oligonucleotides, which anneal to sites flanking the region to be amplified. These oligonucleotides typically have different sequences and are complementary to sequences that lie on opposite strands of the template. Thus they will only anneal to one of the strands. Primers are typically 15 to 30 nucleotides in length and have similar melting temperatures (where the melting temperature is the temperature at which the primer melts and therefore denatures; above this temperature the primer does not anneal and so disassociates from the template). The primer sequence is determined by the sequence of the DNA sample, at the boundaries of the region to be amplified. In the reaction the primers are present in large excess over the template as are the four deoxy-nucleotides triphosphate (dNTPs). When the temperature of the reaction mix is lowered, because of the excess of primers, the formation of primer-template complex is favoured over the reassociation of the two DNA strands. This is called the

annealing step. After this annealing the primers are extended with a thermostable DNA polymerase. Such an enzyme adds on the appropriate dNTP guided by the sequence on the template. This step is called extension. It occurs in the 5′→ 3′ directions.

The typical set of three steps (i.e. denaturation, annealing and extension) is referred to as a cycle. A cycle is repeated many times, typically up to 30- 35. Because the product of one round of amplification serves as templates for the next, each successive cycle essentially doubles the amount of desired DNA product.

The major product of this exponential reaction is a segment of double – stranded DNA whose termini are defined by the 5′ termini of the oligonucleotide primers. This major product is termed the "short product". In addition, longer DNA molecules are generated during the reaction. For example, the products of a successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the second round, these molecules generate DNAs of defined length that will accumulate in an exponential fashion in later rounds of the reaction. Although longer molecules continue to be produced from the original template DNAs in every round, they accumulate only at a linear rate (as the original DNA template remains constant) and

therefore do not contribute significantly to the final mass of target sequences.

Although extremely efficient, exponential amplication of target sequences is not an unlimited process. This is due to product annealing and limited enzyme concentration. If further amplification is required, a sample of the amplified DNA can be diluted and used as the template for further rounds of synthesis in a fresh polymerase chain reaction.

Taq DNA polymerase is isolated from a ubiquitous thermopilic bacterium, *Thermus aquaticus*. It is heat stable at high temperatures and can survive extended incubation at 95°C. It is not inactivated by the heat denaturation step and does not need to be replaced at every round of the amplification cycle. Another commonly used enzyme is Tub DNA polymerase.

AmpliTaq Gold DNA polymerase is the enzyme used for the PCR reactions in this thesis. It has a special feature in that it has an antibody connected to the enzyme's active site, which is stable at lower temperatures and only becomes denatured at 95°C. This property prevents activation of the DNA polymerase enzymes whilst the double stranded DNA are still separating at lower temperatures.

1.6 AIMS AND OBJECTIVES OF THE STUDY

Background

Following this brief review of aetiological agents implicated in the development of prostate cancer, it is clear that genetic factors are of great importance. However, the contribution of underlying genetic factors is less certain. It may be that different genetic aberrations are cumulatively responsible for promoting the pathway of pathogenesis of prostate cancer.

It is postulated that study of genetic mutations may shed some light on the pathogenesis of prostate cancer and provide markers to identify patients at risk of disease progression. The initial study performed by the Imperial Cancer Research Fund, Clare Hall Laboratories (Gray IC *et al*, 1995) postulated that the region at chromosome 10q23-25 contain a prostate cancer tumour suppressor gene. Several groups have narrowed the region containing the tumour suppressor gene, *Pten* to 10q23.3(Li J *et al*, 1997, Steck PA *et al,* 1997, Li D-M and Sun H. 1997).

Various techniques have been used to analyse the frequency of *Pten* mutation in prostate cancer. These techniques produced a wide range of results in the frequency of allele losses. The extent of the tumour suppressor activity of *pten* gene in prostate cancer

is unknown. Hence the rationale for investigating the *Pten* gene in this thesis was to evaluate the magnitude and significance of LOH at *Pten* in prostate cancer DNA. This has led to the hypothesis expressed below, which formed the basis for this research project.

i. **Prostate cancer DNA can be successfully extracted from paraffin embedded radical prostatectomy specimens. Experiments to extra pure cancer cell DNA in prostate cancer are often difficult. This is due to the large concentration of non cancerous DNA contained within stromal epithelial cells which may cause contamination of the DNA extraction process.**

ii. Comparison of SNPs in the *Pten* gene in prostate cancer patients and non-prostate cancer patients will show a difference.

Iii. Analysis of the frequency of LOH within *Pten* gene using microsatellite markers will illustrate a correlation in the frequency of mutation of the *Pten* gene and the different stages of prostate cancer disease progression.

CHAPTER TWO

Polymorphisms in p*ten*

2.1 Introduction

The aim of the first study is to compare the distribution of known polymorphisms in the region of chromosome 10q23 in prostate carcinoma patients with a non-prostate cancer group; and furthermore, to evaluate the significance of the distribution of polymorphisms in the prostate cancer patients in the study.

The population required for the study were selected based on strict criteria. DNA extracted from blood samples from both the prostate carcinoma patients and non-prostate cancer patients were subjected to analysis using three intronic polymorphisms within the *Pten* gene (Figure 2.1): a single-base A→G substitution in intron A 96 bp upstream of exon 2; a 5-bp ATCTT insertion / deletion in intron D 110 bp downstream of exon 4 and a single-base T→G substitution in intron H 32 bp downstream of exon 8.

Association studies were then carried out between the prostate carcinoma patients and non-prostate cancer group using the three polymorphisms in the *pten* gene. Comparisions of the polymorphism distribution with PSA readings at the time of diagnosis and Gleason score of prostate cancer group were made.

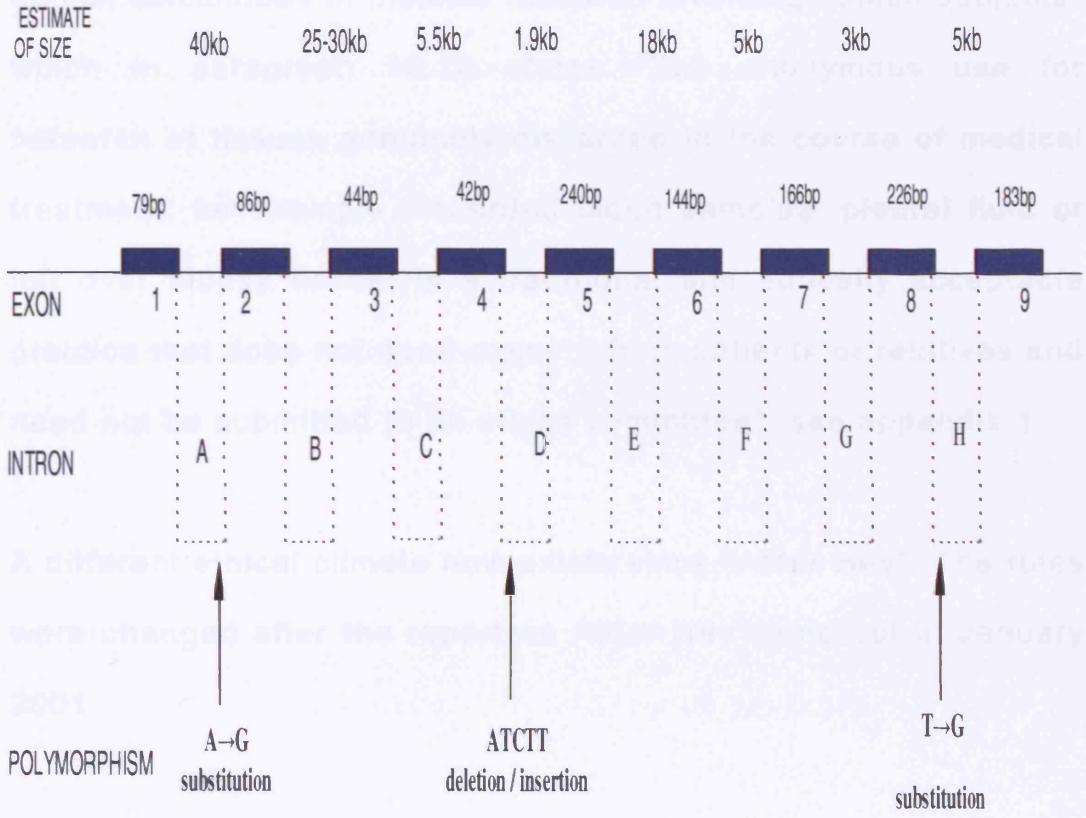


Figure 2.1 Intronic polymorphism variants
The position of the three polymorphisms analysed in Pten: a single-base A→G substitution in intron A 96 bp upstream of exon 2; a 5-bp ATCTT insertion / deletion in intron D 110 bp downstream of exon 4 and a single-base T→G substitution in intron H 32 bp downstream of exon 8.

2.2 Ethics

In 1990 Royal College of Physicians "guidance on the practice of ethical committees in medical research involving human subjects" which in paragraph 13.20 states "The anonymous use for research of tissues genuinely discarded in the course of medical treatment, for example discarded blood samples, pleural fluid or left over biopsy tissue, is a traditional and ethically acceptable practice that does not need consent from patients or relatives and need not be submitted to an ethics committee", see appendix 1.

A different ethical climate now exists since "Alder Hey". The rules were changed after the report on Alder Hey came out in January 2001.

2.3 Case selection in SNPs study

2.3.1 Criteria for selection

The diagnosis of adenocarcinoma of the prostate was made on the basis of investigative procedures that included serum PSA, DRE, bone scans and histological evidence of the disease taken from TURP and TRUS and biopsies. The presence or absence of distant metastases did not affect inclusion in this study. Men under the age of 40 were excluded from the study group.

2.3.2 Selection of cases

The patients were recruited from The Royal Free Hospital. They included patients, newly diagnosed with prostate cancer and patients with prostate cancer on a follow up program.

The control group consist of patients in whom the diagnosis of adenocarcinoma of the prostate had been excluded following DRE, PSA testing and negative prostatic biopsies.

2.3.3 Collection of patient data in SNPs study

I interviewed each patient personally to discuss the proposed study with them and the possible implications to each individual. A verbal consent was obtained. The information requested from each patient is illustrated in appendix 2 and patients' data sheet is illustrated in appendix 3.

2.3.4 Sample collection

Each patient had 3 millilitres of blood withdrawn into a plastic tube containing EDTA to prevent the blood sample from clotting. The sample was stored at 2-8°C for not more than five days to reduce DNA degradation.

The blood samples were obtained from patients attending the prostate labouratory at The Royal Free Hospital. The two groups were divided into patients diagnosed with prostate cancer and patients with no evidence of prostate cancer following investigations. An age-matched control group was used.

2.4 DNA extraction protocol

This consisted of extracting DNA from the blood sample, amplifying the segment of genome of interest by the PCR. In order to ensure the PCR had worked, the PCR product is run on an agarose gel containing ethidium bromide staining and visualised by ultraviolet excitation. The product is purified, sequenced using appropriate primers and run on an acrylamide gel to assess the three SNPs status of the subject.

2.4.1 Preparation of genomic DNA

The DNA was extracted from lymphocytes separated from heparinized blood using Puregene™ DNA isolation kits.

Red cell lysis

Three millilitres of whole blood is added to a 15ml tube containing 9mls RBC lysis solution. The tube is inverted to mix and incubate for 10 minutes at room temperature then centrifuged for 10 minutes at 13 000 revolutions per minute (rpm). As a result of spinning a visible white cell pellet is formed in the bottom of the tube. The RBC lysis solution serves to disrupt the erythrocyte cell wall containing the non-nucleated red blood cells (lacking in genomic DNA) and separate them from white blood cells.

The supernatant, which consist of erythrocyte cell walls, haemoglobin, plasma and platelets, is removed leaving behind the visible white cell pellet and 100-200µl of residual liquid. The tube is vortex vigorously to resuspend the white blood cells in the residual supernatant; this greatly facilitates cell lysis.

Three millilitres of cell lysis solution is added to the tube containing the resuspended cells and pipetted up and down to break down the white blood cell walls and nuclear membranes, thus leaving the DNA accessible. Usually no incubation is required however if cell clumps are visible after mixing, the tubes were incubate at 37° C until the solution is homogeneous.

RNAse treatment

Fifteen microlitre of RNase A solution is added to the cell lysate. The sample is mixed by inverting the tube 25 times and incubated at 37° C for 15 minutes. The Rnase A enzymes destroys the RNA released from the cell lysate and prevents the contamination of the DNA in solution.

Protein precipitation

The sample is cooled to room temperature. One millilitre of Protein precipitation solution is added to the cell lysate. The solution is vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate and then centrifuged at 13 000rpm for 10 minutes. This step allows the cellular proteins to precipitate out to form a tight, dark brown pellet.

DNA precipitation

The supernatant containing the DNA (leaving behind the precipitate protein pellet) is poured into a clean 15ml tube containing 3 millilitres of 100% Isopropanol. The sample was inverted gently 50 times until the white threads of DNA form a visible clump. It is Centrifuged at 13 000 rpm for 3 minutes; the DNA is visible as a small pellet. The supernatant is poured off and the tube drained on clean absorbent paper. Three millilitre of

70% ethanol is added. The tube is inverted several times to wash the DNA pellet and centrifuged at 13 000 rpm for 1 minute. The ethanol is carefully poured off whilst watching the pellet, as it may be loose. The tube is drained on clean absorbent paper and the pellet dried by placing the tube in an incubator at 37°C for 15 minutes.

The final step is to resuspend the pellet of DNA in sterile water by adding 250µl of DNA hydration solution (250µl will give a concentration of 400µg/ml if the total yield is 100µg of DNA) and tapping periodically to help disperse DNA. This DNA is allowed to rehydrate overnight at 23°C and subsequently stored at 2-8°C.

Quantification of DNA by spectrophotometer

The DNA present in 250µl of hydration solution is diluted with sterile water by a ratio 1 in 100 dilution. This step is essential to improve the accuracy of the spectrophotometer reading, which would encounter diffulty if the solution contained high concentration of DNA. The DNA is examined between wavelength 260nm (Optical Density OD_{260}) and 280nm (OD_{280}).

Absorbant A_{260} = A_{260} X Dilutional factor X 50 X 1000 = mg/ml
UV light (1:100)

PCR works best with 30-50 ng of genomic DNA. Therefore further dilution of the working stock was performed.

2.4.2 PCR amplification of SNPs region.

In order to analyse the region containing the polymorphisms, known *Pten* intronic sequence oligonucleotide primers were required to PCR amplify these area of interest. These oligonucleotide primers (Table 2.1) were formulated based on the *Pten* sequence described by Gary IC *et al* (1998) and were synthesised and supplied by The Imperial Cancer Research Fund, Clare Hall Laboratories.

Primers	Oligo nucleotide primers	Sequence of primers	Amplifies segment
A	81167 84305	5'- TACGGTAAGCCAAAAAATGA 3' 5'- TACGGTAAGCCAAAAAATGA 3'	upstream exon 2
B	84314 84315	5'- GGGGGTGATAACATTATCTA 3' 5'- CTTTATGCAATACTTTTCCTA 3'	Downstream exon 4.
C	71429 81694	5'- AACAGATAACTCAGATTGCC 3' 5' CGTAAACACTGCTTCGAAATA3'	Downstream exon 8

Table2.1: Sequence of oligonucleotide primers.
The sequence of oligonucleotide primers runs from 5'⟶ 3' end and is used to amplify the region containing the polymorphisms.

The volumes and concentrations of reagents used for the amplification of genormic DNA in the PCR is illustrated below:

PCR solutions	µl
10 X PCR Buffer (10µl)	2.5
dNTPs (2mMoles.)	2.5
Oligonucleotide primer 1 (10µM)	1.25
Oligonucleotide primer 2 (10µM)	1.25
DNA template (working stock)	2.0
Taq Polymerase (5µl)	0.25
Sterile distilled water	16.25
Volume per PCR reaction	25µl

Experimental procedure: -

Place the requisite numbers of reaction tubes in a tray. For example, if there were 20 DNA samples to be amplified, then 20 reaction tubes need to be set up. Place the reagents stated above into a 0.2ml reaction tube together with the DNA extract to be amplified. Place caps over the tubes and assemble them into the sample block of PCR machine.

The PCR machine was programmed to carry out the following cycles automatically.

PCR Settings

Temperature (°C)	Time (Minutes)	
96	4.00	
95	0.30	Repeat 30X
55	0.15	
72	1.00	
72	5.00	
4	∞	

The samples were initially denatured at 96°C for 4 minutes, then subjected to 30 cycles of 30 seconds denaturising at 95° C, 15 seconds annealing at 55° C and polymerisation at 72° C for I minute allowing for extension at 1 Kb per minute. There is a final step of extension at 72° C for 5 minutes to ensure completion of all amplification events. The PCR product is then cooled to 4° C.

Separation of PCR product by electrophoresis.

This step is to check whether the PCR was successful and free of contamination. 2% Agarose was used as the support matrix for enabling the PCR product to be separated under the influence of an electrical current. A small pore gel results when agarose undergoes change from a liquid to a solid form. Smaller PCR fragments will travel further down the gel in a given time; thus fragments of differing sizes will be separated.

Gel constituents used

2%Agarose	3grams
0.5 TBE (Triss, Borate, EDTA)	150mls
Ethidium bromide	3.5µl

Ethidium bromide is known to contain a planar group that intercalates between the stacked bases of DNA. The fixed position of this group and its close proximity to the bases causes dye bound to DNA to display an increased fluorescent yield compared to dye in free solution. Ultraviolet irradiation absorbed by the DNA at 260nm and transmitted to the dye, or irradiation absorbed at 300nm and 360nm by the bound dye itself, is emitted at 590 nm in the red/orange region of the visible spectrum.

Each electrophoresis gel had a standard marker in the first lane. On electrophoresis this marker separated into fragments of known molecular weight. Thus by using this as a comparison one could estimate the sample fragment sizes. The first electrophoresis well contains standard molecular weight marker of known size. TheΦX174 DNA/*Hae* III Marker was used. They are made by digesting double stranded ΦX174 DNA to completion with *Hae* III.

Experimental procedure

Mix the 2% agarose gel constituents thoroughly in a glass beaker. Heat the glass beaker in a standard domestic microwave, at high power for 5 minutes. Allow the beaker to cool by leaving it standing for 2 minutes. Add 3.5µl of ethidium bromide into 150ml of 0.5 X TBE solution and allow bottle to cool for further 5minutes. Whilst still in the liquid form, pour the solution into perspex receptacle containing two 24 toothed plastic comb. When the liquid set, remove the comb forming two 24 wells.

Mix 4µl PCR product with 2µl of loading buffer (Orange G and 30% Glycerol) and pipet into each well. Pour 0.5 x TBE Into the reservoirs on the assembly to cover the electrodes. Connect the assembly to a power supply and run the gel at 250 volts for 20minutes. Inspect the gel under ultraviolet light wearing UV safe goggles. A Polaroid picture was taken of the illuminated gel in order to provide a permanent record **(Fig 2.2)**.

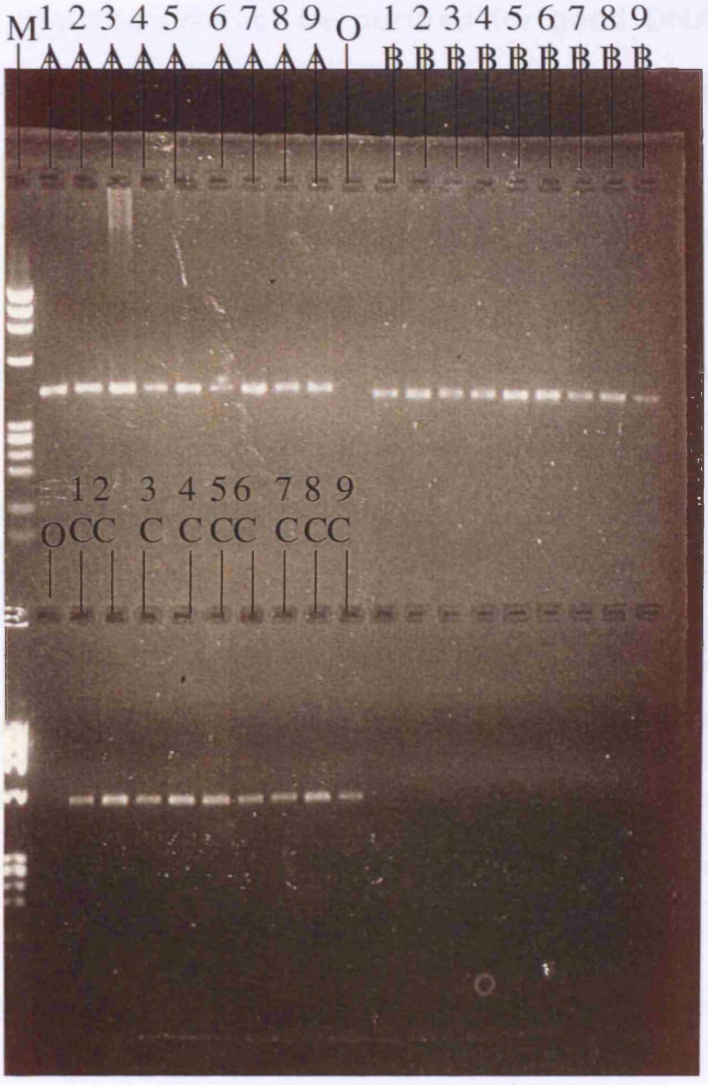


Figure 2.2: **Separation of PCR product by electrophoresis.** Lane one labelled M contains theΦX174 DNA/*Hae* III marker that separates into fragments of known size and molecular weight. This allows for the PCR product fragment size to be estimated and assessed for contaminants. Nine DNA samples were examined on this gel. Primers A, B&C amplifies the region containing the single-base A→G substitution, the 5-bp ATCTT insertion / deletion and the single-base T→G substitution respectively. If the PCR product was contaminated, they would separate out at different molecular weight.

2.4.3 Purification of PCR product

The PCR product is contaminated with unwanted primers and impurities such as salts, enzymes and unincorporated dNTPs. It is essential that the product be purified for good DNA sequence to be obtained. The QIAquick PCR purification protocol is designed to purify PCR products by removing unwanted contaminants using QIAquick spin columns in a microcentrifuge.

The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica-gel membrane. Special buffers listed below are optimised for efficient recovery of DNA and removal of contaminants in each specific application. DNA absorbs to the silica membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with distilled water. Adsorption of DNA to silica membrane depends on pH. Adsorption is typically 95% if the pH is ≤ 7.5, and it reduced drastically at higher pH.

Buffer PB allows the efficient binding of PCR product as small as 100bp and quantitative (99.5%) removal of primers up to 40 nucleotides. It is therefore used to remove oligonucleotide primers after cDNA synthesis or to remove unwanted linkers in cloning experiments.

During the DNA adsorption step, unwanted primers and impurities such as salts, enzymes, and unincorporated dNTPs do not bind to the silica membrane, but flow through the column. Salts are washed away by the ethanol containing Buffer PE. Any residual Buffer PE, which may interfere with subsequent enzymatic reactions, is removed by an additional centrifugation step.

Experimental procedure: -

Centrifuge steps are at ≥10, 000 x g (≈ 13,000 rpm) in a conventional domestic tabletop microcentrifuge.

Add 125mls of Buffer PB to 25µl of PCR product. Place the QIAquick spin column into a 2 ml collection tube.

To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 seconds. Discard the flow through. Place the QIAquick column back in the same tube. To wash, add 0.75mls of Buffer P.E to the QIAquick column and centrifuge for 60seconds. Again, discard the flow through. Return the QIAquick column to the same tube and centrifuge for an additional one minute at maximum speed. Place the QIAquick column in a clean 1.5ml microfuge tube. To elute DNA, add 30µl Buffer E.B to the centre of the QIAquick column and allowed to stand for one minute then centrifuged for one minute leaving an elution of bound DNA.

2.4.4 DNA sequence reaction

The purified PCR product is added to set primers (Table 2.2) and ABI PRISM™ dRhodamine terminator cycle sequencing ready reaction mix in order to incorporate florescence dNTPs. The reaction mix combines the unique properties of AmpliTaq® DNA polymerase, FS and new dRhodamine dye terminators with convenience of the ready reaction mix.

In this format, the dye terminators, dNTPs, enzymes, magnesium chloride, and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing florescence-based cycle sequencing reactions on single or double stranded DNA templates or PCR fragments.

The sequencing enzyme AmpliTaq® DNA polymerase is a variant of *Thermus acquaticus* DNA polymerase that contains a point mutation in the active site. This results in less discrimination against deoxynucleotides, which leads to a greater peak intensity.

This enzyme has a second mutation in the amino terminal domain that virtually eliminates the 5′→ 3′ nuclease activity of AmpliTaq DNA polymerase. The enzyme has been formulated with a thermally stable inorganic pyrophosphate to eliminate problems associated with pyrophosphorolysis.

Primers	Primers Numbers	DNA Sequence of primers
A	81167	5'- CCATGTGGAAGTTATTTTTTA –3'
B	84314	5'- GGGGGTGATAACAGTATCTA –3'
C	81819	5'- GGGTAAATACATTCTTCATACCAGG –3'

Table 2.2: Sequence of primers used for sequence reaction
The sequence of oligonucleotide primers runs from 5'→ 3' end.

The volumes and concentrations of reagents used in the PCR to incorporate florescence dNTPs is illustrated below:

Solution used: -	µl
PCR Product	9.0
dRhodamine terminator ready reaction mix	8.0
Primers A Primers B Primers C	3.0
Total volume	20.0 µl

The PCR machine is programmed to carry out the following cycles automatically.

Temperature (°C)	Time (Minutes)	
96	4.0	
96	0.10	Repeat cycles 25 X
50	0.05	
60	4.00	
4	∞	

2.4.5 Purification of Extension Products

This step is incorporated to remove excess dye terminators by a process called ethanol precipitation.

Experimental procedure: -

Add 2µl of 3M Sodium acetate (PH 5) and 40µl of 95% ethanol to the extension products present in the 0.2ml microcentrifuge tube Mix the solution thoroughly and allow it to sit at room temperature for 10 minutes.

Placed tubes in a microcentrifuge at 4°C for 30 minutes at 13,000 rpm. Remove and discard the supernatant carefully. Add 100µl of 70% ethanol and centrifuge at 13, 000 rpm for 5 minutes. Aspirate the ethanol leaving the DNA extention product as a pellet. The pellet is dried in a SpeedVac vaccum centrifuge.

As a result of this ethanol precipitation, traces of incorporated terminators may be seen at the beginning of the sequence data (up to base 40), but this is usually minimal.

2.4.6 Analysis of extension products on a sequencer

The next stage is to run the extension products on a sequencer to obtain a reading of the nucleotides in order to establish the polymorphisms present within the amplified region.

4µl of sequence dye (blue) is added to the dried DNA pellet and heated at 95°C for 2 minutes then placed on ice.

Acrylamide is used as the support matrix for enabling the oligonucleotide to be separated under the influence of an electrical current. A small pore gel results when acrylamide undergoes change from a liquid to a solid form. Polymerisation of the acrylamide is accomplished by adding ammonium persulphate (APS) and N, N, N′, N′ – tetramethylethylenediamine or TEMED.

The constituents required for the acrylamide gel are listed below with each gel able to support and analyse 32 samples.

Solution used: -

4.2% Acrylamide	50ml
10% APS	250µl
TEMED	35µl

Experimental procedure:-

Clean the gel plates thoroughly with10% alconox then rinse twice with double distilled water. This ensured the plates are clean and reduced interference when the sequences are analysed.

Mix the acrylamide gel constituents together and pour between the two glass plates separated by a small gap and standing in a gel assembly kit. When pouring the gel mixture ensure there are no air bubbles as this causes interference when the DNA sequence are being analysed. Place an inverted 30-toothed comb between the top ends of the plate. When the liquid sets as a gel (a process which takes 1½hours) remove the comb and orientate correctly to form 36 wells for the DNA to be placed.

Mount the gel assembly kit onto the Perkin-Elmer ABI Prism 377 DNA Sequencer. Pour a sufficient amount of 0.5 X TBE into the reservoirs to ensure the electrodes and wells are covered. Connect the assembly kit to a power supply and pre-run at 48°C. for 10minutes. Load the samples into alternate wells and run the gel for 2 minutes and pause the sequencer. Load the remainder of the wells and allow the gel to run at 950 voltages overnight.

2.4.7 Multicomponent analysis of SNP's results

Multicomponent analysis is the process that separates the four different florescent dye colours into distinct spectral components. The four dyes used in the sequencing ready reaction kit are dR110, dR6G, dTAMRA and dROX.

Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI Prism 377 instruments, the collection software collects light intensities from four specific areas on the CCD camera, each area corresponding to the emission wavelength of a particular fluorescent dye. Each of these areas on the CCD camera is referred to as a "virtual" filter, since no physical filtering hardware (like band-pass glass filters) is used. The information that specifies the appropriate virtual filter settings for a particular set of fluorescent dyes is contained in each appropriate ABI Prism collection module file.

In the dRhodamine dyes, dRII0 emits the shortest wavelength and is detected as black, followed by dR6G (green), dTAMRA (blue) and dROX (red) (Table 2.3). Although each of these dyes emits its maximum florescence at a different wavelength, there is some overlap in the emission spectra between the four dyes. The goal of multicomponent analysis is to isolate the signal from each dye so that there is as little noise in the data as possible.

Terminator	Dye Label	Colour of Raw Data on ABI PRISM 377 Gel image
A	Dichlor [R6G]	**Green**
C	Dichlor [TAMRA]	**Blue**
G	Dichlor [R110]	**Black**
T	Dichlor [ROX]	**Red**

Table 2.3: **The (dRhodamine) dye terminators.**
The colour each dye labels emits.

The precise spectral overlap between the four dyes is measured by running DNA fragments labelled with each of the dyes in separate lanes of a gel. These dye-labelled DNA fragments are called matrix standard samples. The instrument file contains tables of numbers with four columns and four rows. These numbers are normalised fluorescence intensities and represent a mathematical description of the spectral overlap that is observed between the four dyes.

Multicomponent analysis of sequencing data is performed automatically by the sequencing Analysis software, which applies a mathematical matrix calculation (using the values in the instrument file) to all sample data.

The sample data are interpreted using the sequencing analysis software. The three intronic polymorphism markers within the *Pten* gene was analysed: a single-base A→G substitution in intron A 96 bp upstream of exon 2(Figure 2.3); a 5-bp ATCTT insertion / deletion in intron D 110 bp downstream of exon 4(Figure 2.4) and a single-base T→G substitution in intron H 32 bp downstream of exon 8(Figure 2.5).

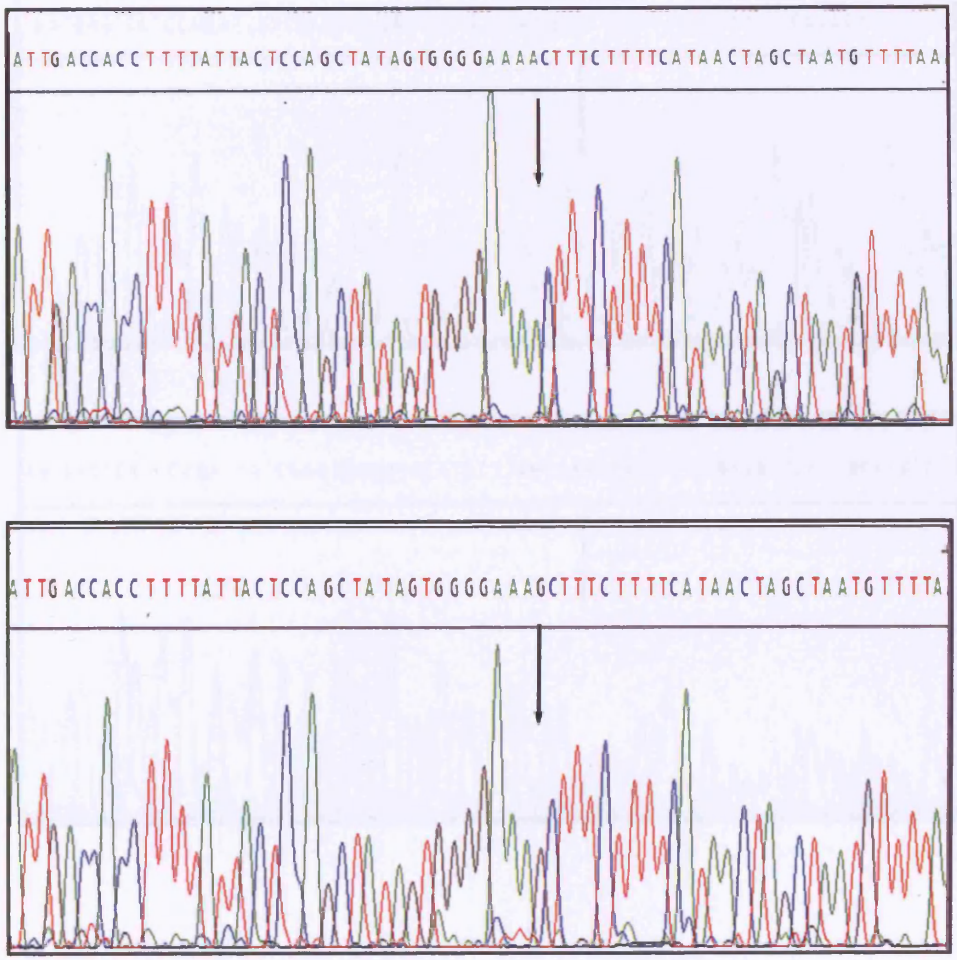


Figure 2.3: **Polymorphism in Pten intron A**

A single-base A→G substitution in intron A is noted by the position of the downward arrows. The colours represent nucleotide bases. Green=A, Black=G, Blue=C and Red =T and the sequence is show above the trace. The numbers seen above the trace are of no signiicance and vary for every sequence analysed by the software.

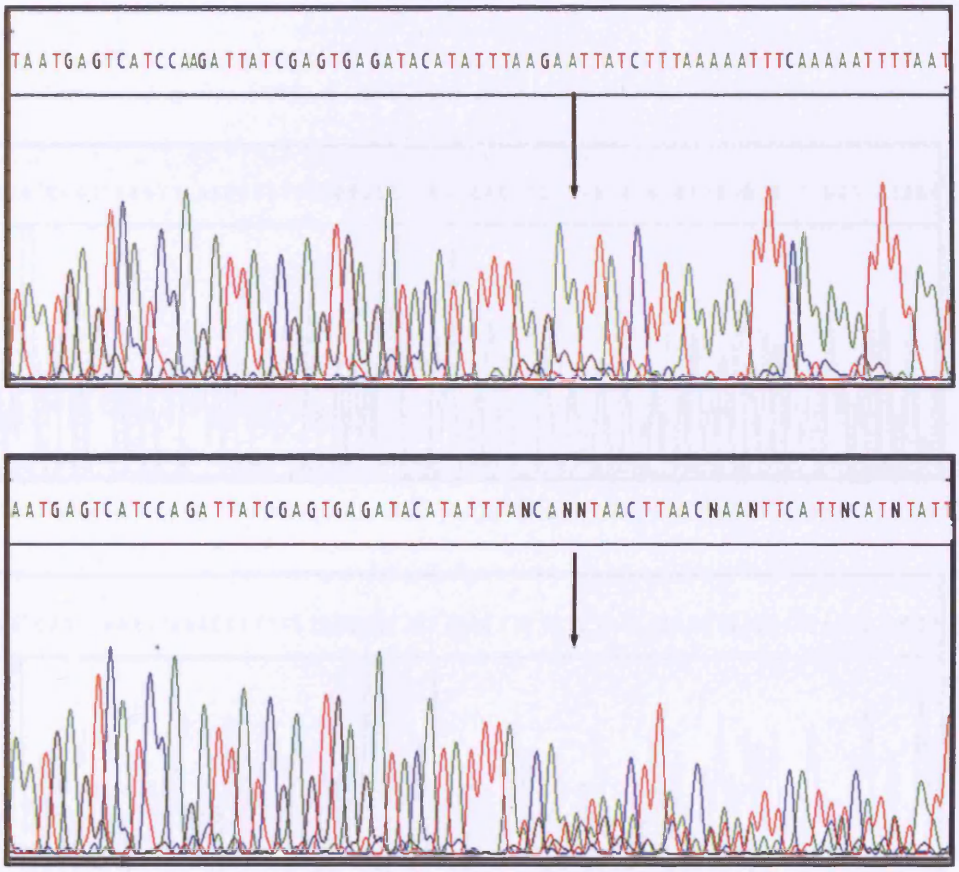


Figure 2.4: **Polymorphism in *Pten* intron D**

A 5-bp ATCTT insertion / deletion in intron D 110 bp downstream of exon 4. The colours represent nucleotide bases. Green=A, Black=G, Blue=C and Red =T and the sequence is show above the trace. The numbers seen above the trace are of no signiicance and vary for every sequence analysed by the software.

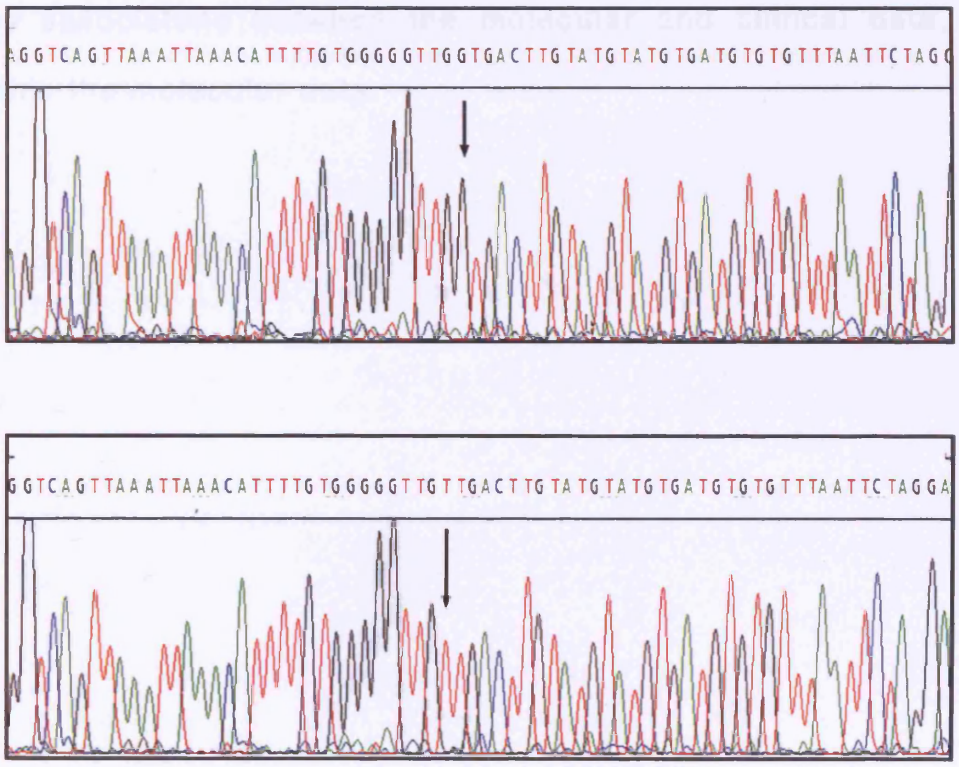


Figure 2.5: **Polymorphism in *Pten* intron H**

A single-base T→G substitution in intron H 32 bp downstream of exon 8. The colours represent nucleotide bases. Green=A, Black=G, Blue=C and Red =T and the sequence is show above the trace. The numbers seen above the trace are of no signiicance and vary for every sequence analysed by the software.

2.5 Statistical Analysis

Mann-Whitney U test, Chi squared test and Fisher's exact test from the statistical package for social science (SPSS) software version 11 were used to determine the statistical significance of any associatons between the molecular and clinical data, and within the molecular data.

CHAPTER THREE

Results and discussions on polymorphism study on the *Pten* gene

3.1 Results of analysis of polymorphisms in *Pten*.

3.1.1 Analysis of study population

A total of 138 blood specimens were studied. Ninety-five of these blood samples were obtained from patients with prostate cancer and fourty three blood samples were from patients with no clinical evidence of prostate cancer following investigations. These acted as the source for the control arm of the study. The sequencing analysis software in the ABI Prism 377 DNA sequencer performed analysis of all the samples data automatically.

The age range of patients with adenocarcinoma of the prostate in the study was 46 – 90 years with a mean age of 70.36years. The age range of the control arm of the study was 40 – 91 years with a mean age of 68.47years.

The PSA at the time of diagnosis for the prostate cancer group ranged from 2 – 425.0µg/l with a mean of 28.4µg/l. The prostate cancer group had a mean Gleason score of 6 with a range of 2 – 10. The PSA at the time of diagnosis for the control group ranged from 0.3 – 22.8µg/l with a mean of 6µg/l. The comparison in PSA, at the time of diagnosis for the cancer group compared to the control groups was statistically significant ($p<0.05$) using Mann-Whitney U test.

3.1.2 Polymorphisms distribution in study population

The distribution of the three polymorphisms studied and the clinical data for each patient is illustrated in Table 3.1.

Table 3:1 Results of polymorphisms

Clinical data for each patient and the position of the three polymorphisms analysed in Pten: a single-base A→G substitution in intron 2; a 5-bp ATCTT insertion / deletion (+/-) in intron 4 and a single-base T→G substitution in intron 8.

ID number 133→137 are missing due to initial numbering error whilst collecting samples from the patients.

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
1	50	3	CA	6	pT1c	A/G	+/-	G/T
2	4.3	0.2	CA	6	pT1b	A/A	-/-	T/T
3	1.7	0.1	CA	3	pT1b	A/G	+/-	G/T
4	1	1.4	CA	3	pT1a	A/G	+/-	G/T
5	6	0.1	CA	5	pT2a	A/A	-/-	T/T
6	5	0.1	CA	5	pT2b	A/G	+/-	G/T
7	31	3.7	CA	7	pT1c	A/G	+/-	G/T
8	33	0.2	CA	7	pT3a	A/G	+/-	G/T
9	8.4	13.9	CA	6	pT1c	A/G	+/-	G/T
10	4.2	0.2	CA	3	pT1c	A/G	+/-	G/T
11	0.2	0.2	CA	4	pT1c	A/A	-/-	T/T
12	4.4	10	CA	7	pT1c	A/G	+/-	G/T
13	27	2	CA	8	pT1c	G/G	+/+	G/G

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
14	5.1	11.3	CA	4	pT1c	A/G	+/-	G/T
15	13	0.2	CA	2	pT1c	A/A	-/-	T/T
16	82	0.2	CA	7	pT1c	A/G	+/-	G/T
17	22.8	12.5	BPH	NA	NA	A/G	+/-	G/T
18	4.1	2	BPH	NA	NA	A/G	+/-	G/T
19	3.2	2	BPH	NA	NA	A/G	+/-	G/T
20	10.5	0.2	CA	7	pT3a	A/G	+/-	G/T
21	26.6	2.2	CA	7	pT1c	A/G	+/-	G/T
22	425.	30.5	CA	6	pT1c	A/G	+/-	G/T
23	17.5	21.1	CA	6	pT1a	A/A	-/-	T/T
24	13	0.2	CA	8	pT2b	A/G	+/-	G/T
25	4.5	4.4	BPH	NA	NA	A/A	-/-	T/T
26	5.4	0.8	CA	5	pT1c	A/G	+/-	G/T
27	12.1	3.4	BPH	NA	NA	A/G	+/-	G/T
28	40	3.3	CA	7	pT1c	A/G	+/-	G/T
29	10.2	0.2	CA	6	pT2b	A/G	+/-	G/T
30	35.6	1.7	CA	5	pT1c	A/A	-/-	T/T
31	17.8	11.5	BPH	NA	NA	A/G	+/-	G/T
32	1.6	0.2	CA	4	pT1c	G/G	+/+	G/G
33	10.8	10.2	BPH	NA	NA	A/G	+/-	G/T

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
34	8.4	8.4	BPH	NA	NA	A/G	+/-	G/T
35	8	38	CA	7	pT1c	A/G	+/-	G/T
36	4.5	2.3	BPH	NA	NA	A/A	-/-	T/T
37	26	177	CA	10	pT1a	G/G	+/+	G/G
38	8.3	6.1	BPH	NA	NA	G/G	+/+	G/G
39	7.1	5.1	BPH	NA	NA	A/A	-/-	T/T
40	6	0.2	CA	7	pT3a	G/G	+/+	G/G
41	10.4	4.5	BPH	NA	NA	A/A	-/-	T/T
42	10.9	0.6	CA	4	pT1c	A/A	-/-	T/T
43	16.9	0.5	CA	6	pT1c	G/G	+/+	G/G
44	7.6	6.2	BPH	NA	NA	A/G	+/-	G/T
45	8.6	0.6	CA	8	pT1c	A/G	+/-	G/T
46	9.9	0.2	CA	5	pT2a	A/G	+/-	G/T
47	203	4	CA	6	pT1b	A/G	+/-	G/T
48	34.2	0.1	CA	6	pT1a	A/G	+/-	G/T
49	19.3	9.4	BPH	NA	NA	A/A	-/-	T/T
50	186	7.1	CA	5	pT1c	A/A	-/-	T/T
51	16.1	13.9	CA	7	pT1c	A/A	-/-	T/T
52	14.2	0.2	CA	7	pT3a	A/G	+/-	G/T
53	4.4	4.3	BPH	NA	NA	A/G	+/-	G/T

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
54	13	0.7	CA	7	pT3a	A/A	-/-	T/T
55	16	0.2	CA	6	pT2a	A/A	-/-	T/T
56	5	0.2	CA	7	pT3a	A/A	-/-	T/T
57	30	0.4	CA	6	pT2b	G/G	+/+	G/G
58	4.3	3.9	BPH	NA	NA	A/G	+/-	G/T
59	31.6	2.7	CA	3	pT3a	A/G	+/-	G/T
60	7.5	2	BPH	NA	NA	A/A	-/-	T/T
61	4.3	1.8	BPH	NA	NA	A/A	-/-	T/T
62	4.1	3.9	BPH	NA	NA	A/G	+/-	G/T
63	3.9	2.9	BPH	NA	NA	G/G	+/+	G/G
64	4.8	1.4	BPH	NA	NA	A/G	+/-	G/T
65	5.2	4.8	BPH	NA	NA	A/G	+/-	G/T
66	6	0.2	CA	4	pT3a	G/G	+/+	G/G
67	15.7	13.8	CA	7	pT1c	G/G	+/+	G/G
68	5.5	3.2	BPH	NA	NA	A/G	+/-	G/T
69	5.1	4.2	BPH	NA	NA	A/A	-/-	T/T
70	4.9	3.0	BPH	NA	NA	A/G	+/-	G/T
71	20	0.2	CA	8	pT2a	A/A	-/-	T/T
72	3.3	1.3	BPH	NA	NA	A/A	-/-	T/T
73	8	0.2	CA	6	pT3a	G/G	+/+	G/G

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
74	6.2	0.5	CA	4	pT1c	A/G	+/-	G/T
75	8	0.1	CA	7	pT2b	A/A	-/-	T/T
76	0.2	0.5	CA	3	pT1a	A/A	-/-	T/T
77	25	0.7	CA	8	pT1b	A/A	-/-	T/T
78	6.2	0.2	CA	6	pT3a	G/G	+/+	G/G
79	4.7	4.4	BPH	NA	NA	A/A	-/-	T/T
80	12.6	0.5	CA	5	pT1a	A/A	-/-	T/T
81	5	0.1	CA	5	pT3a	A/G	+/-	G/T
82	5.2	0.3	CA	7	pT1b	A/G	+/-	G/T
83	9.2	7.1	CA	9	pT1c	A/A	-/-	T/T
84	32	23	CA	7	pT1c	A/A	-/-	T/T
85	48	13	CA	8	pT1a	G/G	+/+	G/G
86	26	0.8	CA	5	pT2b	A/G	+/-	G/T
87	28	0.2	CA	7	pT3a	A/G	+/-	G/T
88	32	0.2	CA	5	pT3a	A/G	+/-	G/T
89	3.7	3.7	BPH	NA	NA	G/G	+/-	G/G
90	24.4	0.2	CA	7	pT3a	A/G	+/-	G/T
91	11.8	0.2	CA	6	pT3a	A/G	+/-	G/T
92	8.6	0.6	CA	4	pT1c	A/G	+/-	G/T
93	6	0.2	CA	7	pT2b	A/G	+/-	G/T

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
94	9	0.2	CA	9	pT2b	A/A	-/-	T/T
95	6.1	0.2	CA	6	pT2a	A/G	+/-	G/T
96	10	0.8	CA	8	pT2a	A/A	-/-	T/T
97	17.5	0.2	CA	6	pT2b	A/A	-/-	T/T
98	2.1	1.2	BPH	NA	NA	A/A	-/-	T/T
99	59	110	CA	7	pT1b	A/A	-/-	T/T
100	6.7	2.2	BPH	NA	NA	A/G	+/-	G/T
101	4.3	0.2	CA	6	pT2b	A/A	-/-	T/T
102	6.4	0.4	CA	8	pT2a	A/G	+/-	G/T
103	9	0.2	CA	5	pT3a	G/G	+/+	G/G
104	6.1	0.1	CA	6	pT3a	G/G	+/+	G/G
105	5	0.2	CA	4	pT2b	A/G	+/-	G/T
106	5.2	0.2	CA	6	pT2a	A/A	-/-	T/T
107	72	0.2	CA	9	pT1b	A/A	-/-	T/T
108	4.2	2.1	BPH	NA	NA	A/G	+/-	G/T
109	4.2	4.2	BPH	NA	NA	A/G	+/-	G/T
110	14.8	8.5	BPH	NA	NA	A/G	+/-	G/T
111	10.9	44.8	CA	7	pT1c	A/G	+/-	G/T
112	4.5	2.9	CA	6	pT1c	A/G	+/-	G/T
113	4.9	4.2	BPH	NA	NA	A/A	-/-	T/T

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
114	18	0.2	CA	6	pT2a	G/G	+/+	G/G
115	2.2	0.2	CA	6	pT3a	A/A	-/-	T/T
116	210	0.2	CA	5	pT2b	A/G	+/-	G/T
117	39	0.5	CA	6	pT3a	A/G	+/-	G/T
118	6	0.2	CA	7	pT2b	A/G	+/-	G/T
119	8.9	3.6	BPH	NA	NA	A/A	-/-	G/G
120	5	3	CA	6	pT2a	A/G	+/-	G/T
121	4.3	0.2	BPH	NA	NA	A/A	-/-	T/T
122	4.1	3.1	BPH	NA	NA	A/A	-/-	T/T
123	1	0.2	CA	2	pT1a	A/A	-/-	T/T
124	3.9	4.4	BPH	NA	NA	A/G	+/-	G/T
125	4.8	1.9	BPH	NA	NA	A/G	+/-	G/T
126	17.4	0.3	CA	6	pT1c	A/G	+/-	G/T
127	10	0.2	CA	6	pT2a	A/A	-/-	T/T
128	252	0.2	CA	5	pT3a	A/G	+/-	G/T
129	12.6	0.5	CA	6	pT2a	A/A	-/-	T/T
130	5.9	0.2	CA	5	pT2a	A/A	-/-	T/T
131	6.7	0.3	CA	6	pT2b	G/G	+/+	G/G
132	13.5	2.2	CA	7	pT2b	A/A	-/-	T/T
138	4.6	5.6	BPH	NA	NA	A/A	-/-	T/T

ID-num	Pre op PSA	Post op PSA	Diagnosis	Gleason Score	TNM Stage	A to G sub intron 2	ATCTT Ins/del intron 4	T to G Sub intron 8
141	12.2	0.2	CA	9	pT3a	A/A	-/-	T/T
142	6.1	4.2	BPH	NA	NA	A/A	-/-	T/T
143	11.1	7.9	CA	7	pT2a	G/G	+/+	G/G
144	5.4	4.2	BPH	NA	NA	A/G	+/-	G/T
145	6.8	3.2	BPH	NA	NA	A/A	-/-	T/T

3.1.3 Comparison of the polymorphisms in the BPH group with the prostate cancer group.

Analysis of the distribution of the polymorphisms at each intron in the prostate cancer group was compared to that of the BPH group (Figure 3.1; 3.2 and 3.3). At intron 2 the Adenine nucleotide (A) substitution for Guanine nucleotide (G) was analysed. Of the 138 samples studied, fivety one were homozygous A/A (33 cancer: 18 BPH), nineteen homozygous G/G (15 cancer: 4 BPH) and sixty-eight heterozygous A/G (47cancer: 21 BPH).

The insertion or deletion of the polymorphic nucleotide sequence ATCTT at intron 4 was analysed. Homozygous insertion of the sequence ATCTT (+/+) was detected in 19 of 138 samples (15cancer: 4 BPH), 51 of 138 samples were homozygous deletion for the sequence ATCTT (-/-)(33 cancer: 18 BPH) and 68 of 138 samples were heterozygous insertion / deletion (+/-) with a distribution of 47cancer and 21BPH.

At intron 8 the Guanine nucleotide (G) substitution for Thymine nucleotide (T) was analysed. Of the 138 samples studied, sixty eight were heterozygous G/T (47cancer: 21 BPH). Fivety one were homozygous T/T (33 cancer: 18 BPH) and nineteen were homozygous G/G (15 cancer: 4 BPH).

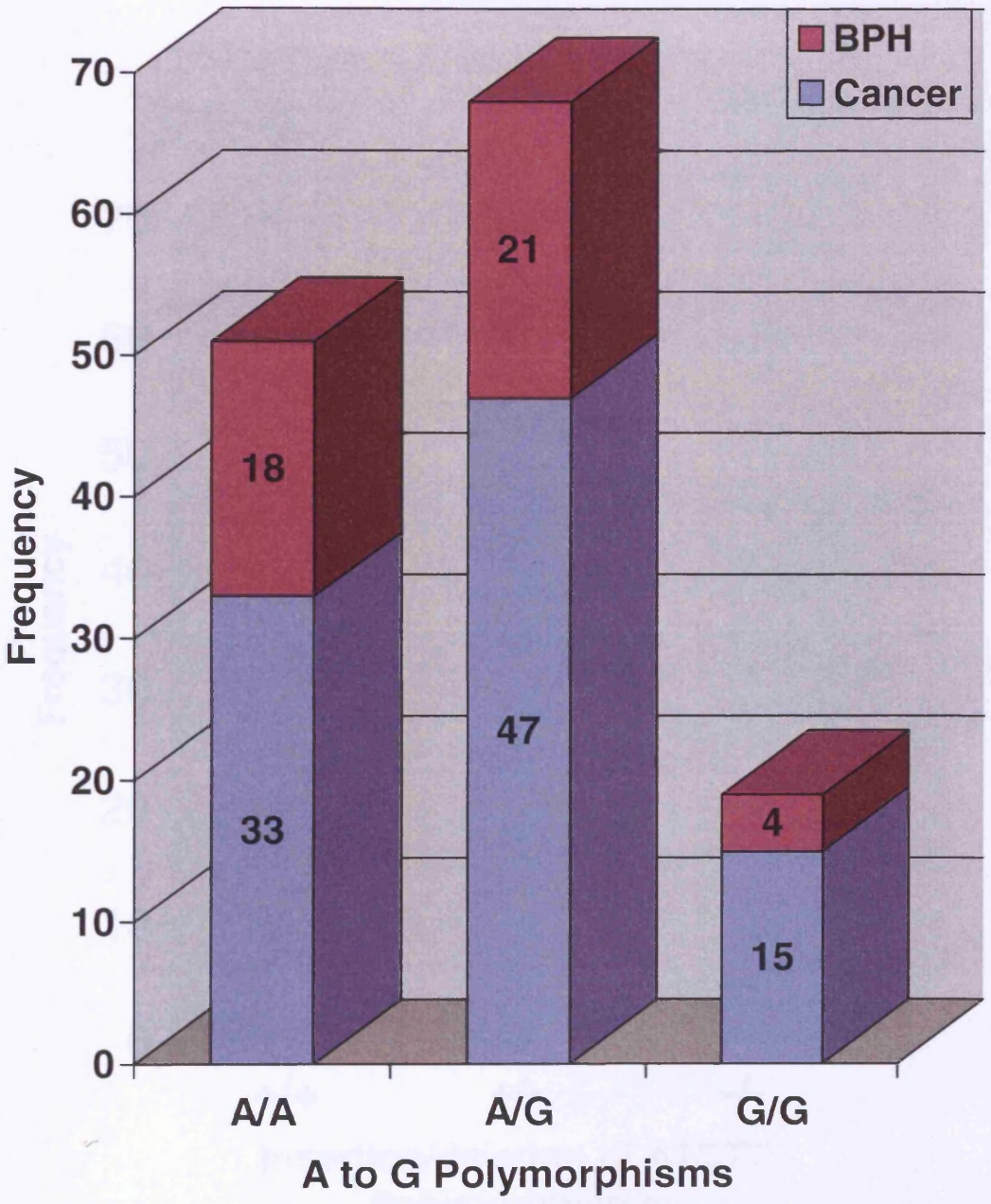


Figure 3.1: Distribution of Adenine nucleotide (A) substitution for Guanine nucleotide (G) polymorphism in intron 2 with the *Pten* gene.
A/A and G/G illustrate homozygous and A/G illustrates heterozygous at that polymorphic site.

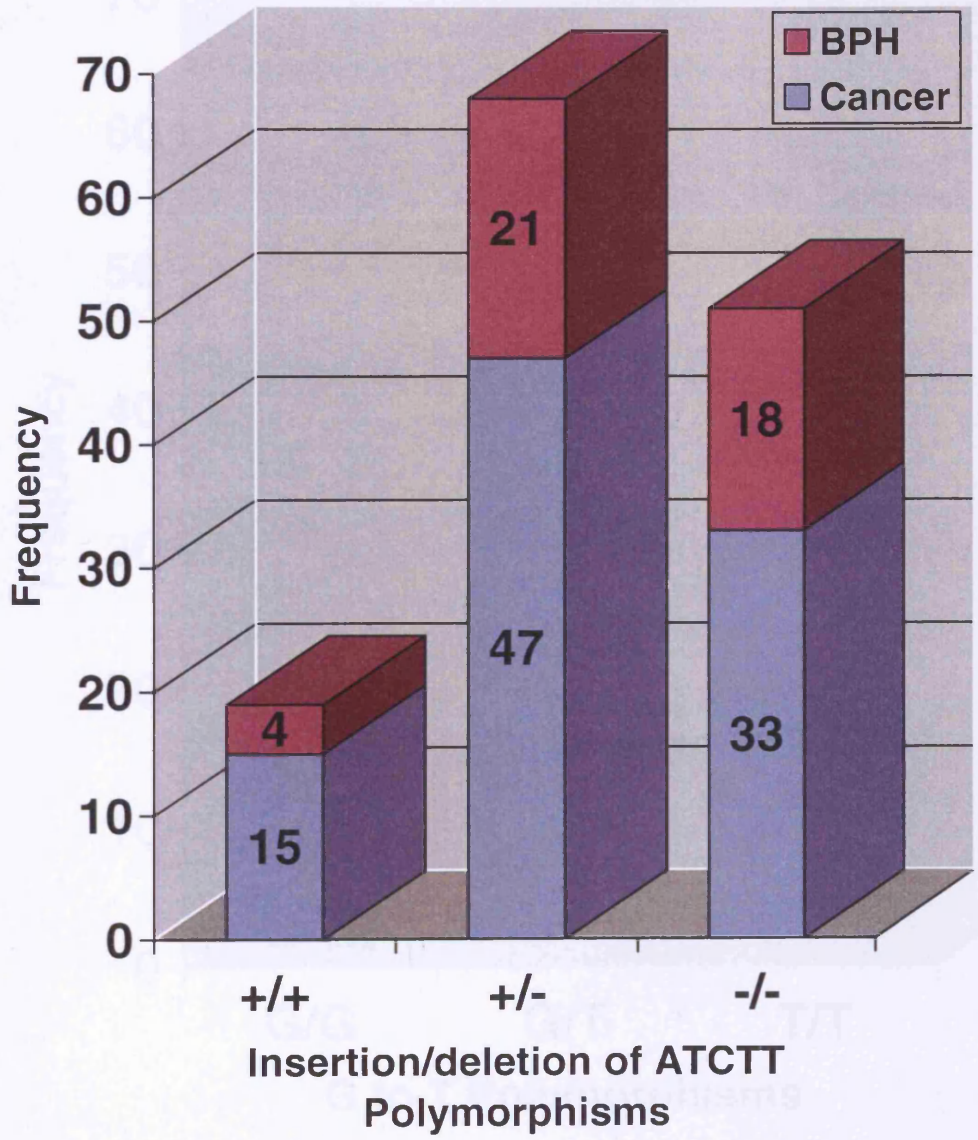


Figure 3.2: **Distribution of ATCTT insertion or deletion polymorphism in intron4 within the *Pten* gene**
(+)=insertion, (-) =deletion, +/+ & -/- illustrates homozygous and +/- illustrates heterozygous at that polymorphic site.

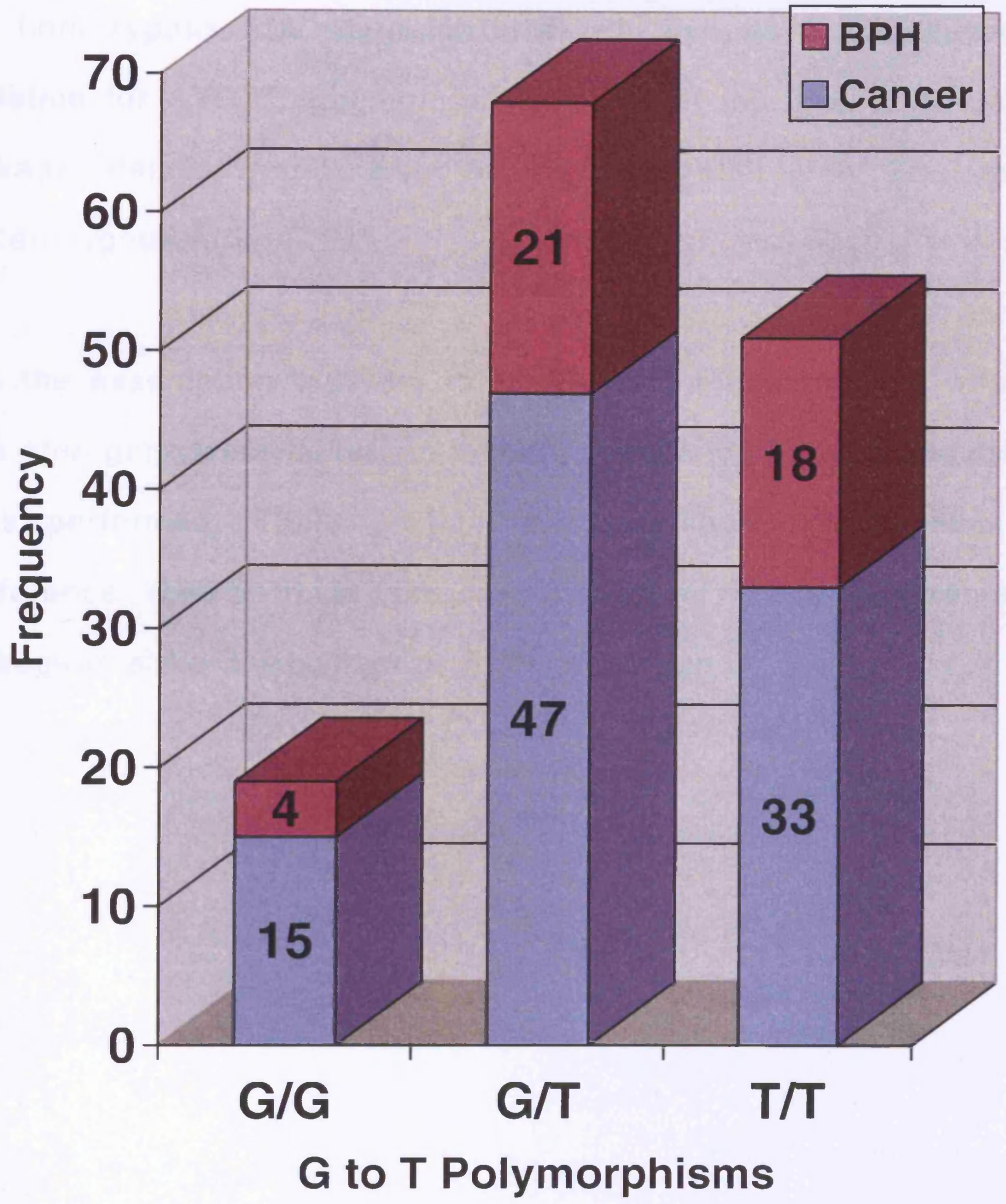


Figure 3.3: Distribution of Guanine nucleotide (G) substitution for Thymine nucleotide (T) polymorphism in intron 8 within the *Pten* gene.
G/G and T/T illustrate homozygous and G/T illustrates heterozygous at that polymorphic site.

These results showed no greater frequency of occurance of a specific polymorphism in either the prostate cancer or BPH population (p=0.518). However, linkage between the three haplotypes investigated was demonstrated. If intron 2 is found to be homozygous A/A, then intron 4 will also have homozygous deletion for ATCTT and homozygous T/T at intron 8. The other linkage demonstrated was homozygous G/G +/+ G/G and heterozygous A/G +/- G/T.

On the assumption that the three intronic polymorphisms within the *pten* gene were linked genetically, analysis of this distribution was performed (Table 3.2). It did not show any significant difference (p=0.518) in the distribution of the polymorphism linkage in either the cancer or BPH population.

POLYMORPHISMS	DIAGNOSIS		TOTAL
LINKAGE	BPH	CANCER	
HETEROZYGOUS (A/G +/- G/T)	21 30.9%	47 69.1%	68
HOMOZYGOUS (A/A –/- T/T)	18 35.3%	33 64.7%	51
HOMOZYGOUS (G/G +/+ G/G)	4 21.1%	15 78.9%	19
TOTAL	43	95	138

Table 3.2: **Distribution of the three linked haplotypes**

Using Pearson Chi-Square test, there was no statistical difference in the distribution (p=0.518).

3.1.4 Correlation of SNPs with Gleason score.

At intron 2 the correlation between each polymorphism and the Gleason score was analysed to assess wheather a particular polymorphism was related to more aggressive disease using Mann-Whitney test. On observation of the graph (Figure 3.4), the polymorphism G/G appears to correspond with higher-grade disease. However, statistical analysis failed to show a significant difference when the G/G was compared to the A/A (p=0.394) and a trend towards significance when the G/G was compared to the polymorphism A/G (p=0.105).

At intron 4 the homozygous insertion of ATCTT/ATCTT (+/+) appears to correspond with higher-grade disease (Figure 3.5). Statistical analysis did not show a significant difference when compared to the homozygous deletion -/- (p=0.394) but showed a trend towards significance when the homozygous insertion of ATCTT/ATCTT (+/+) was compared to the heterozygous insertion/deletion of ATCTT (+/-) (p=0.105).

Observation of the analysis at intron 8 (Figure 3.6) shows that the polymorphism G/G was associated with more aggressive prostate disease but comparisons with the polymorphism G/G showed no statistical clinical significance (p=0.394), however, comparison of

the polymorphism G/G with the polymorphism G/T showed a trend towards significance (p=0.105).

Based on the assumption that the three intronic polymorphisms within the Pten gene were linked genetically, analysis of the correlation of these polymorphisms with the Gleason score was compared (Figure 3.7). Patients who were homozygous G+G appear to have a tendency of having a higher Gleason score within their prostate adenocarcinoma compared to patients who were homozygous A-T and patients who were heterozygous at these three point mutations. However these findings were not statistically significant.

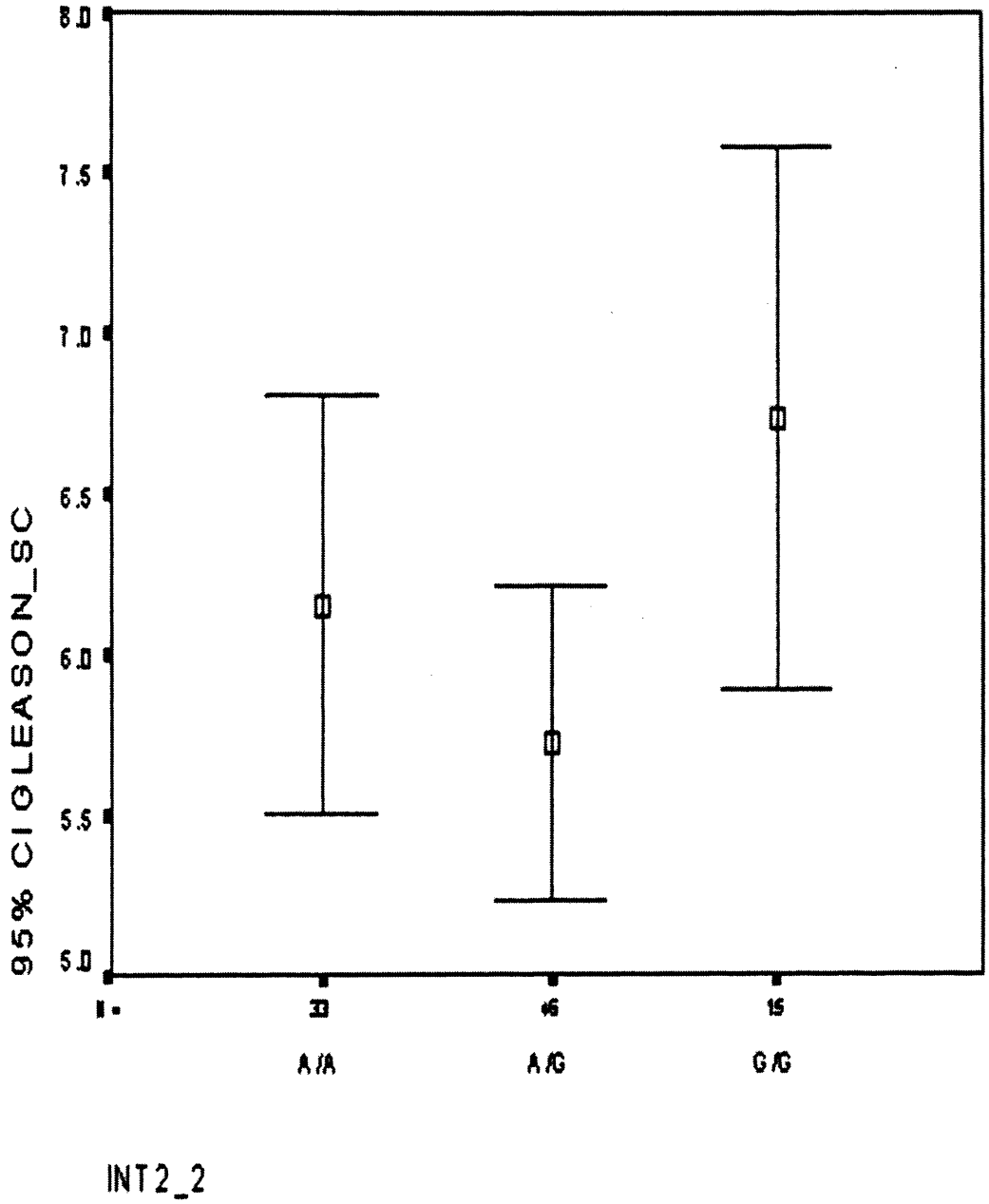


Figure 3.4: **Correlation between each polymorphism at inton 2 and the Gleason score**

Y-axis demonstrates 95% confidence intervals. X-axis demonstares the 95 cancer patients' distribution at intron 2

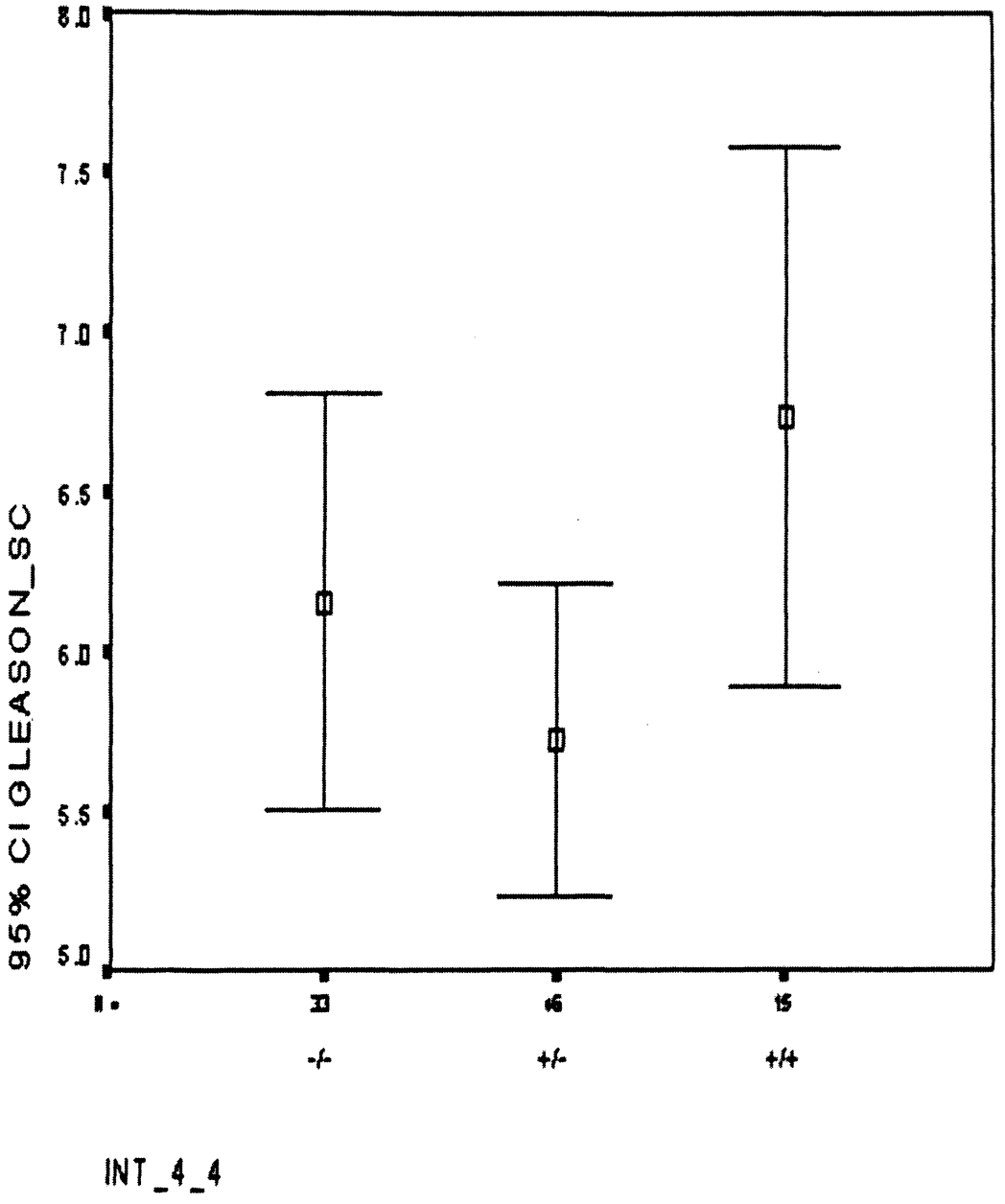


Figure 3.5: **Correlation between each polymorphism at intron 4 and the Gleason score**

Homozygous insertion of ATCTT/ATCTT (+/+) appears to correspond with higher-grade disease however, there was no statistical significant difference when compared to the homozygous deletion -/- (p=0.394).

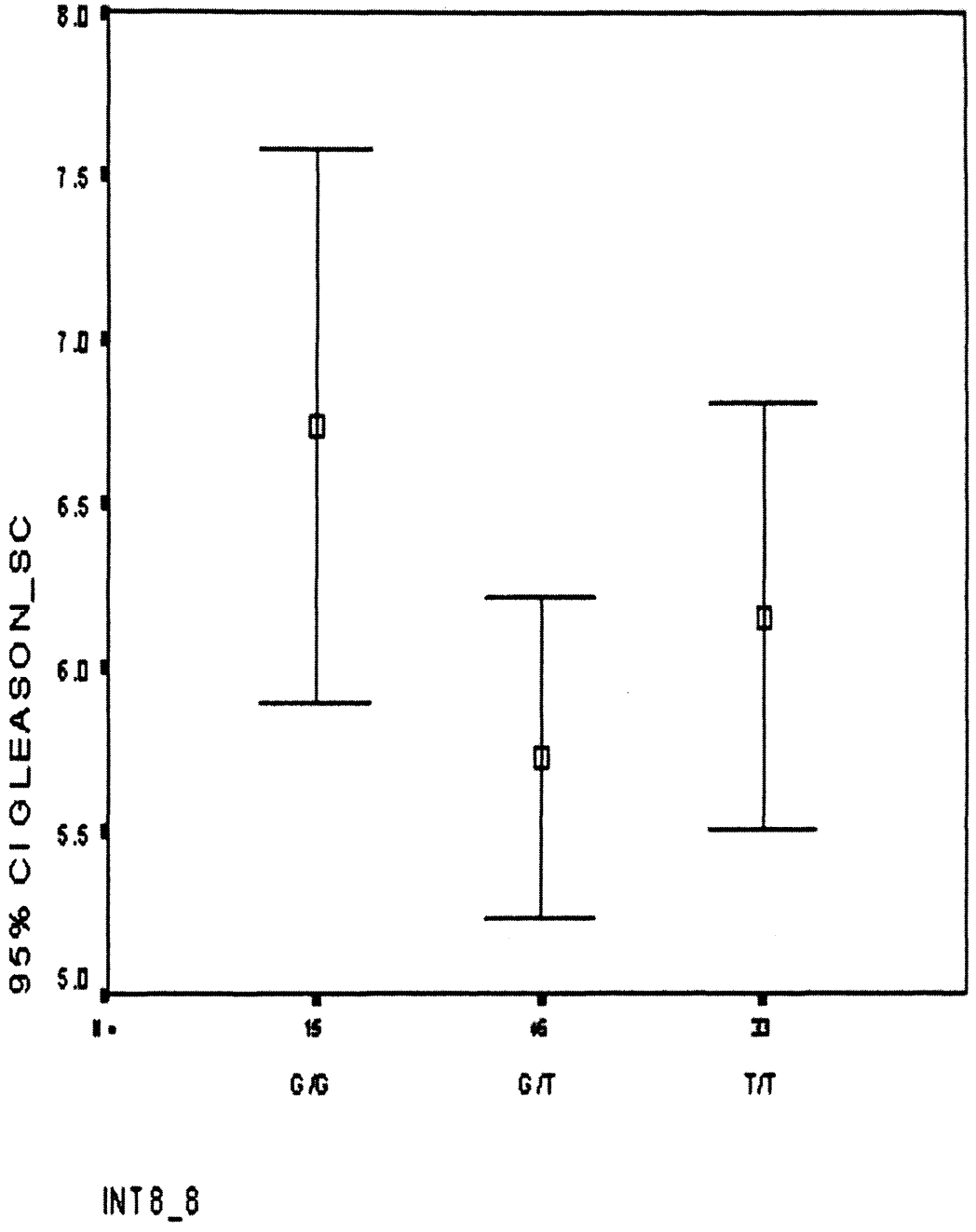


Figure 3.6: **Correlation between each polymorphism at intron 8 and the Gleason score**

Polymorphism G/G was associated with more aggressive prostate disease but there was no statistical clinical significance when compared to the other two groups (p=0.394).

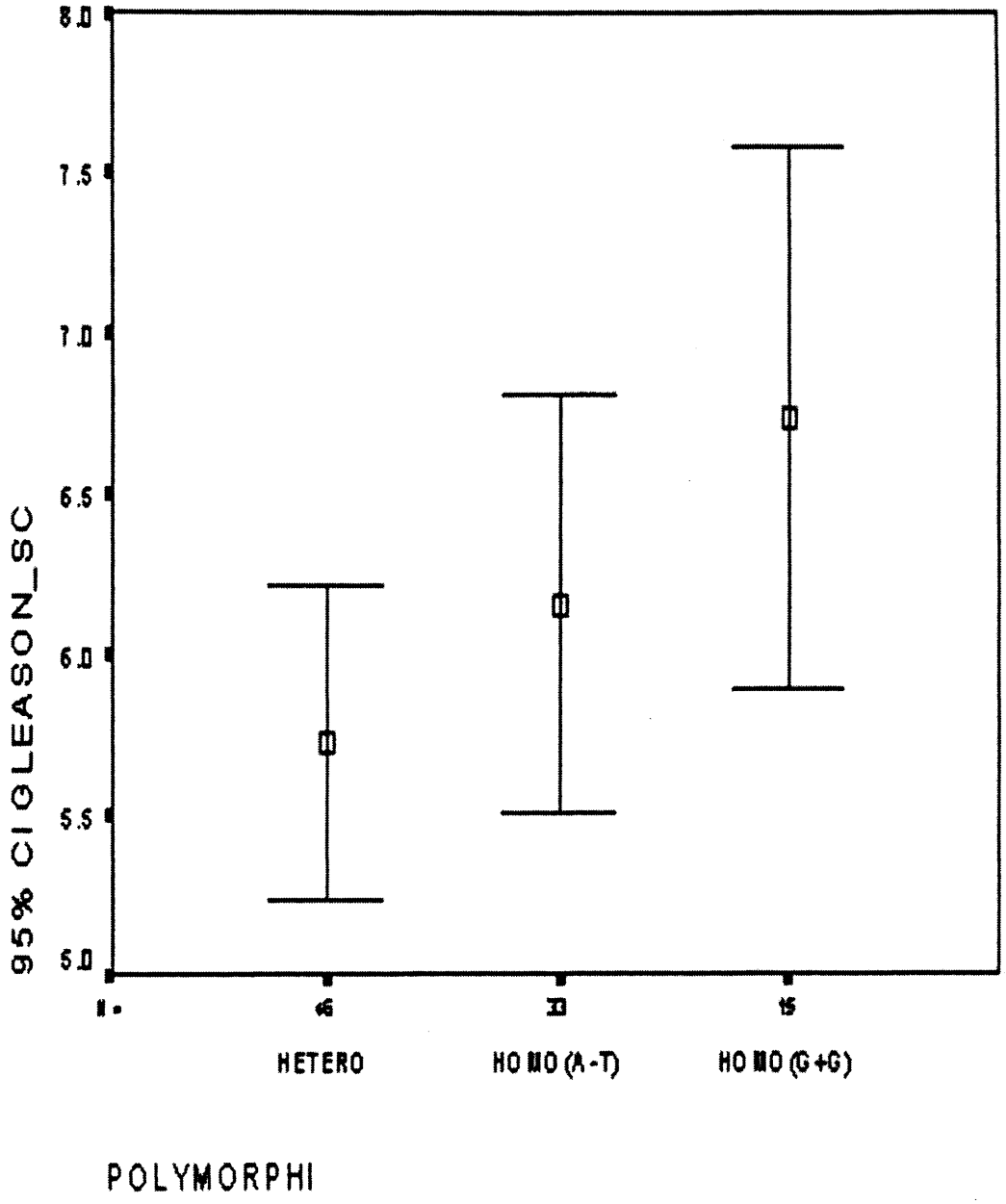


Figure 3.7: **Correlation between the genetically linked polymorphism sites and the Gleason score.**

HOMO (A-T)= Homozygous (A/A –/- T/T), HOMO (G+G)= Homozygous (G/G +/+G/G) &HETERO=Heterozygous(A/G +/- G/T) Patients who were homozygous G+G appear to have a tendency of having a higher Gleason score within their prostate adenocarcinoma compared to patients who were homozygous A-T and patients who were heterozygous at these three point mutations.

3.1.5 Correlation of SNPs with pre-op PSA.

At intron 2 the correlation between each polymorphism and the pre-operative or pre-diagnosis PSA on all 138 samples was analysed to assess wheather a particular polymorphism was related to higher PSA levels at presentation using Mann-Whitney test. On observation of the graph (Figure 3.8), the polymorphism A/G appears to correspond with higher PSA levels at presentation. However, statistical analysis failed to show a significant difference when the polymorphism A/G was compared to the polymorphism A/A (p=0.768) and the polymorphism G/G (p=0.996).

At intron 4 the heterozygous insertion/deletion of ATCTT (+/-) appears to correspond with higher PSA levels at presentation (Figure 3.9). Statistical analysis failed to show a difference when the heterozygous insertion/deletion of ATCTT (+/-) was compared to the homozygous deletion -/- (p=0.768) or homozygous insertion of ATCTT/ATCTT (+/+) (p=0.996).

Observation of the analysis at intron 8 (Figure 3.10) showed that the polymorphism G/T was associated with higher PSA levels at presentation. Statistical analysis showed no clinical significance when the polymorphism G/T was compared with the polymorphism G/G (p=0.917), and with the polymorphism T/T (p=0.574).

Based on the assumption that the three intronic polymorphisms within the Pten gene were linked genetically, analysis of the correlation of these polymorphisms with the pre-operative PSA was compared (Figure 3.11). Patients who were genetically heterozygous at these three point mutations had a tendency to present with a higher pre-operative PSA compared to patients with who were homozygous A-T and patients who were homozygousG+G. However these findings were not statistically significant.

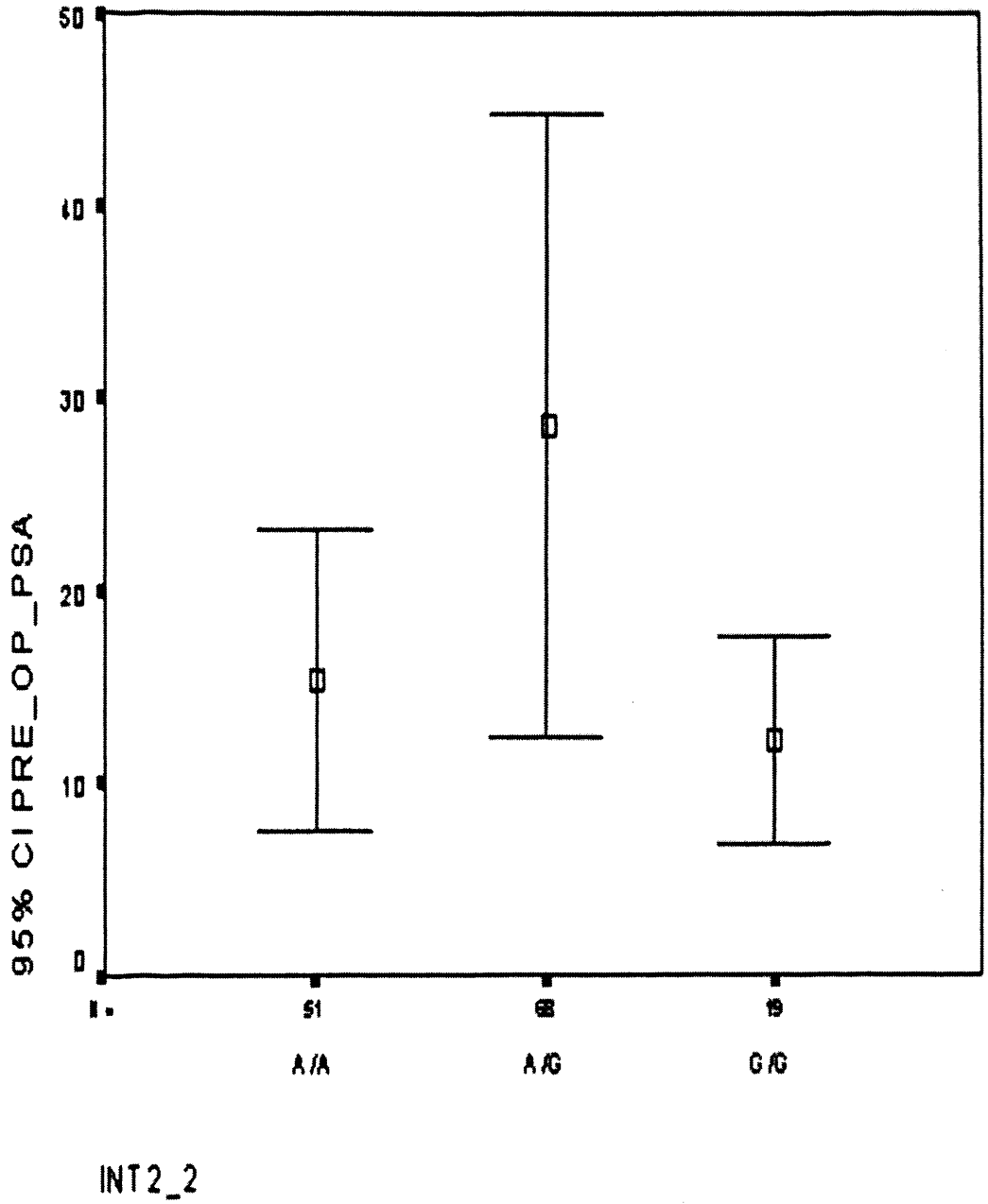


Figure 3.8: **Correlation between each polymorphism at intron 2 and pre-operative PSA**.
This graph includes the PSA on all 138 patients with or without prostate cancer. The polymorphism A/G appears to correspond with higher PSA levels at presentation.

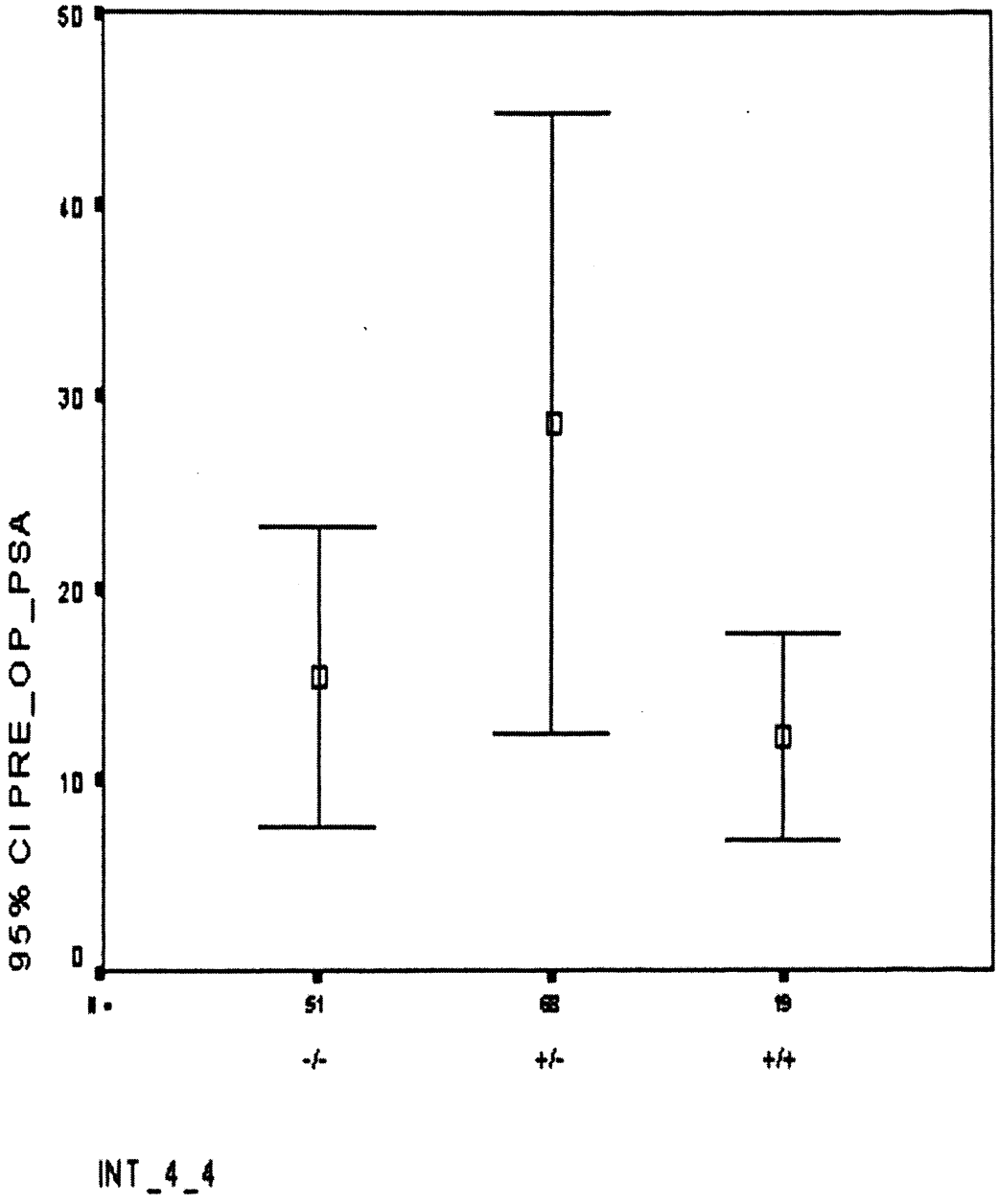


Figure 3.9: **Correlation between each polymorphism at intron 4 and pre-operative PSA.**

This graph includes the PSA on all 138 patients with or without prostate cancer. Heterozygous insertion/deletion of ATCTT (+/-) appears to correspond with higher PSA levels at presentation.

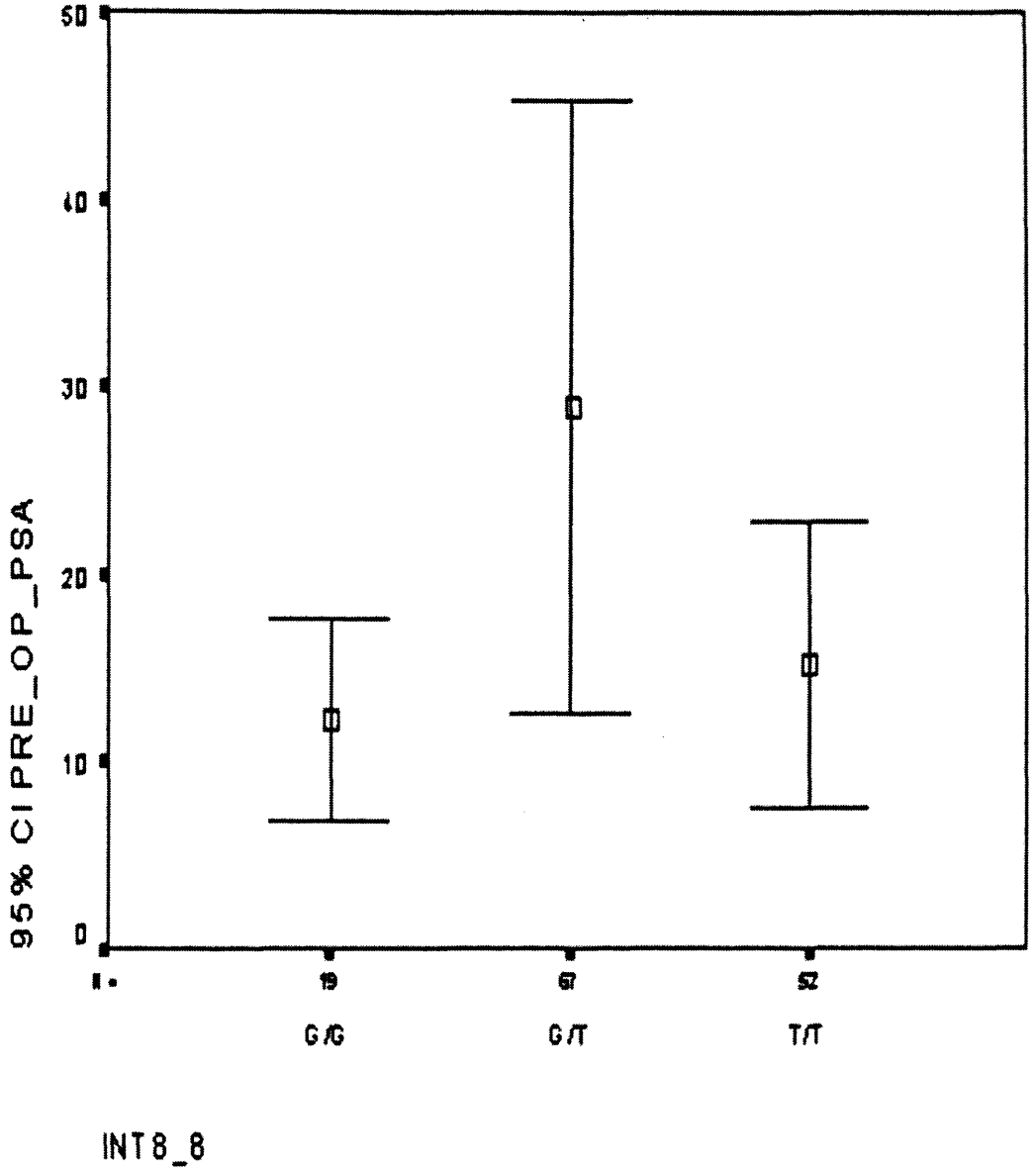


Figure 3.10: **Correlation between each polymorphism at intron 8 and pre-operative PSA.**

This graph includes the PSA on all 138 patients with or without prostate cancer. The polymorphism G/T was associated with higher PSA levels at presentation.

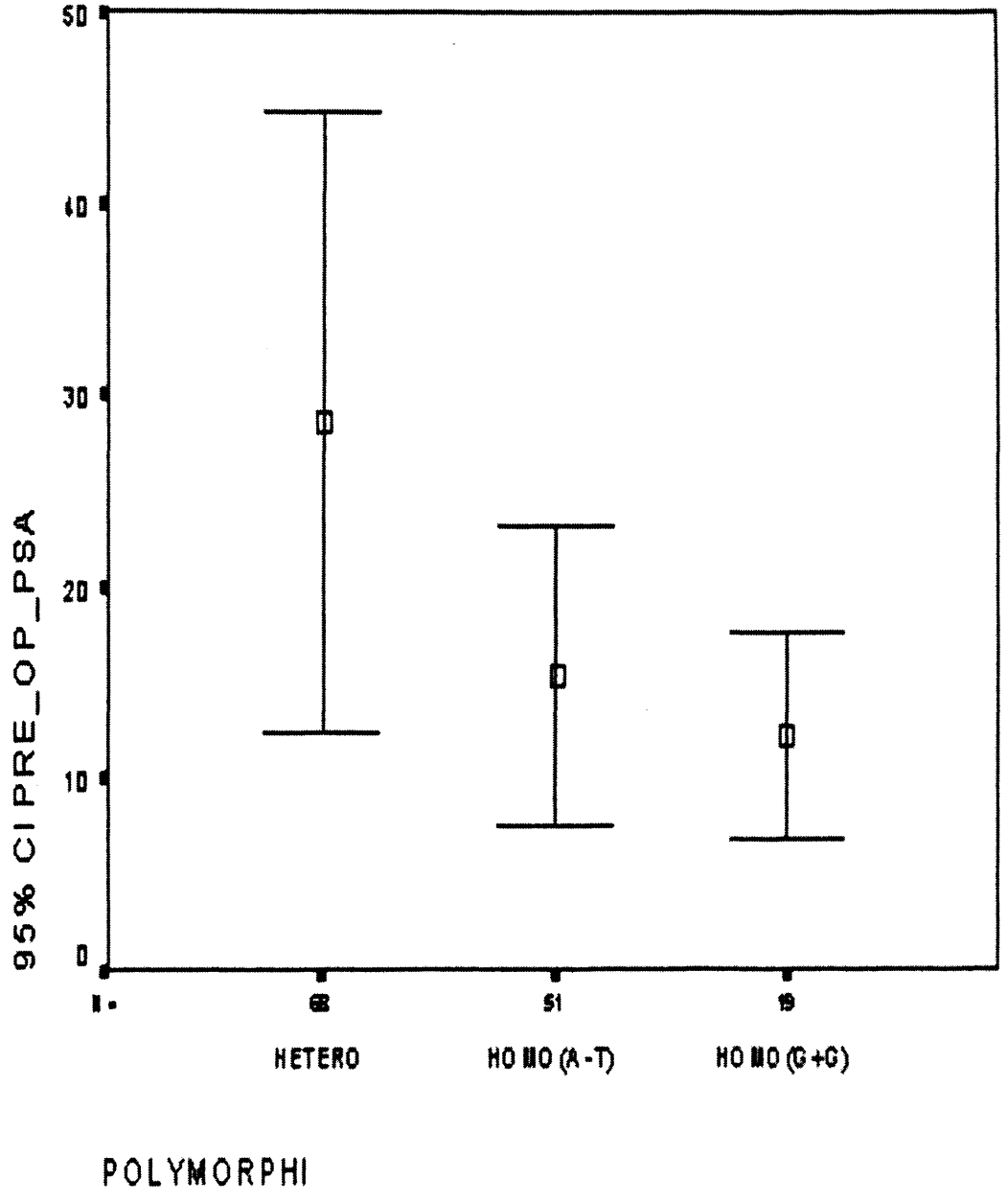


Figure 3.11: **Correlation between the genetically linked polymorphism sites and the Gleason score**

HOMO (A-T)= Homozygous (A/A –/- T/T), HOMO (G+G)= Homozygous (G/G +/+G/G) HETERO=Heterozygous (A/G +/- G/T) Patients who were genetically heterozygous at these three point mutations had a tendency to present with a higher pre-operative PSA compared to patients with who were homozygous A-T /G+G.

3.3 Discussion on SNPs

In prostate cancer, polymorphisms have been identified in two genes, the 17-hydroxylase cytochrome P450 gene (CYP17) and the steroid 5α-reductase type II gene (SRD5A2) that are involved with androgen synthesis (Lunn RM *et al,* 1999). The CYP17 A2 allele contains a T→C transition in the 5' promoter region that creates an additional Sp1-type (CCACC box) promoter site. The SRD5A2 valine to leucine (V89L) polymorphism has been correlated with lower DHT levels. There is evidence that high activity allele (A2) of the CYP17 gene, increase levels of testosterone and may be associated with prostate cancer (Lunn RM *et al*, 1999).

SNPs are an abundant form of genome variation with a low mutation rate per generation. This property makes them the marker of choice for association studies in genetic analysis of complex diseases such as prostate cancer. In this series of experiments, association studies was performed to look for differences in frequency of genetic variants between prostate cancer patients and a control group with no clinical diagnosis of prostate cancer. Such study design has long been used to test the involvement of candidate genes in diseases by linkage (Lander ES and Schork NJ. 1994).

According to the theoretical models, if the genotype of a group of individuals with a common disease and that of a group without the disease are studied, certain genotypes will be consistently associated with those individuals who have the disease (Risch N and Merikangas K 1996). Based on the population genetic phenomenon of linkage disequilibrium (Risch N and Merikangas K 1996), particular genetic markers (haplotypes) in close proximity to a mutation responsible for a disease are often found to be associated with the disease.

Three intronic polymorphisms within the *Pten* gene were analysed: a single-base A→G substitution in intron A 96 bp upstream of exon 2; a 5-bp ATCTT insertion / deletion in intron D 110 bp downstream of exon 4 and a single-base T→G substitution in intron H 32 bp downstream of exon 8. Comparisons between the prostate cancer population and the non-prostate cancer control group did not show any specific association between any of the polymorphism and prostate cancer.

The control group consisted of patients with negative prostate biopsies. This investigative tool has a high sampling error (false-negative) and does not entirely exclude the presence of prostate cancer. A more accurate non-cancer control group would have been obtained from TURP chipping submitted for histological analysis. This method would allow for more accurate sampling of

the patient's prostate tissue and disease state. If this methodology had been adopted, it might have reflected in the results, as the clinical data might have been different.
Attempts were made to identify a correlation between these SNPs and high-grade prostate cancer as well as an association between the SNPs and PSA at presentation. No identifiable correlation was established.

Depending on where a SNP occurs, it might have different consequences at the phenotypic level. SNPs in the coding regions of genes that alter the function or structure of the encoded protein are a necessary and sufficient cause of most of the known inherited monogenic disorders.

By performing these series of experiments, I had set out to illustrate an association between one of the polymorphisms investigated and prostate cancer. If an association had been established, a marker could be developed to identify individuals at risk of developing prostate cancer and may predict subsequent disease behaviour such as the likelihood of disease recurrence or progression. As well as a potential role in the natural history of a disease, SNPs may well determine an individual's response to treatment such as chemotherapy or radiotherapy. To date, no such marker has been found for prostate cancer. The experiments in this chapter have also failed in the quest to discover such a marker in prostate cancer.

CHAPTER FOUR

LOSS OF HETROZYGOUSITY

WITHIN THE *Pten* GENE.

4.2 Introduction

The *Pten* gene is located at chromosome 10q23 and is a putative tumour suppressor gene thought to play a role in development and progression of prostate cancer. *Pten* gene is known to be deleted in 90% of glioblastomas, however, the frequency and significance of *Pten* deletion in prostate cancer remains unknown.

The aim of this work was to determine the frequency of Loss of heterozygousity (LOH) within and around the *Pten* locus when an individuals' normal DNA profile is compared to the DNA obtained from prostate cancer cells of the same individual. The cancer DNA is obtained from archival paraffin embedded radical prostatatectomy specimens.

The study was performed using fluorescence-based allelotyping with four highly informative microsatellite cytosine-adenine (CA)n dinucleotide repeat markers: D10S541, D10S2492, D10S1765 and AFMa086wg9 located within and around the *Pten* locus (Fig 4.1). This study also investigates the significance of these LOH in relation to clinicopathological findings.

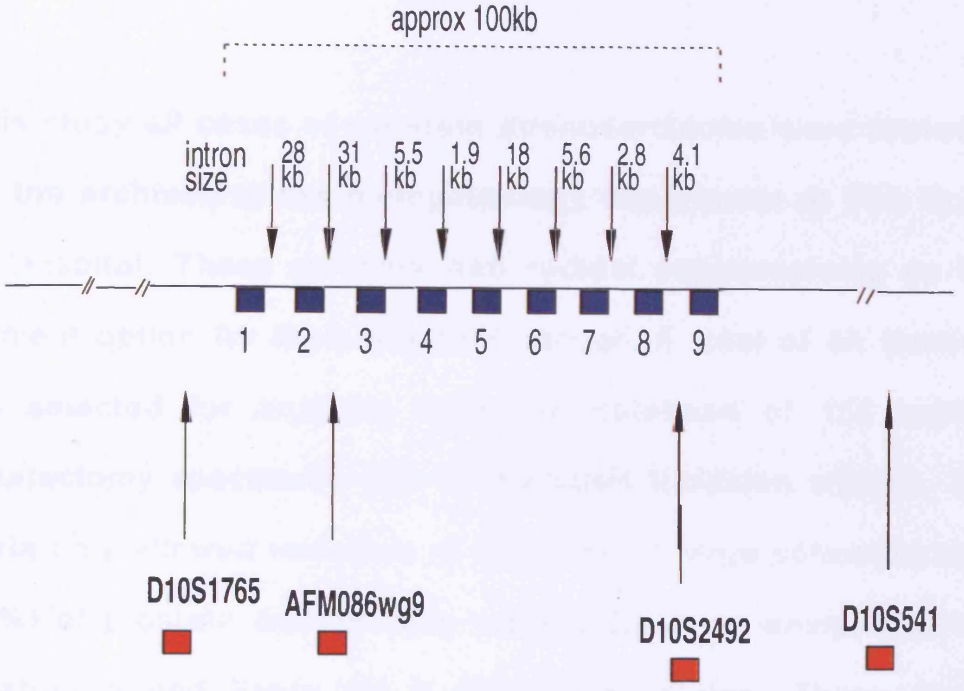


Figure 4.1: **Position of microsatellite markers in *Pten***
Demonstrates the location of the four microsatellite markers within and around the *pten* gene.

4.2 Case selection for LOH study

4.2.1 Criteria for selection

In this study all cases of prostate adenocarcinoma were retrieved from the archives of the histopathology department at The Royal free Hospital. These patients had radical prostatectomy as the treatment option for their prostate cancer. A total of 34 tumours were selected for analysis from the database of 155 radical prostatectomy specimens due to the strict inclusion criteria. The criteria only allowed inclusion of specimen if large concentrations (>70%) of prostate cancer cells were present on analysis of the Hematoxylin and Eosin (H & E) stained slides. These slides mirrors the archival paraffin embedded radical prostatectomy tissue sections.

4.2.2 Criteria for exclusion

The patients with prostate cancer whose archival paraffin embedded radical prostatectomy specimen would have produced a low concentration (<70%) of cancer DNA following manual microdissection of the tissue.

4.3 Source of prostate tissue

Tumour tissue was obtained from archival paraffin embedded radical prostatectomy specimens from the histopathology department at the Royal Free hospital. The histopathologist retrieved and reviewed H & E stained slides in the archives and selected only specimens with large concentrations (>70%) of prostate cancer cells on the slides. Three 3µm thick sections were obtained from the corresponding paraffin-embedded tissue block and were subjected to histological evaluation to ensure the high tumour cell density was maintained. These areas were circumscribed with a marker pen allowing me to focus on the area for manual microdissection (Figure 4.2).

The distribution of disease and exact source of each paraffin block was indicated clearly on a diagram as the entire gland had been submitted for microscopic evaluation allowing for a final diagram of the prostatic diseases to be generated documenting anatomic distribution and multifocality when present (Figure 4.3). The tumour areas are mapped in red ink indicating the geographic and zonal distribution of the neoplasm(s) and the states of the surgical margins, seminal vesicles, and extracapsular tissue. This allows for a highly accurate microscopic characterisation of each minor image sample (proportion of tumour present, histological grade(s), proportions of benign epithelium, stroma, premalignant lesions, etc).

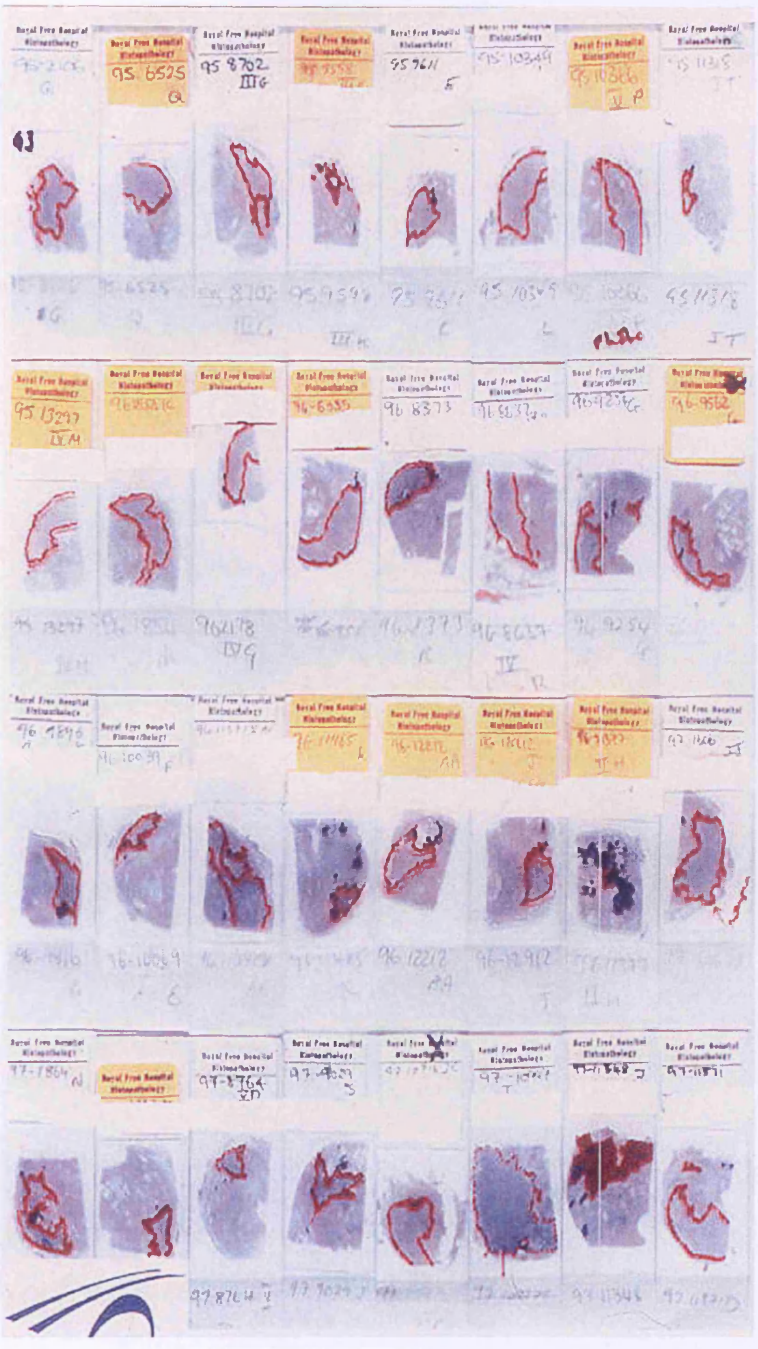


Figure 4.2: **Slides containing prostate cancer stained with Hematoxylin and Eosin.** Demarcate areas of high tumour concentration (>70%).

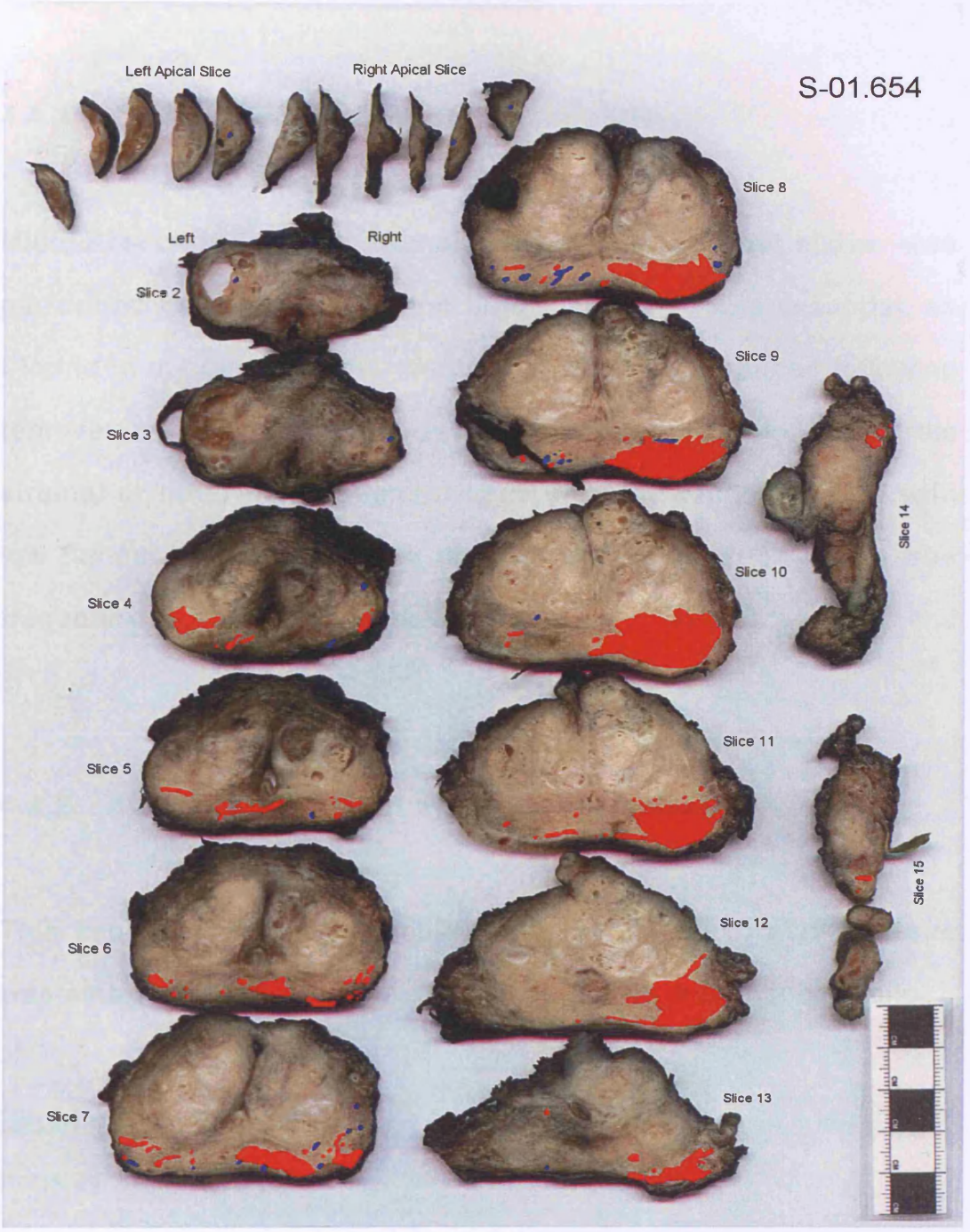


Figure 4.3 Site mapping on prostate specimen

Areas containing adenocarcinoma

Areas containing PIN (High grade).

4.4 DNA extraction protocol

4.4.1 Microdissection of Cancer cells

Microdissection of the demarcated areas on the slides was performed manually with a fine blade. This step was essential, as I found in my earlier experiments that the DNA produced following removal of paraffin was heavily contaminated with DNA from stromal or other non-malignant cells. Microdissection of area with low tumours density was resulting in underestimation of the true frequency of LOH at a locus.

4.4.2. Deparaffination and DNA extraction

This process removes the paraffine, which the prostate tissue was embedded allowing for extraction of DNA from cancer cells.

Solutions used

3mls of 99% Xylene

3mls of Industrial methylated spirits

1ml of Proteinase K

1 X PCR buffer (15mM $MgCl_2$)

Experimental procedure

Soak the slide in Xylene for 5 minutes to deparaffinate the tissue on the slide, then repeat this process. Transfer the slide into a solution of industrial methylated spirits for 5 minutes in order to remove the xylene, and then allow slide to air dry.

Manually microdissect out the area of high tumour density marked out on the slide by the histopathologist. Place the tissue in the solution of 1 X PCR buffer ($MgCl_2$) containing Proteinase K and keep at 55°C in a heating block for 2 days to allow for digestion of cellular protein, forming a cloudy solution. Incubate the solution at 96°C for 10 minutes to deactivate the Proteinase K. Centrifuge the solution at ~13,000 rpm and carefully transfer the supernatant into 1.5ml sterile eppendorf tube. This supernatant is the source for the genomic prostate cancer cell DNA.

In order to analyse the region of LOH in prostate cancer, comparison needs to be made with DNA obtained from non-cancer tissue obtained from the same patient. In this thesis non-cancer DNA was obtained from lymphocytes separated from heparinized corresponding blood using DNA Puregene™ DNA isolation kits as described earlier in chapter 2.4.1 (Preparation of genomic DNA).

4.4.3 PCR of cancer and corresponding blood DNA.

PCR is performed on DNA extracted from the adenocarcinoma cells obtained from the archival prostate tissue and the corresponding blood sample obtained from the patient. The solutions used are similar for both PCR reactions except, the first PCR used genomic DNA obtained from the patients blood and the working solution volume was 25µl compared to the second set of reactions, which used DNA, obtained from prostate cancer tissue and used 50µl solutions in order to dilute out contaminants such as formalin fixatives which inhibits polymerase chain reactions.

Solutions used	µl
DNA template (50ng)	1.0
AmpliTaQ Gold Buffer	2.5
dNTP's (2mM)	2.5
Primer A (10µM)	1.25
Primer B (10µM)	1.25
AmpliTaq Gold Polymerase	0.125
Sterile dH_2O	16.375
Total	25 µl

The oligonucleotide primers were obtained from the Genebank database and synthesised by the ICRF, Clare Hall laboratories. The sequences of the fluorescent primers for microsatellite analysis are listed in Table 4.1.

Markers	Primer	Number	Sequence of primers
D10S541	A	100650	5'-AAGCAAGTAAGTCTTAGAACCACC-3'
	B	103596	5'-GTTCTTCCACAAGTAACAGAAAGC-3'
D10S2492	A	100653	5'-TGCAGTGAGCTGTGAAGATG-3'
	B	106578	5'TTTCTTTGTTTCTCTTACTACCTATGT3'
D10S1765	A	100655	5'-ACACTTACATAGTGCTTTCTGCG- 3'
	B	106581	5'-GTTTCTTCAGCCTCCCAAAGTTGC –3'
AFMa086 wg9	A	100654	5'-AAATGTACGGTTCATTGACTT-3'
	B	106579	5'-TTTCTTGACTGACTACAAATGGGCA3'

Table 4.1: **Sequence of oligonucleotide primers.**
The DNA sequence of the oligonucleotide primers used to sequence the DNA in the region of the four microsatellite markers. The sequence runs from 5'⟶ 3' end.

Experimental procedure

PCR Parameters used were:-

Temperature (°C)	Time (minutes)	
96	10.00	
96	00.15	Repeat cycle 35 X
55	00.30	
72	03.00	
72	10.00	
4	∞	

The samples were initially denatured at 96°C for 10 minutes, then subjected to 35 cycles of 15 seconds denaturising at 96° C, 30 seconds annealing at 55° C and polymerisation at 72° C for 3 minute allowing for extension at 1 Kb per minute. There is a final step of extension at 72° C for 10 minutes to ensure completion of all amplification events. The PCR product is then cooled to 4° C.

4.4.4 Separation of PCR product by electrophoresis.

This step is to check whether the PCR was successful and free of contamination. 2% Agarose was used as the support matrix for enabling the PCR product to be separated under the influence of an electrical current. A small pore gel results when agarose undergoes change from a liquid to a solid form. Smaller PCR fragments will travel further down the gel in a given time; thus fragments of differing sizes will be separated.

Solution used

2% Agarose	3grams
0.5 X TBE (Triss, Borate, EDTA)	150mls
Ethidium bromide	3.5µl

Ethidium bromide is known to contain a planar group that intercalates between the stacked bases of DNA. The fixed position of this group and its close proximity to the bases causes dye bound to DNA to display an increased fluorescent yield compared to dye in free solution. Ultraviolet irradiation absorbed by the DNA at 260nm and transmitted to the dye, or irradiation absorbed at 300nm and 360nm by the bound dye itself, is emitted at 590 nm in the red/orange region of the visible spectrum.

Each gel had a standard marker in lane 1 of the gel. On electrophoresis this marker separated into fragments of known size and molecular weight. Thus by using this as a comparison one could estimate the sample fragment sizes. First well contains standard molecular weight marker of known size. TheΦX174 DNA/*Hae* III Marker was used. They are made by digesting double stranded ΦX174 DNA to completion with *Hae* III.

Experimental procedure

Mix the 2% agarose gel constituents thoroughly in a glass beaker. Heat the glass beaker in a standard domestic microwave, at high power for 5 minutes. Allow the beaker to cool by leaving it standing for 2 minutes. Add 3.5µl of ethidium bromide into 150ml of 0.5 X TBE solutions and allow bottle to cool for further 5minutes. Whilst still in the liquid form, pour the solution into perspex receptacle containing two 24 toothed plastic comb. When the liquid set, remove the comb forming two 24 wells.

Mix 4µl PCR product with 2µl of loading buffer (Orange G and 30% Glycerol) and pipet into each well. Pour 0.5 x TBE Into the reservoirs on the assembly to cover the electrodes. Connect the assembly to a power supply and run the gel at 250 volts for 20minutes. Inspect the gel under ultraviolet light wearing UV safe goggles. A Polaroid picture was taken of the illuminated gel in order to provide a permanent record.

4.4.5 Purification of PCR product

The PCR product is contaminated with unwanted primers and impurities such as salts, enzymes and unincorporated dNTPs. It is essential that the product be purified for good DNA sequence to be obtained. The QIAquick PCR Purification protocol is designed to purify PCR products by removing unwanted contaminants using QIAquick spin columns in a microcentrifuge.

The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica-gel membrane. Special buffers listed below are optimised for efficient recovery of DNA and removal of contaminants in each specific application. DNA absorbs to the silica membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with distilled water. Adsorption of DNA to silica membrane depends on pH. Adsorption is typically 95% if the pH is ≤ 7.5, and it reduced drastically at higher pH.

Buffer PB allows the efficient binding of PCR product as small as 100bp and quantitative (99.5%) removal of primers up to 40 nucleotides. It is therefore used to remove oligonucleotide primers after cDNA synthesis or to remove unwanted linkers in cloning experiments.

During the DNA adsorption step, unwanted primers and impurities such as salts, enzymes, and unincorporated dNTPs do not bind to the silica membrane, but flow through the column. Salts are washed away by the ethanol containing Buffer PE. Any residual Buffer PE, which may interfere with subsequent enzymatic reactions, is removed by an additional centrifugation step.

Experimental procedure: -

Centrifuge steps are at ≥10, 000 x g (≈ 13,000 rpm) in a conventional domestic tabletop microcentrifuge.

Add 125mls of Buffer PB to 25µl of PCR product. Place the QIAquick spin column into a 2 ml collection tube.

To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 seconds. Discard the flow through. Place the QIAquick column back in the same tube. To wash, add 0.75mls of Buffer P.E to the QIAquick column and centrifuge for 60seconds. Again, discard the flow through. Return the QIAquick column to the same tube and centrifuge for an additional one minute at maximum speed. Place the QIAquick column in a clean 1.5ml microfuge tube. To elute DNA, add 30µl Buffer E.B to the centre of the QIAquick column and allowed to stand for one minute then centrifuged for one minute leaving an elution of bound DNA.

For the sequence reaction, 1 in 20 dilution of blood PCR product and 1 in 10 dilution of tissue PCR product is used for gene typing to reduce fluorescence and allow for product to be in the range for suitable for the ABI Prism 377 DNA Sequencer.

4.4.6 Analysis of sequenced fragment

The next stage is to run the extension products on a sequencer to obtain a reading of the nucleotides in order to establish the magnitude of LOH present within the amplified region.

Solutions used

PCR product

Loading buffer containing dextran blue and formamide.

TAMRA 500bp ladder

Experimental procedure

1µl of dilute PCR product and 3µl of loading buffer is added to 55µl buffer plus 10µl of TAMRA 500bp ladder. The samples were denatured immediately prior to gel loading by heating the solution to 95°C for 10 minutes followed by snap chilling in an ice/water bath.

The Longranger gel containing acrylamide is used as the support matrix for enabling the oligonucleotide to be separated under the influence of an electrical current.

Solution used

Longranger gel (Acrylamide)	5mls
5 x TBE	10mls
10% APS	250µl
Urea	18gms
TEMED	35µl

The above was sufficient to run a gel and each gel was able to support and analyse 32 samples. The sequence products were run on the ABI Prism 377 DNA sequencer and the results analysed using Genescan software (version2.1.1) by Perkin-Elmer).

CHAPTER FIVE

Results and discussions on

LOH study on *Pten* gene.

5.1 Results of LOH analysis

5.1.1 Analysis of sample population

The age range of the prostate cancer population studied (at the time of diagnosis of the disease) was 46 – 77 years, with the mean age being 65.21 years (Figure 5.1). The preoperative PSA range was 2.2 – 252.0 µg/l with a mean of 24.13µg/l (Figure 5.2). The distribution was skewed because two patients underwent radical prostatectomy with exceptionally high preoperative PSA of 210µg/l and 252µg/l. If these two patients were omitted from the study, the preoperative PSA range would have been 2 – 39.9µg/l with a mean of 10.7µg/l. Statistically, the value of the 5% trimmed mean for the preoperative PSA was 13.65µg/l. The TRUS of these two patients revealed their prostates to have estimated volumes of 152grams and194grams respectively. The histology of the prostate biopsy specimen revealed adenocarcinoma with Gleason scores of five as well as histological evidence of chronic active prostatitis in both cases. Neither of these two patients had evidence of metastatic spread on bone scan or CT imagining. Further histological analysis of their entire prostate gland and lymph nodes post radical prostatectomy did warrant upstaging of their disease to stage pT2bNoMo and pT3aNoMo respectively. The distribution of the TNM stage and Gleason score for the prostate carcinoma patients at the time of diagnosis is illustrated in Figure 5.3 and Figure 5.4 respectively.

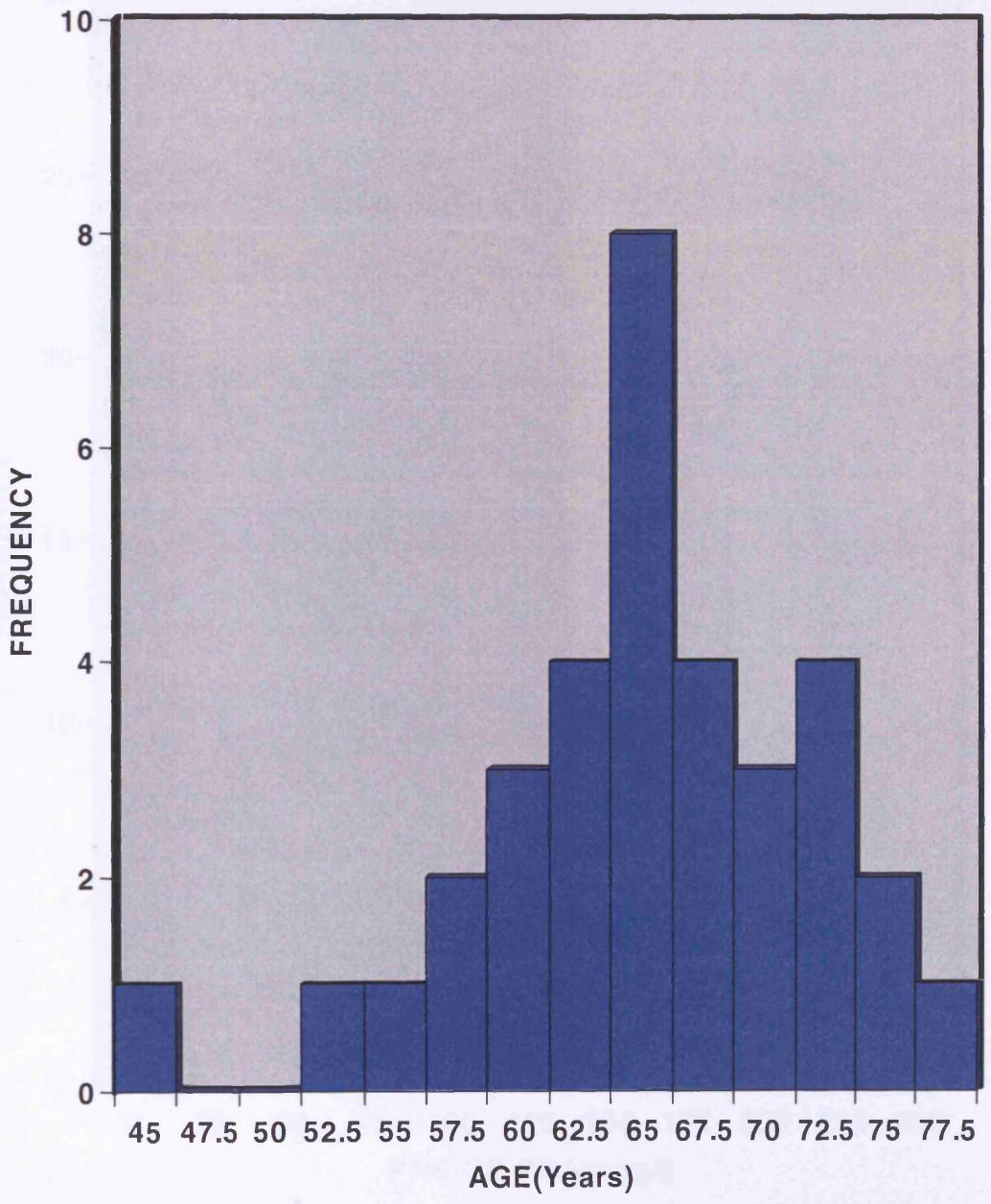


Figure 5.1: Age distribution in LOH study

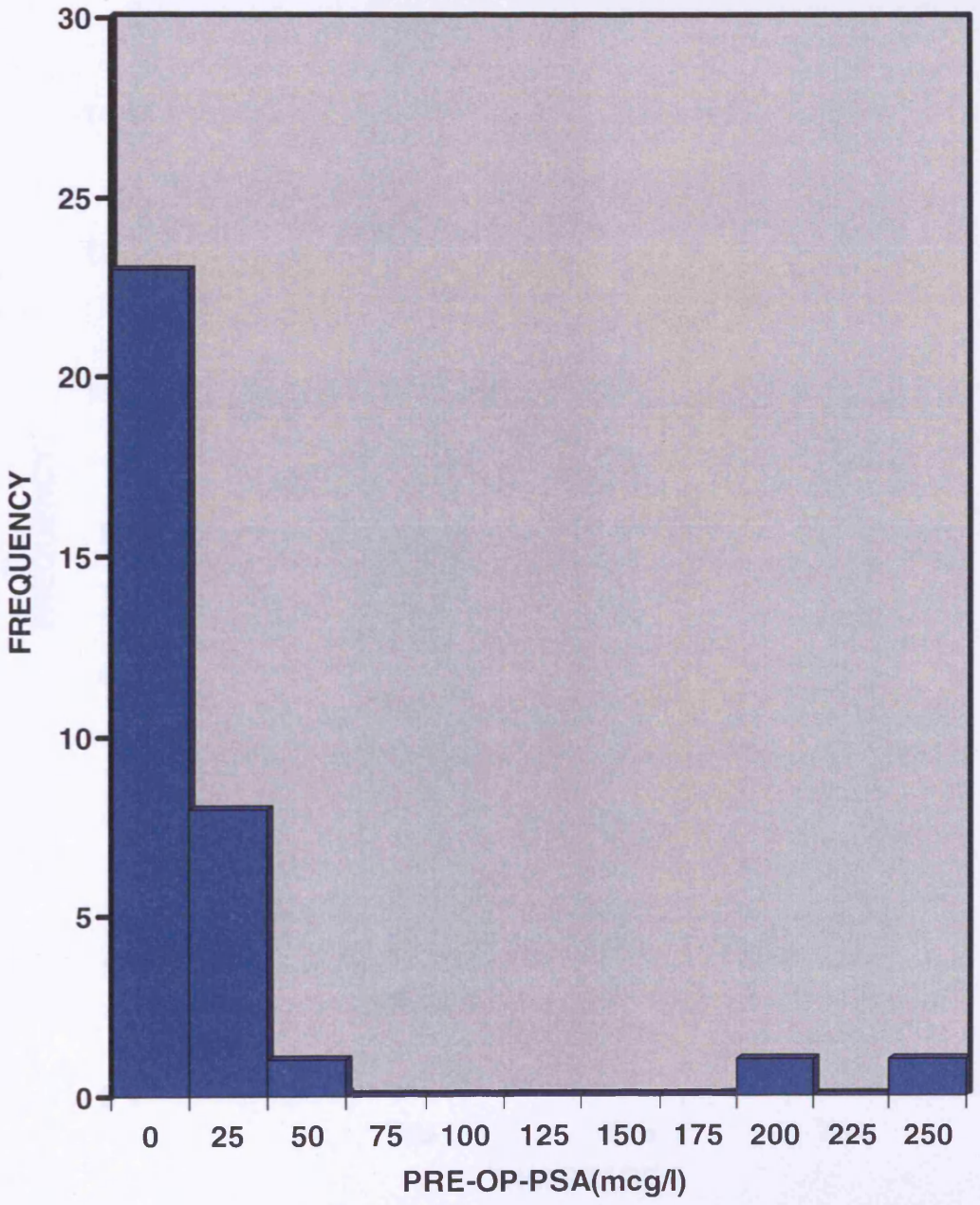


Figure 5.2: Pre-operative PSA distribution in LOH study

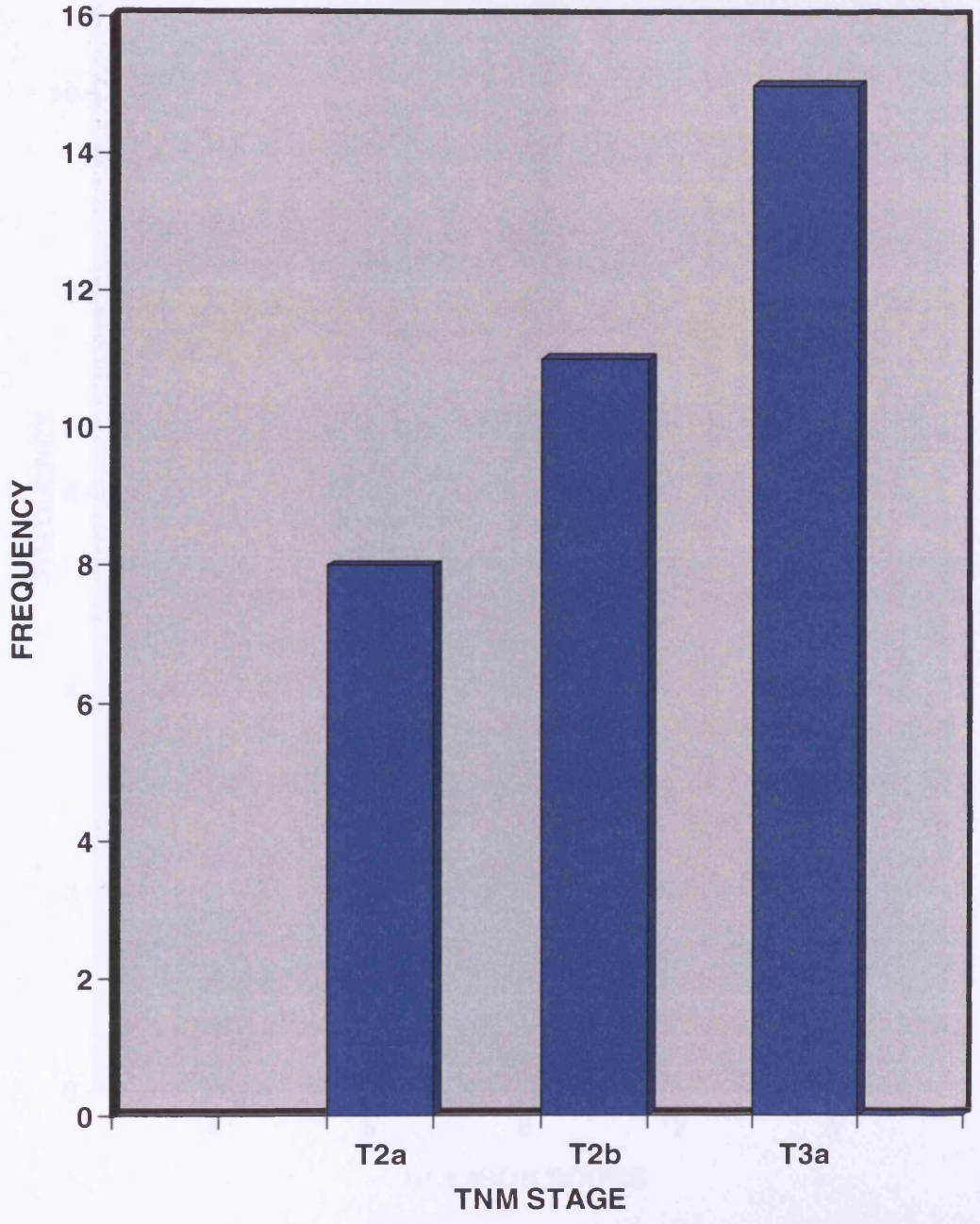


Figure 5.3: TNM staging for the prostate cancer population.

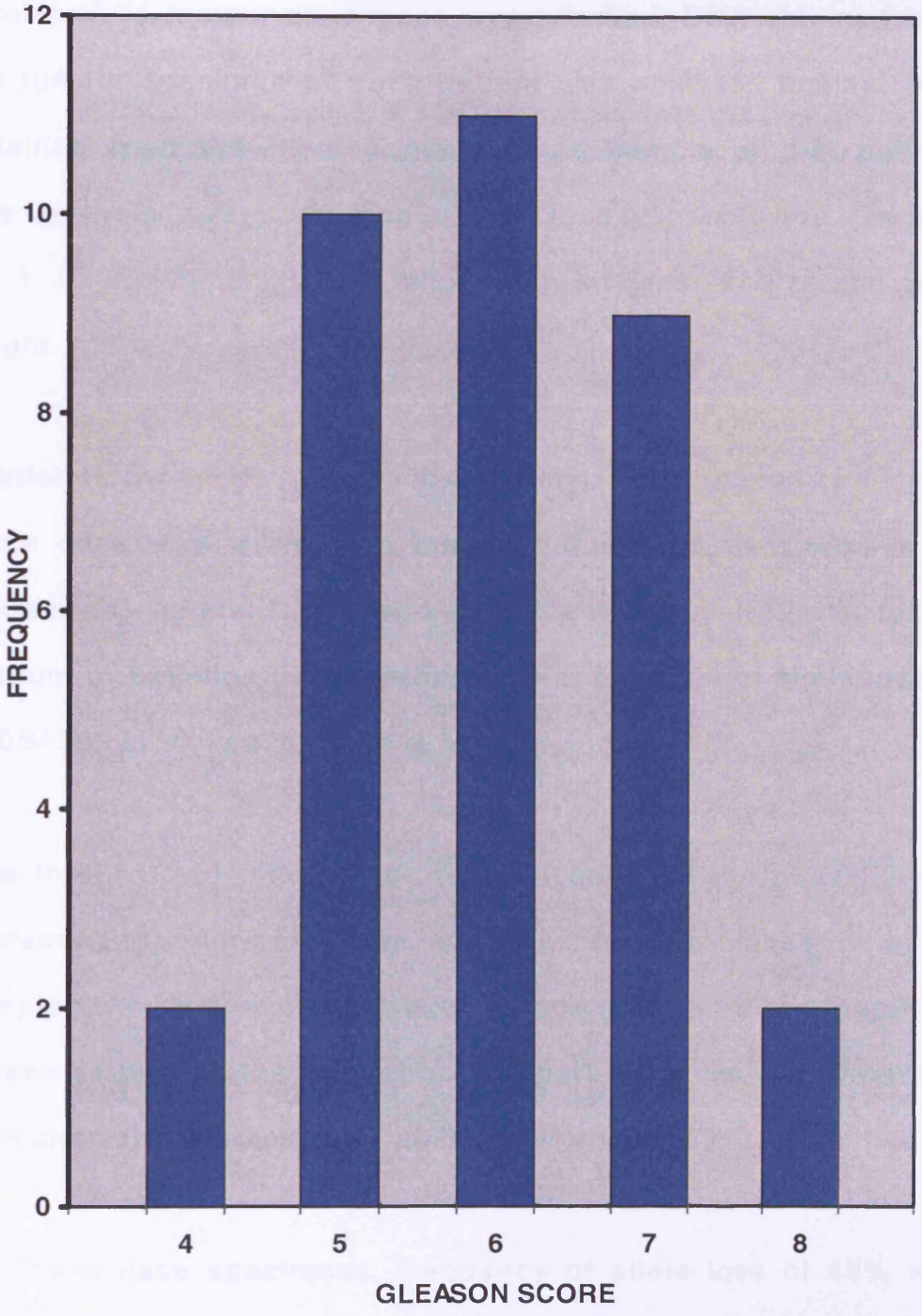


Figure 5.4: **Gleason Score for the prostate cancer population.**

5.1.2 Analysis of LOH study data

A total of 34 tumour specimens were studied. DNA obtained from the tumour specimen of each patient was analysed against DNA obtained from the corresponding blood sample of that patient. The analysis was performed using Genescan software (version 2.1.1; Perkin-Elmer), which allowed for analyses of size and peak height.

In informative cases, allelic loss was scored if the area under one allele peak was reduced in the tumour to less than 50% of its value in the normal DNA (relative to the other allele), after taking account of baseline fluorescence levels. Example of allele loss at D10S1765 is shown in figure 5.5.

This threshold for allelic loss takes account of the possibility of contaminating normal tissue or other tumour clones in some samples. Any loci exhibiting extra bands (alleles) that differed by 2 base pairs from the normal counterpart were excluded from the LOH analysis and classified as "non informative".

For these case specimens, frequency of allele loss of 48% was found at the D10S541 locus. LOH occurred at the D10S1765 locus at a frequency of 39%, at D10S2492 at a frequency of 32% and at the AFMa086wg9 locus at a frequency of 22%. At all four

microsatellite, the mean (range) LOH was 35.25 (22-48) %. Of the 34 case specimens 17(50%) showed LOH in at least one of the informative marker sites (Table 5.1).

Inspection of the extent of allele loss in individual cases reveals that LOH at D10S541 and D10S1765 were often apparently associated with a single event (Fisher exact test: $p< 0.099$). There was no significant association between allele loss at D10S541 or, D10S2492 and AFMa086wg9 and that LOH at each allele appears to be a separate genetic event (Table 5.2). Thus there may be more than two separate regions on 10q which tends to be lost in prostate cancer.

The results of LOH analysis at *Pten* using the four microsatellite markers is illustrated in figure 5.6

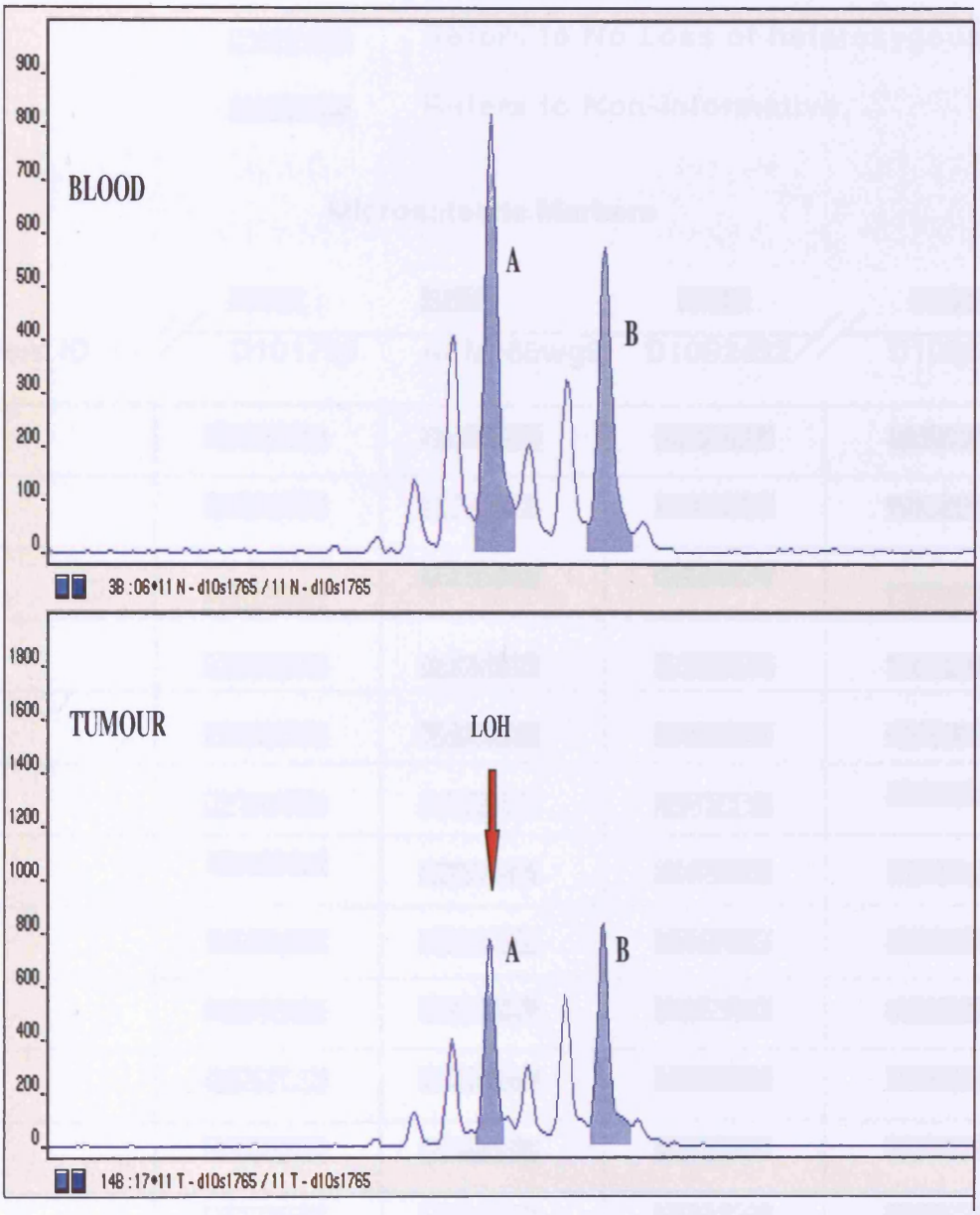


Figure 5.5: Example of allele loss at D10S1765 in patient 14. LOH is demonstrated by the reduction in the peak height (of the magnitude of 50%) in the allele A, of the tumour sample relative to the allele of the blood sample.

Figure 5.6: **Distribution of LOH at the four microsatellite markers**

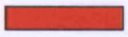
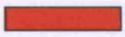
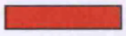
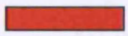
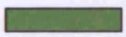
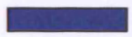
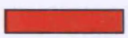
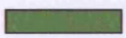
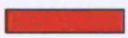
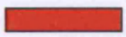
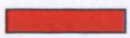
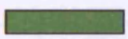
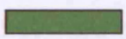
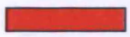
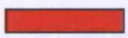
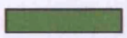
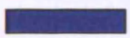
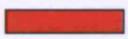
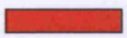
Refers to Loss of heterozygousity

Refers to No Loss of heterozygousity

Refers to Non-informative.

Microsatellite Markers

Patient ID	D101765	AFM086wg9	D10S2492	D10S541
5				
6				
20				
24				
40				
46				
52				
54				
66				
73				
75				
81				
86				
87				
88				
90				
91				

93				
95				
97				
101				
102				
103				
105				
106				
115				
116				
117				
118				
127				
128				
129				
130				
131				

Locus	Number of Tumours	Number Informative	Number with LOH	% With LOH
D10S541	34	25	12	48
D10S1765	34	31	12	39
D10S2492	34	19	6	32
AFMa086wg9	34	18	4	22

Table 5.1: **Frequencies of allele loss at the microsatellite markers studied.**

Number with LOH / Number of informative markers = % with LOH.

		D10S541		
		Loss	No Loss	Total
(a) **D10S1765**	Loss	7	3	10
	No Loss	4	8	12
	Total	11	11	22
(b) **D10S2492**	Loss	3	3	6
	No Loss	6	5	11
	Total	9	8	17
(c) **AFMa086wg9**	Loss	2	4	6
	No Loss	4	6	10
	Total	6	10	16

Table 5.2: **Association between allele losses at (a) D10S541 and D10S1765, (b) D10S541 and D10S2492 and (c) D10S541 and AFMa086wg9.**

Only cases informative at both loci being compared are shown. Fisher's exact test gives for (a) $p< 0.099$ (b) $p=0.627$ and for (c) $p=0.608$.

The association between allele loss at these four loci and pathological information was evaluated. Table 5.3 illustrates the correlation between the frequency of LOH at each microsatellite marker and TNM tumour stage. The frequency of LOH in localised prostate cancer (pT2a to pT2b) was 31.9% (15/49), compared to those for locally advanced disease (pT3a), which was 42.86% (18/42). Using Pearson Chi-square test to analysis these results; there was no demonstrable difference in the frequency of LOH observed in the more advanced stage disease (pT3a) compared with clinically localised prostate disease (T1c to T2b) (p=0.226). However, when the correlation between the frequency of LOH at all 4 microsatellite collectively and TNM tumour stage were analysed. There was a statistical significant trend (p<0.0398).

By contrast, the incidence of LOH and Gleason score showed no correlation (p=0.274), with 60% (3/5) in well-differentiated disease (Gleason score 4), 29.8% (17/57) in moderately differentiated disease (Gleason score 5 & 6) and 43.8% (7/16) in poorly differentiated disease (Gleason score 7&8). The correlation between the frequency of LOH at all 4 microsatellite collectively and Gleason score did not show a statistical significance towards a trend (p=0.798).

Locus	T2a	T2b	T3a	*p*⊕
D10S541	1/2	4/8	6/13	0.341
D10S1765	1/5	3/10	8/14	0.784
D10S2492	0/3	4/9	2/7	0.512
AFMa086wg9	0/2	2/8	2/8	0.0782
Total	2/12 (16.6%)	15/37 (37.1%)	18/42 (42.9%)	0.0398

Table 5.3: **Frequencies of allele loss at each marker in relation to the TNM stage for prostate cancer.** ⊕ χ^2 test for trend. n/N (%). n= number of LOH. N=number of informative markers. Not total number of tumours.

Locus	Gleason Score 4	Gleason Score 5	Gleason Score 6	Gleason Score 7	Gleason Score 8	*p**
D10S541	2/2	4/8	2/5	1/2	0/0	0.341
D10S1765	1/1	4/9	1/10	2/5	1/1	0.784
D10S2492	0/1	4/8	1/5	1/3	0/1	0.512
AFMa086wg9	0/1	0/7	1/5	1/2	0/0	0.078
Total	3/5 (60%)	12/32 (37.5%)	5/25 (20%)	5/12 (41.6%)	1/2 (50%)	0.798

Table 5.4: **Frequencies of allele loss at each marker compared to the Gleason score for prostate cancer.** $*\chi^2$ test for trend. n/N (%). n= number of LOH. N=number of informative markers. Not total number of tumours.

The correlation between the mean number of loci with LOH and prostate cancer stage was analysed (Figure 5.7). The mean number of LOH in locally advanced tumours (1.20 for T3) was relatively more than that for organ confined tumours (0.79 for T1 and T2). The difference was approaching statistical significance ($p<0.065$). However, there was no statistical significance ($p=0.274$), in the relationship between the mean number of loci with LOH and Gleason score in this study. There was no difference in the mean number of LOHs in low-grade tumour (1.50 for Gleason score 4), intermediate grade tumour (0.907 for Gleason 5, 6 and 7) and high-grade tumour (1.00 for Gleason 8) (Figure 5.8).

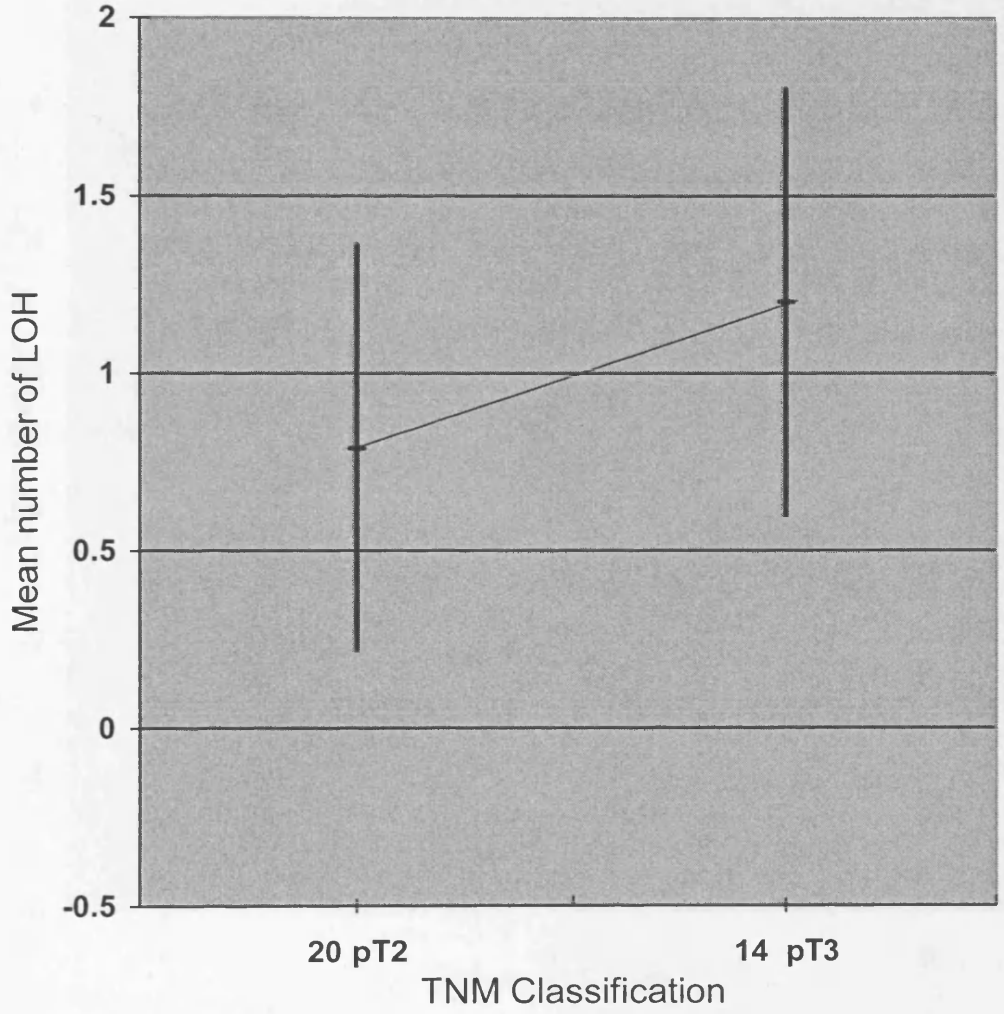


Figure 5.7: **The mean number of LOH at the four loci in relation to pathological stage**

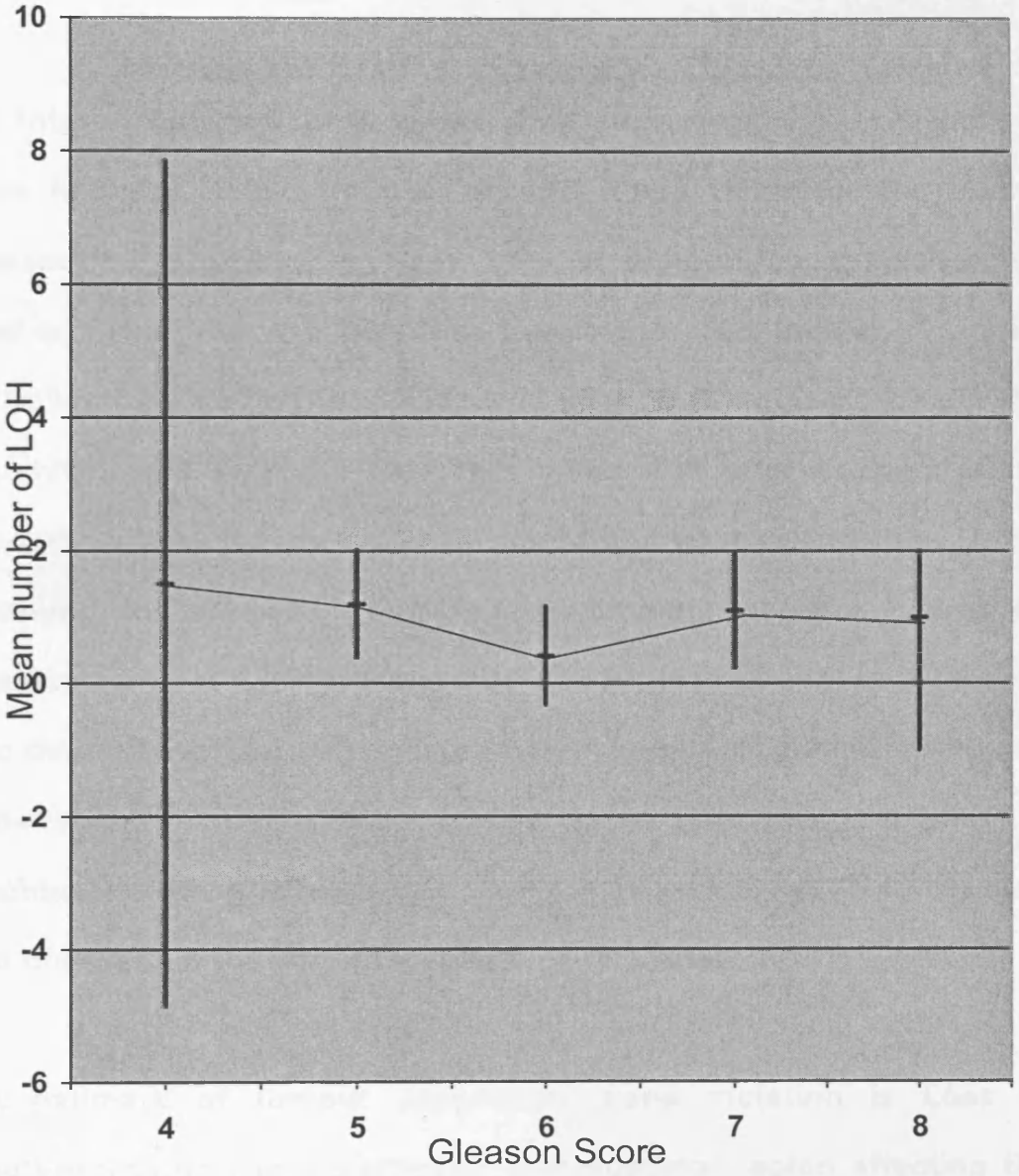


Figure 5.8: **The mean number of LOH at the four loci in relation to Gleason score**

5.2 Discussion

A total of 155 radical prostatectomy specimens were available in the histopathology archives at The Royal Free Hospital at the onset of this project, however only 34 tumours could be selected for analysis from the database due to the strict inclusion criteria. The histopathologist had reviewed all H & E stained slides in the archives and selected only specimens with large concentrations (>70% of a cluster of cells) of prostate cancer cells on the H & E stained slides which mirrors the archival paraffin embedded radical prostatectomy tissue sections. It was essential to adhere to this criterion because my earlier experiments whilst fine-tuning the experimental protocol, discovered significant degree of contamination of the cancer DNA by stromal DNA. This resulted in underestimation of true frequency of LOH.

A hallmark of tumour suppressor gene mutation is Loss of heterozygousity in a particular chromosomal region affecting the homologous wild-type allele. The existence of a putative tumour suppressor gene on chromosome 10q23 is implied by LOH that includes this region in many cases of prostate cancer (Ittmann M 1996) (Trybus TM *et al*, 1996).

Inactivation of tumour suppressor genes is the most common genetic alterations in prostate cancer. Such inactivation is frequently accompanied by loss of portions of a chromosome on which the tumour suppressor gene resides. Loss of portions of long arm of chromosome 10 has been identified in a significant percentage of prostate carcinomas (Ittmann M *et al,* 1996) (Trybus TM *et al,* 1996).

The *Pten* gene covers over 100kb of a 400kb region of allelic loss common to most prostate tumours (Gray IC *et al* 1995). LOH on chromosome 10q was originally observed by a number of groups using restriction fragment length polymorphism (RFLP) analysis (Bergerheim US *et al*, 1991) (Ittmann M *et al,* 1996). Rates of LOH on 10q ranged from 20–42% of informative cases (Bergerheim US *et al*, 1991) (Ittmann M *et al* 1996) using RFLP technique. Microsatellite PCR is now the method of choice for most allelotyping studies. In this thesis the detection frequency of LOH in *Pten* using the panel of four microsatellite markers produced a similar result with LOH ranging from 22- 48% in informative cases.

The detection frequency of *Pten* mutations in prostate cancer was lower than anticipated in this thesis. This finding has also been observed in studies by other authors (Cairns *et al*, 1997; Gray IC *et al* 1998; Feilotter HE *et al* 1998) and could be explained by the

fact that sequencing as a method of mutation detection is unlikely to be 100% efficient. The nature of prostate cancer growth with a lack of normal tumour boundary makes it difficult to be certain about the level of normal tissue contamination of the dissected tumour.

In the LOH experiments in this thesis, efforts were made to reduce the degree of contamination by employing the assistance of a histopathologist to mark out areas of high tumour concentration (>70%) on the H & E stain slides prior to manual microdissection. Effort was also made during interpretation of the results to ensure that allelic loss was scored only if the area under one allele peak was reduced in the tumour to less than 50% of its value in the normal DNA (relative to the other allele), This threshold for allelic loss takes account of the possibility of contaminating normal tissue.

Inspection of the extent of allele loss in individual cases reveal that the microsatellites D10S2492 and AFMa086wg9, present within the 100Kb thought to contain *Pten* gene revealed a frequency of loss of 32% and 22% respectively. Whereas, D10S 541 and D10S1765, which are present on either side of the *Pten* gene showed a higher frequency of LOH at 48% and 39% respectively. Therefore the assumption could be made that D10S541 and D10S1765, D10S2492 and AFMa086wg9 on 10q

appeared to be because of separate distinct event. Thus, there may be at least two separate regions on chromosome 10q that tend to be lost in prostate cancer.

This study suggests that the allelic loss at chromosome 10q23 may be related to the pathogenesis of some cases of prostate cancer as demonstrated with the correlation with tumour stage. This association was found after scoring LOH in three different ways; (i) at least one LOH per chromosome; (ii) the total number of LOHs per chromosome: and (iii) the mean number of LOHs per chromosome.

Using χ^2 test for trend, it demonstrated a statistical significant correlation between the frequency of LOH at all 4 microsatellite collectively and higher TNM tumour stage ($p=0.0398$). The frequency of LOH in localised prostate cancer (T1c to T2b) was compared to those for locally advanced disease (T3a). Using χ^2 test to analysis these results, I was unable to demonstrate a significant correlation between the higher frequency of LOH observed in the more advanced stage disease compared with clinically localised prostate disease ($P=0.52$). However a correlation between advance clinical stage and LOH on 10q has also been noted in other malignant neoplasms including glioblastomas (Fults D *et al*, 1992) and melanomas (Herbst RA *et al* 1994). Perhaps if a larger number of case studies fulfilled the

inclusion criteria and had been available for this study, the results might have shown a clear difference.

The microsatellite analysis in this study showed the stage dependence of loss of 10q.This is in keeping with observations from studies by Gray *et al*, Komiya *et al* and Trybus *et al*, which identified the region at 10q23 between D10S215 and D10S541 to demonstrate LOH in up to 58% of advanced prostate cancers.

Among individual markers, the prevalence of LOH at AFMa086wg9 showed a trend towards correlating with the progression of prostate cancer as defined by a higher stage and grade. No other marker correlated with either stage or grade.

Although the detection frequency of *Pten* mutations in prostate cancer was lower than anticipated the frequency of mutations in other tumour types such as glioblastomas (Rasheed BK *et al* 1997) and endometrial carcinoma (Tashiro H *et al* 1997) was 90% and 50% respectively.

Using microsatellite analysis the frequency of LOH in localised prostate cancer 30.6%, compared to those for locally advanced disease, which was 42.86%. This is comparable to the work by Ittmann M *et al* in which RFLP analysis was performed where overall, approximately 20% of clinically localized prostate cancers

showed LOH on 10q while 50% of the advanced cases showed LOH on RFLP analysis (Ittmann M *et al,* 1996).

Pathological stage and Gleason grade are important predictors of prognosis in patients with primary prostate cancer who undergo radical prostatectomy. Prostate cancer, however, is a remarkably heterogeneous disease. Distinguishing tumours associated with a poor outcome at the time of radical prostatectomy is problematic. The molecular mechanisms of prostate carcinogenesis remain poorly understood.

There are several general possibilities when considering tumour suppressor genes. A possibility is that the ability to detect mutations in candidate genes may be masked by the inability to obtain relative pure tissue samples for analysis, because of tumour heterogeneity and mutifocality. This technical explanation is quite conceivable because most analysis is still performed with relatively large tissue samples that are unlikely to be homogenous.

Another possibility is that the candidate genes may be inactivated by a mechanism other than a coding region mutation, such as a promotor methylation or mutations within regulatory sequences that may affect transcription, translation, or mRNA stability. In

addition, inactivation could occur through alterations of upstream or down stream components in a regulatory pathway. A final possibility is that haploinsufficiency (loss of a single allele) may play an important role in prostate carcinogenesis, perhaps consistent with slow rate of progression and indolent phenotype of most tumours.

From a practical standpoint, the heterogeneity and mutifocality of prostatic lesions, combined with the relatively small size of the prostate, make it difficult to obtain reasonably homogenous material sufficient for molecular analysis. These factors represent significant limitations in identifying regulatory genes associated with prostate carcinogenesis, as well as in defining a molecular pathway for the imitation and progression of prostate cancer. In recent years, these difficulties have been partially circumvented by microdissection and laser capture microscopy approaches that facilitate analysis of individual neoplastic foci (Emmert-Buck MR *et al* 1995; Macintosh CA *et al* 1998), and by cell-sorting approaches that permit the isolation of relatively pure populations of carcinoma cells (Liu AY *et al,* 1999).

Genetic studies may prove useful in the identification of markers of prostate cancer progression. LOH studies of preneoplastic, primary and metastatic prostate cancer samples could identify loci involved in early and late stages of prostate carcinogenesis.

The absence of associations between the clinical data and the allelic loss data is not surprising. LOH in *Pten* is thought to occur as a late event in the pathogenesis of prostate cancer.

Genetic studies are hampered by the limited availability of metastatic prostate tissues and it is particularly rear to have tissue from metastases available in a patient who has undergone radical prostatectomy. Prostatectomy is performed in patients with organ-confined disease. If the disease progresses, the metastatic are not usually biopsied. As a result, data for LOH in metastatic prostate cancer are lacking in the majority of studies on prostate cancer progression.

Studies that have included significant numbers of metastatic lesions have generally been limited to only one chromosomal region (Sakr WA *et al*, 1994; Ueda T *et al*, 1997). As a result, most existing data on LOH in prostate cancer have been derived from localised prostate cancer from prostatectomy specimens, which are most often (approximately 70%) moderately differentiated (Gleason score 4-7) (Gleason DF 1992). While useful for determination of the earlier genetic events in tumour formation, these samples may not be optimal for identification of alterations that predict metastatic potential. Additionally, the most appropriate samples for defining very early genetic alterations in

prostate cancer would be precursor lesions. To date, only a few studies have analysed allele loss PIN and were limited mostly to chromosome 8 (Sakr WA *et al,* 1994; Emmert-Buck MR *et al*, 1995). Thus a comprehensive LOH study on PIN lesions is also lacking.

A number of genetic changes have been shown to occur in prostate tumourigenesis, yet it is difficult to translate this molecular knowledge into widely applicable diagnostic / prognostic criteria in the management and treatment of the disease.

In order to prove this, large quantity of metastatic prostate tissue would be required. This tissue is rarely available as there is no clinical indication for obtaining metastatic prostate tissue except that which is available at the time of fixation of pathological fractures.

My goal was to identify genetic changes associated with histological and clinical progression of prostate cancer. The LOH studies were performed using polymorphic microsatellite markers on microdissected prostate cancer specimens from patients found to have organ confined and locally advanced disease.

In conclusion, the results presented in this thesis provide evidence that the 10q23-24 region may involve a putative candidate tumour-suppressor gene which might be associated with progression of prostate cancer. Moreover, they suggest that the loss of *Pten* may be a late step in the progression of some prostate neoplasms.

5.3 Summary

The objectives in this thesis were to investigate the significance of the *Pten* gene in prostate cancer. In the first series of experiments described in the second chapter, none of the three intronic polymorphisms showed specific associations with the prostate cancer sample group compared with the control group.

By performing these series of experiments I had hoped to illustrate an association between prostate cancer and at least one of the polymorphisms investigated. If an association had been established, a marker could be developed to identify individuals at risk of developing prostate cancer and may predict subsequent disease behaviour such as the likelihood of disease recurrence or progression. As well as a potential role in the natural history of a disease, SNPs may well determine an individual's response to treatment such as chemotherapy or radiotherapy.

The second set of experiments attempted to demonstrate the frequency and significance of LOH within the *Pten* gene using our panel of four microsatellite markers. This produced a result with the frequency of LOH ranging from 22-48% in informative cases. There was also a statistically significant trend between the frequency of LOH at all four mirosatellites collectively and a higher TNM tumour stage ($p=0.0398$). This finding demonstrates

that LOH in the *pten* gene was a late event in prostate cancer pathogenesis.

The number of samples that fulfilled the inclusion criteria was of a limited number. Perhaps, if a larger quantity of prostate cancer sample was available for these studies the results might have demonstrated a significant correlation between a higher frequency of LOH at *pten* and late stage prostate cancer. This would have been an important finding in the quest to identify a genetic marker for prostate cancer. It would allow for genetic analysis of prostate tissue obtained following TRUS and biopsy. Following the diagnosis of adenocarcinoma of the prostate, such a marker could be added to the panel of prognostic markers of disease progression currently available and help in stratify patients into high and low risk of prostate cancer disease progression. This would improve the counselling of patients with the diagnosis of localised adenocarcinoma of the prostate and allow appropriate treatment stategies to be offered depending on the risk group into which the patient is judged to belong.

APPENDIX 1 - ETHICS

Royal Free & Med Sch. Local Research Ethics Committee
Pond Street
Hampstead
London
NW3 2QG

15th August 2005

To whom it may concern

Re: Dr Adeleye Afolabi Ajayi
35B Ashburnham Road,
Kensal Green
London NW10 5SB.

I gather that Dr Ajayi has submitted a thesis in which research was carried out on archived prostate specimens and that the examiners of the thesis are requesting the ethical approval documents for the research.

This research was carried out in 1998. At that time ethics committees were still guided by the Royal College of Physicians guidance 1996 – a copy of which is enclosed. With reference to that guidance I feel that it is highly likely that in 1998 Dr Ajayi would have been advised that his research did not need the approval of an ethics committee. It was only in the year 2000 that ethics committees changed their attitudes on this type of research.

I therefore feel that the award of his degree should not be hindered by the absence of approval from a research ethics committee

Yours sincerely

Dr Michael S Pegg
Chair

Appendix 2: **Patient information sheet for the SNPs and microsatellite study.**

Date interviewed:

Study no:

Hospital number:

Pathology specimen number:

Initials of patient:

Date of birth:

Diagnosis:

Age at first diagnosis:

Date of treatment:

Treatment modality:

Pre diagnosis PSA:

Latest PSA:

Mode of diagnosis:

Pathology findings:

Gleason grade:

Gleason score:

TNM classification of cancer:

Appendix 3: Patient data for the SNPs and microsatellite study

ID-number	Age	Pre op PSA	Post op PSA	Diagnosis	Method	Gleason Score	TNM Stage
1	75	50	3	CA	BIOPSY	6	pT1c
2	76	4.3	0.2	CA	TURP	6	pT1b
3	75	1.7	0.1	CA	TURP	3	pT1b
4	72	1	1.4	CA	TURP	3	pT1a
5	56	6	0.1	CA	RRP	5	pT2a
6	73	5	0.1	CA	RRP	5	pT2b
7	66	31	3.7	CA	BIOPSY	7	pT1c
8	65	33	0.2	CA	RRP	7	pT3a
9	82	8.4	13.9	CA	BIOPSY	6	pT1c
10	82	4.2	0.2	CA	BIOPSY	3	pT1c
11	71	4.5	0.2	CA	BIOPSY	4	pT1c
12	77	4.4	10	CA	BIOPSY	7	pT1c
13	74	27	2	CA	BIOPSY	8	pT1c
14	75	5.1	11.3	CA	BIOPSY	4	pT1c
15	81	13	0.2	CA	BIOPSY	2	pT1c
16	78	8.2	0.2	CA	BIOPSY	7	pT1c
17	91	22.8	12.5	BPH	BIOPSY	NA	NA
18	79	4.1	2	BPH	BIOPSY	NA	NA
19	45	3.2	2	BPH	BIOPSY	NA	NA

ID-number	Age	Pre op PSA	Post op PSA	Diagnosis	Method	Gleason Score	TNM Stage
20	73	10.5	0.2	CA	RRP	7	pT3a
21	75	26.6	2.2	CA	BIOPSY	7	pT1c
22	73	425	30.5	CA	BIOPSY	6	pT1c
23	84	17.5	21.1	CA	TURP	6	pT1a
24	68	13	0.2	CA	RRP	8	pT2b
25	59	4.5	4.4	BPH	BIOPSY	NA	NA
26	70	5.4	0.8	CA	BIOPSY	5	pT1c
27	83	12.1	3.4	BPH	BIOPSY	NA	NA
28	68	40	3.3	CA	BIOPSY	7	pT1c
29	62	10.2	0.2	CA	RRP	6	pT2b
30	68	35.6	1.7	CA	BIOPSY	5	pT1c
31	79	17.8	11.5	BPH	BIOPSY	NA	NA
32	72	1.6	0.2	CA	BIOPSY	4	pT1c
33	77	10.8	10.2	BPH	BIOPSY	NA	NA
34	80	8.4	8.4	BPH	BIOPSY	NA	NA
35	90	8	38	CA	BIOPSY	7	pT1c
36	84	4.5	2.3	BPH	BIOPSY	NA	NA
37	74	26	177	CA	TURP	10	pT1a
38	65	8.3	6.1	BPH	BIOPSY	NA	NA
39	66	7.1	5.1	BPH	BIOPSY	NA	NA
40	63	6	0.2	CA	RRP	7	pT3a

ID-number	Age	Pre op PSA	Post op PSA	Diagnosis	Method	Gleason Score	TNM Stage
41	63	10.4	4.5	BPH	BIOPSY	NA	NA
42	76	10.9	0.6	CA	BIOPSY	4	pT1c
43	71	16.9	0.5	CA	BIOPSY	6	pT1c
44	73	7.6	6.2	BPH	BIOPSY	NA	NA
45	78	8.6	0.6	CA	BIOPSY	8	pT1c
46	66	9.9	0.2	CA	RRP	5	pT2a
47	75	203	4	CA	TURP	6	pT1b
48	82	34.2	0.1	CA	TURP	6	pT1a
49	67	19.3	9.4	BPH	BIOPSY	NA	NA
50	64	186	7.1	CA	BIOPSY	5	pT1c
51	80	16.1	13.9	CA	BIOPSY	7	pT1c
52	76	14.2	0.2	CA	RRP	7	pT3a
53	48	4.4	4.3	BPH	BIOPSY	NA	NA
54	59	13	0.7	CA	RRP	7	pT3a
55	62	16	0.2	CA	RRP	6	pT2a
56	61	5	0.2	CA	RRP	7	pT3a
57	71	30	0.4	CA	RRP	6	pT2b
58	43	4.3	3.9	BPH	BIOPSY	NA	NA
59	71	31.6	2.7	CA	RRP	3	pT3a
60	74	7.5	2	BPH	TURP	NA	NA
61	43	4.3	1.8	BPH	BIOPSY	NA	NA

ID-number	Age	Pre op PSA	Post op PSA	Diagnosis	Method	Gleason Score	TNM Stage
62	90	4.1	3.9	BPH	BIOPSY	NA	NA
63	47	3.9	2.9	BPH	BIOPSY	NA	NA
64	86	4.8	1.4	BPH	BIOPSY	NA	NA
65	42	5.2	4.8	BPH	BIOPSY	NA	NA
66	62	6	0.2	CA	RRP	4	pT3a
67	74	15.7	13.8	CA	BIOPSY	7	pT1c
68	65	5.5	3.2	BPH	TURP	NA	NA
69	78	5.1	4.2	BPH	BIOPSY	NA	NA
70	66	4.9	3.0	BPH	BIOPSY	NA	NA
71	55	20	0.2	CA	RRP	8	pT2a
72	71	3.3	1.3	BPH	BIOPSY	NA	NA
73	57	8	0.2	CA	RRP	6	pT3a
74	78	6.2	0.5	CA	BIOPSY	4	pT1c
75	68	8	0.1	CA	RRP	7	pT2b
76	84	0.2	0.5	CA	TURP	3	pT1a
77	78	25	0.7	CA	TURP	8	pT1b
78	72	6.2	0.2	CA	RRP	6	pT3a
79	74	4.7	4.4	BPH	BIOPSY	NA	NA
80	79	12.6	0.5	CA	BIOPSY	5	pT1a
81	69	5	0.1	CA	RRP	5	pT3a
82	85	5.2	0.3	CA	TURP	7	pT1b

ID-number	Age	Pre op PSA	Post op PSA	Diagnosis	Method	Gleason Score	TNM Stage
83	79	9.2	7.1	CA	BIOPSY	9	pT1c
84	74	32	23	CA	BIOPSY	7	pT1c
85	63	48	13	CA	TURP	8	pT1a
86	74	26	0.8	CA	RRP	5	pT2b
87	63	28	0.2	CA	RRP	7	pT3a
88	73	32	0.2	CA	RRP	5	pT3a
89	76	3.7	3.7	BPH	BIOPSY	NA	NA
90	63	24.4	0.2	CA	RRP	7	pT3a
91	70	11.8	0.2	CA	RRP	6	pT3a
92	79	8.6	0.6	CA	BIOPSY	4	pT1c
93	60	6	0.2	CA	RRP	7	pT2b
94	62	9	0.2	CA	RRP	9	pT2b
95	46	6.1	0.2	CA	RRP	6	pT2a
96	67	10	0.8	CA	RRP	8	pT2a
97	68	17.5	0.2	CA	RRP	6	pT2b
98	85	2.1	1.2	BPH	BIOPSY	NA	NA
99	74	59	110	CA	TURP	7	pT1b
100	70	6.7	2.2	BPH	BIOPSY	NA	NA
101	60	4.3	0.2	CA	RRP	6	pT2b
102	65	6.4	0.4	CA	RRP	8	pT2a
103	72	9	0.2	CA	RRP	5	pT3a

ID-number	Age	Pre op PSA	Post op PSA	Diagnosis	Method	Gleason Score	TNM Stage
104	69	6.1	0.1	CA	RRP	6	pT3a
105	71	5	0.2	CA	RRP	4	pT2b
106	77	5.2	0.2	CA	RRP	6	pT2a
107	76	72	0.2	CA	TURP	9	pT1b
108	45	4.2	2.1	BPH	BIOPSY	NA	NA
109	63	4.2	4.2	BPH	BIOPSY	NA	NA
110	51	14.8	8.5	BPH	BIOPSY	NA	NA
111	85	10.9	44.8	CA	BIOPSY	7	pT1c
112	73	4.5	2.9	CA	BIOPSY	6	pT1c
113	40	4.9	4.2	BPH	BIOPSY	NA	NA
114	67	18	0.2	CA	RRP	6	pT2a
115	65	2.2	0.2	CA	RRP	6	pT3a
116	65	210	0.2	CA	RRP	5	pT2b
117	66	39	0.5	CA	RRP	6	pT3a
118	57	6	0.2	CA	RRP	7	pT2b
119	85	8.9	3.6	BPH	TURP	NA	NA
120	68	5	3	CA	RRP	6	pT2a
121	83	4.3	0.2	BPH	BIOPSY	NA	NA
122	54	4.1	3.1	BPH	BIOPSY	NA	NA
123	82	1.3	0.2	CA	TURP	2	pT1a
124	52	3.9	4.4	BPH	BIOPSY	NA	NA

ID-number	Age	Pre op PSA	Post op PSA	Diagnosis	Method	Gleason Score	TNM Stage
125	60	4.8	1.9	BPH	BIOPSY	NA	NA
126	80	17.4	0.3	CA	BIOPSY	6	pT1c
127	65	10	0.2	CA	RRP	6	pT2a
128	64	252	0.2	CA	RRP	5	pT3a
129	52	12.6	0.5	CA	RRP	6	pT2a
130	64	5.9	0.2	CA	RRP	5	pT2a
131	67	6.7	0.3	CA	RRP	6	pT2b
132	72	13.5	2.2	CA	RRP	7	pT2b
138	68	4.6	5.6	BPH	BIOPSY	NA	NA
141	48	12.2	0.2	CA	RRP	9	pT3a
142	68	6.1	4.2	BPH	BIOPSY	NA	NA
143	68	11.1	7.9	CA	RRP	7	pT2a
144	69	5.4	4.2	BPH	BIOPSY	NA	NA
145	57	6.8	3.2	BPH	BIOPSY	NA	NA

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The expression profile for the tumour suppressor gene *PTEN* and associated polymorphic markers

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Summary *PTEN*, a putative tumour suppressor gene associated with prostate and other cancers, is known to be located within the chromosomal region 10q23.3. Transcription of the *PTEN* gives rise to multiple mRNA species. Analyses by Northern blots, using cell lines which express *PTEN* together with cell lines which have lost the *PTEN* or carry a truncated version of the gene, has allowed us to demonstrate that the pseudogene is not transcribed. In addition, 3′ RACE studies confirmed that the multiple mRNA species arising from the gene probably result from the use of alternative polyadenylation sites. No evidence for tissue- or cell-specific patterns of transcription was found. Analysis by 5′ RACE placed the putative site for the start of transcription around 830 bp upstream of the start codon. A map of the location of the *PTEN* gene with a series of overlapping YAC, BAC and PACs has been constructed and the relative position of eight microsatellite markers sited. Two known and one novel marker have been positioned within the gene, the others are in flanking regions. The more accurate location of these markers should help in future studies of the extent of gene loss. Several polymorphisms were also identified, all were within introns. Four of the common polymorphisms appear to be linked. In blood, DNA from 200 individuals, including normal, BPH and prostate cancer patients, confirmed this link. Only two samples of 200 did not carry the linked haplotype, both were patients with advanced prostate cancer. It is possible that such rearrangements within *PTEN* could be evidence of predisposition to prostate cancer in this small number of cases. © 2000 Cancer Research Campaign

Keywords: PTEN; microsatellite markers; polymorphisms; prostate; transcription

Allelic loss at the chromosomal region 10q23–25 has been reported for prostate and several other cancers (Gray et al, 1995; Bose et al, 1998; Cairns et al, 1998; Feilotter et al, 1998; Maier et al, 1998; Robertson et al, 1998). Analysis of loss of heterozygosity (LOH) in microdissected prostate tumours initially placed the minimum region of loss between the markers D10S1644 (AFMa124wd9) and D10S583 (Gray et al, 1998) and was subsequently refined to a region of 400 kb between D10S1765 and D10S541 (Gray, unpublished data). A putative tumour suppressor gene called *PTEN*, *MMAC1* or *TEP1* (Li and Sun, 1997; Li *et al*, 1997; Steck et al, 1997) has been located within this region and was mutated in glioblastomas, endometrial and prostate tumours (Cairns et al, 1997; Rasheed et al, 1997; Tashiro et al, 1997; Feilotter et al, 1998; Gray et al, 1998). In addition, germline *PTEN* mutations have been found to be associated with Cowden's disease (Liaw et al, 1997) and related syndromes such as Bannayan-Zonana syndrome (Marsh et al, 1997).

PTEN has regions of homology to chicken tensin and auxilin (Li et al, 1997) and incorporates phosphatase domains which are active against both lipid and protein substrates (Myers et al, 1997; Maehama and Dixon, 1998). Evidence for a tumour suppressor function for *PTEN* comes from experiments in which transfection of *PTEN* into a medulloblastoma cell line null for *PTEN* expression caused inhibition of proliferation and anchorage-dependent growth (Cheney et al, 1998). Total gene disruption resulted in embryonic lethality in mice, whereas animals heterozygous for *PTEN* expression showed hyperplastic-dysplastic changes in epithelial tissues and an increased incidence of tumours (Di Cristofano et al, 1998). The tumour suppressor activity of *PTEN* may result from its demonstrated ability to dephosphorylate the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3), thereby restricting cell growth and allowing apoptosis to occur (Maehama and Dixon, 1998; Tamura *et al*, 1998). In addition, the ability of *PTEN* to dephosphorylate focal adhesion kinase may prevent cell migration (Tamura *et al*, 1999), and the inactivation of *PTEN* has recently been associated with increased angiogenesis in prostate carcinomas (Giri and Ittmann, 1999).

Analysis of the *PTEN* mRNA species has revealed a complex pattern of different sized transcripts (Steck et al, 1997; Gray *et al*, 1998) some of which could have arisen from alternate splice events. Additionally a pseudogene for *PTEN* (ψ *PTEN*) has been identified on chromosome 9 which has been claimed to be transcribed (Dahia *et al*, 1998). In this paper we investigate origins of the major mRNA species seen on Northern blots and describe BAC/PAC coverage of the region along with the exact location of several polymorphic markers and identification of single nucleotide polymorphisms.

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MATERIALS AND METHODS

YAC, BAC and PAC isolation

YAC 821D2 was identified from the CEPH Mega YAC library by using the 'infoclone' program (Chumakov *et al*, 1995) to screen for YACs bearing CA repeat markers spanning the minimal region and a clone obtained from the UK HGMP Resource Centre in Cambridge. BAC and PAC clones were identified from Human BAC pools and RPCI Human PAC libraries (Research Genetics) by polymerare chain reaction (PCR) screening with CA repeat markers. BAC146B18 was isolated as a clone from which we could amplify the T7 (telomeric) end of BAC46B12 and whose centromeric end sequence could be amplified from BAC122L22. Insert size was estimated by restriction enzyme digests using infrequently cutting restriction endonucleases, followed by separation by pulsed field gel electrophoresis using manufacturer's recomended conditions (BioRad).

Cell lines

The lymphoblastoid line Bristol 8 (BRI8) has been previously described (Gray *et al*, 1998), DU145, PC3 and A172 were from ATCC, and UMUC3 was provided by Prof. M Knowles (Aveyard *et al*, 1998). A second vial of DU145 was obtained from Dr R Schrock (Introgen Therapeutics Inc.). All lines were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum.

Exon, intron and microsatellite marker amplification

Exons were amplified as previously described (Gray *et al*, 1998) using primers based on intronic sequences. Primer sequences for Chemical Cleavage of Mismatches (CCM) were obtained from BAC sequence data obtained within the laboratory, and the 5′ end of each 3′ primer was PIG-tailed to facilitate CCM (Brownstein et al, 1996). CCM cycling conditions were as follows: 10 min at 96°C followed by 35 cycles of 96°C for 30 s, 49–60°C for 30 s, 72°C for 5 min in a Perkin-Elmer 9600 thermal cycler and using Taq Gold (Perkin-Elmer). Annealing temperature was dependent on primer pair used.

BAC sequencing

BACs were prepared using a modified Qiagen midi prep method. Two hundred millilitres cultures were grown in selective medium and processed by the Qiagen midi prep protocol, except that 15 ml of P1, P2 and P3 were used in place of the recommended 4 ml. After centrifugation DNA was precipitated from the supernatant with isopropanol, the pellet washed with 70% ethanol and resuspended in 1 ml of dH_2O (heavy water) and 0.25 ml of 10 M NH_4Ac. Non-soluble particles were removed by centrifugation and DNA was ethanol precipitated. After centrifugation, the pellet was redissolved in 2 ml of H_2O and 10 ml of QBT buffer. Qiagen protocols were followed thereafter. The DNA was finally resuspended in a total of 120 µl, and 6–8 µl used per sequence reaction. DNA sequence reactions were performed using Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer) and analysed on an ABI377.

Chemical cleavage of mismatches

CCM in heteroduplexes formed between two different alleles was performed using the hydroxylamine method (Rowley *et al*, 1995). The reaction products were analysed on a 6% acrylamide/urea gel with 12-cm plates at 950 volts for 3.5–4 h on an ABI 377 DNA sequencer. The results were analysed using Genescan 2.1.1 software. When cleavage products were detected, PCR products spanning the relevant interval were purified using Qiagen PCR cleanup columns and sequenced using the amplification primers to determine the exact nature of the mismatch.

5′- and 3′-rapid amplification of cDNA ends (RACE)

5′- and 3′-RACE reactions were performed using 0.1–0.2 ng of Marathon-Ready prostate cDNA (Clontech) and Advantage-GC cDNA PCR buffer and polymerase mix (Clontech). PCR reactions were carried out in a 9700 thermal cycler (Perkin-Elmer) in a total volume of 50 µl. Cycling parameters for 5′-RACE were either 94°C for 1 min followed by 35 cycles of denaturation for 30 s and extension for 3 min at 68°C or 'touchdown' PCR where a 30 s denaturation step was followed by 5 cycles of 94°C for 10 s and 72°C for 4 min, 5 cycles of 94°C for 10 s and 70°C for 4 min and 25 cycles of 94°C for 10 s and 67°C for 4 min. When touchdown PCR conditions were used for the primary PCR, the nested PCR parameters were denaturation for 30 s followed by 5 cycles of 94°C for 10 s, 62°C for 30 s, 68°C for 2 min, 5 cycles of 94°C for 10 s, 60°C for 30 s, 68°C for 2 min and 25 cycles of 94°C for 10 s, 59°C for 30 s, 68°C for 2 min. The following primers were used in the primary 5′-RACE reactions: GSP1, 5′-GGCAGAAGCTGCTGGTGGCGGGG; GSP2, 5′-GCCGCCGTGTTGGAGGCAGTAGAAGGGG; GSP3, 5′-AACTGAGCGCAGTCGCGTCCCAGCGC and AP1 (Clontech). In the nested 5′-RACE reactions the primers were NGSP1, 5′-GGAAATGGCTCTGGACTTGGCGG; NGSP2,5′-ACCAACTCTCCGGCGTTCCCAGC; NGSP3, 5′-CAGCGCATAAAGAGTCCTGCCAC and AP2 (Clontech). Cycling parameters for 3′-RACE were 94°C for 1 min followed by 6 cycles of 94°C for 10 s and 67°C for 3 min and 30 cycles of 94°C for 10 s and 64°C for 4 min. The following primers were used in the 3′-RACE reactions in combination with the AP1 and AP2 primers from Clontech: 3′GSP2, 5′-CATCCACAGGGTTTTGACAC; 3′NGSP2, 5′-GGTTGTGTAGCTGTGTCATG; 3′GSP3, 5′-CGGGTTAGGGCAATGGAGGGGAATGC; 3′NGSP3, 5′-CGAGGAATTGGCCGCTGTCACTGC. The RACE products were gel purified (Qiagen) and cloned into pGEM-T Easy Vector (Promega) or sequenced directly.

RNA and Northern analysis

Messenger RNA was extracted from the cell lines UMUC3, BRI8, DU145 and A172 using FastTrack RNA isolation kit (Invitrogen). Five micrograms of mRNA from each cell line were size-fractionated on a 1% formaldehyde agarose gel and transferred to a Hybond N^+ membrane (Amersham) using 20 × SSC (sodium–saline citrate) as transfer buffer. A full length *PTEN* cDNA probe was labelled with ^{32}P-dCTP using Megaprime DNA labelling system (Amersham). A probe fragment was generated for the 3′UTR region by PCR using primers 5′-GACTGAAAGGTTTTCGAGTCC and 5′-GAGGAGCTACAAAGGACTTGG and labelled with ^{32}P-dCTP as described previously (Gray et al, 1998). RNA hybridization was carried out using ExpressHyb

hybridization solution (Clontech) according to the manufacturer's instructions. Membranes were washed in 2 × SSC and 0.1% sodium dodecyl sulphate (SDS) at room temperature for 50 min with several changes of wash solution. X-ray film (Fuji) was exposed to the Northern blots in the presence of 2 screens for 1–3 days.

RESULTS

LOH of the chromosomal region around *PTEN* occurs in several cancer types, and to allow a more detailed analysis of this loss a map of overlapping BACs and PACs was constructed and several microsatellite markers positioned on this map along with the exons/introns of *PTEN* (Table 1). The *PTEN* gene covers approximately 100 kb and the markers D10S2491 and D10S1765 which are located 5′ or telomeric to the gene were found to be identical. D10S608 which has previously been used to map *PTEN* LOH is not located within the designated region and is at least 160 kb telomeric of the *PTEN* gene. Markers AFMa086wg9 (intron 2) and D10S2492 (intron 8) are located within *PTEN*. In addition a further CA repeat was identified from sequencing within intron 8 which was heterozygous in 45% of a sample of blood DNAs from a group of 200 individuals when these were analysed by microsatellite methods.

Comparison of genomic and BAC sequencing with database sequences for intron 1 (Genebank sequence – BAC 265N13) showed that there was probably a deletion in the database submission. Genomic sequences we have obtained from around PTEN have been lodged with Genbank (Accession Nos AF143312, AF143313, AF143314, AF143315, AF143316, AF143317 and AF143318).

In addition to microsatellite markers a search for single nucleotide polymorphisms was undertaken. *PTEN* intronic sequence primers were used to PCR amplify 1–1.5 kb fragments of DNA from 20 normal human blood samples. *PTEN* regions amplified included 1.9 kb downstream of exon 3, approximately 6.7 kb upstream of exon 4, all of intron 4, 1.5 kb downstream of exon 5, and 1 kb downstream of exon 6. These fragments, which covered around 12 kb of sequence in total, were analysed by CCM to identify polymorphisms and potential changes were confirmed by sequencing. Polymorphisms and levels of heterozygosity are listed in Table 2; some of these polymorphisms have been described previously but confirmed by CCM and sequencing during the course of our studies.

Four polymorphic markers located within the *PTEN* gene had a similar frequency of heterozygosity of around 40% and were used to analyse the DNA obtained from the blood of 200 individuals (these included 97 prostate cancer patients, 43 patients with benign prostatic hyperplasia and 60 other non-cancer patients). From this analysis it appeared that two distinct haplotypes existed. For the four markers 1, 4, 5 and 8 respectively (Table 2), the linkage was either G,A,+,G or A,G,–,T. Of the 200 DNAs tested, 45.5% were heterozygous at each of the markers, 12% were homozygous for A,G,–,T haplotype and 41.5% were homozygous for G,A,+,G haplotype. Only two out of 200 individuals did not carry the linked haplotype in their blood DNA and both were cancer patients, their genotypes were A,G,–,G/T and A,G/A,+/–,G/T.

To analyse the complex pattern of transcription found for *PTEN* a number of cell lines were studied. Five cell lines were analysed by PCR for the presence or absence of *PTEN* and *ψPTEN*. To distinguish *PTEN* from *ψPTEN*, primers complementary to intron sequences were used to amplify *PTEN* exons (*ψPTEN* is processed and has no introns). In cell lines where all nine *PTEN* exons were present these exons were sequenced. The B lymphoblastoid cell line BRI8 and the prostate line DU145 contained all *PTEN* exons and carried no mutations. DU145 has been described as carrying a mutation at codon 145 (Li et al, 1997); we were unable to find this mutation in two independent sources of DU145. Consistent with published results the glioblastoma cell line A172 contains only exons 1 and 2, and the bladder cell line UMUC3 has lost all PTEN exons (Li et al, 1997; Steck et al, 1997; Aveyard et al, 1998). PC3 cells used for these studies contained only exons 1 and 2. The presence of the *PTEN* pseudogene in these cell lines was determined using PCR primers from the boundary of exons 3 and 4 and from exon 6, which could only generate a product when no intronic sequences were present. *PTEN* pseudogene sequences were found to be present in all cell lines examined. Multiple *PTEN* transcripts (Steck et al, 1997; Gray et al, 1998) were evident in BRI8 and DU145 cells, but no mRNA transcripts were detected in UMUC3 cells which were null for *PTEN* but positive for the *ψPTEN*. A shortened transcript was detected in the mRNA from the A172 cell line which has only exons 1 and 2 (Figure 1).

Table 1 Microsatellite and *PTEN* content of YACs, BACs and PACs and their alignment in the region of LOH

	Approx size (kb)	D10S 608	D10S 215	D10S 1765 (2491)	Exon 1	Exon 2	AFM a086wg9	Exons 3–8	D10S 2492	8/9 CA	Exon 9	D10S 541
Y738B12	1330	■	■									
Y821-D2	1141		■	■	■	■	■	■	■	■	■	■
P72G8	165		■	■								
P274D21	150			■	■							
B2F20	90			■	■	■						
B46B12	230				■	■	■	■	■	■	■	
B60C5	160						■	■	■	■	■	
B146B18	110											
B122L22	105											■

Black squares indicate the presence and white squares the absence of the marker or exon. Primers used for the amplification of the exons can be found in the Genome Database (http://www.GDB.org) with the exception of CA8/9 which was amplified using the primer pair 5′-CAGCACTTTGG-GAGGCTAAG/5′- TTTCACTTAAAACGTGCAGGGG. B146B18 overlaps B122L22 and B60C5 but does not contain a marker or exon.

Table 2 Polymorphisms in *PTEN* and their frequency in normal DNA samples.

No.	Intron	Position	Polymorphism	Heterozygosity (%)
1	1	– 96 exon 2	A/G	40
2	3	+ 329 exon 3	T/C	15
3	3	+ 3362 exon 3	A/C	6
4	3	– 1606 exon 4	A/G	39
5	4	+ 109 exon 4	ATCTTins/del(+/–)	36
6	4	+ 403 exon 4	T/C	5
7	7	+ 461 exon 6	G/A	6
8	8	+ 32 exon 8	T/G	37

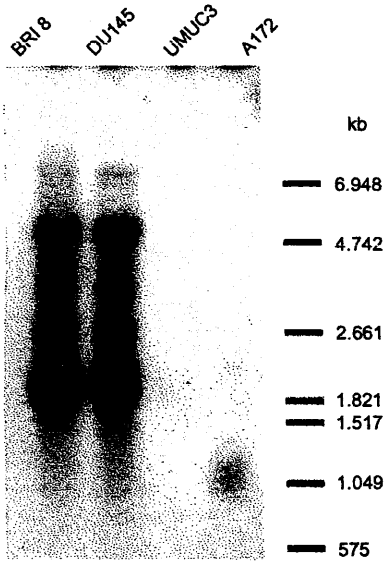

Figure 1 Northern blot analysis of mRNA from cell lines probed with a *PTEN* cDNA clone. BRI 8 – B lymphoblastoid cell line; DU145 – prostate cell line; UMUC3 – bladder cell line; A172 – glioblastoma cell line

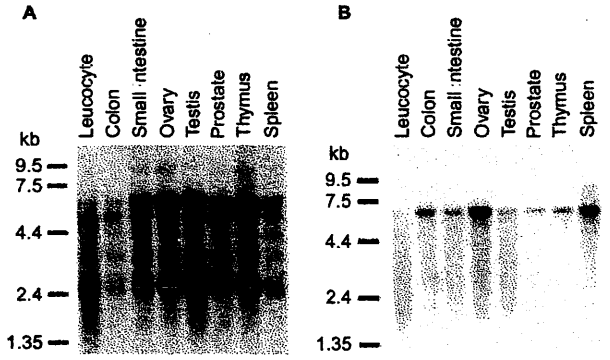

Figure 2 Northern blot analysis of multiple tissue blots probed with: (A) a *PTEN* cDNA; and (B) a probe from sequence located 3 kb downstream of the *PTEN* termination codon

Previously we reported that a 121 bp probe from position 338 to 459 upstream of the ATG start codon of *PTEN* hybridized to a single transcript on Northern blots (Gray et al, 1998), which could be evidence of alternate splicing. However, when this probe was used on a Northern blot containing mRNA from BRI8, DU145, UMUC3 and A172 cells a band of approximately 5.5 kb was detected in cell lines that are both positive and negative for *PTEN*. Exact alignment of Northern blots showed that this band was marginally smaller than the expected 5.5 kb transcript detected with a *PTEN* exon 1 probe (data not shown; Gray et al, 1998).

3′-RACE was employed to confirm the use of alternate polyadenylation sites, which would give rise to multiple transcripts, and extension of transcription beyond the end of the published *MMAC1* sequence (GenBank accession U92436). 3′-RACE products generated using primers 3′NGSP2 (located 204 bp downstream of the *PTEN* stop codon) and AP2 terminated with poly-adenosine (poly-A) tails either 290–300 bp downstream from the stop codon and within 20–40 bp of two AAUAAA elements, or where the published *MMAC1* sequence terminates 910 bp from the stop codon (data not shown). 3′-RACE products produced using the primers 3′NGSP3 and AP2 terminated in the vicinity of the end of the *MMAC1* sequence with a poly-A tail. Longer 3′-RACE products were not generated with the conditions used. However, while this work was in progress a search of the GenBank database revealed one EST, IMAGE clone 361374 (Accession Nos AA017563 and AA017584), which extended 271 bp downstream of the end of the *MMAC1* sequence, indicating that transcription can progress beyond the end of the *MMAC1* sequence. Furthermore, a probe from the 3′ untranslated region located 2471–2680 bp downstream of the termination codon, and prior to a group of polyadenylation signals in the genomic sequence, gave a single band on Northern blots equivalent to the largest mRNA species (Figure 2), providing further evidence for transcription extension.

To identify the putative transcription start site and to explore the possibility that long *PTEN* transcripts may result from alternate splicing involving novel 5′ exons, 5′ RACE was performed using three sets of nested primers: GSP1 + NGSP1, GSP2 + NGSP2, GSP3 + NGSP3. The PCR primer set GSP3 + NGSP3, located 860 nt upstream of the longest published cDNA sequence (Li and Sun, 1997), did not result in a PCR product suggesting the coding sequence does not extend into this region. Several PCR fragments were generated using GSP1 + NGSP1 and GSP2 + NGSP2 which displayed sequence identity with previously published sequence (Li and Sun, 1997). The largest fragment extended 827 bp in the 5′ direction from the start codon, 35 bp further than the *TEP1* cDNA sequence. A second fragment also finished at this point while a third finished 824 bp from the start codon.

DISCUSSION

The *PTEN* gene covers over 100 kb of a 400 kb region of allelic loss common to most prostate tumours (Gray et al, 1995). Although the detection frequency of *PTEN* mutations in prostate

tumours is lower than anticipated (Cairns et al, 1997; Teng et al, 1997; Feilotter et al, 1998; Gray et al, 1998), the frequency of mutations in other tumour types such as glioblastoma and endometrial carcinoma (Liaw et al, 1997; Rasheed et al, 1997; Tashiro et al, 1997; Teng et al, 1997; Duerr et al, 1998; Risinger et al, 1998), taken together with the data from in vitro cell growth experiments and the targeted disruption of *PTEN* in mice (Di Cristofano et al, 1998), support the view that PTEN is a tumour suppressor gene. We have mapped CA repeat markers D10S608, AFMa086wg9, D10S2491 and D10S2492 precisely around PTEN and identified a number of other common intragenic polymorphisms including a novel CA repeat within intron 8 of *PTEN*. These polymorphisms could prove useful for future studies on *PTEN* and will provide a panel of markers for more detailed mapping of LOH. We have also identified the existence, within the population, of two distinct haplotypes covering this region, although no polymorphisms were found within the coding sequence of *PTEN*.

We have attempted to dissect the complex *PTEN* mRNA transcript profile. Two cell lines, UMUC3 and A172, were used to determine whether a known pseudogene on 9p21 (Dahia et al, 1998) contributes to the pattern of *PTEN* mRNA transcripts. Both cell lines retain the pseudogene but UMUC3 has completely lost both copies of the *PTEN* gene and A172 retains exons 1 and 2 of *PTEN*. On Northern blot analysis no transcript was identified for UMUC3 and an altered message profile was found in A172 cells consistent with the truncated copy of *PTEN* present in this cell line. These results indicate that the pseudogene is not transcribed and does not contribute to any of the transcripts identified in cell lines studied. The pseudogene was originally identified by cloning from RT-PCR implying that transcription was taking place (Kim et al, 1998) and has subsequently been reported as occurring as a major mRNA species (Fujii et al, 1999). Although we cannot exclude the possibility that there could be some cell/tissue-specific transcription of the pseudogene, the similarity of the multiple species seen on Northern blots with mRNA from a variety of different tissues (Gray et al, 1998) and the *PTEN*-positive cell lines described here, taken together with data described by others (Dahia et al, 1998) makes it appear more likely that the pseudogene is not transcribed.

We previously reported that a probe derived from the 5′ sequence of *PTEN* detects only a 5.5 kb transcript whereas full length cDNA probes or probes derived from individual exons all give a complex pattern with major RNA species at 2.1 kb, 2.4 kb and 5.5 kb (Gray et al, 1998). When this probe was used to analyse RNA from *PTEN* null cells a single band was seen in both UMUC3 and A172 which was identical to the pattern seen for *PTEN*-expressing cells. Therefore this 5.5 kb band is distinct from the similarly sized transcript detected with probes derived from the *PTEN* coding region. Consequently it is unlikely to have arisen from alternate *PTEN* splicing and is more likely to be the result of cross-hybridization to an unrelated RNA with some sequence similarity to the *PTEN* 5′ UTR.

Published cDNA sequence of the *PTEN* 5′ UTR extends 790 bp upstream of the ATG translation start site. To determine the region of initiation of transcription a commercial prostate cDNA library was analysed by 5′-RACE. The clones obtained extend the longest previously reported cDNA sequence (Li and Sun, 1997) by 38 bases. Primers derived from genomic sequence 5′ to this region and 5′ to the published *TEP1* cDNA sequence did not produce a PCR product. This suggests that the transcription start site is in the region of the sequence described by Li and Sun (1997), which would give a 5′ untranslated sequence of around 827 bp, with a gene sequence of 1209 bp and the first polyadenylation sites at 60 bp and 290 bp from the translation termination signal. These distances correlate with the size of the smaller mRNA species of 2.1 and 2.4 kb seen on Northern analysis (Gray et al, 1998). The larger 5.5 kb species is likely to result from a further polyadenylation site approximately 3 kb downstream of the second polyadenylation signal. This conclusion is supported by Northern blot analysis where the 5.5 kb mRNA species was identified with a probe from a region 3 kb downstream of the termination codon (Gray et al, 1998). cDNA sequences in the database and 3′ RACE experiments confirm that alternative polyadenylation sites are used and that poly-A tails are added at the equivalent position to the 2.1 and 2.4 kb and that longer species are also transcribed. This analysis taken in conjunction with the analysis of *PTEN* null and *PTEN* truncated cell lines suggests that the complex pattern of mRNAs seen on Northern blots arises largely from alternative polyadenylation sites and that no major contribution to this pattern is made by pseudogene transcription, alternate splicing or cross-hybridization to related gene family members. The significance of the variable 3′ UT sequences is unclear but regulatory elements in this region in other genes have been associated with cell differentiation (Rastinejad and Blau, 1993), mRNA stability particularly in cancer cells (Rajagopalan and Malter, 1997) and tissue-specific expression (Coy et al, 1999).

PTEN appears to be an important tumour suppressor gene which is lost in many cancers. In some cases, particularly in glioblastomas and endometrial cancers, the presence of LOH and mutations in the second gene is consistent with the Knudson two-hit hypothesis (Knudson, 1991). In the case of prostate cancer, LOH is common (Gray et al, 1995) but mutations in the remaining gene are rare (Gray et al, 1998), although loss of transcription due to methylation has been reported in some tumour samples (Whang et al, 1998). The detailed genomic map and an analysis of *PTEN* transcripts should assist in the understanding of the genomic changes which contribute to tumour progression by the inactivation of *PTEN* in prostate and other tumours.

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