Functional Analysis of TSPY and Its Role in Prostate Carcinogenesis

A thesis submitted in part for the requirement for
the degree of Doctor of Philosophy

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Abstract

Testis specific protein Y chromosome encoded (TSPY) is a multicopy gene located on the human Y chromosome. It has been reported that the human genome harbours between 20 to 40 copies of the gene. Mammalian homologues of human TSPY were also found to be repetitive. The gene is expressed in human foetal and adult testis. The precise function of the expressed TSPY gene product is not fully defined, but it has been postulated to regulate proliferation of testicular spermatogonia. Up-regulation of TSPY expression has been detected in gonadoblastoma, testicular cancer and prostate cancer. The contribution of abnormal expression of TSPY to prostate carcinogenesis has not been investigated. Prostate cancer (CaP) and benign prostate hyperplasia (BPH) are the most common diseases of the human prostate. Prostate cancer is a disease of the elderly and is the second most common cancer and second most common cause of death from cancer among men in UK.

In this thesis, the role of TSPY in prostate carcinogenesis was studied in four related aspects. First, TSPY protein and transcript expression pattern in CaP compared to Benign Prostate Hyperplasia (BPH) was studied using a combination of immunohistochemistry and mRNA *in situ* hybridisation. Second, the ability of TSPY to regulate cellular proliferation was investigated by transfection experiments of the prostate cancer LNCaP cell line. Third, TSPY genomic copy number in prostate cancer was characterised and compared to BPH. Finally, the downstream genes regulated by TSPY were investigated using high density microarray gene profiling method.

An anti-human TSPY polyclonal antibody was developed for immunodetection of TSPY expression level in resected prostate tissues. In total, 72 cases of patients with prostate cancer and 20 cases of patients affected with BPH were studied by immunohistochemistry. TSPY was predominantly detected in the prostatic epithelium. In the benign gland, TSPY expression was limited to the basal cells compartment. TSPY expression was significantly up-regulated in prostate cancer when compared to BPH ($P<0.0001$). Furthermore, increased TSPY expression level was associated with aggressive disease (tumour with high Gleason score; $P<0.02$) and the presence of bone metastasis at the time of diagnosis ($P<0.028$). To address the functional role of

TSPY in prostate cancer, FLAG-TSPY was cloned and stably transfected into LNCaP cells. The presence of transfected TSPY increased LNCaP proliferation by two fold compared to empty vector control, consistent with a mitogenic function in CaP ($P<0.0001$).

An absolute quantitative real time PCR based on Taqman assay was established and validated. TSPY genomic copy number was determined from comparing 161 samples: CaP-serum (n=47), resected tumour (n=31); BPH-serum (n=27), resected prostate (n=13) and control–serum (n=45). Of the clinical samples analysed, interpersonal variability of TSPY copy numbers was observed with the majority of cases containing between 20 to 50 TSPY copies per genome. Although, there were variability in TSPY copy numbers among individuals, there was no statistically significant correlation between TSPY copy number (serum and prostatic tissue) and the development of prostate cancer.

Studying LNCaP stably transfected with TSPY and empty vector control, the key genes mediating the functional effect of TSPY were identified using Affymetrix oligonucleotide microarray method. In total, 332 genes have been altered 1.5 to 90 fold due to the effect of TSPY over-expression. Ten genes were selected and the gene expression levels were confirmed using semi-quantitative RT-PCR method. Gene clustering analysis has indicated changes in genes that regulate cellular differentiation (NRDG1, NF2, C-MAF and BMP11), apoptotic gene (BAX), cell cycle gene (cyclin G), detoxification gene (GST-2A) and genes linked to adhesion (PCDH7a, PLOD2 and IRS1).

In summary, TSPY is over-expressed in prostate cancer. This abnormal expression is linked to prostate cancer progression and metastasis. The up-regulation of TSPY expression is unlikely to be due to increased copy number. Expression of exogenous TSPY in prostate cancer cells enhanced cellular proliferation. The gene meditates its effect by down-regulating the expression pattern of selected differentiation, apoptosis and adhesion genes. Hence, over-expression of TSPY contributes to prostate carcinogenesis.

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Abbreviations

aa	Amino acid(s)
ABC	Avidin biotin complex
ANOVA	Analysis of Variance
APES	Amino-propyl-tri-ethoxy-silane
AR	Androgen receptor
ARE	Androgen responsive element
ATP	Adenosine 5'-triphosphate
dATP	Deoxyadenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indoylphosphate
BCL2	B cell lymphoma 2
BPH	Benign prostatic hyperplasia
BRCA2	Breast cancer susceptibility gene 2
BSA	Bovine serum albumin
bp	Base pair
CaP	Carcinoma of the prostate
cAMP	Adenosine 3', 5'-cyclic-monophosphate
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative genomic hybridisation
cpm	Counts per minute
Ct	Threshold cycle
DAB	Diamino-Benzidine-Tetrachloride
DAPI	Diamidino-2-phenylindole
DCC	Dextran coated charcoal
DD	Double distilled
DEPC	Diethyl pyrocarbonate
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DRE	Digital rectal examination
Ds	Double stranded
DTT	Dithiothreitol

EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3 phosphate-dehydrogenase
GFP	Green Fluorescent protein
HGPIN	High grade prostate intraepithelial neoplasia
HMW	High molecular weight
HPC	Hereditary prostate cancer
HRP	Horseradish peroxidase
LB	Luria Bertani
I.F.	Immunofluorescence
I.I.F.	Indirect immunofluorescence
I.M.A.G.E.	Integrated molecular analysis of genome and their expression
kb	Kilobase
kDa	KiloDalton
KLH	Keyhole limpet haemocyanin
LMW	Low molecular weight
LOH	Loss of heterozygosity
MM	Mismatch
mRNA	Messenger ribonucleic acid
MSY	Male specific Y chromosome
NBT	Nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
OD_{260}	Optical density at 260 nm
O/N	Overnight
PAGE	Polyacrylamide gel electrophoresis
PAR	Pseudoautosomal region
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PIN	Prostatic intraepithelial neoplasia

PM	Perfect match
PMSF	Phenyl methyl sulfonyl fluoride
PSA	Prostate specific antigen
Rb	Retinoblastoma
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLR	Signal log ratio
SSC	Standard saline citrate
TAE	Tris acetate EDTA (buffer)
TBE	Tris borate EDTA (buffer)
TBS	Tris-buffered saline (buffer)
TCA	Trichloroacetic
TE	Tris EDTA (buffer)
TEMED	*N, N, N′, N′*-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane (buffer)
TSG	Tumour suppressor gene
TTBS	Tween-20 tris-buffered saline (buffer)
Tm	Melting temperature
TMB	Tetramethylbenzidine
TURP	Transurethral resected prostate
UCSC	University of California Santa Cruz
UTR	Untranslated region
WST-1	Tetrazolium salt

Chapter 1
General Introduction

1.1 Introduction

1.1.1 Physiology and anatomy of prostate gland

The human prostate is a male accessory sex gland that weighs approximately 20 gm and is shaped liked a chestnut. The gland lies below the bladder and encircles the upper part of urethra. This anatomical location allows direct deposition of prostatic fluid into urethra during ejaculation. The secreted prostatic fluid is milky in colour and is rich in citric acid, fibrinolysin and enzymes, particularly acid phosphatase. These prostatic secretions play a key role in liquefying semen and activating sperm (Shipley, 1999).

The prostate gland is anatomically divided into four distinct zones (McNeal, 1981): peripheral zone, central zone, transition zone and the non glandular anterior fibromuscular stroma (Figure 1.1). The peripheral zone accounts for 75% of the normal adult glandular tissue in the prostate. The peripheral zone is the most susceptible region to develop prostate cancer. The second major region of the prostate is the central zone which forms 20% of prostate glandular tissue. The central zone is cone-shaped and surrounds the ejaculatory duct. The transitional zone makes up 5% of the prostate glandular tissue and is the primary site for the development of benign prostatic hyperplasia (BPH). The anterior fibromuscular stroma forms a non-glandular part of the gland consisting predominantly of collagenous stroma and muscle fibres.

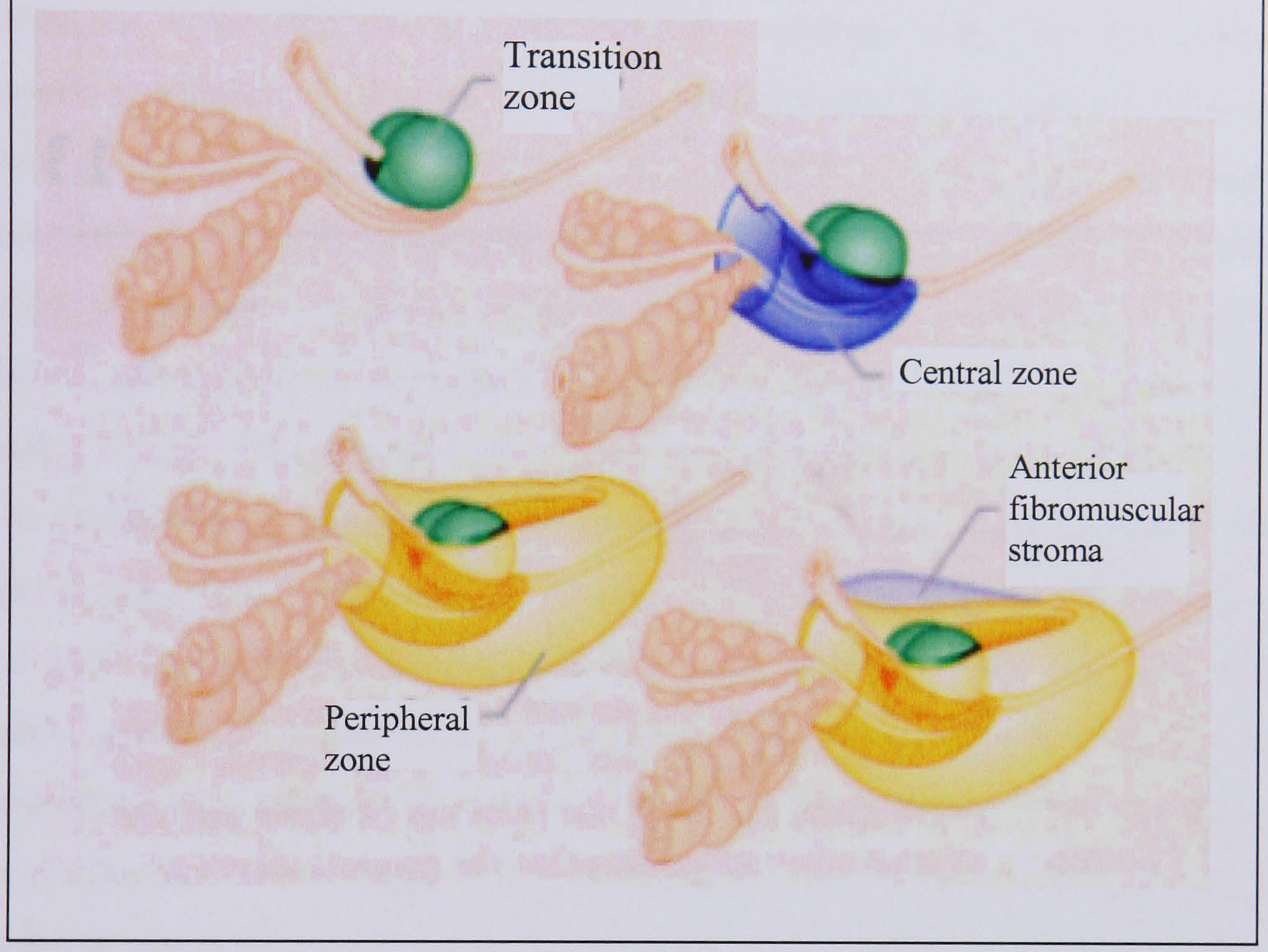

Figure 1.1. Prostate gland zonal anatomy.

The prostate is anatomically divided into four distinct zones; peripheral, central, transitional and non-glandular fibromuscular stroma. The peripheral zone forms the major part of the gland and directly surrounds the prostatic urethra and indirectly surrounds the ejaculatory duct. The central zone is the second largest region of the gland that directly surrounds the ejaculatory ducts. The transitional zone accounts for the minor part of the gland and surrounds the prostatic urethra. The non-glandular fibromuscular stroma is located anterior to the gland (Mundy *et al.*, 1999).

1.1.2 Histology of Prostate gland

The human prostate gland consists of two compartments, ducts and the surrounding stroma. The prostate ductal epithelium is composed of basement membrane lined with three distinct cell types: luminal, basal and neuroendocrine cells (Figure 1.2). These

cells can be distinguished by their morphology and the expression of molecular markers (Abate-Shen and Shen, 2000). The secretory luminal cells represent the predominant cell type in the prostate epithelial compartment (Figure 1.3). They are terminally differentiated and columnar in shape producing prostatic secretory proteins and are positive for the expression of androgen receptor, cytokeratin 8, 18 and cell surface marker CD57. The basal cells, the second major epithelial cell type, are cuboidal in shape and are located between columnar cells and the underlying basement membrane. The basal cells are distinguished from luminal cells by the expression of cytokeratin 5, 14 as well as CD44. These cells are androgen independent, but remain androgen responsive. Analysis of cytokeratin expression patterns in prostate epithelium has identified transient populations of prostatic epithelial cells that have both basal and luminal characteristics (Schalken and van Leenders, 2003). This suggests the existence of prostate stem cells which could give rise to transiently proliferating cells that can divide to generate all types of prostate epithelial cells. The third prostate epithelial cell type is the neuroendocrine cells. This minor cell population is characterised by expression of hormones and secretory proteins such as Chromogranin A, secretogranin II, neurone specific enolase (marker of neuroendocrine differentiation) and serotonin (Abrahamsson, 1999). Chromogranin A is a member of the family of acidic secretory proteins found in the secretory granules of endocrine cells and neurones. The exact function of chromogranin A is not yet clear (Huttner *et al.*, 1991). However, it may have extracellular bioactivity that acts as autocrine and/or paracine regulatory agent in the secretory process. Serotonin is another secretory hormone protein produced by neuroendocrine cells which can be involved in mediating growth activity, regulating morphogenesis and secretory peptide hormones from endocrine cells. In general, the function of neuroendocrine cells in the prostate remains unknown. Nevertheless, they appear essential for growth, differentiation and the regulation of secretory processes in the prostate.

The second compartment of the prostate gland is the stroma. It consists of dense collagen, fibroblasts, smooth muscle fibres, skeletal muscles, blood vessels, neuroendocrine cells as well as sympathetic nerves (Young and Heath, 2000) (Figure 1.3). Interaction between stroma and epithelium plays a vital role in prostate organogenesis. Aberrant interaction between stroma and epithelium contributes to prostatic diseases including CaP and BPH (Chung, 1995).

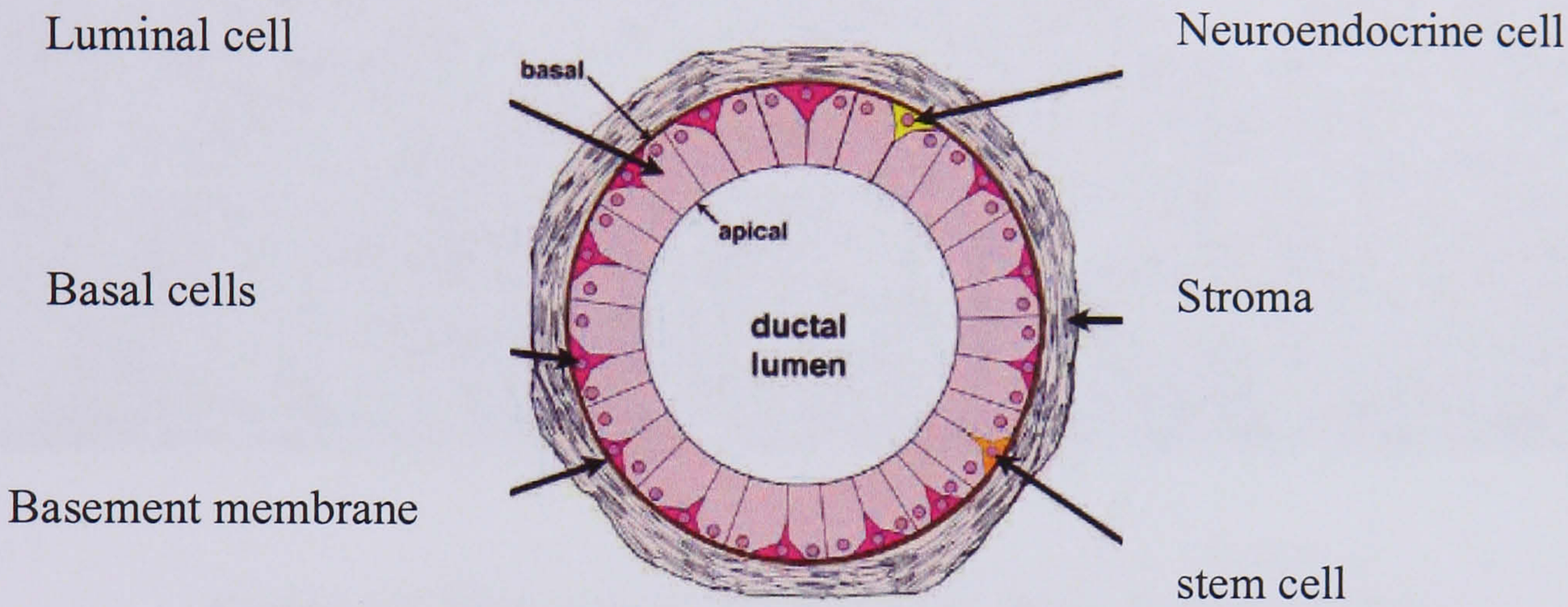

Figure 1.2. Schematic representation of prostate gland.

The normal human prostate is made up of glands surrounded by stroma. The prostatic gland epithelium consists of three distinct cell types lining the basement membrane: luminal secretory cells, basal cells and neuroendocrine cells. The luminal secretory cells are the predominant cell type in prostate epithelium and are columnar in shape. The basal cells are located between the columnar cells and the basement membrane and are cuboidal in shape. The neuroendocrine cells are minor cell types that are present within the prostatic epithelium. The stroma surrounds the basement membrane and mainly consists of fibroblasts, muscle cells, nerve, and blood vessels (Marker *et al.*, 2003).

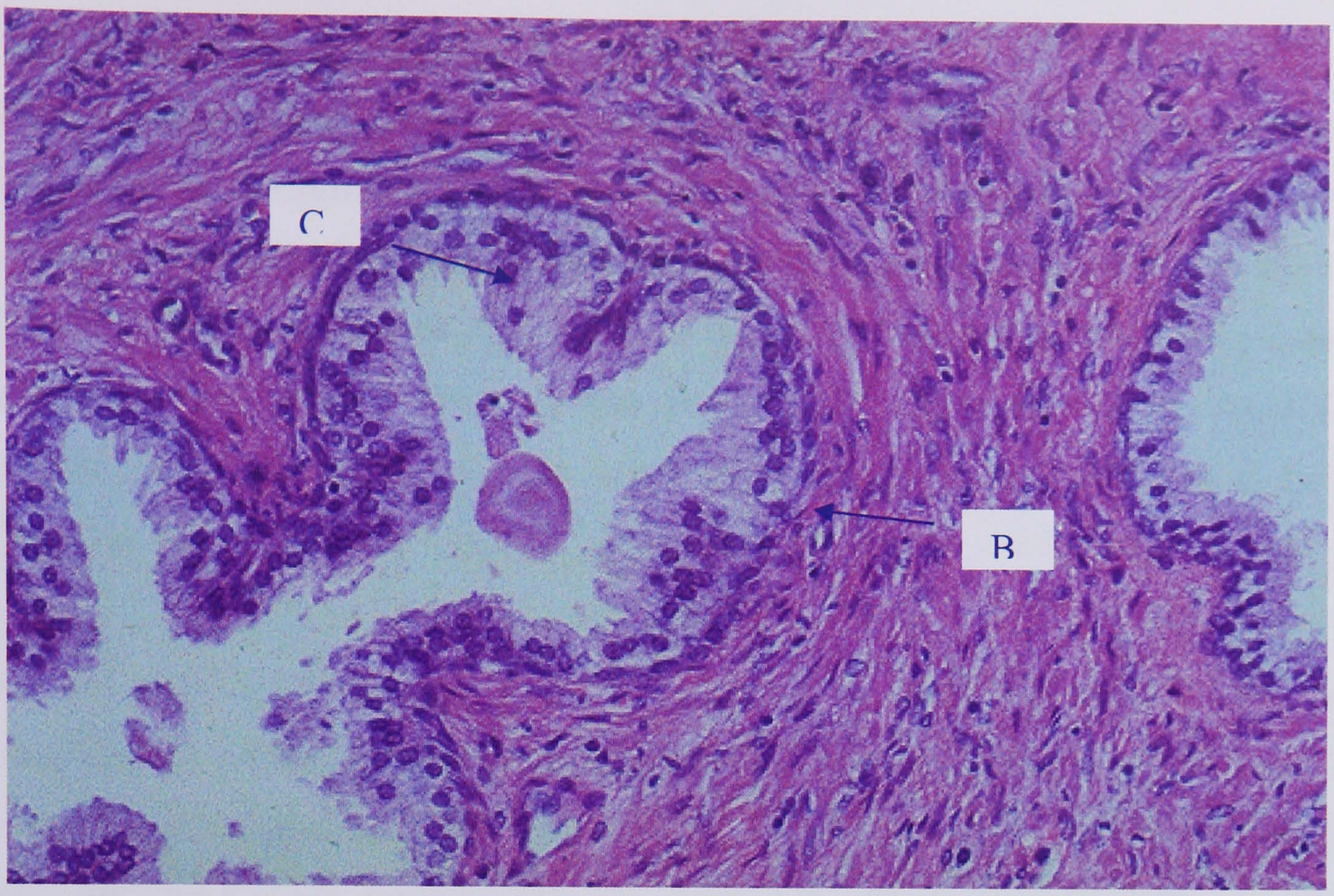

Figure 1.3. Histological structure of normal prostate gland.

The normal human prostate is composed of glandular secretory epithelium surrounded by stroma. In histological cross section of the prostate, glands appear to be rounded to irregular branching in shape. The basement membrane of the gland is lined with two cell layers, tall columnar secretory cells (C) and cuboidal basal cells (B). The glands are surrounded by fibromuscular stroma which accounts for about half of the volume of prostate (http://www.medlib.med.utah.edu). The stroma composed of smooth muscles, nerve supply, fibroblasts, and blood vessels.

1.1.3 Pathology of Prostate Gland

Common pathological conditions of the human prostate gland include prostatitis, benign prostatic hyperplasia, prostate cancer and its precursor lesion, prostatic intraepithelial neoplasm.

1.1.3.1 Benign prostatic hyperplasia

Benign nodular hyperplasia or benign prostatic hyperplasia (BPH) is a non-neoplastic enlargement of the prostate gland. The incidence of BPH increases with age and is detected in approximately 20% of men 40 years of age, increasing to 70% at the age of 60 and 90% at the age of 70 (Arrighi *et al.*, 1991). More than 400, 000 prostates are resected each year in USA (Cotran *et al.*, 1999). BPH does not predispose to prostate cancer. Hence, the disease is not considered to be a pre-malignant lesion (Cotran *et al.*, 1999). On average, prostates affected by BPH weigh 60-100 gm (compared to normal prostate weighing 20g). Less commonly, the prostate can weigh up to 200 gm (10 times normal prostate) in patients with BPH. BPH is characterised by hyperplasia of both stroma and epithelium, resulting in formation of large discrete nodules in the transitional zone of the prostate (Steers and Zorn, 1995). The glandular acini are larger than normal and the epithelium is characterised by numerous infoldings (Figure 1.4).

The development of BPH occurs in two phases, pathological and clinical BPH. The pathological phase is divided into microscopic and macroscopic BPH. Nearly all men throughout the world will develop microscopic BPH if they live long enough. In about half of men with microscopic BPH, enlargement of prostate will progress to macroscopic BPH. In ageing men with macroscopic prostatic enlargement, only about half will progress to produce clinical BPH (Isaacs and Coffey, 1989). Men affected by BPH, develop lower urinary tract symptoms in keeping with bladder outlet obstruction. The hyperplastic nodules compress the prostatic urethra and obstruct the urinary flow, resulting in gradual hypertrophy of the bladder and dilation of ureter. Severe or untreated BPH may eventually lead to recurrent urinary infections, haematuria and ultimately impaired renal function (Underwood, 1998).

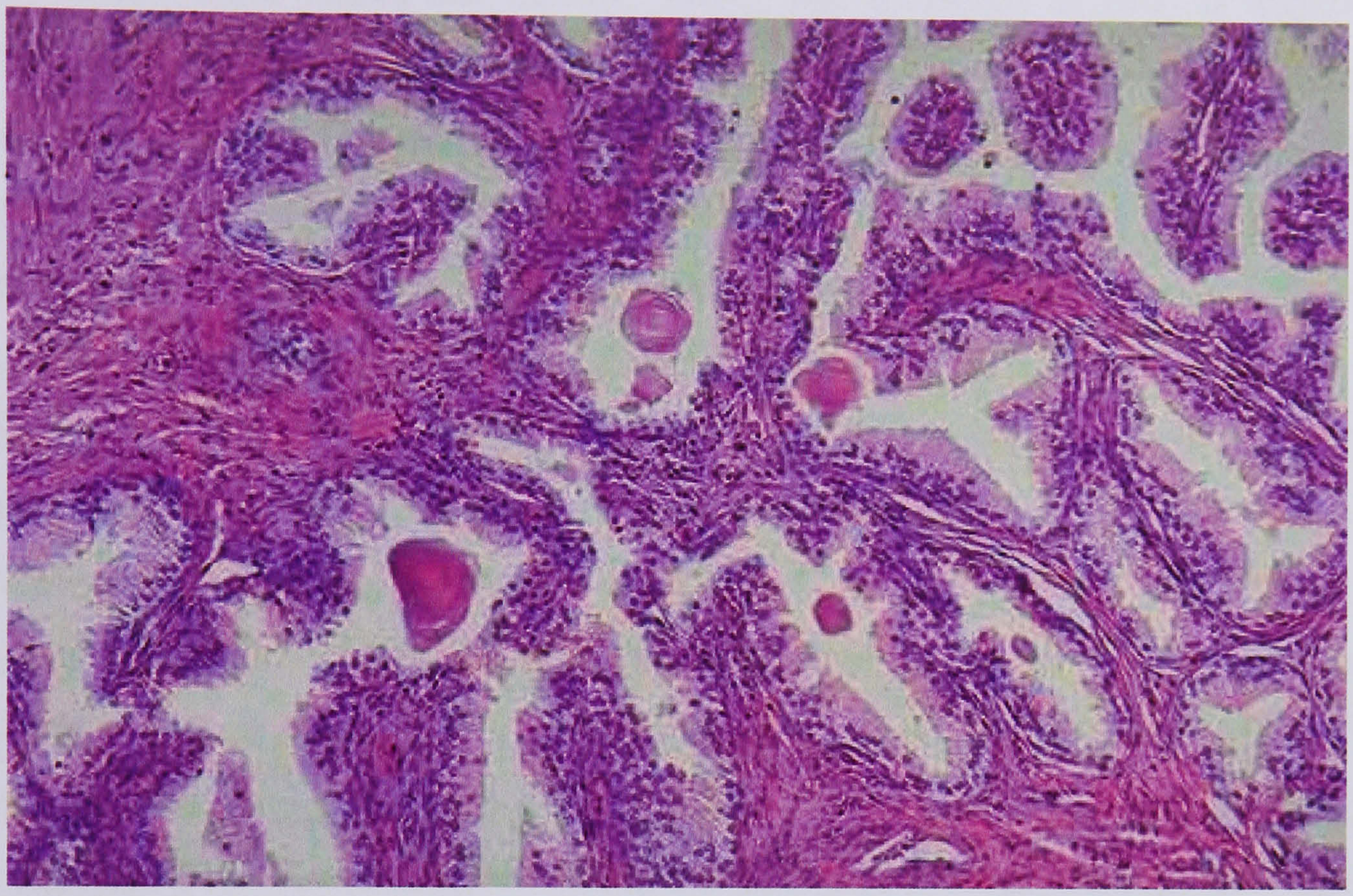

Figure 1.4. Histological morphology of BPH.

Microscopic appearance of BPH resected tissue. The glandular structure of the prostate is preserved. However, enlarged glands separated by stroma are noted. The glands retain glandular basement membrane lined with columnar cells and cuboidal cells (www.medlib.med.utah.edu).

1.1.3.1.1 Aetiology

The cause of BPH is not known. However, age, race, family history and hormone are potential risk factors for BPH. The incidence of BPH increases with increasing age (Berry *et al.*, 1984, Isaacs and Coffey, 1989). The incidence of BPH is highest in African Americans and lowest in native Japanese populations. However, the incidence increases in Japanese immigrants to Western countries due to either environmental or dietary factors (Ekman, 1989). Family history is another risk factor for the development of BPH. Recent studies have indicated that patients with a positive family history of BPH have larger prostates than controls (Sanda *et al.*, 1997). Dietary fat intake, vitamin supplements and obesity, which cause disturbances in hormone balance, have also been suggested as risk factors (Ziada *et al.*, 1999). Mounting evidence suggests that prostate enlargement is related to the action of dihydrotestosterone (DHT), a metabolite of testosterone which plays a vital role in regulating prostate development, growth and differentiation (Droller, 1997). DHT is produced from testosterone by the action of steroid, 5-α-reductase type 2 (Figure 1.5). The enzyme is produced primarily by the stromal cells. DHT can act on both stromal and epithelial cells in autocrine and paracrine manners, respectively. In both cell types, DHT binds to androgen receptor which translocates to the nucleus to regulate transcription of genes regulating growth of epithelial cells and stromal cells. In men with BPH, the use of 5 α-reductase inhibitor significantly reduces prostatic volume (Walsh, 1996).

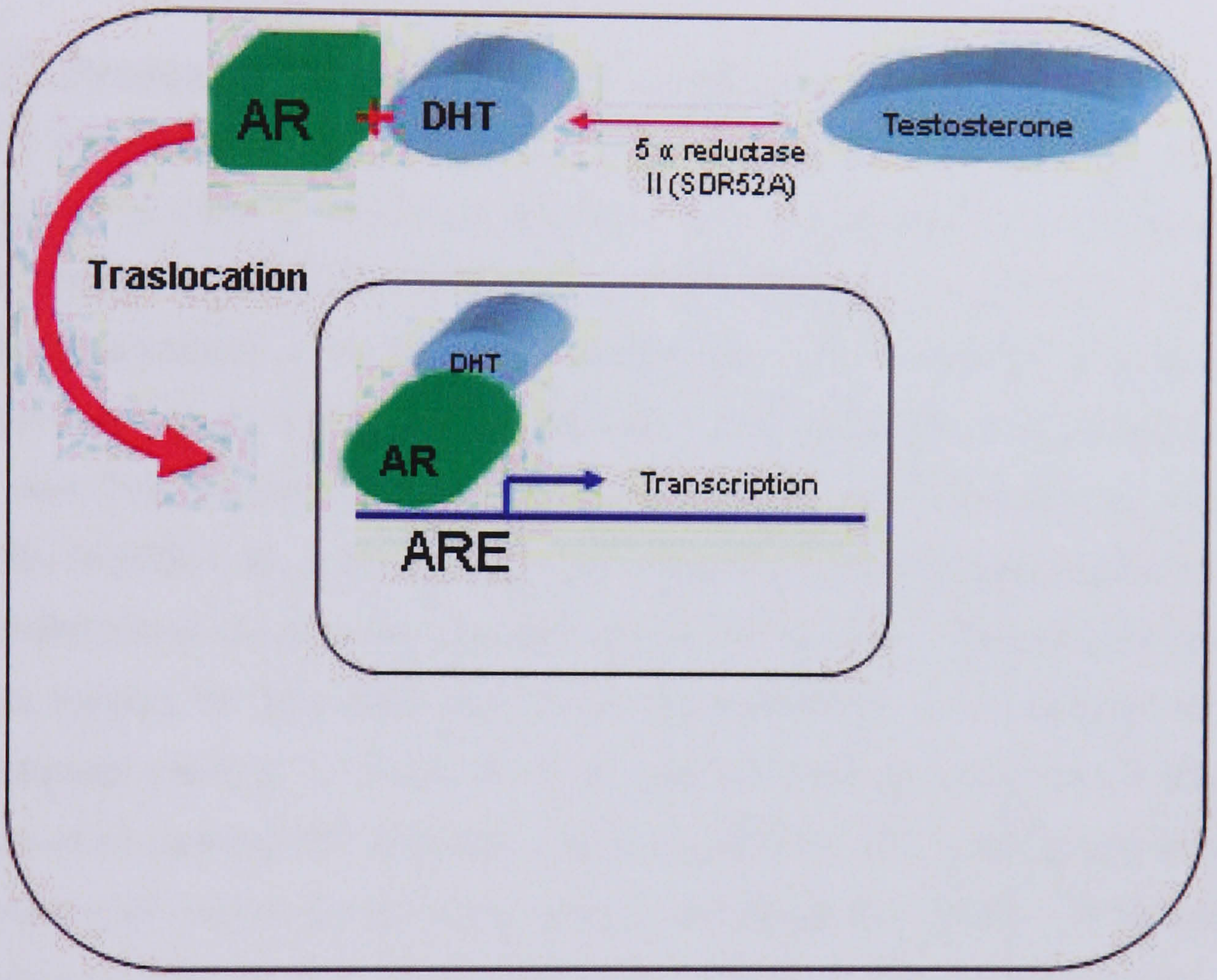

Figure 1.5. Androgen receptor activation.

Testosterone is converted into dihydrotestosterone (DHT) by the action of cellular 5 α- reductase type II (SRD5A2). Active DHT binds to AR and translocates it from cytoplasm to the nucleus. In the nucleus the activated AR binds to androgen response elements of genes that regulate growth of epithelial and stromal cells and regulate their transcription.

1.1.3.1.2 Treatment

If the symptoms from BPH severely affect micturition, the patient can be treated with medication or by surgical procedure. Medication aims to relax smooth muscles within the prostate stroma. Surgical removal of the enlarged part of the prostate by means of transurethral resection is the most reliable treatment to give long term relief from symptoms of BPH.

1.1.3.2 Prostatic intraepithelial neoplasia

Prostatic intraepithelial neoplasia (PIN) has been previously referred to as putative precancerous dysplastic, intraductal dysplasia, large acinar atypical hyperplasia and atypical primary hyperplasia. The incidence of PIN detected in transurethral resected prostates from patients varies from 2.8-33% (Mundy *et al.*, 1999). PIN is divided into two grades; low grade and high grade (HGPIN). Low grade PIN is often detected in young men. The frequency of high grade PIN increases with advancing age (Sakr *et al.*, 1993). HGPIN is the most likely precursor lesion of prostatic adenocarcinoma and has high predictive value as a marker for CaP (Bostwick *et al.*, 1996). Several lines of evidence support the hypothesis that PIN is an intermediate lesion between normal and malignant prostate. In nearly 80 % of confined prostate cancer cases, there is evidence of co-existing PIN (Graham, 1992). In addition, molecular genetic analysis of PIN and CaP lesions reveals many genetic alterations in common. These include aneuploidy as well as loss of heterozygosity (LOH) at several genetic loci. For instance, at 8p12 loci, there is LOH in 64 % and 91 % in PIN and prostate cancer, respectively (Haggman *et al.*, 1997b). Furthermore, it has been suggested that approximately 30% of PIN may progress to invasive cancer over a period of 10 year (Bostwick, 1996). Moreover, cytological features of PIN closely resemble those of invasive carcinoma (Bostwick *et al.*, 1993). Further study has shown that in the prostate transgenic mice model expressing SV40 large antigen driven by the prostate specific promoter probasin, multifocal loci of high-grade dysplasia similar to PIN have developed (Kasper *et al.*, 1998).

The peripheral zone of the prostate is the most common location for PIN development (Bostwick *et al.*, 1993). PIN epithelium is histologically characterised by disruption of basal cell layer and retained basement membrane (Bostwick and Brawer, 1987) (Figure 1.6). The epithelial cells of low grade PIN are crowded and irregularly spaced

and cells of HGPIN are more crowded and characterised by nuclear and nucleolar enlargement. PIN differs from prostate carcinoma in that PIN lesions do not invade normal stroma and PIN can only be detected by biopsy samples (Haggman *et al.*, 1997a).

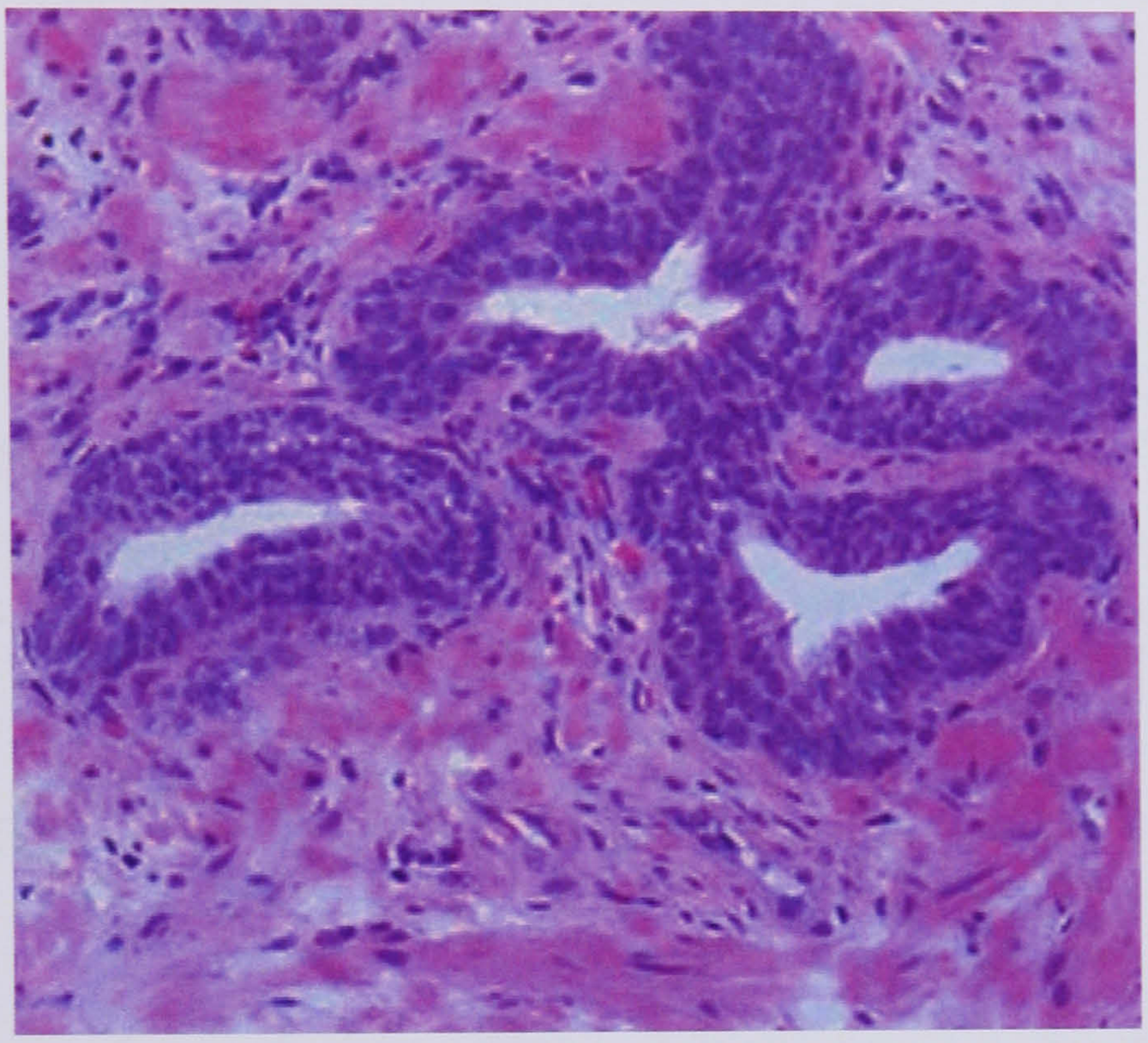

Figure 1.6. Morphological structure of prostatic intraepithelial neoplasia (PIN).

PIN is characterised by atypical basal cell hyperplasia, preservation of basement membrane, replacement of normal luminal secretory cells with neoplastic cells and crowded epithelial cells. (www.medlib.med.utah.edu).

1.1.3.3 Prostate cancer

Prostate cancer is the third most common cancer in men in the world (Quinn and Babb, 2002a, Quinn and Babb, 2002b) and is the second most common cancer in men in UK. It is a rapidly growing global health problem as the numbers of detected and reported prostate cancer cases are continuously increasing. Although there are more than 1.5 million persons diagnosed with prostate cancer, there are more than 500,000 new cases reported every year worldwide (Parkin *et al.*, 2001). Prostate cancer is a disease of the elderly with approximately 75% of cases diagnosed in patients over 65 years old (Dijkman and Debruyne, 1996). It has been anticipated that more than 1.25 million new cases of prostate cancer will be detected annually by year 2050. Thus, by the middle half of the century, prostate cancer will be the second most common cancer in men. Mortality from prostate cancer has increased and it accounts for 204,000 deaths per year. It has been estimated that this number will double in less than 50 years. In England, 23,109 new cases of prostate cancer were reported in the year 2000 and 9,004 deaths were confirmed due to prostate cancer (Cancer registrations in England, 2003).

Clinical symptoms associated with locally advanced prostate cancer and/or metastatic disease are bone pain, spinal cord compression and urinary tract obstruction. At the initial stage of prostate cancer formation, tumours originate in the peripheral zone, invade and spread through several routes; direct local invasion, lymph and blood vessels. Prostate cancer can spread directly from within the gland and to the extra-capsular adjacent structures. Invasion of prostatic stroma towards the peri-urethral tissue and base of the bladder is common. Extension through the prostate capsule to the seminal vesicle is also common. Lymphatics vessels provide an important route of dissemination of prostatic carcinoma, leading to metastasis in lymph nodes. These may result in lymphatic obstruction and oedema of the legs. Prostate cancer can spread through the vascular route and metastasise to bone, lung and liver. The most frequent sites of bone metastasis are pelvis, spine and ribs.

1.1.3.3.1 Diagnosis of CaP

Several diagnostic tools are available to aid the detection of prostate cancer in men. Careful digital rectal examination (DRE) is a useful direct method for early detection

of prostatic carcinoma, since the posterior location of most tumours (peripheral zone), renders them palpable. DRE is carried out by inserting a gloved finger up the rectum of a patient by a physician to detect any lumps or hardening of the prostate gland. DRE alone is not sufficient to detect CaP early disease. Transrectal ultrasonography is another important diagnostic tool for early detection as well as assessment of local spread of the tumour and transrectal biopsy is required to confirm the diagnosis. Several procedures are used to detect prostate metastasis. Pelvic lymphadenectomy is used to detect microscopic metastasis to the lymph nodes. Metastasis to bone can be detected by radionuclide bone scanning. The introduction of a blood test for prostate specific antigen (PSA) in 1986 had accelerated the efficiency of detecting prostate cancer at early stages. PSA is a serine protease which functions to cleave and liquefy the seminal coagulum formed after ejaculation. The PSA protein is produced by the normal gland in low amounts (4 ng/ml) and the serum levels of PSA increase with age. Furthermore, PSA levels can be spuriously elevated in BPH and inflammation of the prostate (prostatitis). It has been estimated that nearly 25% of men with abnormal DRE and PSA tests actually they do not have CaP. Combination of DRE and PSA testing can increase the detection efficiency of CaP up to 85% (Jones, 1997). It is now suggested that men should have an annual PSA test at the age of 50 for white men and age 40 for black persons. Once the CaP is diagnosed, histological grading and staging of tumour are determined to assess the degree of differentiation, size and location of tumour.

1.1.3.3.2 Gleason classification of CaP

Gleason system is widely accepted for histological assessment of prostate tumour grade (Gleason, 1992). Based on glandular pattern and degree of differentiation as inspected under low magnification, prostate cancer is divided into five distinct grades on a scale of 1-5. Grade 1 represents the best differentiated tumour in which the neoplastic glands appear uniform and tend to be packed into well circumscribed nodules. Occasionally the glands are somewhat larger with a papillary pattern. The glands are lined with a single uniform layer of columnar epithelium. The basal cell layer is absent. The nuclei of the tumour cells are large, vacuolated and contain one or more large nucleoli. On the other hand, grade 5 tumours lack glandular differentiation with tumour cells infiltrating the stroma as nest of cells (Figure 1.7). Because CaP is

markedly heterogeneous in nature, two grades are used to describe an individual tumour, with primary grade for the dominant pattern and a secondary grade for the second commonest pattern. The primary and secondary grades are added to obtain the Gleason sum score. In tumours that are homogeneous, the primary and the secondary grades are the same and can be added up to give the Gleason score. The most well differentiated tumour has a Gleason score of 2 and the least differentiated tumours are score 10. The low grade tumours progress slowly and high grade tumours grow rapidly.

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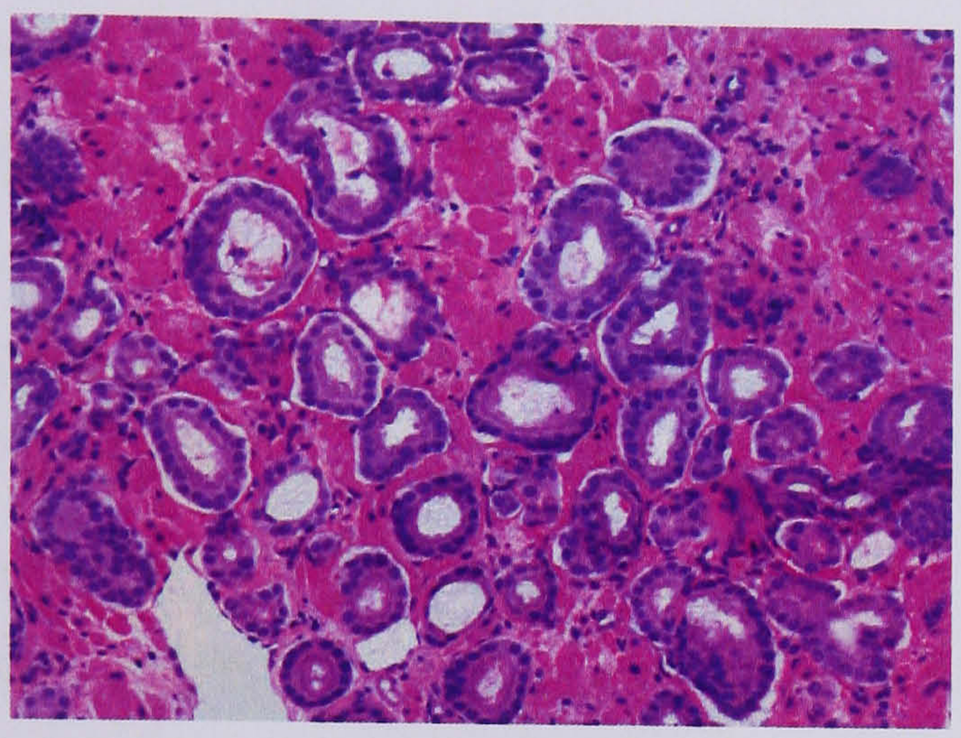

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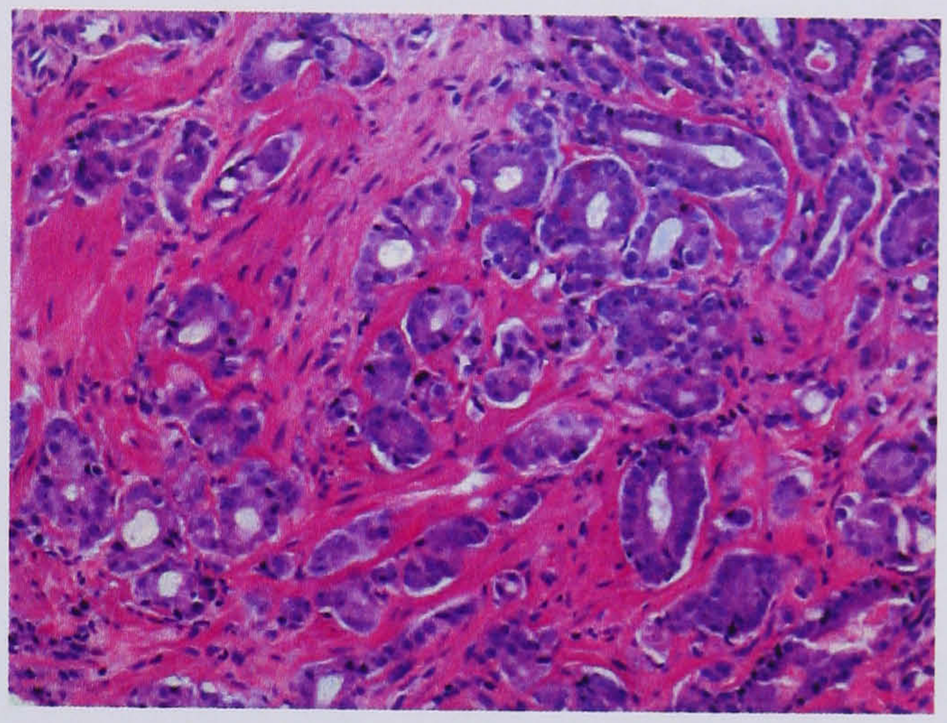

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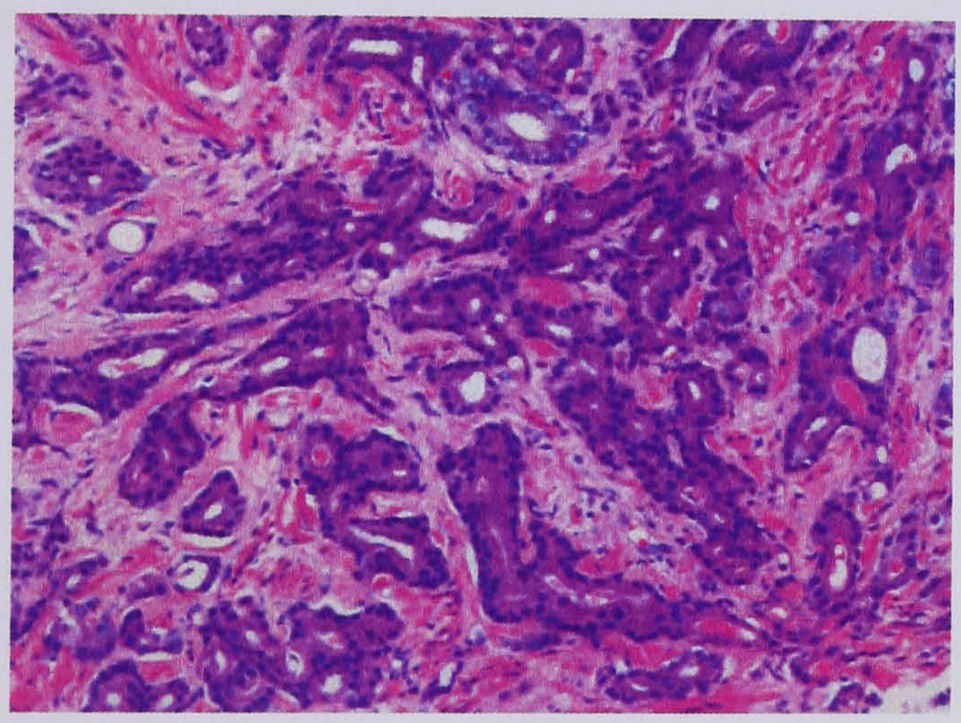
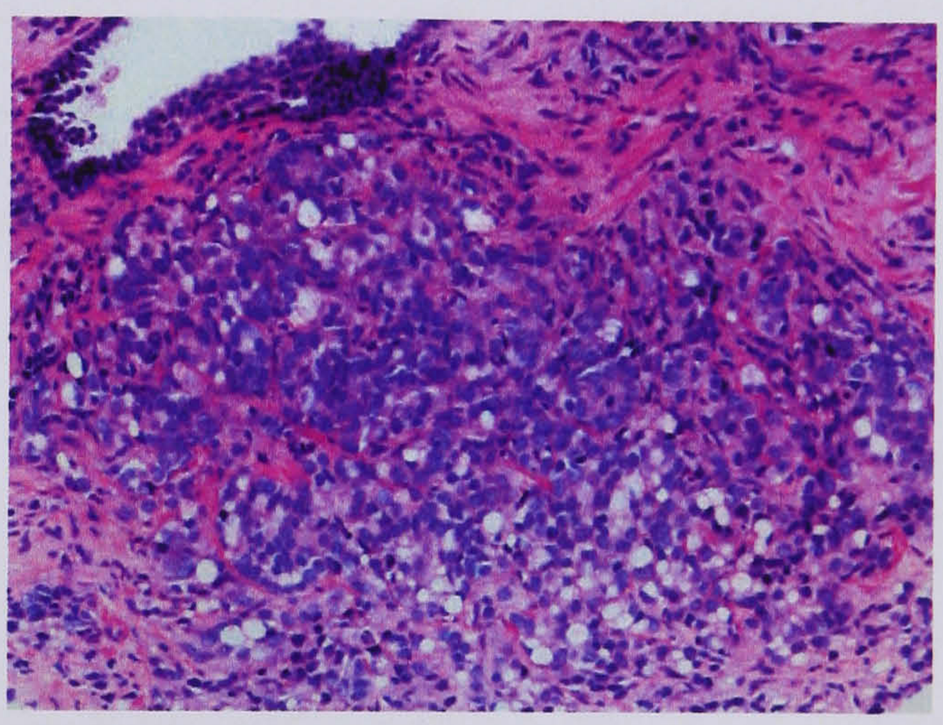


Gleason grade 4



Gleason grade 5

Figure 1.7. Histological features of prostate cancer with Gleason patterns (single grades) from 2-5.

In Gleason grade 2, the cancer glands are slightly variable in size, shape and spacing. The glands spread though the surrounding stroma. Gleason grade 3 is the most detected pattern. The malignant glands are variable in size, shape and spacing. The glands are discrete and infiltrate the stroma. The most distinguishable feature of Gleason grade 4 is fused glands. The glands are no longer recognised as individual units. The tumours in Gleason grade 5 grow as solid sheets with loss of gland features (http://prostate-help.org/cagleas.htm).

1.1.3.3.3 Pathological staging

Clinical staging of prostate cancer is the assessment of tumour size and location and is important in determining the appropriate form of cancer therapy. Currently, there are two different staging systems for prostate cancer: ABCD (Whitmore-J. Wett) system (Whitmore, 1984) and the tumour nodes metastasis (TNM) system (Schroder *et al.*, 1992) (Table 1.1). The TNM staging system is now more widely used and describes the tumour size (T) and whether the cancer has spread to lymph node (N) or metastasised to distant sites. Tumour size is assessed on the scale of 1-4. Generally, T1 tumour is confined to the prostate gland and so small that it cannot be detected by either DRE or ultrasound. T2 lesions are localised in the prostate and are detectable by DRE and TRUP. T3 and T4 prostate cancers have spread beyond the prostate into adjacent organs such as bladder, seminal vesicles and pelvic side wall. Involvement of prostate cancer in the lymph nodes is graded on the scale N0-N3. N0 means that the tumour has not spread to the lymph node. The number and the size of lymph nodes involved indicate whether the cancer is N1, N2 or N3. Metastasis to different organs is rated 0 or 1. M0 means no metastasis has occurred and M1 means metastasis has been detected at a distant location.

Whitmore-J Wet	TNM-1992	Description
No tumour palpable		
A1	**T0**	**No local tumour detectable.**
A2	**T1**	**Local tumour cannot be evaluated.**
	T1a	**Tumour found in <5% of excised tissue.**
	T1b	**Tumour found in >5% of excised tissue.**
	T1c	**Tumour confirmed by needle biopsy.**
Intracapsular	palpable tumour	
B1	**T2a**	**Tumour limited to half of 1 lobe or less.**
B2	**T2b**	**Tumour has spread to half of 1 lobe but not both.**
B3	**T2c**	**Tumour has spread to both lobes.**
Extra capsular tumour		
C1	**T3a**	**Unilateral extra capsular spread.**
C2	**T3b**	**Bilateral extra-capsular spread.**
	T3c	**Tumour has spread to one or both seminal vesicles.**
	T4	**Tumour has invaded adjacent structure other than seminal vesicles.**
Disseminated tumour		
D1	**Nx**	**loco-regional lymph nodes can't be evaluated.**
	N0	**No lymph node involvement.**
	N1	**Metastasis in 1 node, <2 cm.**
	N2	**One node only > 2 cm or <=5 cm; multiple <=5 cm.**
D2		
	Mx	**Distant metastases cannot be evaluated.**
	M0	**No distant metastasis.**
	M1	**Distant metastasis present.**
	a=	**Lymph node on the other than regional one.**
	b=	**Skeletal.**
	c=	**Other sites.**
D3		**Resistant to hormone therapy.**

Table 1.1. TNM staging system for prostate cancer

1.1.3.3.4 Treatment

Treatment of prostate cancer depends on the disease stage, Gleason score, age of the patient and PSA level. Several different methods are available to treat CaP, including watchful waiting, surgery (prostatectomy and orchidectomy), radiotherapy and endocrine therapy. Localized prostate cancer that has not spread to adjacent organs can be treated by surgical removal of the entire prostate (radical prostatectomy) or radiotherapy using high energy source of X-ray. Another option is watchful waiting, alternatively called observation or surveillance. The patient receives no active treatment unless symptoms appear or the disease progression is suggested by serial PSA or DRE. Endocrine therapy is the mainstay for treatment of advanced and/or metastatic disease. Androgen deprivation can be achieved by surgical castration (orchidectomy) or medical castration by administration of oestrogens or synthetic agonists of luteinizing hormone-releasing hormone. Oestrogen inhibits testicular androgen synthesis directly by suppression of pituitary luteinizing hormone secretion which in turn causes the reduction of testicular release of testosterone. Steroidal anti-androgens and 5 α-reductase inhibitors are other available treatment options.

1.1.3.3.5 Aetiology of prostate cancer

The cause(s) of CaP are not known. However, several risk factors are associated with CaP such as age, race, family history, environmental factors and hormone levels (Chan *et al.*, 1998a). Age is a strong risk factor contributing to prostate cancer. The incidence of prostate cancer increases with age more than any other cancers. It is common among men between the age of 50 and 70 years. This trend can be explained by accumulation of genetic changes with age (Chan *et al.*, 1998b). Positive associations between CaP and dietary intake of animal fat, red meat and dairy products have been reported (Kolonel, 1996). It has been suggested that dietary fat intake influences the levels of hormones such as testosterone, which in turn affect the growth of prostate. Race is one of the well established risk factor for CaP. In USA, the disease is 66% more common and twice as likely to be fatal among African-American than Caucasian. Japanese and Chinese men have lower rates of CaP (Giovannucci, 2001).

Familial predisposition to development of prostate cancer has been observed since 1956 (Gonzalgo and Isaacs, 2003). Men with a first degree relatives affected by prostate cancer are at higher risk of developing the disease than those without such a family history. Familial prostate cancer studies have found that patients have 3 fold excess risk of developing CaP if their first or second degree relatives have CaP. The risk tends to increase with increased numbers of relatives with CaP (Carter *et al.*, 1992) and inherited CaP tends to develop at an earlier age compared to sporadic CaP (Bova *et al.*, 1998). A cohort twin study of CaP risk among 44,788 pair from Sweden, Denmark and Finland has indicated that more than 40% of CaP cases were attributed to inheritance and that highly penetrant susceptibility genes account for only 10% of CaP cases (Lichtenstein *et al.*, 2000). Hereditary CaP does not differ clinically from disease that arises sporadically. Genome wide linkage analysis in familial prostate cancer cases has led to identification of CaP susceptibility loci. So far, seven susceptibility loci associated with prostate cancer have been identified. This suggests that the predisposition factors to prostate cancer are heterogeneous and may involve multiple genes and variable phenotypic expression. Three susceptibility loci on chromosome 1 have been identified; 1q24-25 hereditary prostate cancer 1 (HPC1) (Smith *et al.*, 1996); 1q42.2-43, predisposing for prostate cancer (PCaP) (Berthon *et al.*, 1998) and 1q36; cancer of prostate and brain (CaPB) (Gibbs *et al.*, 1999). Other loci have been identified on chromosome 16 (16q23.2) (Suarez *et al.*, 2000), chromosome 17 (17p or, hereditary prostate cancer 2, HPC2) (Tavtigian *et al.*, 2001), chromosome 20 (20q13 or HPC20) (Berry *et al.*, 2000) and chromosome X (xq27-28 or HPCX) (Xu *et al.*, 1998). Putative susceptibility genes in these regions have rarely been identified. Demonstrating linkage of currently known candidate genes have proven problematic (Simard *et al.*, 2002).

The familiar clustering may also occur because of polymorphism in genes that are important for prostate development and function. Polymorphism involving genes coding for AR and 5 α-reductase type II (SRD5A2) have been associated with increased risk of developing CaP. Different allele length of repetitive elements or substitution polymorphism can modulate susceptibility to CaP. AR gene is located on chromosome Xq11.2. The gene product is composed of three domains, N-terminus (transactivation) domain, DNA binding domain (DBD) and C-terminus (ligand binding) domain. The transactivation domain encoded by exon 1 has nucleotide repeat

variants (CAG) with different transcriptional activities. The CAG sequence varies in length from 11-31 repeats in healthy men. There is an inverse relationship between the number of CAG repeats and the transcriptional activity of AR. Differences in prostate cancer risk among different ethnic groups may be related to polymorphic variability of the *AR* gene CAG repeats. Interestingly, Black American men have the highest CaP incidence and mortality rates in USA and they are also found to have shorter CAG repeat length in *AR* compared to other ethnic groups (Chang *et al.*, 2002). SRD5A2 gene is another polymorphic gene mapped to chromosome 2 locus. Substitution polymorphisms in SRD5A2 gene, A49T and V89L have been reported to confer increased risk of CaP. These genetic polymorphisms may affect the catalytic activity of the enzyme, thus increasing the likelihood of neoplastic transformation. (Makridakis *et al.*, 1999, Nam *et al.*, 2001).

Several supporting evidences indicate that sex hormones can initiate or promote development of CaP (Wilding, 1995). Growth and maintenance of the normal prostate epithelium depend on both circulating testosterone and its conversion to DHT within the prostate. Prolonged administration of high levels of testosterone induces prostate cancer in rats (Noble, 1977). Androgen removal induces tumour regression. Men who underwent castration before puberty and those with congenital abnormalities in androgen metabolism do not develop prostate cancer (Haas and Sakr, 1997). Studies have demonstrated that high circulating levels of testosterone, low oestrogen levels and low levels of sex hormones binding globulin (SHBG) may increase the risk of developing CaP (Gann *et al.*, 1996). SHBG binds to circulating testosterone thereby decreasing its bioavailability.

Other risk factors such as vasectomy, sexual activity, smoking, alcohol consumption, physical activities and social class have not been reliably shown to affect prostate cancer risk.

1.1.4 Prostate carcinogenesis

Prostate cancer development (carcinogenesis or tumourigenesis) as for other types of cancer is a multi-step process that is caused by multiple genetic and epigenetic alterations in genes involved in the regulation of diverse cellular biological processes

including proliferation, division, apoptosis, differentiation, senescence, DNA repair, invasion, metastasis, motility and adhesion. Transformation of normal cells into malignant derivates requires cellular acquisition of six capabilities; self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, limitless replication potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Several lines of evidences support the cancer multi-step process. Histopathological analyses of a number of human cancers in different organs have revealed multiple stages of tumour development such as dysplasia and carcinoma *in situ* (Foulds, 1958) and some of these lesions appear to represent an intermediate step in a process through which cells evolve progressively from a normal state via a series of pre-malignant lesions into invasive cancer. Hence, accumulation of genetic changes during the multi-step process confers progressive growth advantages that result in invasive cancer (Nowell, 1976). In the initial stages of prostate cancer, the tumour is localised within the prostate capsule and as it acquires more aggressive characteristics, the cancer advances locally and invades adjacent structures including the seminal vesicles, followed by distant metastasis (mainly affecting bone). This transition to metastasis is followed by a shift from androgen dependence to androgen independence, which is induced by androgen deprivation therapy (Figure 1.8).

Figure 1.8. Prostate carcinogenesis model

Prostate cancer development and progression. PIN is considered to be the earliest precursor for prostate cancer formation. In the initial stages of CaP, the tumour is localised within the prostate. Upon acquiring further genetic alterations, the tumours invade adjacent organs and metastasise to distant sites. The transition to metastasis is followed by shift from androgen dependent to androgen independent.

1.1.5 Chromosomal, genetic and epigenetic changes in CaP

Accumulation of multiple chromosomal genetic and epigenetic alterations are associated with prostate cancer development. Chromosomal aberrations that are frequently linked to CaP are ploidy, aneuploidy, duplication or deletion of whole or part of chromosome. The chromosomal regions most commonly lost in prostate cancer are 6q, 8p, 10q, 13q, 16q and 18q (DeMarzo *et al.*, 2003, Elo and Visakorpi, 2001, Zenklusen *et al.*, 1994)(Table 1.2 and Table 1.3). Chromosomal gains at 7p, 7q, 8q and Xq are common changes linked to hormone-refractory and metastatic prostate cancer (Alcaraz *et al.*, 1994).

Chromosomal loss	Chromosomal gain
8p	**8q**
6q	**7 gain**
7q	**X gain**
9p	**11p**
10q	**199**
12p	**3q**
13q	**2p**
16q	**Y gain**
17p	
18q	
Y loss	

Table 1.2. Chromosomal alterations associated with prostate carcinogenesis

Chromosomal abnormalities that are frequently linked to prostate cancer development (Ozen and Pathak, 2000).

Epigenetic events can affect gene expression without altering DNA sequence and this may occur through DNA methylation. Many gene promoters harbour CpG rich region of DNA knowing as CpG islands. Abnormal methylations of CpG islands located within gene promoters are associated with suppressed transcription activity and have been implicated in many of tumour types (Jones, 2002). Gene prometers that have been found to be methylated in CaP are glutathione S-transferase protein (GTS), RAS associated domain family protein 1 isoform A (RASSF1A), ENDRB encoding endothelin receptor type B (Nelson *et al.*, 1997) and CD44, a cell adhesion molecule (Lou *et al.*, 1999).

Chromosome	Locus	Gene	Function	Mechanism of activation and inactivation
Chromosome 1	1q24-25	HPC1(RNAseL)	RNAseL inhibitor	Mutation
Chromosome 3	3p21	RASSF1A	Tumour suppressor gene	Hypermethylation
Chromosome 4	4q26-27	bFGF	Growth	Over-expression
	4q25	EGF	Growth	Over-expression
Chromosome 5	5p13	AMACR	Lipid metabolism	Over-expression
Chromosome 6				
6q loss	6q15-22			
	6q23-24	BMP6	induces cell growth	Over-expressed
6p				
	6p12	VEGF	Angiogenesis	Over-expression
Chromosome 7				
7 aneousomy	7q21-15			
	7q21			
	7q31	MET	Tyrosine kinase receptor	Over-expression
		Caveolin 1	Metastatic suppressor gene	Hypermethylationa
Chromosome 8				
8p loss	8p21	NKX3.1	Transcription factor	Deletion
	8p22	FEZ1	Tumour suppressor gene	Mutations
		MSR1	Macrophage scavenger receptor	Mutations
8q gain	8q21			
	8q23-4	C-MYC	Nuclear transcription factor	Amplification
		EIF3S3	Translation initiation factor	Amplification and over-expression
		PSCA	Prostate stem cell antigen	overexpresed
Chromosome 9	9q21	P16	Cell cycle regulator	Deletion and hypermethylation
Chromosome 10				
10q loss	10q23-24	PTEN	Lipid phosphatase	Mutations
	10q24	MXI-1	Transcriptional repressor	Mutations
	10q26	FGF8	Growth	Over-expressed
10p				
	10p	KLF6	Cell proliferation	Mutation
Chromosome 11				
Epigenetic	11q13	GSTP1	Detoxifying enzyme	Hypermethylated
	11p11	KAI1	Metastatic suppressor gene	Down regulated
	11p13	CD44	Cell adhesion molecules	Hypermethylated
Chromosome 12				
	12q12-13	P27	Cell cycle regulator	Hypermethlyation and deletion
	12q22-23	EGF-1	Growth	Over-expression
Chromosome 13				
13q loss	13q14	RB1	Tumour suppressor gene	Hypermethylation and deletion
	13q21-22	EDNRB	Endothelin receptor type B	Hypermethylated
	13q33			
	13q12.3	BRCA2	Transcription factor	Mutation
Chromosome 16				
16q loss	1622.1	E-cadherin	Adhesion molecule	Hyeprmethylation
	16q23-24			
Chromosome 17				
17q loss	17q21	BRCA1	Transcription regulator	Mutation
	17p11.2	HPC2	Prostate cancer susceptibility gene	Mutations
		P53	Nuclear transcription factor	Mutations
Chromosome 18	18q21	Bcl-2	Apoptosis	Over-expression
Chromosome 19	19q13.2	TGF-β	Growth regulator	Over-expressed
Chromosome X				
Xq12-13 gain	Xq12-13	AR	Transcription factor	Over-expressed Amplification Mutations
Chromosome Y				
	11p	TSPY	Growth regulator	Over-expressed

Table 1.3. Chromosomal loci linked to prostate cancer.

1.1.6 Genes associated with CaP

Cellular transformation from normal to malignant phenotype is caused by an accumulation of somatic and inherited genetic changes in two major classes of cellular genes: dominantly acting growth inducing proto-oncogenes and growth inhibitor tumour suppressor genes (TSGs). Proto-oncogenes are defined as a family of genes that under normal circumstances encode for proteins that critically regulate cellular growth. When they are mutated or activated, they can promote carcinogenesis (Stedman, 2000). Mechanisms of oncogene activation include activating mutations that cause constitutive functional protein product (e.g., RAS), gene deletion resulting in a truncated protein that is constantly active (e.g., truncated tyrosine kinase receptor lacking ligand binding domain), abnormal over-expression of oncogene transcript or protein due to gene amplification or gene translocation to hyperactive genomic loci. Proto-oncogenes usually regulate broad biological processes such as growth factor receptor signalling, intracellular signal transduction and transcription. The oncogenes are relatively easy identified by their ability to transform immortalised cells such as the mouse NIH3T3 cell line to grow in soft agar, loss of contact inhibition, serum independent growth and *in vivo* tumour formation (Mitchell, 1991).

Anti-oncogenes, more commonly called tumour suppressor genes (TSGs), promotes multiple physiological functions and suppress cellular transformation. There is no distinct definition of TSGs, but they share common characteristics; inactivation mutation and/or inherited mutation contribute towards carcinogenesis while normal functional TSG suppresses *in vitro* cellular growth (Haber and Harlow, 1997). Evidence of existence of TSGs arise from somatic cell hybridization which showed that fusion of tumour cells with normal cell results in non-tumuorigenic hybrids (Sager, 1988). This indicates that tumour cells have acquired genetic sequence(s) that are capable of suppressing neoplastic phenotype. A second clue was provided by Knudson's work (Knudson, 1971). He proposed the two hits theory: in familial cancer, a germline mutation of one allele is inherited and a second mutation is acquired via somatic mutation. In sporadic cancer, both alleles are required to be somatically mutated. Loss of TSG function can result from inactivating point mutation, gene deletion, loss of expression due to promoter hyper-methylation and insufficient level

of gene product caused by presence of only one functional copy of TSG (haploinsufficiency).

In addition to oncogenes and TSGs, other classes of genes are found to play roles in prostate carcinogenesis including DNA repair gene, telomerase, angiogenic genes, detoxification genes and metastasis suppressor genes (adhesion molecules) (Table 1.4).

Genes associated with CaP	
Ki-67	Metastasis suppressor genes
PCNA	**KA1**
Cyclin dependent kinase inhibitors	**Nm23**
P27	Immortalization
P16	**Telomerase**
P21	Growth factors
Tumour suppressor genes	**FGF, α, β and 8**
RB1	**TGF-α**
P53	**KGF**
PTEN	**TGF-β1**
NKX3.1	**BMP-6**
Apoptosis	Growth factor receptor
Bcl-2	**EGF-R**
Angiogenesis	**P180-C-ErbB3/C-erb-3**
Hif-1	**C-met**
D31, CD34	**Prolactin receptor**
VEGF	Growth factor binding proteins
Adhesion	**IGFBP-2**
E-cadherin	**IGFBP-3**
CD44	Nuclear receptors
PSCA	**AR**
PSMA	**Estrogen receptor-β**
α-catenine	Carcinogen detoxification
C-CAM	**GSTP1**
Ep-CAM	Intermediate filaments
Cyclooxygenases	**Cytokeratin 5, 14**
COX-2	
Oncogenes	
C-MYC	
ERBB2/ HER-2/Neu	

Table 1.4. Genes involved in prostate cancer development.

Genes that are linked to prostate cancer development. They play diverse roles in normal cellular processes and their alterations lead to carcinogenesis (Nelson *et al.*, 2001b).

1.1.6.1 Role of oncogenes in CaP

Amplification and over-expression of several oncogenes have been found to be associated with CaP including growth factors, C-myc, EIF3S3 and MET1. Several growth factor families have been implicated in normal prostate growth and in prostate carcinogenesis. In general, growth factors regulate diverse cellular processes, including proliferation, motility, differentiation and apoptosis. The main growth factor families involved in the prostate are Insulin Growth Factor (IGF) family, Epidermal Growth Factor (EGF), Transforming Growth Factor-α (TGF-α), Fibroblast Growth Factor (FGF) family and Vascular Endothelial Growth Factors (VEGF). These growth signalling systems can exert inhibitory and inductive influence on prostate growth, in paracrine and /or autocrine manner affecting stromal and epithelial cells.

The family of Fibroblast Growth Factors (FGF) consists of structurally related peptides (Benharroch and Birnbaum, 1990). bFGF gene located on 4q26-27 and KGF located on 15q15-q21.1 are members of FGF family. These peptide growth factors ligand bind to FGF receptors (1-4) (Partanen *et al.*, 1991) and exert their cellular function and play a role in normal and malignant prostate development (Hellawell and Brewster, 2002). Elevated levels of bFGF have been reported in prostate cancer (Cronauer *et al.*, 1997). FGF8, alternatively referred to as androgen induced growth factor, is another member of FGF family located at 10q24 and has been found to be over-expressed in prostate cancer (Leung *et al.*, 1996). FGF8 increased expression correlated with increased Gleason score and pathological stage of CaP (Dorkin *et al.*, 1999). Other members of growth factor family are Epidermal Growth Factor (EGF) and Transforming Growth Factor- α (TGF- α). EGF and TGF- α are structurally similar and bind to EGFR and modulate its function (Carpenter and Cohen, 1979, Ullrich *et al.*, 1984). EGF is located at 4q25. Over-expression of EGF, TGF-α and EGFR have been detected in prostate cancer (Morris and Dodd, 1990, Turkeri *et al.*, 1994, Yang *et al.*, 1993).

ERBB2 (HER-2/neu) is another oncogene and a member of the epidermal growth factor receptor (EGFRs) family located at 17q11.2-q12. Neu is a tyrosine kinase receptor that has been found to activate androgen receptor signalling pathways in the presence of low levels of androgens (Craft *et al.*, 1999, Yeh *et al.*, 1999). This implicates ERBB2 as potentially important in the development of androgen-

independent prostate cancer. Furthermore, ERBB2 is over-expressed in prostate cancer (Gu *et al.*, 1996, Myers *et al.*, 1994, Signoretti *et al.*, 2000) and the expression level correlates with advanced disease stages and high Gleason scores (Sadasivan *et al.*, 1993). Amplification of ERBB2 gene has also been reported in prostate cancer (Ross et al., 1997).

Other oncogenes linked to CaP are Insulin Growth Factor I and II. They are another member of growth families. IGF I and II exert their effects by binding to their receptors to modulating cellular growth, division, adhesion, motility and apoptosis (Macaulay, 1992). Regional loss of imprinting of the IGF-II gene has been observed in prostate cancer (Jarrard *et al.*, 1995). A link between high plasma IGF-I level and high risk of developing prostate cancer has been observed (Chan *et al.*, 1998a, Wolk *et al.*, 1998). Up-regulation of IGF1R level has been reported in resected prostate tumour (Kurek *et al.*, 2000).

TGF-β is a superfamily of proteins that contains 5 isoforms (TGFβ 1-5) *(Derynck et al.*, 1985). The gene is located at 19q13.2. These ligands bind to 3 different receptors, TβR1, TβRII and TβRIII. TGF-β plays an important role in regulating prostate cellular growth and can inhibit cellular proliferation as well as induce apoptosis in prostate epithelial cells (Martikainen *et al.*, 1990). Loss of both TβR I and TβRII receptors correlates with prostate tumour stage, survival and recurrence rate (Kim *et al.*, 1998). Bone morphogenic proteins (BMPs) are secreted signalling molecules that are member of TGF-β superfamily. BMPs represent almost one third of the TGF-β superfamily with more than 30 members already described (Ducy and Karsenty, 2000). They play a role in regulating cellular function including proliferation, differentiation, apoptosis, morphogenesis and organogenesis (Graff, 1997). One member of BMP family, BMP-6 gene, located at 6p23-24, has been found to be implicated in prostate cancer metastasis to the bone. BMP-6 over-expression appears to be restricted to tumours showing evidence of bony metastasis with no expression detected in organ confined prostate cancer. BMP-6 expression also correlates with increased recurrence states and decreased survival (Thomas and Hamdy, 2000).

Other member of the growth family is Vascular Endothelial Growth Factor (VEGF). VEGF is a glycoprotein located on 6p12 and exists in at least 5 isoforms (Ferrara and

Davis-Smyth, 1997). They mediate their effects via tyrosine kinase receptors located in the cytoplasmic membrane of endothelial cells. VEGF plays a role in prostate growth and deregulated expression can induce angiogenesis and permit tumour growth. Increased VEGF expression has been reported to correlate with increased stage and Gleason score in CaP (Jackson *et al.*, 1997, West *et al.*, 2001).

One of the frequently genetically altered oncogene in CaP is C-MYC. The gene encodes a transcription factor that plays a significant role in regulating various cellular processes such as growth, proliferation, differentiation and apoptosis. The c-MYC oncogene is located at chromosomal band 8q24 which is one of the most frequently amplified regions in advanced prostate cancer (Elo and Visakorpi, 2001). C-MYC gene has been found to be amplified in 8% of primary prostate carcinoma and in 11-35% of advanced prostate tumours (Bubendorf *et al.*, 1999, Jenkins *et al.*, 1997, Nupponen *et al.*, 1998, Reiter *et al.*, 2000). Amplification of MYC gene has been shown to correlate with over-expression of C-MYC gene product as well as tumour grade and poor prognosis (Sato *et al.*, 1999). *In vitro* and *in vivo* functional studies of c-MYC have demonstrated that abnormal MCY expression can contribute to prostate cancer. *In vitro* knock out of c-MYC by anti-sense technique causes anti-proliferative effect in prostate cancer cell lines (Balaji *et al.*, 1997). Transgenic mice over expressing c-MYC develop prostate epithelial abnormalities similar to low-grade PIN lesions in human (Zhang *et al.*, 2000).

Another oncogene that inhibits apoptosis and has been implicated in CaP is Bcl-2. The gene is located at 18q21.3 and encodes an integral outer mitochondrial membrane protein. Bcl-2 is expressed only in basal cells in normal prostate epithelium (McDonnell *et al.*, 1992). High level expression of Bcl-2 has been detected in prostate cancer especially after androgen withdrawal and in hormone-refractory tumours (McDonnell *et al.*, 1997, Stattin *et al.*, 1996). It has been demonstrated that Bcl-2 protects prostate cells against apoptosis and increases their tumourigenicity (Raffo *et al.*, 1995). Inhibition of Bcl-2 expression has been found to trigger apoptosis in prostate cancer cells (Dorai *et al.*, 1999) and delay the progression of hormone-refractory tumour after castration in the xenograft mouse model (Miyake *et al.*, 1999).

Other putative candidate oncogenes that is found to be linked to CaP are EIF3S3 and MET. EIF3S3 is a subunit of a translation initiation factor eIF3. EIF3S3 is located at 8q23 and is amplified in 30% of hormone-refractory prostate cancers and in 50% of metastatic lesions. EIF3S3 amplification has been shown to be associated with high Gleason score (Saramaki *et al.*, 2001). MET is located at chromosome 7 (7q31) and encodes a tyrosine kinase receptor for hepatocyte growth factor (HGF). MET over-expression at protein and mRNA levels have been detected in high grade tumours and metastases (Humphrey *et al.*, 1995, Knudsen *et al.*, 2002, Pisters *et al.*, 1995).

1.1.6.2 Role of TSGs in CaP

Loss of functions of several tumour suppressor genes have been detected in CaP. Ras Associated Domain Family Protein 1 Isoform A (RASSF1A) is a candidate TSG located on chromosome 3p21. LOH of this region and silencing of the remaining RASSF1A allele is a frequent event in prostate tumourigenesis. The RASSF1A promoter is hypomethylated in normal prostate tissue, whereas RASSF1A hypermethylation has been reported to occur in 60-70% of CaP (Kuzmin *et al.*, 2002). RASS1FA methylation is more frequent in high grade prostate cancer (Liu *et al.*, 2002). The gene is a candidate tumour suppressor gene and has been found to induce cell cycle arrest (Mathe, 2004).

NKX3.1 is a putative prostate specific tumour suppressor gene located on 8p2 and encodes a prostate specific homobox protein essential for normal development of prostate (Sciavolino *et al.*, 1997). NKX3.1 gene product binds DNA and represses expression of PSA gene (Chen *et al.*, 2002). Neither mutations nor loss of expression of NKX3.1 have been detected in CaP (Voeller *et al.*, 1997, Xu *et al.*, 2000). In animal models, NKX3.1 expression is detected in early stages of murine prostate development as well as in all subsequent stages of prostate epithelial differentiation. NKX3.1 knock-out mice have defects in prostate ductal morphogenesis and secretory protein production. PIN and dysplasia were also detected and increased in severity with age (Bhatia-Gaur *et al.*, 1999). Conditional loss of NKX3.1 in adult mice has been shown to induce PIN lesions, thus supporting the role of NKX3.1 in prostate carcinogenesis (Abdulkadir *et al.*, 2002)..

PTEN (phosphatase and tensin homologue) is another tumour suppressor gene located on chromosome 10q23. PTEN encodes 403 aa protein that shows homology to the protein tyrosine phosphatase family and to two cytoskeleton proteins, tensin and auxilin. The gene product has phosphatase activity for proteins and lipids. PTEN can regulate G2 cell cycle arrest via negative regulation of PI3K-Atk signalling pathway and is frequently mutated and deleted in prostate cancer cell lines and tumours (Li *et al.*, 1997, Ramaswamy *et al.*, 1999). Loss of PTEN expression correlates with high Gleason score and advanced stage (McMenamin *et al.*, 1999). In the PTEN knockout mice model, PTEN heterozygous (+/-) mice develop PIN in 50% of cases indicating that haploinsufficiency of PTEN prostate carcinogenesis (Kwabi-Addo *et al.*, 2001).

Another putative tumour suppressor gene is Kruppel like factor 6 (KLF6), located on chromosome 10p15 and encodes a Zinc finger transcription factor that has been implicated in CaP carcinogenesis. Approximately, 77% LOH has been detected in primary prostate tumours. KLF9 expression in culture cells decreased cell proliferation and increased p21 expression (Narla *et al.*, 2001).

The tumour suppressor gene CDKN1B is located on chromosome 12p12, a region frequently deleted in advanced CaP (Kibel *et al.*, 1998). The gene encodes for a cyclin dependent kinase inhibitor. The p27 protein regulates cell cycle progression from G1 to S phase via inhibition of the cyclin E/cdk2 complex. P27 inactivation occur through aberrant phosphorylation and/or ubiquitination resulting in loss of expression or altered sub-cellular localisation (Loda *et al.*, 1997). Decreased expression of P27 is common in early prostate cancer and is associated with increased risk of disease recurrence in patients following radical prostatectomy (Cote *et al.*, 1998). P27 expression level is affected by PTEN, which negatively regulates PI3K-Akt signalling pathway (Graff *et al.*, 2000). Thus, decreased levels of p27 can be a consequence of PTEN loss.

The RB1 tumour suppressor gene is located on chromosome 13q. Loss of this region occurs in at least 50% of prostate cancers (Cooney *et al.*, 1996, Melamed *et al.*, 1997). Mutation of Rb1 gene and loss of expression have been reported in clinically localised as well as more advanced prostate carcinoma (Bookstein *et al.*, 1990, Cunningham *et*

al., 1996). RB1 encodes a nuclear phosphoprotein that plays a key role in negative regulation of cell proliferation during G-S phase transition (Weinberg, 1995).

Another cell cycle regulator is P16 TSG located at 9q21. Most studies have reported that p16 is rarely mutated in primary prostate carcinoma, but mutated in advanced metastatic disease (Chen *et al.*, 1996, Tamimi *et al.*, 1996). It has been suggested that in primary tumour, inactivation of p16 may occur by deletion of one allele, combined with promoter methylation of the remaining allele (Jarrard *et al.*, 1997).

P53 tumour suppressor gene located on 17p13.1, is involved in regulating multiple cellular biological processes. Loss of 17p has been detected in 50% of metastatic prostate cancers and 65% of androgen refractory cancers (Cher *et al.*, 1994, Saric *et al.*, 1999). Mutations in P53 occur infrequently in early invasive carcinoma (Voeller *et al.*, 1994). In contrast, p53 mutations are detected in advanced stage of CaP as well as recurrent metastasis disease (Bookstein *et al.*, 1993, Eastham *et al.*, 1995). P53 encodes 53KDa nuclear phosphoprotein that regulates the cell cycle and induces growth arrest at the G-S1 phase (Vogelstein and Kinzler, 1992). In addition, the gene regulates differentiation, senescence and maintains genomic stability (Aloni-Grinstein *et al.*, 1995, Atadja *et al.*, 1995).

1.1.6.3 Other genes

In addition to the role of oncogenes and tumour suppressor genes in prostate carcinogenesis, other genes that regulate normal cellular processes such as adhesion, detoxification and maintaining telomeres length have been implicated in CaP. Several genes involved in invasion and metastasis of cancerous cells have been characterised and are called metastasis suppressor genes. These genes do not affect the cell growth of primary tumour, but can inhibit development of distant metastasis. E-cadherin metastasis tumour suppressor gene is a member of the Cadherin transmembrane protein family that regulates cell adhesion (Hajra and Fearon, 2002). Although no mutations have been detected in E-cadherin gene so far (Otto *et al.*, 1993, Umbas *et al.*, 1994, Umbas *et al.*, 1992), decreased expression of E-cadherin was detected in prostate cancer and it is associated with high grade disease (Umbas *et al.*, 1994).

Furthermore, E-cadherin has been shown to play a role in the suppression of invasion and metastasis of prostate cancer (Bryden *et al.*, 2002). Other member of the cadherin signalling pathway such as α-catenin are also found to be altered in CaP. DC44 is another metastatic tumour suppressor gene and is down-regulated in high grade and metastatic prostate cancer (Gao *et al.*, 1997).

Another gene that has been frequently found to be altered in CaP is Glutathione-S-Transferase isoform Pi (GSTP1). The gene belongs to a super-family of enzymes responsible for detoxifying environmental electrophilic carcinogens and oxidants, preventing oxidant and electrophilic DNA damage which may cause mutations (Nelson *et al.*, 2001a). The gene is located at 11q13. In normal prostate epithelium, GSTP1 is expressed in basal cells but not in the columnar secretory cells. Hypermethylation of the regulatory region of GSTP1 locus is the most common somatic genetic alteration reported in prostate cancer and represents an early event in neoplastic transformation. GSTP1 methylation occurs in approximately 70% of PIN lesions and in more than 90% of prostate carcinomas, but not in normal or BPH tissues (Lee *et al.*, 1994).

Telomere sequences located at the tips of chromosomes contain hundreds of tandem sequences of which 50-200 nucleotides are shortened at each cell division. In contrast, cancer cells do not show loss of telomere length during cell division. Telomerase is a ribonucleoprotein enzyme that functions to maintain the telomere length. The protein consists of two components: an RNA moiety encoded by gene located at 3q26 and a catalytic subunit (TERT, reverse transcriptase) located at 5p15.33. Telomerase is associated with cellular immortalization and cancer. In normal cells the activity of telomerase is barely detectable. However, malignant tumour cells often have detectable activity of telomerase. Telomerase activity has been detected in 84% of prostate cancers (Sommerfeld *et al.*, 1996). This suggests that telomerase plays an important role in the development of prostate cancer.

1.1.7 Y chromosome abnormalities in CaP

Y chromosome abnormalities are one of many cytogenetic abnormalities reported in human prostate cancers. However, the frequencies of Y chromosomal loss and/or gain

vary. Loss of Y chromosome is among one of the most common cytogenetic abnormalities detected in prostate cancer (Arps *et al.*, 1993, Brothman *et al.*, 1991, Lundgren *et al.*, 1992, Micale *et al.*, 1992, Webb *et al.*, 1996, Zitzelsberger *et al.*, 1996). Loss of Y chromosome was detected in 10-50% of prostate cancers (Alers *et al.*, 1998, Alers *et al.*, 1997, Alers *et al.*, 2000, Breitkreuz *et al.*, 1993, Brothman *et al.*, 1999, Jordan *et al.*, 2001, Konig *et al.*, 1996, Lundgren *et al.*, 1992, Takahashi *et al.*, 1996, Zitzelsberger *et al.*, 1996). Interestingly, loss of Y chromosome was detected in 89% of hormone-refractory prostate cancers (Haapala *et al.*, 2001) and Y aneuosomy was closely associated with metastatic prostate cancer (Takahashi *et al.*, 1996). Y chromosome gain has been detected in 5-30% of prostate cancer (Baretton *et al.*, 1994, Brothman, 1997, Brothman *et al.*, 1999, Brown *et al.*, 1994, Cher *et al.*, 1994, Konig *et al.*, 1996, Qian *et al.*, 1997, Visakorpi *et al.*, 1994) and is also associated with poor prognosis and less favourable survival outcome (Takahashi *et al.*, 1996).

1.1.7.1 The Structure of Y chromosome

The Y chromosome is the smallest chromosome in the human genome. It is 63 mb in length and represents only 2-3% of the haploid genome. Early chromosome banding studies have identified different regions: pseudoautosomal parts and non-recombining region (NRG), also called male specific Y chromosome (MSY). The pseudoautosomal regions (PARs) constitute 5% of the total Y chromosome length and are divided into two separate distal parts, PAR1 and PAR2. PAR1 is located at the terminal region of the short Y chromosome (Yp) arm and is 2.6 Mb in length. PAR2 is located at the tip of the long arm (Yq) and is 420 kb in length. The PAR regions on the Y chromosome pair and exchange genetic material with the PAR region on the X-chromosome during meiosis. PAR1 harbours 10 genes that function in different pathways including bone growth, melatonin synthesis, cytokine signalling and cell surface antigens. PAR2 contains only two genes.

MYS region constitute 95% of the Y chromosome and is made up of heterochromatin and euchromatin. Approximately, two thirds of MYS region consists of heterochromatin. The rest constitutes euchromatin region. The heterochromatin region comprises distal Yq. This part of the Y chromosome is considered to be genetically inert and polymorphic in length in different male populations. It is composed of

mainly highly repetitive sequences (Quintana-Murci and Fellous, 2001). The euchromatin region consists of the short arm of the paracentromeric region, the centromere and the paracenteric long arm. The euchromatin region is divided into 3 classes: X-transposed, X-degenerate and ampliconic. These classes constitute 156 known transcription units including 78 protein-coding genes which collectively encode for 27 distinct proteins. The X-transposed sequences show 99% identity to the X chromosome. The X-degenerate sequences are remnants of the ancient autosomes from which X and Y chromosome originated. The ampliconic class includes large regions (30% of MYS euchromatin) where sequence pairs show greater than 99.9% identity which is maintained by frequent non-reciprocal recombination (gene conversion) (Rozen *et al.*, 2003, Skaletsky *et al.*, 2003). This region contains nine families of genes encoded for Y specific proteins, the copy number of these genes ranges from 2 to 35 copies (Hawley, 2003) (Table 1.5).

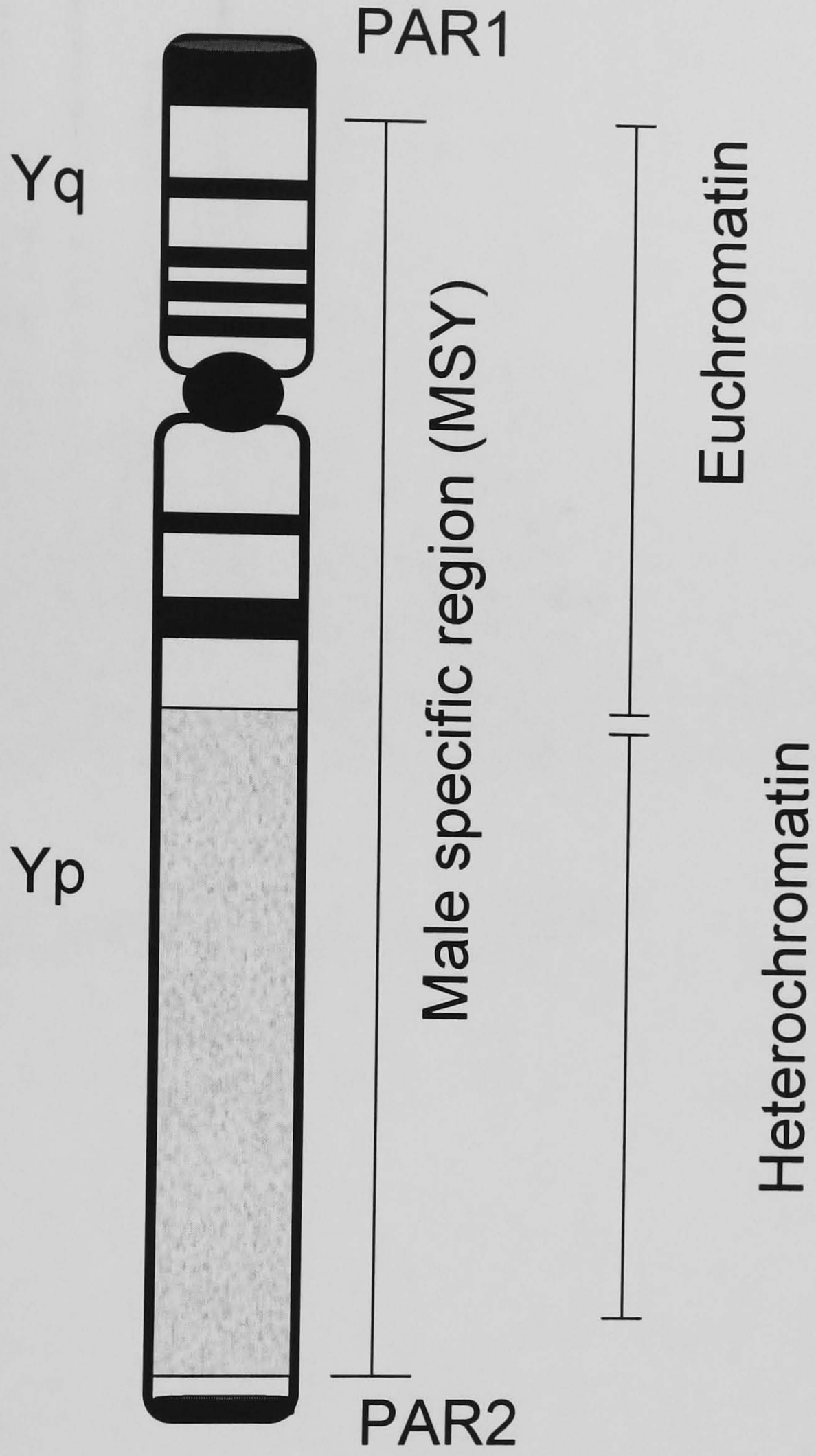


Figure 1.9. Y chromosome structure

Schematic representation showing the structure of Y-chromosome. The Y chromosome is 63 Mb in length and is divided into two pseudoautosomal regions (PAR1 and PAR2) and male specific region (MSY). PAR1 and PAR 2 region each contains 10 genes and 2 genes, respectively. The MSY part consists of Euchromatin and heterochromatin regions. Heterochromatin region is inert part. The genes within the euchromatin region are categorised into three classes: X-transposed, X-degenerated and ampliconic.

Gene symbol	Gene Name	Copy number	Map	Gene Function
PAR1				
PGPL	**Pseudoautosomal GTP-binding protein-like**	**1**	**Yp11.31**	**Encodes 445 aa and have motifs for GTP binding protein domain**
SHOY/PHOG	**Short stature homeo-box/pseudoautosomal homeo-box-containing osteogenic gene**	**1**	**Ypter-p11.2**	**Involved in short stature of Turner syndrome**
XE7	**XE7.**	**1**	**Ypter-p11.2**	**DNA segment on X and Y chromosome, 155 expressed sequence**
CSFR2A	**GM-CSF receptor 2, α subunit**	**1**	**Yp11.3**	**A cytokine that regulates productions, differentiation and function of granulocytes and macrophages,**
IL3RA	**Interleukin 3 receptor α-subunit**	**1**	**Yp11.3**	**Over-expressed in leukaemia and a marker for leukaemia stem cells**
SLC25A6 (ANT3Y)	**Mitochondrial solute carrier family 25**	**1**	**Yp**	**ADP/ATP translocase**
ASMT	**Acetylserotonin methyltransferase**	**1**	**Yp11.3**	**Enzyme catalyses the synthesis of melatonin, a hormone that decrease body temperature and facilitate sleeping**
ASMTL	**ASMT-like**	**1**	**Yp11.3**	**Homologue of ASMT gene**
TRAMP	**TRAMP**	**1**	**Yp11**	**ACE-like transposable element, ALCE**
MIC2 (CD99)	**MIC2**	**1**	**Ypter-p11.2**	**Surface antigen detected by monoclonal antibody, involved in cellular adhesion**
X-transposed				
TGIF2LY	**TGFb induced factor 2 -like**	**1**	**Yp11.2**	**Member of TGIF homebox transcription factor family, involve in regulating transcription**
PCDH11Y	**Protocadherin 11 Y linked**	**1**	**Yp11.2**	**Member of proto-cadherin family that play role in cell adhesion**
X-degenerated				
RPS4Y	**Ribosomal protein S4 Y isoform**	**1**	**Yp11.3**	**Encodes ribosomal protein S4, a component of ribosomal S40 subunit.**
SRY	**Sex determining region Y**	**1**	**Yp11.3**	**Intronless gene that encodes transcription factor, initiate male sex determination**
ZFY	**Zinc finger Y**	**1**	**Yp11.32**	**Transcription factor**
PRKY	**Protein kinase Y**	**1**	**Yp11.2**	**Member of cAMP- serine/threonine protein kinase family**
AMELY	**Amelogenin Y**	**1**	**Yp11.2**	**Member of amelogenin extra cellular matrix protein family involved in biomineralization of tooth enamel development**
DFRY (US9PY)	**Drosophila fat facets related Y(Ubiquitin specific protease 9)**	**1**	**Yq11.2**	**Degrades specific ubiquitin**
DBY	**Dead box 3 Y**	**1**	**Yq11**	**Ecodes DEAD box protein that are putative RNA helicase**
UTY	**Ubiquitous TPR motif Y**	**1**	**Yq11**	**Encodes tetraicopeptide protein that is involved in protein-protein interactions**
NLGN4Y	**Neuroligin 4 Y**	**1**	**Yq11.221**	**Cell adhesion molecule at the synapses, expressed in prostate**
CYorf15A	**Chromosome Y open reading frame 15A**	**1**	**Yq11.222**	**Encodes protein of unknown function**
CYorf15B	**Chromosome Y open reading frame 15 B**	**1**	**Yq11.222**	**Encodes protein of unknown function**
TB4Y	**Thymosin β4 isoform**	**1**	**Yq11.221**	**Functions in actin squestration**
SMCY	**Selected mouse cDNA Y**	**1**	**Yq11.221**	**Human homologue of mouse encoding for minor histocompatibility H-Y antigen**
EIF1AY	**Eukaryotic translation initiation factor 1A**	**1**	**Yq11.222**	**Similar to Eukaryotic initiation factor**
Ampliconic				
TSPY	**Testis specific protein Y-encoded**	**M**		**Proliferation of spermatonia of testis**
PRY	**PTP-BL related Y**	**M**	**Yq11.223**	**Similar to tyrosine protein phosphatase PTPBL**
TTY1	**Testis transcript Y1**	**M**	**Yp11.2**	**Cytoplasmic protein of Unknown function**
TTY2	**Testis transcript Y2**	**M**	**Yp11.2**	**Cytoplasmic protein of unknown function**
BPY1 (VCY)	**Basic protein Y1**	**M**	**Yq11.22**	**Positively charged nuclear protein of unknown function**
BPY2	**Basic protein Y2**	**M**	**Yq11**	**Positively charged nuclear protein of unknown function**
CDY	**Chromodomain Y**	**M**	**Yq11.223**	**Expressed in testis and encodes protein with histone acetyltranferase catalytic domain**
XKRY	**XK related Y**	**M**	**Yq11.221**	**Similar to membrane transport protein, X linked Kell blood group precursors**
HSFY	**Heat shock transcription factor Y linked**	**M**	**Yq11.221**	**Regulation of transcription**
RBM	**RNA binding motif**	**M**	**Yq11.23**	**Expressed in testist and function in RNA processing**
DAZ	**Deleted in azoospermia**	**M**	**Yq11**	**Involved in speramtogenesis**
TB1L	**Transducin bet like 1 Y linked like**	**M**	**Yp11.2**	**Homologue to WD40 repeat-containing protein family that meditates protein-protein interactions**
PAR2				
IL9R	**Interleukin 9 receptor**	**1**	**Yq12**	**Growth factor for T cells**
SYBL1	**Synaptobrevin-like 1**	**1**	**Yq**	**Protein transport**

Table 1.5. Y chromosome genes

Description of functions, band location, copy number, symbols and abbreviations of all the genes located on Y-chromosome (Lau *et al.*, 2000, Skaletsky *et al.*, 2003)

1.1.7.2 Role of Y chromosome in diseases

Although the Y chromosome harbours limited number of genes, some of these genes have been found to be linked to sex determination, fertility and diseases. The human SRY gene mapped to the short arm of Y chromosome, in close proximity to the PAR1 boundary is responsible for male sex determination (Sinclair *et al.*, 1990). The gene consists of one exon, encoding for 204 amino acids protein and contains a conserved DNA binding domain (high mobility group, HMP). SRY is required for initiation, development and differentiation of testes. The role of Y-chromosome in spermatogenesis was suggested by the presence of gross cytogenetic deletions in patients with azoospermia (absence of mature spermatozoa in semen fluid). Azoospermia loci were postulated in Y chromosome and called AZF (Tiepolo and Zuffardi, 1976). Y chromosome play a role in male sex oncogenesis. It has been suggested that Y-chromosome harbours both oncogenes and TSGs, one example is the gonadoblastoma locus (GBY). Gonadoblastomas are rare gonadal neoplasms that are composed of aggregates of germ cells and sex cord elements (Scully, 1970). These neoplasms arise within the dysgenetic gonads of individuals who possess Y chromosome in their genome (Vergnaud *et al.*, 1986). The disease occurs in individuals who are phenotypically female known as sex-reversed females. The frequency of occurrence of gonadoblastoma in patients who have gonadal dysgenetic and Y chromosome exceeds 30% (Iezzoni *et al.*, 1997). In contrast to 46, XY sex-reversed females, 45, X females who have dysgenetic gonads do not develop gonadoblastoma. Page, postulated the existence of a GBY locus on the Y chromosome that predisposes the dysgenetic gonads of XY sex-reversed females to develop gonadoblastoma (Page, 1987). GBY candidate gene may act as an oncogene only in a dysgenetic gonad, while it may regulate spermatogenesis in the normal testis. Using a panel of DNA from sex-reversed and gonadoblastoma patients, the GBY locus was mapped to the short arm of Y chromosome and on the long arm (Disteche *et al.*, 1986, Magenis *et al.*, 1984, Petrovic *et al.*, 1992). A refined deletion map analysing material from gonadoblastoma patients localised GBY to a small region in the short arm of the Y chromosome (Vollrath *et al.*, 1992). This GBY critical region was estimated to be 1-2 Mb in size; within this, TSPY is thought to be the most likely candidate gene for GBY. There are multicopies of TSPY within the GBY critical regions.

1.1.8 Role of TSPY in male sex cancers

In the testes, TSPY transcript expression is detected during testes organogenesis and persists throughout adulthood. Immunohistological staining of normal testis for TSPY protein confirms its expression primarily in the cytoplasm of spermatogonial cells around the basal lamina of seminiferous tubules of the adult testis (Figure 1.10). TSPY expression was also detected in the nuclei of a relatively small proportion of spermatogonial cells. Hence, it has been hypothesised that TSPY regulates the normal proliferation of spermatogonia and marks the entry of the spermatogonia into meiotic differentiation. Expression analysis studies have shown that TSPY is over-expressed in spermatogonia at early stages of testicular cancer, in carcinoma *in situ* of the testis and as well as in seminomas. These results suggest that TSPY is aberrantly expressed in the tumours with germ line origins (Schnieders *et al.*, 1996). Further studies have shown that TSPY was also found to be over-expressed in sex-reversed patients with gonadoblastoma (Hildenbrand *et al.*, 1999). Recently, TSPY was found to be over-expressed in prostate clinical samples compared to BPH. Using *in situ* mRNA hybridization and immunostaining techniques, up-regulated expression of TSPY was detected in prostate cancer (Lau *et al.*, 2003).

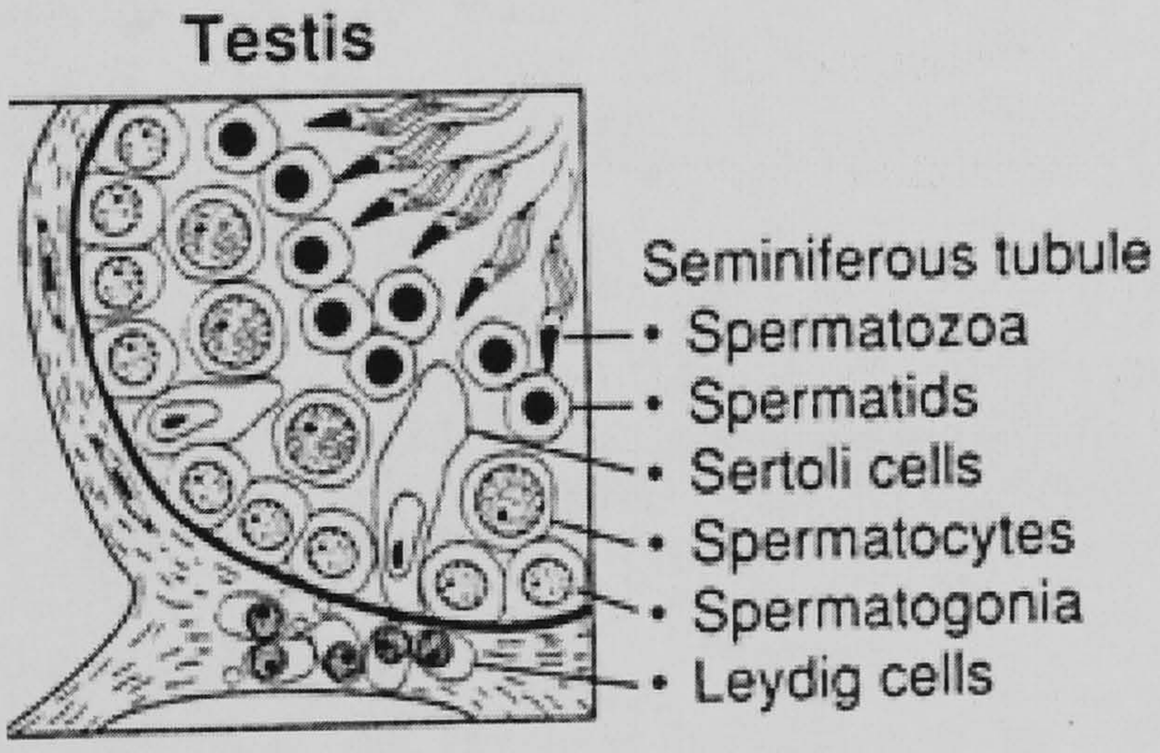


Figure 1.10. Cross section of testis seminiferous tubules

Sperms are generated from testicular spermatogonial cells. Spermatogonial cells divide to generate spermatogonia cells and cells that will differentiate to spermatocytes that eventually will generate mature sperm (Damjanov and McCue, 1996).

1.1.8.1 TSPY Expression Regulation

Molecular mechanism regulating TSPY expression in not yet known. However, several evidences are accumulating indicating several potential genes involved in regulating TSPY expression. Indirect evidence has demonstrated that TSPY can be potentially expressed in different organs and tissues. A transgenic mouse harbouring 1.3 Kb of 5' flanking region of human TSPY regulatory gene fused with simian virus 40 (SV40) large antigen Tag was generated and characterised. The Tag gene product under the control of TSPY regulatory region was expressed in variable tissues including spleen, seminal vesicle, pituitary gland, adrenal glands as well as testis (Tascou *et al.*, 2003). This indicates that the expression of TSPY may not be tissue specific. It has been found that TSPY transcript expression was up-regulated upon addition of increasing amount of androgen analogue R1881 to androgen receptor expressing prostate cancer cell line (LNCaP) that was cultured in medium depleted of hormones (Lau and Zhang, 2000). This observation was confirmed by semi-quantitative RT-PCR and Northern blot method, supporting a potential role for TSPY in regulating prostate growth and carcinogeneis. Recently, Li *et al* have found out that TSPY promoter was among promoter regions bound by C-MYC gene product. Thus, this provides evidence that C-MYC can regulate the transcription level of TSPY (Li *et al.*, 2003). Epigenetic changes are frequent phenomena that can modulate the expression level of transcript through methylation of CpG islands that are common in the promoter region of a gene. Hypermethylation of these islands can suppress normal transcription of the gene and block its expression. The role of methylation was examined in the prostate cell line ND1 by treatment with 5'-aza'3 deoxycytidine (a demethylating agent). TSPY expression is normally not detectable in ND1 cell line and is re-expressed following demethylation treatment. Hence, TSPY expression is affected by the methylation status of the TSPY promoter (Dasari *et al.*, 2002).

1.1.8.2 Genomic TSPY structure

Testis specific protein Y chromosome encoded (TSPY) is a multicopy gene that is located on Y chromosome. Each copy is embedded in 20 Kb tandem repeat units (DYZ5) along the Y chromosome (Manz *et al.*, 1993). Approximately, 20-40 copies of TSPY clustered on the short arm of the human chromosome (Yp). Additional copies are also present on the long arm of the Y chromosome. TSPY gene family

members occur in at least six locations on the human Y-chromosome and each cluster contains a unique combination of variants (TSPYA, B, C, D, E and F). The majority of TSPY copies map to interval 3C on Yp11.2 (TSPYA). Minor locus (TSPYB) exists in an interval proximal to 3D, close to the centromere (Dechend *et al.*, 2000). A new member of TSPY gene family (TSPYq1) was isolated from subinterval 6 E (Ratti *et al.*, 2000). TSPY consists of six exons and five introns distributed over 2.8 Kb (Schnieders *et al.*, 1996) (Figure 1.11). Individual human TSPY members of the cluster are not identical, but rather show microheterogeniety of up to 10% at the genomic level and about 3% at the transcript level. Homologues of human TSPY contain multicopies and are located on the Y chromosome in rodents and other mammals including great apes, pigs, cattle, sheep, goats and horses (Conrad *et al.*, 1996, Glaser *et al.*, 1998, Kim *et al.*, 1996, Mazeyrat and Mitchell, 1998, Schempp *et al.*, 1995, Vogel *et al.*, 1997a, Vogel *et al.*, 1997b).

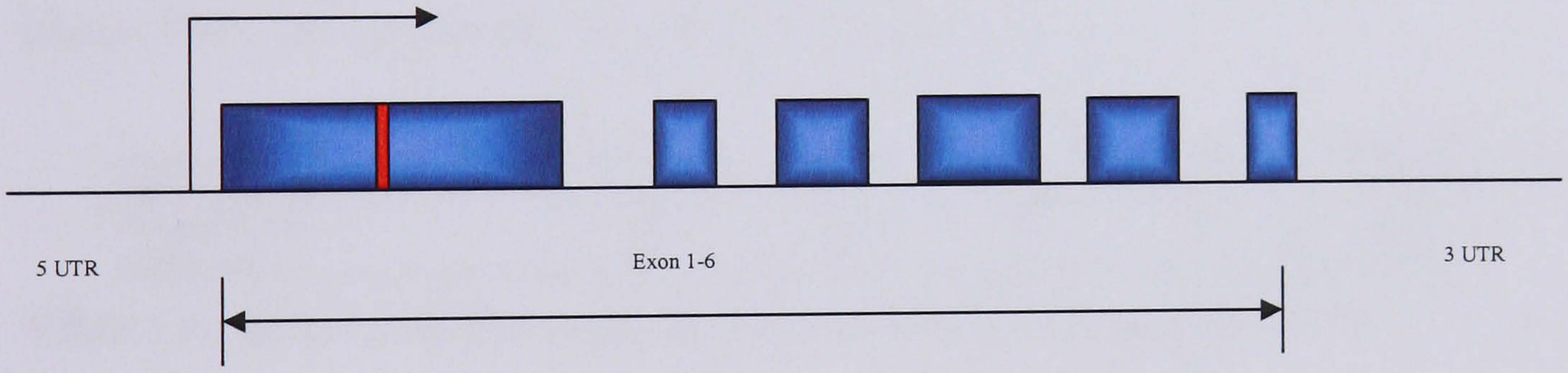


Figure 1.11. Genomic structure of TSPY.

The gene is 2.8 kb in length and consists of 6 exons spaced by 5 introns. The 3 and 5 untranslated regions (UTR) were identified. Small duplication in exon 1 is represented in red and transcription imitation site is indicated.

Male specific bovine genomic homologues of the human TSPY are located on the Y chromosome and are also repetitive. The gene is part of a clustered array of 50-200 repeated sequences on the Y chromosome resembling the arrangement present in the human genome (Jakubiczka *et al.*, 1993). The bovine TSPY homologue (bTSPY) is 1266 bp in length and consists of seven exons (Figure 1.12). Microheterogeneous sequence variation is found between TSPY family members (Jakubiczka *et al.*, 1993). Bovine genomic DNA TSPY shows 53% of DNA homology to human TSPY. In Japanese monkey, TSPY gene was also cloned and contained an open reading frame of 246 amino acids. This coding fragment shared 89% nucleotide sequences identity and 81% amino acid identity with the human homologue of TSPY. The rat homologue of human TSPY (rTSPY) has been isolated and characterised. The rTSPY genomic size is 2624 bp and the gene consists of 6 exons and 5 introns and is organised similarly to human TSPY (Dechend *et al.*, 1998). Mouse TSPY (mTSPY) has been identified and was found to consist of 6 exons. Sequence comparison between mouse and rat TSPY has revealed that 92% of coding exons are identical. The putative rat TSPY protein is 88% identical to the murine TSPY. Rat TSPY is 47% identical to human TSPY (Mazeyrat and Mitchell, 1998) (Table 1.6).

Amio acid homology	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5
Mouse/Human	63	86	73	57	44
Rat/Human	65	88	70	59	55

Table 1.6. Amino acid homology between mouse, human and rat TSPY.

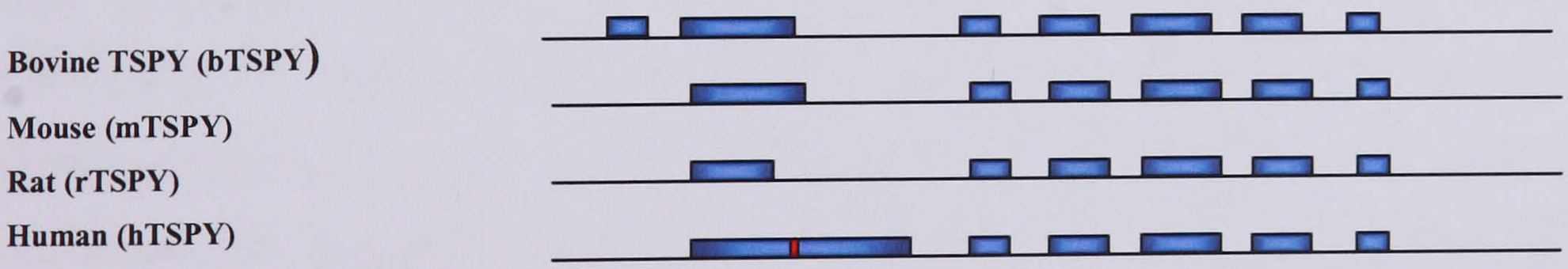


Figure 1.12. Genomic structure of hTSY, bTSPY, mTSPY and rTSPY.

Genomic structure of TSPY from bovine, mouse, rat and human. All consist of 6 exons except for bovine TSPY which consists of 7 exons.

1.1.8.3 TSPY haplotype

Several variants of the TSPY gene have been identified including base substitutions and 18 bp tandem duplication (Krick *et al.*, 2003). Nucleotide substitutions have been described in exon 1 and exon 3 (135G-A silent and 584 C-G) (Table 1.7). The 18 duplications in exon 1 (221-238) were detected at loci TSPY-A and TSPY-B, whereas TSPY-D, TSPY-E and TSPY-F harbour only 18 alleles. TSPY-A locus contains the majority of TSPY gene copies whereas TSPY-B, C, D, E loci contain fewer copies. Each locus on Yp (TSPY A-D) contains both G and A variants at nucleotide 135. The interval 18 tandem duplication at position 221-238 in TSPYA-B does not affect the ORF of TSPY major.

Locus	135G-A	584 C-G	Duplication 18 bp
TSPY A (Yp)	**G/A**	**C**	**36**
TSPY B (Yp)	**G/A**	**G**	**36**
TSPY C (Yp)	**G/A**	**C**	**18**
TSPY D (Yp)	**G/A**	**C**	**18**
TSPY E (Yq)	**G**	**C**	**18**
TSPY F (Yq)	**G**	**G**	**18**

Table 1.7. Haplotypes identified in TSPY transcripts

TSPY sequence variants isolated from prostate and testis cDNA including base substation, and short tandem duplication (Krick *et al.*, 2003).

1.1.8.4 Transcriptional & post-translation variants

Three transcripts have been described in testicular specimens: a major transcript called TSPY ^{major} and two shorter transcripts TSPY A and B that were generated from alternative splicing of exon 1 (Schnieders *et al.*, 1996) (Figure 1.13). In addition to the three transcripts detected, other TSPY alternative splice forms have been detected from a collection of normal testes, testicular cancer and prostate cancer (Lau *et al.*, 2003). TSPY major transcript (full length) encodes for 308 a.a. Western blot indicated that TSPY is a phosphoprotein and two protein forms exist, 33 kDa unphosphorylated protein and 38 kDa phosphorylated protein (Schnieders *et al.*, 1996). TSPY protein structure suggests the presence of a bipartite nuclear localisation signal (NLS) at the N-terminus. This NLS is similarly observed in Xenopus oocyte protein nucleoplasmin (Dingwall and Laskey, 1991). Similar bipartite signals are found in proteins that demonstrate nuclear localisation such as transcription factors, polymerases,

topoisomerases and nuclear hormone receptors (Dingwall and Laskey, 1991). The NLS appears to be conserved in bovine TSPY suggesting a functional significance (Figure 1.14).

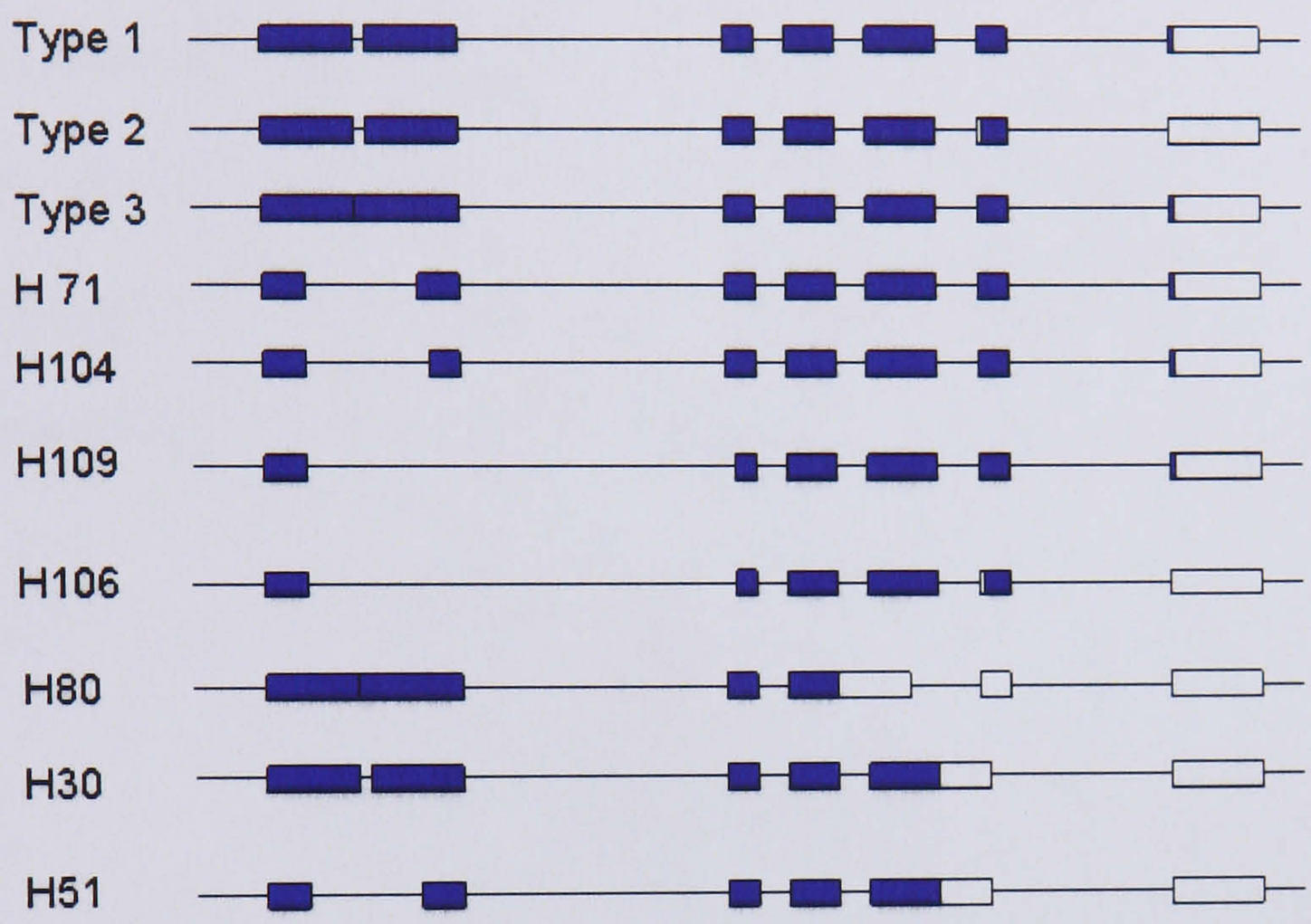


Figure 1.13. TSPY alternative splice forms.

TSPY alternative splice forms isolated from testis and prostate cDNA (Lau *et al.*, 2003).

10	20	30	40
MRPEGSLTYR	VPERLRQGFC	GVGRAAQALV	CASAKEGTAF
50	60	70	80
RMEAVQEGAA	GVESEQAALG	EEAVLLLDDI	MAEVEVVAEE
90	100	110	120
EGLVERREEA	QPRQQAVPGP	GPMTPESALE	ELLAVQVELE
130	140	150	160
PVNAQARKAF	SRQREKMERR	RKPHLDRRGA	VIQSVPGFWA
170	180	190	200
NVIANHPQMS	ALITDEDEDM	LSYMVSLEVE	EEKHPVHLCK
210	220	230	240
IMLFFRSNPY	FQNKVITKEY	LVNITEYRAS	HSTPIEWYPD
250	260	270	280
YEVEAYRRRH	HNSSLNFFNW	FSDHNFAGSN	KIAEILCKDL
290	300	308	
WRNPLQYYKR	MKPPEEGTET	SGDSQLLS	

PKC phosphorylation site

8	TYR
33	SAK
269	SNK

Casein kinase II phosphorylation site

33	SAKE
107	SALE
174	TDED
233	TPIE
300	TSGD

Nuclear localisation signal

127	NLS

Figure 1.14. TSPY putative phosphorylation sites.

Potential phosphorylation sites and their corresponding kinases have been predicted using phosphobase site (http://phospho.elm.eu.org).

1.1.8.5 TSPY function

Although TSPY gene product is postulated to regulate cellular proliferation of spermatogonia, it biological function is not yet known. Detailed database and comparison sequence analysis reveals that TSPY shows homology members of super-family containing SET and NAP proteins (Schnieders *et al.*, 1996). TSPY shows homology to SET oncogene. SET was initially identified in patient with acute undifferentiated leukaemia who harboured chromosomal translocation on chromosome 9 (von Lindern *et al.*, 1992). This leads to fusion of CAN with SET to create SET-CAN chimeric that encodes 155 KD putative oncoprotein. C-SET gene encodes 32 KD protein that is widely expressed in numerous tissues and cell types, including testis (Adachi *et al.*, 1994). SET has also been identified as the template-activating factor, TAF-1 (Nagata *et al.*, 1995) and is a potent inhibitor of protein phosphatase 2A (PP2A) (Li *et al.*, 1996). PP2A is an abundant, multifunctional serine/threonine-specific phosphatase that stimulates DNA replication (Lin *et al.*, 1998). PP2A antagonises cyclin B complexes to regulate cell cycle progression at the G2 stage. Furthermore, SET and related protein, nucleosome assembly protein 1(NAP-1), interact specifically with mitotic cyclin B (Kellogg *et al.*, 1995). Nucleosomal assembly proteins are histone chaperones that transport histone protein from cytoplasm into the nucleus across the nuclear membrane. Deposited histone into the replicative DNA is required to form nucleosomes and regulate the cell cycle. NAP-1 was found to be located in the cytoplasm during the G2 phase of the cell cycle and it is found in the nucleolus in the S phase (Ito *et al.*, 1996). NAP-1 plays a role in regulating gene expression, through its affect on the maintenance of chromatin in a dynamic state. A genome wide analysis study of NAP-1 deficiency in *Saccharinyces cerevesiae* yeast has indicated that 10% of all genes were affected, some being up regulated and other being down-regulated. Approximately, 35% of NAP-1 regulated genes are clustered together, suggesting that NAP-1 might function in large chromosome domains (Ohkuni *et al.*, 2003). Further study in *S. cerevesisae* has shown that NAP-1 also participates in the control of mitotic events through an interaction with ClB2, a protein belonging to the cyclin B family (Kellogg *et al.*, 1995). NAP-1/2 knock out mice are embryonic lethal at mid gestation stage. There is accompanied overproduction of neural precursor cells suggesting a role for NAP-1 in regulating neuronal cell proliferation (Rogner *et al.*, 2000).

While the exact function of TSPY remains to be fully examined, there is mounting evidence to suggest a role for TSPY in development and pathogenesis of male associated accessory organs, including the testis and prostate. In this thesis, work has been carried out to test our hypothesis:

TSPY plays a key role in human prostate carcinogenesis with aberrant gene expression or genetic alteration.

1.2 Overall aims

To investigate the role of TSPY in prostate cancer and study its functional role in prostate cancer development.

1) To investigate TSPY expression product profile in clinical prostate cancer compared to BPH using IHC method and generated peptide TSPY polyclonal antibody.

2) To determine the functional effect of expressing TSPY in LNCaP cell lines.

3) To investigate if TSPY gene copy number is associated with prostate cancer.

4) To identify altered genes associated with LNCaP cells over-expressing TSPY using microarray method.

Chapter 2
General Materials and Methods

2.1 Molecular biology

2.1.1 Cloning TSPY gene

A full length TSPY cDNA I.M.A.G.E. bacterial clone was selected from the I.M.A.G.E. consortium (accession number, 5269330) web site and purchased from the HGMP Resource Centre in the UK. The clone is part of an adult testis cDNA library generated by unidirectional insertion of cDNA fragments into the ECOR I and Not I restriction site of PT7T3-pac vector (Figure 2.1). The clone was maintained in DH10B host bacteria. The selected clone contained 924 bp insert of TSPY. The bacterial clone was streaked from slab culture into LB agar (1% Bacto-tryptone plate Petri dish containing 100 μg/ml Ampicillin and incubated O/N at 37°C to form separate individual colonies each arising from a single bacterial cell. Midi scale plasmid DNA was prepared from a single selected bacterial colony and the presence of the TSPY insert was confirmed by restriction digestion and sequencing (Molecular Biology Unit, Newcastle upon Tyne University) using universal T7 promoter and T3 promoter primers.

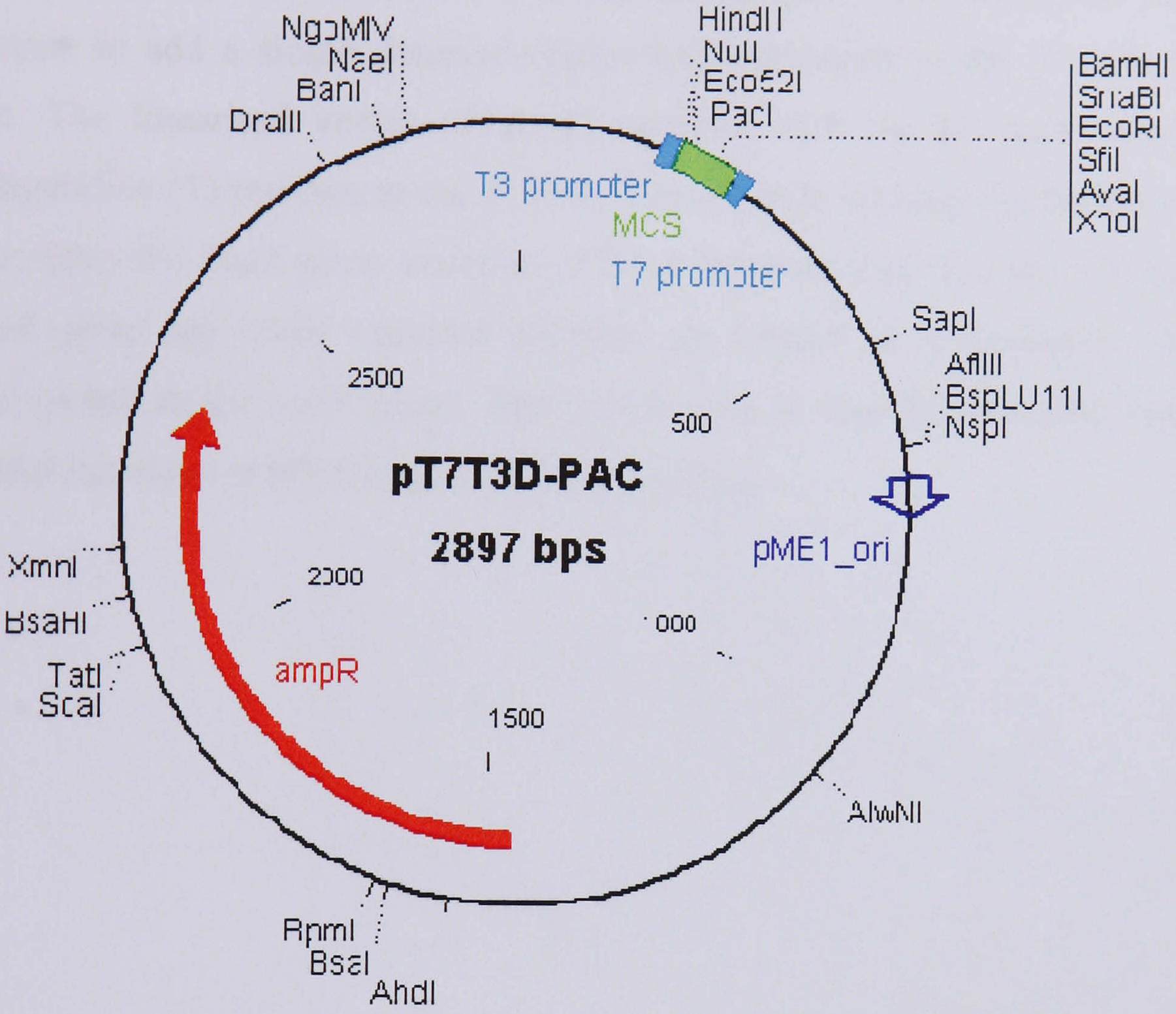


Figure 2.1. PT7T3d-pac vector.

The vector is 2.9 kb in length and the multiple cloning site is flanked by T3 promoter and T7 promoter. The vector contains an ampicillin resistance gene as a selectable marker.

2.1.1.1 TA cloning and adding FLAG tag at 5'end of TSPY cDNA

TA cloning kit provides a one-step cloning procedure for the direct insertion of a PCR product into a plasmid vector (PCR2.1).The kit depends on the ability of Taq DNA polymerase to add a single deoxyadenosine (A) nucleotide to the 3' ends of PCR product. The linearised vector (PCR2.1) supplied with the kit have overhanging deoxythymidine (T) residues at the 3' ends. The multiple cloning site for the vector is located within the LacZ gene. Insertion of DNA fragment into this site will inactivate the LacZ gene and white bacterial colonies are formed in the presence of X-Gal solution (substrate for LacZ gene). Thus, LacZ gene is used as selectable marker for successful insertion of DNA fragment into the vector.

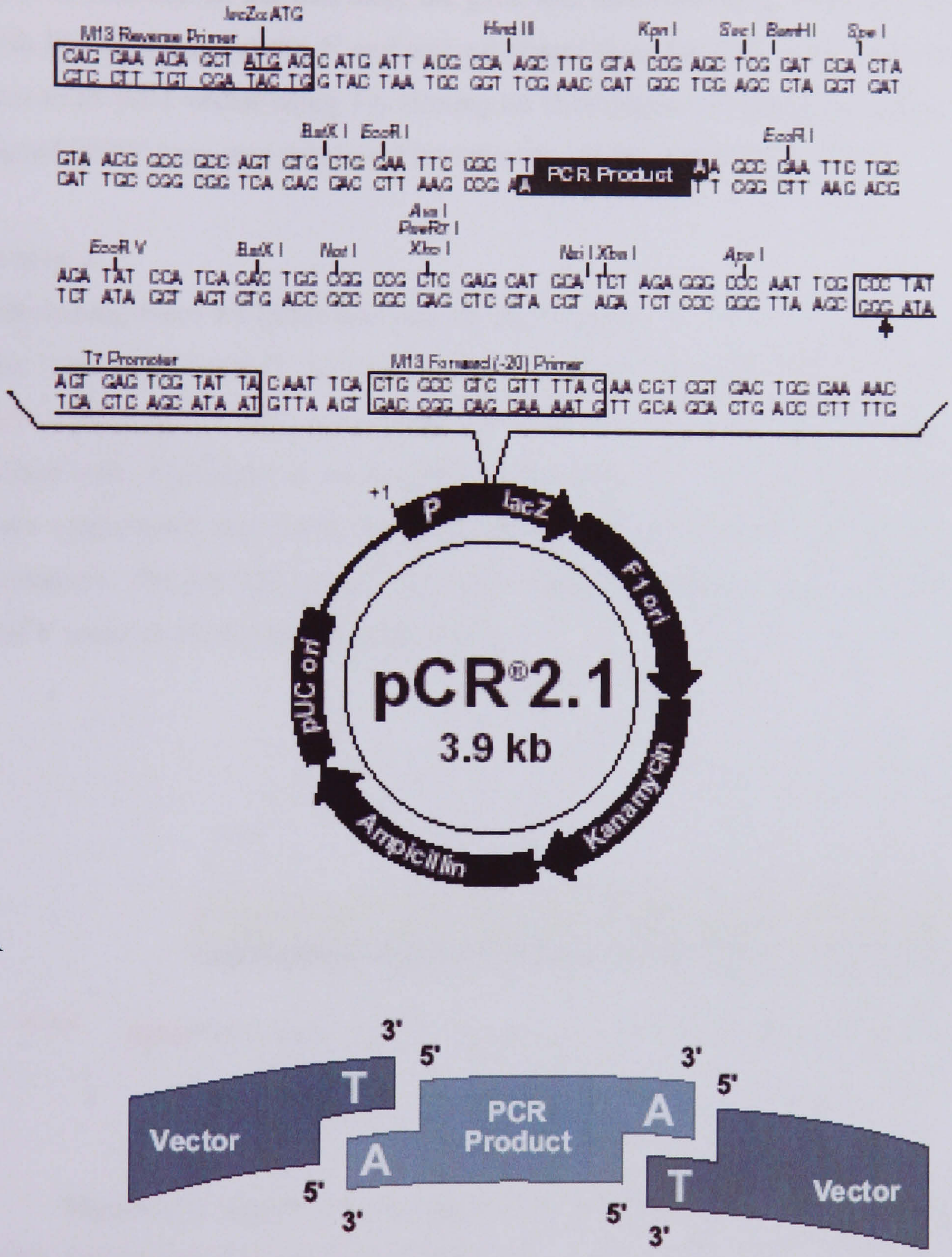


Figure 2.2. TA cloning vector.

(A) The vector is 3.9 kb in length and two antibiotic selectable markers included in the vector, ampicillin and Kanamycin. The multiple cloning site is located within the LacZ gene and is used as a marker for selecting bacterial colonies that contain that insert. (B) The linearised TA cloning vector has overhanging 3' ends with T nucleotide and the PCR amplified fragment with A nucleotide added by Taq polymerase.

To clone the full length of TSPY coding region, two sequential subcloning strategies were carried out. In the first step, the gene was simultaneously amplified and tagged with FLAG epitope at the 5' end and subcloned from the I.M.A.G.E. vector (PT7T3-pac) to PCR2.1 vector using TA cloning kit (Invitrogen). In the second step, a FLAG tagged TSPY gene was subcloned into the mammalian pcDNA3.1 vector.

In step 1

Subcloning from PT7T3D-pac into PCR2.1 vector

The following 5' end FLAG tagged TSPY forward primer 5'-ATG GAC TAC AAA GAC GAC GAT GAC AAA GAG GTC GTG CAG GAG GGG-3' and reverse primer with stop codon at the end, 5'- GGG ACT CCC AGT TGT TGA GTT GA-3' were synthesised and purchased from Thermohybaid (Thermo BioSciences GmbH, Germany). The IMAGE clone insert was used as a template to amplify the 924 bp TSPY insert in PCR reaction (Figure 2.3).

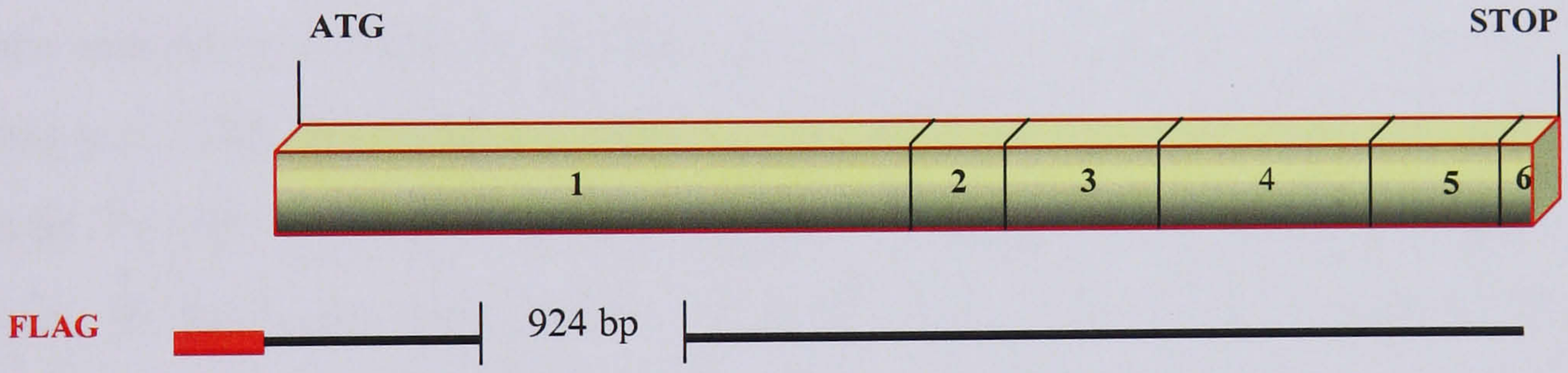


Figure 2.3. Schematic representation of TSPY cDNA exon structure.

TSPY full length (924 bp) that includes 6 exons was subcloned into pcDNA3 vector and a short FLAG-tag was added at the 5' end.

2.1.1.2 Geneclean

Geneclean is a very basic method is used to recover nucleic acid fragments from agarose gel for further DNA manipulation. In this method, the required fragment was excised from the gel, transferred into an eppendorf tube and weighed. Three volumes of 3 M NaI were added. The tube was placed on 55 °C water bath for 5 minutes until the gel dissolved. Then, 5 µl of Glass milk (Biolabs) that binds DNA was added and the tube was placed on ice for 10 minutes. The tube was centrifuged at 14000 g for 1 min and the supernatant was discarded. The pellet was resuspended in 3 volumes of New wash solution and centrifuged at 14000 g for 1 min, the supernatant was discarded. The previous step was repeated twice. Finally, the pellet was resuspended in 15 µl of ddH_2O heated to 55 °C and supernatant containing the DNA was separated from Glass milk by centrifugation at 14000 rpm for 1 min.

2.1.1.3 DNA Ligation

Once the TSPY fragment was recovered, it was inserted into PCR2.1 vector in a ligation reaction. A ligation mixture was set up by adding a linearised PCR2.1 plasmid and DNA fragment at a ratio of 1:3 respectively, 1 µl of 10X ligation buffer (60 mM Tris-HCl, pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml bovine serum albumin, 70 mM mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol and 10 mM spermidine), 1 µl of T4 DNA ligation enzyme (Invitrogen) and ddH_2O to a final volume of 10 µl. The reaction was incubated at 14 °C O/N.

2.1.1.4 Transformation

TOP10 competent cells (Invitrogen) were thawed out on ice (50 µl). Three microlitres of overnight ligation mixture were added to the cells and incubated on ice for 30 min. The cells were heat shocked for 45 sec at 42 °C and placed on ice for 2 min. Then 500 µl of SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added and the cells were incubated at 37°C for 1hr in a shaking incubator to allow expression of antibiotic resistance marker. From these transformed cells, 100 µl of bacterial culture was spread onto LB agar plate containing 100 µg/ml ampicillin and 40mg/ml X-Gal solution (400 mg X-Gal in 10 ml dimethylformamide). The plate was incubated O/N

at 37°C to allow formation of colonies. Selection for the correct colonies with insert is based on the colour of the colonies. Insertion of the DNA fragment into the Lac Z cloning site will inactivate the gene and colonies that harbour the insert will be white in colour.

2.1.1.5 Restriction digestion

Successful insertion of PCR product into flanked EcoR I restriction site in TA vector was further checked by restriction digestion of plasmid isolated from bacterial clones. All restriction digestion reactions were carried out in a final volume of 20 µl. Restriction enzyme master mix was prepared by adding the following: 2 µl Restriction enzyme buffer (Promega), 0.2 µl 100 X BSA, 1 µl EcoR I restriction enzyme (5 units) and 1 µg of plasmid DNA. Reactions were incubated at 37°C for 90 min. Digestion product size was checked on 1% agarose gel.

Step 2

Subcloning FLAG tag TSPY gene from PCR2.1 into pcDNA3.1 vector

The FLAG-tagged TSPY (FLAG-TSPY) fragment was digested out from the pCR2.1 plasmid using EcoRI restriction enzyme to release the FLAG-TSPY fragment. The digestion product was visualized on 1% agarose gel and gel extraction was performed as given in section 2.1.1.. The pcDNA3 vector (Invitrogen) was also digested with the same restriction enzyme and the vector was dephosphorylated to remove the phosphate groups at the 5' ends of the linearised pcDNA3.1 plasmid DNA to prevent self ligation (Figure 2.4). The dephosphorylation step was carried out by adding 20 µl EcoRI-digested pcDNA3.1 product, 2.5 µl 10X Alkaline phosphatase buffer (Promega), 2 units of Alkaline phosphatase (Promega) in a tube and incubating at 37 °C for 30 min. The FLAG-TSPY fragment, the linearised and dephosphorylated pcDNA3 plasmid were ligated, transformed and colonies were screened and selected using colony PCR method.

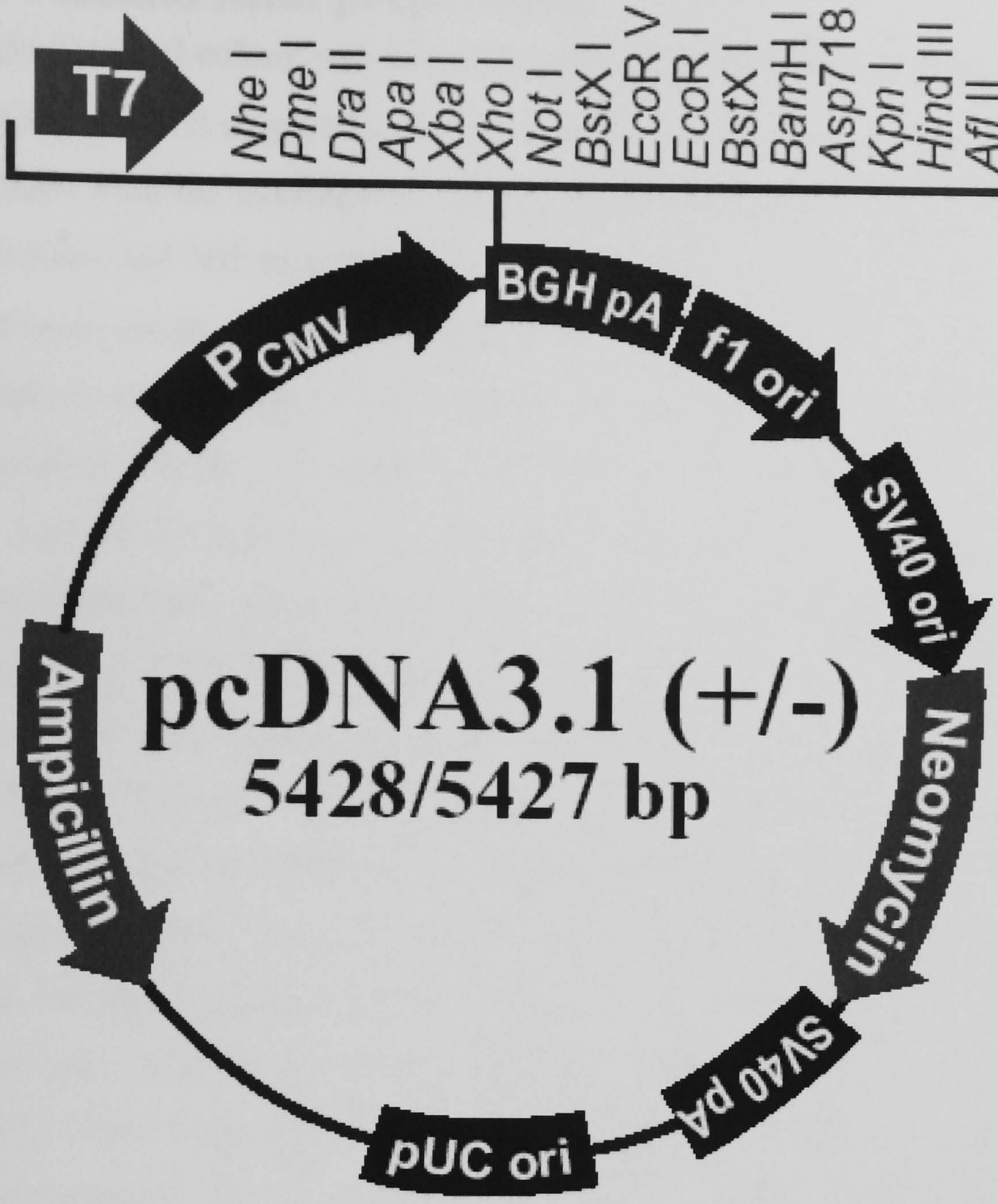


Figure 2.4. pcDNA3.1 mammalian expression vector.

The vector is approximately 5.4 kb in length and contains a neomycin selection marker for generating stable clones in mammalian cells. It also includes ampicillin selectable maker for selection in bacterial cells and an origin of replication for propagating the vector in bacterial cells. A strong CMV promoter derives expression of downstream cloned genes.

2.1.2 Plasmid Midi preparation

A single bacterial colony was inoculated into 5 ml LB medium containing 100µg/ml Ampicillin and left to grow at 37 °C overnight in a shaking incubator. One hundred microlitres from the overnight bacterial growth were re-inoculated into fresh 100 ml LB medium and left to grow O/N at 37 °C with vigorous shaking. The bacterial culture was centrifuged to pellet the cells at 10000 rpm for 10 minutes at 4°C and the supernatant was discarded. The bacterial cell pellets were resuspended in 3 ml cell resuspension solution (12.5 mM Tris-HCl, 2.5 mM EDTA and 100 µg /ml RNAase A). Then, 3 ml of cell lysis solution (200 mM NaOH and 1% SDS) were added and the tubes inverted many times. Immediately, 3 ml of neutralizing solution (3 M Potassium acetate and 11.5 % Glacial acetic acid) were added and mixed by gentle inversion many times. Tubes were centrifuged at 10000 rpm for 20 minutes. The supernatants were filtered through Miracloth and 10 ml of resin solution (66.64 g Guanidine hydrochloride (SIGMA), 33.33 ml Neutralizing solution, 1.5 ml NaOH and 1.5 g Diatomaceous Earth) were added and mixed with the samples. The mixture was filtered through Promega Wizard columns. The DNA bound to the columns was washed twice using 20 ml column washing solution (10 mM Tris-Cl, 100 mM NaCl, 2.5 mM EDTA and 50 % ethanol). Then, columns were placed into eppendorf tubes and centrifuged at 14000 rpm for 2 minutes to remove any traces of washing solution. DdH_2O was warmed to 65 °C and 300 µl of was added to each column and left at room temperature for 2 minutes. The DNA was eluted by centrifuging at 14000 rpm for 2 minutes. The DNA concentration was determined using a spectrophotometer (Wallac).

2.1.3 Plasmid Mini-preparation.

One bacterial colony was picked and transferred into 5 ml LB containing 100 µg/ml Ampicillin in a universal tube. The tube was incubated at 37 °C O/N with shaking. The next day, the content of overnight culture was sequentially transferred into eppendorf tube and centrifuged at 14000 rpm for 20 sec. The supernatant was discarded and the pellet was resuspended in 100 µl of solution I (250 mM Glucose, 125 mM Tris-cl and 50 mM EDTA, pH 8). Then, 200 µl of solution II (200 mM NaOH and 1 % SDS) was added and the tube was mixed by inversion. Immediately, 200 µl of Solution III (3 M Potassium acetate and 11.5 % Glacial acetic acid) was

added and mixed again by inversion. The tube was centrifuged at 14000 rpm for 10 min. The supernatant was transferred into fresh tube and 500 µl of phenol : chloroform : isoamylalcohol mixture (ratio 25:24:1, respectively) was added and mixed by shaking. The tube was centrifuged at 14000 rpm for 3 min. The upper aqueous layer was transferred to a fresh tube and 900 µl of 100 % Ethanol was added and the tube was kept at -80 °C for 10 min. The DNA was precipitated by centrifuging at 14000 rpm for 10 min. The DNA precipitate was washed with 70% Ethanol, air dried and resuspended in 50 µl of ddH_2O.

2.1.4 Conventional PCR

Polymerase chain reaction method selectively amplifies a specific segment of nucleic acid using specific pair of primers that bind to the target sequence. The method was set up as follow: into the PCR HYBAID omnitubes, the following PCR mix was added; 5 µl of 10 X PCR buffer (160 mM $(NH_4)_2SO_4$., 670 mM Tris-HCL, pH, 8.8 and 0.1% Tween-20), 1.5 µl of 5 mM $MgCL_2$, 2 µl of 5 mM dNTPs (Sigma), 50 pmole/µl of forward and reverse primers, 0.25 units of Taq DNA polymerase (BIOLINE), 0.1-0.5 µg DNA and ddH_2O up to 50 µl. The tubes were inserted into HYBAID PCR EXPRESS thermal cycler. The following 3 stages of PCR cycles were carried out: denaturation step at 95 °C for 3 min, amplification step consisting of 3 steps at 95 °C for 1 min, 65 °C for 1min and 72 °C for 30 sec, the cycle number was between 30-35 cycles and extension step at 72 °C for 10 min. The PCR product was mixed with 6X loading buffer (0.25% Bromophenol blue, SIGMA, 0.25% Xylene Cyanole FF, SIGMA, 30% Glycerol, BDH and 50 mM EDTA, pH 8) and loaded into 1% agarose gel.

2.1.5 Agarose gel electrophoresis

It is a method that is used to separate DNA fragments based on their size to determine the length of the separated DNA fragments by comparison to a standard marker with known fragment sizes. In this method the agarose gel was prepared by adding 1-2 g of agarose electrophoresis grade (GibcoBRL) into 100 ml 1 x TAE electrophoresis buffer (242 g Tris (TRIZMA BASE, SIGMA), 57.1 ml glacial acetic acid (BDH), 100 ml of 0.5 M EDTA (pH, 8.0) and ddH_2O to final volume of 1 litre) depending on the product size. The mixture was heated using a microwave until boiling to dissolve the

agarose. Then, the gel was allowed to cool to 50 °C before adding 5 µl of 10 mg/ml Ethidium bromide (SIGMA). Immediately, the gel was poured into glass plate and left to solidify for 10 minutes. The gel was placed in electrophoresis tank and filled with 1 x TAE buffer. Samples were mixed with 6x loading buffer and 5 µl of DNA Hyper ladder markers (BIOLINE) IV or V were loaded into each well and the gel was exposed to 100 V. for 30 minutes. The gel was exposed to U.V. source and molecular analysis software was used to view and capture pictures of the gel.

2.1.6 Colony PCR

Colony PCR is a simple PCR based method that was developed to check the presence, absence or the orientation of insert during DNA cloning procedure. Colonies were selected and transferred into eppendorf tubes containing 100 µl of ddH_2O, mixed and 10 µl were streaked onto plates. The cells were boiled for 10 min to release plasmid DNA and the tubes were pulse centrifuged to collect cell debris. Colony PCR was carried out by adding 3 µl of boiled bacterial supernatant into a standard PCR master mix and the presence or absence of the insert was determined by using TSPY forward and reverse primers. The insert orientation in each clone was identified using TSPY reverse primer and T7 promoter primer. Once the correct clone was identified, a maxi DNA plasmid was prepared using Qiagen endotoxin free plasmid maxi preparation kit following the manufacture's instructions.

2.2 Mammalian Cell Culture

2.2.1 Cell lines

Three mammalian cell lines were used: the androgen-responsive human prostate carcinoma cell line, LNCaP purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, U.S.A.). The androgen unresponsive human prostate carcinoma cell line, PC3M purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury Wiltshire, U.K.). NIH Swiss mouse embryo fibroblast cells 3T3, were also purchased from the European Collection of Animal Cell Cultures.

2.2.2 Thawing cells

Frozen cells in a cryovial were thawed at 37°C. Immediately, the contents were removed into 9 ml of pre-warmed complete medium in a universal tube. Cells were

resuspended and the tube was centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 5 ml complete medium. The suspended cells were transferred into 75 cm^2 vented flask and complete medium was added to a final volume of 15 ml. Cells were incubated at 37°C in humidified conditions supplemented with 5 % CO_2. Medium was changed the next day and the cell condition and viability was observed and reported.

2.2.3 Cell culture

Each cell line was grown in its recommended medium. LNCaP and PC3M were cultured in RPMI (Sigma) and NIH/3T3 was cultured in DMEM (Sigma). Both RPMI 1640 medium containing 25 mM HEPES buffer and DMEM were supplemented with 20 mM L-Glutamine (Sigma), 10 % heat inactivated foetal calf serum (Sigma), Penicillin (100 IU/ml; Sigma) and streptomycin (100 µg/ml; Sigma). Cell lines were routinely maintained in 100 cm dishes or 75 cm^2 flasks and incubated at 37 °C in the presence of 5 % CO_2. Media were changed three times a week and the cells were sub cultured very often when they were 70% confluent. The medium was discarded and the cells were washed with 1X PBS. They were covered with 0.2% Trypsin/EDTA solution and incubated at 37 °C for 2-10 minutes until the cells detached and floated freely. The Trypsin was inactivated by adding 10 ml of complete medium and cells were resuspended and centrifuged at 2000 rpm for 5 min. The cell pellet was resuspended in 10 ml complete medium. The cell number was determined using a haemocytometer and were split into 10 cm dishes at required densities. The cell lines were routinely tested for mycoplasma contamination using Mycoplasma PCR kit.

2.2.4 Freezing cells

Frozen stocks of all cultured cells were generated. Cells were grown to 90% confluent in 10 cm dish. The cells were detached and the cell pellet was resuspended in 1-2 ml of freezing medium (10% FCS, 10% DMSO and basal medium). Cell suspension was aliquoted into 1 ml cryovial tubes, frozen gradually and stored in liquid nitrogen.

2.3 Transfection

2.3.1 Transient Transfection

Cells were counted and 1 X 10^5 cells/well were plated into 6 well plate. After incubation for 24 hr, the cells were washed with 1 X PBS and 1 ml of RPMI 1640 basal medium containing 3 μg of Plasmid DNA and 9 μl of Superfect (Qiagen) were added to each well. The cells were incubated at 37 °C for 3 hr before the mixture was removed and replaced with 3 ml of RPMI 1640 complete medium. The cells were cultured for additional 48 hr before they were put forward for further analysis.

2.3.2 Stable transfection

In 10 cm dish, 0.5 X10^6 cells were plated and left for 24 hr. The medium was removed, cells were washed with 1X BPS and 4 ml of basal medium was added. DNA and superfect complex was prepared by adding 30 μg of FLAG-TSPY-PCDNA3 plasmid DNA or PCDNA3 vector DNA only into 300 μl of basal medium in a microfuge tube. Then, 90 μl of Superfect (Qiagen) was added, mixed and the DNA-Superfect complex was left for 10 min at RT. Basal medium was added to the microfuge to a final volume of 1000 μl. The mixture was added to dishes and the cells were incubated at 37 °C for 3 hrs. The medium was replaced with complete medium and the cells left at 37 °C for another 48 hrs. Geneticin was added at concentration of 500 μg/ml to the cells and they were left for 2-3 weeks until visible colonies were formed (killing curve for LNCaP cell line had already been carried out and the minimum concentration of the antibiotic required to kill all the cells in a period of 3 weeks was pre-determined).

2.3.3 Ring cloning

Colonies formed were marked. The cells were washed in 1X PBS and 2 μl of 0.2 % Trypsin /EDTA was used to detach each colony from the dish. The detached cells from selected colonies were placed into 48 well plate and left for another 1 week to grow. Stable clones were then replated onto 12 well plate in duplicate for screening and expanding. The presence of exogenous constitutive expressed FLAG-TSPY in each clone was assessed using Western blot method. Clones that showed strong and weak expression were selected, expanded and frozen stock were generated.

2.4 Biochemical methods

2.4.1 Western blot

2.4.1.1 Protein lysis

Cells were washed in PBS and 100-800 µl of 1 X SDS-sample buffer (125 mM Tris-Cl, pH 6.8, 2% SDS, 10% Glycerol, 10% β-mercaptoethanol and 0.001% Bromophenol Blue) was added according to cell number and left for 2 minutes. The cells were sheared by pipetting and transferred into eppendorf tubes. The cell lysates were boiled for 10 min, centrifuged at 14000 rpm for 1 minute and 15 µl volume was loaded into 12 % SDS-PAGE gel.

2.4.1.2 12 % SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

BIO-RAD Mini Protean II apparatus was used to set up 12 % SDS-PAGE. Running gel (375 mM Tris-HcL, pH 8.8, 0.1 % SDS, 12% acrylamide/bis-acrylamide gel, 0.1% Ammonium persulphate and 0.1% TEMED) was loaded between two glass plates and left to set for 15 minutes. A stacking gel (125 mM Tris-HCl, pH 6.8, 0.1 % SDS, 6% acrylamide/bis-acrylamide gel, 0.1% Ammonium persulphate and 0.1% TEMED) was loaded into the top of the running gel and left to set for 10 minutes. The plate set was transferred into electrophoresis tank containing running buffer (25 mM Tris-HCl, 190 mM glycine and 0.1% SDS). The gel was electrophoresed at 200 V for 45 minutes to allow separation of proteins.

2.4.1.3 Immunodetection

Proteins on the gel were transferred into Hybond C++ membrane (Amersham Biosciences) using the Bio-Rad protein transfer apparatus. The gel and the filter were sandwiched between two Whatman paper and two mats. A transfer tank was filled with transfer buffer (25 mM Tris-HCl, 150 mM glycine and 10% methanol) and the proteins were transferred overnight by applying 30 V. The filter was blocked in 5% Marvel skimmed milk dissolved in TBS buffer (20 mM Tris-HCl and 500 mM NaCl) for 1 hr with shaking at RT. The membrane was washed twice in 1 X TTBS (20 mM Tris-HCl, 500 mM NaCl and 0.1% tween-20) for 10 minutes before placing it into a

plastic bag filled with 1% Marvel in TTBS buffer and the diluted primary antibody. The antibody was left to hybridise for 1hr shaking at RT. Unbound antibody was removed by washing the membrane twice in 1x TTBS for 5 minutes before adding 1% blocking solution with 1:1000 dilution of secondary antibody conjugated to horse radish peroxidase (Dako). The membrane and the secondary antibody were left shaking at RT for 1 hr. Then, the membrane was washed twice in 1X TTBS for 10 minutes and once in 1X TBS for 10 minutes. ECL Western blotting detecting reagent kit (Amersham Biosciences) was used to develop the signal.

2.4.1.4 Stripping membrane

After the signal development, the membrane was washed twice in 1x TTBS. Then, placed in 50 ml stripping solution (62.5 mM Tris-HCl, pH 6.7, 2% SDS and 0.78% β-mercaptoethanol) and left shaking for 30 minutes at 50 °C. The membrane was washed twice in 1 X TTBS for 10 minutes and re-used for further few times.

Chapter 3
Expression and Functional Analysis of TSPY in Prostate Cancer

3.1 Introduction

The expression of TSPY transcript is detected in testes during early foetal stage and persists in the adult males (Lau *et al.*, 2000). The gene product was previously thought to be organ specific and the testes were the only tissues to express TSPY. Subsequent studies have detected TSPY expression in prostate glands (Lau *et al.*, 2003). In normal adult testes, TSPY expression is predominantly detected in cells lining the seminiferous tubules, specifically spermatogonia and to a lesser degree in early spermatocytes (Schnieders *et al.*, 1996). Spermatogonia cells are the stem progenitor cells that either divide to generate new spermatogonia or differentiate to produce mature spermatocytes. TSPY protein expression was mainly detected in the cytoplasm of spermatogonia while a few cells expressed the gene product in the nucleolus. Aberrant expressions of TSPY gene product have been reported in sex-reversed females developing gonadoblastoma, testicular cancer and recently in prostate cancer. Hildenbrand has used TSPY anti-fusion protein antibody in immunohistochemistry method on the Turner mosaic patient (45X/46XY) gonads. The patient had developed a unilateral microscopic gonadoblastoma and had a Y chromosome lacking the distal Yq arm. Strong TSPY immunoreactivity was detected in gonadoblastoma tumour cells of the gonad (Hildenbrand *et al.*, 1999, Lau *et al.*, 2000). Furthermore, TSPY was strongly expressed in testicular seminoma and carcinoma *in situ* of the testis (Schnieders *et al.*, 1996).

3.2 Aims:

1) To characterise TSPY expression in human prostate cancer.

2) To define the functional significance of TSPY over-expression in prostate cancer.

3.3 Materials and Methods

3.3.1 Patient samples

For immunohistochemistry, prostate tumour specimens were obtained from Freeman Hospital, Newcastle upon Tyne, U.K. between years 1989 to 1995. Seventy two patients with locally and advanced prostate cancer and 20 patients with BPH were examined. The samples were collected with patient consent. Clinical and pathological reports were collated for each case and in most cases, follow up status was available. Normal adult testis was used as a positive control. Biopsies from female liver, lung and kidney were used as negative controls. For validation of TSPY expression at mRNA level, tissue *in situ* hybridisation was carried out in selected cases of CaP and BPH.

3.3.2 Tissue preparation

Tumour specimens were fixed in formalin and embedded in paraffin. The tumours were sectioned at a thickness of 4 μm and adhered onto APES coated slides. The sections dehydrated in graded ethanol, deparaffinized in xylene, stained by Haematoxylin & Eosin and graded according to Gleason's score system (Gleason, 1992) by a pathologist. Six of the cases were grade 2-5 and 66 cases were grade 6-10. The tumours were also classified according to TNM staging criteria (Schroder *et al.*, 1992) and 30 cases were T1/T2 and 24 cases were T3/T4.

3.3.3 TSPY expression analysis

3.3.3.1 Generating TSPY polyclonal antibody

TSPY peptide (-AVPGPGPMTPGC-) corresponding to amino acid sequence 96-106 of the TSPY protein (accession number AAB51693) was selected with the assistance of Dr. Jo Gray, Molecular Biology Unit, University of Newcastle upon Tyne). The synthesised peptide was conjugated to Keyhole Limpet Haemocyanin (KLH) via cystine residue. Pre-immunised serum was collected from two different rabbits prior to injecting conjugated peptide. First bleed and final bleed were also collected and tested for the presence of anti-peptide antibody using Enzyme Linked Immunosorbent Assay (ELISA), dot blot and Western blot.

3.3.3.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a very sensitive and simple method to detect and quantify antibody or antigen in solutions using an enzyme linked to antibody and a substrate that forms a coloured reaction product. ELISA method was used to detect the presence of TSPY specific immunoglobulin generated from both rabbit sera. The procedure is as follow: wells of 96 well Immunol 2 plates (BD Biosciences) were coated with 0.5 µg of synthetic TSPY peptide dissolved in 50µl of 0.1 M Carbonate buffer, pH 9.2. The plates were incubated O/N at 4 °C. Excess peptides were removed by washing the wells three times with 300 µl of PBS/0.1%Tween-20. Non-specific sites on the bases of the wells were blocked by addition of 300 µl of 3% fat skimmed Marvel milk dissolved in PBS/0.1%Tween 20 and incubating for two hours. The wells were rinsed once with PBS/0.1%Tween. The pre immune-serum and final bleed serum were diluted in PBS, 1:50, 1:200, 1:400, and 1:800, 1:32000, 1:12800 and 1:125600 in PBS. Fifty microlitres of diluted sera were added in triplicate to each well coated with peptide. Wells that are not coated with the TSPY peptide but blocked with Marvel milk were used as negative controls. The plate was incubated at RT for 2 hr. Wells were washed three times in PBS/0.1 Tween to remove excess serum and unbound primary antibodies. A Swine anti-rabbit antibody conjugated to Horse radish peroxidase was diluted 1:1000 in PBS and 50 µl was added to each well and left for two hrs. Unbound secondary antibody was removed by washing three times in PBS/0.1%Tween. The presence of TSPY antibody was detected by adding 100 µl of Tetramethylbenzidine (TMB) Solution (1 tablet of TMB into 1ml DMSO, 9 ml 0.05 M Phosphate-Citrate buffer, pH, 5.0 and 2 µl of 30% H_2O_2) to each well and incubating for 30 min at RT. The reaction was stopped by adding 50 µl of mM H_2SO_4. The plate was read in ELISA plate reader using a 450 nm filter, data was collected from the reader using Dynex Revelation software.

3.3.3.3 Dot blot

Dot blot is another method for detecting the presence of specific protein (antigen or antibody) in a solution. A spot of solution containing antigen is dotted onto special protein binding membrane and the antibody is allowed to bind. The presence of protein/antibody complex is visualised using a secondary antibody conjugated to horse radish peroxidase and ECL detection reagent. In the current study, dot blot

method was used to detect the presence of TSPY antibody in rabbit serum. TSPY peptide and non-specific peptide were dissolved in PBS and diluted to 10 µg, 1 µg, 0.1 µg, 0.01 µg and 0.001 µg and 1 µl of each diluted peptide solution was spotted onto Hybond C++ membrane (Amersham) and left to dry for 15 min (Figure 3.1). The membranes were washed in TBS (20 mM Tris-HCl and 500 mM NaCl)) for 5 min and blocked in blocking solution consisting of 5 % Marvel non-fat milk dissolved in TBS for 1 hr. This step is essential to block non-specific binding sites on the filters. The filters were washed twice in TTBS (20 mM Tris-HCl, 500 mM NaCl and 0.1% Tween-20) for 10 min. Crude serum absorbed with TSPY specific peptide, non-TSPY peptide and non absorbed crude serum were added to the membranes at 1:2000 dilution in 1% blocking solution and incubated for 1 hr on shaking platform. The membranes were washed twice in TTBS to remove unbound antibodies and non-specific bound proteins. Swine anti-rabbit immunoglobulin conjugated to horse radish peroxidase was diluted in 1% blocking solution and added to the membranes and incubated for 1 hr. The membrane was washed twice in TTBS and one in TBS and the signal was developed in a form of dots on the membranes using ECL kit (Amersham).

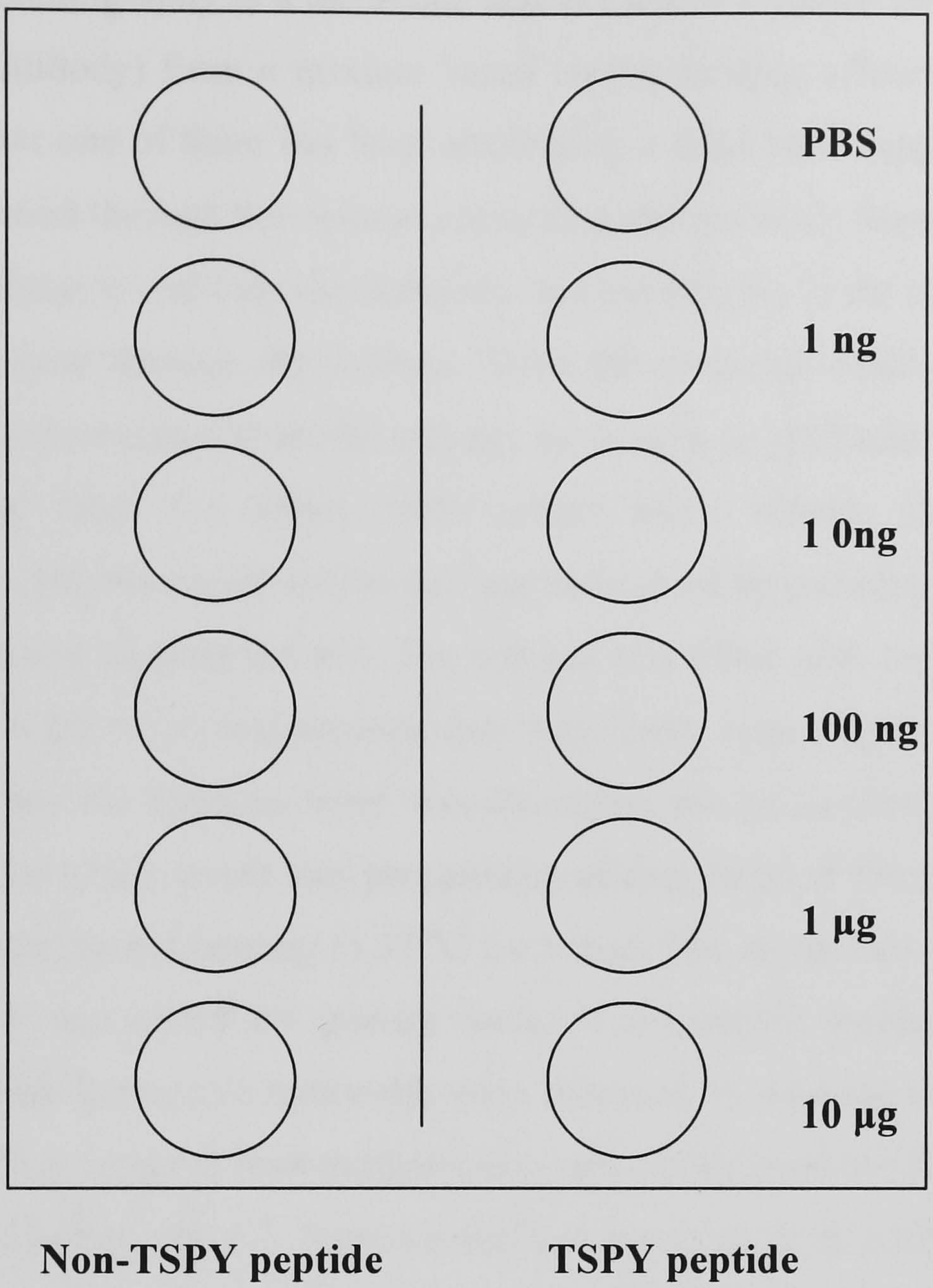


Figure 3.1. Schematic representation of dot blot

The presence of TSPY antibody in rabbit crude serum was tested using dot blot method. TSPY and non-TSPY peptide (TIP60) were serially diluted and dotted onto filters. PBS was used as negative control. Eight membranes were prepapred for every rabbit serum and various treatment protocols were carried out as following: (A) Rabbit serum diluted to 1:2000 was absorbed with 1µg TSPY peptide, (B) Rabbit serum diluted to 1:2000 was absorbed with 10µg TSPY peptide, (C) Rabbit serum diluted to 1:2000 was absorbed with 100µg TSPY peptide, (D) Rabbit serum diluted to 1:2000 was absorbed with 1 µg non-TSPY peptide, (E) Rabbit serum diluted to 1:2000 was absorbed with 10 µg non-TSPY peptide, (F) Rabbit serum diluted to 1:2000 was absorbed with 100 µg non-TSPY peptide, (G) Unabosorbed Crude immune serum was used as positive control and (H) pre-immune serum was used as negative control.

3.3.3.4 Affinity Chromatography

Affinity Chromatography is a technique that is used to separate and purify molecules (protein or antibody) from a mixture based on the binding affinity between the two molecules after one of them has been attached to a solid inert support substance. The mixture is passed through the column containing the molecule (ligand) attached to the stationary substance and only the molecule that has affinity to the ligand will bind and the rest will pass through the column. Once the molecule (antibody) of interest is captured, it is dissociated from the column by using low pH buffer. TSPY antibodies were purified from the rabbit crude serum using affinity chromatography. A disposable polystyrene column (Pierce) was assembled by inserting Polyethylene disk at the bottom and capping the end. The column was filled with 1ml of Affi-gel beads (Bio-Rad) and left to set and separate into two layers, upper aqueous and lower solid gel beads. Once the aqueous layer was discarded, the gel is washed in 10 ml TBS. Meanwhile, the rabbit serum was prepared by adding 10 µl of 5% sodium azide to the TSPY crude serum and heating to 37 ºC for 5 min. The serum was passed through the column twice and eluted by gravity force. Non specific molecules bound to the column or weak interactive molecules were removed by washing the column twice in 20 ml TBS. The captured immunoglobulins were eluted from the column by adding 8 ml of Elution buffer, pH 2.7. Approximately, 8 fractions, 1 ml each were collected in tubes containing 100 µl of neutralising buffer (1M Tris-HCL, pH 8; 1.5 M NaCl; 1 mM EDTA and 0.5 % azide). Only the first 3 fractions were pooled and added to the centriplus tubes (Peirs) and centrifuged for 45 min at 3000 rpm to concentrate the antibody. Aliquots of elutes were collected after each step, mixed in 750 µl of ddH_2O and 200 µl Bio-Rad protein assay kit and the optical density (O.D.) at 595 nm was reported.

3.3.4 Immunohistochemistry (IHC)

Immunohistochemistry is a method used for detecting, semi-quantifying and locating the presence of specific protein in tissues or cells. Fixed cells or tissues on glass slides are prepared and primary antibody is added to each slide to bind to its specific antigen. The slide is treated with a secondary antibody that specifically recognises the primary antibody and is linked to an enzyme. The immunoreactivity is detected by addition of enzyme chromogenic substrate and visualising the staining under the bright field

microscope. Purified TSPY specific peptide antibody was used to detect TSPY protein expression level using IHC method. Different paraffin embedded prostate sections from different patients on APES coated slides were immersed in xylene for 10 minutes, rehydrated in graded series of ethanol at 100%, 70 % and 50% ethanol and washed in ddH_2O for 5 minutes. Endogenous non specific hydrogen peroxide activity sites on the tissue sections were blocked in 0.5% H_2O_2 (SIGMA), prepared in 180 ml of methanol, for 10 minutes. The slides were washed again in ddH_2O and incubated in PBS until processed for antigen retrieval. A pressure cooker (PC) was filled with 1.5 litre of 10 mM Citrate buffer pH and heated in a microwave for 16 minutes until boiling. The slides were inserted into PC and placed in the microwave and heated for another 4 minutes. After the sections were cooled, non specific serum binding sites on the tissues were blocked in 10% normal goat serum prepared in PBS and incubated for 1 hr at RT. The optimal dilution of TSPY affinity purified peptide polyclonal antibody was determined by using serial purified TSPY antibody and sections from prostate cancer specimens found to express TSPY gene product. TSPY antibody was diluted 1:1000 in PBS and 200 µl of the it was added to the slides. The sections were incubated O/N at 4 °C. Unbound primary antibody was discarded and slides were washed twice in PBS for 10 min. The biotinylated goat secondary antibody (DAKO) generated against rabbit immunoglobulin was diluted 1:250 in PBS and 200 µl was transferred onto each slide and left at RT for 30 minutes. Excess unbound antibody was removed by washing twice in PBS for 10 min. Immunodetection was performed using Vectastain Avidin Biotin Complex kit (ABC kit, Vector laboratories, CA, USA) following the manufacture's instructions. Each tissue section was covered with 200 µl of Abiding–Biotin complex for 30 min at RT. Extra reagents were washed out twice in PBS. The immunostaining signal was detected using chromogen substrate Diamino-benzidine tetrachloride (DAB) solution which was prepared by dissolving a white urea tablet and brown DAB tablet (Sigma) in ddH_2O, and 200 µl was placed on each section for 20 minutes. The DAB reaction was stopped by inserting the slides in ddH_2O for 5 min. The sections were counterstained in Harris' Haematoxylin for 1 minute and destained in 1 % HCL diluted in Ethanol for 10 seconds. Excess stains were removed by sequential immersion of slides in ddH_2O. The sections were dehydrated, placed in xylene and mounted with DPX (BDH). The signal was detected using Leica light microscope at 10X magnification. The immuno-specificity of the TSPY antibody signal was

evaluated by using different approaches; omitting the primary antibody (TSPY antibody) and replacing it with PBS, substituting the antibody with pre-immune serum, immunoabsorbing the purified TSPY antibody using 100 fold molar excess of TSPY specific peptide and using female tissue sections (liver, lung and kidney) as negative controls. Positive immunodetecting using the purified TSPY antibody was further confirmed by using testis sections as a positive control.

3.3.4.1 Evaluation of immunohistochemical staining

The immunostaining signal intensity of hyperplastic and malignant prostate epithelium was evaluated semi-quantitatively by a pathologist and the following scoring system was adopted; a score of 0 was assigned if staining was absent in tumour cells, a score of + was assigned if weak stainings were detected in <10% of tumour cells, score ++ if moderate staining intensities were observed in 10-50% of tumour cells and score +++ if strong staining was seen in >50% of tumour cells.

3.3.5 Tissue *In Situ* Hybridisation (TISH)

TISH is a semi-quantitative method for detecting and localising a specific transcript within morphologically preserved tissues or cells preparations using labelled riboprobe.

3.3.5.1 Patient material

Slides of paraffin embedded prostate sections removed from patients suffering from CaP and PBH were collected from Freeman Hospital. Fifty eight cases of CaP and 30 cases of BPH were used in the current study.

3.3.5.2 Riboprobe synthesis:

An I.M.A.G.E clone number 173557 in pT3T7 vector harbouring 0.6 kb TSPY cDNA was ordered from HGMP resource centre. The vector was completely linearised using EcoR I or Not I. In eppendorf tubes, the following were added; 14 µl of ddH_2O, 2 µl of 10 X restriction buffer, 1 µl of 20 X BSA, 1 µg of DNA, 1 µl Not I (10 U/µl) or 1 µl EcoR I (8 U/µl). The tubes were incubated at 37 °C for 90 minutes. The digestion products were mixed with 3 µl of loading buffer and transferred into 1 % agarose gel.

3.3.5.3 Phenol extraction and ethanol precipitation.

Double distilled water was added to each sample to a final volume of 400 µl. Equal volumes of phenol : chloroform : isoamyl alcohol (25:24:1) were added to each sample and mixed. The sample was centrifuged for 3 minutes at 14000 rpm and the supernatant was transferred into a new tube. The DNA was precipitated by adding 40 µl of 3 M sodium acetate and 1 ml of 100 % ethanol. The tube was left at –80 °C for 10 minutes. Then, centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol and dried. The pellet was resuspended in 20 µl ddH_2O.

3.3.5.4 Digoxigenin labelling cDNA

One microgram of linearized plasmid was transferred into eppendorf tube containing 2 µl of 10 X transcription buffer (Roche), 2 µl 10 X Digoxigenin RNA labelling mixture, 0.5 RNAguard, 2.5 µl T3 or T7 RNA polymerase and DEPC ddH_2O to a final volume of 20 µl. The mixture was placed at 37 °C for 2 hrs. The synthesised riboprobe was ethanol precipitated and resuspended in 20 µl DEPC ddH_2O.

3.3.5.5 Riboprobe quantitation

A control RNA labelled riboprobe (Roche) and the TSPY synthesised riboprobe were diluted in DEPC ddH_2O into 20ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 0.1 pg/µl and 0.01 pg/µl. One microlitre from each dilution was transferred into Nylon membrane (Amersham). The membrane was baked at 80 °C for 2 hrs. Then it was washed in washing buffer for 2 min at RT. The membrane was blocked in 10 ml of blocking solution (1X washing buffer, 0.1 % Triton-X 100 and 5 % Marvel milk) for 30 min at RT. Then, an anti-digoxigenin-alkaline phosphatase was diluted to 1/1000 in a fresh blocking solution, added to the membrane and left shaking at RT for 1 hr. The membrane was washed twice in washing buffer for 5 min, incubated in 10 ml of NBT/BCIP at RT for few min and washed in ddH_2O for 5 min. The concentration of the riboprobe was extrapolated from the intensity and the concentration of the control dot.

3.3.5.6 *In Situ* Hybridisation

Paraffin embedded tissue sections from patients with benign and malignant tumours were dewaxed twice in 1 X xylene for 10 min, dehydrated twice in 100 % ethanol, once in 70 % and 50 % for 5 min. The sections were washed sequentially in 1 X PBS for 5 min, 1X PBS + 0.3 % Triton X-100 for 15 min and 1 X PBS for 5 min. The tissues were permeabilized using 20 µg/ml proteinase K (100 mM Tris, pH, 7.4 and 5 mM EDTA, pH, 8.0) at 37 °C for 30 min, then fixed in 4 % paraformaldehyde in PBS for 5 min. at 4 °C. The sections were washed twice in 1X PBS for 5 min with shaking and acetylated in 0.1 M triethanolamine and 0.25 % acetic anhydride for 10 min. The tissues were washed twice in 1 X PBS for 5 min. The sections were prehybridized at 50 °C for 1 hr in 50 % formamide, 10 % dextran sulfate, 1 X Denhard's solution, 4 X SSC, 10 mM DTT, 250 µg/ml *E. coli* t-RNA and 100 µg/ml sonicated salmon sperm DNA. A fresh hybridisation mixture containing 20 ng/ml TSPY Digoxigenin labelled riboprobe was added to each section and placed at 50 °C overnight. The tissues were washed at increased stringency in 50 % formamide containing 2 X SSC (0.3 M NaCl and 0.03 M Sodium citrate), 1 X SCC and 0.5 SSC at 50 °C for 30 min.

3.3.5.7 Immunodetection

The tissues were washed twice in washing buffer for 10 min at RT, then blocked in 1 X washing buffer + 5% normal sheep serum at RT for 30 min. A fresh solution containing 1 X washing buffer, 0.1% Triton-X 100, 1% sheep serum and an anti-Digoxigenin antibody conjugated with alkaline phosphatase at 1/500 dilution was added to the sections and left at RT for 2 hrs. The tissues were washed twice in washing buffer for 5 min. The sections were covered with NBT/BCIP colour substrate solution (0.34 mg/ml Nitroblue Tetrazolium salt, NBT and 0.17 mg/ml 5-Bromo-4-Chloro-3-Indoylphosphate, BCIP) and left overnight at RT, rinsed in TE buffer (10 mM Tris-Cl, pH, 8.0 and 1 mM EDTA), ddH_2O and counterstained in 0.1% nuclear fast red (12.5 g Aluminium Sulfate, SIGMA, 0.25 g Nuclear fast red, SIGMA and 250 ml ddH_2O).

3.3.6 WST-1 cell proliferation assay

A non-radioactive colourimetric assay for the quantitation of cell proliferation and viability. The assay is based on cleavage of Tetrazolium salt (WST-1) to Formazan dye by mitochondrial dehydrogenases (Figure 3.2). The amount of dye produced from viable cells is quantified by measuring absorbance at 450 nm using microtiter plate (ELISA) reader. The assay is carried out as follows: cells were detached, counted and $8x10^3$ cells in 100 µl of full media were placed in 60 wells of 96 well plate. The cells were incubated at 37°C for 24hr. Then, the cells were washed twice in PBS and 100 µl of basal medium was added. After, 48 hr of incubation, 10 µl of WST1 reagent was added to each well and left at 37°C for 1–4 hrs. The plate was inserted into microtiter plate (ELISA) reader using a filter suitable for wave length of 450 nm. In each assay, samples were performed in 18 replicates. The readings were subtracted from background control (blank). Dynex Revelation software was used to collect the data.

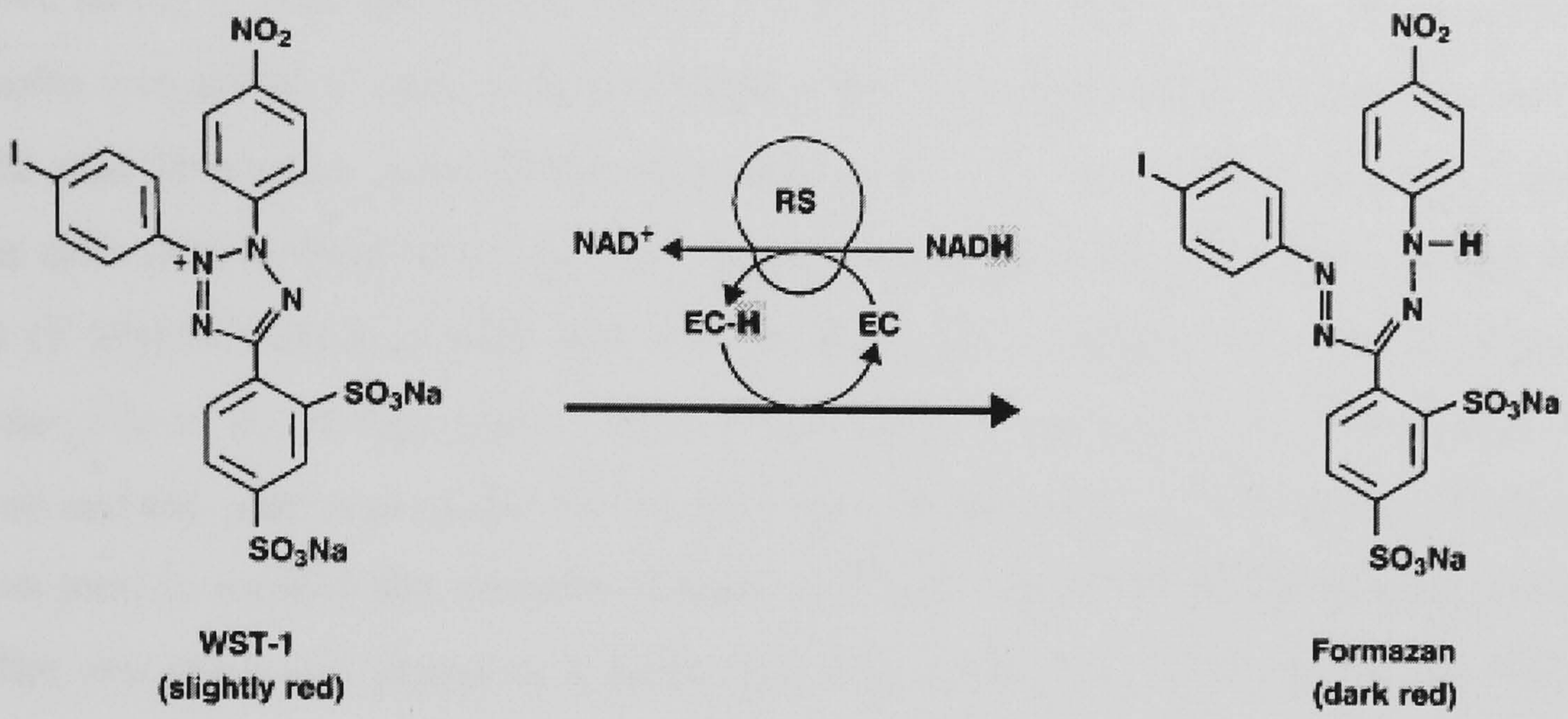


Figure 3.2. Cell proliferating WST-1 reagent

WST-1 assay is a non radioactive test that is an alternative to th thymidine incorporation assay. It is used to determine proliferation rate of viable cells. The assay is based on the cleavage of WST1 reagent (tetrazolium salt) by mitochondrial dehydrogenase to formazan that is darker in colour and can be quantified at specific wavelength.

3.3.7 ^{3}H Thymidine incorporation assay

The Thymidine assay is a very sensitive and simple assay. This assay measures the cell proliferation through incorporation of tritiated Thymidine into the DNA of replicating cells (S-phase). In this assay, cells were detached, counted and $8x10^3$ cells in 200 µl of full media was inserted in 60 wells of 96 well plate. The cells were left at 37°C for 24 hrs. Then, the wells were washed twice in PBS and 200 µl of basal media were added to each and left for another 24 hrs at 37 °C. The next day, 200 µl of full media was added to each well, combined with 0.6 µ MCi/well H^3 Thymidine and 1 µM cold Thymidine. After 24 hrs of incubation at 37 °C, the media was removed and the cells were washed twice in PBS. The cells were detached and lysed by adding 50 µl of Trypsin/EDTA to each well and the plate was incubated in 37°C incubator. After 1 hr of incubation, 200 µl of 12.5 % Trichloroacetic (TCA) was added to each well and the plate was cooled on ice for 2 hrs. An automated cell harvester (Wallac) was used to transfer the contents of each well onto a glass fibre filter (Wallac). The filter was dried and placed in a plastic bag filled with 5 ml of β-scintillation fluid. Then, the filter was inserted into a liquid scintillation counter and the level of β emission was determined and reported as count per second using MicroBeta software. Samples were performed in 24 replicates. The readings were subtracted from the readings of the blank control.

3.3.8 Colony formation assay

This assay determines the ability of single cells to grow in isolation and form colonies. Cells were trypsinised, counted, diluted and 500 cells were seeded into 100 mm dish in 10 ml full media plus selective media. Cells were left for 2-3 weeks to form colonies. The medium was removed and the cell were washed twice in PBS, and then fixed in 4 % paraformaldyhyde dissolved in PBS for 30 min with shaking. The cells were washed two times in PBS before adding 5 ml of 4% Methylene Blue (SIGMA) dissolved in 50% ethanol. The cells were left shaking for 30 min to stain. Cold water was used to remove excess dye, the cell were dried and the number of colonies formed were counted and colony formation efficiency was calculated by dividing the counted colony number by initial plated cell number, multiplied by 100. Samples were tested in triplicate and the assay was performed 3 times.

3.3.9 Immunofluorescence

Sterile cover glasses sized 22x22 mm were inserted in wells of 6 well plate. Cells were seeded on the cover glasses and left for 24-72 hrs at 37°C to grow. The medium was discarded and the cells were washed twice in ice cold PBS. Then, cells were fixed in 1 ml pre-cooled methanol at –20 °C for 20 minutes. The methanol was removed and the cells were air dried. The cells were blocked in 600 µl of I.F. blocking solution (10 % Goat Serum, 50 mM Tris and 0.4 % Triton-X) at RT for 1 hr. Then, 600 µl of Anti-FLAG M2 monoclonal antibody (SIGMA) diluted 1:200 in I.F. solution (0.3 % Goat Serum, 50 mM Tris and 0.4 % Triton-X) was added and left at 4 °C O/N. The next day, the cells were washed three times in 50 mM Tris solution for 5 minutes before adding 600 µl of Goat anti mouse immunoglobulin-FITC F $(ab')_2$ diluted 1:20 in I.F. solution. The cells were left at RT for 1 hr in dark. The cells on the cover glass were washed three times in 50 mM Tris solution for 5 minutes, dried and mounted on slides using Vectashield mounting medium with DAPI. The edges of the cover glass were sealed using nail vanish. The slides were examined using fluorescent Leica microscope.

3.3.10 Statistics

All statistical analyses were performed with the statistical software package SPSS. Fisher's exact test was used to compare different TSPY expression intensities between CaP and BPH, between CaP and clinico-pathological parameters. Correlation between immunoreactivity and age was carried out using Spearman's correlation test. Survival analysis was performed using the Kalpan-Meier Method and long rank test was applied to determine statistical significance. Proliferation rate comparison between clones with and without transfected TSPY were performed using Student's T test. P values of less than 0.05 were considered statistically significant.

3.4 Results

3.4.1 Validation of anti-TSPY antibody

A rabbit polyclonal TSPY anti-peptide antibody was generated as described in the materials and methods. Rabbit terminal bleeds were tested using ELISA, dot blot and Western blot. In the ELISA method, the curve of pre-immune serum shows basal detection levels binding activity. On the other hand, constant binding activity was detected using immunised rabbit terminal bleed crude serum from dilution 1:50-1:800, followed by a sharp decrease in antibody detection (Figure 3.3).

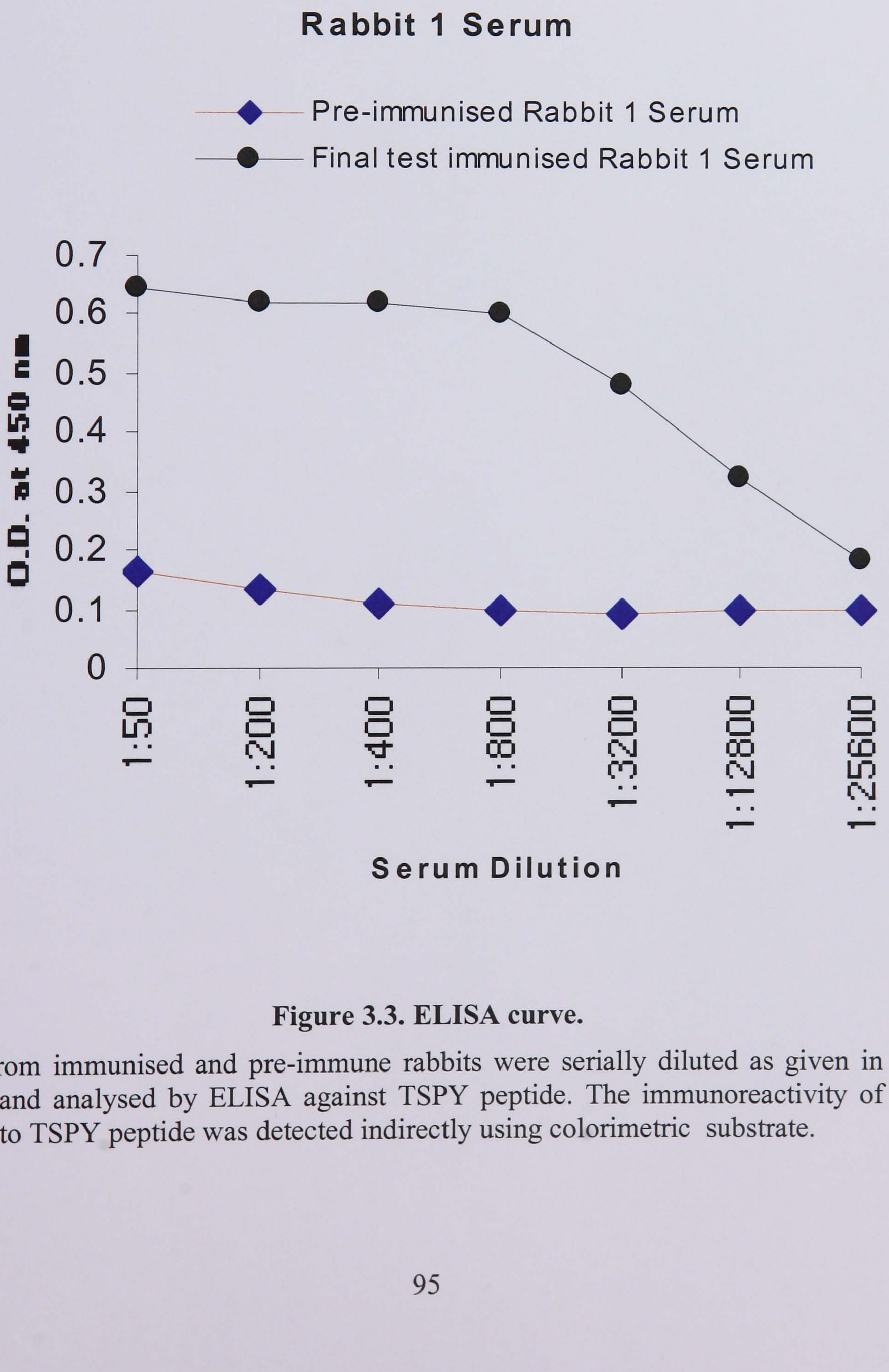


Figure 3.3. ELISA curve.

Sera from immunised and pre-immune rabbits were serially diluted as given in the graph and analysed by ELISA against TSPY peptide. The immunoreactivity of the serum to TSPY peptide was detected indirectly using colorimetric substrate.

3.4.2 Detection of rabbit TSPY polyclonal antibody using dot blot.

Successful generation of TSPY immunoglobulin against TSPY peptide was confirmed using dot blot analysis. Only the serum from immunised rabbit with or without absorption to non-TSPY peptide can detect TSPY bound to filter. (Figure 3.4)

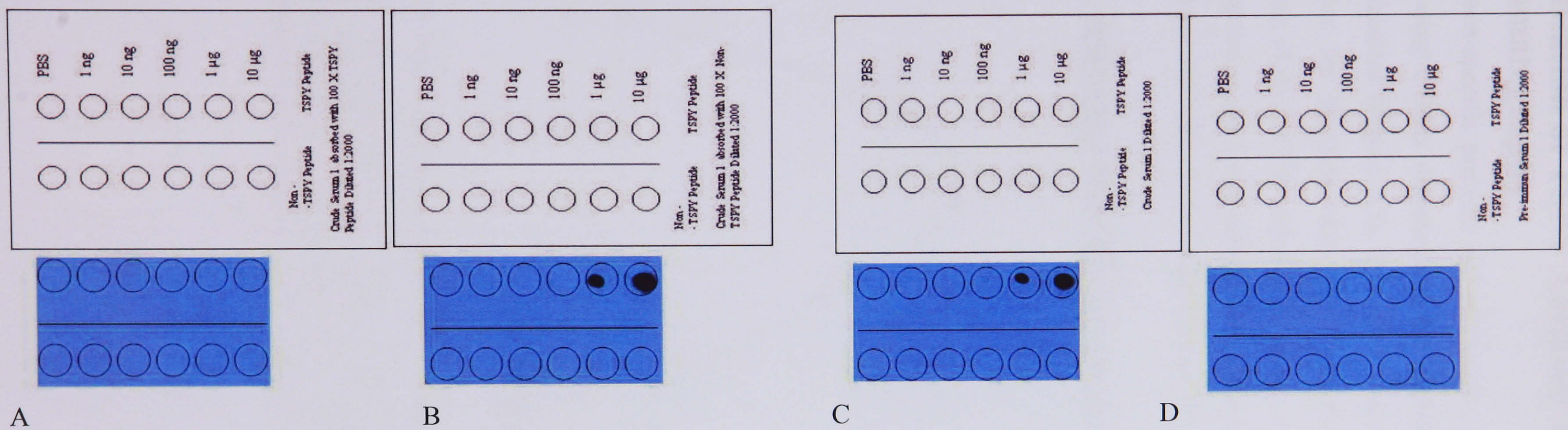


Figure 3.4. Detection of TSPY antibody in rabbit serum using dot blot technique.

TSPY specific peptide and non-TSPY peptide (TIP60) were serially diluted and dotted onto filters. Rabbit crude serum immunised with TSPY peptide was absorbed with 100 µg of TSPY peptide or non-TSPY peptide and added onto membranes. (A) No signals were detected with serum absorbed with TSPY. (B) Signals were detected when serum absorbed with non-TSPY peptide was used. (C) Immunised rabbit crude serum was used as control. (D) Pre-immune serum was used as negative control.

3.4.3 Purification of TSPY Antibody by Affinity Chromatography

To eliminate non-specific proteins and antibodies in the serum, TSPY rabbit serum was purified using affinity chromatography. Eluted immunoglobulins from affinity column was detected (Figure 3.5). The specificity of the purified antibody and the optimal dilution for Western blot was tested using PC3M cell line transiently transfected with FLAG-TSPY and empty vector. A clear specific protein band for TSPY was detected in the lysate derived from cells transfected with FLAG-TSPY but not in cells transfected with empty vector control (Figure 3.6).

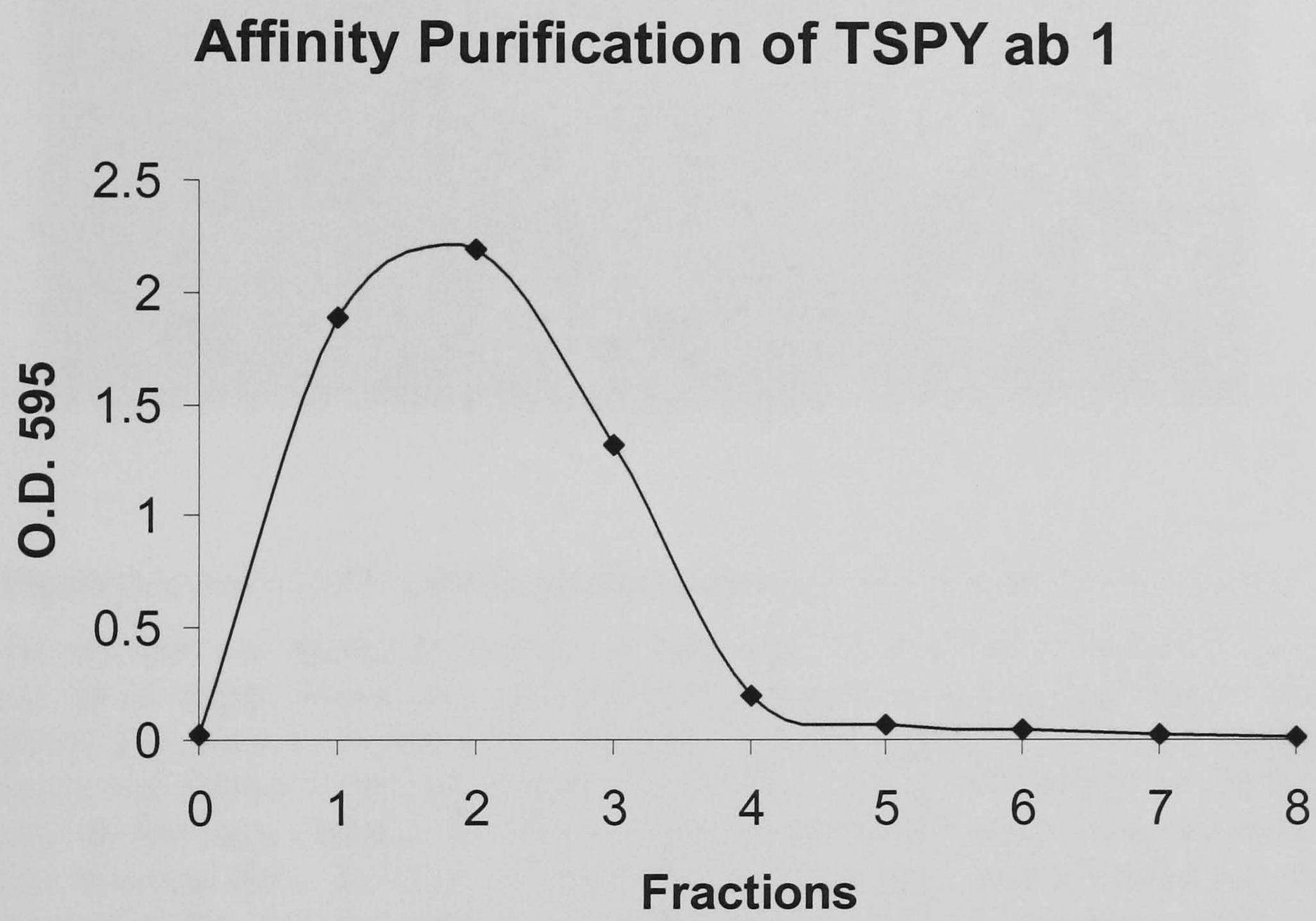


Figure 3.5. TSPY antibody purification using Affinity Chromatography.

TSPY antibody was purified from rabbit crude serum using affinity chromatography method. Eight fractions were collected from eluted solution and each fraction was mixed with Bio-Rad protein detection kit to determine the faction containing TSPY antibody. Only the first 3 fractions were pooled together and concentrated.

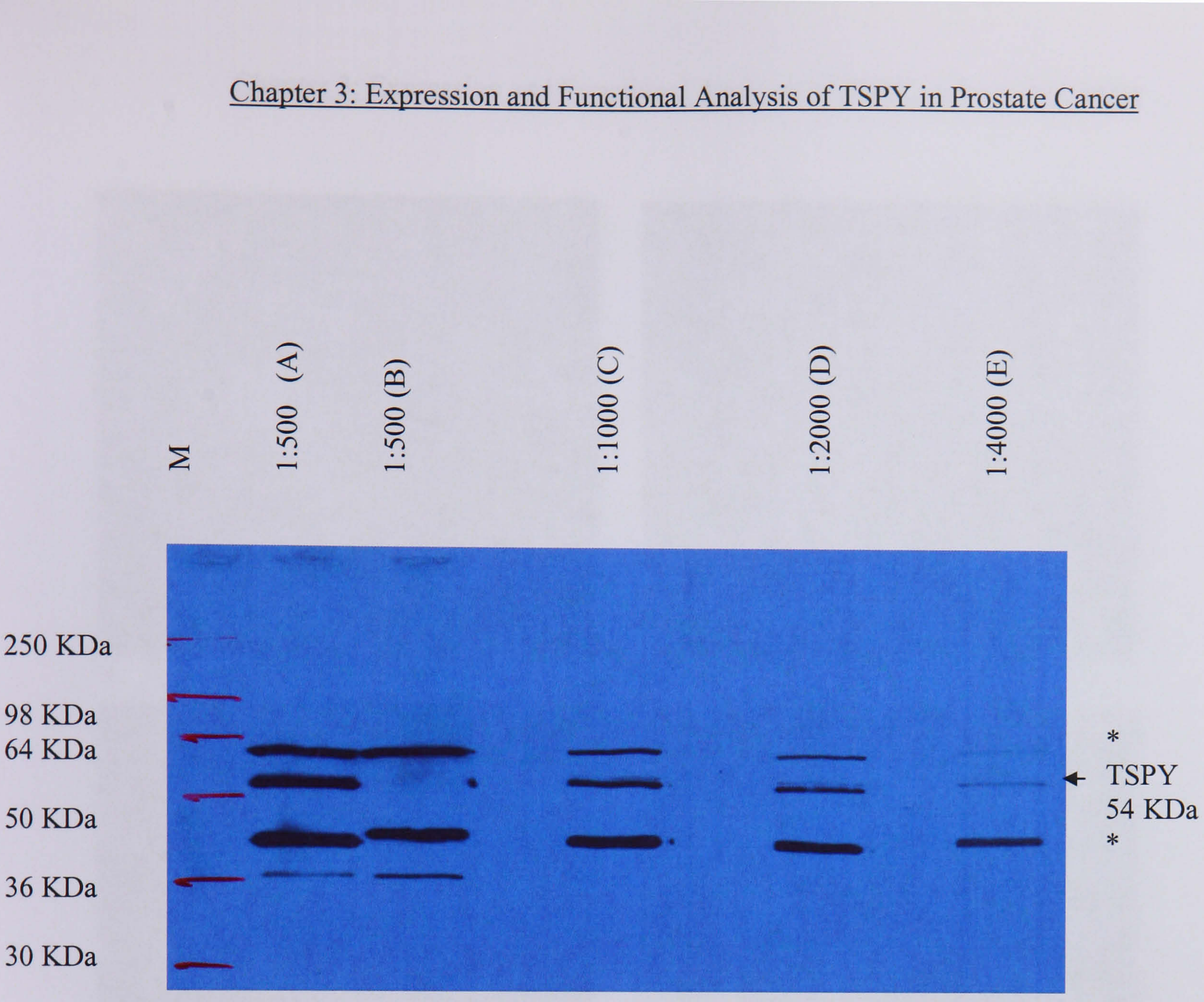


Figure 3.6. Anti-TSPY affinity purified antibody titration for Western blot.

PC3M cell line was transiently transfected with either FLAG-TSPY–PCDNA3 (A, C, D and E) or empty vector (B), and left for three days to grow. Cell lysates were collected and used in Western blot analysis. Affinity purified TSPY anti-peptide antibody was diluted 1:500 (A), 1:1000 (C), 1:2000 (D) and 1:4000 (E) and was used as primary antibody. Dilution 1:1000 was selected as the optimal dilution and used in further Western blots. SeeBlue standard protein broad range marker (BioRad) was loaded in lane M. Top and bottom bands (*) represent non-specific proteins detected by antibody.

3.4.4 Cellular localisation of TSPY

Cellular localisation of FLAG-TSPY transiently transfected into LNCaP cell line was determined using indirect immunofluorescence. Cells transfected with FLAG-TSPY were compared to clones transfected with empty vector (negative control). Cellular localisation was determined after 3 days. FLAG-TSPY was found to be located in the cytoplasm in all transfected cells. Strong peri-nuclear membrane located TSPY was observed (Figure 3.7).

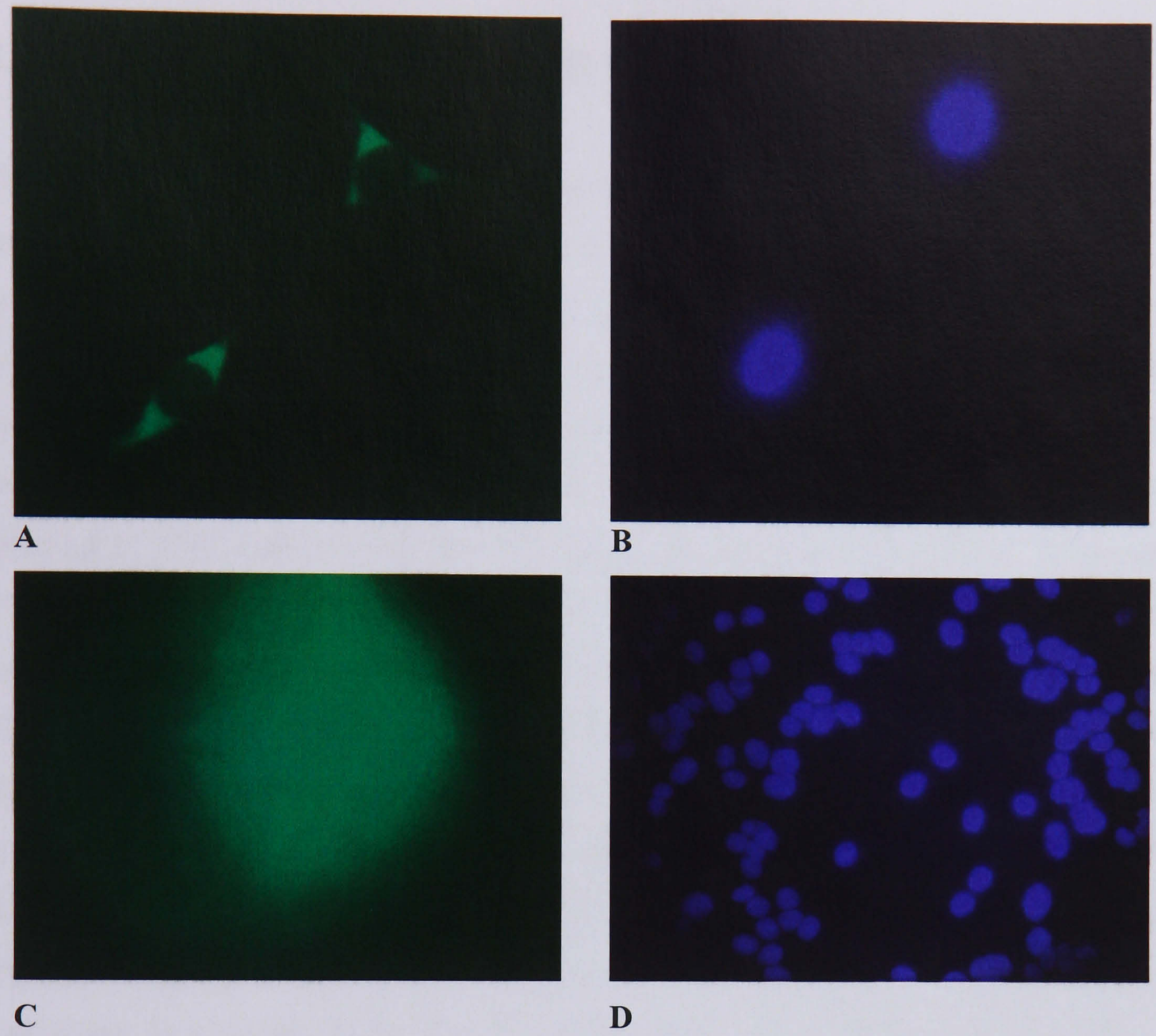
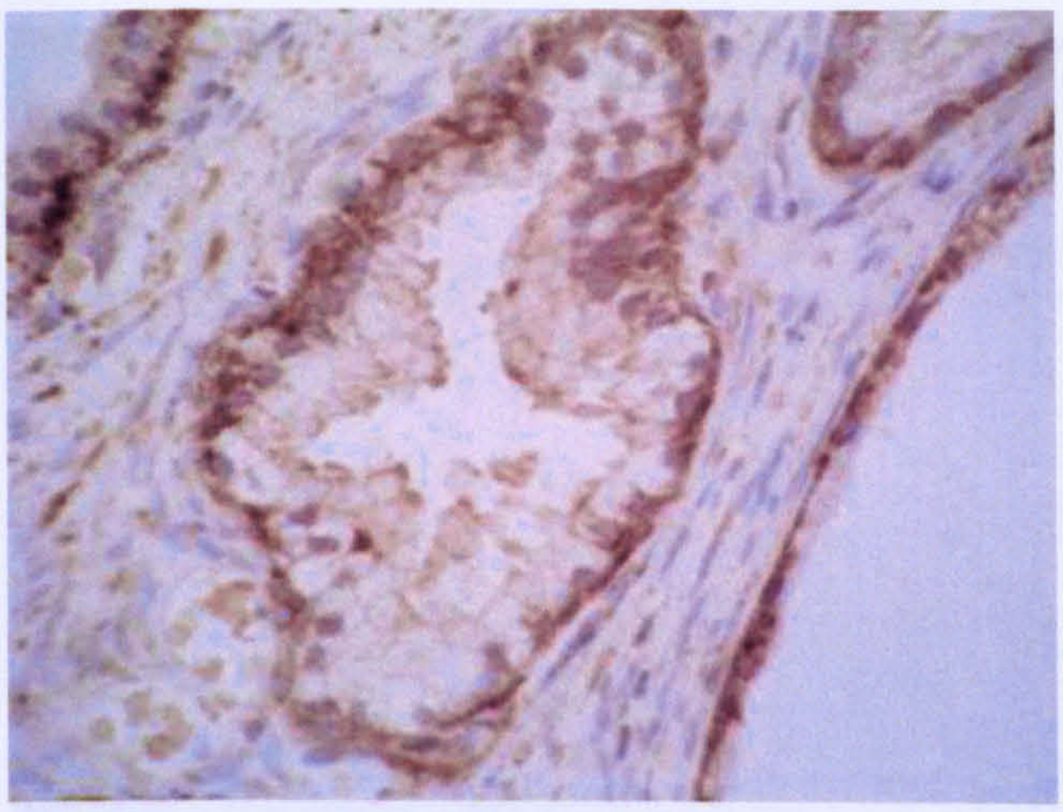


Figure 3.7. Cellular localisation of FLAG-TSPY in LNCaP cells

LNCaP cells were transiently transfected with either FLAG-TSPY or empty vector (negative control) and left to grow for three days. Anti-FLAG monoclonal antibody was diluted and used to detect cellular localisation of recombinant TSPY protein. Cytoplasmic fluorescence signal was detected in LNCaP cell line expressing exogenous FLAG-TSPY (a). Nuclear DAPI staining of cells (b). No background signal was detected in the negative control. Other controls were included such as omitting the primary antibody, secondary antibody or both antibodies (data not shown). LNCaP transfected with empty vector (c). Neclear DAPI staining of cells (D).

3.4.5 Immunodetection of TSPY expression in clinical samples

The optimal dilution of the affinity purified anti-TSPY peptide specific rabbit polyclonal antibody was determined using a series of paraffin embedded tissue sections from one BPH patient. Dilution 1:1000 was the optimal concentration. This concentration was used in all subsequent IHC to determine the level of TSPY expression in CaP compared to BPH cases. In total, 92 cases of prostate paraffin embedded sections were used for immunostaining, 72 cases were from patients affected by CaP and 20 cases were from patients suffering from BPH. In general, TSPY immunoreactivity was present in all BPH and CaP tissue sections. The expression was predominantly localised to cytoplasm of the epithelium in CaP and BPH. The expression profile in BPH was different from CaP (Figure 3.8). In all BPH cases, moderate cytoplasmic expression was detected within the glandular prostate epithelial basal cells and the expression within secretory epithelial cells was weak to absent. Similar trends of expression were also observed in histological normal and hyperplastic granular prostate epithelium adjacent to prostate malignant epithelium in CaP cases. In contrast, in the majority of CaP cases, the expression was moderate to strong in tumour cells. Statistical analysis has confirmed that TSPY was preferentially over-expressed in CaP compared with BPH ($P<0.0001$) (Table 3.1). The expression intensity was correlated to the clinical and pathological parameters available. The 72 CaP were sub-divided according to Gleason grade into six cases of Gleason grade 2-5 and 66 cases of Gleason grade 6-10. The expression was weak in only 1 case with Gleason 2-5 and 3 cases with higher grade (6-10). The expression was moderate in five cases with G 2-5, but strong expression was not detected (Table 3.1). Moderate to strong expression was detected in 63 high grade CaP cases. A positive statistically significant correlation between TSPY expression and Gleason score was confirmed ($P<0.02$). In the CaP group, 26 patients had positive bone scans signifying the presence of bone metastasis. Increased TSPY expression was significantly associated with the presence of bone metastasis ($P<0.028$) (Table 3.1). Pathological stages for most of the cases were available. TSPY expression was not found to be associated with clinical stage ($P<0.4$). In addition, in this small cohort of patients with prostate cancer, no correlation was observed between TSPY expression and survival outcome or patient age (data not shown).



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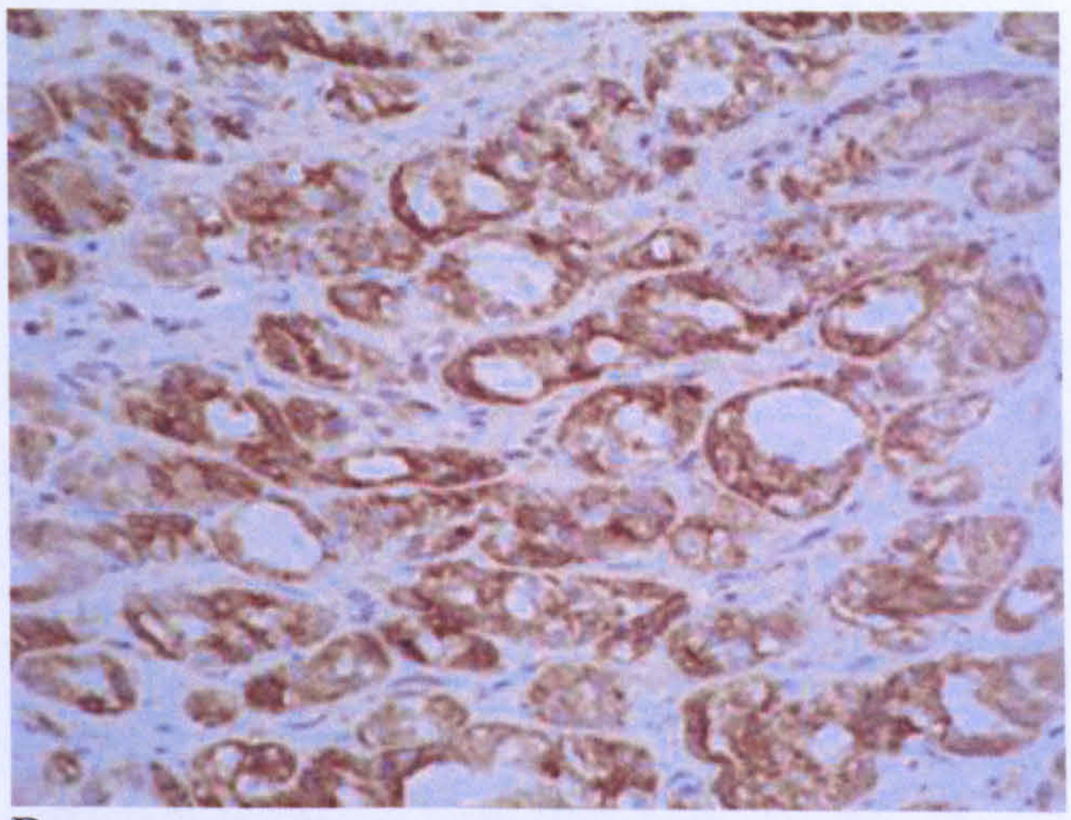

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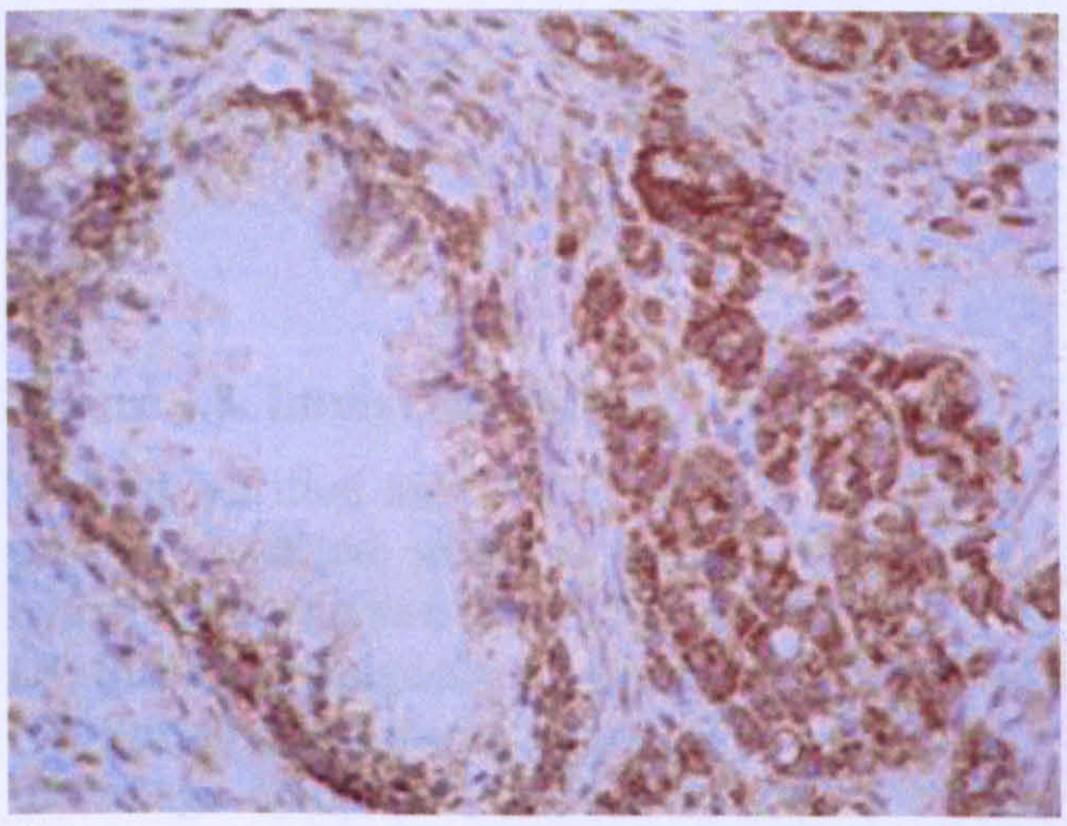

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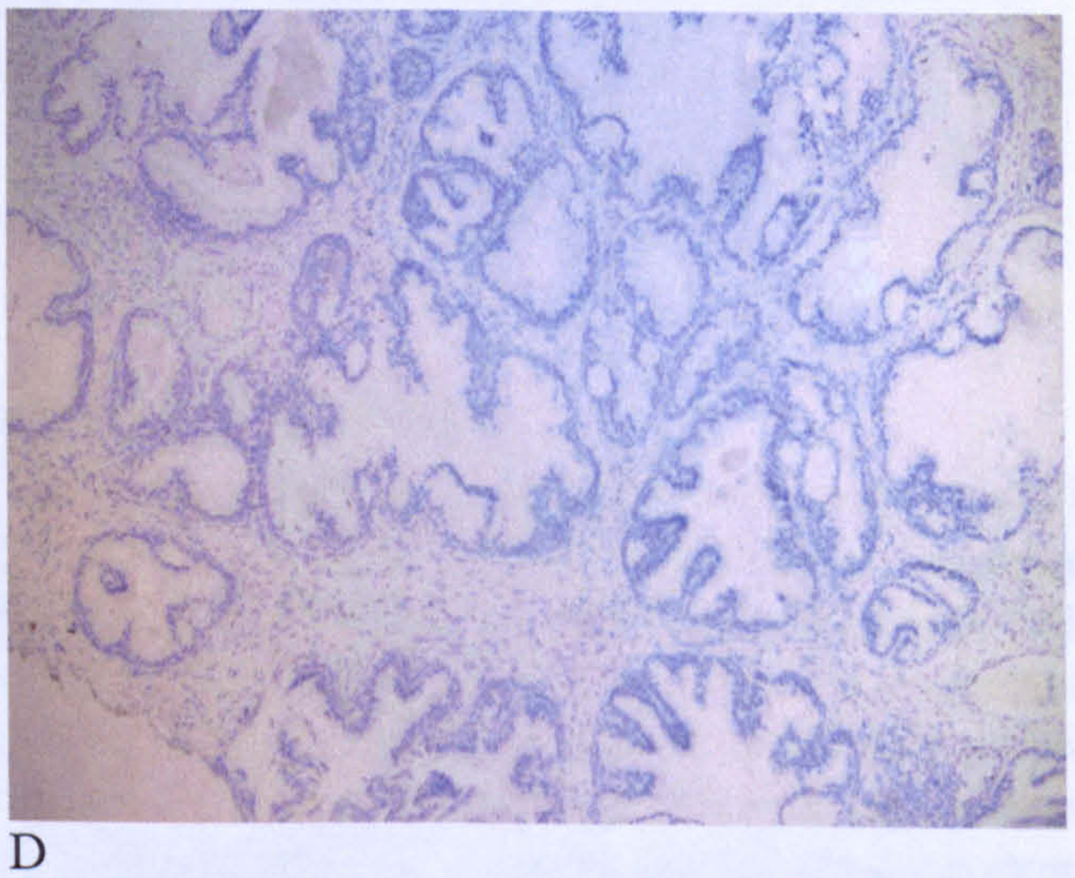
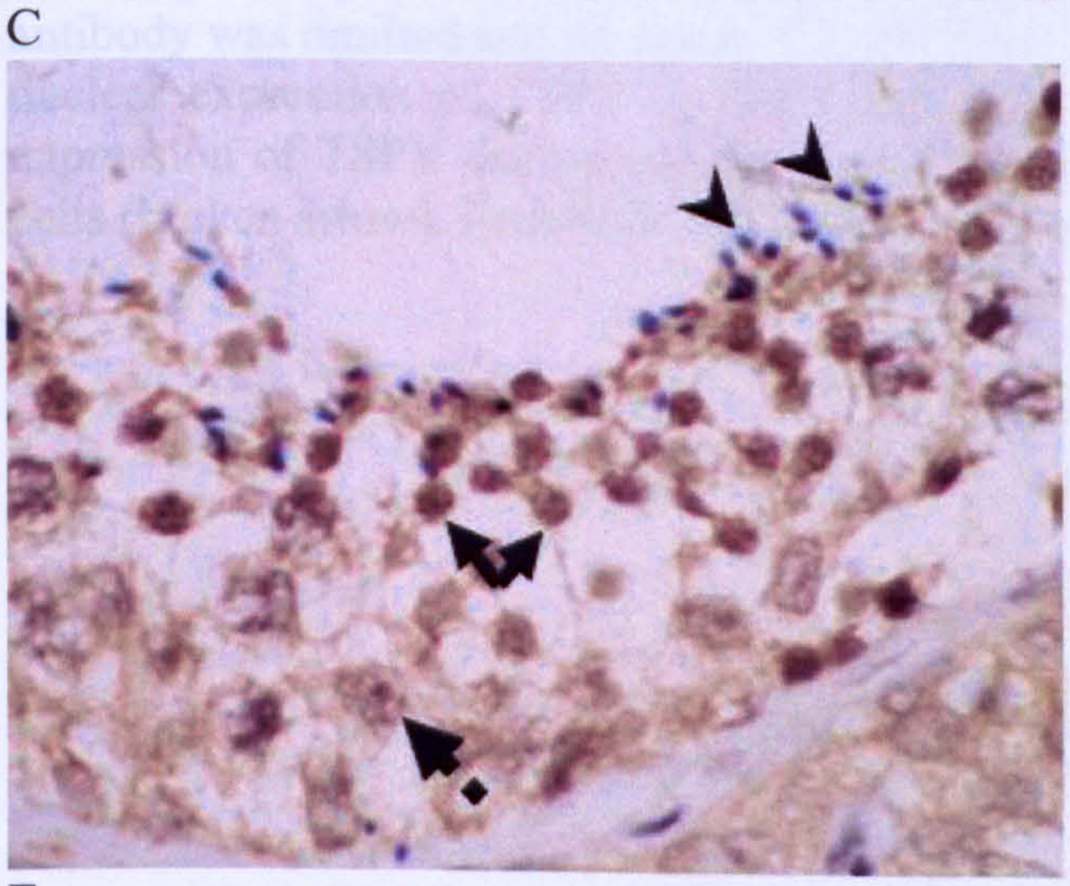
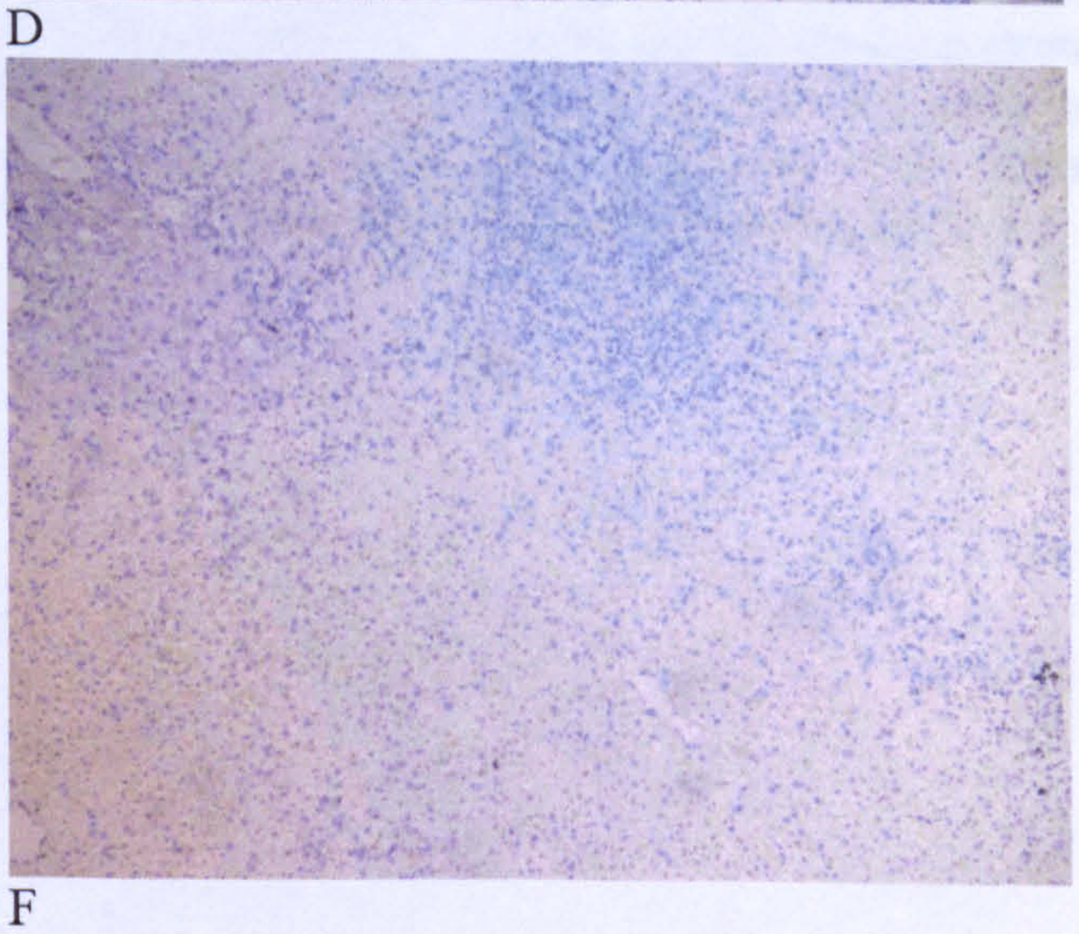


D



E



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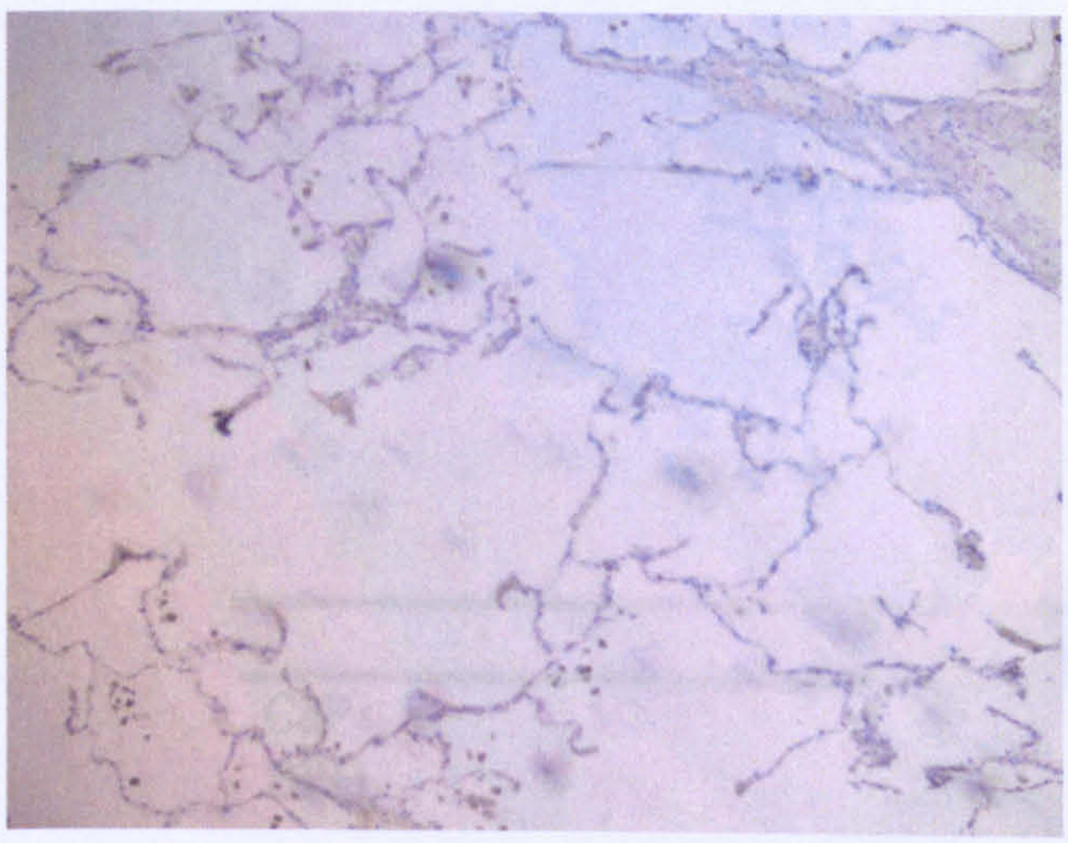
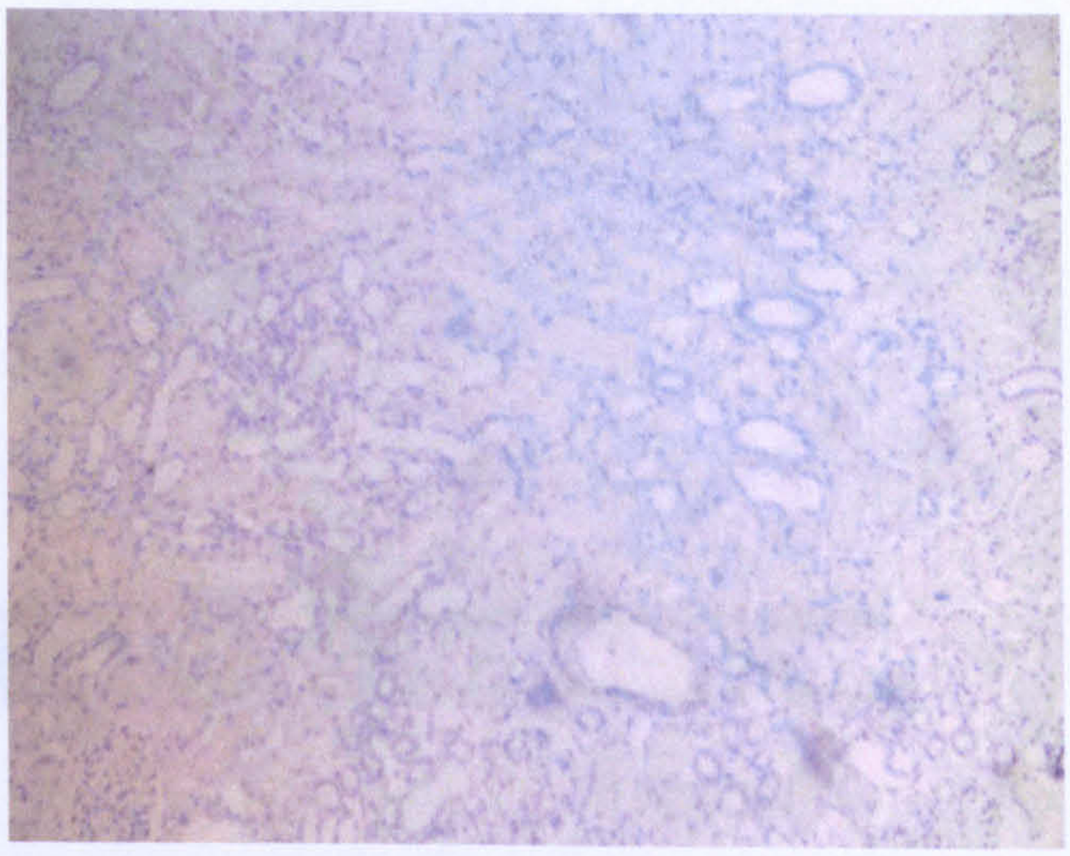


G



H

Figure 3.8. Immunostaining of prostate sections using TSPY antibody.

Affinity purified anti TSPY peptide was used in IHC method to determine TSPY expression level in prostate resected sections from patients with BPH and CaP. (A) BPH gland showing moderate cytoplasmic expression of TSPY in basal cells, with minimal expression in the sceretory epithelial cells. (B) Strong cytoplasmic expression in Gleason grade 3 prostate cancer, no evidence of nuclear expression. (C) Strong cytoplasmic expression of TSPY in Gleason 4 prostate cancer as shown in the right side of the image compared to the adjacent BPH gland on the left. (D) BPH section in which the primary antibody was omitted and no signal was detected. (E) Section of testis as positive control showing strong nuclear expression of TSPY in speramtocytes (double headed arrow), mature speramtides show no expression of TSPY (arrow heads), weak nuclear and cytoplasmic expression is present in the sertoli cells (broken arrow). Female liver (F), lung (G) and kidney (H) tissues were used as negative controls.

		Weak	Moderate	Strong	P -value
CaP		4	40	28	
BPH		20			P<0.0001
Gleason score					
2-5 G	(n=6)	1	5		
6-10 G	(n=66)	3	35	28	P<0.026
Metastasis					
Bone scan	(n=26)		12	14	
None	(n=29)	3	18	8	P<0.028
Clinical Stage					
T1	(n=11)	1	6	4	
T2	(n=14)	1	7	6	
T3	(n=16)		12	4	
T4	(n=8)	1	4	3	P<0.44

Table 3.1. Immunostaining pattern of TSPY in prostate.

TSPY expression intensity was classified into weak, moderate and strong. Prostate cancer cases were categorised according to expression profile, clinical and pathological data available. Statistical significance correlation between expression level and Gleason score, metastasis or clinical stage was determined using Fisher's exact test.

3.4.6 TSPY mRNA expression in clinical samples

3.4.6.1 Probe synthesis

TSPY sense and anti-sense riboprobes were generated using *in vitro* transcription (Figure 3.9). The riboprobe was diluted, semi-quantified using dot blot by comparing the intensity of riboprobe signal to standard control with known quantity of labelled probe (Roche) (Figure 3.10).

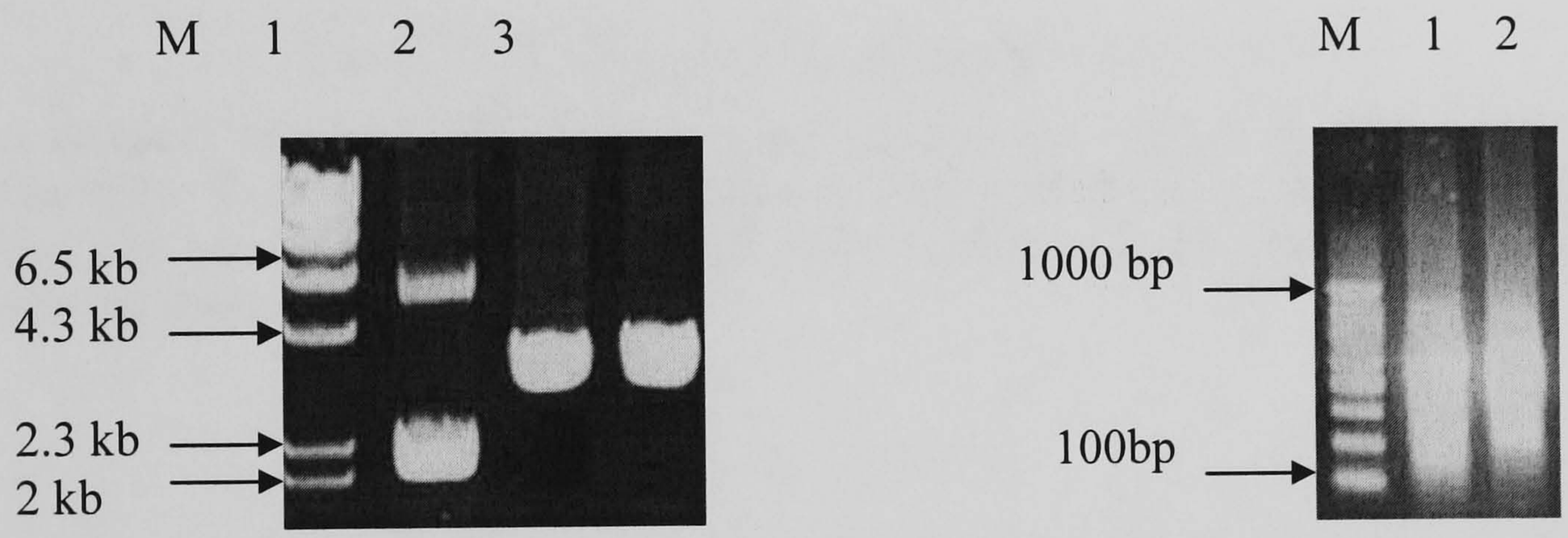


Figure 3.9. Generating labelled probes

Expression and cloning vector harbouring partial TSPY gene sequence including 3' end was linearised using EcoR I or Not 1 (A); Hind III cut Lamda DNA marker (M); lane 1, uncut plasmid; lane 2, EcoR I cut plasmid and lane 3, Not I cut plasmid. (B) Labelled riboprobe product (M) 1kb ladder marker; lane 1, sense riboprobe; lane 2 anti-sense riboprobe.

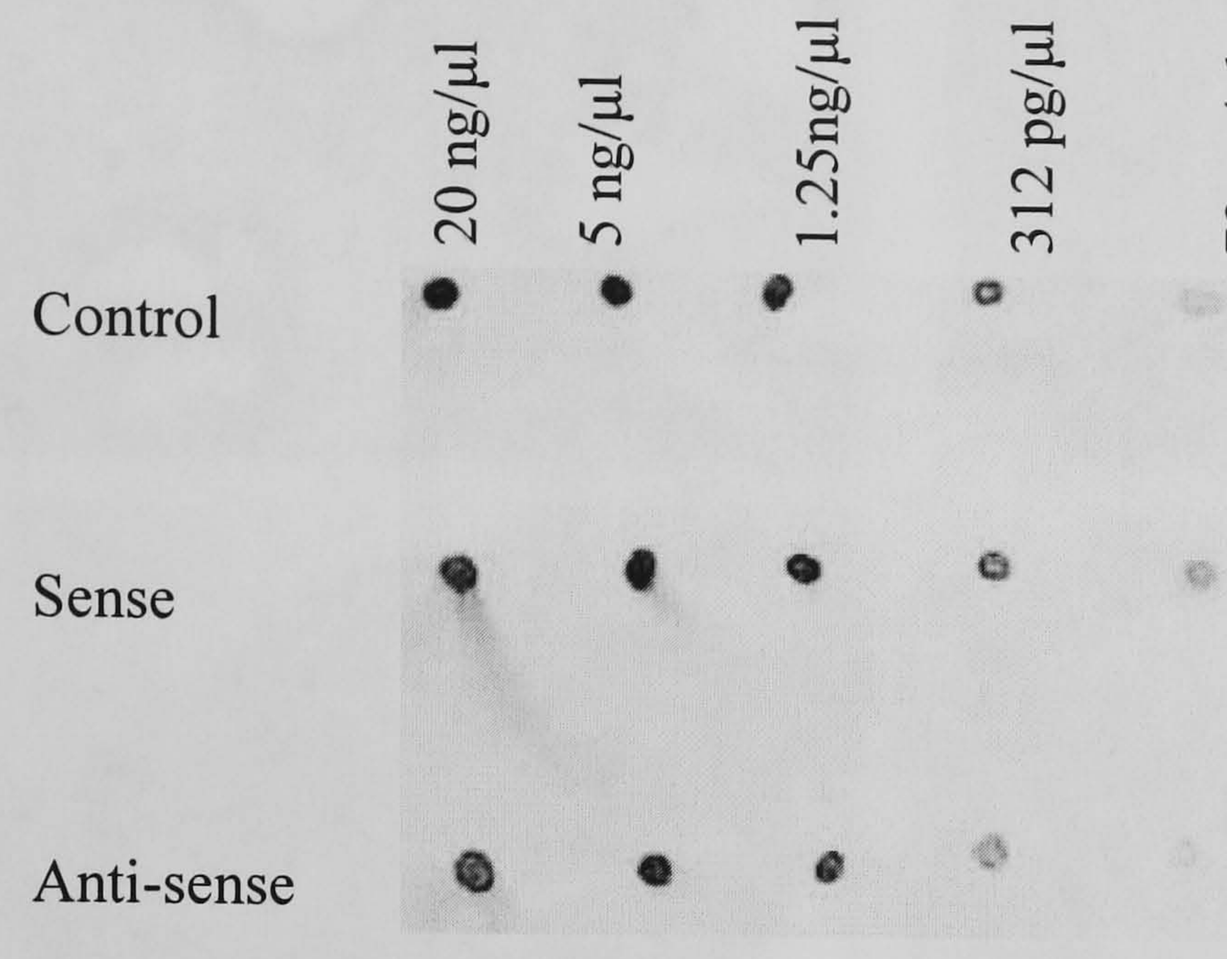


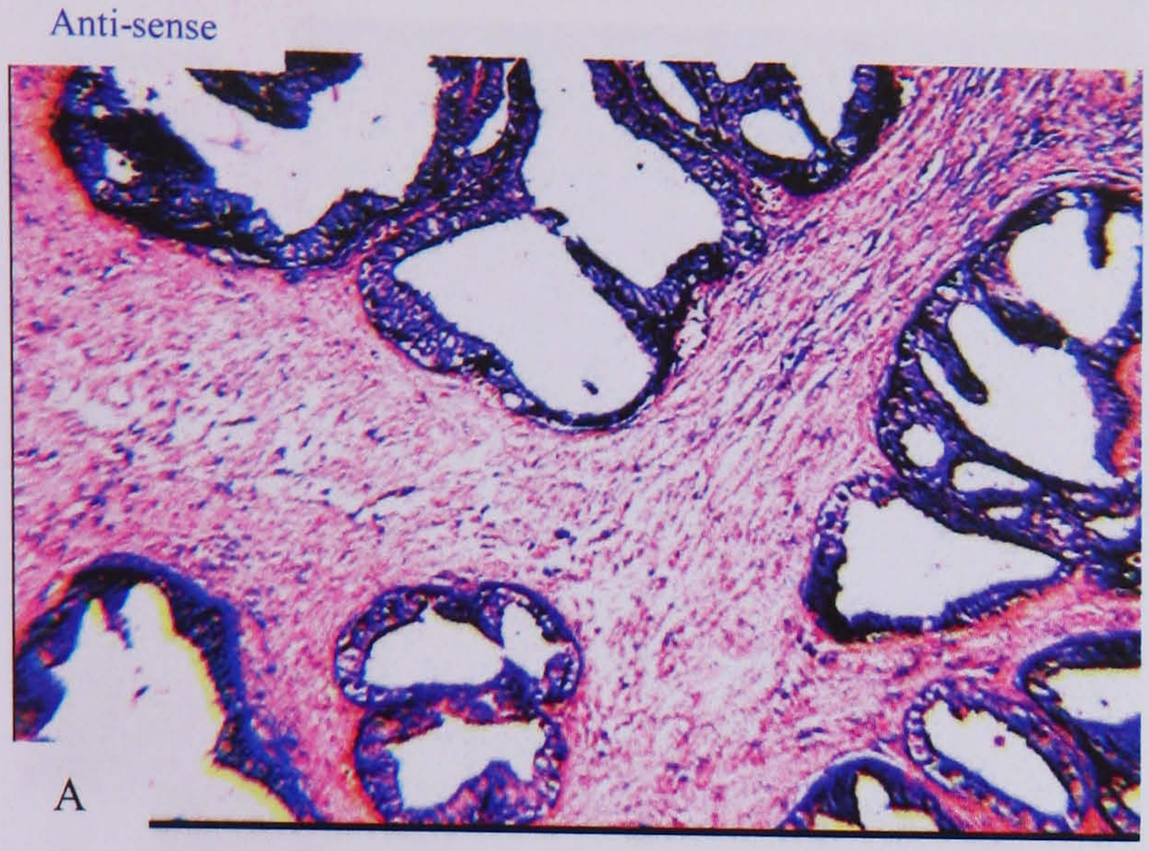
Figure 3.10. Riboprobe quantitation using dot blot.

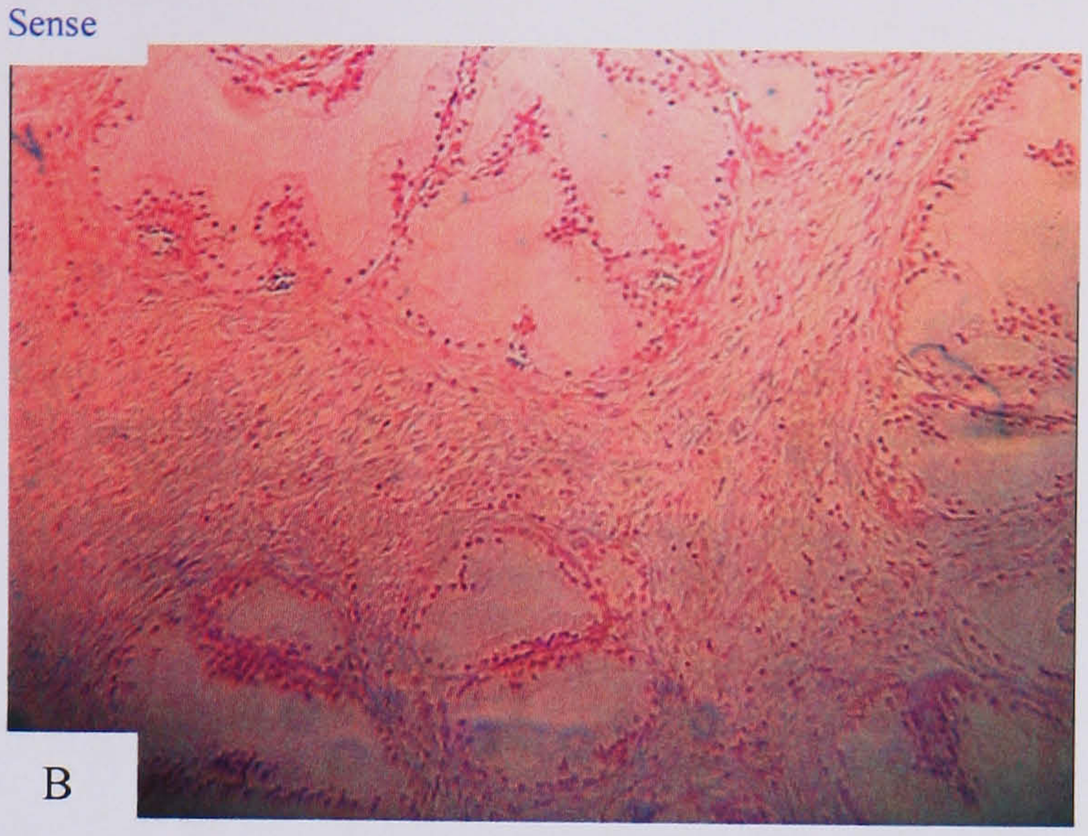
A standard labelled probe of known concentration was diluted serially 1:4 and dotted onto filter. Sense and anti-sense riboprobes were also diluted and dotted on the same filter and an estimate of synthesised riboprobe quantity is determined by comparison with the control.

3.4.6.2 TSPY mRNA expression in prostate

For each case of CaP or BPH, both sense and anti-sense riboprobes were generated. In keeping with IHC analysis, TSPY mRNA expression was observed predominantly in the prostate epithelium and a low level of non–specific signal was detected in the stroma (

Figure **3.11**). The mRNA expression was located in the basal cell and luminal cells of the benign epithelium in BPH and CaP. Similar to immunodetected TSPY expression, significant signals were observed in the malignant epithelium. Hence, the pattern of TSPY expression at both protein and mRNA levels are consistent.

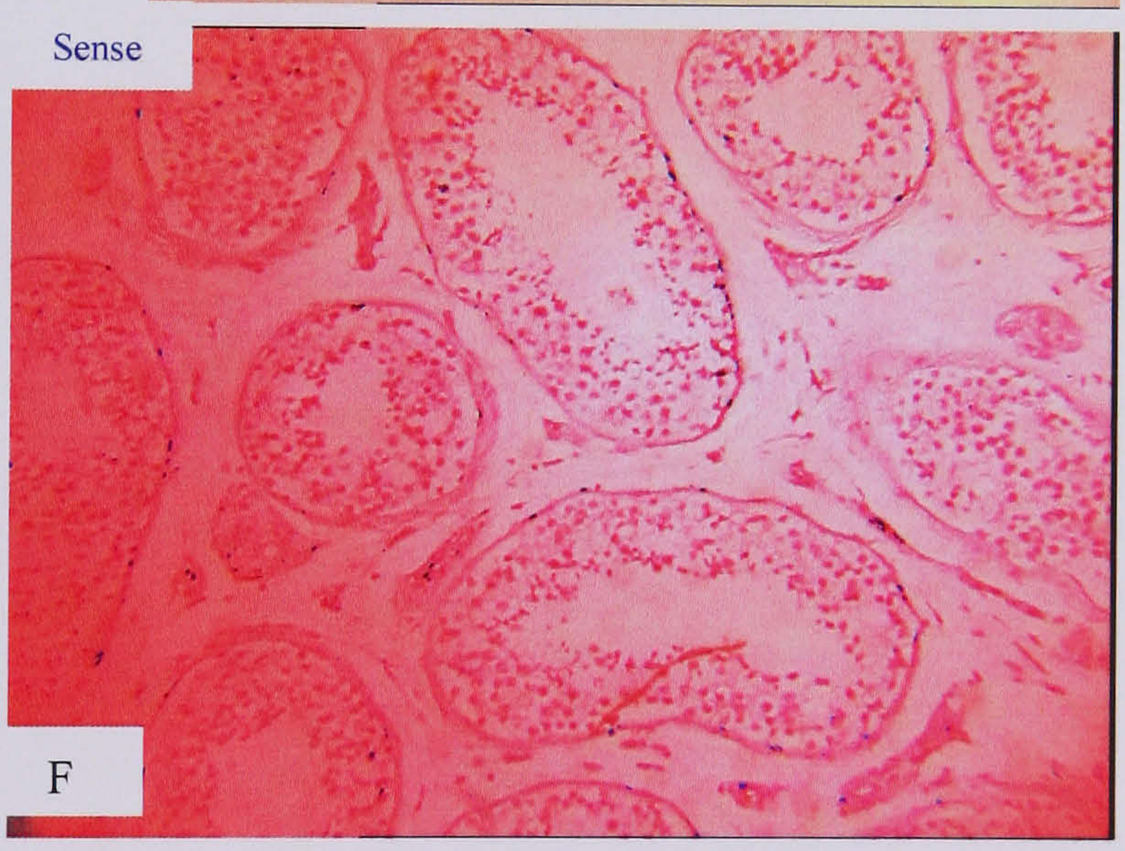

Anti-sense
A

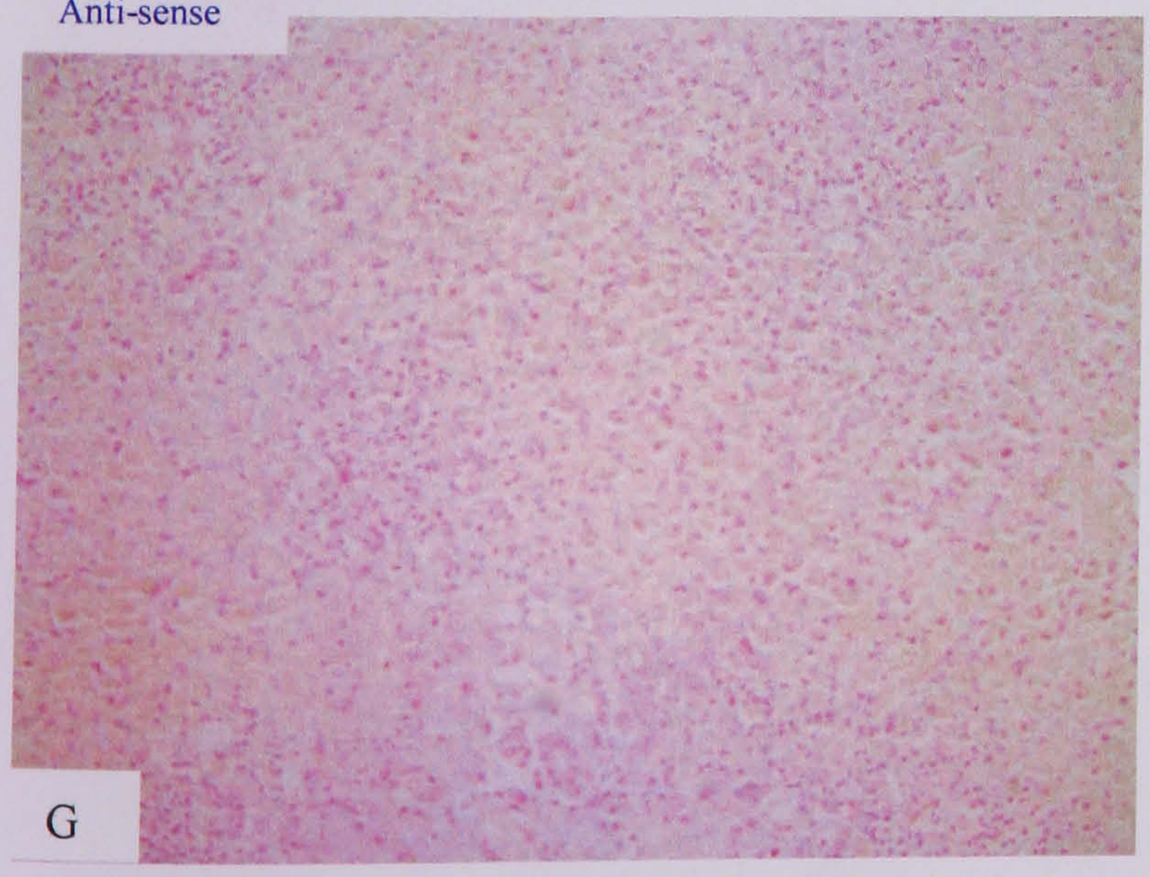

Sense
B

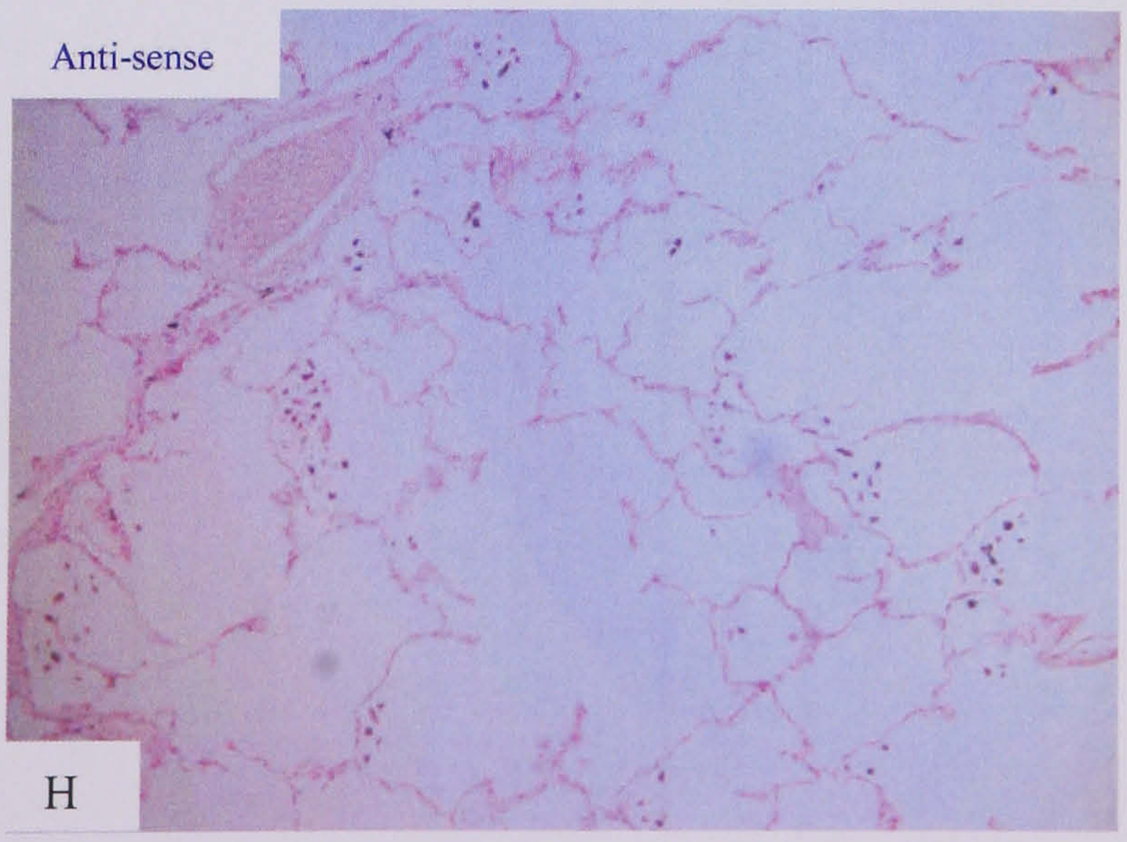

Anti-sense
C


Sense
D


Anti-sense
E


Sense
F


Anti-sense
G


Anti-sense
H

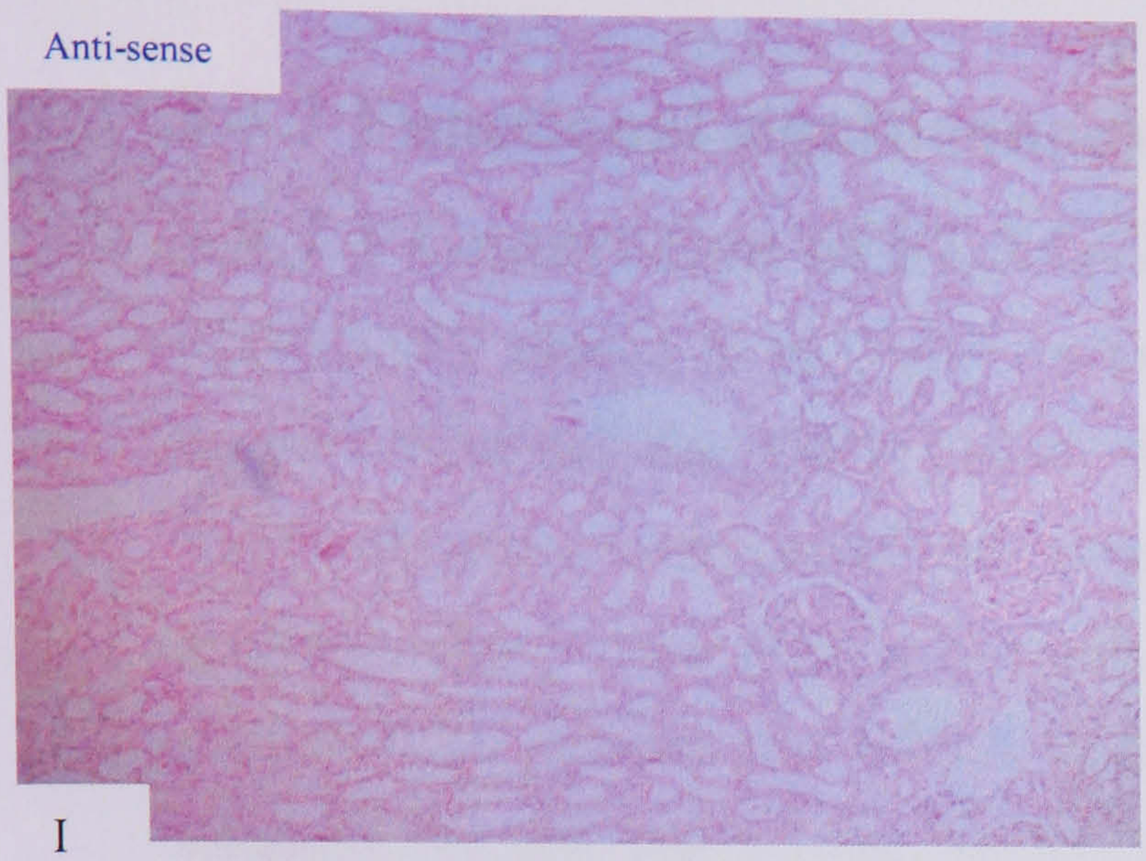


Figure 3.1. TSPY expression pattern in prostate sections using TISH.

Expression patterns of TSPY mRNA and tissue location were detected in CaP and BPH using TISH method. Anti-sense probe and sense probe (control) were used for each case. The expression of TSPY transcript was detected in the epithelium of BPH gland (A). TSPY was expressed in basal cells and luminal cells. Non-specific stromal staining was observed. Expression of TSPY was also shown in the malignant epithelium (C). Normal testis sections were used as positive control (E). Female tissue sections from liver (G), lung (H) and kidney (I) were used as negative controls.

3.4.7 Functional significance of TSPY over-expression

3.4.7.1 Generation of FLAG-TSPY LNCaP expressing cells

FLAG-TSPY cloned in pCDNA3 vector was transfected into the human prostate cancer LNCaP cell line. Stable LNCaP clones were selected, screened and tested for TSPY expression using anti-FLAG monoclonal antibody. In total, 70 clones were screened using Western blot analysis and 14 clones expressed TSPY at variable levels (Figure 3.12). Clones that contain empty vector were also selected and used as reference for comparison. Two positive clones were selected for functional analysis (Figure 3.13).

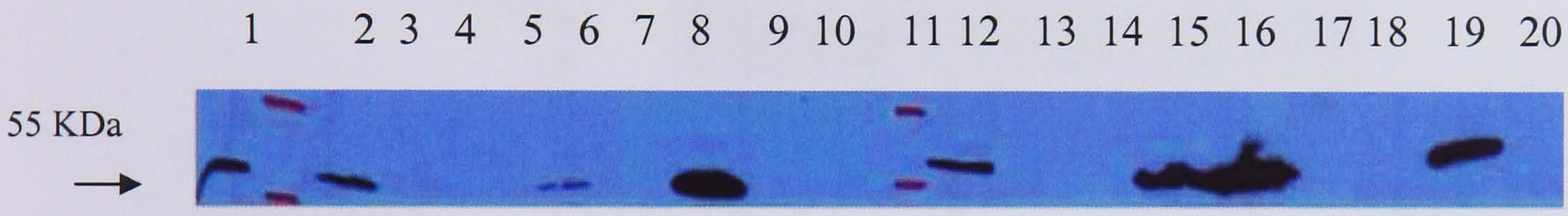


Figure 3.12. Screening LNCaP stable clones for expression of FLAG-TSPY

LNCaP stable clones transfected with FLAG-TSPY were screened for expression of exogenous TSPY using mouse monoclonal anti-FLAG-antibody. LNCaP transiently transfected with FLAG-TSPY was used as positive control (1 and 12). LNCaP transiently transfected with empty vector was used as negative control (10 and 20). Clones that either expressed TSPY or not were identified (3-9 and 13-19). SeeBlue protein marker was used (2 and 11)

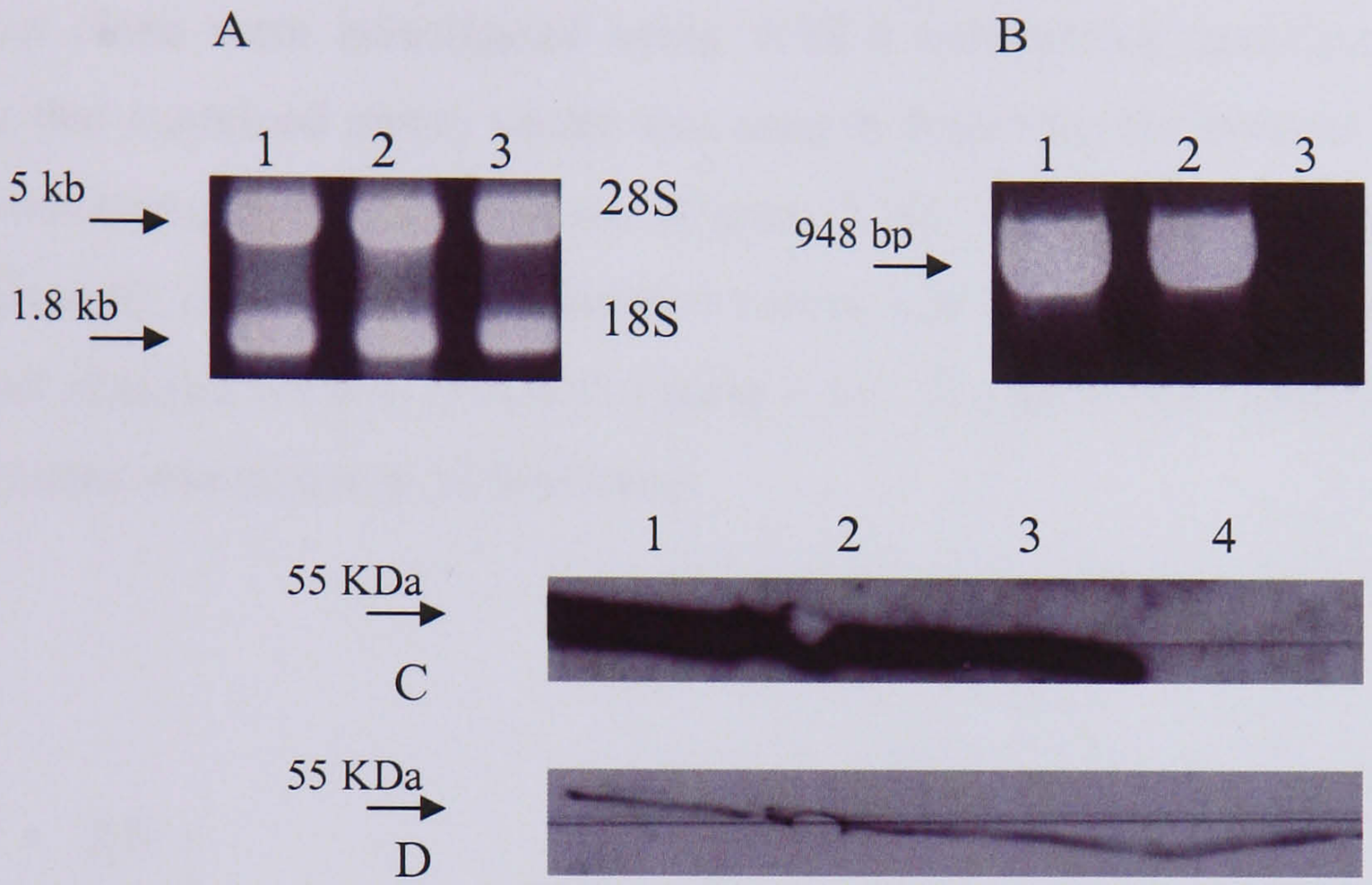


Figure 3.13. Selected FLAG-TSPY expression clones

Quality of total RNA isolated from LNCaP stable clones expressing FLAG-TSPY and one clone with empty vector were checked using agarose gel electrophoresis (A). The presence of recombinant TSPY was assessed using FLAG-forward primer and TSPY reverse primer in RT-PCR (B). Lane 1 and 2 are stable clones expressing FLAG-TSPY and lane 3 is an empty vector as a control. Western blot analysis of two stable clones expressing FLAG-TSPY and empty vector (C). Lane 1, transiently transfected FLAG-TSPY into LNCaP cell line, Lane 2, 3, 4 are two stable clones and one control clone respectively. The membrane was stripped and probed with α-tubulin antibody to check for loading (D).

3.4.7.2 TSPY over-expression promotes *in vitro* LNCaP cell growth

1) WST-1 assay

The proliferative rate of two independent clones expressing FLAG-TSPY and a control clone were investigated using WST-1 colorimetric proliferation assay. The clone that contained empty vector was used to determine the optimal seeding number and incubating time for the assay (Figure 3.14). Presence of exogenous TSPY has significantly increased the proliferative rate of LNCaP by nearly two fold compared to control (Student's t test, $P<0.000$, Figure 3.15). The assay was performed 6 times and each clone was plated in 18 replicates.

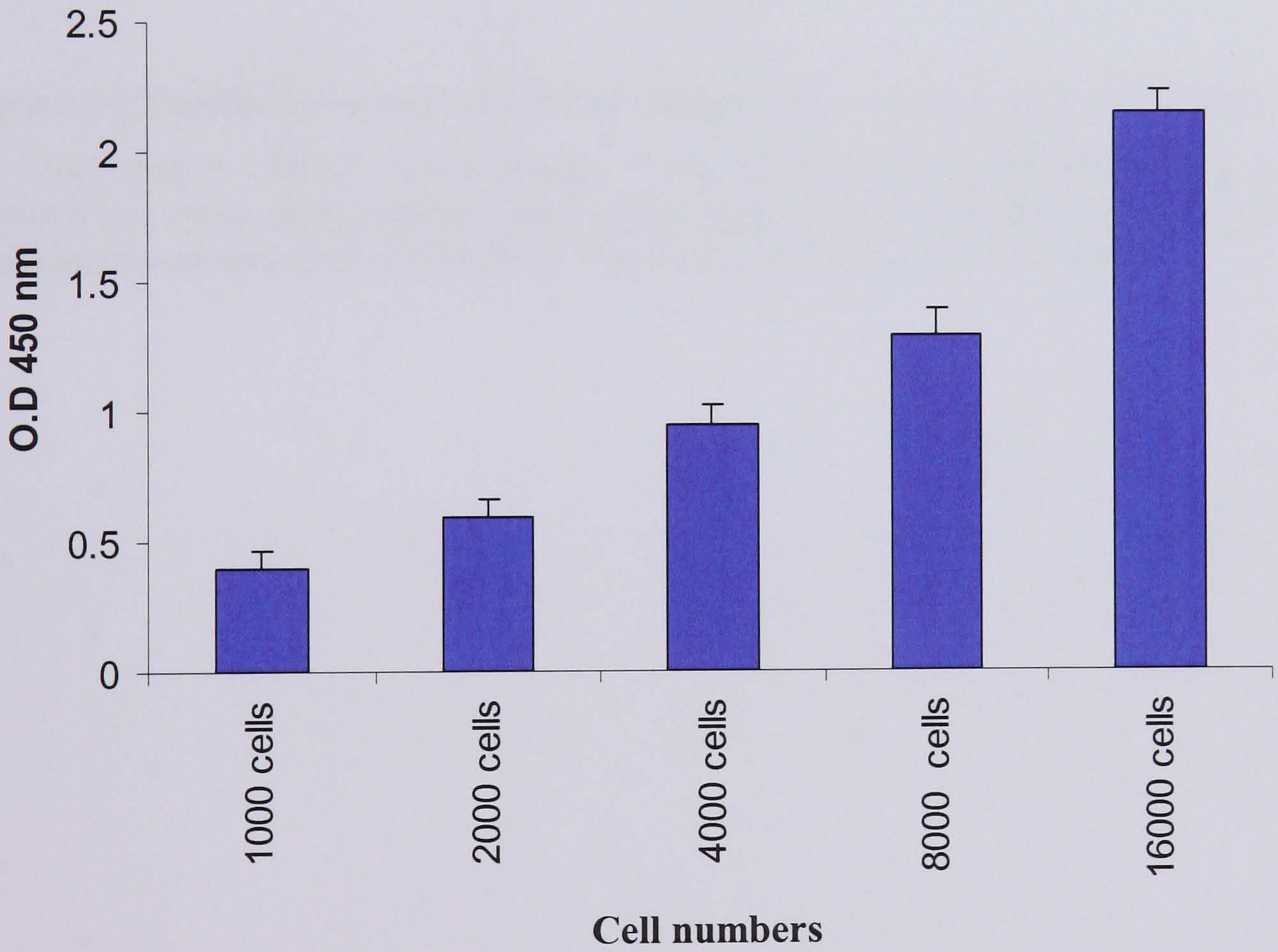


Figure 3.14. Cell proliferation assay.

Optimisation of cell number to be seeded for cell proliferation assay. LNCaP Cells were seeded as indicated and left for 24 hr to adhere, followed by serum starvation for 24 hr prior to addition of full medium for 24 hr followed by adding WST-1 reagent. Cell number 8000 was the optimal.

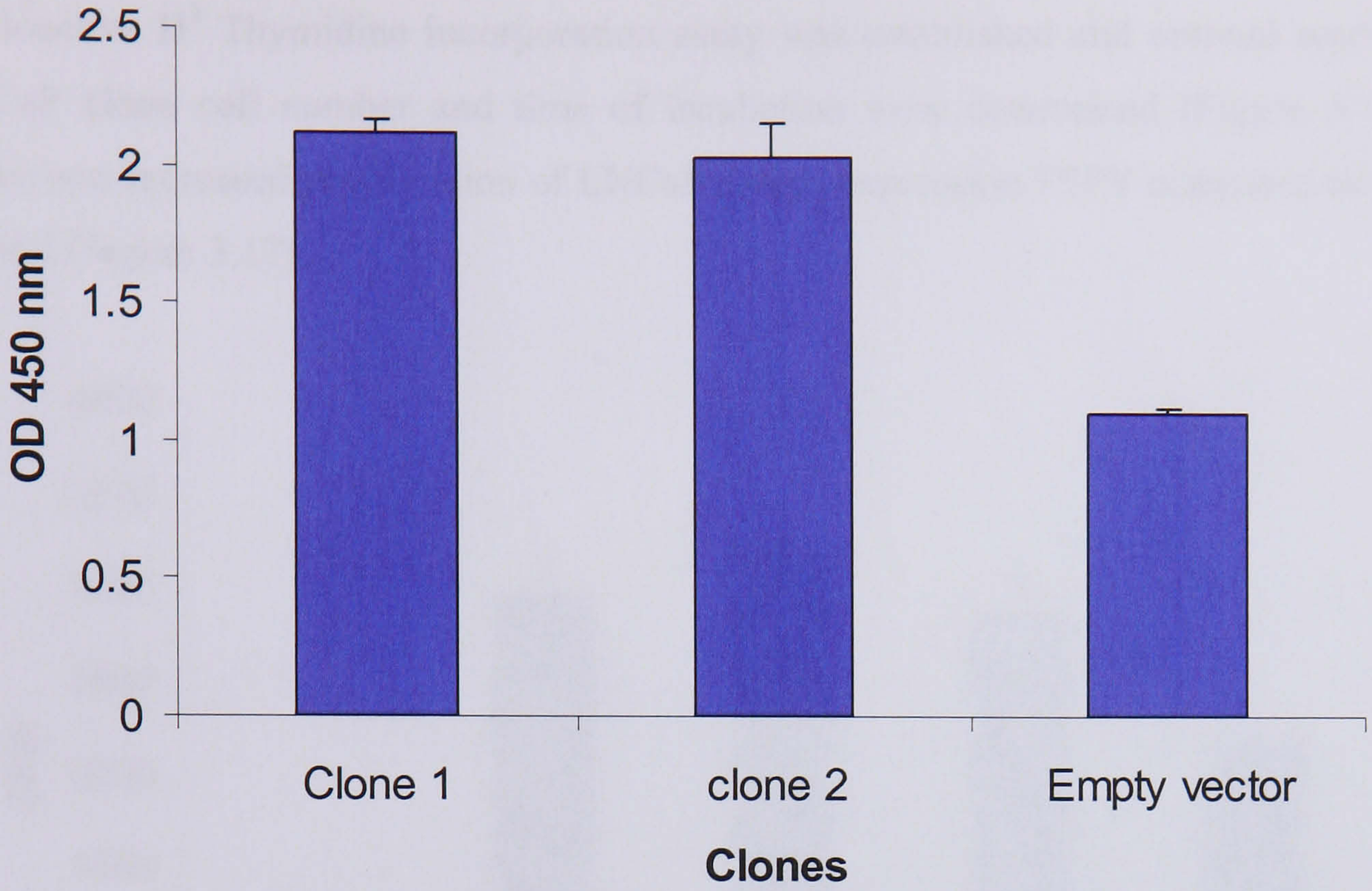


Figure 3.15. Proliferation rate of LNCaP clones over-expressing FLAG-TSPY.

Two independent LNCaP stable clones were selected and proliferation rate was compared to a clone that contained only stable empty vector. Two fold increase in cell proliferation was observed ($P<0.0001$). The assay was performed six times.

2) Thymidine incorporation assay for DNA synthesis

Radioactive H^3 Thymidine incorporation assay was established and optimal seeding LNCaP clone cell number and time of incubation were determined (Figure 3.16). There was increased proliferation of LNCaP clones expression TSPY compared to the control (Figure 3.17).

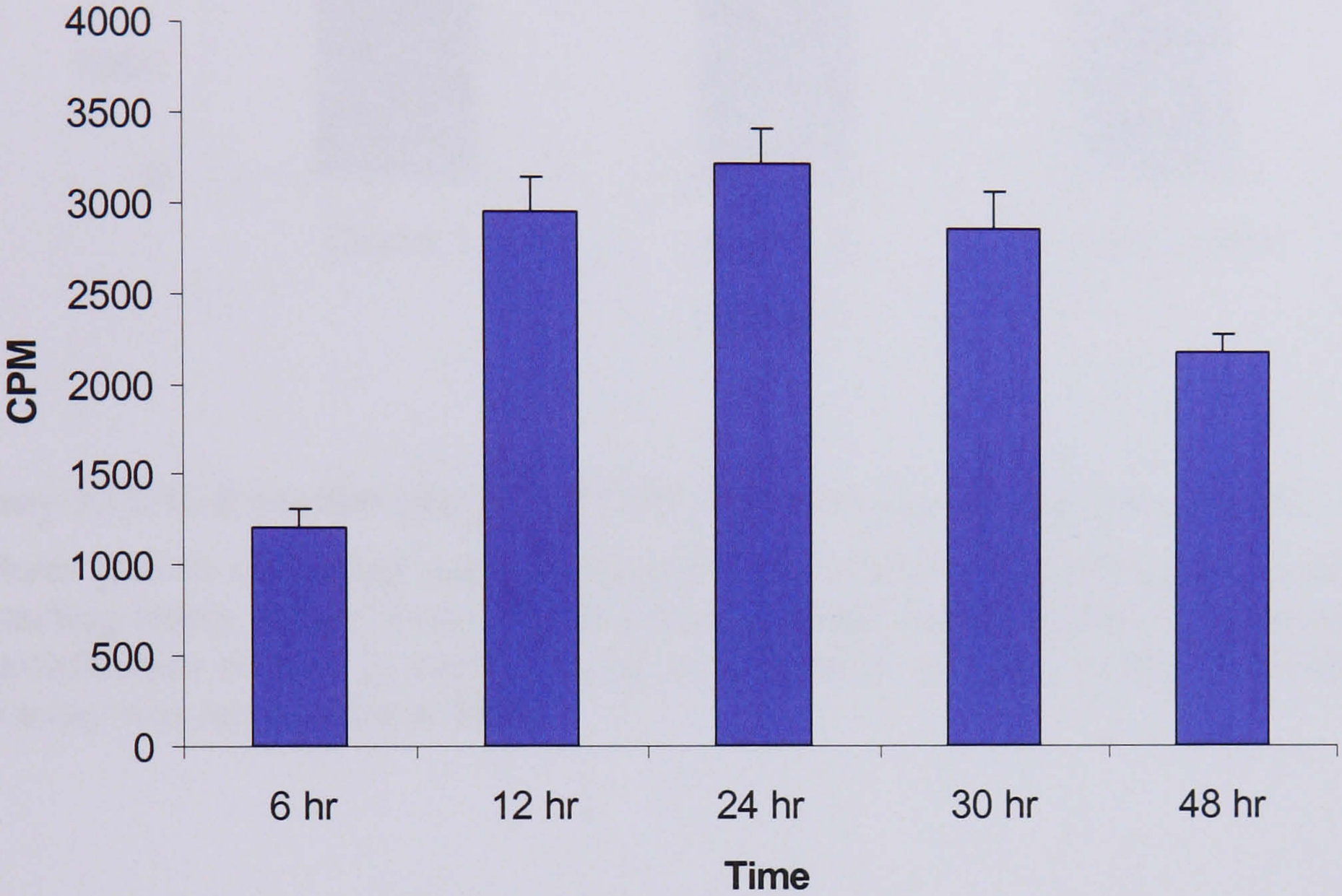


Figure 3.16. Optimal incubation time for Thymidine incorporation assay.

Optimisation of incubation time with radioactive Thymidine. LNCaP cells were plated in 24 replicates and left to adhere and grow for 24hr. Cells were serum starved for 24 hr. Full medium was added and H^3 radioactive Thymidine was added and cells incubated for 6, 12, 24, 30 and 48 hrs. Incubation for 24 hr was the optimal time.

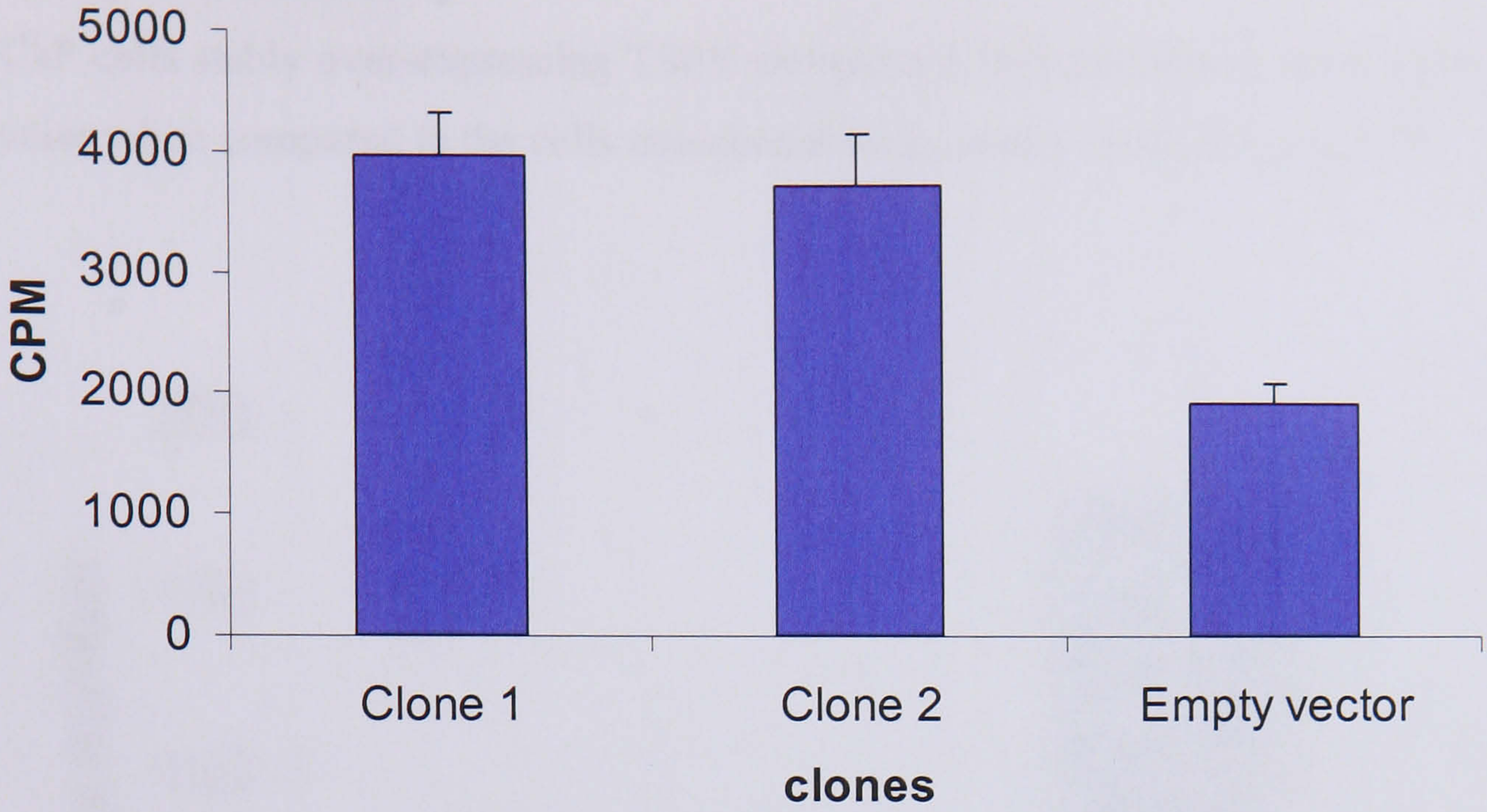


Figure 3.17. Cell proliferation of LNCaP clone over-expressing FLAG-TSPY

Cellular growth of LNCaP cells expressing FLAG-TSPY was compared to LNCaP containing empty vector. Two LNCaP different clones were used and 2 fold increase in proliferation relative to control vector was observed (Student's t test, $P<0.0001$). The assay was repeated three times.

3) Colony formation assay

LNCaP cells stably over-expressing TSPY showed a 3 fold increase in the number of colonies when compared to the cells transfected with control vector (Figure 3.18).

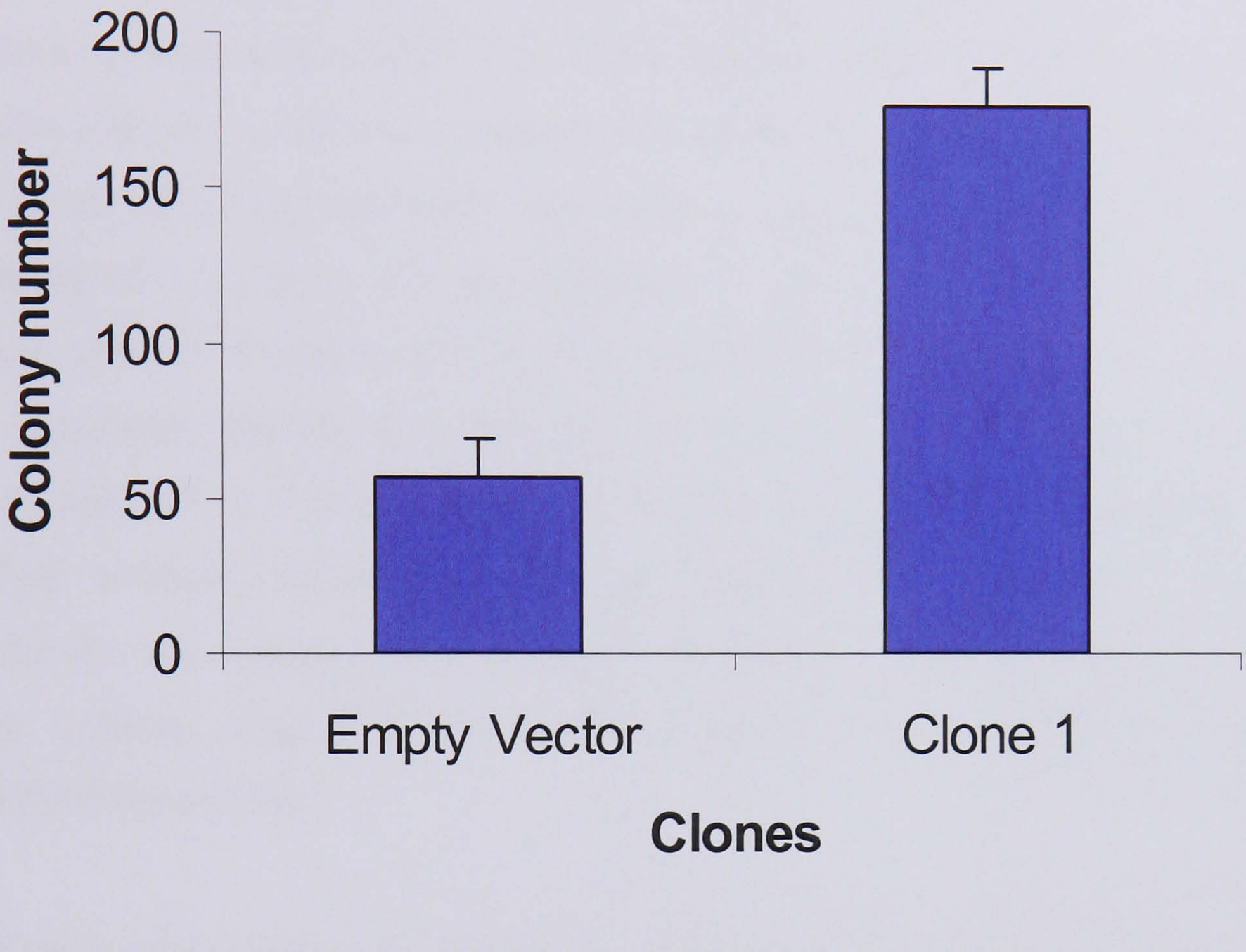


Figure 3.18. Colony formation ability of LNCaP cells over-expressing FLAG-TSPY

Colony forming ability of FLAG-TSPY LNCaP clone compared to a clone with an empty vector. Up to three fold increase in colony forming ability in clone expressing recombinant TSPY was observed (Student's t test, $P<0.0001$). The assay was carried out three times.

3.5 Discussion

An anti-TSPY rabbit polyclonal antibody was generated, validated and applied to determine the levels of TSPY expression in clinical prostate specimens. Seventy two cases of CaP and 20 cases of BPH were studied and immunostained for TSPY expression. The expression pattern within the prostate epithelium of normal and hyperplastic glands was similar. The TSPY immunoreactivity was moderate in the basal cells and weak to absent in luminal cells which are differentiated and have a low rate of proliferation and relatively high apoptotic index. In contrast, basal cells are undifferentiated and have a high proliferative capacity and low apoptotic index (Schalken and van Leenders, 2003). This indicates that TSPY level of expression is higher in prostate dividing cells than non-dividing cells. Similar trend of expression has been detected in a study carried out by Lau et al, 2003. He generated a rabbit polyclonal antibody raised a gainst a recombinant TSPY protein. Although he examined the expression profile of TSPY protein in a limited number of clinical prostate sections using IHC, he detected similar level of TSPY expression in normal/BPH epithelium.

Among most of the CaP cases studied in the current study, over-expression of TSPY was observed in 68/72 (95%) of the malignant epithelium. TSPY expression in prostate cancer was significantly higher than in BPH ($P<0.0001$, Fisher's exact test). Comparable patterns of TSPY expression were also observed between the BPH control and the foci of benign glands adjacent to the malignant epithelium in the CaP cases. In the benign prostatic epithelium, there is a balance between low rate of cell proliferation and apoptosis. This contrasts with 7 to 10 fold increase in the rate of proliferation in prostate cancer (Abate-Shen and Shen, 2000). This is in consistent with observations reported by IHC analysis carried out by Lau et al, 2003. He reported a preferential elevation of TSPY expression in adenocarcinoma cells of the prostate compated to normal/BPH epithelial cells.

Clinical and pathological details for the series of prostate cancers were corroborated with TSPY expression pattern. Malignant specimens were divided according to their Gleason sum scores into score: 2 to 5 and 6-10. There was a strong positive association between increased TSPY expression and high Gleason sum scores. Similar

findings were reported by Lau et al, 2003. He detected that increased TSPY expression correlated with high tumour grade, suggesting that its abberant expression contributes to prostate carcinogenesis.

Bone metastasis is frequently associated with advanced prostate cancer and in the current study, there was a strong correlation between TSPY expression and bone metastasis. Hence, TSPY may have a role in prostate carcinogenesis and disease progression. Within this relatively small study, TSPY expression was not found to be associated with survival outcome; a larger study with many case of prostate cancer, particularly cases with relatively low Gleason scores, is required.

TSPY immunoreactivity in clinical prostate tissues was detected as a cytoplasmic protein. There was no apparent nuclear TSPY immunoreactivity observed in prostatic sections. Indirect immunofluorescence analysis has shown that exogenous FLAG-TSPY expression in prostate LNCaP cells was also predominantly located in the cytoplasm. Thus, TSPY appears to be a cytoplasmic protein in prosate tissues. In this study, normal testes sections were used as a positive control, TSPY immunoreactivity was detected in the cytoplasm and nucleoplasm of spermatogonial cells. This is consistent with observation reported by Schnieder et al 1996: (1) cytoplasmic expression of TSPY in spermatogonial cells within the normal testis; (2) ocassoinal nuclear staining in a subpopulation in spermatogonial cells. In addition, similar findings were also reported by Hilderbrand et al, 1999 and Lau et al, 2000. They studied the expression of TSPY in gonadoblastoma resected sections using IHC. TSPY was located in the cytoplasm of germ cell tumour with occasional nuclear staining of TSPY in few cells. This indicates that TSPY can be expressed in nucleus and cytoplasm of cells depending on cell type. The presence of nuclear localisation signal in the TSPY protein sequence further support this findings. The significance of this nuclear/cytoplasmic is still not unknown.

Preferential over-expression of TSPY in adenocarcinoma compared to normal and BPH was also detected using TISH. Up-regulation of TSPY mRNA correlated with increased Gleason grade. Although luminal staining was detected in BPH and normal glands, in general there was elevated level of TSPY expression in malignant lesions

compared to normal/BPH glands. Similar trend of expression were also observed by Lau et al., 2003 study.

To address the role of TSPY gene in prostate cancer development, stable LN cells constitutively expressing exogenous FLAG-TSPY were established and characterised. When compared to the empty vector control cells, LNCaP-TSPY expressing cells have an increased proliferative rate and enhanced ability to form colonies in *in vitro* assays.

TSPY is a candidate oncogene located on the Y chromosome that has been postulated to be involved in testicular cancer. TSPY protein shows homology to SET oncogene. The SET gene was originally identified as a component of SET-CAN fusion gene produced by somatic translocation in acute undifferentiated leukaemia (von Lindern *et al.*, 1992). The function of SET oncogene is not yet fully known. However, several possible functions have been proposed. SET is found to specifically interact with cyclin B, but the significance of this interaction is not known (Kellogg *et al.*, 1995). SET has been found to interact with p21 and regulate G1/S transition by modulating the activity of E-CDK2 (Estanyol *et al.*, 1999). SET was confirmed to be a potent inhibitor of protein phosphatase 2A (PP2A) which is a serine threonine phosphatase involved in regulating cell proliferation, differentiation and transformation (Mumby and Walter, 1993). Thus, SET may play a role in regulating the cell cycle and its fusion with CAN protein contributes to leukaemia.

3.6 Conclusion

TSPY is over-expressed in clinical prostate cancer and correlated with high grade tumours. Over-expression of TSPY in LNCaP cells enhances cellular proliferation and colony formation in keeping with a vital role for TSPY in the development of prostate cancer.

Chapter 4

Genomic TSPY Copy number and prostate cancer

4.1 Introduction

TSPY gene was first discovered in humans by (J Arnemann, 1987). Subsequently, its homologues were detected in other mammals including: apes, cattle, sheep, goats, horses, pigs and rodents (Jakubiczka *et al.*, 1993, Kim *et al.*, 1996, Mazeyrat and Mitchell, 1998, Vogel *et al.*, 1997b, Zhang *et al.*, 1992). Using semi-quantitative Southern blotting, 20-50 copies have been detected in the human genome (J Arnemann, 1987, Manz *et al.*, 1993, Schnieders *et al.*, 1996, Tyler-Smith *et al.*, 1988) and most of TSPY copies have been found to map to Yp11.2 (Schempp *et al.*, 1995). However, additional TSPY copies are located at Yq11.23 (Schempp *et al.*, 1995). Each copy is embedded within DZY5 repeat units organised in array of 20 kb genomic sequence (Manz *et al.*, 1993). The full length genomic sequence of TSPY has been identified, cloned and characterised by two independent groups (Arnemann *et al.*, 1991, Zhang *et al.*, 1992). The gene is 2.8 kb in length and consists of 6 exons and 5 introns.

As described in the previous chapter, TSPY was found to be over-expressed in CaP. The molecular mechanism of preferential expression is not yet known. The presence of TSPY as a multicopy gene suggests that increased gene copy number might be responsible for TSPY abnormal expression.

Several different methods are available for determining gene copy number from clinical materials. Southern blot method is quantitative, but it requires a large amount of DNA ($\geq$10 μg) and the experiment takes several days to complete. Comparative genomic hybridisation (CGH) is one of the most widely used methods to detect increase or decrease in gene copy number across the whole genome but at relatively low resolution. Fluorescent *in situ* hybridisation (FISH) is frequently used to detect copy number at cellular level but is not suitable for high throughput analysis. It is also difficult to study gene copy number of more than 25 copies with FISH. In contrast, quantitative PCR based techniques allow amplification and quantification of low starting genomic materials. Quantitative (real time) PCR is therefore the most common assay that is currently used in oncology for gene dosage determination.

4.1.1 Quantitative Real Time PCR

Quantitative Real Time PCR is simple, rapid, specific and efficient method. The method is based on the detection and quantification of a fluorescent reporter. The assay monitors the fluorescence emitted during each PCR cycle (i.e., real time), which increases in direct proportion to the amount of PCR product in the reaction. This method specifically records the fluorescence released during the exponential phase of PCR. The detection of the first significant increase in the amount of PCR product during the exponential phase of PCR correlates with the initial amount of the target template, reviewed in (Bustin, 2000). There are 4 different available real time PCR assay formats. The simplest method is DNA-binding dye which specifically binds to DNA double strands but not to single strands. The other three (molecular beacon, hybridisation and hydrolysis probe) are based on probes designed to bind to specific sequence within the amplicon.

4.1.1.1 DNA binding dye

This assay is a simple quantitative method, relying on the ability of a SYBER green fluorescent dye to bind to double-stranded DNA (dsDNA) but not to single-stranded DNA. Therefore, only dsDNA-bound dye emits fluorescence upon excitation. Unbound dye usually exhibits very low levels of fluorescence (Figure 4.1). The main advantages of this assay are, simplicity, low cost and the fact that there is not need for additional specific probes. However, the dye binds to any double stranded DNA including both specific and non-specific products. In addition, more dyes will bind longer DNA strand than shorter, rendering quantitation inaccurate.

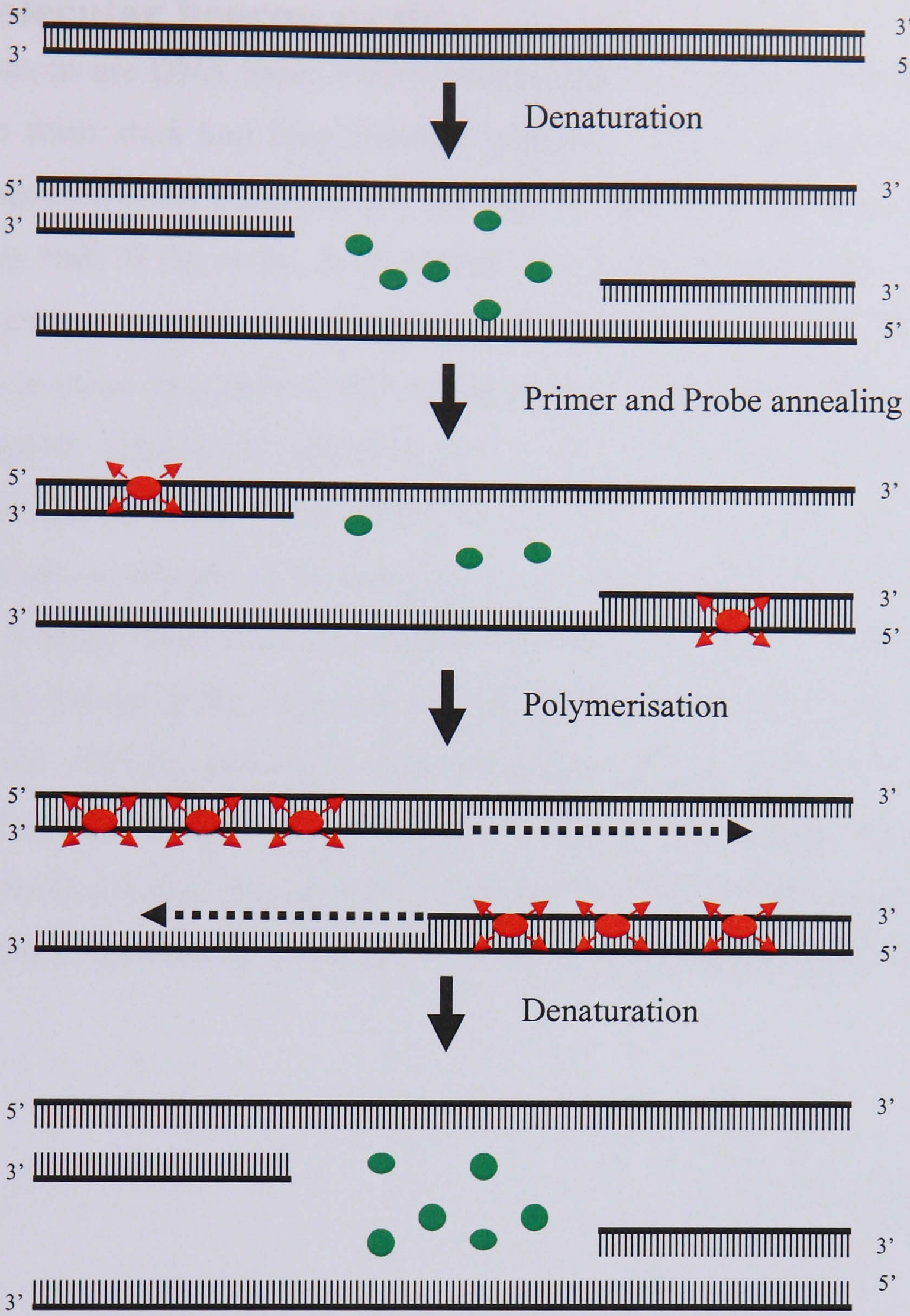


Figure 4.1. Dye incorporation method.

During PCR denaturation step, unbound SYBR Green I dye exhibits little fluorescence. At the annealing step, the dye molecules bind to the double stranded DNA, resulting in light emission upon excitation. During polymerisation step more dye molecules bind to the newly synthesised DNA and the increase in fluorescence can be detected in real time. In the denaturation phase, the dye molecules detach from the DNA and the fluorescence signal returns to background level.

4.1.1.2 Molecular beacon probe

Molecular beacons are DNA hybridisation probes that are synthesised to be partially self anneal to form stem and loop (hairpin structure). The loop part of the probe recognises a specific DNA sequence and the stem is formed by self annealing of the complementary ends of the probe. A fluorescent dye is linked to one end of the probe arm and a quencher is attached to the other end. The hairpin structure of the probe keeps the dye in close proximity to the quencher, hence suppressing the emission of fluorescent signals. During the annealing step of PCR, the molecular beacon arms detach and the probe binds to its target DNA. This separates the dye from the quencher, leading to emission of fluorescence upon excitation (Figure 4.2). This assay is specific, as a single nucleotide mismatch between the probe and the target sequence will prevent the beacon probe from hybridising and the probe will remain in hairpin form. The main obstacle associated with molecular beacons method is the probe design. The molecular probe, may fold to a conformation that displaces the quencher from the dye leading to high background fluorescence. On other hand, if the two ends are bound strongly, the binding of the probe to its target sequence may be impaired.

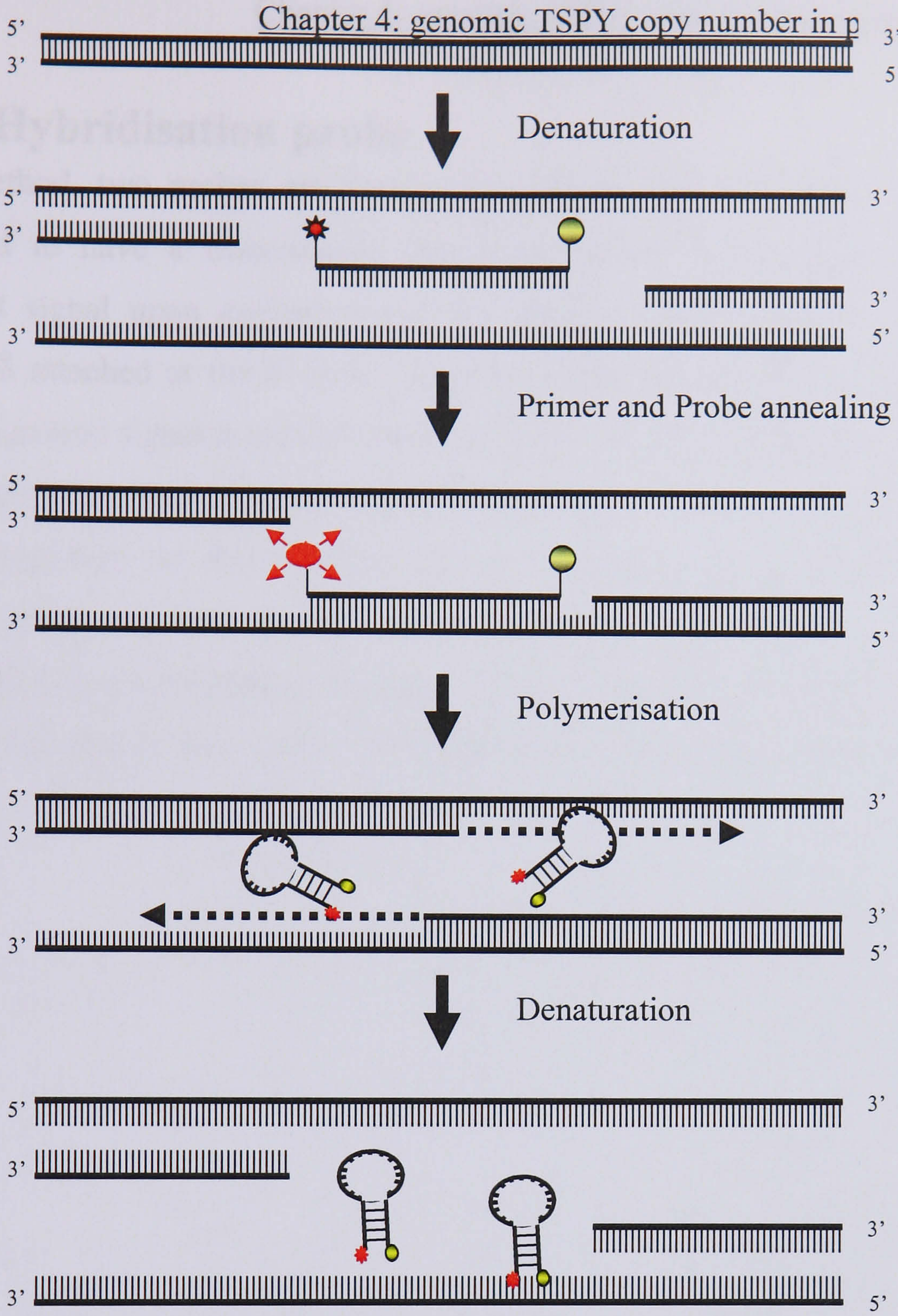


Figure 4.2. Molecular beacon assay.

The assay is based on hybridisation of a probe (a short synthetic oligonucleotide) that specifically recognises the target sequence; fluorescent dye is attached to one end of the probe and a quencher is linked to the other end. In solution, the probe, assumes a hairpin structure due to the annealing of the two complementary ends. This brings the fluorescent dye close to the quencher suppressing emission of fluorescence. During the denaturisation step of PCR, the probe dissociates at the two ends. This is followed by the annealing phase when the molecular beacon binds to its specific DNA target (amplicon) and generates a fluorescent signal. If the DNA target is absent, the probe will anneal to its complementary ends and no fluorescent emission is produced. At polymerisation step, temperature is increased and the probe dissociates from its target, re-form the hairpin structure and fluorescence is suppressed. At every new PCR cycle, the probe hybridises to its target at the annealing step and the intensity of the fluorescent signal indicates the amount of amplicons accumulated at the end of the previous cycle.

4.1.1.3 Hybridisation probe

In this method, two probes are designed to obtain high specificity. One probe is synthesised to have a fluorescence donor dye at the 3' end that emits a green fluorescent signal upon excitation and the other probe is produced with acceptor fluorophore attached at the 5' end. In solution, the two probes are apart and only green background signal is emitted from the donor. At the annealing step of the PCR, the two probes are bound to their target sequence head to tail so that the donor dye is in close proximity to the acceptor. Upon excitation of the donor, fluorescence resonance energy transfers to the acceptor resulting in emission of red fluorescence (Figure 4.3). One of the major advantages of this assay is its high level of specificity as fluorescent signals will only be detected when both probes bind to their specific target.

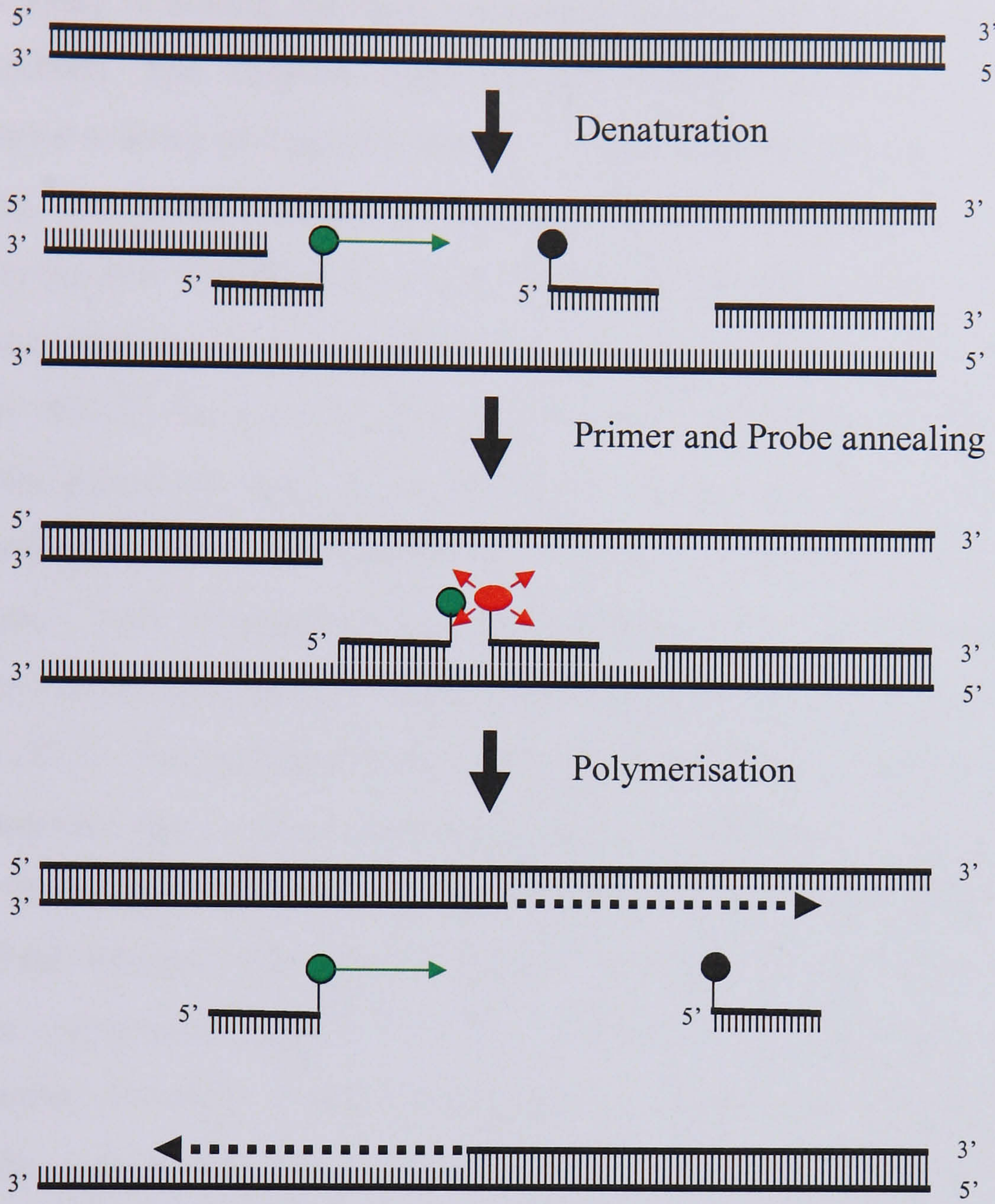


Figure 4.3. Hybridisation probe method.

At the denaturation step, both probes remain apart, a low level of emission signal is present. During annealing step both probes hybridise to target sequence in a head to tail arrangement. The two dyes come into close proximity and the emitted energy from one dye excites a second dye that emits a fluorescent signal. At the polymerisation step, both probes separate and the fluorescent signal returns to background level.

4.1.1.4 Taqman hydrolysis assay

Taqman assay is among the most commonly used quantitative real time fluorescent PCR methods. The Taqman assay using hydrolysis probe is based on the 5'-3' exonuclease activity of Taq polymerase. A short fluorescent oligonucleotide probe is generated. The probe contains a fluorescent reporter (FAM, 6-carboxy-fluorescein) and quencher dye (TAMRA, 6-carboxy-tetramethyl-rhodamine) covalently attached at the 5' end and the 3' end, respectively (Figure 4.4). The close proximity of the quencher dye to the reporter dye quenches the fluorescent signal of the reporter. During the extension phase of the PCR cycle, the fluorescent probe hybridises to its target sequence and is hydrolysed by the 5'-3' exonuclease activity of DNA Taq polymerase. This cleavage of the probe separates the fluorescent dye from the quencher dye resulting in fluorescence of the reporter dye (Lie and Petropoulos, 1998, Walker, 2002). The fluorescent signal is normalised by dividing the emission intensity of the reporter dye by the emission intensity of a passive reference dye (ROC, 6-carboxy-X-rhodamine) included in the Taqman buffer to obtain a ratio known as Rn (normalised reporter). ΔR represents the normalised reporter signal (Rn) minus the base line signal established in the first 15 PCR cycles. Threshold cycle (Ct) is the cycle number in which a statistically significant fluorescence signal is first detected during the exponential phase of PCR (Figure 4.4). Ct value is the core of Taqman assay and can be converted into quantitative results by constructing a standard curve.

The Taqman assay is very sensitive, simple and reproducible. The assay is commonly used in a wide range of applications. It is used for the analysis of viral DNA copy number (Sofi Ibrahim *et al.*, 2003), bacterial and fungal infections (Reiss *et al.*, 1998, Rondini *et al.*, 2003), detection of gene amplification in human neuroblastoma cancer (Raggi *et al.*, 1999) and breast cancer (Bieche *et al.*, 1999).

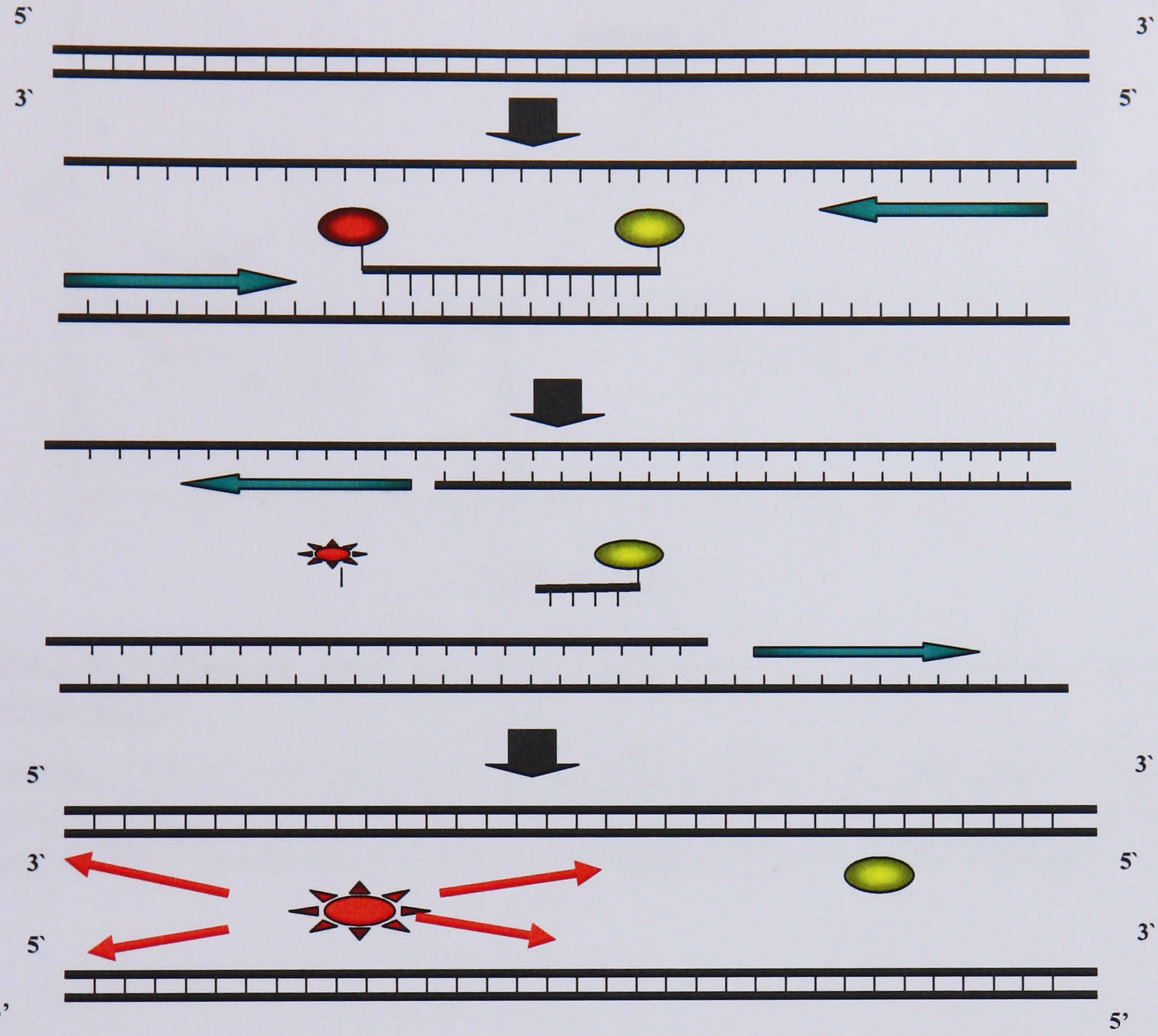


Figure 4.4. Taqman assay.

The assay is based on a hydrolysis probe. Taqman probe is generated harbouring a fluorescent dye at one end and a quencher at the other end. The close proximity of the quencher to the dye suppresses the fluorescent signal. At the annealing phase of PCR, the primer and the probe bind to their target DNA. During the polymerisation phase, the probe is hydrolysed releasing the dye from the quencher, which results in emission the of fluorescent signal.

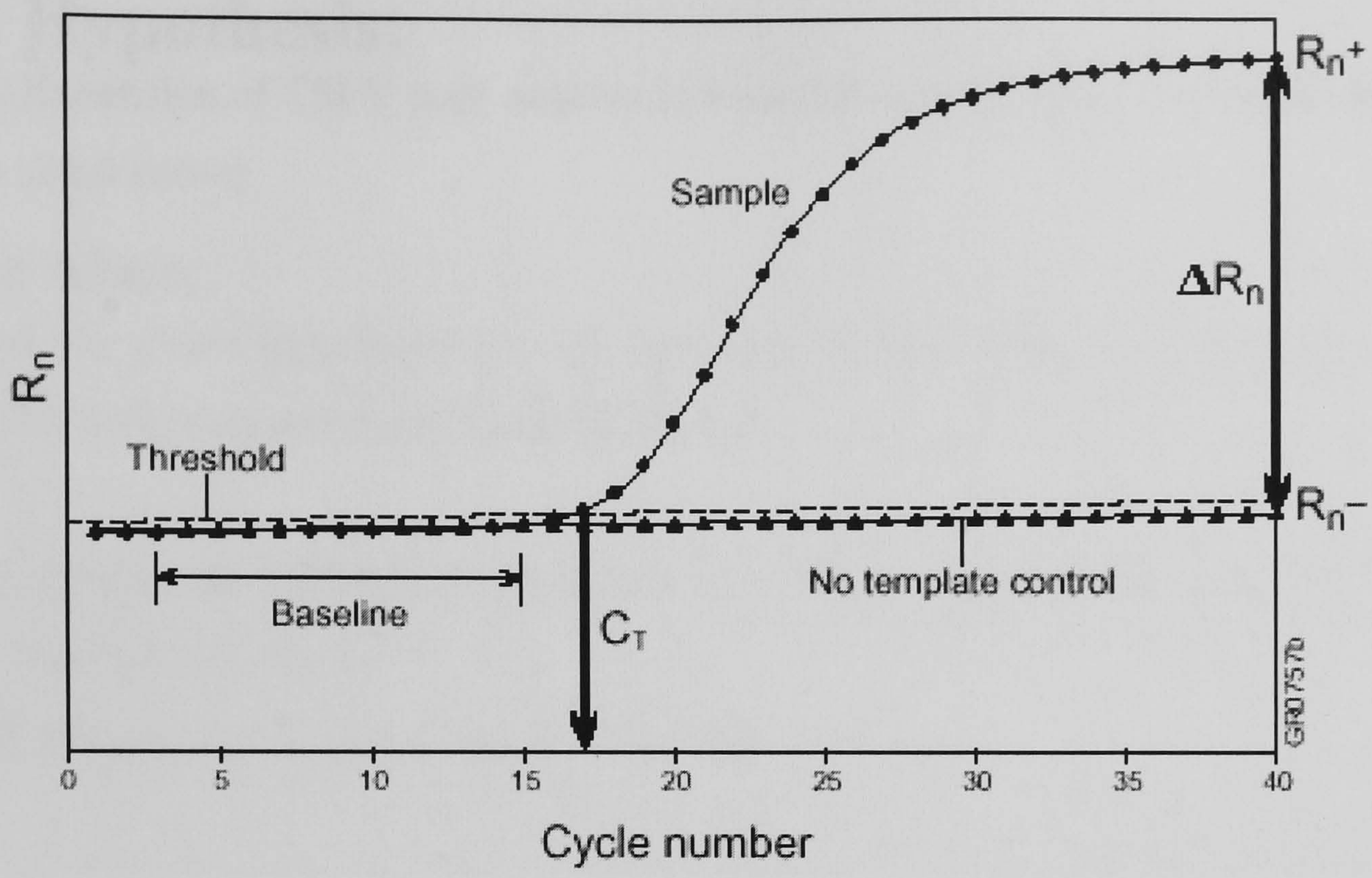


Figure 4.5. Schematic representation of real time PCR amplification plot using Taqman assay.

Threshold cycle (Ct) is cycle number at which a statistically significant increase in fluorescent signal above the basal level is first detected at the start of the exponential phase of PCR. It is a direct reflection of the initial quantity of target template.

4.2 Hypothesis:

1) Expansion of TSPY copy number is a mechanism for TSPY over-expression in prostate cancer.

4.2.1 Aims:

To test the above hypothesis by real time PCR method based on Taqman assay to study the following cohort of clinical materials:

1) Blood samples from patients with CaP compared to patients with BPH and a control population.
2) Resected benign and malignant prostate tissues.

4.3 Materials and Methods

4.3.1 Clinical samples

1) Blood Samples

Blood specimens from 119 individuals were collected and organised into three groups: CaP, n=47; BPH, n=27 and 45 healthy control adult males from North East England. All relevant clinical data for each case was collected. The ages ranged from 56-91 years for the CaP group and from 51-79 years for the BPH group. In the group of control men, the age was generally below 50 years. as the development of BPH is inevitable in the ageing male. Blood was also collected from 4 healthy adult females. These samples were used as negative controls.

2) Prostate tissues

Prostate tissues from patients undergoing TURP were collected. The prostate tissues were snap frozen in liquid nitrogen and stored at -80 °C until further use. Clinical details were obtained. Forty four resected prostate samples were collected: CaP, n=31 and BPH, n=13. Corresponding blood samples were also available for six of the cancer cases and 11 of the BPH cases. The ages ranged from 58-74 and 57-70 years for the CaP and BPH groups, respectively. Two female bladder cancer cell lines (T24 and RT112) were also included as negative controls.

4.3.1.1 Genomic DNA isolation

Genomic DNA was extracted from blood, resected prostatic specimens and cultured human cell lines using QIAamp DNA blood mini kit (QIAGEN. The whole blood was centrifuged and separated into three layers; plasma at the top, buffy coat containing white blood cells in the middle and red blood cells at the bottom. Only, the buffy coat was selectively retained and up to 1 ml volume was stored at -80 °C until processed. Frozen buffy coats were thawed and 200 µl were transferred into 15 ml Falcon tubes and 1X PBS was added to a final volume of 2 ml. White blood cells were digested to release genomic DNA by adding 200 µl of QIAGEN protease stock solution (20mg/ml) and 2.4 ml of AL buffer to the samples and mixing thoroughly by vortexing at least 3 times for 5 seconds each time. The tubes were incubated at 70 °C for 10 minutes. Then, 2 ml of 100 % ethanol was added to the samples and mixed by vortexing. Cellular lysates were divided into 2 halves and the first halves were placed

into QIAamp Midi columns inserted in 15 ml Falcon tube and centrifuged at 3000 rpm for 3 minutes. The filtrates were discarded and the other halves of the mixtures were loaded again into the columns and tubes were spun at 3000 rpm for 3 minutes. The filtrates were discarded and the genomic DNA bound to the columns were washed sequentially in two different buffers. First, 2 ml of AW1 buffer were added to the columns and the tubes were centrifuged at 5000 rpm for 1 minute. Then, 2 ml of AW2 buffer was added to the columns and the tubes were centrifuged at 5000 rpm for 15 minutes. The QIAamp columns were placed in clean Falcon tubes and 200 µl of ddH_2O was added and tubes were incubated at room temperature for 5 minutes and centrifuged at 5000 rpm for 5 minutes. The eluted DNA was dissolved and its concentration was measured using spectrophotometer (Wallac). Genomic DNA from 50 mg solid tumour tissue specimens and from human male and female cancerous cell lines was isolated following the QIAamp DNA mini kit manufacturer's instruction.

4.3.2 Quantitative Real Time PCR

4.3.2.1 Primers and probe design and optimisation

Primers and probes for Taqman assay were designed from TSPY genomic DNA sequence deposited in the NCBI database (accession number, 1932824). The TSPY sequence region including intron 1 and exon 2 was selected for oligonucleotide design (Figure 4.6). Primer express software (Perkin Elmer, PE) was used to select the best Taqman probe and primers that fit PE criteria for Taqman assay. These criteria are several. First, the probe is always designed before primers and the primers are selected to be as close as possible to the probe without overlapping it. Second, the melting temperature of the probe is usually higher than the primers by 10 °C and is in the range of 68-70 °C. Thirdly, keeping the GC contents in the range 30-80 % for both probe and primers is highly recommended. Fourthly, it is essential to avoid oligos that contain runs of 4 or more identical nucleotides especially Gs. Fifthly, probes with G at the 5' ends were avoided and the probe that has more Cs than Gs were selected. Finally, primers that have more than two Gs and/or Cs at the 3' ends were deselected. The Taqman probe was synthesised to contains a fluorescent reporter (FAM, 6-carboxy-fluorescein) attached to the 5' end of the probe and a quencher dye (TAMRA, 6-carboxy-tetramethyl-rhodamine) covalently linked at the 3' end. All the probes and primers were purchased from PE (Table 4.1).

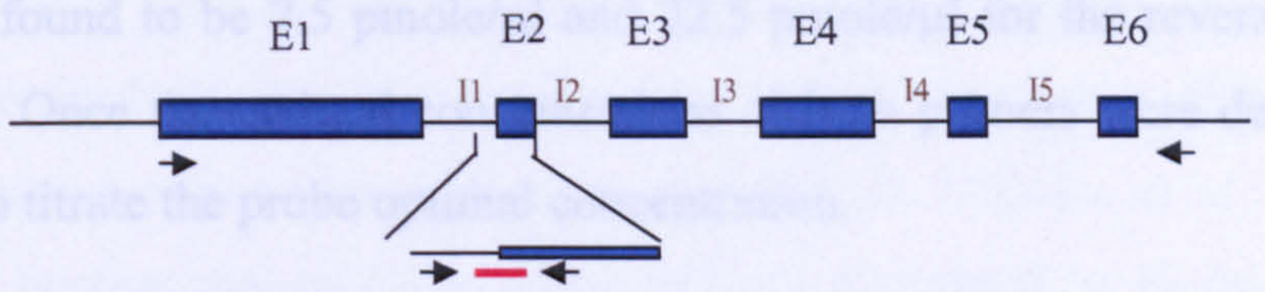


Figure 4.6. TSPY genomic structure.

Exons are highlighted in blue boxes and numbered E1 to E6. The line drawn between the exons represents introns. Intron 1 and exon 2 boundary were selected for designing Taqman probe and primers. Arrows indicate forward and reverse primer and the probe is shown in red box. Primers for conventional PCR were also indicated. Forward primer was selected at the beginning of exon 1 and reverse primer was chosen at the 3' UTR region.

Primers and probes		Sequences (5'-3')
Real time PCR		
	Forward primer	TCTCTTTGACCTAAATCAGATTGCAA
	Reverse primer	CTGACCATGTAGCTCAGCATGTCT
	Taqman probe	CCAGATGTCAGCCCTGATCACTGACG
Conventional PCR		
	Forward primer	ATGCGCCCTGAGGGCTCGCTG
	Reverse primer	TTTTCCTTTAGTTCCTGTGCATAAGA

Table 4.1. Primer and Taqman probe sequences.

4.3.2.2 Primer concentration optimisation

To establish the quantitative real time PCR Taqman assay for determining TSPY genomic copy number, the optimal probe and primers concentrations were determined following PE Taqman assay manual instructions. The forward and reverse primers

were titrated to determine the minimum concentration of both primers that will give the maximum signal above background in real time PCR method. Both primers were diluted to 22.5, 7.5 and 1.25 pmole/ µl, mixed in 9 different primer combinations were with PCR mix and carried out in triplicate. The optimal concentration for the forward primer was found to be 7.5 pmole/µl and 22.5 pmole/µl for the reverse primer, i.e a ratio of 1:3. Once the optimal concentrations of both primers were determined, they were used to titrate the probe optimal concentration.

4.3.2.3 Probe concentration optimisation

The purpose of this procedure was to determine the minimum Taqman probe concentration that will generate a fluorescent signal at the lower PCR cycle number. The PCR master mix was prepared and the optimal primer concentrations from the previous step were used. The probe was added at 0.65, 1.25, 1.87, 2.5, 3.12, 3.75, 4.37, 5 and 5.6 pmole/µl concentration to PCR mix in triplicate. The optimal probe concentration was found to be 5 pmole/µl.

4.3.2.4 Establishing TSPY absolute quantitative standard curve

To establish an absolute quantitative standard curve, a DNA sample with known genomic TSPY copy number was obtained through collaboration with Dr. Mark Jobling at the Department of Genetics, University of Leicester. The genomic DNA copy number had previously been determined using pulse field gel electrophoresis method (PFGE). The DNA was diluted 10 fold from 10^5 to 10^2 copies and stored at -20°C until used. The diluted samples were added to the PCR mix that contained the TSPY primers and probe in triplicate and recommended PCR cycles were used. TSPY absolute quantitation standard curve was established for each PCR run and copy numbers of each unknown sample was extrapolated from the standard curve.

4.3.2.5 Normalisation

To rule out any effect of inaccuracies in determining the DNA concentration and errors due to dilution and pipetting variations, normalisation is integrated as an

essential step in the real time PCR quantitative method. The determined genomic quantity is normalised to a reference gene. A single copy reference gene RNASE P control kit that contains pre-designed, synthesised primers and probe was purchased from PE. The endoribonuclease RNase P gene is a ribonucleoprotein complex consisting of two subunits, an RNA catalytic subunit and a protein binding subunit. The enzyme catalyzes the site-specific cleavage of precursors tRNA to generate mature 5' termini reviewed in (Christian *et al.*, 2002, Gopalan *et al.*, 2002, Jarrous, 2002). It is an essential and ubiquitous enzyme expressed in all cells and cellular compartments that synthesised tRNA (Gopalan *et al.*, 2002). The RNase P complex is encoded by a single-copy gene and hence is used as a reference for normalisation.

4.3.2.6 Real time amplification

The real time PCR reaction was carried out in a final volume of 25 µl. The optimised primers (300 nM forward primer and 900 nM reverse primer) and probe (200 nM) concentrations were added to 12.5 µl Taqman Universal PCR Master mix that contains 10X Taqman Buffer, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 5 mM $MgCl_2$, 0.5 Units AmpliTaq Gold, and 0.5 Units of AmpErase Uracil N-glycosylase (UNG). Each sample was done in triplicate and transferred into a MicroAmp Optical 96 well plate. Each assay included a standard curve DNA diluted from 10^5 to 10^2 copies, no template control, 100 ng of female genomic DNA as negative control, 100 ng of unknown DNA from different clinical sources and 100 ng of known genomic DNA as a calibrator. The plate was placed in ABI PRISM 5700 sequence detector system. A two steps PCR was performed, an initial step at 50 °C for 2 min. and 95 °C for 10 min and a second step of 40 cycles at 95 °C for 15 sec. and 60°C for 1 min. Amplification data was collected and analysed using GenAmp 5700 SDS sequence software (Applied Biosystems), normalised to the reference gene and the normalised copy number of each sample was determined.

4.3.3 Statistical analysis

Statistical package SPSS was used and graphs were produced using Prisms software. Non-parametric statistical method (Mann Whitney U test and Kurskal Wallis test) were applied to determine the statistical significance difference between means of

TSPY copy number from the different groups. Parametric test (Student's t test and ANOVA test) were also used. Comparison between pulse field gel electrophoresis (PFGE) and Taqman method was tested using Pearson's correlation coefficient. P values <0.05 were taken as statistically significant.

4.4 Results

4.4.1 Conventional PCR on resected prostate cancer samples

Prior to determining the TSPY copy number, all the solid clinical prostate tumour samples were tested for the presence or deletion of the structural genomic unit of TSPY using conventional PCR. As described in materials and methods, a forward primer was selected at 5'end of the TSPY gene and a reverse primer at its 3'UTR. The two primers amplify a 2.680 kp fragment from genomic DNA in PCR reaction. All of the 31 cases of CaP and 13 cases of BPH contain the full length genomic TSPY (Figure 4.7). Conventional PCR was also carried out using genomic DNA extracted from prostate cell lines, LNCaP as positive control.

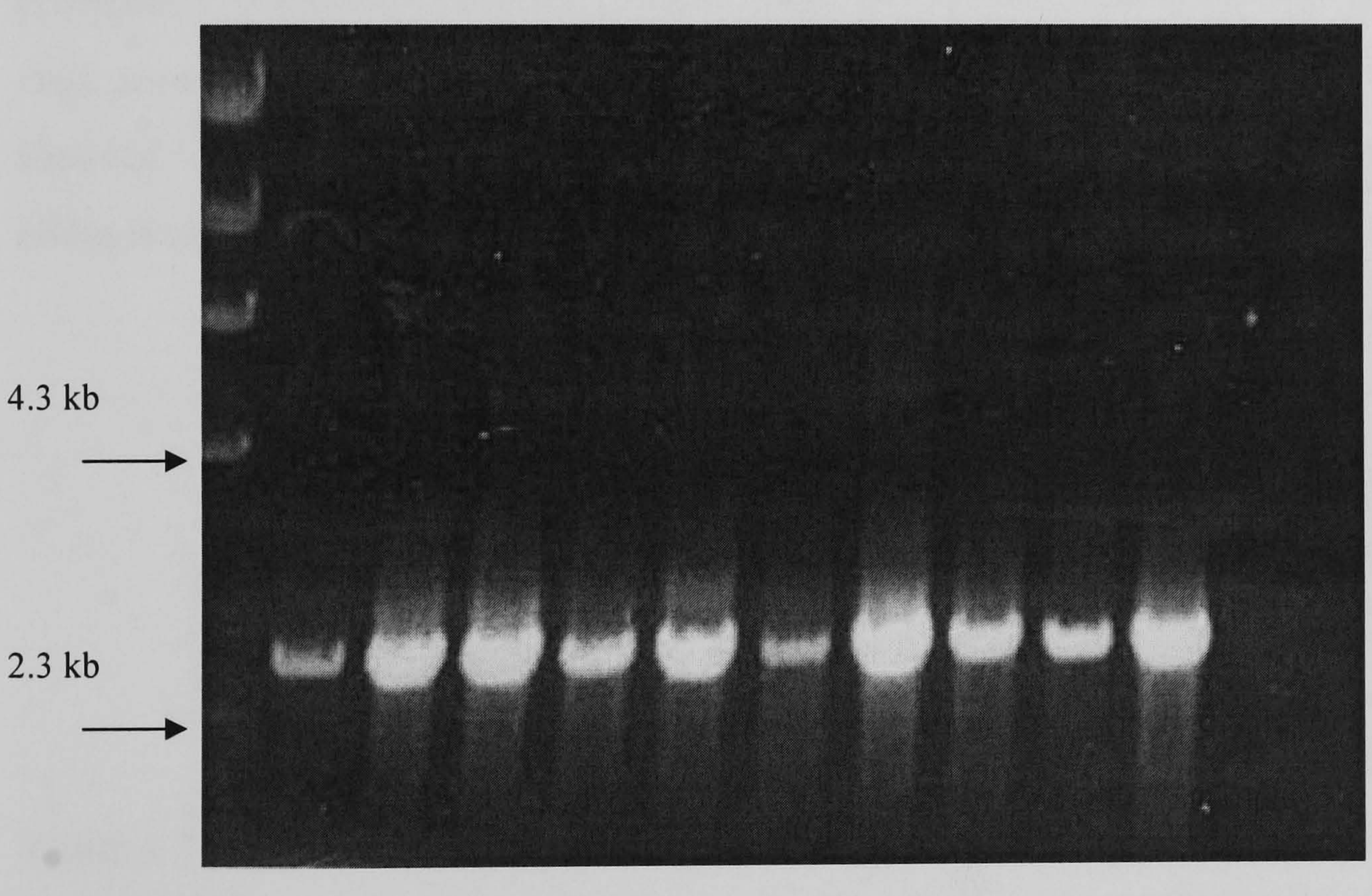


Figure 4.7. Genomic PCR products from different prostate samples.

Genomic DNA amplification from clinical prostate specimens using TSPY exon 1 forward primer and exon 6 reverse primer. Both primers amplify 2.68 kb size. Lane 1, Hind III digested marker; Lane 2, LNCaP; Lane 3-11 tumour DNA; Lane 12 female genomic DNA as a negative control and lane 13 water control.

4.4.2 Taqman assay

Optimal TSPY primer and probe concentrations were used in Taqman assay to establish an absolute quantitative standard curve. Authenticity of the TSPY amplicon product (85 bp) was confirmed by sequencing the DNA product generated by PCR (data not shown).

4.4.2.1 Validation and evaluation of the standard curve.

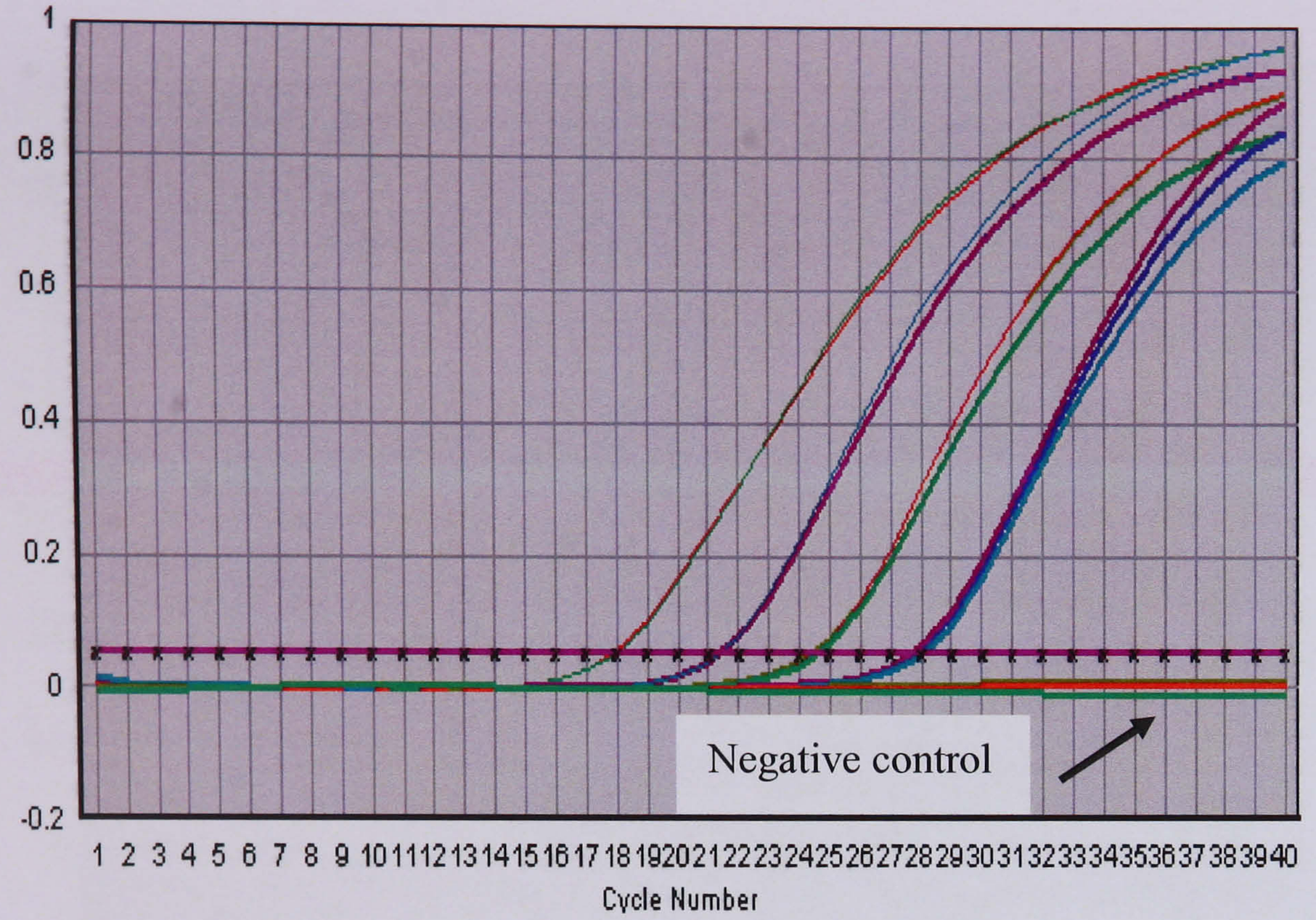
The TSPY absolute standard curve was established (Figure 4.8) and validated using 4 independent DNA samples obtained from Dr. Mark Jobling (Table 4.2). The TSPY DNA copy number for these samples had been previously determined using pulse field gel electrophoresis (PFGE) method (Dr. Jobling). As shown in Table 4.2, the established quantitative real time PCR assay was used to assay for the TSPY gene copy number for all 4 samples. The copy number determined by both methods was identical (Pearson's correlation coefficient, r=1). Absolute standard curve for endogenous control (RNASE P) was established (Figure 4.9).

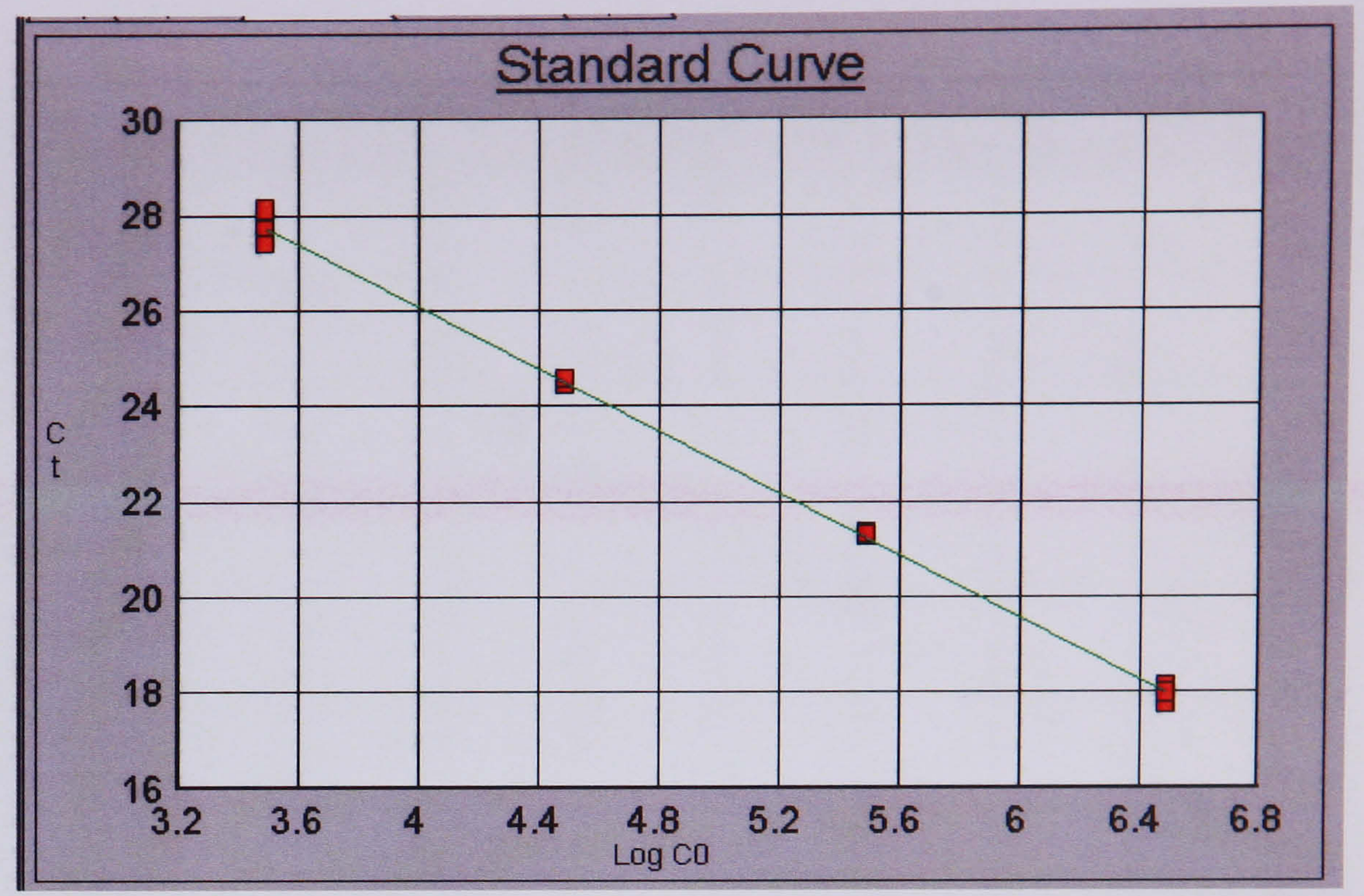
	Copy number determined by PFGE	Copy number determined by Real Time PCR
Sample 1	20	20
Sample 2	28	28
Sample 3	33	33
Sample 4	40	40

Pearson's correlation coefficient, r=1

Table 4.2. Validation of the established absolute quantitative standard Curve.

The established absolute quantitative standard curve based on Taqman real time PCR was validated by determining the TSPY genomic copy number from samples with known gene copy number.

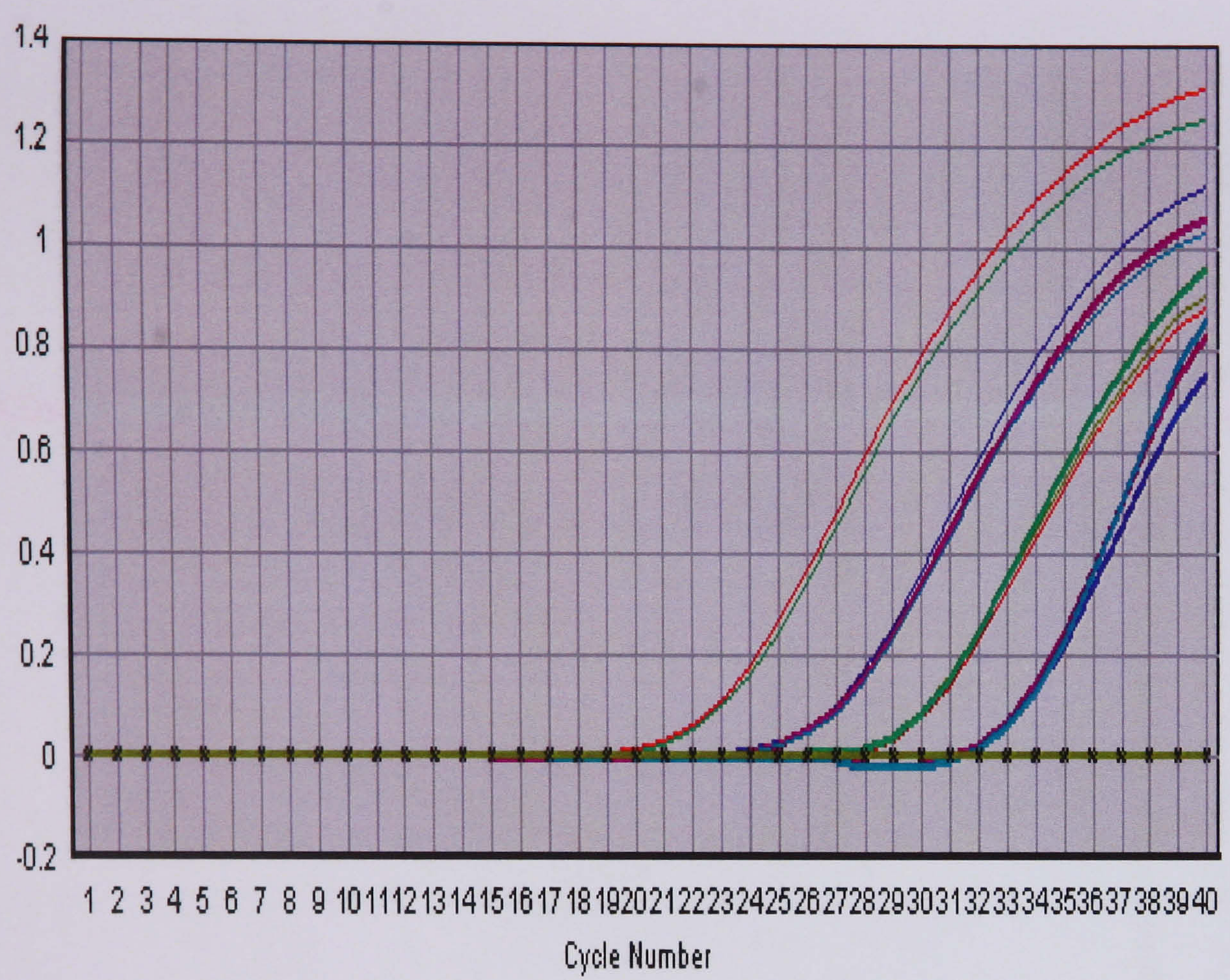




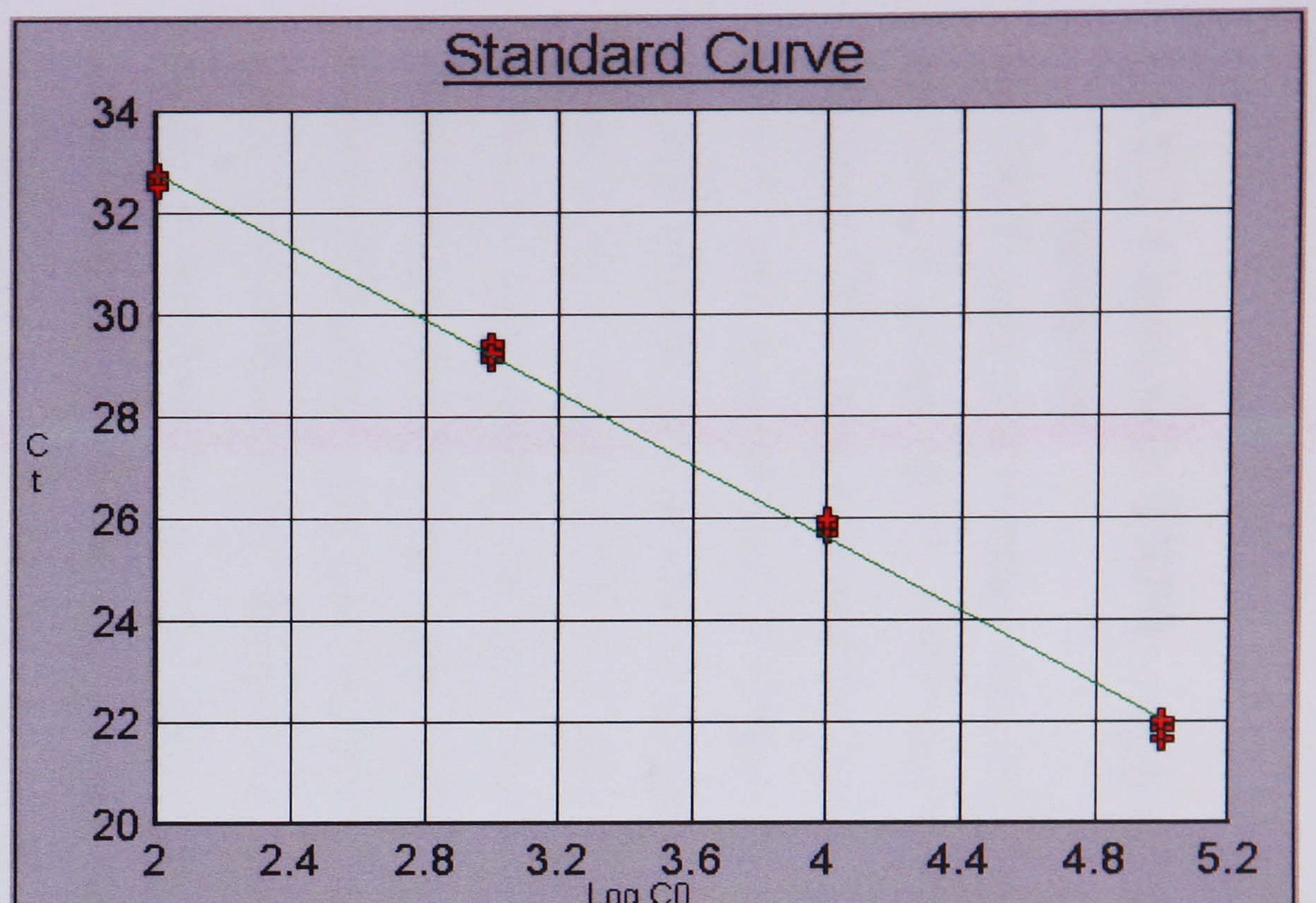
A B

Figure 4.8. Amplification plot and standard curve for genomic TSPY.

Quantitative Real Time PCR based on Taqman Assay was carried out using optimised TSPY probe and primers in GenAmp5700 sequence detecting system. **A**: Genomic DNA with known TSPY copy number was serially diluted 10 fold and each dilution was amplified in triplicate. The PCR cycle number is plotted versus change in ΔRn. ΔRn represents the difference between normalized reporter signal (Rn) and baseline signal established in the first 15 PCR cycles. Rn is obtained by dividing the fluorescence signal of reporter dye by the fluorescence signal of the passive dye included in the Taqman buffer. The ΔRn increases during PCR cycles as TSPY amplicon increases until the reaction reaches a plateau, negative control sample (obtained from female volunteer) is indicated in by arrow below the threshold line. **B**: Standard curve plot represents log TSPY copy number versus Ct (threshold cycle). Ct represents the PCR cycle numbers at which a statistically significant fluorescence signal is first detected during the exponential phase of the PCR and is a direct reflection of the amount of the input template. The Ct values of unknown DNA samples are converted into gene copy number using the established standard curve.



A



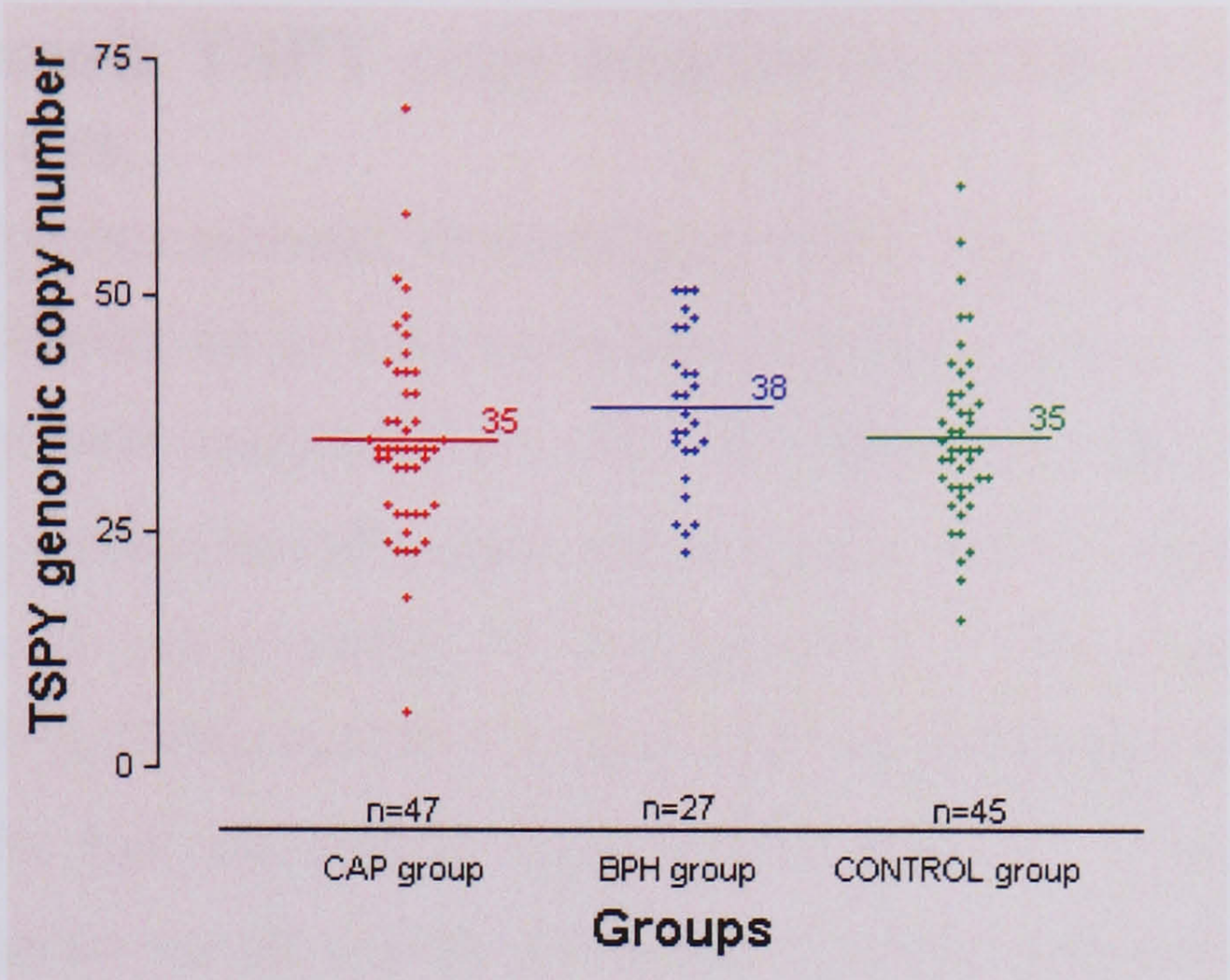
B

Figure 4.9. Amplification plot and standard curve for endogenous control (RNASE P).

A: amplification blot generated by ABI 5700 SDS software using RANSE P (single copy number gene) Taqman probe and primers as endogenous control for normalisation. The genomic DNA was serially diluted and apllied in triplicate. **B**: Absolute quantitative genomic standard curve was constructed using RNAE P control.

4.4.2.2 Genomic TSPY copy number analysis in blood samples

The TSPY gene copy number was determined from 119 blood samples: CaP, BPH and control group, n=47, n=27 and n=45, respectively. In the CaP group, the minimum TSPY gene copy number was 6 and the maximum was 70 with a mean of 35 ± 11 and a median of 34 (Figure 4.10). The statistical description of TSPY genomic average copy number in the three groups of blood samples is summarised in Figure 4.10, C. Figure 4.10, (C) shows that in the majority of the CaP cases, 43 (91%), the copy number was from 20-50 copies. Only 2 cases (4%) had less than 20 copies per genome and remaining 4 (9%) caese had more than 50 copies per person. In the control group, gene copy number ranged from 16 to 62 copies, with an average 35 ± 9 and median of 34. Most of the control cases, 42/45 (93%) contained 20-50 copies. In the BPH group, the lowest copy number was 23 and the highest was 51 copies with an average of 38 ± 8 and median of 38; 24 /27 (89%) BPH cases contained 20-50 copies per individual. Only 3 cases (11 %) had copies between 51 and 60. Statistical analysis using one way ANOVA has shown that there is no significant difference between the average copy numbers in all three groups ($P < 0.31$) (Table 4.3).



A

B

	CaP n=47	BPH n=27	Control n=45
Minimum Value	6	23	16
Maximum Value	70	51	62
Mean	35	38	35
Median	34	38	34
Std. Deviation	11	8	9
Std. Error	1.586	1.615	1.360

C

TSPY Copy Number	CaP n=47	BPH n=27	Control n=45
1-20 copy	2	--	2
21-30 copy	12	5	11
31-40 copy	21	11	22
41-50 copy	8	8	7
51-60 copy	3	3	2
61-70 copy	1	--	1

Figure 4.10. TSPY genomic copy number from blood samples.
(A) TSPY genomic copy numbers were determined from DNA isolated from the blood of 47 CaP, 27 BPH and 45 normal cases. Each dot represents copy number and horizontal lines signify the mean copy number for each group. (B) Statistical description for Figure A. TSPY genomic average copy number, range and S.D. from clinical blood samples, CaP, BPH and control are given. (C) Distribution of increasing frequency of TSPY genomic copy number among the three categories of blood samples (CaP, BPH and control).

	CAP v BPH	CAP v Control	*BPH v Control*	CaP v BPH v normal
Statistical test	Mann Whitney U T test P<0.07	Student's t test P<0.90	Mann Whitney U test P<0.09	ANOVA P<0.31

Table 4.3. Statistical analysis of TSPY genomic copy number in all blood samples.

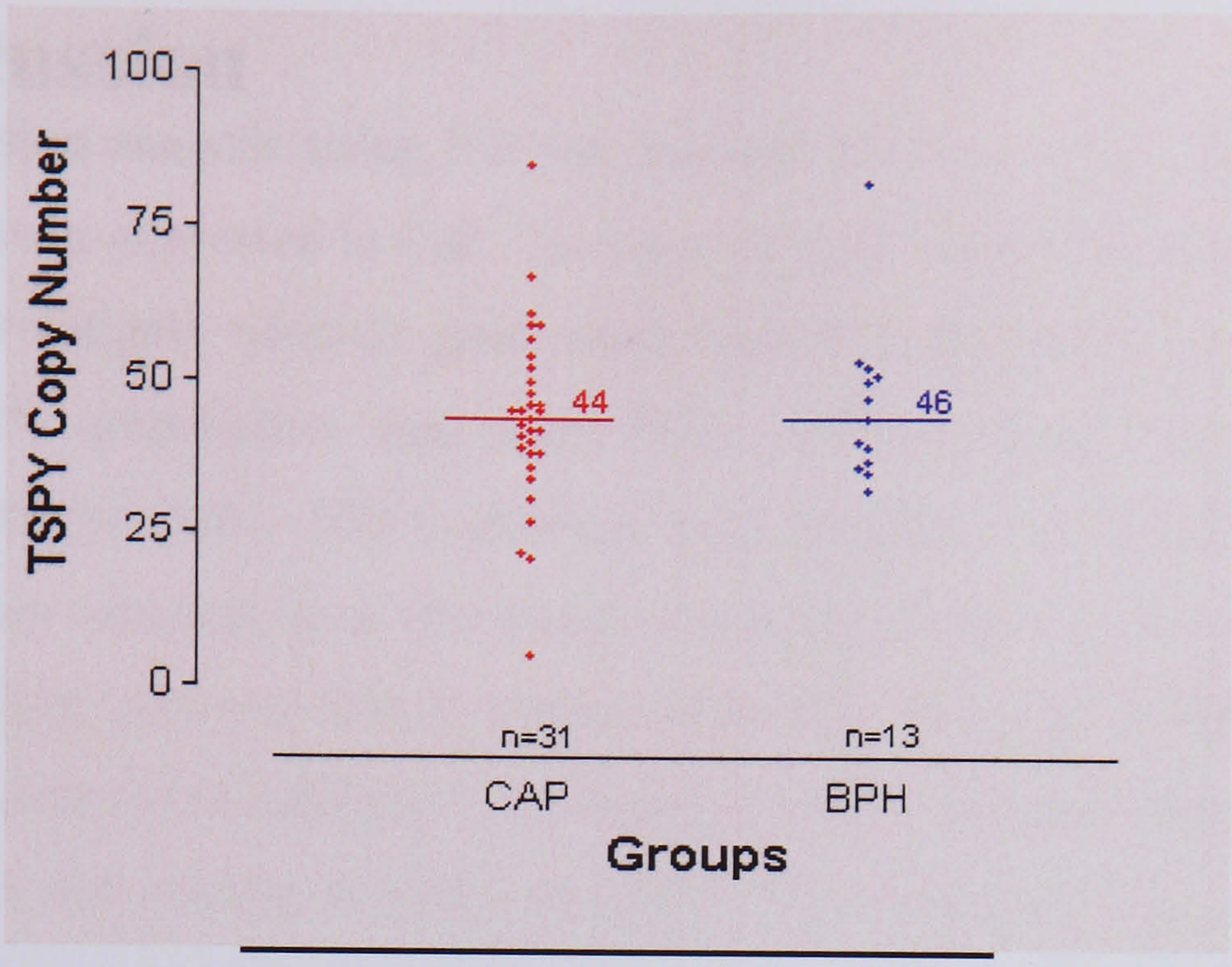
4.4.2.3 Genomic TSPY copy number in solid prostate tumours.

Using resected prostate material, abnormal gene TSPY copy number was examined as a potential mechanism for its over-expression in prostate cancer. Forty four resected prostate samples were analysed: CaP, n=31 and BPH, n=13. In the CaP group, the maximum copy number was 85 copies and minimum was 6 copies per genome with an average 44 ± 15 and a median of 44 (Figure 4.11). Moer than two third of the cancer cases, 23/31 (74%), had 20-50 copies. Only, 8 (26 %) samples had >50 copies and 1 cases (3%) had less than 20 copies (Figure 4.11, C). In the BPH group, the highest copy number was 82 and the lowest was 32 copies with a mean of 46 ± 13 and a median of 44. The TSPY copy number ranged from 20 to 50 in 9/13 (69%) cases. The remaining 4 cases (31%) had more than 50 copies of TSPY genome. Statistical analysis using Mann Whitney U test revealed no significant difference in TSPY copy number in both groups ($P<0.95$).

4.4.2.4 TSPY copy number in paired blood-tissue samples

TSPY gene copy dosage was determined from 6 resected prostate cancer tissue samples obtained by TURP and compared to its matched paired blood specimens. Eleven paired blood-tissue specimens were also collected from BPH patients. Comparing the copy number for each paired sample revealed no significant statistical variation in TSPY copy number in patients affected by CaP (Wilcoxon signed ranks test, two tailed, $P<0.92$) and in patiensts with BPH (Wilcoxon signed ranks test, two tailed, $P<0.44$).

A



B

	CaP n=31	BPH n=13
Minimum	6	32
Maximum	85	82
Median	44	44
Mean	44	46
Std. Deviation	15	13
Std. Error	2.67	3.59

C

Copy Number	CaP	BPH
1-20 copy	1	--
21-30 copy	3	--
31-40 copy	7	6
41-50 copy	12	3
51-60 copy	5	3
61-70 copy	2	--
>70 copy	1	1
Total	31	13

Figure 4.11. TSPY genomic copy number from prostate solid tumours

(A) Genomic TSPY copy number from 31 cases of CaP and 13 cases of BPH were determined using Taqman assay. The horizontal line represents the mean copy number for each group. (B) Statistical description of Figure A. Genomic TSPY copy number from the prostate solid tumours. Range, mean, median and S.D. are given. (C) Distribution of increasing frequency of genomic TSPY copy number in resected prostate tissues: CaP and BPH.

4.5 Discussion

TSPY expression analysis using IHC on resected prostate cancer tissues has shown that TSPY is over-expressed in CaP. The cause of TSPY deregulated expression is not known. To investigate whether gene amplification is the cause, an absolute TSPY genomic DNA quantitative real time PCR method using Taqman assay was established and validated. TSPY genomic copy number was determined from 161 clinical samples obtained from the blood of normal persons, CaP and BPH patients and solid prostate tumours. Blood samples from 47 CaP cases, 27 BPH cases and 45 normal individuals were obtained. Six copies of TSPY was the lowest copy number reported in all the clinical samples assessed. The maximum copy number was 70 copies per genome detected in a CaP patient. In general, it has been noticed that the majority of cases studied had 20-50 copies of TSPY per genome. Although there was variation in TSPY copy number, there were no statistically significant differences in copy numbers of TSPY among the three groups, control, BPH and CaP using the current DNA extraction method from resected tissues. Furthermore, TSPY copy number analysis in paired blood and tissue samples revealed no significant correlation between TSPY copy and tumour formation. This might suggest that abnormal TSPY copy number might not be the underlying cause for TSPY over-expression in prostate cancer. However, prostate cancer is heterogeneous and multi-focal in nature and different grades of tumours can coexist within resected prostate, in addition to normal glands, hindering DNA extraction from homogeneous cancerous cells difficult to obtain. While the selection of locally invasive cancer with significant tumour infiltration is expected to minimise this contamination effect, prostate tissue generated by laser captured microdissection will facilitate the extraction of highly pure homogenous cancerous samples, allowing more accurate determination of TSPY copy number in resected prostate tissues.

Homologues of human TSPY are multicopy and located on Y chromosome in rodents and other mammals including the great apes, pigs, cattle, sheep, goats and horses (Conrad *et al.*, 1996), (Kim *et al.*, 1996), (Vogel *et al.*, 1997a, Vogel *et al.*, 1997b), (Glaser *et al.*, 1998, Mazeyrat and Mitchell, 1998)). Male specific bovine genomic homologue of the human TSPY is repetitive. The gene is part of a clustered array of

50-200 repeated sequences on the Y chromosome resembling the arrangement present in the human genome (Jakubiczka *et al.*, 1993).

Besides TSPY, there are further 9 genes known to be present as multicopy genes on the Y chromosome. The functions of these genes are not fully known. There are many examples of human multiple copy genes in the autosomal chromosomes. The tandem repeated multi-gene families encoding large rRNAs, 5S rRNA and U1 and U2 constitute 2 % of the human genome. The human tandem repeated arrays of ribosomal RNA are distributed on different chromosomes and include nearly 500 copies of the gene (Gonzalez *et al.*, 1988). The multicopy human U2 small nuclear RNA genes are located on chromosome 17 in arrays of tandem repeats varying in number from 6 to more than 30 repeats (Pavelitz *et al.*, 1995). The snRNA forms part of a large spliceosome complex that is involved in removal of introns from pre-mRNA (Valadkhan and Manley, 2003). The neuronal apoptosis inhibitory protein (NAIP) located on chromosome 5 (5q13.1) is another multicopy gene, (Roy *et al.*, 1995). On the same chromosome, there is a further multicopy gene, BTF2p44 (5q13.3), which forms a subunit in the RNA polymerase II complex important in transcription and transcription–mediated DNA repair (Carter TA, 1997). The human major histocompatibility complex located at chromosome 6 contains a multicopy gene families including human leukocyte antigen class I (HLAI), MHC class I related chain (MIC) and haemochromatosis candidate genes (Kulski *et al.*, 2000).

This is the first report describes establishing real time PCR assay to determine TSPY gene copy number in prostate tumour specimens. Two independent studies have reported establishing real time PCR methods for accurate detection and determining human and mammalian TSPY gene copy numbers for other research objectives. Pierce and his colleagues have described a successful and accurate method for determining the human sex of embryos using real time PCR for TSPY gene, based on molecular beacon assay (Pierce *et al.*, 2000) as part of their ongoing research on pre-implantation genetic diagnosis. Wang and his colleagues have also developed quantitative real time PCR based on Taqman assay for detecting and determining mice genomic TSPY copy number from male liver cells engrafted into female liver tissues (Wang *et al.*, 2002).

4.6 Conclusion

The majority of cases assessed in the present study had between 20 to 50 TSPY copies per genome. There were no significant differences between the TSPY copy number in CaP patients compared to persons with BPH or to normal individuals. In addition, there was no correlation between TSPY copy numbers either with increasing Gleason score or increasing age of individuals. Thus, gene amplification might not the underlying cause of TSPY over-expression in CaP.

Chapter 5
Gene expression profiling in LNCaP cells over-expressing TSPY

5.1 Introduction

TSPY expression pattern analysis in CaP has revealed that TSPY is over-expressed in prostate cancer and the expression correlates with advanced CaP and bone metastasis. Furthermore, increased proliferation rate in LNCaP cells over-expressing TSPY has been detected by *in vitro* proliferation assays and colony forming assay. Gene expression alterations associated with over-expressing of TSPY are not yet known. In the last few years, a high density gene profiling GeneChip (Microarray) method has been developed and applied to comparatively analyse the gene expression within a genome (Shalon *et al.*, 1996). It provides a powerful tool for screening biological samples for alterations in the mRNA expression accompanying certain physiological or pathological changes. The microarray is an ordered array of specific and known sequences of synthetic oligonucleotide or cDNA that are spotted onto discrete loci on a glass, silicon or nylon surface (Harkin, 2000). The chip technique is based on hybridisation of a target labelled probe to nucleic acid attached to a solid support.

Applications of microarrays are diverse. One of the early applications of DNA microarray technology was the study of transcriptional profiles of tumours and investigating the global patterns that accompany malignant transformation and correlation with clinical phenotypes. Several studies have established transcript profiling of primary tumours for diagnostic, prognostic and treatment purposes. Welsh *et al* used DNA microarray to identify candidate molecular markers for ovarian cancer (Welsh *et al.*, 2001). Dhanasekaran used DNA microarray to derive a prognostic marker for prostate cancer (Dhanasekaran *et al.*, 2001). DNA microarray has been used in elucidating complex biochemical pathways. The GeneChip array is commonly used to identify the signalling pathways that play a role in development, differentiation and tumourigenesis.

Two formats of microarray are in current use: cDNA and oligonucleotide microarrays. Although both cDNA and oligonucleotide microarrays are used to analyse the profile of global gene expression, fundamental differences exist between these two methods (Figure 5.1).

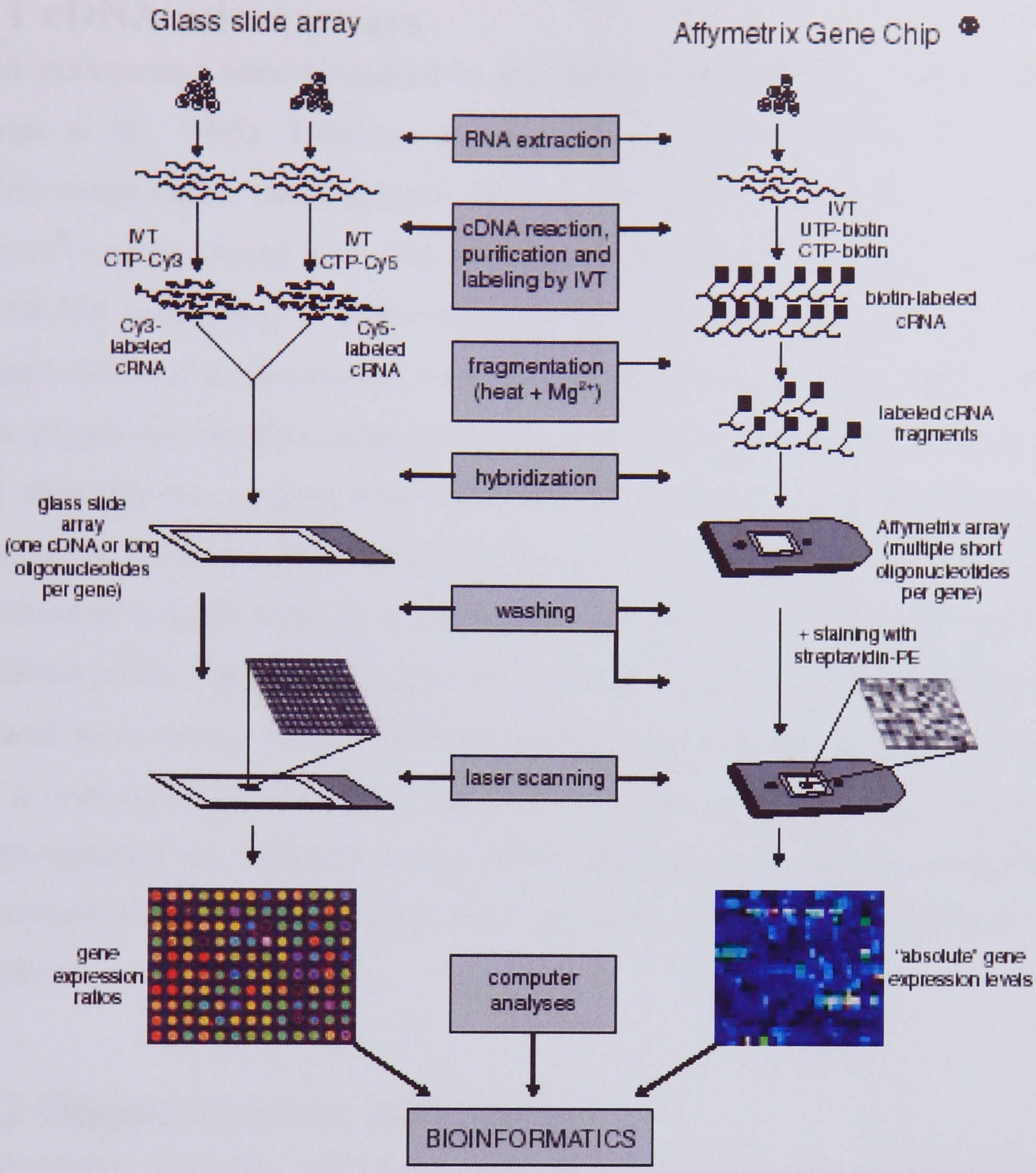


Figure 5.1. Oligonucleotide and cDNA microarray.

Comparison between cDNA and oligonucleotide microarray. In cDNA microarray, total RNA from test sample and reference sample are labelled separately with two different fluorescent dyes, combined and added to a glass slide dotted with cDNA. The slide is excited with laser and the genes that are up-regulated, down regulated or unchanged are colour coded, red, green or yellow, respectively. Affymetrix oligonucleotide is based on synthesised short oligos attached to glassslide. The total RNA is labelled with biotin. Avidin conjugated fluorescent dye is added and the signal is quantified (Staal *et al.*, 2003).

5.1.1 cDNA microarrays

cDNA microarrays were pioneered by the Brown Laboratory at Stanford University (Schena *et al.*, 1995). They are generated by amplifying cDNA clones in PCR reaction using vector based primers flanking the cDNA cloning site. The amplified products (ranging in size from 100-2000 bp) are purified from the gel and re-arrayed into 96-384 well plate. A robot precisely deposits a defined quantity of double standard cDNA at predetermined locations on a solid support such as glass, silicon or nylon. Expression profiling using cDNA microarray is accomplished by labelling total RNA from the test samples with either fluorescent Cye3 or Cye5-dUTP in reverse transcription reaction. The fluorescent labelled cDNA probes are combined and hybridised to a single array in a competitive hybridisation reaction. The detection of hybridised probe is achieved by laser excitation of the individual fluorescence marker followed by scanning with a confocal scanning laser microscopy. The ratio of cye3-cye5 is normalised and digitally colour coded as red, green or yellow. The genes that are up-regulated are indicated in red colour and genes that are down-regulated are represented in green and genes that show no change in expression profile are shown as yellow.

5.1.2 Oligonucleotide microarray

Commercially available Affymetrix oligonucleotide chips are generated by either attaching pre-synthesised oligonucleotide probes to a chip or *in situ* synthesis of oligonucleotide probes on the chip (Figure 5.2). In the latter method, the probe arrays are produced by light directed chemical synthesis that combines solid phase chemical synthesis with photolithography fabrication techniques. A series of photolithographic masks are used to define chip exposure sites followed by specific chemical synthesis steps. High density arrays of oligonucleotide with each probe in a predefined position in the array are produced. Multiple probe arrays are synthesised simultaneously on the chip. Gene expression changes are detected by labelling extracted total RNA from test and reference samples as follows: the first strand cDNA is synthesised using olig-dT primer containing T7 polymerase site in reverse transcription reaction. A biotin labelled cRNA is generated by *in vitro* transcription of cDNA in the presence of biotinylated dNTP (Figure 5.3). The labelled probe is fragmented and hybridised into two separate oligonucleotide microarrays. A streptavidin conjugated fluorescent

marker is added to the bound biotinylated probe. The bound labelled probe is detected by laser excitation of fluorescent marker and the intensity of the fluorescent signal is scanned using confocal laser microscopy. The fluorescent signal intensity is quantified. This represents the alteration in correspondent transcription gene levels.

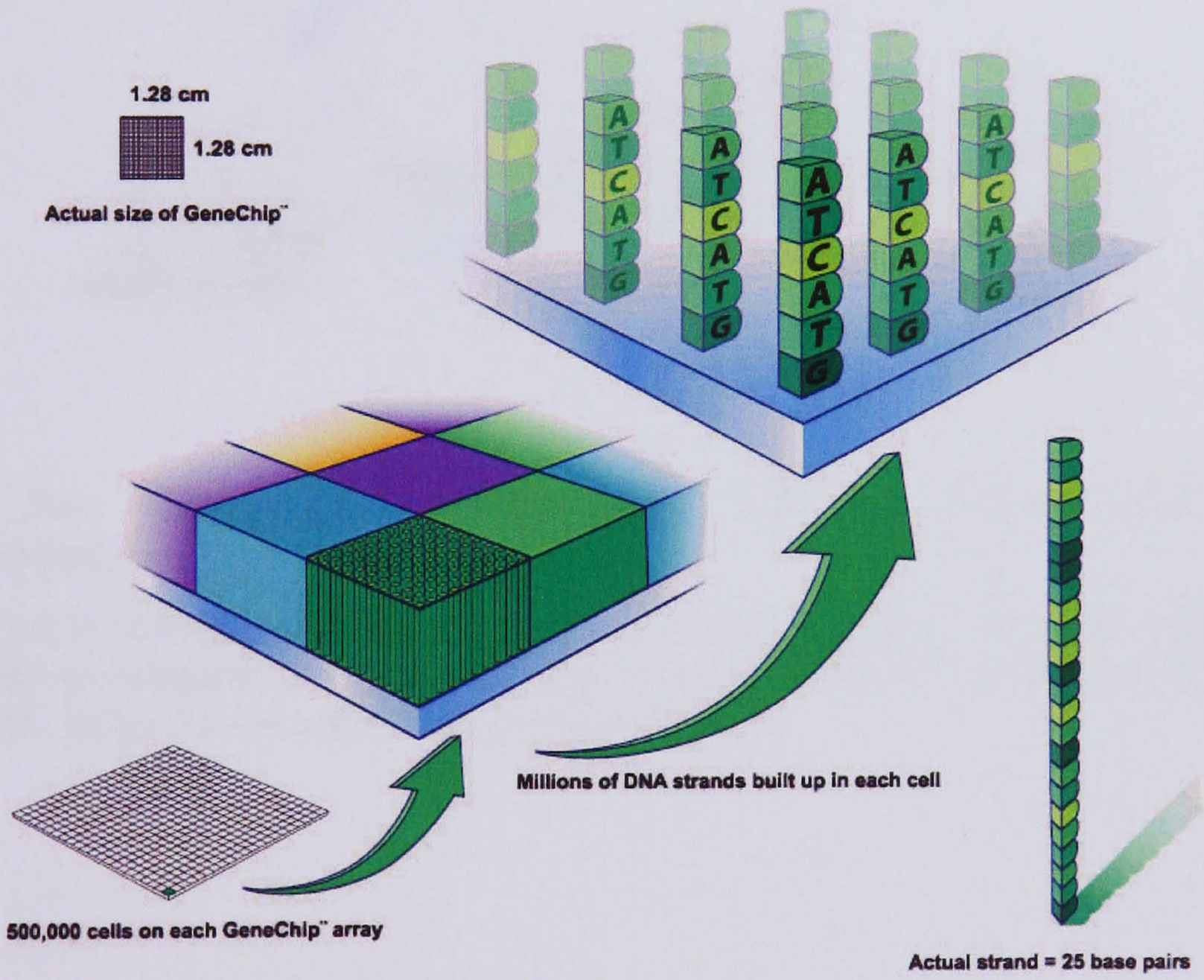


Figure 5.2 Oligonucleotide chip.

Commercially available Affymetrix oligonucleotide chip is based on attaching short synthesised 25 bp oligonucleotide. In general, 11 paired probes are synthesised for each gene (http://www.affymetrix.com/index.affx).

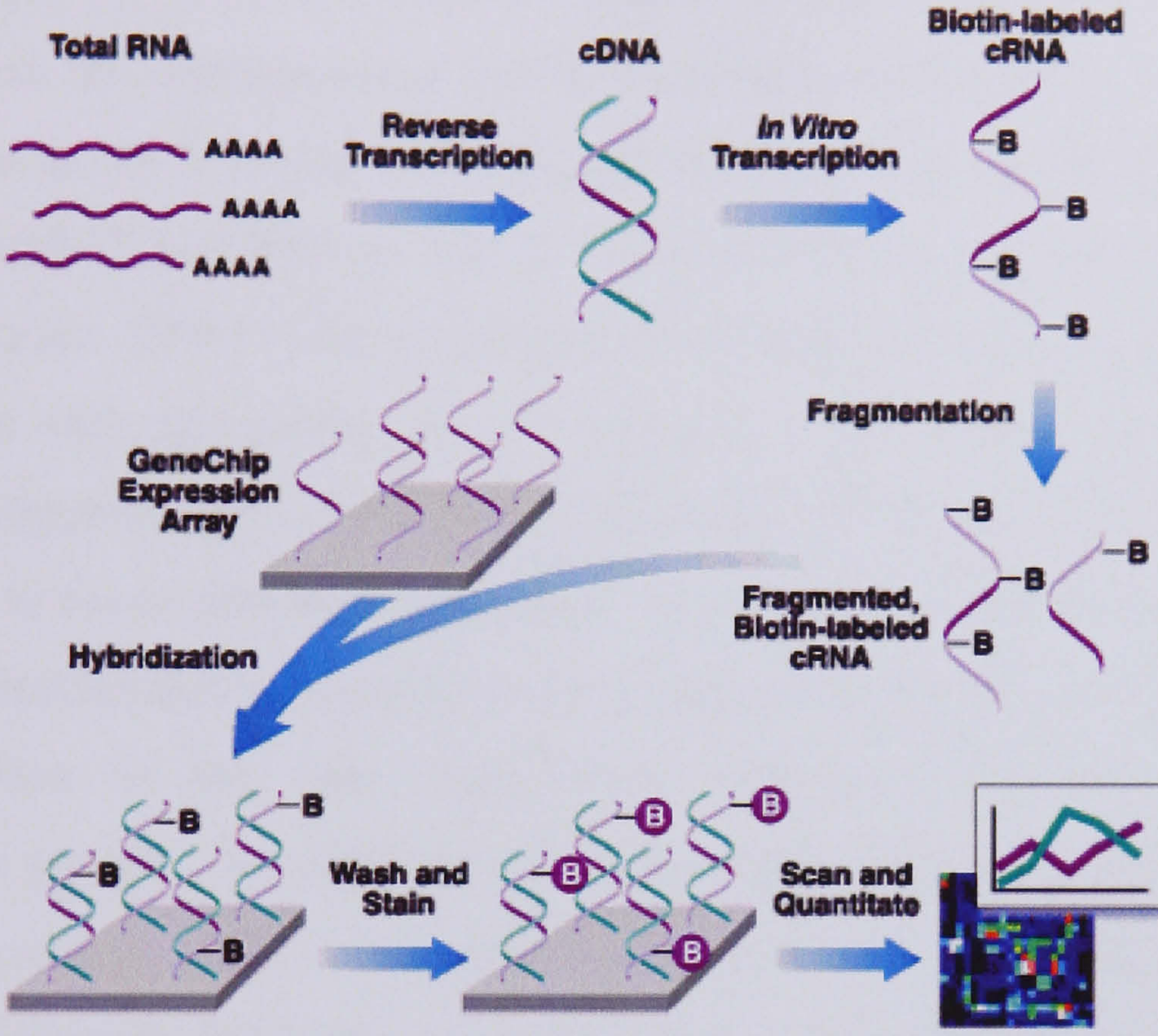


Figure 5.3. Oligonucleotide Affymetrix GeneChip probe labelling and hybridisation.

Total RNA is labelled using biotin, fragmented and hybridised onto the chip, washed and avidin conjugated to fluorescent dye is added. Excited signal is collected and quantified. (http://www.affymetrix.com/index.affx).

5.1.3 Affymetrix GeneChip human genome U133

The Affymetrix oligonucleotide GeneChip human genome U133 consists of a square glass in a plastic cartridge set and is composed of two chips: chip A and chip B. Both microarrays contain in total 1,000,000 unique synthetic oligonucleotides that were generated from the corresponding 39,000 transcript variants which in turn represent 33,000 human genes. The oligos were designed from DNA sequences deposited in the Gene Bank, dbEST and RefSeq. The HU-133 set contains a set of 100 control genes (GAPDH, β-actin. STAT1) for normalisation. Each probe set is composed of 11 pair probes and in each pair probe, one probe sequence is identical to the corresponding target gene sequence and is termed a perfect match (PM) and another probe has one base change in the middle of the sequence and is called mismatch probe (MM). This approach forms the core of microarray probe design and allows accurate quantfication and subtraction of the real signal from non-specific background and cross hybridisation signals. The difference in signal hybridisation within a probe pair and the intensity ratio is used as an indicator for abundance of the specific target gene. Some probes are selected from gene regions that are shared by gene multiple variants. In other cases, a unique sequence that distinguishes between variants is selected for probe design. The probes on the HG-U133 set are designed to detect the anti-sense strand of the gene of interest and these probes are annotated with the "_at" extension. There are three different types of probe sets. Probe sets that are unique to the target gene and only specifically hybridise to the corresponding gene sequence are annotated with "_a_at" extension. In other cases, the probe set is generated from target sequence that is shared by gene multiple variants and named as "_s_at". Occasionally, it is not possible to select either a unique probe set "-a-at" or a probe that detects multiple variants "_s_at". In such cases, some of the probe pairs in probe sets are designed from a gene region that is similar or identical to unrelated sequences. These mixed probe sets are annotated with "_x_at" extension.

5.1.3.1 Advantages of Affymetrix GeneChip.

Oligonucleotides design is based entirely on gene sequences deposited in the NCBI and RefSeq databases and no PCR step is required thus decreasing the possibility of probe mix up. The use of multiple oligonucleotides enables splice variants and closely related members of a gene family to be distinguished. GeneChip can distinguish

transcripts that are up to 90 % identical. Shorter nucleic acid fragments are more accessible for hybridisation. The main disadvantage with this method is the need for special equipment and GeneChips are expensive to purchase.

5.2 Aims

1) To apply LNCaP-TSPY clone in gene profiling experiment, determine candidate genes and to validate their regulation by TSPY.

2) To cluster genes into pathways and generate a model for TSPY effector genes.

5.3 Materials and methods

5.3.1 RNA Isolation

LNCaP cells that constitutively express exogenous FLAG-TSPY and empty vector were seeded into 10 cm dishes and left to grow for 3 days until the cells were 70% confluent. Total RNA was isolated from both clones using Trizol reagent (Invitrogen). The cells were washed twice in BPS and 3 ml of the reagent was added to the cells and left at RT for 5 minutes. The cell lysate was transferred into an eppendorf tube and 200 µl of chloroform was added, mixed and the tubes were spun at 14000 rpm for 15 minutes. The upper aqueous layer containing RNA was transferred into a fresh tube and 500 µl Isopopranol was added and mixed. The tube was centrifuged for 15 minutes at 14000 rpm. The RNA pellet was washed in 70% ethanol and left to dry, before adding 100 µl of DEPC ddH_2O. The total RNA concentration was determined using spectrophotometer (Wallac). The quality and integrity of RNA was checked by loading 1 µg of total RNA into 1% agarose gel and by RT-PCR using GAPDH primers.

5.3.2 Microarray method

Approximately, 10 µg of RNA from each clone was handed over to Dr. Peter Heiko at the Centre for Life, University of Newcastle upon Tyne. All the necessary experiments from probe preparation, hybridisation and washing were carried out by Heiko's group. Only Affymetrix GeneChip human Genome U133 set A that contains 22,285 genes was used. The cut off point for fold expression changes was taken as 1.5.

5.3.3 Primer design

All subsequent analyses from Microsuite data output was performed using Affymetrix web site. The probe set ID number was used to obtain the corresponding region of the gene used to generate the probe. The DNA sequence was inserted into NCBI BLAST to confirm the identity of the gene. Once the corresponding gene has been confirmed, Map viewer was used to obtain relevant information available on the gene. UCSC is used to investigate exon, intron, 5' and 3' UTR regions of genes and their isoforms. Once the gene structure has been investigated, a pair of primers was designed using primer 3 software. On average, 25-30 mers primers length was selected (Table 5.1).

Genes	5'-3' primers	Product size
BAX-F	CCTTTTGCTTCAGGGGATGATTG	145
BAX-R	CACCAGTTTGCTGGCAAAGTAGAAA	
PCDH7-F	GCAAACAGATGCGTCTACATCCATA	309
PCDH7-R	CCGCCTCTTTAAGAATGGAATTTCT	
BMP11-F	TCATGGAGCTTCGAGTCCTAGAGAA	430
BMP11-R	AGAGGTTAAGTTACCCACCCACCAA	
IRS 1-F	AGACCTGGATTTGGTCAAGGACTTC	486
IRS 1-R	GGCCAGCTAAGTCCTTAAGGTTGAA	
MAF-F	TGCACTTCGA CGACCGCTTC TCCGA	360
MAF-R	TGGCTAGCTGGAATCGCGTGTCAGA	
NF2-F	TTATGAGGAGAAGGAAAGCCGATTC	444
NF2-R	TGGCTCTCTTGATATCTGGTCCATC	
NDRG1-F	GTCCTTATCAACGTGAACCCTTGTG	376
NRDG1-R	TCTTGAGGAGAGTGGTCTTTGTTGG	
PLOD2-F	ATGGCTTGATTTTATCCGGGAGTT	444
PLOD2-R	TTCCTCTCATCTTCTCAGCCACAAC	
Cyclin G-F	AAGGGCTGAGTTTGATTGAGGCTAC	318
Cyclin G-R	CCATCCGTTTTATGTCAGAAGCAGT	
GST-F	AAATGATCCTCCTTCTGCCCGTA	279
GST-R	ACTTCTTCACTGTGGGCAGGTTG	
GAPDH-R	GGATTTCCATTGATGACAAGCTTC	147
GAPDH-F	GCCTGGTCACCAGGGCTGCTTT	

Table 5.1. Primer pair of each gene selected from the microarray analysis results used for RT-PCR.

Primer pairs of genes that were evaluated by RT-PCR assay to confirm microarray data. Primer sequence and product length is given. GAPDH was used as reference gene.

5.3.4 Semi-quantitative RT-PCR

Single stranded cDNA was synthesised in a reverse transcription reaction using 2µg of total RNA extracted from LNCaP clones with FLAG-TSPY and empty vector. In an eppendorf tube the following were added and mixed: 4 µl of 5X Transcriptase buffer, 2µl 5mM dNTPs, 1.5 µl OligdT16, 0.3 µl MMLV transcriptase enzyme (Promega), total RNA and ddH_2O to a final volume 20 µl. The mixture was incubated at 37 °C for 1 hr and the enzyme was inactivated by boiling the sample for 5 minutes. One microgram of RNA was used in subsequent RT-PCR. PCR cycle conditions were identical for all the primers used. Initial one denaturation step at 95 °C for 3 min, then 95 °C for 30 sec, 65 °C for 20 sec and 72 °C for 30 sec and a final step at 72 °C for 5 min. RT-PCR was performed for 25 cycles to begin with then increased to 30 cycles and finally to 35 cycles depending on the transcript level. The RT-PCR products were loaded into 1% agarose gel and the gel image pictures were captured. GelBandQuantitation software was used to determine the relative quantity of RT-PCR product for each gene from LNCaP clones with and without FLAG-TSPY compared to the endogenous reference house keeping gene (GAPDH).

5.3.5 Gene ontology to determine gene altered in pathways

Data analysis on Affymterix web site was used to analyse the results generated from the microarray data. Gene ontology mining tools were used to determine the genes and pathways altered under the current conditions investigated. All the genes listed are categorised according to their biological process. Genes that have a similar functional processes are grouped together.

5.4 Results

5.4.1 Validation of LNCaP cell sample for gene profiling

Both LNCaP clones with TSPY-FLAG and empty vector were prepared as given in the materials and methods for gene profiling using Affymetrix GeneChip. Before carrying out profiling, RNA quality and expression of FLAG-TSPY were validated using RNA gel, RT-PCR and Western blot (Figure 5.4).

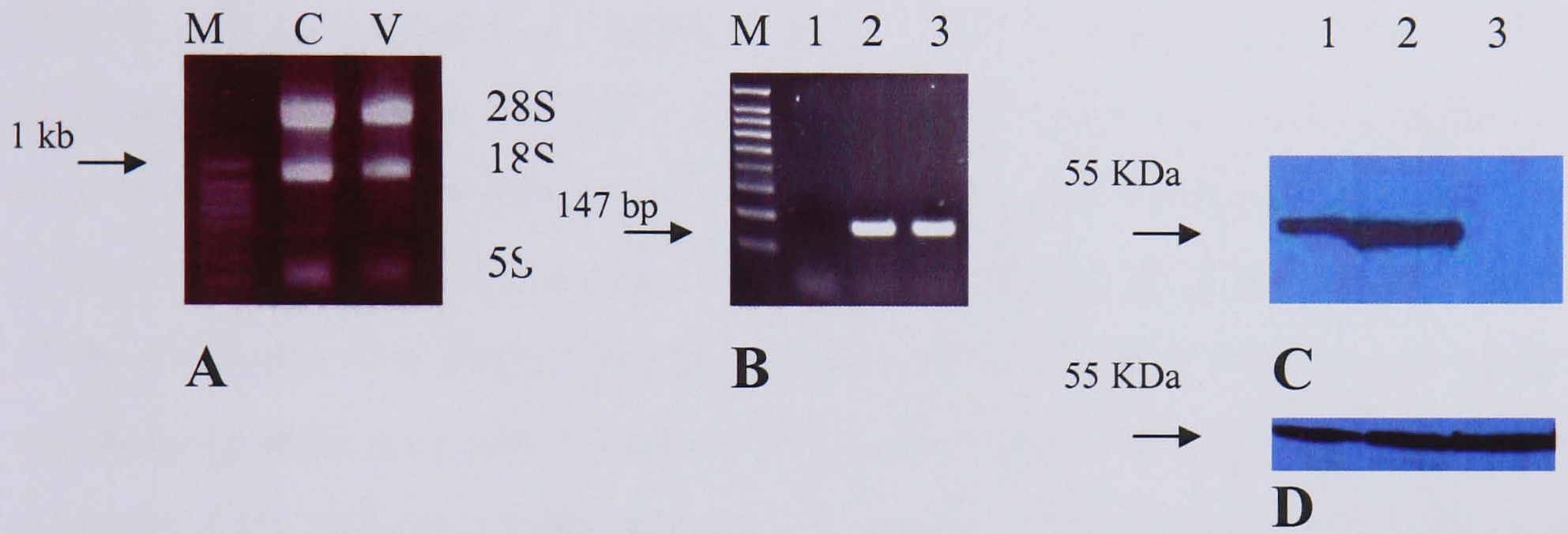


Figure 5.4. Validation of LNCaP cell sample for gene profiling.

(A) RNA gel, the quality of RNA isolated from LNCaP clones with FLAG-TSPY and empty vector was assessed on agarose gel. C indicates clone and V represents vector. (M) 1 Kb ladder marker. (B) RT-PCR, cDNAs prepared from both clones amplified using GAPDH primers, M represents 1 kb ladder, lane 1 water control; lane 2, LNCaP clone with TSPY and lane 3, clone with empty vector. (C) The presence of TSPY FLAG protein was checked using Western blot analysis. Lane 1, LNCaP transiently transfected with FLAG-TSPY, lane, 2 stable LNCaP Clone expressing FLAG-TSPY and lane 3, LNCaP stable clone with empty vector. (D) α-tubulin marker.

5.4.2 Microsuite data output

Global Gene expression level from LNCaP cell line that expressed FLAG-TSPY was compared to LNCaP cell line that contained only empty vector using Affymetrix GeneChip human genome U133 chip A (22,285 genes). The output data was exported into Affymetrix microarray suite (MA) software and analysed (Figure 5.5). TSPY expression was prominent in the output analysis. TSPY expression increased by 69 fold. In total, the expression profiles of 332 genes (excluding TSPY) were also significantly altered (increased or decreased) due to presence of exogenous TSPY in LNCaP cell line relative to control LNCaP cells (appendix and Table 5.2) Ninety eight gene expressions altered by 2-10 fold and 3 gene transcripts changed by more than 10 fold. Two to 10 fold decreased transcript expressions were observed in the 63 genes (Table 5.2) and an increase of 2-10 fold transcript expressions were detected in 35 genes (Table 5.2). Three gene transcripts had decreased by 10-90 fold. Only genes that demonstrated two fold or more decreased changes in transcript levels with change p-value >0.99 and gene transcripts that increased two and more fold with change p-value <0.0006 were selected for further evaluation.

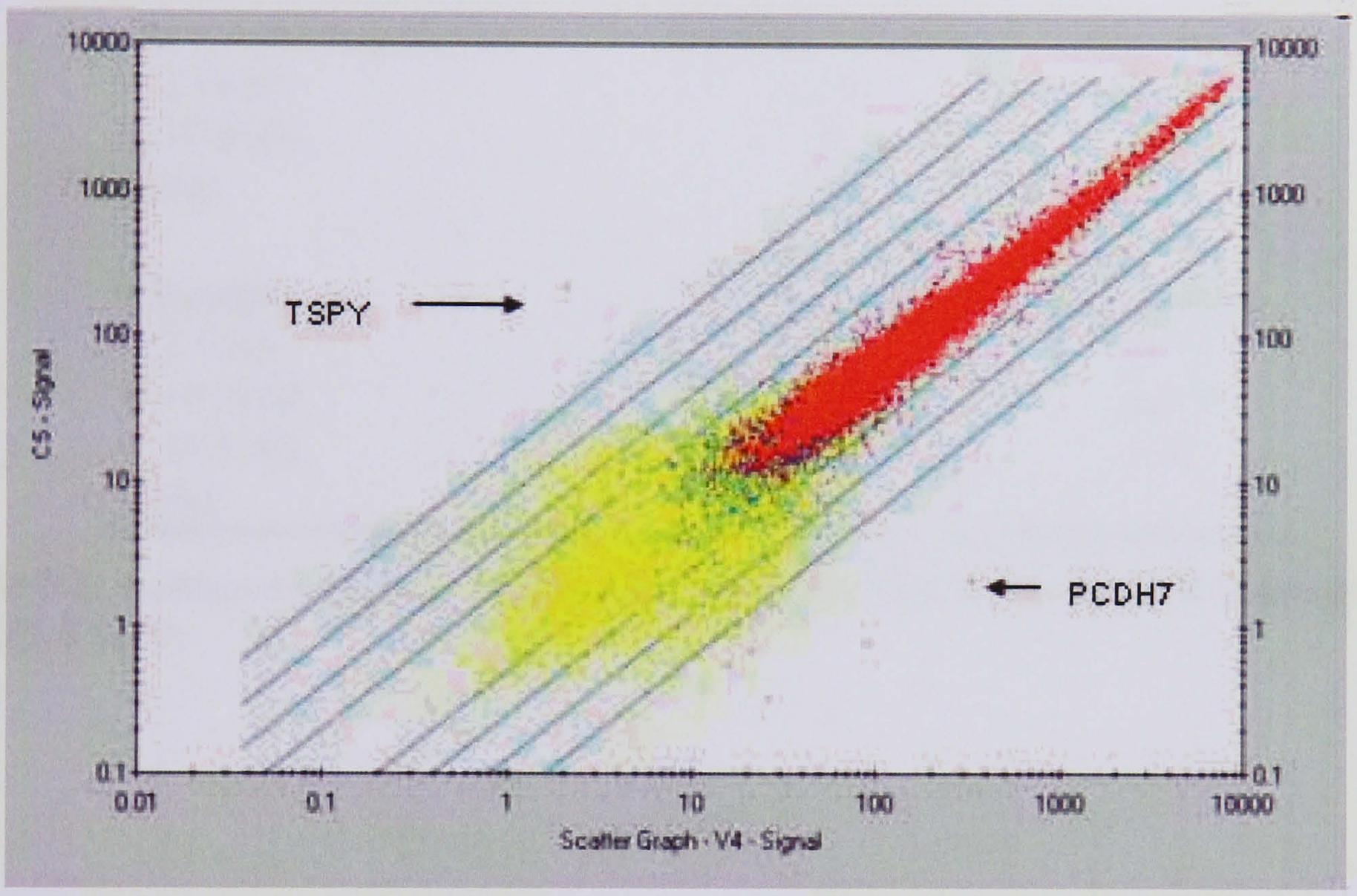


Figure 5.5. Scatter blot representing comparative analysis from LNCaP two clones.

The test RNA sample (LNCaP (C5) that constitutively expressed FLAG-TSPY) is labelled with one fluorescent dye (red) and reference sample RNA (LNCaP (V4) empty vector as reference for comparison) labelled with another fluorescent dye (yellow). The changes in transcript between the two samples are coloured blue. The two inner diagonal lines represent the lowest folds of change and any line above the left line indicates increased folds of expression and decreased fold of gene expressions are lined below the right line. Two examples are highlighted, TSPY and PCDH7.

Decreased expression	Number of genes	Percentage
< 2 Fold	138	68 %
2-10 Fold	63	31 %
> 10 Fold	3	1 %
Total	204	

Increased expression	Number of genes	Percentage
< 2 Fold	93	73 %
2-10 Fold	35	27 %
Total	128	

Changes	Number of genes	Percentage
< 2 Fold	231	69 %
2-10 Fold	98	30 %
> 10 Fold	3	1 %
Total	332	

Table 5.2. Summary of gene expression numbers altered due to the expression of FLAG-TSPY.

5.4.3 Genes expression altered by two fold or more.

Although the MA suite analysis showed expression alterations in many genes that play variable roles in regulating cell cycle, growth, adhesion, apoptosis, migration, replication, DNA repair damage, angiogenesis (appendix), only those transcripts that altered by 2 or more fold are considered for further analysis and verification. Genes that were described as hypothetical were deselected. Ten genes out of 101 genes that altered 2 fold up to 90 fold were selected and their sequences were investigated thoroughly using NCBI, BLAST, Affymetrix web site and DNAstar software. The identity of each gene and the DNA segment of the gene selected for probe design were inspected. These genes are, BAX, PCDH7, BMP11, IRS-1, MAF, NF2, NRDG1, PLOD2, GST-A2 and cyclin G (Table 5.4.). Up to 90 fold decreased in PCDH7 transcript level was observed from cell expressing FLAG TSPY compared to empty vector (Table 5.3). Decreased PLOD2 expression was the second highest change to be noticed. Initially, Mek5c gene was increased by 4 fold and upon closer examining the Mek5c probe sequences and the gene sequence deposited into Genbank database, it was confirmed that the Mek5c Genbank sequence used for designing the GeneChip probe for Mek5C was contaminated with vector sequence. The majority of the probe sequence pairs were corresponding to the vector multiple cloning sites. Upon this finding, Mek5C was deselected for further analysis. Only two genes with up-regualted expression, GST-A2 and cyclin G, were selected for validation,.

Gene	Gene expression fold changes Identified by microarray assay	RT-PCR
NF2	-2	---
BMP11	-2	-2.7
NRDG1	-2.1	-3
MAF	-2.3	-3.7
Bax-delta	-3.5	-2.7
IRS-1	-5	-2
PLOD2	-14	-11
PCDH7	-90	-10
Cyclin G	+2	0
GST A2	+3.7	+2.8

Table 5.3. Comparison of gene expression changes from microarray and RT-PCR.

The expression level for each the 10 genes generated by the microarray analysis was confirmed using simi-quantitative RT-PCR.

Gene Name	Gene Symbol	Chromosome location	Cellular localisation	Function
BH-protocadherin	PCDH7A	4p15	Membrane	Adhesion and cell-cell interaction
Growth differentiation factor 11	GDF11	12q13.13	Extra cellular	Growth and differentiation
N-MYC down regulated gene 1	NDRG1	8q24.3	Cytoplasmic	Growth and differentiation
BCL2 associated X protein	BAX	19q13.3	Cytoplasmic	Pro-apoptosis
Neurofibromatosis type 2 protein isofrom Mer151	NF2	22q12.2	Cytoplasmic and nucleus	Cell proliferation and motility
Musculaponeurotic fibrosacrcom	c-MAF	16q22.23	Nucleolus	Cell growth and regulating transcription
Procollagen-Lysine, 2-oxoglutarate 5- dioxygenase (lysine hydroxylase) 2	PLOD2	3q23.24	Extra cellular and cytoplasmic	Collagen stabilisation
Insulin receptor substrate 1	IRS-1	2q36	Cytoplasm	Signal transduction
Glutathione S- transferase protein	GST A2	6p12.1	Cytolpamsic	Detoxification of electrophonic compounds
Cyclin G2	CCNG2	4q21.1	Cytoplasmic Nuclear	Cell cycle regulator

Table 5.4. Description of the assessed genes

Description of genes altered by presence of TSPY are given including gene name, symbol, chromosome location, cellular localisation and function.

5.4.4 Semi-quantitative RT-PCR method to confirm microarray data

The expression patterns of the 10 candidate genes were verified using semi-quantitative RT-PCR assay (Figure 5.6). Pairs of primers were designed for each gene. Transcript expression level of each gene from LNCaP clone expressing FLAG-TSPY was compared to LNCaP clone containing only empty vector. A house keeping gene (GAPDH) was used as a reference gene for normalising. Each gene transcript level from both LNCaP clones relative to GAPDH internal control was measured using GelBandQuantitation software. All 10 genes were assessed and semi-quantitative RT-PCR confirmed the expression changes detected by GeneChip assay except for NF2 gene. The correct NF2 RT-PCR product was not detected.

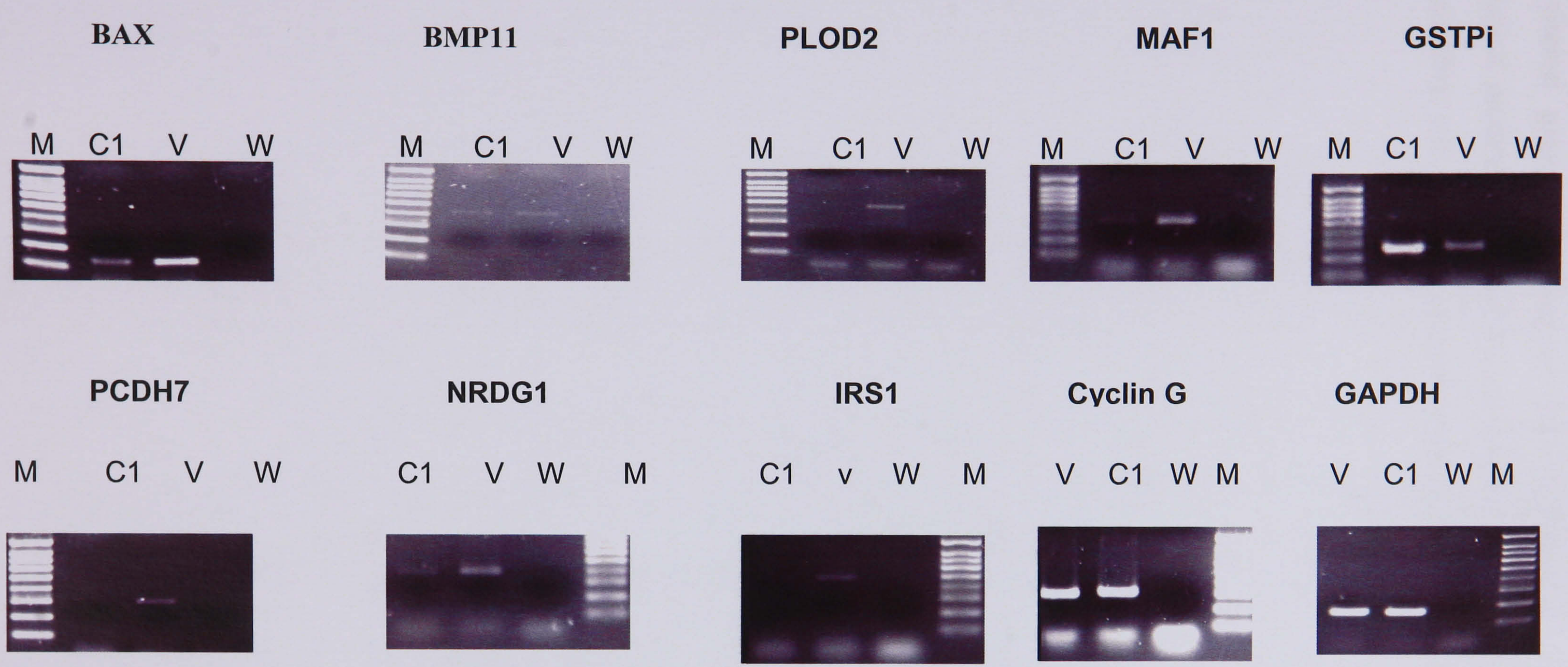


Figure 5.6. Semi-quantitative RT-PCR products for each gene selected from the GeneChip data output.

RT-PCR product for each gene was loaded into agarose gel next to DNA marker. The band size was checked and the band quantity was determined in relation to reference housekeeping gene (GAPDH). M indicates Bioline marker (1 Kb ladder), C1 describes clone that expressed FLAG-TSPY, V indicates empty vector and W is a symbol for water control.

5.4.5 Clustering genes into functional pathways

Initial analysis using ontology gene mining tools from Affymetrix web site has sorted the altered genes according to their biological processes. The major potential biological processes affected by expressing TSPY are apoptosis, cell differentiation, cell motility, cell adhesion, cell communication and signal transduction (Figure 5.7).

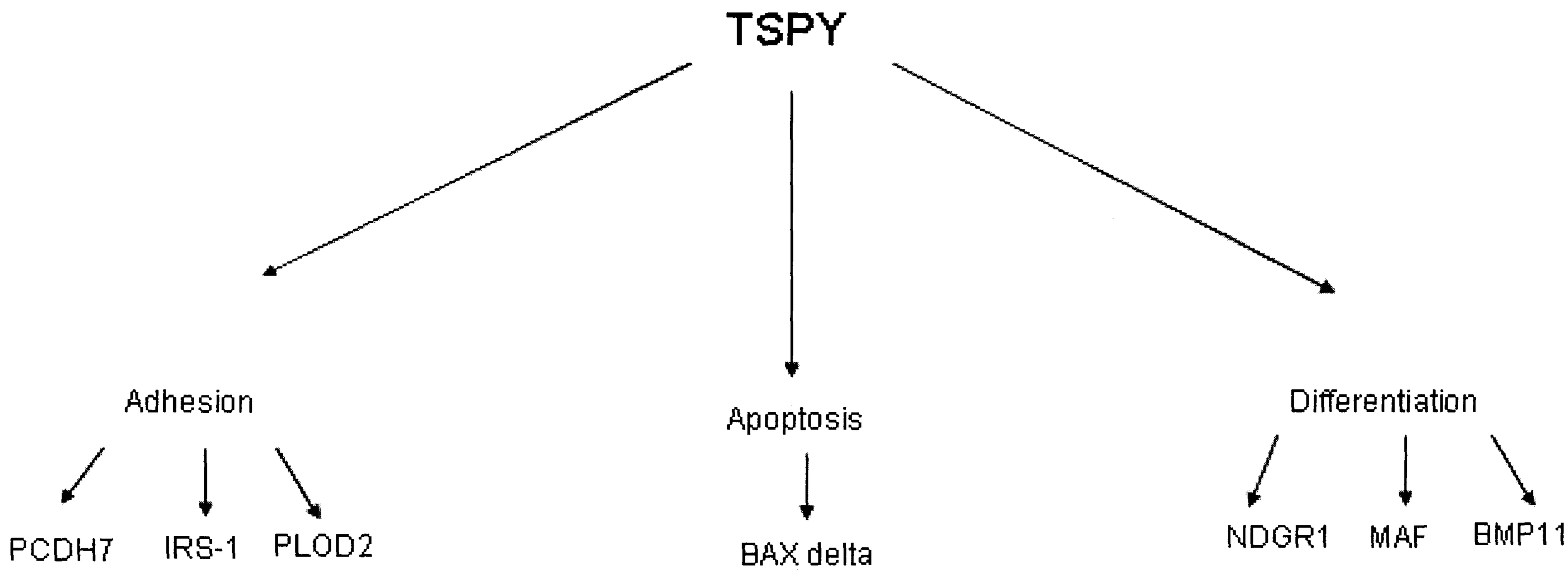


Figure 5.7. Proposed model for TSPY functional pathways.

Genes that were altered due to the effect of TSPY were analysed using gene ontology tool available on the Affymetrix web site to cluster them into functional pathways and infer possible molecular mechanisms of TSPY function.

5.5 Discussion

TSPY over-expression in prostate LNCaP cell line was shown to increase *in vitro* cellular proliferation and ability to form colonies. The molecular mechanism of TSPY mediated proliferation is not yet known. To better understand the cellular biological effect of TSPY, global gene expression profile from LNCaP cells expressing TSPY was compared to LNCaP cells stably transfected with empty vector using Affymetrix oligonucleotide GeneChip (U133 set A) method. The chip contains 22,285 probes representing different genes. Analysis of microarray gene output results has detected alterations in expression of 333 genes (appendix). A threshold for the level of change in gene expressions were taken as 2 fold. In total, 101 genes that showed 2 fold and more changes (increase or decrease) were selected. Of these, only 10 genes were selected for verification and further analysis. Over-expression of TSPY was confirmed with 69 fold up-regulation of transcript level in TSPY LNCaP transfected clone. The selected genes form part of diverse pathways that potentially regulates different biological processes including differentiation, apoptosis, adhesion, cell communication and signal transduction. Using gene ontology mining tools available on the Affymetrix web site, clustering of the candidate genes into distinct pathways was performed.

The first gene to be inspected was PCDH7 isoform A. Its expression was decreased by nearly 90 fold due to the presence of exogenous TSPY. BH-Protocadherin 7 (Heart and brain) isoform a (PCDH7) is a member of the cadherin superfamily. The superfamily is a diverse and large group of membrane associated glycoproteins. At least 80 members of cadherin superfamily sharing common structural features, small cytoplasmic domain, transmembrane domain and large extracellular domain consisting of repeats of cadherin motifs. The cadherin motif is composed of 110 amino acid and is suggested to play role in Ca++ dependent homophilic cell-cell adhesion (Geiger and Ayalon, 1992, Grunwald, 1993). Cadherin superfamily is classified into 4 subfamilies: classic cadherins, desmosomal cadherins, cadherin related proteins and protocadherins (PCDH) (Suzuki, 1996a, Suzuki, 1996b). PCDH subfamily consists of at least 60 members (Frank and Kemler, 2002). PCDH proteins are expressed in various tissues including stomach, brain, heart and thyroid gland and seem to function as cell-cell recognition, adhesion and as a component of signal

transduction pathway. They are also involved in development and tumourigenesis (Yoshida *et al.*, 1998). BH- Protacadhein 7 is one member of PCDH family and is predominantly expressed in heart and brain and thus is named BH-PCDH7. The gene is located in 4p15 and encodes a protein with an extracellular domain containing 7 repeats of cadherin motif (EC1-7). Alternative splicing yields three isoforms with unique cytoplasmic tails. These isoforms are, a, b and c. PCDH7 isoform a is 1069 aa, isoform b is 1072 aa and isoform c is 1200 aa. The protein product of PCDH7C was found to interact with protein phosphatase 1 alpha catalytic subunit (Yoshida *et al.*, 1999). Expression of PCDH7 C has been reported in human lung carcinoma cell line A549, HELA cells S3 and human gastric cancer cell lines, MKN28 and KATO-III. This locus is reported to show frequent loss of heterozygosity in head and neck squamous cell carcinoma. The role of PCDH7 in prostate cancer is not yet investigated. However, TSPY has a potential ability to down regulate expression level of PCDH7 isoform a and this might indicate that TSPY can be involved in regulating attachment and adhesion. The current findings tend to suggest that TSPY decreases the ability of the cell to attach and adhere and thus facilitate motility and migration of prostate cells and this may participate in prostate cancer development and progression.

Further evidence supporting the role of TSPY as a potential regulator of attachment and adhesion is the observation of decreased levels of expression of PLOD2 by 14 fold due to the presence of FLAG-TSPY. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2 (PLOD2) is lysyl hydroxylase catalyses the hydroxylation of lysyl residues on collagens (a post-translational enzyme of collagen biosynthesis). Collagen is a vital part of the extra cellular matrix. Formation of hydroxylysines in collagens are essential for the stability of extra cellular matrix (van der Slot *et al.*, 2003). Thus, decreased level of PLOD2 can destabilize the extra cellular matrix and consequently facilitate detachment and movement of cells.

Additional confirmation that TSPY can play a role as a potential regulator of the process of adhesion is noticed by decreased level of expression of IRS-1 gene by 5 fold in LNCaP cells. Insulin receptor substrate 1 (IRS 1) is a substrate of the insulin receptor tyrosine kinase and a putative participant in insulin signalling. The protein functions as a docking protein involved in binding and activating other signal transduction molecules after being phosphorylated on tyrosine by the insulin receptor

kinase (Sun *et al.*, 1991). It has been reported that expression of IRS-1 in LNCaP increased cell adhesion and decreased cell motility (Reiss *et al.*, 2001). As TSPY over-expression reduces the IRS-1 transcript level, this may lead to increased detachment and cellular motility.

A fourth line of support for the potential role of TSPY as a regulator of the adhesion mechanism is provided by the decreased expression level of NF2 by 2 fold. Neurofibromatosis type 2 protein isoform Mer151 (NF2). NF2 or Merlin is a candidate tumour suppressor gene that encodes 587-amino acid protein with high similarity to ERM family proteins (Ezrin, Radixin and moesin) that link the cytoskeleton components with proteins in the cell membrane (Trofatter *et al.*, 1993, Xiao *et al.*, 2003). Over-expression of wild type merlin protein has been found to alter F-actin organization, decrease motility, cause abnormalities in cell spreading and inhibit cell growth (Gutmann *et al.*, 1999). The NF2 gene is implicated in the development of sporadic schwannomas and meningiomas (Szijan *et al.*, 2003). Apparently, expression of TSPY has a negative influence on NF2 expression and decreased expression of NF2 forms part of the mechanism by which TSPY regulates adhesion. Although microarray data has shown 2 fold decrease in NF2 transcript, semi-quantitative RT-PCR failed to detect NF2. This can be caused by error in primer design and future design of a new primer pair is needed. From all the above evidence, TSPY is implicated in regulation of cell adhesion and motility.

Changes in genes that regulate cellular differentiation, proliferation and apoptosis were also detected from the GeneChip data output. TSPY caused a 2 fold decrease in expression of GDF11. Growth differentiation factor 11 (GDF11) also known as bone morphogenetic protein 11 (BMP11) is a member of the bone morphogenetic protein (BMP) family and the TGF-β superfamily. Secretory BMPs proteins mediate their effect by binding to serine-threonine kinase receptors and transducing signals via smads proteins inducing biological cellular changes (Miyazono *et al.*, 2001, von Bubnoff and Cho, 2001). They play a role in regulating cellular proliferation, apoptosis, differentiation, morphogenesis and adhesion. Thus TSPY contributes to regulating cellular proliferation through intermediate effectors such as GDF11.

Another gene down-regulated by TSPY is NDRG1. The gene is member of the N-myc down regulated gene family. The protein encoded by this gene is a cytoplasmic protein involved in stress responses, hormone responses, cell growth, and differentiation (Lachat *et al.*, 2002). The expression of NDRG1 is repressed by N-myc and C-myc. NDRG1 protein expression is down-regulated in neoplastic cells, and is up-regulated in differentiating cells. NDRG1 is a tumour metastasis suppressor gene and its expression has been found to be inversely linked to the progression of prostate cancer (Bandyopadhyay *et al.*, 2003). It has been reported that the gene product level is linked to induction growth arrest and inhibition of cellular proliferation (Piquemal *et al.*, 1999). The current results from microarray data analysis indicate that TSPY can decrease cellular differentiation and increase cellular proliferation in keeping with the suppressed expression of NRDG1.

Another gene that has been reported to regulate differentiation is C-MAF. C-MAF is an avian muscloaponeurotic fibrosarcoma oncogene homologue that belongs to MAF transcription factors, a unique subclass of basic-leucine zipper transcription (bZIP) factors (Nishizawa *et al.*, 1989). Members of the MAF family appear to play important roles in the regulation of differentiation (Blank and Andrews, 1997). The microarray data has shown that c-MAF expression is down regulated by more than 2 fold in cells over-expressing TSPY. Thus TSPY can induce proliferation by inhibiting differentiation through down regulation of C-MAF.

As well as alterations of gene expressions related to differentiation, a cell cycle regulator gene (cyclin G2) has been found to be up-regulated by two fold in LNCaP cells over-expressing TSPY relative to cells containing empty vector. Cyclin G2 has been identified as p53 target gene (Okamoto and Prives, 1999) and it increased expression is associated with induction of p53 by DNA damage. The gene product is a novel cyclin negatively regulating the cell cycle progression, contrary to the characteristics of conventional cyclins and its expression is up-regulated as cells undergo cell cycle arrest or apoptosis (Bennin *et al.*, 2002). Ectopic expression of Cyclin G2 inhibits cell proliferation in Hela cell line, HEK293 and Chinese hamster ovary cells (Bennin *et al.*, 2002). Cyclin G2 expression has been detected in kidney, spleen and prostate (Horne *et al.*, 1996). Although cyclin G2 gene expression profile

from genechip analysis has indicated a change in the gene expression, there was no alteration in expression between tested sample and control as tested by RT-PCR.

Regulation of apoptosis is another mechanism for controlling proliferation and it is the counter act of proliferation and ability to suppress apoptosis is another way of regulating proliferation. Over-expression of TSPY down regulates BAX delta isoform expression by nearly 4 fold. BAX is Bcl 2 associated X protein. Six alternatively spliced transcript variants, which encode different isoforms, have been reported for this gene: α, β, γ, δ, ε and ζ. The protein encoded by this gene belongs to the BCL2 protein family. BCL2 family members form hetero- or homodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities (Oltvai *et al.*, 1993). This protein forms a heterodimer with BCL2, and functions as an apoptotic activator (Oltvai *et al.*, 1993). The expression of this gene is regulated by the tumour suppressor P53 and has been shown to be involved in P53-mediated apoptosis (Miyashita and Reed, 1995). From all the above, it seems that TSPY plays a potential role in suppressing apoptosis through down regulating BAX gene.

Up-regulation of GST A2 in LNCaP cells over-expressing TSPY compared to control was detected by genechip and validated by RT-PCR. As mentioned in the first chapter of the thesis, Glutathione S transferases enzymes are detoxification enzymes. Six distinct classes of GST have been identified: alpha, Kappa, Mu, Omega, pi and zeta. GST 2A belongs to alpha class and is located on chromosome band 6p21.1. This class of enzyme have peroxidase activity protecting cell from free oxygen radicals (Board and Webb, 1987).

5.6 Conclusion

Microarray analysis tends to suggest that TSPY is a putative regulator of adhesion, apoptosis, differentiation and proliferation. Down-regulation of pro-apoptotic gene, differentiation gene and adhesive molecules underlie the molecular mechanism by which TSPY exerts its functional cellular effect.

Chapter 6
General discussion

6.1 General discussion

Prostate cancer is a multi-staged disease caused by accumulation of multiple genetic changes in several genes regulating normal cellular proliferation, differentiation, apoptosis, adhesion and metastasis. Gene amplification and/or over-expression are among the common polygenetic alterations contributing to transforming normal cellular prostate cells to a malignant form. During the cellular transformation, cells acquire enhanced abilities to continuously divide, resist growth inhibition, inhibit apoptosis, overcome senesce, metastasise and induce angiogenesis. Evolutiong of cancerous clones from their normal parental cells is accelerated by genomic instability and consequently chromosomal aberrations. Sex chromosome abnormalities (aneuploidy) are common events associated with CaP. Gain of Y chromosome has been frequently detected in CaP. Although Y chromosome is the smallest human chromosome harbouring a limited number of genes, the functions of the majority of Y-chromosomes genes are not known. Y chromosome has been postulated to participate in male sex organ development and oncogenesis. TSPY, a sex specific organs (testis and prostate) gene is a candidate gene for the gonadobalstoma locus on short arm of Y chromosome. TSPY gene is polymorphic in copy number and over-expression of TSPY has been detected in gonadoblastoma and testicular cancer. Although the exact function of the gene product is not yet known, it has been suggested that the gene can regulate proliferation of spermatogonia (testicular dividing cells). In the current study, the role of this gene in prostate carcinogenesis was investigated using the following approaches. First, the expression levels of TSPY were analysed in prostate cancer compared to BPH. Second, CaP functional analysis was carried out on LNCaP stably transfected to over-express TSPY. Third, TSPY genomic copy numbers in CaP and BPH were determined to test if expansion of copy numbers may be an underlying cause for its over-expression in prostate cancer. Finally, global transcriptional analysis of LNCaP cells expressing TSPY was investigated using the Affymetrix GeneChip oligonucleotide microarray method.

TSPY genetic alterations (over-expression and/or amplification) and its role in prostate carcinogenesis were investigated. In the current study, anti-TSPY peptide polyclonal antibody was generated and used in IHC to detect and quantitate TSPY expression in clinical prostate tissue specimens. The expression of TSPY gene

product was primarily detected in the prostate epithelium of normal, BPH and malignant glands. Stromal expression was not observed in prostate gland sections. The expression of TSPY protein was predominantly located in the cytoplasm of prostate cells. Nuclear expression was not observed in any prostate sections investigated. In similar but limited study analysing TSPY expression pattern in prostate cancer, TSPY expression was also located in the cytoplasm of prostate cells without nuclear expression (Lau *et al.*, 2003). Similar findings were observed from lummunofluorescence analysis of LNCaP cells stably-transfected to express FLAG-TSPY transgene. FLAG-TSPY protein is predominantly located in the cytoplasm with no signal detected in the nucleus. In spite of the fact that TSPY protein structure analysis indicated presence of potential nuclear localisation signal in the N-terminus of the gene product, it seems that in prostate, TSPY functions in the cytoplasm and only proteins that are located only in the cytoplasm and those are expressed in both the nucleus and cytoplasm may interact with it. Whether TSPY expression is cell cycle phase specific remains to be explored using bio-imaging system and GFP tagged to TSPY gene. Although Schnieders *et al* have detected similar pattern of cytoplasmic location of TSPY in testicular spermatogonial cells using IHC, nuclear and cytoplasmic expression was detected in subpopulations of spermatogonia of the testis. It seems from the current observations that TSPY is generally expressed in the cytoplasm and nuclear expression is tissue specific controlled by tissue specific gene(s).

IHC expression analysis has shown that in normal and BPH epithelium, moderate TSPY expression was detected in the basal cells and expression was close to absent in luminal cells. This pattern of expression was also observed in BPH and normal glands adjacent to the malignant epithelium. It is apparent that TSPY is expressed in proliferative cells of the prostate epithelium and is absent in differentiated cells. In a separate study carried out by Shcienders *et al*, it has been shown that TSPY is expressed in proliferative cell of testis (spermatogonia) and weak expression was detected in cells entering differentiated phase (early spermatogonia) and no expression was detected in terminally differentiated spermatocytes. Overall, TSPY expression is associated with the proliferative status of the cells where it is expressed in proliferarive cells and absent in non-proliferative cells (differentiated cells). In addition, it has been observed from the current expression analysis that TSPY is over-

expressed in prostate cancer compared to BPH. The same was observed by TSPY transcript analysis using TISH, where there was preferential expression of TSPY in malignant glands compared to BPH glands. Furthermore, a similar trend of TSPY expression was reported by Lau study, where he detected over-expression of TSPY in CaP lesions compared to BPH glands. This tends to suggest that up-regulation of TSPY can increase cellular proliferation and thus contribute to CaP development. Functional analysis has demonstrated that TSPY over-expression in LNCaP increased cellular proliferation as confirmed by the proliferation assays and colony forming assay. Thus, TSPY over-expression can cause enhanced cellular growth in LNCaP cell line and its over-expression is linked to prostate cancer development. Furthermore, increased TSPY expression was observed in high Gleason score prostate cancer which is rapid growing tumour and tends to be undifferentiated compared to low Gleason score that is slow growing and well differentiated.

Strong correlation has also been detected between the expression profile of TSPY and metastases. This tends to indicate that over-expression of TSPY associated with prostate cancer progression can lead to alterations in genes that are involved in metastasis, specifically genes regulating adhesion which will be discussed later. It is well known that prostate cancer metastasises to the bone at high frequency in patients with advanced disease status. Approximately, 90% of advanced stage prostate cancer patients develop bone lesions causing morbidity that includes bone pain, immobility and spinal cord compression (Bubendorf *et al.*, 2000, Rubin *et al.*, 2000). Metastasis is a multi-step process that includes growth in the primary organ, neoangiogenesis, detachment, intravasation, immune evasion, attachment to distal organ, extravasation at the new site and growth of secondary neoplasms (Tantivejkul *et al.*, 2004).

The current observation that TSPY is over-expressed in CaP tends to suggest that this abnormal expression might be a direct consequence of gene amplification. Gene amplification is among one of the common events associated with prostate carcinogenesis (Quinn and Babb, 2002, Saramaki *et al.*, 2001). Increased gene copy number has frequently been reported in CaP and can lead to increased gene expression (Linja *et al.*, 2004). To investigate whether the over-expression of TSPY detected in the current study is related to increased gene copy (amplification), quantitative real time PCR based on Taqman assay method using a standard curve approach was

established and used to determine the genomic TSPY copy number from prostate clinical samples (blood and tumours). The exact genomic TSPY copy number for each case was identified. TSPY genomic copies were always present in all samples tested. The minimum copy number detected in CaP was 6 gene copies and the maximum was 85 copies. These extreme values (high and low) were infrequently detected in the cases assessed. In spite of the extreme value of copy number, the most frequently reported copy number range in all cases investigated was 20-50 copies. Similar findings were reported by Manz study where he estimated TSPY copy number to be between 20 and 50, using semi-quantitative Southern blot. Upon comparing TSPY copy number in prostate cancer cases relative to BPH, it was found that although there were inter-individual variations in copy number among cases, there were no amplification of TSPY in CaP relative to BPH samples. This strongly suggests that TSPY polymorphism in copy number is not linked to prostate carcinogenesis and over-expression of TSPY in prostate cancer is not due to gene amplification and therefore another mechanism is responsible for abnormal expression of the gene. Several evidences have been accumulated indicating other transcription factors including androgen receptor and c-myc can potentially play a role in regulating TSPY transcription. Up-regulation of TSPY expression was detected in LNCaP induced by DHT (Lau *et al.*, 2000). A recent study has also shown that c-myc is a potential transcription factor that binds to TSPY promoter region. The effect of this binding is not yet known (Li *et al.*, 2003).

Although TSPY over-expression is found to be linked to prostate carcinogenesis, the molecular consequences of abnormal expression remain unknown. Global gene expression using Affymetrix Genechip method was performed to identify genes with altered expression by TSPY over-expression in LNCaP prostate cancer model cell line. Several hundred genes have been altered by TSPY. Ten genes related to carcinogenesis were clustered into pathways that mediate three major cellular biological processes, differentiation, apoptosis and adhesion. Evasion of apoptosis, suppression of differentiation and deregulated adhesion are among the common cellular alterations associated with cancer development (Hanahan and Weinberg, 2000). It is generally accepted that net tumour growth and tumour progression are regulated by the balance between proliferation and apoptosis. Acquired resistance to apoptosis forms an essential step in prostate carcinogenesis. Bcl-2 oncoprotein and its

family of related genes products such as Bax play a central role in regulating apoptosis. Blc-2 expression has been shown to be an independent prognostic marker in prostate cancer (Moul *et al.*, 1996). Bcl-2 is an anti-apoptotic protein and BAX is a pro-apoptotic protein. The proteins physically interact with each other to form homo- and heterodimers. When Bax predominates, programmed cell death is accelerated and the death repression activity of Bcl-2 is counteracted. Thus the ratio of Bcl-2 to Bax determines survival or cellular death (Oltvai *et al.*, 1993). Over-expression of Bax protein in prostate cancer LNCaP cell line induced apoptosis (Honda *et al.*, 2002, Lowe *et al.*, 2001). Down-regulation of BAX expression mediated by over-expression of TSPY in LNCaP cell line may lead to suppression of apoptosis and thus enhanced proliferation. Both protein products are located in the cytoplasm of the cell. Whether TSPY protein product can interact directly with BAX protein or indirectly through intermediate, still remains elusive. Interacting ability between TSPY and BAX can be evaluated using immunoprecipitation.

Inhibition of cellular differentiation genes can be a primary event that initiates tumourigenesis. NDRG1 is differentiation gene that has been reported to be abnormally expressed in different types of cancer. It has been found that the gene is deregulated during colon epithelial cell differentiation and is down regulated during colorectal neoplasm (van Belzen *et al.*, 1997). Functional analysis studies have shown that NDRG1 suppresses colon cancer metastasis by inducing colon cancer cell differentiation (Guan *et al.*, 2000). The gene has also been found to be deregulated during keratinocyte differentiation and is linked to skin carcinogenesis (Gomez-Casero *et al.*, 2001). The expression is significantly inversely correlated to Gleason grading and metastasis to lymph node and bone. Our data has shown that over-expression of TSPY in prostate cancer cell line modulates differentiation through down-regulating the NDRG1 differentiation gene. Another differentiation gene is C-MAF transcription factor that has an important role in embryonic development and cellular differentiation. It is expressed in a variety of tissues including, spleen, kidney and liver (Ogata *et al.*, 2004). C-MAF plays a significant role in myeloid cell development and induces differentiation in the myeloid cell progenitor (Hegde *et al.*, 1999). The role of C-MAF in prostate carcinogenesis has not been fully investigated. Although the role of BMP11 in prostate cancer is no yet investigated, the gene plays a vital role in regulating kidney organogenesis, neurogenesis and anterior/posterior of

the axial skeleton during embryogenesis (Esquela and Lee, 2003). Functional analysis of BMP11 and its role in the carcinogenesis of prostate is not yet known. Overall, TSPY over-expression contributes to prostate carcinogenesis by suppressing apoptosis and differentiation and thus enhancing proliferation.

Metastasis of tumour is a major cause of cancer-related death (Ahmad and Hart, 1997). Cancer metastasis occurs as a result of disruption of cell-cell and cell-extracellular matrix (ECM) interactions (Cohen *et al.*, 1997). In the early events of metastasis, cancer cells detach from the tumour primary site and evade ECM. Functional down-regulation of intercellular adhesion between malignant cells is a pre-requisite for tumour invasion. Development of abnormalities in cell adhesion of prostate cancer cells is one of the prominent steps in progression of prostate cancer. Adhesion molecules play a central role in mediating cell-cell and cell-matrix adhesion. Protocadherins are transmembrane glycogoproteins belonging to the Cadherin superfamily which critically regulate cellular adhesion, cytoskeletal organisation and morphogenesis. Although the role of Pcdh7 in prostate cancer is not yet known, it seems that TSPY has a significant effect on modulating PCDH7 expression by deregulating its expression and thus permitting detachment. This provides evidence that TSPY can potentially regulate adhesion of cells.

Extra cellular matrix (ECM) is composed of two classes of macromolecules. The first class is called glycosaminoglycans which is made up of polysaccharide chains covalently linked to protein to from proteoglycans. The second class is made up of fibrous proteins like collagen. Glycosaminoglycans form dehydrated gel like substance in which members of fibrous proteins are embedded. Collagen and polysaccharides that are secreted locally and assembled into organised meshwork. Collagen is predominately synthesised by fibroblast and constitute major protein of the matrix. Collagen molecule is long triple helix structure that is composed of three polypeptide chains called α that are wound around each other to form super helix. Collagen helixes are assembled to from collagen fibres which give the ECM strength and resilience (Alberts B., 2002). PLOD2 is lysyl hydroxylase enzyme that catalyses the hydroxylation of lysine residues during the post-translational modification of collagen protein. The hydroxylysine residue formed during lysyl hydrolysine reaction has two important functions: first, the hydroxy group serves as an attachment for a

carbohydrate unit and second it is essential for the stability of intermolecular collagen crosslink. Deficiency of PLOD activity causes Enhler-Dnalos syndrome, characterised by hyper flexibility of the joints and hyperelasticity of the skin (Brinckmann *et al.*, 1998). The role of PLOD2 in the carcinogenesis of prostate is not yet known, but over-expression of TSPY can destabilise ECM by changing collagen cross linking.

Discovering candidate proteins that can potentially interact with the TSPY protein will facilitate better understanding of its molecular function, influencing its protein activity, stability, post-translational modifications and its contributions to prostate carcinogenesis. The molecular mechanism of TSPY expression regulation is still unknown and investigating potential candidate regulatory transcription factors can highlight its abnormal expression in prostate diseases status. Searching for TSPY functional inhibitors can be potential target for designing intervention therapy at early stages of prostate cancer.

In this thesis, the role of TSPY in prostate carcinogenesis has been examined and confirmed with abnormal TSPY expression seen in high grade and metastatic disease. Hence, TSPY can be a potential molecular marker for prostate cancer diagnosis, prognosis and designing best strategy for therapy intervention.

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Appendix

List of the 333 genes whose expressions are altered due to the presence of FLAG-TSPY.

Fold	Decreased gene expression
1.5	Hypothetical protein, DKFZp586K0922.
1.5	Hypothetical protein, DKFZp434D0421.
1.5	Membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10) (MME), transcript variant 1bis.
1.5	Cyclin-dependent kinase 5, regulatory subunit 1 (p35).
1.5	Androgen-regulated serine protease TMPRSS2 precursor.
1.5	Similar to reticulon 1.
1.5	Phosphoprotein enriched in astrocytes 15.
1.5	Adaptor-related protein complex 1, sigma 2 subunit (AP1S2).
1.5	Cytoplasmic dynein intermediate chain 2C.
1.5	Potassium inwardly-rectifying channel, subfamily J, member 3 (KCNJ3).
1.5	CD151 antigen (CD151).
1.5	Hypothetical protein, FLJ13052.
1.5	Thimet oligopeptidase 1 (THOP1).
1.5	WW domain binding protein-2.
1.5	Hypothetical protein, FLJ00003.
1.5	Dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related).
1.5	GTP-binding protein ROC1 (ROC1).
1.5	Trinucleotide repeat containing 3.
1.5	Hypothetical protein, MGC11256.
1.5	Hypothetical protein AF053356_CDS3.
1.5	Ceroid-lipofuscinosis, neuronal 3, CLN3 protein (CLN3).
1.5	Putative sterol reductase SR-1 (TM7SF2).
1.5	Adenylate kinase 1.
1.5	Rho GTPase activating protein 6 (ARHGAP6), transcript variant 2.
1.5	Clathrin, light polypeptide (Lcb).
1.5	Patched (Drosophila) homolog.
1.5	Serine carboxypeptidase 1 precursor protein (HSCP1).
1.5	Wee1+ (S. pombe) homolog.

1.5	Hypothetical protein, FLJ12387.
1.5	TBP-like 1 (TBPL1).
1.5	Ectonucleoside triphosphate diphosphohydrolase 6 (putative function) (ENTPD6).
1.5	Latent transforming growth factor beta binding protein 3 (LTBP3).
1.5	Similar to rat HREV107, clone MGC:1240.
1.5	Bromodomain-containing 4.
1.5	Hypothetical protein, FLJ10312.
1.5	Interferon regulatory factor 7 (IRF7), transcript variant c.
1.5	Mitogen-activated protein kinase kinase kinase 8 (MAP3K8).
1.5	CDC42-binding protein kinase beta (DMPK-like) (CDC42BPB).
1.5	Mouse Mammary Turmor Virus Receptor homolog.
1.5	Monoamine oxidase A.
1.5	Insulin-like growth factor 1 receptor.
1.5	Phospholipase C, beta 4.
1.5	Hypothetical protein, MGC2601.
1.5	Electron transfer flavoprotein-ubiquinone oxidoreductase.
1.5	Lysosomal sialidase.
1.5	Spinocerebellar ataxia 1 (olivopontocerebellar ataxia 1, autosomal dominant, ataxin 1) (SCA1).
1.5	Endothelin receptor type B-like protein.
1.5	Tumour necrosis factor receptor superfamily, member 6.
1.5	KIAA0212 gene product (KIAA0212).
1.5	Chromosome 19, cosmid R31180.
1.5	Putative mitochondrial inner membrane protein import receptor (hTIM44).
1.5	Ubiquitin specific protease 12.
1.5	Cluster Incl. H93026:yv06a08.s1 Homo sapiens cDNA, 3 end /clone.
1.5	Tropomyosin 1 (alpha).
1.5	Glucose phosphate isomerase.
1.5	SH3 domain-containing protein SH3P18.
1.5	Homolog of yeast ubiquitin-protein ligase Rsp5; potential epithelial sodium channel regulator.
1.5	KIAA0699 protein.
1.5	Ca2-activated neutral protease large subunit (CANP).

1.5	Hypothetical protein, FLJ22569.
1.5	Monoamine oxidase A (MAOA), nuclear gene encoding mitochondrial protein.
1.5	Cyclin E2 (CCNE2).
1.5	Sec61 homolog.
1.5	Emerin (Emery-Dreifuss muscular dystrophy), clone MGC:2126.
1.6	Damage-specific DNA binding protein 2 (48kD) (DDB2).
1.6	RNA binding motif protein 9.
1.6	BRG1-Associated Factor 250a (BAF250a).
1.6	Midline 1 (MID1).
1.6	BCL2-like 1 (BCL2L1).
1.6	Hypothetical protein, FLJ11856.
1.6	Breast cancer 2, early onset (BRCA2).
1.6	Hypothetical protein, FLJ13754.
1.6	Putative gene product (13CDNA73).
1.6	U2 small nuclear ribonucleoprotein auxiliary factor (65kD) (U2AF65).
1.6	Homeodomain-interacting protein kinase 2 (HIPK2).
1.6	Neuropeptide Ypeptide YY Y2 receptor.
1.6	Hypothetical protein, FLJ10955.
1.6	Hypothetical protein, FLJ11937.
1.6	Tax interaction protein 1.
1.6	Alternative splicing product of glucocerebrosidase pseudogene.
1.6	Mannosidase, alpha, class 1A, member 1 (MAN1A1).
1.6	DEADH (Asp-Glu-Ala-AspHis) box polypeptide 17 (72kD).
1.6	CCAAT-box-binding transcription factor (CBF2).
1.6	Glutaminase-interacting protein 3.
1.6	Variable charge protein on X with two repeats (VCX-2r).
1.6	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2).
1.6	Biotinidase.
1.6	Nucleosome assembly protein 1-like 3 (NAP1L3).
1.6	Adaptor-related protein complex 1, sigma 1 subunit (AP1S1).
1.6	Sperm specific antigen 2 (SSFA2).

1.6	Clone RP1-221C16 on chromosome 6, contains genes for novel histone, 1, 2B, 2A and 4 family members.
1.6	Nucleobindin 2 (NUCB2).
1.6	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha.
1.6	Synaptojanin 1 (SYNJ1).
1.6	3-Hydroxyisobutyryl-Coenzyme A hydrolase (HIBCH).
1.6	Guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1.
1.7	Ras homolog gene family, member, clone MGC:5612.
1.7	Hypothetical protein, FLJ00115.
1.7	Spermidinespermine N1-acetyltransferase (SAT).
1.7	Seven trans-membrane domain protein (AD3LPAD5).
1.7	Jun B proto-oncogene (JUNB).
1.7	Serumglucocorticoid regulated kinase-like (SGKL).
1.7	Similar to adaptor-related protein complex 1, sigma 1 subunit.
1.7	Cathepsin O.
1.7	Interferon induced transmembrane protein 3 (1-8U).
1.7	Hypothetical protein, DKFZP586D211.
1.7	Insulin-like growth factor 1 receptor.
1.7	Zinc finger protein 294.
1.7	B-cell translocation gene 1, anti-proliferative (BTG1).
1.7	Small GTPase (RAB5C), member of RAS oncogene family.
1.7	SMA4.
1.7	B-TRCP variant E3RS-IkappaB.
1.7	Leukemia inhibitory factor receptor (LIFR).
1.7	RAP2B, member of RAS oncogene family (RAP2B).
1.7	Sin3-associated polypeptide, 18kD.
1.7	Hypothetical protein, DKFZp564F112.
1.7	Protein kinase C, delta (PRKCD).
1.7	Clone RP4-761I2 on chromosome 6, contains gene for enhancer of filamentation (HEF1).
1.7	Hypothetical protein, KIAA1115 protein.
1.7	Adaptor-related protein complex 1, sigma 1 subunit (AP1S1).
1.7	Interferon-stimulated protein, 15 kDa (ISG15).

1.7	Hypothetical protein FLJ10350 (FLJ10350).
1.7	Nucleosomal binding protein 1 (NSBP1).
1.7	Mannosidase, alpha, class 1A, member 1.
1.9	Deiodinase, iodothyronine, type I (DIO1).
1.9	Likely ortholog of mouse variant polyadenylation protein CSTF-64.
1.9	Meis1 (mouse) homolog (MEIS1).
1.9	Adducin 3 (gamma).
1.9	Potassium intermediatesmall conductance calcium-activated channel, subfamily N, member 2 (KCNN2).
1.9	DEADH (Asp-Glu-Ala-AspHis) box polypeptide 17 (72kD) (DDX17), transcript variant 2.
1.9	Hypothetical protein, FLJ11648.
1.9	Adducin 3 (gamma).
1.9	UDP-glucose dehydrogenase (UGDH).
1.9	Neuropilin 1.
1.9	Hypothetical protein, FLJ21079.
2.0	UDP-glucuronosyltransferase type 1 (UGT2B28).
2.0	Neurofibromatosis type 2 protein isoform Mer151 (NF2).
2.0	NADPH-cytochrome P450 reductase.
2.0	Hypothetical protein, FLJ12806.
2.0	Hypothetical protein form IMAGE clone 3629896.
2.0	PCDH7 (BH-Pcdh)c.
2.0	Hypothetical protein, FLJ22490.
2.0	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (Id-1H).
2.0	Hypothetical protein, FLJ14154.
2.0	Spermidinespermine N1-acetyltransferase.
2.1	Similar to granulin.
2.1	Growthdifferentiation factor-11 (GDF11).
2.1	N-myc downstream regulated (NDRG1).
2.1	Nuclear receptor interacting protein 1 (NRIP1).
2.1	Melanoma antigen, family C, 1 (MAGEC1).
2.1	Neurochondrin.
2.1	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain.

2.1	RAB5C, member RAS oncogene family (RAB5C).
2.1	Hypothetical protein from IMAGE clone 341654.
2.1	Clone RP11-110H4 on chromosome 5, contains a pseudogene similar to GUSB (glucuronidase, beta).
2.3	Aldehyde dehydrogenase 1 family, member A3 (ALDH1A3).
2.3	Hypothetical protein, FLJ21159.
2.3	Similar to CAGF9 mRNA, trinucleotide repeat containing 9.
2.3	DEAD-box protein p72 (P72).
2.3	IL8-related receptor (DRY12).
2.3	Short form transcription factor C-MAF (c-maf). v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog.
2.3	h=Hypothetical protein, FLJ20449.
2.5	B-cell translocation gene 1, anti-proliferative.
2.5	Endothelin receptor.
2.5	Adducin 3 (gamma) (ADD3), transcript variant 2.
2.5	Spermidinespermine N1-acetyltransferase.
2.5	gb:W58342 /FEA=EST gi:1365125 est:zd25a08.s1 putative gene product
2.6	Transforming, acidic coiled-coil containing protein 1 (TACC1).
2.6	Annexin A1 (ANXA1).
2.6	Similar to CAGF9 mRNA, trinucleotide repeat containing 9.
2.6	Calciumcalmodulin-dependent protein kinase II beta subunit mRNA, complete cds. (CaM kinase) II beta.
2.6	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1 (HS3ST1).
2.8	Similar to CAGF9, trinucleotide repeat containing 9.
2.8	Hypothetical protein, FLJ20160.
2.8	SWISNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (SMARCA1).
2.8	Ras-like GTP-binding protein (RAB27A) gene.
2.8	RAB27A, member RAS oncogene family.
3.0	Nuclear receptor interacting protein 1.
3.0	RAB27A, member RAS oncogene family.
3.2	Rab27a, member RAS oncogene family.
3.2	Hypothetical protein, DKFZP586D0624.
3.2	E1B-55kDa-associated protein 5.
3.5	(BAX delta) mRNA, complete cds. BCL2-associated X protein.

3.5	G antigen, family B, 1 (prostate associated) (GAGEB1).
3.5	Proline rich calmodulin-dependent protein kinase mRNA, complete cds. (CaM kinase) II beta.
3.7	ATPase, Na+K+ transporting, beta 1 polypeptide (ATP1B1).
3.7	Cytochrome P450, subfamily VIIB (oxysterol 7 alpha-hydroxylase), polypeptide 1 (CYP7B1).
4.0	Ankyrin 2, neuronal (ANK2), transcript variant 1.
4.0	BAC clone GS1-99H8.
4.3	Similar to carnitine palmitoyltransferase I.
4.6	Neuropeptide Y receptor Y1 (NPY1R).
4.9	Glycoprotein Ib (platelet), beta polypeptide.
4.9	ATPase, Na+K+ transporting, beta 1 polypeptide.
4.9	Insulin receptor substrate 1 (IRS1).
5.3	PCDH7 (BH-Pcdh)b.
7.0	G protein-coupled receptor 85 (GPR85).
13.9	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2 (PLOD2).
48.5	BH-protocadherin (brain-heart) (PCDH7).
90.5	BH-protocadherin (brain-heart) (PCDH7).
1.5	Hypothetical protein, FLJ22795.
1.6	TATA box binding protein (TBP)-associated factor, RNA polymerase II, N, 68kD (RNA-binding protein 56) (TAF2N).
1.9	Similar to Bicaudal D (Drosophila) homolog 1.
2.5	Colon Kruppel-like factor (CKLF).
8.6	Hypothetical protein, FLJ10775.

Fold	Increased gene expression
1.5	S100 calcium-binding protein P (S100P).
1.5	Homeobox protein NKX3.1.
1.5	Growth factor receptor-bound protein 14 (GRB14).
1.5	Ribosomal protein L37a.
1.5	Histone fold protein CHRAC17; DNA polymerase epsilon p17 subunit.
1.5	ES1 (zebrafish) protein, human homolog of (C21ORF33).
1.5	Fibroblast growth factor 13 (FGF13).

1.5	EST
1.5	Ets homologous factor (EHF).
1.5	Hypothetical protein, DKFZp564O0523.
1.5	7-Keto-8-amino-pelargonic acid synthetase (bioF).
1.5	Mitochondrial solute carrier.
1.5	Cyclin G2 (CCNG2).
1.5	Non-POU-domain-containing, octamer-binding.
1.5	Fatty-acid-Coenzyme A ligase, long-chain 3 (FACL3).
1.5	P53 cellular tumour antigen.
1.5	Hypothetical protein, DKFZp586I2022.
1.5	Hypothetical protein, expressed in osteoblast (GS3686).
1.5	Hypothetical protein, FLJ13302.
1.5	Calcium binding protein P22 (CHP).
1.5	TG-interacting factor (TALE family homeobox) (TGIF).
1.5	Lipoprotein-associated coagulation inhibitor.
1.6	C MAX protein.
1.6	Highly similar to HSFIB1 Human mRNA for fibronectin (FN precursor). fibronectin 1.
1.6	Androgen-regulated short-chain dehydrogenasereductase 1 (ARSDR1).
1.6	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 2 (lysosomal integral membrane protein II).
1.6	CGI-82 protein.
1.6	Phosphoglucomutase 1 (PGM1).
1.6	Hum-a-tub2 alpha-tubulin Tubulin, alpha, brain-specific.
1.6	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 2 (lysosomal integral membrane protein II).
1.6	Consensus includes gb:BE138647 /CLONE KIAA0741 gene product
1.6	CGI-67 protein (LOC51104).
1.6	Prolyl oligopeptidase.
1.6	Tumor necrosis factor receptor superfamily.
1.6	Hypothetical protein, FLJ20700.
1.6	Arginase II.
1.6	Progesterone membrane binding protein.
1.6	EST.

1.6	Hexokinase 2.
1.6	MAX-interacting protein 1 (MXI1).
1.6	Vitamin A responsive; cytoskeleton related (JWA).
1.6	Similar to HEPATOMA-DERIVED GROWTH FACTOR.
1.6	Hypothetical protein, KIAA0878.
1.6	PTPL1-associated RhoGAP 1 (PARG1).
1.7	ATP-binding cassette, sub-family B (MDRTAP), member 11 (ABCB11).
1.7	Hypothetical protein, FLJ12687.
1.7	28S rRNA sequence, length 5025 bases, middle target bases 1666-3330.
1.7	P53 regulated PA26 nuclear protein (PA26).
1.7	Platelet-derived growth factor receptor-like (PDGFRL).
1.7	C type II Golgi membrane protein.
1.7	Fibronectin 1.
1.7	Homogentisate 1,2-dioxygenase (homogentisate oxidase) (HGD).
1.7	V-myc avian myelocytomatosis viral oncogene homolog (MYC).
1.7	Human serum albumin (ALB) gene.
1.7	Helix-loop-helix zipper protein (max).
1.7	Integrin, alpha 6 (ITGA6).
1.7	Single-stranded-DNA-binding protein (SSBP2).
1.7	Hypothetical protein, DKFZP564M1462.
1.7	18S rRNA sequence, length 1969 bases, 5 prime target bases 1-646
1.7	Argininosuccinate synthetase (ASS).
1.7	Glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID) (GNS).
1.7	Lanosterol 14-alpha demethylase (CYP51P2) processed pseudogene.
1.7	Hypothetical protein, FLJ23516.
1.7	Hypothetical protein, KIAA0367.
1.7	General transcription factor IIH, polypeptide 3 (34kD subunit).
1.7	Golgi membrane protein GP73 (LOC51280).
1.7	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial) (HMGCS2).
1.7	Cyclin G2.
1.7	18S rRNA gene, complete (_5, _M, _3 represent transcript regions 5 prime, Middle, and 3 prime respectively).

1.9	Utrophin (homologous to dystrophin) (UTRN).
1.9	Utrophin (homologous to dystrophin) (UTRN).
1.9	Sushi-repeat-containing protein, X chromosome (SRPX).
1.9	G protein-coupled receptor.
1.9	TUP1-like enhancer of split gene 1 (TUPLE1) .HIR (histone cell cycle regulation defective, S. cerevisiae) homolog A.
1.9	Frizzled (Drosophila) homolog 4 (FZD4),
1.9	Methylcrotonoyl-Coenzyme A carboxylase 2 (beta).
1.9	Homeodomain protein NKX3.1 isoform v1 (NKX3A).
1.9	CD24 signal transducer.
1.9	Protein kinase, cAMP-dependent, catalytic, beta.
1.9	Hypothetical protein, DKFZp762I1914.
1.9	Syndecan-1 gene (exons 2-5).
1.9	Homolog of mouse quaking QKI (KH domain RNA binding protein).
1.9	CD24 antigen (small cell lung carcinoma cluster 4 antigen).
1.9	Adehyde dehydrogenase 5 (ALDH5).
1.9	Cathepsin C (CTSC).
1.9	Integrin, alpha 6.
1.9	Homeodomain protein NKX3.1 isoform v3 (NKX3A).
2.0	Cyclin G2.
2.0	Ribonuclease, RNase A family, 4 (RNASE4).
2.0	CD24 antigen (small cell lung carcinoma cluster 4 antigen).
2.0	RAN binding protein 16 (RANBP16).
2.0	Tumour antigen (L6).
2.0	Zinc finger protein 91 (HPF7, HTF10) (ZNF91).
2.0	Aldehyde dehydrogenase 3 family, member B2 (ALDH3B2).
2.1	CD24 gene, cell surface antigen.
2.1	EST
2.1	Hypothetical protein, FLJ20300.
2.1	CED-6 protein (CED-6).
2.1	Regulator of G-protein signalling 2, 24kD (RGS2).
2.1	Hypothetical protein, FLJ14085.

2.3	Similar to CD24 signal transducer.
2.3	T-cell receptor Ti rearranged gamma-chain mRNA V-J-C region, complete cds. T cell receptor gamma locus.
2.3	AKAP450 anchoring gene.
2.3	(clone HGP08) T cell receptor gamma-chain mRNA, C2 region.
2.3	Similar to Homo sapiens CED-6 protein (CED-6).
2.5	Non-biotin containing subunit of 3-methylcrotonyl-CoA carboxylase.
2.5	PTB domain adaptor protein CED-6.
2.5	Acyl-Coenzyme A dehydrogenase, long chain.
2.5	Protocadherin alpha 9 (PCDHA9).
2.6	CD24 signal transducer.
2.6	Hypothetical protein, KIAA0924.
2.6	Spondin 2, extracellular matrix protein (SPON2).
2.8	T-cell receptor aberrantly rearranged gamma-chain mRNA from cell line HPB-MLT. T cell receptor gamma constant 2
3.0	Vimentin.
3.5	T-cell receptor gamma chain VJCI-CII-CIII region mRNA, complete cds. TCRG T cell receptor gamma locus
3.7	Glutathione S-transferase A2 (GSTA2).
3.7	MAP kinase MEK5c.
4.0	Hypothetical protein, FLJ20706.
4.6	Phosphatidylcholine 2-acylhydrolase (cPLA2).
4.9	Ornithine aminotransferase (gyrate atrophy).
4.9	Albumin.
8.0	Signal sequence receptor, gamma (translocon-associated protein gamma) (SSR3).
68.6	Testis-specific protein (TSPY).
1.5	Heterogeneous nuclear ribonucleoprotein H1.
1.5	EST.
1.6	HCF-binding transcription factor Zhangfei (ZF).
1.7	(Hsp40) homolog, subfamily C, member 8.
1.9	Hypothetical protein, DKFZp434C1722.
1.9	Prostate stem cell antigen (PSCA).