U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

EXPRESSION ANALYSIS AND REGULATION OF STEAP1 IN PROSTATE CELL LINES AND IN *TISSUE MICROARRAYS* OF PROSTATE TUMORS

Patrícia Margarida Ferreira Saraiva Baptista Arinto

Dissertation for a Master's Degree in Oncology
Molecular Oncology

Porto, 2012

Patrícia Margarida Ferreira Saraiva Baptista Arinto

EXPRESSION ANALYSIS AND REGULATION OF STEAP1 IN PROSTATE CELL LINES AND IN *TISSUE MICROARRAYS* OF PROSTATE TUMORS

Dissertation for applying to a Master's degree in Oncology – Specialization in Molecular Oncology submitted to the Institute of Biomedical Sciences Abel Salazar, University of Porto

Supervisor: Cláudio Jorge Maia Baptista, PhD
Category/Grade: Assistant Professor
Affiliation: Faculty of Health Sciences, University of Beira Interior
Co-supervisor: Carlos Alberto da Silva Lopes, MD, PhD
Category/Grade: Cathedratic Professor
Affiliation: Institute of Biomedical Sciences Abel Salazar – University of Porto

ACKNOWLEDGEMENTS

To my supervisor, Professor Cláudio Maia for all dedication, availability and support that showed throughout the preparation of this work.

To Professor Carlos Lopes, director of Pathology department of Hospital Geral Santo Antonio, for their willingness and cooperation in this master thesis.

To Professor Carmén Jerónimo, coordinator of the group of cancer epigenetic of genetic service, of IPO Porto, for all the availability, suggestions and knowledge sharing.

To cancer epigenetic group of genetic service, of IPO Porto, in particular to Mafalda, Sara and Pedro, and to all my colleagues at the Health Sciences Research Centre (CICS) of the University of Beira Interior, for availability and help that they have provided in the laboratorial execution in this work.

To Arnaud for the availability, assistance and all the time dispensed to help me during the work in the Pathology department, Hospital Geral Santo António.

To ladies of the secretariat of Pathology service of Hospital Geral Santo António for help me in accessing clinical information.

To my friends, for the companionship, aid and especially for the great friendship that they have shown during this stage of my life.

LIST OF ACRONYMS

ACT – α1-antichymotrypsin
ANT – Adjacent normal tissue
AR – Androgen receptor
BPH – Benign prostatic hyperplasia
BSP – Bisulfite sequencing PCR
DAB – 3,3-Diaminobenzidine
DHT – 5α-Dihydrotestosterone
DNA – Deoxyribonucleic acid
DU145 - Androgen-independent prostate cancer cells
FAS - Fatty acid synthetase
% fPSA - Free-to-total PSA ratio
GSTP1 – Glutathione S-transferase 1
HIF-1 – Hypoxia-inducible factor-1
LNCaP – Androgen-sensitive human prostate adenocarcinoma cells derived from left supraclavicular lymph node metastasis
mRNA – messenger ribonucleic acid
mSTEAP1 – murine STEAP1
mTOR - mammalian target of rapamycin
OCT-4 – Octamer-binding transcription factor 4
PBS – Phosphate Buffered Saline
PBS-T – Phosphate Buffered Saline – Tween 20
PC3 – Prostate adenocarcinoma cell line derived from grade IV androgen independent bone metastasis
PCA3 – Prostate cancer gene 3
PCR - Polymerase chain reaction
PIN – Prostatic intraepithelial neoplasia
PNT1A – Human post pubertal prostate normal, immortalized with simian virus 40
PNT2 – Normal prostate epithelium cell line, immortalized with simian virus 40
PSA - Prostate specific antigen
PSA-V – PSA velocity
PTEN – Phosphatase and tension homolog
RER^{+} - Replication error
RNA - Ribonucleic acid
STEAP1 - Six transmembrane epithelial antigen of the prostate 1
TMA – Tissue microarray

XMRV – Xenotrophic MuLV-related virus

LIST OF FIGURES

LIST OF TABLES

.

ABSTRACT

The *six transmembrane epithelial antigen of the prostate 1 (*STEAP1) gene was firstly identified as over-expressed in prostate cancer, but since then, several studies have shown that STEAP1 is also over-expressed in several types of tumors. As STEAP1 expression is almost absent in normal tissues, it has been pointed STEAP1 as a potential biomarker and immunotherapeutic target for cancer. However, the clinical significance of STEAP1 expression in prostate cells remains to be clarified.

At the moment, very little is known about the regulation of STEAP1 in prostate cells, and the mechanisms underlying the over-expression of STEAP1 in prostate cancer remain unknown. In addition, another STEAP1-related gene is encoded by human genome, referred to STEAP1B gene. This gene originates two different transcripts, STEAP1B1 and STEAP1B2, probably resulting from alternative splicing. However, no studies are found in the literature reporting the characterization of STEAP1B expression in prostate cells.

Therefore, the main aims of this study was to analyze the STEAP1, STEAP1B1 and STEAP1B2 mRNA and STEAP1 protein expression in neoplastic and non-neoplastic prostate cell lines by real-time PCR and Western blot, respectively; and to evaluate if epigenetic mechanism is involved in regulation of STEAP1 gene expression by bisulfite sequencing PCR. In order to evaluate the clinical significance of STEAP1 expression in prostate cancer, tissue microarrays were constructed and STEAP1 immunoreactivity was determined by immunohistochemical method. A score scale for STEAP1 immunoreactivity was established in order to establish associations between STEAP1 immunoreactivity and histologic diagnosis or the clinic-pathological data of patients.

Our results show that STEAP1 is highly expressed in LNCaP and PC3 neoplastic cells when compared to PNT1A and PNT2 non-neoplastic cells. On the other hand, STEAP1B1 and STEAP1B2 showed a slight expression in prostate cell lines. Regarding the methylation rate of STEAP1 gene promoter region, no significant differences were found between neoplastic (LNCaP) and non-neoplastic (PNT1A and PNT2) prostate cells, suggesting that STEAP1 over-expression in LNCaP cells should not be explained by hypomethylation at promoter region of STEAP1 gene. However, due to amplification of STEAP1 and STEAP1B gene using some sets of primers, these results should be confirmed through another strategy.

By immunohistochemistry technique, it was found that STEAP1 is expressed in cell membrane and cytoplasm of epithelial cells, and it is also visible a weaker staining in

stromal cells. Our results show that STEAP1 is over-expressed in prostate cancer and PIN lesions when compared to adjacent normal tissue or benign prostate hyperplasia, suggesting that STEAP1 gene may become over-expressed even before cancer development. Finally, it was found that there is a relationship between the STEAP1 immunoreactivity and Gleason score, but not with other clinic-pathologic data such as age, total-PSA, free-PSA, TNM and bone metastasis.

In summary, we conclude that STEAP1 is over-expressed in neoplastic cell lines when compared to non-neoplastic prostate cells, but its over-expression doesn't seem to involve epigenetic mechanisms. STEAP1 is over-expressed in human prostate cancer cases and PIN lesions, and its immunoreactivity correlates positively with Gleason score. However, more studies are required to clarify the clinical significance of STEAP1 expression in prostate cancer.

RESUMO

O gene *six transmembrane epithelial antigen of the prostate 1* (STEAP1) foi inicialmente identificado como um gene sobre-expresso no cancro da próstata. Desde então, vários estudos têm mostrado que o STEAP1 é também sobre-expresso em vários outros tipos de tumores. Como a expressão do STEAP1 está praticamente ausente em tecidos normais, tem sido apontado como um potencial biomarcador e alvo imunoterapêutico para o cancro. Contudo, o significado clínico da expressão do STEAP1 nas células da próstata continua por clarificar.

Neste momento, muito pouco é conhecido sobre a regulação do STEAP1 em células da próstata, e os mecanismos que conduzem à sobre-expressão do STEAP1 no cancro da próstata continuam por ser identificados. Para além disto, um outro gene relacionado com o STEAP1 é codificado pelo genoma humano, designado por STEAP1B. Este gene origina dois transcritos diferentes, o STEAP1B1 e o STEAP1B2, provavelmente resultantes de splicing alternativo. Contudo, não são encontrados estudos na literatura descrevendo a caracterização da expressão do STEAP1B em células da próstata.

Assim sendo, os principais objetivos do nosso estudo são analisar a expressão do mRNA STEAP1, STEAP1B1 e STEAP1B2 e da proteína STEAP1 em linhas celulares neoplásicas e não neoplásicas da próstata, através de PCR em tempo real e Western blot, respectivamente; e avaliar se mecanismos epigenéticos estão envolvidos na regulação da expressão do gene STEAP1 através de PCR de Sequenciação bissulfito. Para avaliar o significado clínico da expressão do STEAP1 em carcinoma de próstata, microarrays de tecido foram construídos e a imunoreactividade do STEAP1 foi determinada pela técnica de imunohistoquímica. Uma escala semi-quantitativa para a imunorecatividade do STEAP1 foi estabelecida com o objetivo de estabelecer associações entre a imunoreactividade do STEAP1 e o diagnóstico histológico ou os dados clinico-patológicos dos doentes.

Os nossos resultados mostram que o STEAP1 é altamente expresso nas células neoplásicas LNCaP e PC3 em relação às não neoplásicas PNT1A e PNT2. Por outro lado o STEAP1B1 e STEAP1B2 apresentaram uma reduzida expressão nas linhas celulares da próstata. Em relação ao rácio de metilação da região promotora do gene STEAP1, não foram encontradas diferenças significativas entre as células malignas (LNCaP) e benignas (PNT1A e PNT2), sugerindo que a sobre-expressão do STEAP1 nas células LNCaP não parece ser explicada pela hipometilação da região promotora do gene STEAP1. Contudo, devido à amplificação dos genes STEAP1 e STEAP1B usando alguns

dos pares de primers, estes resultados precisam de ser confirmados usando uma outra estratégia.

Por imunohistoquímica verificou-se uma marcação positiva para o STEAP1 na membrana e no citoplasma das células epiteliais, embora seja visível também uma marcação mais fraca nas células do estroma. Os nossos resultados mostram que o STEAP1 é sobre-expresso no cancro da próstata e em lesões de PIN quando comparados com tecido normal adjacente ou hiperplasia benigna da próstata, sugerindo que o gene STEAP1 pode começar a ser sobre-expresso antes do desenvolvimento do cancro. Por fim, foi encontrada uma relação entre a imunoreactividade do STEAP1 e os valores de Gleason, mas não com outros dados clinico-patológicos como a idade, PSA total, PSA livre, TNM e metástases ósseas.

Em suma, nós concluímos que o STEAP1 é sobre-expresso em linhas celulares neoplásicas da próstata quando comparados com as linhas celulares não neoplásicas, mas esta sobre-expressão não parece envolver mecanismos epigenéticos. O STEAP1 é sobre-expresso em casos de cancro de próstata humana e lesões de PIN, e a sua imunoreactividade correlaciona-se positivamente com o valor de Gleason. Contudo mais estudos são necessários para clarificar o significado clínico da expressão do STEAP1 no cancro da próstata.

TABLE OF CONTENTS

INTRODUCTION

1 - Prostate anatomy and physiology

The male reproductive system consists of the testis, a serie of ducts, accessory glands and supporting structures. The ducts include the epididymides, ductus deferens and urethra. The accessory glands include the seminal vesicles, prostate gland and bulbourethral glands. The supporting structures comprise the scrotum and penis (Seeley 2004) (Figure 1 and Figure 2).

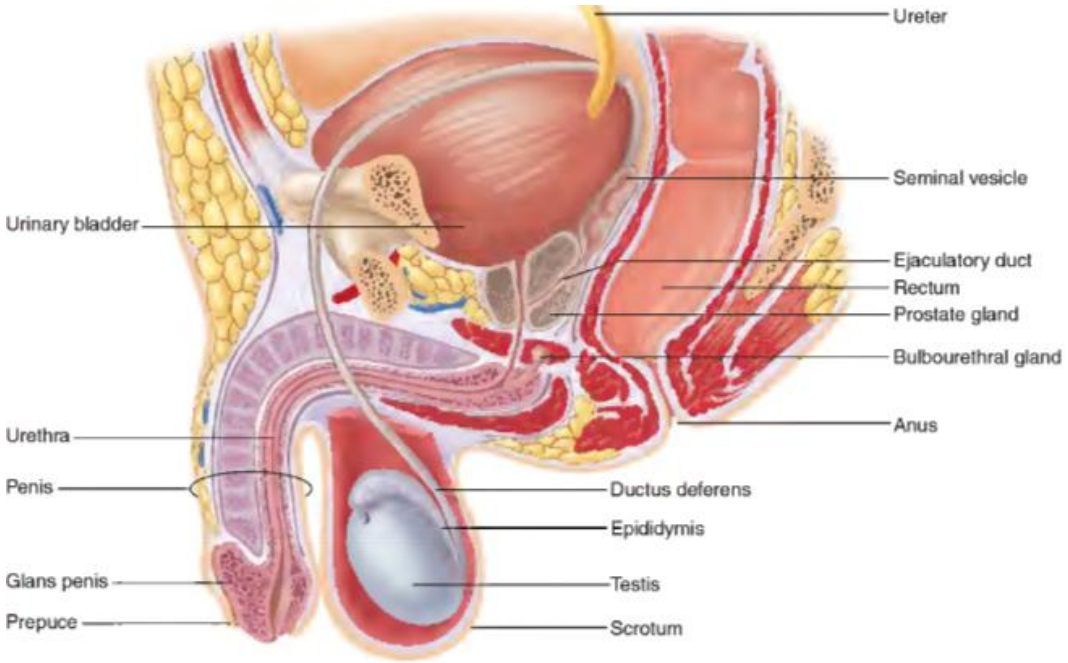

Figure 1: Sagittal section of the male pelvis showing the male reproductive structures, [adapted from (Seeley 2004)].

The prostate gland consists of glandular and muscular tissue and it has the size and shape of a walnut, with about 4 cm long and 2 cm wide. It is dorsal to the symphysis pubis at the base of the urinary bladder, where it surrounds the prostatic urethra and ejaculatory ducts (Seeley 2004).

The urethra courses through the prostate to become the *membranous urethra* at the apex of the prostate. In the substance of this gland, the urethra merges with the ejaculatory ducts and, at this region, angles forwards (Young 2007).

So, this gland is composed of a fibrous connective tissue containing distinct smooth muscle cells and numerous fibrous partitions. Covering these muscular strata, there is a layer of columnar cells that secretes prostatic fluid. A range from 15 to 30 small prostatic

ducts open into the prostatic urethra, secreting the seminal and prostate fluids and spermatozoa (Seeley 2004).

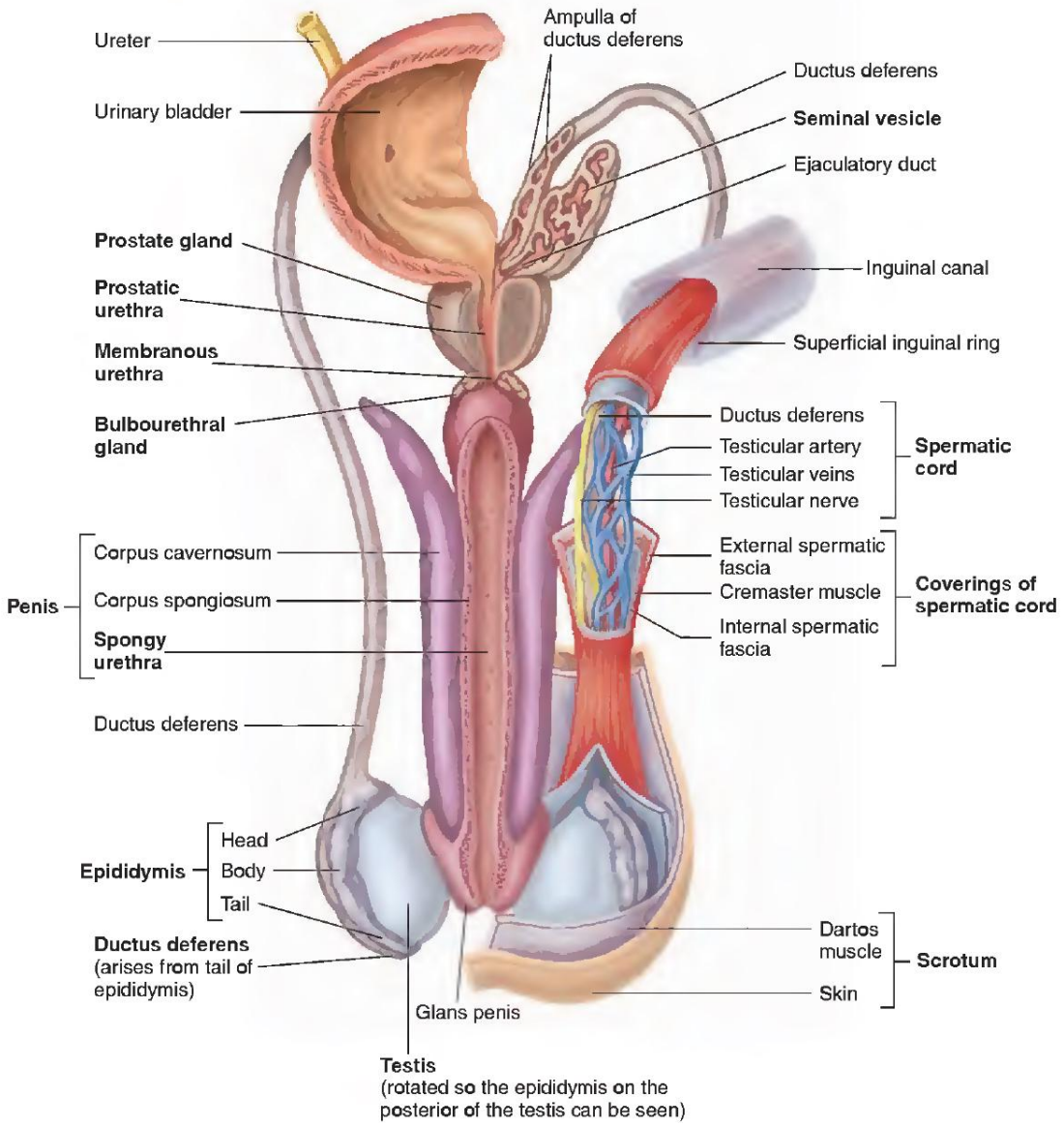

Figure 2: Frontal view of the testes, epididymis, ducts deferens and glands of male reproductive system [adapted from (Seeley 2004)].

The secretions from prostate, seminal vesicles, bulborurethral glands and urethral mucous glands help to neutralize the acidic urethra. Moreover, the secretions of the prostate and seminal vesicles also help to neutralize the acidic secretions of the testes. The prostatic secretions are also important in the transient coagulation of semen, because they contain clotting factors that convert fibrinogen from the seminal vesicles to the fibrin, resulting in coagulation. After ejaculation, the coagulated material keeps the semen as a single, sticky mass for a few minutes, and then fibrinolysin from the prostate causes the coagulum to

dissolve, thereby releasing the sperm cells to make their way up the female reproductive tract (Seeley 2004).

Before ejaculation, the ducts deferens begin to contract rhythmically to boost sperm cells and testicular fluid from the epididymis to the prostatic urethra, where they blend with seminal and prostatic secretions, which are released as a result of contractions of the seminal and prostate glands (Seeley 2004). In figure 3 is shown an image of a normal prostate.

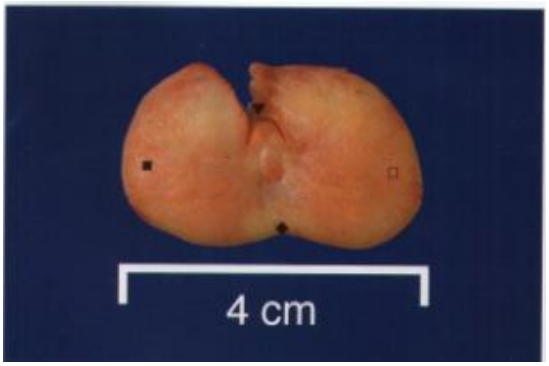

Figure 3: Normal prostate, gross. This is a transversal (axial) section through a healthy prostate. There is a central urethra (▼) at the depth of the cut made to open it anteriorly at autopsy, with the left lateral lobe (■), the right lateral lobe (□), and the posterior lobe (◆).
[Adapted from (Klatt 2006)].

The prostate consists of branched tubulo-acinar glands embedded in a fibromuscular stroma. On the other hand, in this gland, there also is a partial capsule enclosing the posterior and lateral aspects of the prostate but the anterior and apical surfaces are bounded by the *anterior fibromuscular stroma*, which consists, as the name implies, only of collagenous stroma and muscle fibers (Young 2007).

In the past, the prostate was considered to have a huge number of ill-defined lobes. However, this terminology has been replaced by the concept of prostate zones. Thus, this gland is now described as consisting of four zones of unequal size (Young 2007) (Figure 4 and Figure 5):

- The **transition zone** surrounds the proximal prostatic urethra and comprises about 5% of the glandular tissue.
- The **central zone** (20%) encloses the ejaculatory ducts.
- The **peripheral zone** makes up the bulk of the gland (approximately 70%).
- The **anterior fibromuscular stroma** contains no glandular tissue and is situated anteriorly.

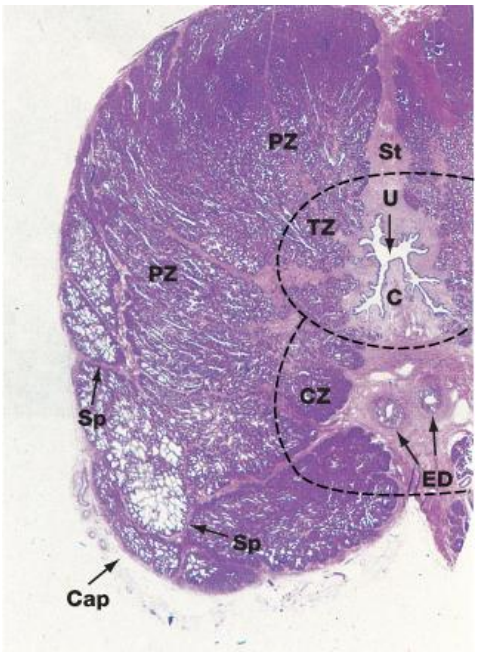

Figure 4: Prostate gland H&E x 5, [adapted from (Young 2007)]. The urethral (**U)** is localized centrally and surrounded by a fibrous stroma (**St)**. The ejaculatory ducts (**ED)** are also found in this central stroma as they course towards their junction with the prostatic urethra. The zones of the prostate are not clearly demarcated from each other anatomically. Partial fibrous septa (**Sp)** separate the gland into lobules. The transition zone (**TZ)** surrounds the first part of the prostatic urethra. The central zone (**CZ)** is posterior to the transition zone and encircles the ejaculatory ducts. The peripheral zone (**PZ)** makes up the main bulk of the prostate. The ducts of the peripheral zone glands empty into the postero-lateral recesses of the urethra on either side of the ***verumontanum*** **(urethral crest, C)**. Most cases of carcinoma of the prostate arise in the peripheral zone while the transition zone incorporates almost all cases of BPH. At this case, the anterior fibromuscular stroma appears continuous with the capsule and its content of muscle fibers cannot be discerned.

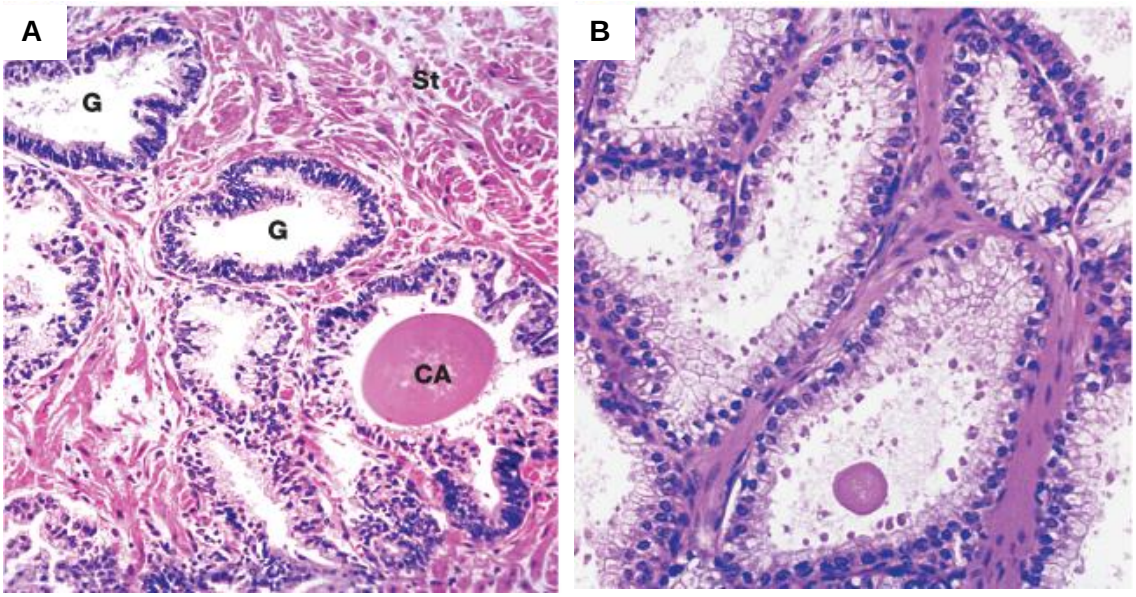

Figure 5: A – Human prostate H&E x 30. B – Human prostate H&E x 50 [adapted from (Young 2007)].

In figure 5A, it is possible to see that the branching nature of the prostatic gland **(G)** is more apparent. The prostate is irregularly shaped with the epithelium forming folds, which allow for expansion of the glands by secretions. This fact gives the epithelium a papillary appearance. The secretory product of the prostate, which makes up about half of the seminal fluid volume, is thin and milky, rich in citric acid and hydrolytic enzymes, especially fibrinolysin, which liquefies the coagulated semen after deposition within the female genital tract. Lamellated glycoprotein masses called ***corpora amylacea*** **(CA)** are a feature of aging, becoming progressively calcified to form prostatic concretions. The stroma of the prostate consists of dense collagen, fibroblasts and haphazardly arranged smooth muscle fibers which, like those of the seminal vesicles and the rest of the tract, are innervated by the sympathetic nervous system that stimulates powerful contractions during ejaculation. Towards the apex of the gland the anterior fibromuscular stroma also contains skeletal muscular fibers (Young 2007).

In figure 5B, the epithelium of the prostate gland is composed by a double layer of cells. The luminal layer is composed of columnar secretory cells with basal nuclei. Between the secretory cells and the basement membrane, there is a layer of flattened basal cells, which probably act as reservoir of cells that replace the dead ones. The basal cell layer is often incomplete and may be hard to stain with H&E in sections. However, these cells produce a type of high molecular weight-keratin that is not found in the secretory cells (Young 2007).

Age-related changes have been detected in the prostate gland after 40 years old. By 60 years old, there is a clear decrease in blood flow, an increased thickness in the epithelial cell lining of the prostate gland, and a decrease in functional smooth muscular cells in the wall of the prostate. Although the alterations in this gland do not decrease fertility, there is a substantial increase in the incidence of benign prostatic hyperplasia (BPH) that can create difficulty in urination because it compresses the prostatic urethra (Seeley 2004).

2 - Prostate pathology

Nowadays, there are three pathologic processes that affect the prostate gland with emphasis to being discussed: inflammation, BPH and tumors. BPH is the most common alteration in prostate and it can be considered as a "normal" process of aging. Prostatic carcinoma is also an extremely frequent lesion in man and, therefore, it needs to be carefully considered. On the other hand, the clinical significant of inflammatory process is reduced and can be easily treated (Kumar 2005).

2.1 - Benign Prostatic Hyperplasia

BPH is an extremely common disorder in men over 50 years old (Foster 2000; Ramsey 2000). The etiology of BPH is not completely understood, but it seems to be multifactorial and endocrine controlled. BPH consists of two components: static (related to absolute size of the prostate gland) and dynamic (associated to prostate smooth muscle contractions), that clinically may result in lower urinary tract symptoms of BPH (urinary frequency, urgency, sensation of incomplete emptying, weak stream and straining to initiate urination) (Facio 2010).

Histological studies of BPH can be seen in approximately 20% of men with 40 years old, increasing to 70% by age 60 and to 90% by age 70. Only 50% of those who have microscopic evidence of nodular hyperplasia have clinically detectable enlargement of the prostate, and of these individuals, only 50% develop clinical symptoms (Kumar 2005).

There are some evidences that associate this prostatic enlargement with the action of androgens. For example, prepubertal castration prevents the development of nodular hyperplasia. 5α-dihydrotestosterone (DHT), a metabolite of testosterone, is the ultimate mediator of prostatic growth. It is synthesized in this gland by circulating testosterone by the action of the enzyme 5α-reductase type 2. This enzyme is mainly localized in the stromal cells; hence, these cells are the main site for the synthesis of DHT (Wong 2000). Once synthesized, DHT can act in an autocrine signaling on the stromal cells or in

paracrine signaling by diffusing into nearby epithelial cells. In both cases DHT binds to nuclear androgen receptor (AR), which acts as a transcription factor regulating the expression of several growth factors that are mitogenic to the epithelial and stromal cells (Figure 6).

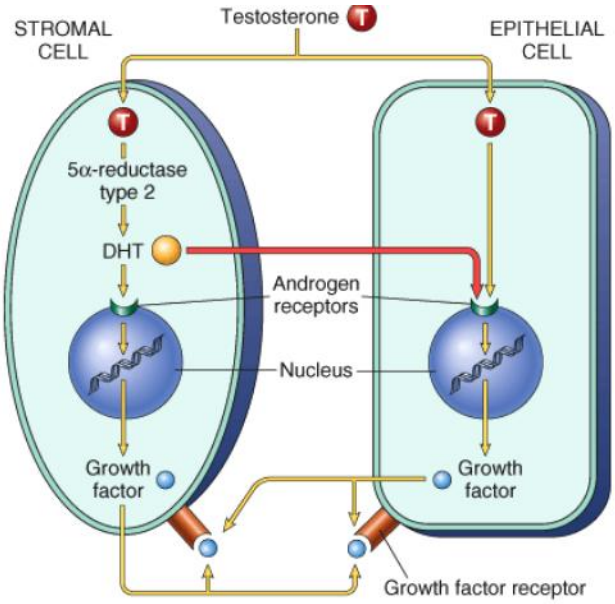

Figure 6: Simplified scheme of pathogenesis of nodular prostatic hyperplasia – Role of stromal cells to generate DHT, [adapted from (Kumar 2005)].

DHT is 10 times more potent than testosterone because it has greater affinity for the AR. While DHT appears to be the major trophic factor mediating prostatic hyperplasia, estrogens also appear to play an important role, perhaps by rendering cells more susceptible to the action of DHT (Wong 2000).

The role of DHT in the developing of BPH is supported by clinical observations in which an inhibitor of 5α-reductase is given to man with this condition. Therapy with 5α-reductase inhibitor reduces the DHT content of the prostate, and consequently, there is a decrease in prostatic volume and urinary obstruction. However, not all patients benefit from androgen-depriving therapy, suggesting that prostatic hyperplasia may be etiologically heterogeneous, and in some cases, other factors besides androgens may be involved (McConnell 2003).

Some studies have demonstrated that BPH are originated almost exclusively in the transition zone of prostate gland. Fairly discrete nodules in the periurethral region of the prostate are formed, and when sufficiently large, compress and narrow the urethral canal to cause partial or complete obstruction of the urethra. The first nodules are composed almost entirely of stromal cells; later, predominantly epithelial nodules arise (Kumar 2005). On cross-section of the affected prostate, there are nodules that can vary in color and consistency. In nodules with primarily glandular proliferation, the tissue is yellow-pink with

a soft consistency and a milky white prostatic fluid flows from these structures. In those primarily due to fibromuscular involvement, each nodule is pale gray, tough, does not exude fluid, and is less clearly demarcated from the surrounding prostatic capsule (Kumar 2005) (Figure 7).

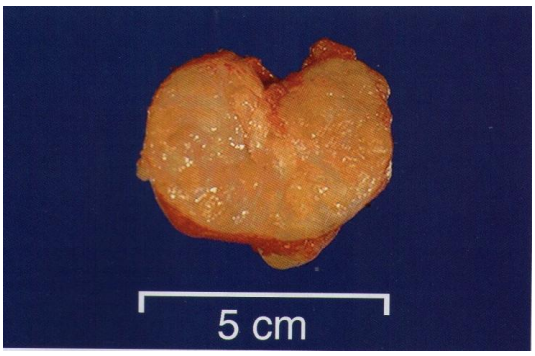

Figure 7: Gross appearance of prostatic hyperplasia, which is most pronounced in the lateral lobes [adapted from (Klatt 2006)].

Microscopically, the main feature of BPH is the presence of nodules due to glandular proliferation and fibrous or muscular proliferation of the stroma. The proportion of these elements varies from nodule to other, ranging from only stromal fibromuscular nodules to fibroepithelial nodules with a glandular predominance. Glandular proliferation takes the form of aggregations of small to large of cystically dilated glands, lined by two layers, an inner columnar and an outer cuboidal or flattened epithelium, based on an intact basement membrane (Figure 8). Two histologic changes associated with BPH are found: (1) foci of squamous metaplasia and (2) small areas of infarction. The former tend to occur in the margins of the foci of infarction as nests of metaplastic reactive squamous cells that can be confused with adenocarcinoma of the prostate or urothelial carcinoma involving the prostate (Kumar 2005).

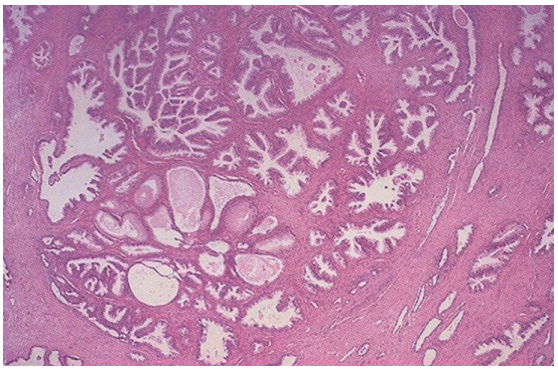

Figure 8: Prostatic hyperplasia, microscopic. Prostatic hyperplasia can involve glands and stroma, although the former are usually more prominent. A large hyperplastic nodule with numerous glands is present here. There is still stroma between the glands. The glands are larger than normal, with more complex infoldings, but still lined by a double layer of uniform columnar cells and basal cuboidal cells that show no atypia. The transitional zone often enables an initial increase in these hyperplastic nodules, although the bulk of prostatic enlargement often comes later from pronounced nodular growth in the peripheral zone [adapted from (Klatt 2006)].

2.2 - Prostate Cancer

Prostate cancer is one of the most commonly diagnosed tumors and the second leading cause of cancer-related death in European and American men, with approximately 220 000 new cases of prostate cancer and 30 000 deaths estimated to arise in the US alone in 2010 (Andriole 2009; Schröder 2009). Concerning Portugal, the last statistics available revealed PCa as the most incident neoplasia in men, with 5140 cases in 2008, however being the third most lethal cancer (Ferlay 2010).

There is a large discrepancy between the incidence of the disease and mortality rate. The increase of incidence rate can be ascribed to the combination of an aging male population and the use of early testing, based on more sophisticated measurement of prostate specific antigen (PSA) serum levels. Besides PSA levels in plasma, it has been identified novel serum markers such as prostate cancer gene 3 (PCA3) (Hessels 2003).

Prostate cancer is a disease with a long natural history, with progression commonly related to stage and grade of tumor and lack of differentiation (Selley 1997). Several reports have described that more than 65% of all prostate cancers are diagnosed in men over 65 years old (Madu 2010). Compared with the White population, the incidence of prostate cancer is approximately 60% higher in Black men, while native Japanese and Chinese population have a low risk of incidence and mortality (Stanford 1999). African-American men generally are diagnosed with more advanced stages of prostate cancer and at an earlier age. Besides to genetics–characteristics, social and environmental factors (especially diet and lifestyle) may act as the determining factors, which may explain why some individuals have higher risk for developing prostate cancer than are others (Madu 2010).

Prostate adenocarcinoma typically proceeds through a series of defined stages, from prostatic intraepithelial neoplasia (PIN), to invasive and metastatic cancer (Tomuleasa 2010).

The niche is a cell environment that provides critical signals to maintain stem cells and to support their undifferentiated phenotype of progenitor cells. These relevant signals include the Hedgehog, Wnt or Notch pathways, which all of them are important in early ontogenesis and cell differentiation and proliferation control. In cancer, the cell-cell and cell-matrix interactions are overlaid on top of other features of tumor physiopathology microenvironment, including the presence of hypoxia, low pH and nutrient deprivation. Fluctuations of these parameters have profound effects on the activity of cancer stem cells and their potential niche (Tomuleasa 2010).

Hypoxia is an important characteristic of the niche because it is intrinsically linked to the formation of neovasculature and the regulation of the production of proangiogenic factors. It is well characterized that several genes are expressed in hypoxic environment, and the most of these genes are controlled by hypoxia-inducible factor-1 (HIF-1) (Hill 2009). The gene expression may be altered toward an immature phenotype, under hypoxic conditions (3 - 5% O_2), promoting de-differentiation of prostate tumor cells into more “stem-like” ones (Das B 2008).

The hypoxic cells express higher levels of the embryonic stem-ness gene octamer-binding transcription factor 4 (OCT-4) due to the interactions between HIF-transcription factors (HIF-1α and HIF-2α). OCT-4 is a direct target of HIF-2α, and its induction could contribute to the formation and maintenance of cancer stem cells (KoshIji 2004). It should also be pointed out that the Myc and OCT-4 oncogenes were two of the four genes that are shown to be able of inducing fibroblasts to revert to a stem cell phenotype (Maherali 2007).

A number of potentially carcinogenic viruses have been detected in human prostatic tissues, such as the oncogenic human papovavirus BK or the human gammaretrovirus Xenotrophic MuLV-related virus (XMRV) in premalignant lesions (Urisman 2006; Das D 2008). These studies have improved the hypothesis of prostate oncogenesis, where PIN is preceded by an inflammatory atrophy with prostatic epithelial cells showing an increased Ki-67- marked proliferation (Tomuleasa 2010) (Figure 9).

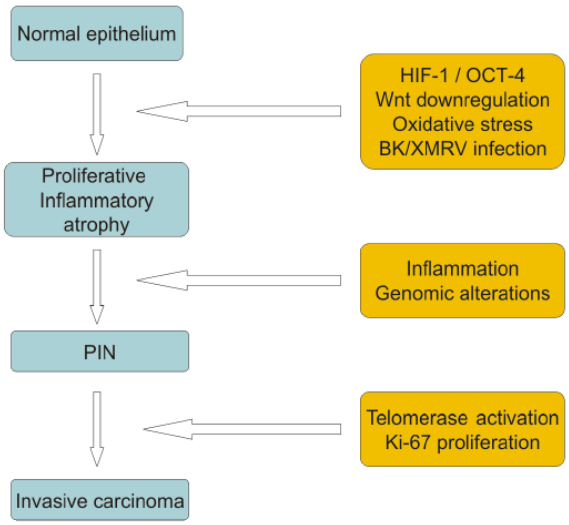

Figure 9: A model for prostate oncogenesis. The normal epithelium (under oxidative stress, Wnt down-regulation or human gammaretrovirus XMRV infection) have an increased proliferative potential and eventually lead to the appearance of the PIN. The high proliferative potential and telomerase activation determine the evolution from PIN to invasive, metastatic and treatment-resistant carcinoma [adapted from (Tomuleasa 2010)].

In approximately 70% of the cases, prostate carcinoma arises in the peripheral zone of the gland, normally in a posterior location, usually rendering it palpable on rectal examination (Figure 10) (Byar 1972).

Characteristically, on cross-section of the prostate, the neoplastic tissue is gritty and firm, but when it embedded within the prostatic substance, it may be extremely difficult to visualize and be more readily apparent on palpation. Proliferation of prostate cancer cells occurs by direct local invasion and through the bloodstream and lymph. Local extension most commonly involves the seminal vesicles and the base of the urinary bladder, which may result in urethral obstruction (Potter 2000). Hematogenous spread of cancer occurs chiefly to the bones, but some lesions spread widely to viscera (Saitoh 1984).

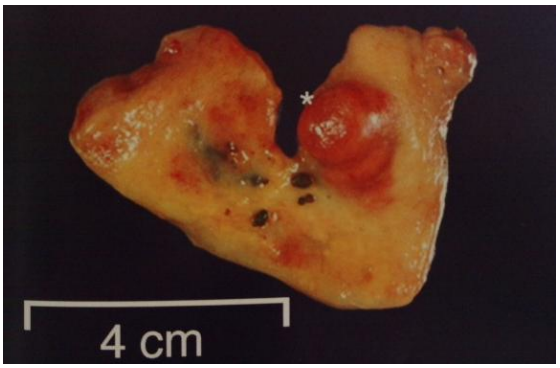

Figure 10: Adenocarcinoma, gross. This axial section reveals a single prominent nodule (*) that proved to be adenocarcinoma. Such a nodule may be palpable by digital rectal examination or may be detected on ultrasound. Some small dark glandular concretions are also seen in the adjacent normal prostate. This prostate is not significantly enlarged, and no nodules are present [adapted from (Klatt 2006)].

Histologically, the most lesions are adenocarcinomas that produce well-defined, readily demonstrable gland patterns (Epstein 1995; Epstein 2002). The neoplastic glands are typically smaller than benign ones and they are lined by a single uniform cuboidal layer or low columnar epithelium. In contrast to benign glands, prostate cancer glands are more crowded, characteristically lacking branching and papillary infolding (Figure 11). The outer basal layer of cells, typical of normal and hyperplastic glands, are absent in prostate cancer. The cytoplasm of the tumor cells ranges from pale to clear, as seen in benign glands, to a distinctive amphophilic appearance. Nuclei are large and often contain one or more large nucleoli (Figure 12). There is some variation in nuclear size and shape, but in general, pleomorphism is not marked. Mitotic figures are extremely uncommon (Rubin 2002).

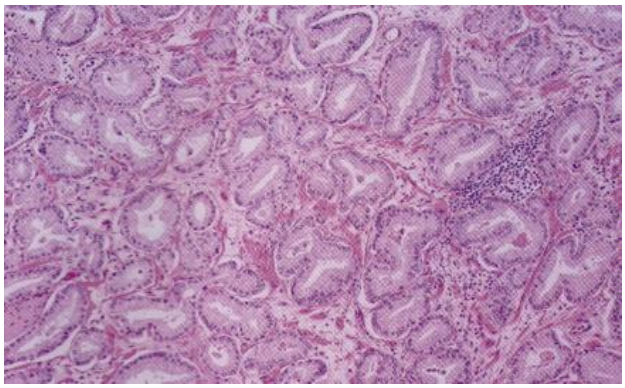

Figure 11: Adenocarcinoma, microscopic (a). It is possible to observe that the glands of the carcinoma are small, irregular, and crowded, with no intervening stroma [adapted from (Klatt 2006)].

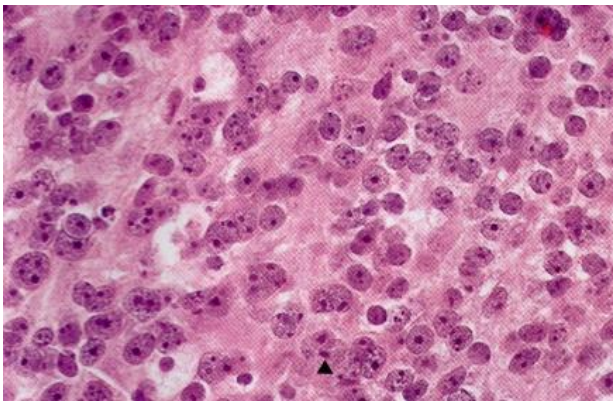

Figure 12: Adenocarcinoma, microscopic (b). Prominent nucleoli (▲) are a histological feature of prostatic adenocarcinoma [adapted from (Klatt 2006)].

PIN, firstly described in 1969 (McNeal 1969), is a neoplastic proliferation of prostatic epithelial cells that is confined to pre-existing prostatic ducts or acini (glands). PIN was initially termed intraductal dysplasia by McNeal and Bostwick in 1986. In recent years, many studies have shown that high-grade PIN is the major precursor of prostate cancer (McNeal 1986).

The population incidence of high-grade PIN and prostate adenocarcinoma seem to be parallel. Previous autopsy studies revealed that high-grade PIN had a low prevalence in men during their third decade of life (7% in African Americans vs. 8% in Caucasians) and had an increase with aging (91% in African Americans vs. 67% in Caucasians) (Sakr 1996).

Preferentially, high-grade PIN and carcinoma involve the peripheral zone of the prostate (Bostwick 1996; Häggman 1997), and lately many biomarkers and molecular changes, such as TMPRSS2-ERG gene fusion, have been described in both entities (Cerveira 2006; Perner 2007). The *TMPRSS2-ERG* gene fusion has been found in 30 to 79% of prostate cancer (Tomlins 2005). In some studies, TMPRSS2-ERG gene fusion is

associated with severe disease (Perner 2006; Attard 2008), but not in others (Gopalan 2009). Additionally, up-regulation of the mammalian target of rapamycin (mTOR) pathway promotes prostate cancer. The expression of 14-3-3 sigma protein, which regulates the mTOR pathway, increase progressively from high-grade PIN through Gleason score 6, Gleason score 7, and high-grade prostate cancer (Evren 2011).

The replication error (RER^+) phenotype leads to the inactivation of many tumor suppressor genes. In one study, the RER^+ phenotype was found in 42% of prostate tumors, 16% of PIN lesions and 4% of noncancerous prostate tissues. In this study was also found microsatellite instability in 12%, 35%, and 53% of normal, PIN, and cancerous tissues, respectively (Miet 1999).

Fatty acid synthetase (FAS) expression, which is thought to be an early event in malignant transformation, has been shown to be over-expressed in high-grade PIN and prostate cancer, but no FAS expression was observed in normal tissue (Swinnen 2002). Similarly, p53 mutations were detected in 14% of PIN and 25% of prostate cancer lesions (Yasunaga 1998). Bcl-2, a proto-oncogene that inhibits apoptosis, is expressed in many prostate cancers, and it has also been reported by Baltaci et al. (Baltaci 2000) to be expressed in both low-grade PIN and high-grade PIN. In high-grade PIN have also been described abnormalities in chromosomes, chromatin structure, and deoxyribonucleic acid (DNA) processing enzymes. More than half of high-grade PIN lesions may have chromosomal anomalies, including gains of chromosomes (decreasing order of frequency) 8, 10, 7, 12, and Y (Emmert-Buck 1995; Qian 1995). One study discovered the loss of heterozygosity on chromosome 8p12-21 in 63% of PIN lesions in patients with prostate tumor (Emmert-Buck 1995). Telomerase, which can contribute to cancer cell immortalization, is active in some high-grade PIN foci (Koeneman 1998). DNA topoisomerase II-alpha staining by immunohistochemistry is intermediate between benign tissue and prostate cancer (Willman 2000). High-grade PIN also displays epigenetic changes, including hypermethylation of the several genes, such as glutathione S-transferase 1 (GSTP1) (Brooks 1998).

High-grade PIN is characterized by a proliferation of secretory cells with significant cytologic atypia within the prostate glands and acini. These secretory cells are enlarged with an increased nuclear/cytoplasmic ratio and prominent nucleoli. The cytoplasm of the high-grade PIN cells tends to be staining positively for α-methylacyl-CoA. Most of these features are shared by invasive prostate cancer (Bostwick 1987; Bostwick 2004; Montironi 2007). However, in contrast to prostatic adenocarcinoma in which the basal cells are absent, in high-grade PIN the basal cell layer is retained although and it is frequently

discontinuous on hematoxylin and eosin stain (Bostwick 1987) (Figure 13). Four main architectural patterns of high-grade PIN have previously been described (tufting, micropapillary, cribriform, and flat), but these are thought to not vary clinically, and differentiation seems to have only diagnostic value (Bostwick 1993; Montironi 2007).

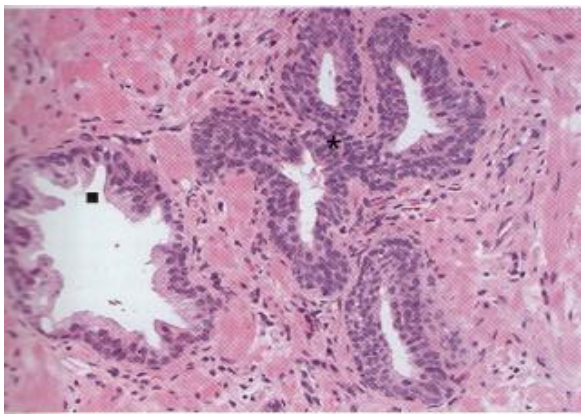

Figure 13: Prostatic intraepithelial neoplasia, microscopic. PIN is a precancerous cellular proliferation and it is found in a single acinous or small group of prostatic acini. A normal prostatic gland (■) is seen on the left for comparison, with the acini showing PIN (*) on the right. The PIN can be low grade or high grade (as seen here). The finding of PIN suggests that prostatic adenocarcinoma may also be present, and an adenocarcinoma accompanies high-grade PIN about half the time [adapted from (Klatt 2006)].

2.2.1 - Risk factors

Usually, prostate tumor is a disease of men over 50 years old, but in men who are at increased risk, the recommendations are beginning to start the screening for prostate cancer near of 40 years old to detect uncommon cases of this type of cancer before they become incurable (Boyle 2003; Kumar 2005).

Some risk factors, such as age, race, family history, hormone levels and environmental influences, are supposed to play important roles in prostate cancer (Yip 1999; Boyle 2003; Grönberg 2003; Nelson 2003).

A number of diet factors appear to be linked to prostate cancer, particularly fat and fiber intake. It has been found that prostate cancer incidence is highly correlated with total fat intake, particularly animal rather than vegetable fat (Jones 1993; Marchand 1994). The evidence about other diet factors is contradictory, being suggested that soya intake as a potential protective factor (Messina 1994), and deficiency in vitamin A may lead to increase risk of prostate cancer (Pienta 1993).

It is accepted that there is a little variation in the prevalence of microscopic data of prostate tumors around the world, but there are considerable differences in the rates of progression to clinically evidence disease. This fact is further shown by migrants moving

to higher risk areas acquiring the local incidence rate (Pienta 1993; Haas 1994). Black men have a higher risk of prostate cancer than white men. They are more susceptible to develop prostate cancer at an earlier age and to have aggressive tumors that grow quickly. The exact reasons for these differences are not known and may involve socioeconomic and environmental factors. Hispanic men have a lower risk of developing prostate cancer and dying from the disease than white men. Prostate cancer occurs most often in North America and northern Europe. It also appears that prostate cancer is increasing among Asian people living in urbanized environments, such as Hong Kong, Singapore, and North American and European cities, particularly among those who have a more western lifestyle (Cancer.Net 2012).

As with BPH, it is believed that androgens have a fundamental role in prostate cancer progression. Neoplastic epithelial cells, similar to their normal counterparts, express AR. It seems that the role of hormones in this malignancy is essentially tolerant because androgens are required for the maintenance of the prostatic epithelium. AR gene mutations have been mentioned in only a minority of prostate cancers. However, AR gene amplification may also influence androgen-sensitivity of prostatic epithelium. The AR gene is polymorphic, with individuals having variable lengths of CAG repeats. Some studies have shown that prostate cells with short CAG repeats have an increased sensitivity to androgens (Kumar 2005).

A number of studies have established that genetic factors are involved in some cases of prostate tumor. Some authors claim that these are of two types: hereditary prostate cancer which is distinguished by early age at onset and autosomal dominant inheritance within families; and familial prostate cancer in which members of the same family have the disease. Hereditary prostate cancer results from a gene and confers a greatly increased susceptibility to the disease. It may account for a substantial amount of early onset disease, and overall about 9% of this type of tumor (Bova 1993). Family history of prostate cancer has been identified in many studies as a obvious risk factor, accounting with approximately 10% of all the cases (Dillman 1994; Ellis 1994; Narod 1995). It has been suggested that familiar prostate cancer may be more hostile than that found in the general population, because it tends to occur at early ages and accounts for a substantial fraction of the disease in younger men (Bova 1993; Li 1993).

The development of this cancer, in approximately 10% of white American men, has been linked to it germ line inheritance susceptibility genes. In one third of these familiar cases, a susceptibility gene has been mapped to chromosome 1q24-25. Putative cancer-suppressor genes that are lost early in prostate carcinogenesis have been localized to

chromosomes 8p, 10q, 13q, and 16q. In many cases, the identity of the relevant genes at these loci is unknown. p53 mutations in primary prostate cancer are relatively low and they are more frequently detected in metastatic disease, suggesting that p53 mutations are late events in prostate carcinogenesis (Jussi 2001; Rhodes 2003). Other tumor-suppressor genes that are thought to play a role in this disease include phosphatase and tension homolog (PTEN). Prostate cancers also show a relatively frequent loss of E-cadherin and CD44 (DeMarzo 2003; Rhodes 2003). In this kind of illness, one of the most common genetic alterations is the hypermethylation of GSTP1 gene promoter. More than 90% of prostate cancers show hypermethylation of this gene, which turn off its expression (Kumar 2005).

2.2.2 - Grading and Staging

Among the various grading systems described, the Gleason system is the best known. According to this system, prostate cancers are stratified into five grades on the basis of glandular patterns and differentiation degree as observed under low magnification. Grade 1 represents the well-differentiated tumors, in which the neoplastic glands are uniform with round appearance and are packed into well-circumscribed nodules. By contrast, grade 5 tumors show no glandular differentiation, and the tumor cells infiltrate the stroma in the form of cords, sheets, and nests (Figure 14). The other grades fall in between (Gleason 1974; McNeal 1990; Epstein 2001).

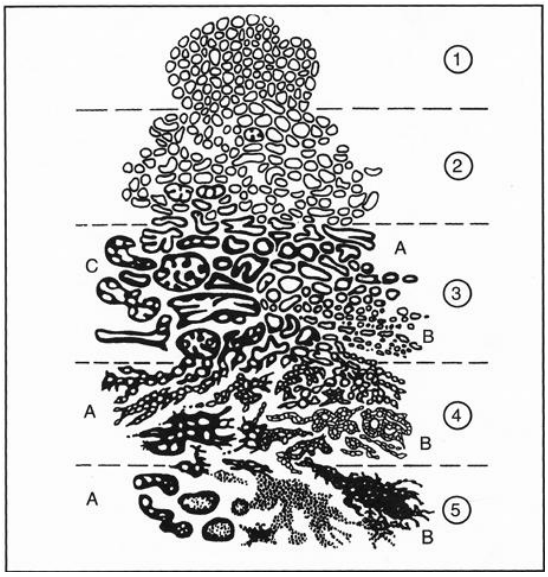

Figure 14: Gleason grades 1 to 5, [adapted from (Humphrey 2008)].

Nowadays, it is believed that most tumors contain more than one pattern, in which case one assigns a primary grade to the dominant pattern and a secondary grade to the subdominant pattern. The two numeric grades are then added to obtain a combined

Gleason grade or score. Thus, for example, a tumor with a dominant grade 3 and a secondary grade 4 would achieve a Gleason score of 7 (Gleason 1974; McNeal 1990; Epstein 2001).

There is consensus that a Gleason score of 1+1=2 is a ranking that should not be diagnosed regardless of the type of specimen, with extremely rare exception. Most cases in which were diagnosed as Gleason score 1+1=2 would today be referred to as adenosis (atypical adenomatous hyperplasia) (Epstein 2011).

However, there are several reasons why the Gleason score 2-4 should not be assigned to cancer on needle biopsy: poor reproducibility even amongst experts; poor correlation with prostatectomy grade with almost all cases showing higher grade at resection; a diagnosis of Gleason score 3-4 may misguide clinicians and patients into believing that they had has an indolent tumor (Steinberg 1997; Epstein 2000). Most of the lesions that appear to be very low grade on needle biopsies are diagnosed by urological pathologists as Gleason score 2+3=5 or 3+2=5 (Epstein 2011).

Gleason pattern 3 cancer consists of variably sized individual glands. A further area of divergence from the original Gleason system is the controversial area of cribriform Gleason pattern 3. Within Gleason's original illustrations of his cribriform pattern 3, he depicted large cribriform glands, which the consensus panel would uniformly diagnose as cribriform pattern 4. The criteria used to diagnosed cribriform pattern 3 were rounded shape, well circumscribed glands of the same size of normal glands. Conceptually, one would except the change in grade from pattern 3 to pattern 4 to be reflected in a distinct architectural paradigm shift where cribriform as opposed to individual glands are formed, rather the merely a subjective continuum of differences, in size, shape and contour of cribriform glands (Epstein 2011).

Ill-defined glands, with poorly formed glandular lumina also demand the diagnosed of Gleason pattern 4. Only a cluster of such glands, where a tangential section of Gleason pattern 3 glands cannot account for the histology, would be acceptable as Gleason pattern 4. Nevertheless, it was noted that in most cases of ill-defined glands with poorly formed glandular lumina are accompained by fused glands. Very small, well-formed glands are still within the spectrum of Gleason pattern 3. This definition differs from Gleason's original description of pattern 4 which only included the hypernephromatoid pattern (Gleason 1966).

The original schematic diagram of Gleason pattern 4 consisted almost entirely of cribriform patterns without depicting fused glands or ill-defined glands with poorly formed

glandular lumina. Gleason patterns 4 are closely resembling renal cell carcinoma (hypernephromatoid pattern) makes up only a very small percentage of Gleason pattern 4 cases (Epstein 2011).

Gleason pattern 5 consists of individual or cords of cells and sheets of tumor. Although typically one sees comedonecrosis with solid nests, occasionally one can see necrosis with cribriform masses that by themselves might be cribriform pattern 4. If there is true comedonecrosis, the consensus was that these patterns should be regarded as Gleason pattern 5. One must be stringent as to the definition of comedonecrosis, requiring intraluminal necrotic cells and/or karyorrhexis, especially in the setting of cribiform glands (Figure 15) (Epstein 2011).

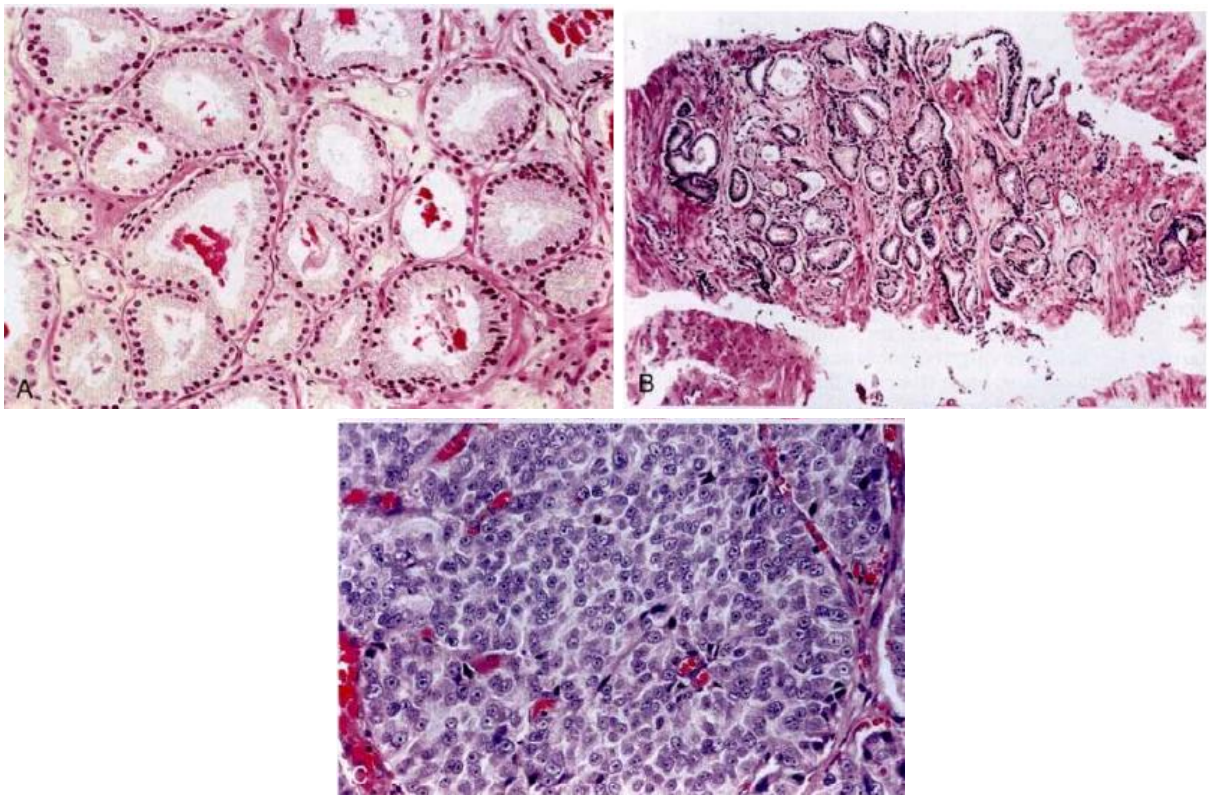

Figure 15: Different Gleason score of adenocarcinomas. A, Low-grade (Gleason score 1 + 1 = 2) prostate cancer consisting of back to back, uniformly sized malignant glands. Glands contain eosinophilic intraluminal prostatic crystalloids, this feature that is more commonly seen in cancer than in benign glands and more frequently seen in lower grade than in higher grade prostate cancer. B, Needle prostate biopsy with variably sized, more widely dispersed glands of moderately differentiated (Gleason score 3 + 3 = 6) adenocarcinoma. C, Poorly differentiated Gleason score (5 + 5 =10) adenocarcinoma composed of sheets of malignant cells, [adapted from (Kumar 2005)].

Staging of prostatic cancer is important in the choice of the appropriate form of therapy. The most common staging system is the TNM system. Stage T1 refers to cancer which is found incidentally either on trans-urethral resection done for BPH symptoms (T1a and T1b depending on the extent and grade) or on needle biopsy, typically performed for elevated

serum PSA levels (stage T1c). Stage T2 is defined as organ-confined cancer. Stage T3a and T3b tumors exhibit extraprostatic extension, with and without seminal vesicle invasion, respectively. Stage T4 shows direct invasion of contiguous organs. Any spread of tumor the lymph nodes, regardless of extent, is eventually associated with a fatal outcome, such that the staging system merely records the presence or absence of this finding (NO/N1) (Epstein 1994; Matzkin 1994) (Table 1).

Table 1: TNM system for staging the prostate cancer (Sobin 2009).

Primary tumor	Tx	Tumor has not evaluated or not evaluable	
	T0	No evidence of tumor	
	T1	Tumor clinically not apparent by palpation or ultrasound	
		T1a	Incidental finding of tumor in <5% of prostate tissue resected
		T1b	Incidental finding of tumor in >5% of prostate tissue resected
		T1c	Tumor diagnosed after PSA elevation
	T2	Tumor confined to prostate	
		T2a	Tumor involving up to half of one lobe of the prostate
		T2b	Tumor involving more than half a lobe of the prostate (not both)
		T2c	Tumor involving both lobes of the prostate
	T3	Tumor with extraprostatic extension	
		T3a	Extension through the prostate capsule
		T3b	Invasion of seminal vesicles
	T4	Tumor fixed or invading adjacent structures (colo vesical, external urethral sphincter, rectum, parede pelvic ósteo-muscular)	
Lymph Nodes	Nx	Regional lymph nodes were not evaluated or not evaluable	
	N0	No evidence of regional lymph node compromise	
	N1	Metastasis to regional lymph nodes	
Metastasis	Mx	Distant metastasis were not evaluated or not evaluable	
	M0	No evidence of distant metastasis	
	M1	Distant metastasis	
		M1a	Non-regional lymph nodes
		M1b	Bone
		M1c	Other locations

2.2.3 - Genes regulation by Epigenetic Mechanisms

Epigenetics changes may lead to alteration of gene expression, since they do not involve a modification in the DNA sequence or the proteins encoded by their genes (Jones 1999). Epigenetic phenomena is mediated by a variety of molecular mechanisms, including posttranslational histone modifications, histone variants, adenosine 5'-triphosphate (ATP)-dependent chromatin remodeling complexes, polycomb/trithorax protein complexes, small and other noncoding RNAs such as short interfering RNA (siRNA) and microRNAs (miRNAs), and DNA methylation (Tost 2008).
DNA methylation is highly related with several chromatin modifications. On the other hand, enzymes that modify DNA and histones have been shown to interact directly with it and they link with local DNA methylation and local chromatin structure (Tost 2009).

Cancer is possibly the best-studied disease with a strong epigenetic component (Jones 2002; Laird 2005). In tumors, a global loss of DNA methylation (hypomethylation) of the genome is observed (Feinberg 1983) and it has been suggested as an initiator and propagator of oncogenesis by inducing chromosome instabilities and transcriptional activation of oncogenes and prometastatic genes such as r-ras (Ehrlich 2002). The overall decrease in DNA methylation is accompanied by a region and gene-specific increase of methylation (hypermethylation) of multiple CpG islands (Jones 2002; Laird 2005).

Hypermethylation of CpG islands in the promoter region of the tumor suppressor or otherwise cancer related gene is often associated with transcriptional silencing of the associated gene. The number of gene-associated promoters that are known to become hypermethylated during carcinogenesis is rapidly growing. Genes of numerous pathways involved in signal transduction (adenomatosis polyposis coli (APC)), DNA repair (O-6-methylguanine-DNA methyltransferase (MGMT), mutL homolog 1 (MLH1), and breast cancer 1 (BRCA1)), detoxification (GSTP1), cell cycle regulation (p15, p16, and retinoblastoma protein (RB)), differentiation (myogenic differention 1 (MYOD1)), angiogenesis (thrombospondin 1 (THBS1) and von Hippel-Lindau tumor suppressor (VHL)) and apoptosis (Caspases, p14, and death-associated protein kinase (DAPK)) are often inappropriately inactivated by DNA methylation. So far no single gene has been identified that is always methylated in a certain type of cancer. Both hypo- and hypermethylation are found in the same tumor, but the mechanisms that lead to both phenomena have not yet been elucidated (Frigola 2006).

A new dimension has recently been added to epigenetic cancer research with the demonstration of long-range gene silencing by epigenetic modifications (Frigola 2006). Long-range epigenetic silencing seems to be a prevalent phenomenon during

carcinogenesis, as a recent survey identified 28 regions of copy-number-independent transcriptional deregulation in bladder cancer that are potentially regulated through epigenetic mechanisms (Stransky 2006).

Although important genetic factors are involved in cancer, such as BRCA1 and p53 mutations in familial cancers, epigenetic changes occur at higher frequency when compared to genetic alterations and may play an important role in early-stage of human neoplasia (Goelz 1985; Costello 2000).

2.2.4 – Biomarkers of prostate cancer

According to the National Cancer Institute, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease" (Figure 16). A biomarker may be objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes, or pharmacologic responses to a particular treatment or condition (Atkinson AJJr 2001). Biomarkers are extensively used as analytical tools to assess biological parameters for a rapid and comprehensive therapeutic analysis. In addition, biomarker measures can further the development and evaluation of new therapies (Rolan 1997).

Biomarkers that represent highly sensitive and specific indicators of disease pathways are frequently used as substitutes for outcomes in clinical trials where they can be used to predict and evaluate the clinical risk and/or benefit of a treatment, which is the optimal objective of all therapeutic interventions (Lagakos 1992).

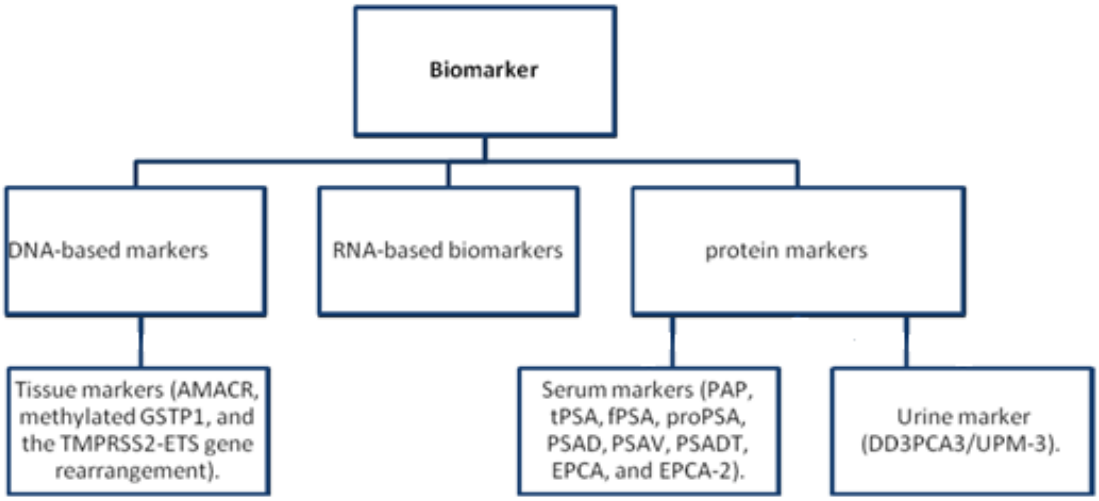

Figure 16: General classification of biomarkers based on their description, [adapted from (Ludwig JA 2005)].

Usually, the cancer biomarkers are classified into three different categories: prognostic, predictive, and pharmacodynamic. Prognostic biomarkers predict the natural course of the cancer, to distinguish the tumor's outcome; help to determine whom to treat, how aggressively to treat, and which candidates will likely respond to a given drug and the most effective dose. Predictive biomarkers evaluate the probable benefit of a particular treatment. Pharmacodynamic biomarkers assess the imminent treatment effects of a drug on a tumor and can possibly determine the proper dosage in the early stages of clinical development of a new anticancer drug (Sawyers 2008).

Among the various proteins used as biomarkers (described in figure 16), which is more used in assessing prostate cancer in its early stage, is the PSA. However, the utilization of PSA as biomarker has been presented several limitations and others proteins have been also pointed as potential biomarkers. Recently, some emphasis has been given to six transmembrane epithelial antigen of the prostate 1 (STEAP1), which was identified as over-expressed in prostate cancer.

2.2.4.1- PSA

PSA is a serine protease that has been used extensively in clinical practice since 1988. It is the best-known member of the kallikrein family, which is the largest proteases group in human genome that is clustered in a 300-kilobase region on chromosome 19q13.4. In clinical practice, the levels of PSA in serum have been used extensively for risk stratification of prostate cancer. The levels of PSA are also used for monitoring disease recurrence after initial treatments and for evaluating response to cancer treatments (Kohli 2010).

In serum, PSA is bound primarily by α1-antichymotrypsin (ACT), an endogenous protease inhibitor, and also by another similar inhibitor, α2-microglobulin. PSA was initially thought to be solely synthesized by epithelial cells of the prostate, and thus, it could be used as a biomarker for prostate cancer diagnosis (Chu 1997). However, PSA has also been found in a variety of human normal and tumor cell lines, and in several biological fluids synthesized by numerous cells (Chu 1997; Polascik 1999; Rao 2008).

Despite uncertainty regarding the value of routine PSA testing, it has been widely used. Furthermore, there are problems with sensitivity and specificity using standard PSA testing strategies (Abrahamsson 1997).

The sensitivity of PSA for detection of prostate cancer ranges from 63% to 83%. The specificity is lower in men with larger prostate glands or those with lower urinary tract symptoms (Harris 2001).

The main advantage of PSA testing is its sensitivity. However, the main disadvantage of this test is its low specificity, because some common pathological conditions such as BPH and prostatitis can also increase the levels of PSA (Oh 2003).

In order to overcome the problems associated with sensitivity and specificity, a variety of diagnostic parameters and tests have been proposed. However, none of them have been proved to be beneficial or superior to current PSA reference ranges. Refinements include lowering the cutpoint for defining an abnormal PSA level to below 4.0 ng/mL (e.g.,below 3.0 ng/mL), age-specific PSA, PSA velocity (PSA-V), volume-adjusted PSA densities, the free-to-total PSA ratio (%fPSA), and bound/ complexed PSA (Labrie 1992).

The PSA-V is the most useful if three or more measurements are obtained over an interval of 1 to 3 years (Mettlin 1994). An abnormal PSA-V has been defined if the levels of PSA increase more than 0.75 ng/mL per year. Because prostate inflammation contributes to PSA elevations, the use of PSA-V change after antibacterial therapy for infectious/inflammatory symptoms might help differentiate benign and malignant conditions in men with elevated PSA (Stenman 1994).

PSA circulates in two forms: free and complexed with molecules such as ACT. Men with prostate cancer tend to have a lower percentage of their PSA in the free form compared with men without prostate cancer (Stenman 1994). The free/total PSA measurements might help to differentiate between prostate cancer and normal prostate conditions among men with PSA concentrations between 4.0 ng/mL and 10 ng/mL. To be useful, a negative value for %fPSA would need to reduce the probability of prostate cancer to a low enough level that men would be willing to forego biopsy (Hoffman 2000). PSA complexed with ACT, α2-macroglobulin or α1-protease inhibitor as well as human glandular kallekrein-2 might hold promise when evaluating the need for repeat biopsies in men with a mildly elevated PSA and negative initial biopsy (Wilt 2003).

PSA levels are normally elevated in older men relative to younger one, regardless of the absence or presence of cancer. Therefore, a continuous rise in PSA levels over time may be more indicative of cancer than moderately increased of PSA (Carter 1992). Higher PSA values have also been observed in African American men with newly diagnosed prostate tumor when compared with newly diagnosed Caucasian men (Moul 1995).

The levels of PSA in serum can independently predict a pathological stage. However, the serum PSA level alone may not adequately calculate pathological stage because the relationship between pathological stage and serum PSA varies by tumor grade, volume, and origin site (Partin 1993; Stamey 1993). Nevertheless, comparative studies have demonstrated that PSA and its related testing can increase the detection rate of prostate cancer in men with no symptoms (Mettlin 1991; Catalona 1993). It has also been shown that the stage distribution of cancers detected through PSA is much more favorable (Madu 2010).

2.2.4.2 - STEAP1

The STEAP family of proteins is a relatively new family comprising four members named STEAP1 to STEAP4. STEAP1 and STEAP2 are mainly expressed in the prostate, whereas the expression of STEAP3 and STEAP4 in human tissue is highest in bone marrow, followed by placenta and fetal liver (Ohgami 2006).

STEAP1 protein is composed by 339 aminoacids with a molecular weight of 36 KDa, and it is localized on chromosome 7q21, a region close to the telomeric sequences. STEAP1 was identified as over-expressed in prostate cancer and in several cancer cell lines of various origins, such as pancreas, colon, breast, testicular, cervical, bladder and ovarian carcinoma, acute lymphocytic leukemia and Ewing sarcoma. STEAP1 is absent in normal human tissues except in prostate and bladder (Hubert 1999).

STEAP1 protein presents a molecular topology of six transmembrane domains with intracellular N- and C- terminals and three extracellular and two intracellular loops (Hubert 1999). Its structure prediction and location at cell–cell junctions suggests that STEAP1 may act as a channel, or a transport protein in tight or gap junctions, or in cell adhesion (Hubert 1999; Kobayashi 2007). In fact, recent data showed that STEAP1 is involved in intercellular communication between adjacent cells in culture, and that it seems to favor tumor development (Challita-Eid 2007) (Figure 17).

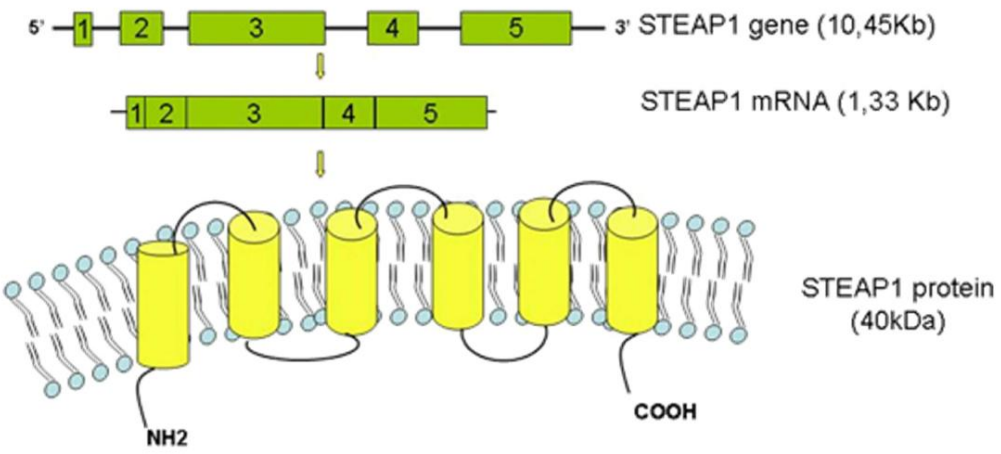

Figure 17: STEAP gene organization, mRNA transcripts and predicted protein structure. Green boxes correspond to exons, [Adapted from (Santos 2010)].

A detailed *in silico* analysis allow us to identify another gene related to STEAP1, which is known as STEAP1B. This gene is localized on chromosome 7p15.3 and may originate two different transcripts, namely STEAP1B1 and STEAP1B2. STEAP1B1 is the longer transcript and may encode the longer isoform. STEAP1B2 transcript uses an alternate in-frame splice site in the 5' coding region and an alternate 3' exon with a distinct 3' coding region and 3' UTR, compared to variant 1. STEAP1B1 and STEAP1B2 have a predicted molecular weight of 42 KDa and 30 KDa, respectively. The STEAP1B2 isoform lacks an internal segment near the N-terminus and has a shorter and distinct C-terminus, when compared to STEAP1B1 isoform (http://www.ncbi.nlm.nih.gov/gene?term=STEAP1B). Of our knowledge, no studies have been conducted regarding the expression of STEAP1B in human prostate cells.

At this time, very little is known about the regulation of STEAP1. Previously, our research group has demonstrated that STEAP1 is down-regulated by 17β-estradiol (E_2) in MCF-7 breast cancer cells (Maia 2008). Recently, we also demonstrated that DHT and E_2 down-regulates STEAP1 expression in LNCaP prostate cancer cells (Gomes (in press)). However, the molecular mechanisms underlying the over-expression of STEAP1 in prostate cancer remain unknown.

As described previously, cancer presents a strong epigenetic component, which may lead to deregulation of gene expression without involve a modification in DNA sequence (Jones 2002; Laird 2005).

Regarding the STEAP gene family, Tamura and Chiba demonstrated that CpG islands in STEAP4 promoter region were frequently methylated in DU145, androgen-independent prostate cancer cells, and this may down-regulate STEAP4 gene expression (Tamura

2009). *In silico* analysis of the promoter region and first intron of STEAP1 gene, it was observed that this region also presents a CpG islands (see Figure A1 in appendix), raising the question whether STEAP1 may also be regulated by methylation mechanisms.

As STEAP1 is highly expressed at cell membrane in several types of tumors but not in most vital organs in human, turned STEAP1 into a potential target for antitumor immunotherapy (Gomes 2012). In fact, STEAP1 peptide epitopes can be used to stimulate cytotoxic T and helper T lymphocytes, which kill STEAP1 expressing tumor cells (Alves 2005). In addition, murine STEAP1 (mSTEAP1)-based vaccination was able to induce a specific CD8 T-cell response against a newly defined mSTEAP1 epitope that prolonged the overall survival rate in tumor-challenged mice very significantly (Garcia-Hernandez 2007).

Although several studies have been pointed STEAP1 as a potential biomarker in human cancer, especially in prostate and Ewing tumors (Li 2004; Valenti 2008; Grunewald 2011), the relationship between STEAP1 expression in prostate cells and clinic-pathological data are scarce and more studies are required.

AIMS

Considering the incidence and the mortality of prostate cancer and some of the limitations of the PSA test as a marker of this disease, we believe that it is important to study novel putative biomarkers in prostate cancer.

Although it is well characterized that STEAP1 is over-expressed in prostate cancer cell lines, the mechanisms that conduct its over-expression remains unknown. In addition, no studies are found in the literature regarding the characterization of STEAP1B expression in prostate cells.

Several studies have been pointed STEAP1 as a potential biomarker or therapeutic target in cancer, but the clinical significance of STEAP1 expression in prostate cancer remains poorly understood. In order to better understand the STEAP1 and STEAP1B expression, regulation and its clinical significance in prostate cancer, the following aims were delineated:

- To analysis the STEAP1, STEAP1B1 and STEAP1B2 messenger ribonucleic acid (mRNA) and STEAP1 protein expression in human prostate cell lines;
- To evaluate whether promoter region of STEAP1 gene has different methylation pattern between neoplastic and non-neoplastic prostate cells;
- To analysis the STEAP1 expression in tissue microarrays (TMAs) of human prostate and compare its immunoreactivity with the clinical profile, biochemical, and pathological data of patients.

MATERIALS AND METHODS

1 – STEAP1 gene and protein expression in prostate cell lines and its regulation by epigenetic mechanisms.

1.1 - Cell lines culture

In this study were used four cell lines of prostate, LNCaP and PC3 (neoplastic cell line: LNCaP – Androgen-sensitive and PC3 – androgen independent), PNT1A and PNT2 (non-neoplastic cell lines) that were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). All cell lines were cultured in recommended medium with RPMI 1640 phenol-red (Invitrogen) in the presence of 10% FBS and 1% penicillin/streptomycin (Invitrogen) in a humidified atmosphere at 37ºC containing 5% CO_2.

1.2 - Mycoplasma testing

In order to ensure that cell lines culture was mycoplasm-free, 100µl of cell culture medium was removed to an eppendorf. The medium was incubated at 95ºC for 5 min and then was centrifuged for 5 min at 10000rpm.

Next, PCR reactions were carried out with the purpose to detect mycoplasm contamination in culture medium. A mixture was prepared for the first PCR with 8.05µl of Miliq water, 2.5µl of 10x buffer ($(NH_4)_2SO_4$ and $MgCl_2$), 0.75µl of $MgCl_2$ (50 mM), 1µl of dNTPs (10mM), 7.5µl of primers mix (10µM) (Table 2), 0.2µl of Taq polymerase (Fermentas) (5U/µl) and 5µl of culture medium. The tubes were incubated at 94ºC for 10 min followed by 35 cycles of 30 sec at 94ºC, 2 min at 55ºC, and 1 min at 72ºC, followed by a final elongation for 8 min at 72ºC on a PCR system. Amplified PCR products were analyzed on a 2% agarose gel and the results were visualized in an image analyzer (UVITEC - Cambridge).

As the samples were negative in the first PCR, it was prepared a second PCR with 12.05µl Miliq water; 2.5µl of 10x buffer ($(NH_4)_2SO_4$ and $MgCl_2$); 0.75µl of $MgCl_2$ (50 mM); 1µl of dNTPs (10mM); 7.5µl of primers mix (10µM); 0.2µl of Taq polymerase (Fermentas) (5U/µl) and 1µl of amplification product of the first PCR. The samples were incubated at 94ºC for 10 min followed by 30 cycles of 30 sec at 94ºC, 2 min at 55ºC, and 1 min at 72ºC, followed by a final elongation of 8 min at 72ºC on a PCR system. Amplified products were analyzed on a 2% agarose gel.

Table 2: Sequences of the specific primers used to mycoplasm test**.**

	Sequence (5'-3')	Sequence (5' – 3')
1º PCR	MycoF1t	ACA CCA TGG GAG TTG GTA AT
	MycoR1cat	CCT CAT CGA CTT TCA GAC CCA AGG CAT
	MycoR1	CTT CAT CGA CTT TCA GAC CCA AGG CAT
	MycoR1ac	CTT CAT CGA CTT CCA GAC CCA AGG CAT
	MycoR1tt	CTT CTT CGA CTT TCA GAC CCA AGG CAT
	MycoF1	ACA CCA TGG GAG CTG GTA AT
Nested PCR	MycoF2	GTT CTT TGA AAA CTG AAT
	MycoF2cc	GCT CTT TCA AAA CTG AT
	MycoF2a	ATT CTT TGA AAA CTG AT
	MycoR2	GCA TCC ACC AAA AAC TCT
	MycoR2cc	GCA TCC ACC ACA AAA CTC
	MycoR2at	GCA TCC ACC AAA TAC TCT

1.3 - Extraction of total ribonucleic acid (RNA) and cDNA synthesis

Total RNA was isolated from LNCaP, PNT1A, PNT2 and PC3 cells using Tri reagent (Sigma). For this, 500 µl of this reagent was added to cells and 40 µl of a chloroform solution. The solution was homogenized by vortex for about 15 seconds and stands at room temperature for 15 minutes. Next, samples were centrifuged at 12000 x g for 15 minutes at 4ºC, in order to promote the separation of the different components in different phases: a reddish organic phase consisting of proteins; a white intermediate phase that is the DNA; and an upper aqueous phase containing RNA. This aqueous phase was removed for into a clean 1.5 ml microcentrifuge tubes and 100 µl of isopropanol solution was added to precipitate RNA and remained for about 10 minutes at room temperature. Samples were centrifuged at 12000 x g for 10 minutes at 4ºC and the supernatant was discarded. Total RNA pellet was washed twice, by adding 200 µl of a solution of 75% ethanol, then, was homogenizated in vortex mixing, and centrifuged at 7500 x g for 5 minutes at 4ºC. Finally, pellets were dried for 5 minutes at room temperature, and dissolved in 20 µl of H2O DEPC-treated.

In order to evaluate the quantity of total RNA, its optical density at 260 nm and 280 nm was determined (Pharmacia Biotech, Ultrospec 3000), and the integrity of RNA was verified through agarose gel electrophoresis.

For cDNA synthesis, 1 µg of total RNA was subjected to reverse transcription for PCR, according to the manufacturer's instructions. Briefly, total RNA was denatured for 5 min at

65ºC in a 10 µl reaction containing random hexamer primers (100 µM) and DEPC-treated water. A mix was prepared for a 10 ul reaction containing reverse transcriptase buffer RiboLock™ Rnase Inhibitor (20 u/µl); dNTPs (10mM) and M-MuLV Reverse Transcriptase (20 u/µl). This mixture was added to the previously denatured RNA. Reverse transcription was carried out at 37ºC for 60 min and was stopped after 15 min at 75ºC.

1.4 – Conventional polymerase chain reaction (PCR) and Real-Time PCR

Conventional PCR was used to evaluate the quality of cDNA samples, which were assessed using a GAPDH housekeeping gene. Then, as first approach, the expression of STEAP1, STEAP1B1 and STEAP1B2 genes were carried out by PCR, using specific primers (Table 3). PCR reactions were performed in a 25 ul reaction volume containing 1 µl of synthesized cDNA; DreamTaq buffer (KCl, $(NH_4)_2SO_4$ and $MgCl_2$ 20mM); dNTP (10 mM) (Amersham); $MgCl_2$ (50mM); 50 nM of each primer and 0,125 U DreamTaq™ polymerase (Fermentas). Reaction tubes were kept on ice to prevent non-specific amplification and incubated for 5 min at 94ºC, followed by 30 cycles of 30 sec at 94ºC, 30 sec at 56ºC, and 1 min at 72ºC, and a final elongation step of 5 min at 72ºC on a Tprofissional Basic Gradient - PCR system (Biometra). Amplified products were analyzed on a 1% agarose gel and the results were visualized in an image analyzer (UVITEC - Cambridge).

Real-time PCR was used to quantify the expression of STEAP1 mRNA levels in neoplastic (LNCaP, PC3) and non-neoplastic cells (PNT1A, PNT2). Real time PCR reactions were carried out using 1µl of cDNA synthesized, in a 20µl reaction containing 10µl SYBR Green Supermix (Biorad) and the appropriate concentration of each primer: 1.2µl STEAP1 primer (300nM) and 1.2ul GAPDH primer (300nM), (Table 3). After an initial denaturation at 95ºC during 5 min, cycling conditions (35 cycles) were the following: 95ºC for 10 sec, annealing temperature at 60ºC for 30 sec and 72ºC for 10 sec, on an IQ5 Multicolor Real Time PCR Detection System (Bio-Rad). The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95ºC with 10 sec holds at each temperature (0.05.C/s). Samples were run in triplicate in each real-time PCR assay. Fold differences were calculated following the mathematical model proposed by Pfaffl using the formula $2^{-(\Delta\Delta Ct)}$ (Pfaffl 2001). The results presented were obtained by a Log_2 transformation of real-time PCR data.

Table 3: Details of specific primers used for amplication of STEAP1, STEAP1B1 and STEAP1B2.

Gene name	Oligo name	Sequence (5'-3')	Amplicon size (bp)
STEAP1 (NM_012449.2)	hSTEAP1_980fw hSTEAP1_1181rv	TTCCCTTCTACTGGGCACAA GACGTCTTCCCAACCATGTC	202
STEAP1B1 (NM_001164460.1)	hSTEAP1B_isof1fw hSTEAP1B_isof1rv	CTGGAAGCCTGGTAGCTTTG GGCTGGCTGCTGATAAAATG	162
STEAP1B2 (NM_207342.2)	hSTEAP1B_isof2fw hSTEAP1B_isof2rv	CGATTATTTGCAAACAGCCC GGGAAGTTGCTAAAGGGTGA	173
GAPDH (NM_002046)	hGAPDH_74fw hGAPDH_149rv	CGC CCG CAG CCG ACA CAT C CGC CCA ATA CAA TCC G	75

1.5 - Protein extraction and Western Blot

Prostate cell lines were lysed in 100µl of Radioimmunoprecipitation assay buffer (RIPA) (150mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50mM Tris, 1mM EDTA, 1% Protease cocktail and 1mM PMSF). The total proteins (supernatant) were recovered after a 12000 x g centrifugation for 20 min at 4ºC. Quantification of total protein extracts was assessed using the Bradford method (Biorad Protein Assay).

Western Blot was carried out to determine the expression of STEAP1 protein in prostate cell lines. Firstly, the proteins were denaturated for 5 min at 100ºC and ran on a polyacrylamide gel (12.5% resolving gel and 4.7% stacking gel), for 90 min, at 140V. The resolving gel was constituted by 4.15ml acrylamide (40%); 3.75ml Tris-HLC 1.875M pH 8.8; 1.85ml H_2O; 0.1ml SDS 10%; 125µl PSA 10% and 15µl TEMED, and the stacking gel by 0.875ml acrylamide (40%); 0.625ml; 3,45ml H_2O and 1 ml SDS 10%; 125µl PSA 10% and 15µl TEMED.

PVDF membrane was activated in methanol (5 sec.), H_2O (5 min) and electrophoresis buffer (10mM CAPS in methanol 10%, pH 11) (5 to 15 min). Proteins were electro-transferred from gel to PVDF membrane in CAPS electrophoresis buffer at 750 mA for 30 min. Membrane was blocked during 1h, at room temperature, in blocking solution (TBS-T 5% milk) before incubation overnight with a rabbit polyclonal antibody against human STEAP1 (H-105, Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:300 with TBS-T 1% milk. After that, membrane was washed three times in washing buffer (TBS-T 0.5% milk) for 45 min, and then, incubated with alkaline phosphatase conjugated with the secondary antibody (1:20000 dilution) for 1 h at room temperature. The membrane was washed again during 45 min, under the same conditions; at last, this membrane was air

dried and incubated with 800 µl ECF reagent for 2 – 3 min, and exposed to chemiluminescence's detection in a Molecular Imager FX (Biorad, Hercules, USA).
The same membranes probed with anti-STEAP1 were incubated with mouse anti α-tubulin antibody diluted 1:5,000 (Sigma) and then with goat anti-mouse IgG phosphatase alkaline conjugated secondary antibody diluted 1:20,000 (Abcam, Massachusetts,MA). The expression levels of STEAP1 and α-tubulin proteins were quantified by densitometry using Quantity One software (Biorad, Hercules). The signal of STEAP1 was normalized to the corresponding α-tubulin signal.

1.6 - DNA extraction and quantification

DNA was extracted from LNCaP, PNT1A and PNT2 cells using the Gentra Puregene Cell Kit (QIAGEN). The cells were centrifuged for 5 sec at 13400 x g and the supernatant was discarded by pipetting. The solution was homogenized by vortex to resuspend the cells in the residual supernatant. Next, 300µl of Cell Lysis Solution was added to resuspended cells with up and down pipetting. 100µl of Protein Precipitation Solution was added to samples and then, they were homogenized and vortexed for 20 sec at a high speed, which was followed by further centrifugation for 1 min at 13400 x g. After to add 300µl isopropanol into a clean 1.5ml microcentrifuge tubes, the supernatant from the previous step was dropped carefully. The tubes were mixed by gently inverting about 50 times. Then, the samples were centrifuged for 1 min at 13400 x g and the supernatant was discarded. Subsequently, 300µl of 70% ethanol was added to wash the DNA pellet, which was centrifuged for 1 min at 13400 x g and so, the supernatant was discarded. After drying the DNA at room temperature for 15 min, 50µl DNA Hydration Solution was added and homogenized by vortex during 5 sec at medium speed. The tubes were incubated at room temperature overnight with gentle shaking.

To access the quantity of DNA, its optical density at 260 nm and 280 nm was quantified (Pharmacia Biotech, Ultrospec 3000), and the integrity of DNA was verified through an agarose gel electrophoresis.

1.7 - Bisulfite modification of DNA

Sodium bisulfite treatment performs a chemical modification in cytosine bases. It is based on the different sensitivity of cytosine and 5-methylcytosine to deamination by bisulfite, where unmethylated cytosines are converted to uracil and that methylated cytosines remain as cytosines. This method is crucial in Epigenetic, because it allows the analysis of methylation levels in CpG islands in genomic DNA. Bisulfite modification consists mainly in four steps: an initial denaturation to allow a better efficiency of the bisulfite's action; a

sulphonation resulting in the formation of a sulphonated cytosine derivate; a hydrolytic deamination step to convert this compound into uracil sulphonate; and the last step is based in a alkali desulphonation to remove bisulfate adducts from the uracil ring.

Sodium bisulfate modification was performed using the EZ DNA Methylation-Gold™ Kit [Zymo Research, Orange, CA, USA] according to the manufacturer's instructions. 1µg of DNA (in a total volume of 20µl), extracted from LNCaP, PNT1A and PNT2 cells, was submitted to the modification process. Briefly, 130µl of CT Conversion Reagent were included with DNA in a PCR tube. This mix was then incubated in PCR system, during 10 min at 98ºC, and subsequent stage of 180 min at 64ºC for DNA denaturation and sodium bisulfite conversion. After incubation, DNA was recovered in a Zymo-Spin™ IC Column using 600µl of M-Binding Buffer and centrifuged for 30 sec at 10000 rpm. The column was washed with 100µl of M-Wash Buffer and centrifuged once again in same condition as the previous step. Following the M-Wash Buffer was discarded, and then 200µl of M-Desulphonation Buffer were added for 20 min incubation's period at room temperature. After discard the M-Desulphonation, the DNA was washed with M-Wash Buffer. In the end, the column was inserted in a 1.5ml-recovery tube and DNA was eluted in 30 + 30µl of distilled water. The final product was kept at -20ºC.

1.8 - Bisulfite sequencing PCR (BSP)

Using the program Methyl primer express v1.0**,** five pairs of primers were designed in order to amplify the gene region containing CpG island (end of the promoter, first exon and the beginning of the first intron) (Table 4).

Table 4: Sequences, amplicons sizes and cycling conditions used in PCR for amplification of the specific primers of STEAP1.

Oligo name	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature
STEAP1_met_-338fw STEAP1_met_+23rv	AAAGTGTGATTTGGGAATGTT CTCCAAACCAACTACAAACCC	412	61º
STEAP1_-206fw STEAP1_+13rv	TGGGGAGTTTTAGTTTTTAAGG TCAAAAAACTCACCCACTCTT	219	62º
STEAP1_-113fw STEAP1_+100rv	TTTGTAGTTGGTTTGGAGGTTT TACACCCCTCCCAAAACTAA	213	62º
STEAP1_-8fw STEAP1_+325rv	AAGAGTGGGTGAGTTTTTTGAA TTACCATCAAAATCCAACCTAAA	333	60º
STEAP1_+230fw STEAP1_+487rv	GGGGAGAGTTAGGGATTTATAG CACCCAAATCCTAAAAAAATAA	257	54º

After bisulfite modification, STEAP1 gene was amplified with all pairs of primers. In this PCR were evaluated different annealing temperatures in order to determine the best one of each primer pair.

PCR reactions were performed in a 20ul reaction volume containing 2µl of DNA (modified), 2µl of 10x Dynazyme II Hot Start Reaction Buffer, 0.4µl dNTP mix (10 mM each dNTP), 0.5µl of each primer (250nM each primer), and 0.24µl of Dynazyme II HotStart DNA polymerase (2U/µl) (Frilabo). Reactions tubes were kept on ice at all times and incubated for 10 min at 94ºC, followed by 35 cycles of 30 sec at 94ºC, 30 sec at annealing temperature (Table 4) and 30 sec at 72ºC, followed by a final elongation of 10 min at 72ºC on a PCR system. Amplified products were analyzed on a 2 % agarose gel and the results were visualized in an image analyzer (UVITEC - Cambridge).

1.9 - Direct sequencing (Bisulfite) and analysis of sequences

PCR products were purified with kit Illustra GFX (GE Healthcare). The columns were put in the respective “collection tube”, next 500ul of “Capture buffer type 3” was added to the column and then was joined it the amplification product. The columns were centrifuged during 1 min at 12000 rpm. The flow-through was discarded and the columns were placed back into the collection tube. Next, 500ul of “Wash Buffer type 1” was added to each column. Subsequently, the columns were again centrifuged for 1 min at 12000 rpm. The PCR Clean-up Columns were placed into a new 1.5ml microcentrifuge tube, and 30ul of “Elution Buffer type 6”, was added, incubated at room temperature over 5 min and, at last, the columns were centrifuged for 5 min at 12000 rpm. Purified products were analyzed on a 2 % agarose gel and the results were visualized in an image analyzer.

After purification, the purified PCR products were sequenced using Sequencing PCR kit (Applied Biosystems). Two tubes were used for each sample, one for the forward reaction, and another for the reverse. Sequencing PCR reactions were performed in a 10 ul reaction volume containing 1.9µl of Big Dye Buffer, 1µl Big Dye Mix v1.1, 0.35µl of each primer (350nM each primer) and 1 to 3 ul of DNA that depend on the intensity of each sample previously visualized on agarose gel. Reaction tubes were kept on ice and incubated for 2 min at 96ºC, followed by 30 cycles of 15 sec at 96ºC, 15 sec at 50ºC, and 4 min at 60ºC, on a PCR system.

The PCR products were purified with Illustra Sephadex G-50 fine. The columns were put in the respective tube of 2mL and was added the Sephadex to each column. The columns were centrifuged for 4 min at 4400 rpm. The columns with sephadex were placed into a new 1.5ml microcentrifuge tube and were added 10ul of Sequencing PCR product. The

columns were centrifuged again during 4 min at 4400 rpm and the sephadex was removed of the columns. Finally, it was added 12ul of formamide to purified product and transferred to sequencer tubes.

After sequencer analysis, it was determined the ratio of methylation by the formula [height C / (height C + height T)] x 100.

2 – STEAP1 protein expression in human prostate cancer

2.1 - Patients and tissue samples

The human tissue samples (N=102) are derived from prostatectomy and were gently provided by pathology service of “Hospital Geral de Santo António – Porto”. The patients did not receive preoperative chemotherapy or any other type of treatment. The detailed characteristics of patients are described in Table A1 in appendix.

2.2 - Construction of TMAs

For the construction of a TMA, the number of cases and cores that were used in the generation of the TMA were determined and then it was reviewed all the slides and was marked the areas of interest. For this, TMA building was used a core size of the 2 mm and a maximum of 12 cores per case. Liver tissues were placed in TMA block for quality control and to address tumor heterogeneity.

The block from which a core was taken is referred to as the donor block, and the area of this donor block from which the core was removed for TMA, was selected by a pathologist. The block, in which the cores were placed, is referred to as the recipient block. Next, we cut sections of 4µm of each block, using a microtome, and placed in a solution of cold water and ethanol to be extension of the paraffin followed by a solution of warm water (42ºC). Finally, they were removed with the help of a slide and were left to dry (Figure 18). Prior to performe immunohistochemistry technique, the slides (with respective sections of each TMA) were placed in “*hothouse*” at 60ºC overnight, which allowed the melting of the paraffin.

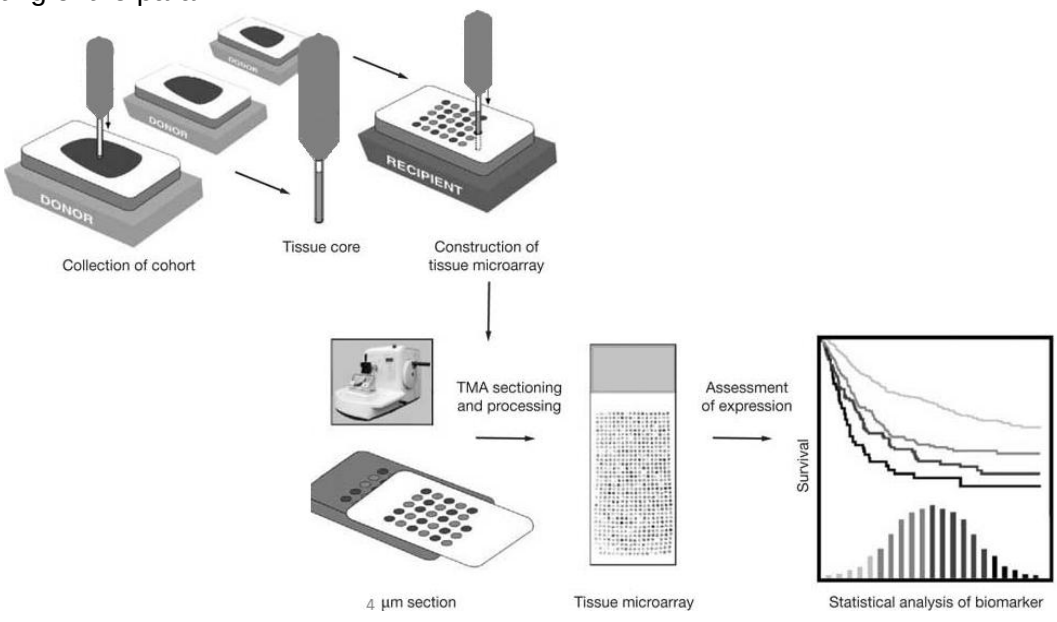

Figure 18: Steps involved in TMA construction [adapted from (Giltane 2004)].

2.3 - Immunohistochemistry and analysis of STEAP1 immunoreactivity

The immunohistochemical analysis was carried out in TMAs of human prostate cancer, BPH, PIN and non-neoplastic tissue samples. Firstly, the slides for immunohistochemical were deparaffinized in xilol and rehydrated in decreasing solution of ethanol. In order to block the activity of endogenous peroxidases, 2ml of H_2O_2 (3%) were added to each slide. After 5 min, these slides were washed in PBS-T (Phosphate Buffered Saline-Tween 20) during 2 min, and added goat serum 5% (Santa Cruz Biotechnology, Santa Cruz, USA) to prevent any nonspecific binding sites for 30 min, a new wash in PBS-T was done for 5 min with agitation, and then, the samples were incubated with primary antibody α-STEAP1 (H-105, Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:50 in PBS (Phosphate Buffered Saline) with 1% BSA for 1 h at room temperature. The slides were washed again for 10 min with agitation, and they were incubated with secondary antibody α-rabbit (diluted 1:20 in PBS with 1%BSA) for 1 h at room temperature. After new wash for 10 min in PBS-T with agitation, the slides were incubated with "ExtrAvidin-Peroxidase" for 30 min. Then, it was washed again in PBS-T for 10 min with shaking and 100µl 3,3-Diaminobenzidine (DAB) (Sigma) were added in each slide, until the development of appropriate coloration. The slides were passed through water and immersed in hematoxylin (Hematoxylin 7211, Richard-allan Scientific) for 1 – 2 min. Finally, the slides were washed for 10 sec, and subjected to a process of gradual dehydration, in which it was added 1 – 2 drops of the permanent mounting medium (Entellan, Merck (103602)) and covered with a coverslip.

STEAP1 immunoreactivity was assessed semiquatitatively using a grade score system, based on the intensity of staining and percentage of stained cells. The intensity was divided into "0" (no staining), "1" (low staining) and "2" (high staining). The percentage of stained cells was divided into "0" (no stained cells), "1" (up to 25% of stained cells), "2" (between 25-50% of stained cells) and "3" (more than 50% of stained cells).

In order to calculate the final score, the percentage of stained cells and intensity were multiplied, giving the following scores (0, 1, 2, 3, 4 e 6). Then, these score values were grouped into low immunoreactivity (score 0 and 1), moderate immunoreactivity (score 2 and 3), and high immunoreactivity (score 4 and 6).

2.4 – Statistical analysis

Association between STEAP1 immunoreactivity and histologic diagnosis or clinic-pathologic data of patients was analyzed by Chi-square statistical test. All data were

analyzed using SPSS version 17.0 software. $p<0.05$ was considered to be statistically different.

RESULTS / DISCUSSION

1 - Expression analysis of STEAP1 in human prostate cell lines.

To analysis the expression of STEAP1 genes in non-neoplastic (PNT1A and PNT2) and neoplastic (LNCaP and PC3), PCR reactions were performed using specific primers to STEAP1, STEAP1B1 and STEAP1B2 gene. A negative control was used for each primers set, containing only the mix without cDNA (Figure 19).

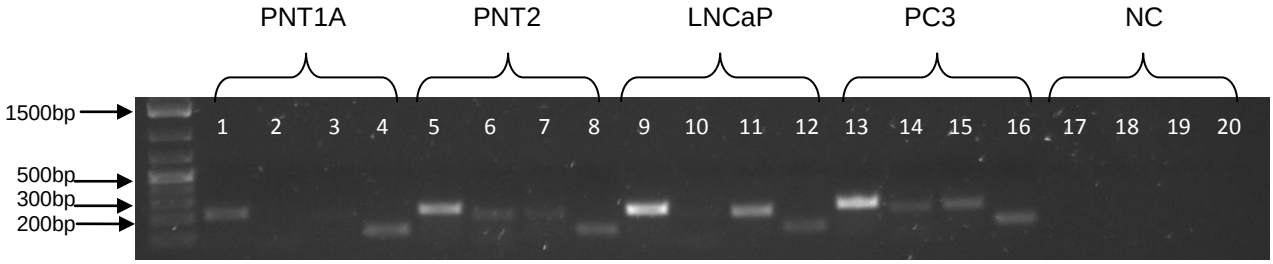

Figure 19: PCR analysis of STEAP1, STEAP1B isoform 1 and STEAP1B isoform 2 mRNA expression in PNT1A, PNT2, LNCaP and PC3 prostate cell lines. 1, 5, 9 and 13 – STEAP1; 2, 6, 10 and 14 – STEAP1B1; 3, 7, 11 and 15 – STEAP1B2; 4, 8, 12 and 16 – GAPDH; 17, 18, 19 and 20 – STEAP1, STEAP1B1, STEAP1B2 and GAPDH negative control, respectively.

As depicted in figure 19, the expression of STEAP1 was detected in all cell lines, but neoplastic cell lines (LNCaP and PC3) seems to present higher levels of STEAP1 than non-neoplastic cell lines (PNT1A and PNT2). STEAP1B1 mRNA is slightly expressed in PNT2 and PC3 cells, but no expression was detected in LNCaP and PNT1A cells. Regarding the STEAP1B2 expression, it was observed that LNCaP and PC3 cells present a higher level when compared to PNT1A and PNT2 cells.

The results obtained by conventional PCR showed that the STEAP1 mRNA is more expressed in all prostate cell lines when compared to STEAP1B1 or STEAP1B2, suggesting that the main physiologic roles may be attributed to STEAP1. However, it is necessary to analyze the expression of STEAP1B1 and STEAP1B2 proteins using specific antibodies. In case of expression of these proteins, it is necessary to develop functional studies in order to clarify the role of each STEAP1 isoform in prostate cells.

Considering these results obtained by conventional PCR, it was quantified the expression of STEAP1 by Real Time PCR in prostate cell lines. The data showed that STEAP1 is highly expressed in LNCaP, followed by PC3, PNT1A and PNT2 (Figure 20).

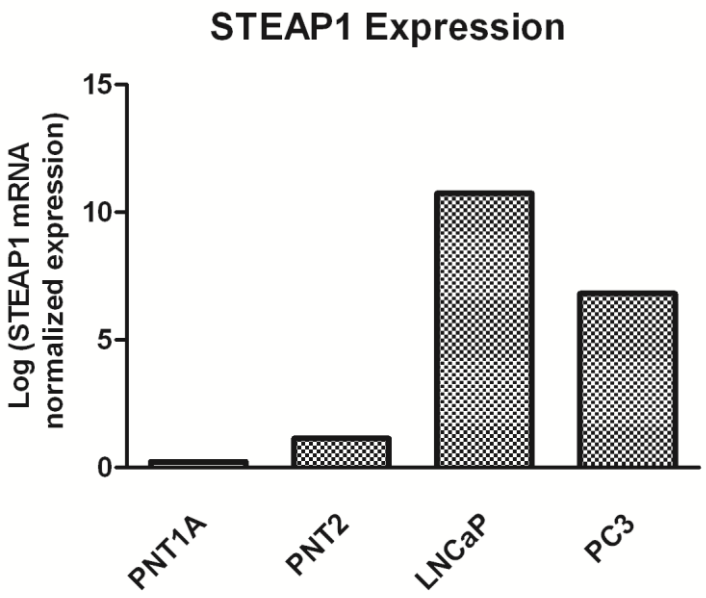

Figure 20: Analysis of STEAP1 mRNA expression in PNT1A, PNT2, LNCaP and PC3 cells by Real-time PCR. STEAP1 mRNA expression was normalized with GAPDH housekeeping gene.

As expected, our results show that STEAP1 is over-expressed in neoplastic prostate cell lines when compared to non-neoplastic. As previously described by (Hubert 1999), LNCaP present higher levels of STEAP1 than PC3 cells. Taking into account that LNCaP cells are AR-positive and PC3 are AR-negative, this differential expression suggests that STEAP1 expression may be regulated by androgens. However, contrarily as expected, our research group demonstrated that STEAP1 gene is slightly down-regulated by androgens in LNCaP cells (Gomes (in press)).

In order to confirm that the higher levels of STEAP1 mRNA in LNCaP cells also corresponds to higher levels of protein, western blot analysis was carried out using total protein extracted from LNCaP, PC3, PNT1A, and PNT2 cells. The results show one immunoreactive band of 36 KDa in LNCaP and PC3 cells, whereas in PNT1A cells two immunoreactive bands of 36 and 30 KDa were detected (Figure 21). Regarding the PNT2 cells, no immunoreactive bands were detected. The immunoreactive band of 36 KDa corresponds to the molecular weight of STEAP1, as previously reported by others (Hubert 1999; Vaghjiani 2009).

Our results show that STEAP1 protein is also over-expressed in LNCaP cells in comparison with the other cell lines. . In opposition to observed at mRNA level, only a slight expression of STEAP1 protein is detected in PC3 cells, suggesting that others mechanisms may be involved, such as regulation of translation, and stability of mRNA and/or protein. Regarding the immunoreactive band of 30 KDa detected in PNT1A cells, it

is liable to speculate that may correspond to STEAP1B2 isoform, which is in accordance with the predicted molecular weight for STEAP1B2 protein. However, it is necessary develop others approaches to confirm it, as for example proteomics and development of specific antibodies against each STEAP1 isoform.

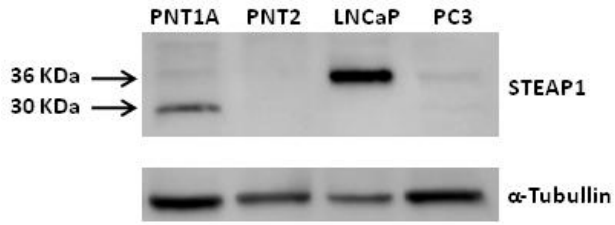

Figure 21: Western blot analysis of STEAP1 protein expression using an anti- STEAP1 polyclonal antibody (1:300) in PNT1A, PNT2, LNCaP and PC3 cell lines.

2 - Analysis of methylation of DNA in cell lines (LNCaP, PNT1A and PNT2)

As described above, STEAP1 gene is over-expressed in LNCaP cells when compared to non-neoplastic cells (PNT1A and PNT2). However, the mechanisms underlying its up-regulation in cancer remain to be explored. As epigenetic mechanisms have been described as involved in prostate cancer, our aim was to assess whether epigenetic mechanism are involved in regulation of STEAP1 gene expression.

As described in Materials and Methods, the confirmation of cell-cultures mycoplasm-free was carried out by PCR (appendix, Figure A2). After bisulfite modification, we sequenced part of the STEAP1 gene, namely where there are more CpG islands. Through the five pairs of primers, it was possible, in total, to analyze the ratio of methylation of 61 CpG islands (see Figure A3 of appendix). In figure 22 are depicted the schematic representation of each CpG island analyzed.

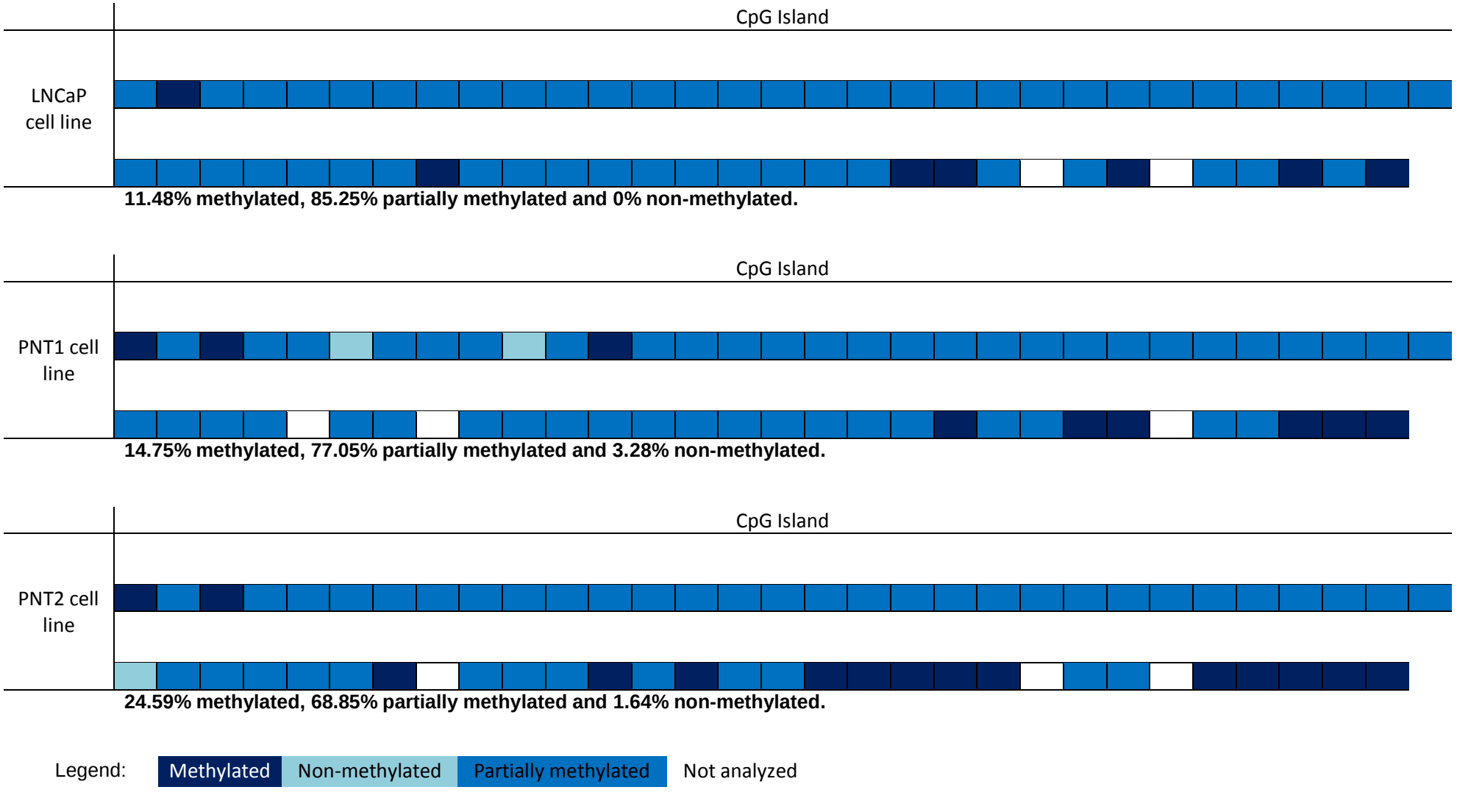

Figure 22: CpG methylation in promoter region of STEAP1 gene. The methylation status of CpG was analyzed in LNCaP, PNT1A and PNT2 cells.

Some primers did not work properly well and for this reason in each cell line, there is always “CG” dinucleotides that we cannot analyze. However, it seems there is no significant difference in DNA methylation of “CpG” island between neoplastic (LNCaP) and non-neoplastic (PNT1A and PNT2) prostate cells. So, the over-expression of STEAP1 mRNA should not be explained by hypomethylation at promoter region of STEAP1 gene. During sequencing, we found that some “CpG” island were not present in the sequence of STEAP1 gene. Probably, this may be due to the existence of the other gene, STEAP1B1. In fact, by overlapping the sequences of STEAP1 and STEAP1B genes (see Figure A4 of Appendix) it was observed that both sequences differ in a few nucleotides and some of them coincide exactly with additional “CG” dinucleotide, which are detected during the STEAP1 sequencing. However, these results should be confirmed through another strategy.

3 - Expression analysis of STEAP1 in human prostate TMAs

3.1 – Clinic and pathological characteristics

Tissue samples from 63 prostate cancer, 41 adjacent normal tissue (ANT), 7 PIN lesions, and 39 BPH were analyzed. The clinical characteristics of patients are illustrated in table 5.

Table 5: Clinic-pathological data of patients from which tissue samples were obtained.

Clinic-pathological features	Prostate cancer	BPH
Patients (n)	63	39
Mean age (years)	64 (41 – 83)	70 (41 – 88)
Total-PSA (ng/mL), mean	8.93 (3.24 – 77.91)	3.73 (0.17 – 15.4)
Gleason (n) %		
<7	(16/63) 25.4%	n.a
≥7	(47/63) 74.6%	n.a
TNM (n) %		
T2a	(3/32) 9.4%	n.a
T2b and T2c	(20/32) 62.5%	n.a
T3	(9/32) 28.1%	n.a
Bone Metastasis (n) %		
Absence	(23/25) 92%	n.a
Present	(2/25) 8%	n.a

(The lack of some values is due to the absence of some data in the clinical records of patients. Abbreviations: BPH – Benign Prostatic Hyperplasia, n.a – Not Applicable).

3.2 - TMAs

In order to analyze the STEAP1 expression in neoplastic tissue and non-neoplastic tissues of the prostate, immunohistochemistry method was carried out in TMAs of human prostate. With the aid of TMA block constructs, which correspond to tissues of the prostate tumor (see Figure A5 - A and B in appendix) and BPH (see Figure A5 - C and D in appendix), several slides were analyzed using a specific antibody against STEAP1 protein through of immunohistochemistry technique (see Figures A6, A7 and A8 in appendix).

3.3 - STEAP1 protein expression in prostate tissues

STEAP1 immunoreactivity was classified according to a score scale established for this parameter, which takes into account the intensity of the signal and percentage of stained cells. Representative images of positive cases with low, moderate and high STEAP1 immunoreactivity are shown in figure 23.

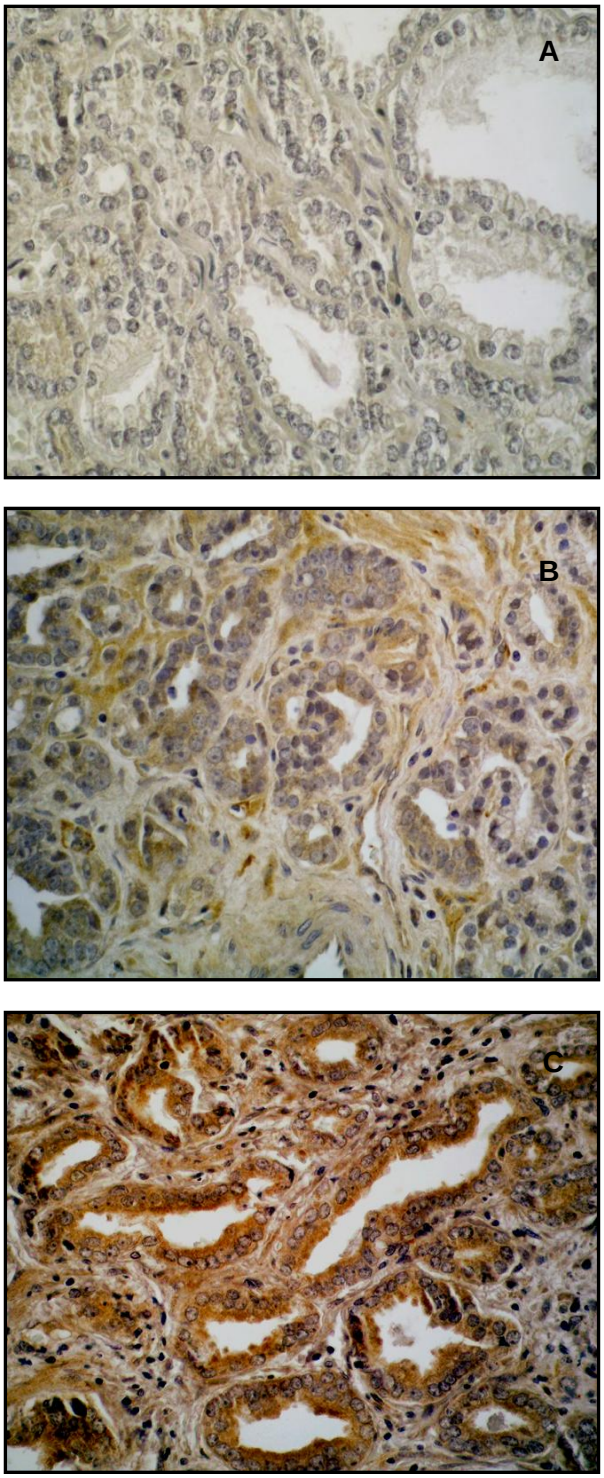

Figure 23 - STEAP1 immunoreactivity in prostate cancer TMAs. Representative images of: A - Low, B - Moderate and C - High STEAP1 immunoreactivity are shown in prostate carcinoma.

In order to evaluate whether STEAP1 immunorecativity differs between histologic diagnosis, several cases were analyzed using immunochemistry method. A representative picture of STEAP1 immunoreactivity in ANT, BPH, PIN lesions, and prostate cancer are showing in figure 24.

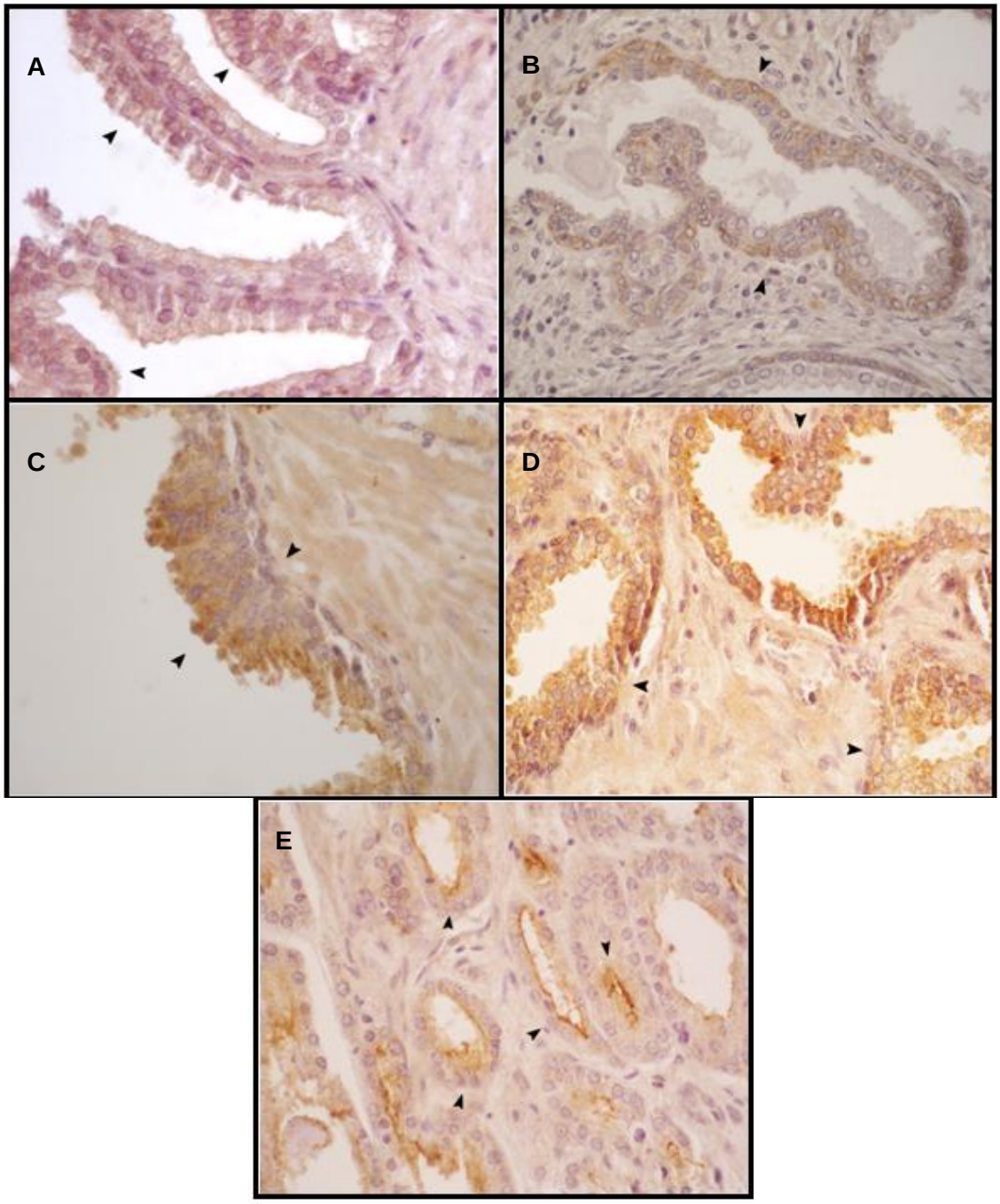

Figure 24 - The immunohistochemical expression of STEAP1 in adjacent normal glands tissue, BPH, PIN and prostate cancer. A - Image that depict the STEAP1 expression in non-neoplastic prostate tissue, (with reasonable staining). B - Image representative of STEAP1 expression in benign hyperplasia prostate tissue, (with a reasonable staining). C - Images representatives of STEAP1 expression in PIN tissue. D and E - Representatives images of STEAP1 expression in prostate tumor tissue, (D – There is a stronger staining in the cytoplasm of cells, E – There is a strong staining in the membrane of cells). These images are observed in an optic microscope, ate a magnification of 40x.

Regarding to the BPH, the results shows that STEAP1 is expressed in basal cells (Figure 24, image B). Taking into account that basal cells may act as reservoir of stem cells and

STEAP1 and STEAP2 are differently expressed in mesenchymal stem cells from bone morrow (Vaghjiani 2009), it is liable to speculate that STEAP1 may be involved in differentiation process of prostate cells. However, more studies are required to clarify the expression and role of STEAP1 in prostate stem cells. Tumor tissues positive for STEAP1 shows a brown staining in cytoplasm (Figure 24, image D) and membrane (Figure 24 image E) of the epithelial cells. These results are consistent with previous studies, which indicate that STEAP1 is localized in plasma membrane of epithelial cells of the prostate, especially in cell junctions (Hubert 1999; Challita-Eid 2007). Besides cell membrane, STEAP1 is also uniformly expressed in cytoplasm of epithelial cells. However, we observe that the STEAP1 expression in cell membrane is similar to found in cytoplasm, i.e., there is no statistical difference between cell membrane and cytoplasm (data not shown). Even with low intensity, it is also observed that STEAP1 is expressed in stromal cells.

As described in material and methods, the STEAP1 immunoreativity in prostate cells was grouped into three groups, low, moderate and high. Using the qui-square statistical test (IBM SPSS Statistic 20), it was possible to investigate the differences of STEAP1 immunoreactivity between the different types of histologic diagnosis (Table 6). This analysis showed that STEAP1 is over-expressed in high percentage of tumor tissues and PIN lesions when compared to BPH or ANT. These results are in agreement with previously described by Li and colleagues (Li 2004). However they only evaluated the STEAP1 expression in prostate cancer and BPH, and their results are not clearly understood.

Table 6: Distribution of STEAP1 immunoreactivity in ANT, BPH, PIN lesions, and prostate cancer**.**

	STEAP1 immunoreactivity			
Histologic diagnosis	**Negative or low**	**Moderate**	**High**	**P (Chi-square)***
Prostate cancer	11.11% (7/63)	36.51% (23/63)	52.38% (33/63)	----------
PIN	0% (0/7)	14.29% (1/7)	85.71% (6/7)	0.236
BPH	97.44% (38/39)	2.56 % (1/39)	0% (0/39)	<0.0001
ANT	73.17% (30/41)	19.51% (8/41)	7.32% (3/41)	<0.0001

* *p value* corresponds to comparisons between prostate cancer and each histologic diagnosis.

Of our knowledge, this is the first report showing that STEAP1 is over-expressed in PIN lesions. Comparing the STEAP1 immunoreactivity between tumors and PIN lesions, no statistical differences were obtained, suggesting that the deregulation of STEAP1 expression may occur even before the development of cancer.

3.4 - Relationship between STEAP1 immunoreactivity and the clinic-pathological data

We further assessed the association between STEAP1 expression and clinic-pathological data of the patients, such as age, total-PSA and free-PSA value, Gleason score, stage and presence of bone metastases (Table 7).

Table 7: Correlations between STEAP1 expression in prostate tissues samples and clinico-pathological data**.**

		STEAP1 immunoreactivity			
Variable	**(n)**	**Negative or low**	**Moderate**	**High**	**P (Chi-square)**
Age					
<55 years	63	0 % (0/9)	11.11% (1/9)	88.89% (8/9)	0.076
55 to 65 years		9.09% (3/33)	48.48% (16/33)	42.42% (14/33)	
>65 years		19.05 % (4/21)	28.57 % (6/21)	52.38% (11/21)	
Total-PSA					
< 4 ng/ml	36	16.67% (1/6)	16.67% (1/6)	66.67 % (4/6)	0.892
4 to 10 ng/ml		16% (4/25)	36% (9/25)	48% (12/25)	
>10 ng/ml		20% (1/5)	40% (2/5)	40% (2/5)	
Free-PSA					
< 10%	27	10% (1/10)	30% (3/10)	60% (6/10)	0.490
10 to 15%		0% (0/8)	37.5% (3/8)	62.5% (5/8)	
15 to 20%		25% (1/4)	50% (2/4)	25% (1/4)	
20 to 25%		40% (2/5)	20% (1/5)	40% (2/5)	
Gleason					
< 7	63	31.25% (5/16)	43.75% (7/16)	25% (4/16)	**0.004**
≥ 7		4.26% (2/47)	34.04% (16/47)	61.7% (29/47)	
TNM					
T2a	32	33.33% (1/3)	33.33% (1/3)	33.33% (1/3)	0.372
T2b and T2c		5% (1/20)	55% (10/20)	45% (9/20)	
T3		11.11% (1/9)	22.22% (2/9)	66.67% (6/9)	
Bone metastasis					
Absence	25	8.70% (2/23)	43.48% (10/23)	47.83% (11/23)	0.908
Present		0% (0/2)	50% (1/2)	50% (1/2)	

Most patients are between 55 and 65 years old. Patients under 55 years old have mainly a high expression of STEAP1, patients between 55 and 65 years are moderate expression and over 65 years old have a high expression of STEAP1 (p=0.076). Comparing the values of PSA with the STEAP1 expression, was observed that most patients presented a PSA value between 4 and 10 ng/ml, and all PSA levels are correlation with high expression of STEAP1 (p=0.892). Most of the patients with Gleason scores below 7 have a moderate expression of STEAP1, whereas patients with Gleason scores equal or superior to 7 present mostly a high STEAP1 expression (p=0.004). As for the stage, most patients have a stage T2b and T2c with a moderate STEAP1 expression while patients with a stage T3a have a high expression (p=0.372). The largest part patients has no bone metastases, which a largely relates to a moderate or high expression of STEAP1 (p=0.908).

Looking at the outcome of the ratios obtained with chi-square test and the significance (p value) that we obtained, we could affirm that the levels of STEAP1 are associated with “Gleason score”. However, it was not observed an association between the variable “STEAP1 expression” and the variables “age”, “total-PSA”, “free PSA value”, “staging” and “bone metastases”. The size of some samples and the fact that not all patients analyzed have all information available and described, may be on the basis of the lack of association between certain variables. So, this was considered a limitation of this study.

CONCLUSION

In this study, we can conclude that STEAP1 is over-expressed in neoplastic cells lines when compared to non-neoplastic prostate cells, being highly expressed in LNCaP cells.

Although the regulation of STEAP1 does not seem to involve epigenetic mechanisms, it is necessary to use other approach to confirm it.

STEAP1 is over-expressed in human prostate cancer cases and PIN lesions when compared ANT or BPH. STEAP1 expression in prostate cancer seems to correlates positively with Gleason score, but not with age, total-PSA, free-PSA, TNM and bone metastasis.

FUTURE PERSPECTIVES

To reach all the aims of this thesis is still needed to complete some gaps. Concerning to the STEAP1 regulation by epigenetic mechanisms, it is required to use another strategy to evaluate whether there are differences in methylation pattern between LNCaP and non-neoplastic prostate cells. For example, the PCR products could be cloned into expression vector and transformed with competent cells. Next, plasmid vector should be extracted from several colonies and sequenced in order to estimate the methylation pattern of STEAP1 gene promoter region. This strategy has been used by several authors to determine the methylation degree in promoter region of genes (Suh 2002; Liu 2010).

Besides the involvement of epigenetic mechanisms in regulation of STEAP1 gene, it would be important to explore other possible mechanisms that could be involved in its over-expression in prostate cancer, such as to evaluate the stability of STEAP1 mRNA and protein between neoplastic and non-neoplastic prostate cells.

Regarding the expression of STEAP1 protein in prostate cancer tissues, would be important to increase the number of samples in order to better clarify the clinical significance of STEAP1 expression in prostate cancer. In addition, it would be interesting to develop studies in order to analyze the impact of STEAP1 protein expression in patient's outcome.

REFERENCES

Abrahamsson, P. A., Lilja, H., Oesterling, J.E. (1997). "Molecular forms of serum prostate-specific antigen. The clinical value of percent free prostate-specific antigen." Urol Clin North Am 24(2): 353–365.

Alves, P. M., Faure, O., Graff-Dubois, S., Cornet, S., Bolonakis, I., Gross, D.A., Miconnet, I., Chouaib, S., Fizari, K., Soria, J.C., Lemonnier, F.A., Kosmatopoulos, K. (2005). "STEAP, a prostate tumor antigen, is a target of human $CD8^+$ T cells." Cancer Immunol Immunother 55(12): 1515-1523.

Andriole, G. L., Crawford, E.D., Grubb Rl, 3rd., Buys, S.S., Chia, D., Church, T.R., Fouad, M.N., Gelmann, E.P., Kvale, P.A., Reding, D.J., Weissfeld, J.L., Yokochi, L.A., O'brien, B., Clapp, J.D., Rathmell, J.M., Riley, T.L., Hayes, R.B., Kramer, B.S., Izmirlian, G., Miller, A.B., Pinsky, P.F., Prorok, P.C., Gohagan, J.K., Berg, C.D (2009). "Mortality results from a randomized prostate-cancer screening trial." N Engl J Med 360(13): 1310–1319.

Atkinson AJJr, C. W., DeGruttola, V.G., DeMets, D.L., Colburn, W.A., Downing, G.J., Hoth, D.F., Oates, J.A., Peck, A.C., Schooley, R.T, Spilker, B.A., Woodcock, J., Zeger, S.L. (2001). "Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework". Biomarkers Definitions Working Group. Clin Pharmacol Ther 69(3): 89–95.

Attard, G., Clark, J., Ambroisine, L., Fisher, G., Kovacs, G., Flohr, P., Berney, D., Foster, C.S, Fletcher, A., Gerald, W.L., Moller, H., Reuter, V., De Bono, J.S., Scardino, P., Cuzick, J., Cooper, C.S. (2008). "Duplication of the fusion of TMPRSS2 to ERG sequences identifies fatal human prostate cancer." Oncogene 27(3): 253-263.

Baltaci, S., Orhan, D., Ozer, G., Tolunay, O., Gögõüs, O. (2000). "Bcl-2 proto-oncogene expression in low- and high-grade prostatic intraepithelial neoplasia." BJU Int 85(1): 155-159.

Bostwick, D. G. (1996). "Prospective origins of prostate carcinoma. Prostatic intraepithelial neoplasia and atypical adenomatous hyperplasia." Cancer 78(2): 330-336.

Bostwick, D. G., Amin, M.B., Dundore, P., Marsh, W., Schultz, D.S. (1993). "Architectural patterns of high-grade prostatic intraepithelial neoplasia. " Hum Pathol 24(3): 298-310.

Bostwick, D. G. and Brawer, M.K. (1987). "Prostatic intra-epithelial neoplasia and early invasion in prostate cancer." Cancer 59(4): 788-794.

Bostwick, D. G. and Qian, J. (2004). "High-grade prostatic intraepithelial neoplasia." Mod Pathol 17(3): 360-379.

Bova, G. S., Beaty, T.H., Steinberg, G.D., et al (1993). "Hereditary prostate cancer: epidemiologic and clinical features." J Urol 150: 797–802.

Boyle, P., Severy, G., Gilles, G.G. (2003). "The epidemiology of prostate cancer." Urol Clin North Am 30(2): 209-217.

Brooks, J. D., Weinstein, M., Lin, X., Sun, Y., Pin, S.S., Bova, G.S., Epstein, J.I., Isaacs, W.B., Nelson, W.G. (1998). "CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia." Cancer Epidemiol Biomarkers Prev 7(6): 531-536.

Byar, D. P. and Mostofi (1972). "Carcinoma of the prostate: prognostic evaluation of certain pathologic features in 208 radical prostatectomies." Cancer 30(1):5-13.

Cancer.Net (2012). "Prostate Cancer - Risk Factors and Prevention." American Society of Clinical Oncology.

Carter, H. B., Pearson, J.D., Metter, E.J., Brant, L.J., Chan, D.W., Andres, R., Fozard, J.L., Walsh, P.C. (1992). "Longitudinal evaluation of prostate-specific antigen levels in men with and without prostate disease." JAMA 267(16): 2215-2220.

Catalona, W. J., Smith, D.S., Ratliff, T.L., Basler, J.W. (1993). "Detection of organ-confined prostate cancer is increased through prostate-specific antigen-based screening." JAMA 270(8): 948-954.

Cerveira, N., Ribeiro, F.R., Peixoto, A., Costa, V., Henrique, R., Jerónimo, C., Teixeira, M.R. (2006). "TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions." Neoplasia 8(10): 826-832.

Challita-Eid, P. M., Morrison, K., Etessami, S., An, Z. , Morrison, K.J., Perez-Villar, J.J., Raitano, A.B., Jia, X.C., Gudas, J.M., Kanner, S.B., Jakobovits, A. (2007). "Monoclonal

antibodies to six-transmembrane epithelial antigen of the prostate-1 inhibit intercellular communication in vitro and growth of human tumor xenografts in vivo." Cancer Res 67(12): 5798-5805.

Chu, T.M. (1997). "Prostate-specific antigen and early detection of prostate cancer." Tumor Biol 18(2): 123-134.

Costello, J. F., Frühwald, M. C., Smiraglia, D. J., Rush, L.J., Robertson, G.P., Gao, X., Wright, F.A., Feramisco, J.D., Peltomäki, P., Lang, J.C., Schuller, D.E., Yu, L., Bloomfield, C.D., Caligiori, M.A., Yates, A., Nishikawa, R., Su Huang, H., Petrelli, N.J., Zhang, X., O'Dorisio, M.S., Held, W.A., Cavenee, W.K., Plass, C. (2000). "Aberrant CpGisland methylation has non-random and tumour-type-specific patterns." Nat Genet 24(2): 132–138.

Das, B., Tsuchida, R., Malkin, D., Koren, G., Baruchel, S., Yeger, H. (2008). "Hypoxia enhances tumor stemness by increasing the invasive and tumorigenic side population fraction." Stem Cells 26(7): 1818–1830.

Das, D., Wojno, K., Imperiale, M.J. (2008). "BK virus as a cofactor in the etiology of prostate cancer in its early stages." J Virol 82(6): 2705-2714.

DeMarzo, A. M., Nelson, W.G., Isaacs, W.B., Epstein, J.I. (2003). "Pathological and molecular aspects of prostate cancer " Lancet 361(9361):955-964.

Dillman, R. O. and Williams, T. (1994). "Value of prostate cancer screening in a comprehensive community cancer centre." Cancer Invest 12(5): 456–462.

Ehrlich, M. (2002). "DNA methylation in cancer: too much, but also too little." Oncogene 21(35): 5400–5413.

Ellis, W. J. and Lange, P.H. (1994). "Prostate cancer." Endocrinol Metab Clin North Am 23(4): 809–824.

Emmert-Buck, M. R., Vocke, C.D., Pozzatti, R.O., Duray, P.H., Jennings, S.B., Florence, C.D., Zhuang, Z., Bostwick, D.G., Liotta, L.A., Linehan, W.M. (1995). "Allelic loss on chromosome 8p12-21 in microdissected prostatic intraepithelial neoplasia." Cancer Res 55(14): 2959-2962.

Epstein, J. I. (1995). "Diagnostic criteria of limited adenocarcinoma of the prostate on needle biopsy." Hum Pathol 26(2):223-229.

Epstein, J. I. (2000). "Gleason score 2-4 adenocarcinoma of the prostate on needly biopsy: a diagnosis that should not be made." Am J Surg Pathol 24(4): 477-478.

Epstein, J. I. and Netto, G.J. (2002). Biopsy Interpretation of the Prostate. Four edition. Philadelphia, Lippincott Williams & Wilkins.

Epstein, J. I. (2011). "Update on the gleason grading system." Ann Pathol 31(5 Suppl): S20-S26.

Epstein, J. I., Walsh, P.C., Carmichael, M., Brendler, C.B. (1994). "Pathological and clinical findings to predict tumor extent of non-palpable (stage T1c) prostate cancer." JAMA 271(5):368-374.

Epstein, J. I. and Potter, S.R. (2001). "The pathologic interpretation and significance of prostate biopsy findings: implications and current controversies." J Urol 166(2):402-410.

Evren, S., Dermen, A., Lockwood, G., Fleshner, N., Sweet, J. (2011). "mTOR-RAPTOR and 14-3-3sigma immunohistochemical expression in high grade prostatic intraepithelial neoplasia and prostatic adenocarcinomas: a tissue microarray study." J Clin Pathol 64(8): 683-688.

Facio, F., Kashiwabuschi, R., Nishi, Yutaro., Leao, R., McDonnell, P., Burnett, A. (2010). "Benign Prostatic Hyperplasia. Clinical Treatment Can Complicate Cataract Surgery." Int Braz J Urol 36(5): 563-570.

Feinberg, A. P. and Vogelstein, B. (1983). "Hypomethylation distinguishes genes of some human cancers from their normal counterparts." Nature 301(5895): 89–92.

Ferlay, J., Parkin, D.M., Steliarova-Foucher, E. (2010). " Estimates of cancer incidence and mortality in Europe in 2008". European Journal of Cancer 46: 765-781.

Foster, C. S. (2000). Pathology of benign prostatic hyperylasia. Prostate Suppl 9:4-14.

Frigola, J., Song, J., Stirzaker, C., Hinshelwood, R.A., Peinado, M.A., Clark, S.J. (2006). "Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band." Nat Genet 38(5): 540–549.

Garcia-Hernandez, M. L., Gray, A., Hubby. B., Kast, W.M. (2007). "In vivo Effects of Vaccination with Six-Transmembrane Epithelial Antigen of the Prostate: A Candidate Antigen for Treating Prostate Cancer." Cancer Res 67(3): 1344-1351.
Giltane, J. M. and. Rimm, D.L. (2004). "Technology Insight: identification of biomarkers with tissue microarray technology." Nat Clin Pract Oncol 1(2): 104-111.

Gleason, D. F. (1966). "Classification of prostate carcinomas." Cancer Chemother Rep 50: 125-128.

Gleason, D. F. and Mellinger, G.T. (1974). "Prediction of prognosis for prostatic adenocarcinoma by combined histologic grading and clinical staging." J Urol 111(1):58-64.

Goelz, S. E., Vogelstein, B., Hamilton, S. R., Feinberg, A.P. (1985). "Hypomethylation of DNA from benign and malignant human colon neoplasms." Science 228(4696): 187–190.

Gomes, I.M., Maia, C.J., Santos, C.R. (2012). "STEAP proteins: from structure to applications in cancer therapy." Mol Cancer Res 10(5): 573-587.

Gomes, I.M., Santos, C., Socorro, S., Maia, C.J. Six transmembrane epithelial of the prostate 1 is down-regulated by sex hormones in prostate cells. The Prostate (In press).

Gopalan, A., Leversha, M.A., Satagopan, J.M., Zhou, Q., Al-Ahmadie, H.A., Fine, S.W., Eastham, J.A., Scardino, P.T., Scher, H.I., Tickoo, S.K., Reuter, V.E., Gerald, W.L. (2009). "TMPRSS2-ERG gene fusion is not associated with outcome in patients treated by prostatectomy." Cancer Res 69(4): 1400-1406.

Grönberg, H. (2003). Prostate cancer epidemiology. Lancet. 361(9360): 859-864.

Grunewald, T., Diebold, I., Esposito, I. Plehm, S., Hauer, K., Thiel, U. da Silva-Bittkus, P., Neff, F., Unland, R., Müller-Tidow, C., Zobywalski, C., Lohring, K., Lewandrowski, U., Sickmann, A., Prazeres da Costa, O., Görlach, A., Cossarizza, A., Butt, E., Richter, G.H., Burdach, S. (2011). "STEAP1 Is Associated with the Invasive and Oxidative Stress Phenotype of Ewing Tumors." Mol Cancer Res 10(1): 52-65.

Haas, G. P. (1994). "Epidemiology of early prostate cancer (introduction)." In vivo 8(3): 403-405.

Häggman, M. J., Macoska, J.A., Wojno, K.J., Oesterling, J.E. (1997). "The relationship between prostatic intraepithelial neoplasia and prostate cancer: critical issues." J Urol 158(1): 12-22.

Harris, R. P., Lohr, K.N., Beck, R., Fink, K., Godley, P., Bunton, A. (2001). "Screening for Prostate Cancer [Internet]." (Prepared by the Research Triangle Institute-University of North Carolina Evidence-based Practice Center under Contract no. 290-97-0011). Rockville, MD: Agency for Healthcare Research and Quality 16.

Hessels, D., Klein Gunnewiek, J.M., Van Oort, I., Karthaus, H.F., Van Leenders, G.J., Van Balken, B., Kiemeney, L.A., Witjes, J.A. Schalken, J.A. (2003). "D3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer." Eur Urol 44(1): 8–15.

Hill, R. P., Marie-Egyptienne, D.T., Hedley, D.W. (2009). "Cancer stem cells, hypoxia and metastasis, ." Semin Radiat Oncol 19(2): 106–111.

Hoffman, R. M., Clanon, D.L., Littenberg, B., Frank, J.J., Peirce, J.C. (2000). "Using the free-to-total prostate-specific antigen ratio to detect prostate cancer in men with nonspecific elevations of prostate-specific antigen levels." J Gen Intern Med. 15(10): 739–748.

Hubert, R. S., Vivanco, I., Chen, E., Rastegar, S., Leong, K. , Mitchell, S.C., Zhou, Y., Kuo, J., Raitano, A.B., Jakobovits, A., Saffran, D.C., Affar, D.E. (1999). "STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumours." Proc. Natl. Acad. Sci. USA 96(25): 14523-14528.

Humphrey, P. A., Dehner, L.P., Pfeifer, J.D. (2008). Washington Manual of Surgical Pathology Reproductive tract - Prostate, Lippincott Williams & Wilkins. 1st edition.

Jones, G. W. (1993). "Prostate cancer. Magnitude of the problem: American College of Surgeons Database and Epidemiologic Study." Cancer 71(3 Suppl): 887-889.

Jones, P. A. and Baylin, S. B. (2002). "The fundamental role of epigenetic events in cancer." Nat Rev Genet 3(6): 415–428.

Jones, P. A. and Laird, P.W. (1999). "Cancer epigenetics comes of age." Nat. Genet. 21(2): 163-167.

Jussi, P. E. and Visakorpi, T. (2001). "Molecular genetics of prostate cancer." Ann Med 33(2):130-141.

Klatt, E. C. (2006). Robbins and Cotran ATLAS OF PATHOLOGY. The male genital tract. S. Elsevier. Second edition.

Kobayashi, H., Nagato, T., Sato, K., Aoki, N., Kimura, S., Murakami, M., Iizuka, H., Azumi, M., Kakizaki, H., Tateno, M., Celis, E. (2007). "Recognition of prostate and melanoma tumor cells by six-transmembrane epithelial antigen of prostate-specific helper T lymphocytes in a human leukocyte antigen class II-restricted manner." Cancer Res. 67(11): 5498-5504.

Koeneman, K. S., Pan, C.X., Jin, J.K., Pyle, J.M. 3rd, Flanigan, R.C., Shankey, T.V., Diaz, M.O. (1998). "Telomerase activity, telomere length, and DNA ploidy in prostatic intraepithelial neoplasia (PIN)." J Urol 160(4): 1533-1539.

Kohli, M. and Tindall, D.J. (2010). "New developments in the medical management of prostate cancer." Mayo Clin Proc 85(1): 77-86.

Koshlji, M., Kageyama, Y., Pete, E.A., Horikawa, I., Barrett, J.C., Huang, L.E. (2004). "HIF-1αinduces cell cycle arrest by functionally counteracting Myc." EMBO J 23(9): 1949-1956.

Kumar, V., Abbas, A.K., Fausto, N. (2005). Pathologic Basis of Disease 7th edition, chapter 21: 1047-1056.

Labrie, F., DuPont, A., Suburu, R., Cusan, L., Tremblay, M., Gomez, J.L., Emond, J. (1992). "Serum prostate specific antigen as pre-screening test for prostate cancer." J Urol 147(3 Pt 2): 846–851.

Lagakos, S. W. and Hoth, D.F. (1992). "Surrogate markers in AIDS: where are we? Where are we going?" Ann Intern Med 116(7): 599–601.

Laird, P. W. (2005). "Cancer epigenetics." Hum Mol Genet 14 Spec No 1: 65–76.

Li, F. P. (1993). "Molecular epidemiology studies of cancer in families." BR J Cancer 68(2): 217-219.

Li, L., Li, J., Shen, Z., Liu, W., Chen, Z. (2004). "Clinical significance of six-transmembrane epithelial antigen of the prostate expressed in prostatic carcinoma." Zhonghua Nan Ke Xue 10(5): 351-354.

Liu, M., Peng, Y., Wang, X., Guo, Q., Shen, S., Li, G. (2010). "NGX6 gene mediated by promoter methylation as a potential molecular marker in colorectal cancer." BMC Cancer 10(160): 1-10.

Ludwig JA, W. J. (2005). "Biomarkers in cancer staging, prognosis and treatment selection." Nat Rev Cancer 5(11): 845-856.

Madu, C. O. and Lu, Y. (2010). "Novel diagnostic biomarkers for prostate cancer." J Cancer 1: 150-177.

Maherali, N., Shridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K., Hochedlinger, K. (2007). "Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution." Cell Stem Cell 1(1): 55-70.

Maia, C. J., Socorro, S., Schmitt, F., Santos, C.R. (2008). "STEAP1 is over-expressed in breast cancer and down-regulated by 17beta-estradiol in MCF-7 cells and in the rat mammary gland." Endocr 34(1-3): 108-116.

Marchand, L. L., Kolonel, L.N., Wilkens, L.R., Myers, B.C., Hirohata, T. (1994). "Animal fat consumption and prostate cancer: a prospective study in Hawaii." Epidemiology 5(3): 276-282.

Matzkin, H., Patel, J.P., Altwein, J.E. Soloway, M.S. (1994). "Stage T1a carcinoma of the prostate." Urology 43(1): 11-21.

McConnell, J. D., Roehrborn, C.G, Bautista, O.M. Andriole, G.L. Jr, Dixon, C.M., Kusek, J.W., Lepor, H., McVary, K.T., Nyberg, L.M. Jr, Clarke, H.S., Crawford, E.D., Diokno, A., Foley, J.P., Foster, H.E., Jacobs, S.C., Kaplan, S.A., Kreder, K.L., Lieber, M.M., Lucia, M.S., Miller, G.J., Menon, M., Miliam, D.F., Ramsdell, J.W., Schenkman, N.S., Slawin,

K.M., Smith, J.A. (2003). "The long-term effect of Doxawsin, Finasteride, and combination therapy on clinical progression of benign prostatic hyperplasia." N Engl J Med 349(25): 2387-2398.

McNeal, I. T., Villers, A.A., Redwine, E.A. Freiha, F.S., Stamey, T.A. (1990). "Histologic differentiation, cancer volume, and pelvic lymph node metastasis in adenocarcinoma of the prostate." Cancer 66(6): 1225-1233.

McNeal, J. E. (1969). "Origin and development of carcinoma in the prostate." Cancer 23(1): 24-34.

McNeal, J. E. and Bostwick., D.G. (1986). " Intraductal dysplasia: A premalignant lesion of the prostate." Hum Pathol 17: 64-71.

Messina, M. J., Persky, V., Setchell, K.D., Barnes, S. (1994). "Soy intake and cancer risk: a review of the in vitro and in vivo data." Nutr Cancer 21(2): 113-131.

Mettlin, C., Lee, F., Drago, J., Murphy, G.P. (1991). "The American Cancer Society National Prostate Cancer Detection Project. Findings on the detection of early prostate cancer in 2425 men." Cancer 67(12): 2949-2958.

Mettlin, C., Littrup, P.J., Kane, R.A., Murphy, G.P., Lee, F., Chesley, A., Badalament, R., Mostofi, F.K. (1994). "Relative sensitivity and specificity of serum prostate specific antigen (PSA) level compared with agereferenced PSA, PSA density, and PSA change." Cancer. 74(5): 1615–1620.

Miet, S. M., Neyra, M., Jaques, R., Dubernard, P., Revol, A.A., Marçais, C. (1999). "RER(+) phenotype in prostate intra-epithelial neoplasia associated with human prostate-carcinoma development." Int J Cancer 82(5): 635-639.

Montironi, R., Mazzucchelli, R., Lopez-Beltran, A., Cheng, L., Scarpelli, M. (2007). "Mechanisms of disease: high-grade prostatic intraepithelial neoplasia and other proposed preneoplastic lesions in the prostate." Nat Clin Pract Urol 4(6): 321-332.

Moul, J. W., Sesterhenn, I.A., Connelly, R.R., Douglas, T., Srivastava, S., Mostofi, F.K., McLeod, D.G. (1995). "Prostate-specific antigen values at the time of prostate cancer diagnosis in African-American men." JAMA 274(16): 1277-1281.

Narod, S. A., Dupont, A., Cusan, L., Diamond, P. (1995). "The impact of family history on early detection of prostate cancer." Nat Med 1(2): 99-101.

Nelson, W. G., DeMarzo, A.M., Isaccs, W.B (2003). "Prostate cancer." N Engl J Med 349: 366-381.

Oh, W. K., Hurwitz, M., D'Amico, A.V., Richie, J.P., Kantoff, P.W. (2003). "Neoplasms of the Prostate." Cancer Medicine. 6th Edition. Chapter 108.

Ohgami, R. S., Campagna, D.R., McDonald, A., Fleming, M.D. (2006). "The STEAP proteins are metalloreductases." Blood 108(4): 1388-1394.

Partin, A. W., Yoo, J., Carter, H.B., Pearson, J.D., Chan, D.W., Epstein, J.I., Walsh, P.C. (1993). "The use of prostate specific antigen, clinical stage and Gleason score to predict pathological stage in men with localized prostate cancer." J Urol 150(1): 110-114.

Perner, S., Demichelis, F., Beroukhim, R., Schmidt, F.H., Mosquera, J.M., Setlur, S., Tchinda, J., Tomlins, S.A., Hofer, M.D., Pienta, K.G., Kuefer, R., Vessella, R., Sun, X.W., Meyerson, M., Lee, C., Sellers, W.R., Chinnaiyan, A.M., Rubin, M.A. (2006). "TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer." Cancer Res 66(17): 8337-8341.

Perner, S., Mosquera, J.M., Demichelis, F., Hofer, M.D., Paris, P.L., Simko, J., Collins, C., Bismar, T.A., Chinnaiyan, A.M., De Marzo, A.M., Rubin, M.A. (2007). "TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion." Am J Surg Pathol 31(6): 882-888.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res 29(9): e45.

Pienta, K. J. and Esper, P.S. (1993). "Risk factors for prostate cancer." Ann Intern Med 118(10): 793-803.

Polascik, T. J., Oesterling, J.E., Partin, A.W. (1999). "Prostate specific antigen. A decade of discovery-what we have learned and where we are going." J Urol 162(2): 293-306.

Potter, S. R., Epstein, J.I., Partin, A.W. (2000). "Seminal vesicle invasion by prostate cancer: prognostic significance and therapeutic implications." Rev Urol 2(3):190-195.

Qian, J., Bostwick, D.G., Takahashi, S., Borell, T.J., Herath, J.F., Lieber, M.M., Jenkins, R.B. (1995). "Chromosomal anomalies in prostatic intraepithelial neoplasia and carcinoma detected by fluorescence in situ hybridization." Cancer Res 55(22): 5408-5414.

Ramsey, E. W. (2000). "Benign prostatic hyperylasia: a review." Can J Urol 7(6): 1135-1143.

Rao, A. R., Motiwala, H.G., Karim, O.M. (2008). "The discovery of prostate-specific antigen." BJU Int 101(1): 5-10.

Rhodes, D. R. Sanda, M.G., Otte, A.P. Chinnaiyan, A.M., Rubin, M.A. (2003). "Multiplex biomarker approach for determining risk of prostate-specific antigen-defined recurrence of prostate cancer." J Natl Cancer Inst 95(9):661-8.

Rolan, P. (1997). "The contribution of clinical pharmacology surrogates and models to drug development: a critical appraisal." Br J Clin Pharmacol 44(3): 219–225.

Rubin, M. A., Zhou, M., Dhanasekaran, S.M. Varambally, S., Barrette, T.R., Sanda, M.G., Pienta, K.J., Ghosh, D., Chinnaiyan, A.M. (2002). "a-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer." JAMA 287(13): 1662-1670.

Saitoh, H., Hida, M., Shimbo, T. Nakamura, K., Yamagata, J., Satoh, T. (1984). "Metastatic patterns of prostatic cancer: correlation between sites and number of organs involved." Cancer 54(12):3078-3084.

Sakr, W. A., Grignon, D.J., Haas, G.P., Heilbrun, L.K., Pontes, J.E., Crissman, J.D. (1996). "Age and racial distribution of prostatic intraepithelial neoplasia." Eur Urol 30(2): 138-144.

Santos C, Socorro S, Maia C. STEAP1 (Six Transmembrane Epithelial Antigene of the Prostate 1). Atlas Genet Cytogenet Oncol Haematol. 2010; 14(5):482-484.

Sawyers, C. L. (2008). "The cancer biomarker problem." Nature 452(7187): 548-552.

Schröder, F. H., Hugosson, J., Roobol, M.J., Tammela, T.L., Ciatto, S., Nelen, V., Kwiatkowski, M., Lujan, M., Lilja, H., Zappa, M., Denis, L.J., Recker, F., Berenguer, A., Määttänen, L., Bangma, C.H., Aus, G., Villers, A., Rebillard, X., Van Der Kwast, T., Blijenberg, B.G., Moss, S.M., De Koning, H.J., Auvinen, A. (2009). "Screening and prostate-cancer mortality in a randomized European study." N Engl J Med 360(13): 1320–1328.

Seeley, R. R., Stephens, T.D. and Tate, P. (2004). Anatomy and physiology. "Reproductive System". sixth edition, chapter 28: 1017-1031.

Selley, S., Donovan, J., Faulkner, A., Coast, J., Gillatt, D. (1997). "Diagnosis, management and screening of early localised prostate cancer." Health Technol Assess 1(2): 3 - 9.

Sobin, L., Gospodarowicz, M. and Wittekind, C. (2009). TNM Classification of Malignant Tumours. Seventy Edition.

Stamey, T. A., Dietrick, D.D., Issa, M.M. (1993). "Large, organ confined, impalpable transition zone prostate cancer. Association with metastatic levels of prostate specific antigen." J Urol 149(3): 510-515.

Stanford, J. L., Stephenson, R.A., Coyle, L.M., Cerhan, J., Correa, R., Eley, J.W., Gilliand, F., Hankey, B., Kolonel, L.N., Kosary, C., Ross, R., Severson, R., West, D. (1999). "Prostate Cancer Trends " National Cancer Institute NIH Pub No 99-4543.

Steinberg, D. M., Sauvageot, J., Piantadosi, S., Epstein, J.I. (1997). "Correlation of the prostate needly biopsy and radical prostectomy Gleason grade in academic and community setting." Am J Surg Pathol 21(5): 566-576.

Stenman, U. H., Hakama, M., Knekt, P., Aromaa, A., Teppo, L., Leinonen, J. (1994). "Serum concentrations of prostate specific antigen and its complex with alpha 1-antichymotrypsin before diagnosis of prostate cancer." Lancet. 344(8937): 1594–1598.

Suh, E. R., Ha, C.S., Rankin, E.B., Toyota, M., Traber, P.G (2002). "DNA Methylation Down-regulates CDX1 Gene Expression in Colorectal Cancer Cell Lines." Journal of Biological Chemistry 277(39): 35795-35800.

Stransky, N., Vallot, C., Reyal, F., Bernard-Pierrot, I., de Medina, S.G., Segraves, R., de Rycke, Y., Elvin, P., Cassidy, A., Spraggon, C., Graham, A., Southgate, J., Asselain, B., Allory, Y., Abbou, C.C., Albertson, D.G., Thiery, J.P., Chopin, D.K., Pinkel, D., Radvanyi, F. (2006). "Regional copy number-independent deregulation of transcription in cancer." Nat Genet 38(12): 1386–1396.

Swinnen, J. V., Roskams, T., Joniau, S., Van Poppel, H., Oyen, R., Baert, L., Heyns, W., Verhoeven, G. (2002). "Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer." Int J Cancer 98(1): 19-22.

Tamura, T. and Chiba, J. (2009). "STEAP4 regulates focal adhesion kinase activation and CpG motifs within STEAP4 promoter region are frequently methylated in Du145, human androgen-independent prostate cancer cells." Int J Mol Med 54: 599-604.

Tomlins, S. A., Rhodes, D.R., Perner, S., Dhanasekaran, S.M., Mehra, R., Sun, X.W., Varambally, S. Cao, X., Tchinda, J., Kuefer, R., Lee, C., Montie, J.E., Shah, R.B., Pienta, K.J., Rubin, M.A., Chinnaiyan, A.M. (2005). "Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer." Science 310(5748): 644-648.

Tomuleasa, C., Kacsó, G., Soriţău, O., Şuşman, S., Irimie, A., Virág, P. (2010). "Cellular interactions in prostate cancer genesis and dissemination. Looking beyond the obvious." Rom J Morphol Embryol 51(3): 427–435.

Tost, J. (2008). "Epigenetics." Horizon Scientific Press, Norwich, UK.

Tost, J. (2009). DNA Methylation: Methods and Protocols. DNA Methylation: An Introduction to the Biology and the Disease-Associated Changes of a Promising Biomarker. H. Press. 507.

Urisman, A., Molinaro, R.J., Fischer, N., Plummer, S.J., Casey, G., Klein, E.A., Malathi, K., Magi-Galluzzi, C., Tubbs, R.R., Ganem, D., Silverman, R.H., Derisi, J.L. (2006). "Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant." PloS Pathog 2(3): e25.

Vaghjiani, R.J., Talma, S., Murphy, C.L. (2009). "Six-Transmembrane Epithelial Antigen of the Prostate (STEAP1 and STEAP2) - Differentially expressed by Murine and Human Mesenchymal Stem Cells." Tissue Enginnering 15(00): 1-11.

Valenti, M. T., Dalle Carbonare, L., Donatelli, L., Bertoldo, F., Giovanazzi, B., Caliari, F.,Lo Cascio, V. (2008). "STEAP mRNA detection in serum of patients with solid tumours." Cancer Letters doi:10.1016/j.canlet.2008.07.037.

Willman, J. H. and Holden, J.A. (2000). "Immunohistochemical staining for DNA topoisomerase II-alpha in benign, premalignant, and malignant lesions of the prostate." Prostate 42(4): 280-286.

Wilt, T. J. (2003). "Prostate Cancer: Epidemiology and Screening." Rev Urol 5 (Suppl6): S3-S9.

Wong, Y. C. and Wang, Y.Z. (2000). "Growth factors and epithelial-stromal interactions in prostate cancer development." Int Rev Cytol 199: 65-116.

Yasunaga, Y., Shin, M., Fujita, M.Q., Nonomura, N., Miki, T., Okuyama, A., Aozasa, K. (1998). "Different patterns of p53 mutations in prostatic intraepithelial neoplasia and concurrent carcinoma: analysis of microdissected specimens." Lab Invest 78(10): 1275-1279.

Yip, I., Heber, D., Aronson, W. (1999). "Nutrition and prostate cancer." Urol Clin North Am, 26(2): 403-411.

Young, B., Lowe, J.S., Stevens, A., et al. (2007). Wheater's Functional Histology - A text and colour atlas. Male reproductive system Elsevier. 5th edition

APPENDIX

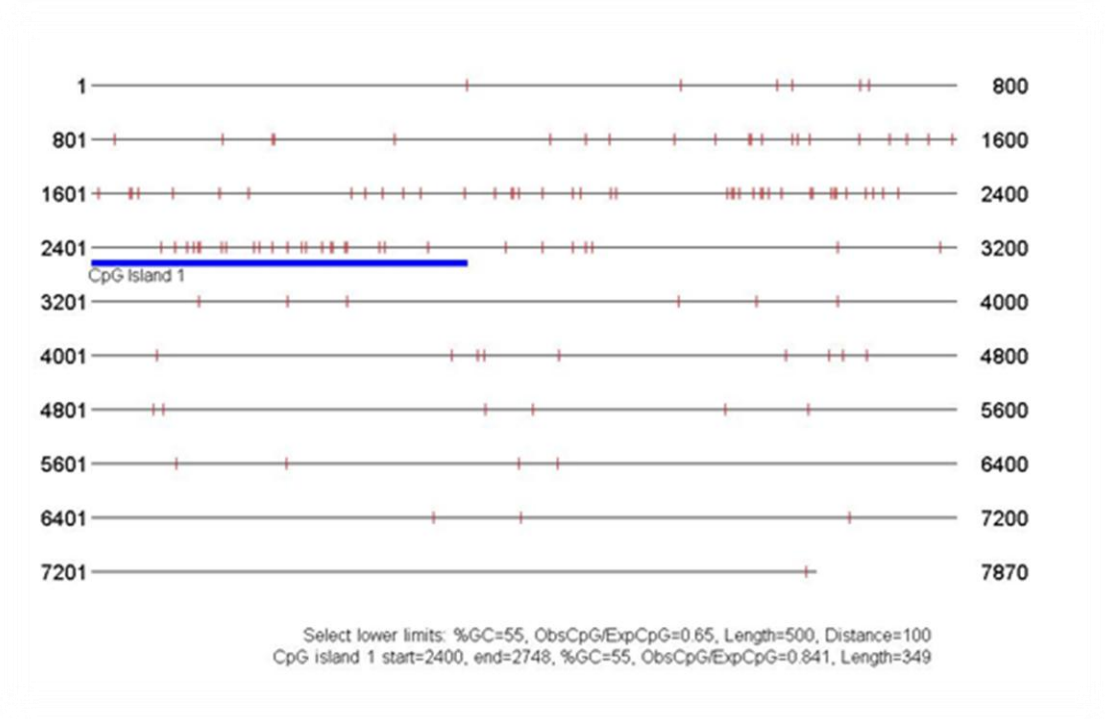

Figure A1: Location of region rich in CpG islands, in STEAP1 gene.

In order to ensure that cell lines culture was mycoplasm-free PCR reactions were carried out.

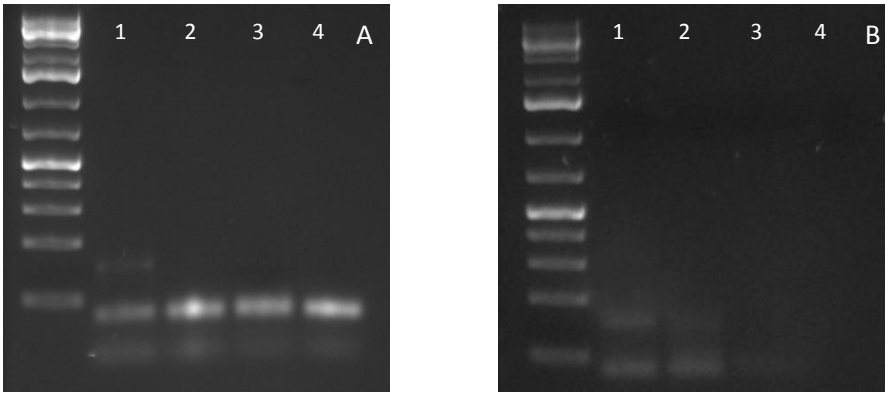

Figure A2: Running of the amplified nested-PCR products, in agarose gel. A – First PCR, B – Second PCR. In both, 1 – LNCaP, 2 – PNT1A, 3 –PNT2 and 4 negative control.

TTTTTATAAGAAGTGTAGAAATAAAATTGTTAAAGTTTAATTATAAAATAAAAATATTTGGGGTTTGATTTTA
AATGTTTTAATTAGAAAAGGAAAAGTAGTTTTAAGTGTAGTTTGTTATATAGTTATATTTATATGAATATATA
TTATTATGTTAATATAATAATAATTTTTTGTTTTTTTTTATATGGAAAATAGATTGTGAAAAATAGAATGATTT
TTGTTTTATTAAATGTTAAATAATAAAAGTATATCGTTTTGTTTGATTATTTAAGATTTATGATTATTTTTAA
AATTTAATAATGTTTAGTTAAAAAGTTTTAATTTTTTGTAGTTTGTGTTATATATATAAAGATTATATAGATT
TTTTTTTTAAAATTTTTAAATTATTTTTTAGTTTTATTAGAAATAATAGGTGTAAGATTTTATTTTTATTATA
AGGTGTTAGTTTACGGTAATTTAAGTTTTAAAGGGTTTTAAATTAATTTAAGGAAAGTTAGTGATTATAGAAA
ATATTTATATTAAAATATGTTTTGTTAAACGGATGTTTGAAGGCGGTATGTTAGTTAAGAGTTATTATTATTT
TTTAATTTTAAGTATTTAGGGATATAAATATTGCGGAAGGTCGTAGGGTTTTTTGTTTAGGAAAATTAGAGAA
TTTTGTTTATTTGTTTATTTGTTGATTTTTTTTTTATTATTGTTTTATGATTTTGTTAAATTTTTTTTTGCGA
GAAATATTTAAGAATGATTAATAAAAAAAAATAATAATAATTAAAAAAAAAAAAGTAAAAAAAAAAAAAAAAA
AAAAAAAAAAATATGAATAGTTTTCGGTGTTTTAGTAGTGAATAAAAAATAATTATGGAAAATGTGGTTGCGC
GTTTATTTTTTTTTGTGTTTTTTATTTAGTTTAGTTTTGAGAATGTTGTATATTAAATTTTTAGAATTTTGTA
ATTTATGTTTTAGATTTTATTTTGAGGTTGGTTTTAACGGAGAGAAGTAAATTTTTTGTTTAAGTTGTTTTTT
TTTAAAATTTTGGAATATTAATAGTTAATTTTATTTTAGGTTTGGAATATTTTATTGAGTTAGGATATGGGAA
GTTGTATTTAAAAGTTAAGGTAAGGAAGGGAGGGACGGAGTAAATATGGGGTTTAAGGGTTTTTTTTTCGGTT
GGGGAGTAGGGTTTGTGCGATTAAAGGTATATAATTTGAATAATTTAGGGAGTAAGTTATTTTTAGAAATTTA
GTTTCGGATTTTTTAAATTGTTAATTATAGTTATTTTAGGATCGGTTGTTAGGTTTTAAATAAGGTGGTTGGG
CGCGGTGGTTTACGTTTGTAATTTTAGTATTTTGGGAGGTCGAGGCGGGTGGATTACGAGGTTAGGAATTTAG
GATTAGTTTGGTTAAGATGGTGAAATTTTCGTTTTTGTTGAAAATATAAAAATTAGTCGGGTATAGTGGTAGG
CGTTTGTAATTTTAGTTATTCGGGAGGTTGAGGTAGGAGAATCGTTTGAATTCGGGGTTGGAGGTTGTAGTGA
GTTAAGATCGCGTTATCGTATTTTAGTTTGGGTAATAGATTGAGATTTCGTTTTAAAAAAAATAAAAATAAAA
TAAAAATAAATAAGTTAACGTGTGGGAGGGTTTGTAAGTTTTAAACGGTTTATTTTAAAGTGTGATTTGGGAA
TGTTTTTATATTTTATGGGAATTTGTTATAAATGAGAATTTTAGATTTTATTTTAGATATATTGAATCGGAAA
TTGTGGGCGGATAGTTTAATAATCGGTGTTTTAATAAGTTTTCGGGTAATTTTATATACGTTAAAGTTTGAGA
ATTATTGTTTTAGAGAAATGTATAAACGTTTTTTAGTGGAGATTAAATAAAAGGCGAGGGGTTGGAGGGCGCG
AAACGGATTTTAGGTGTAGGGTAGACGGTATTTGAGAGGAAATTGGGAGAGGTCGGAGTGCGATTTGGGGAGT
TTTAGTTTTTAAGGACGTTTCGGAATTTTGCGGACGCGGGCGTTAGTAGGTGGCGTTGGACGCGTAACGGAT
AAGGAGGCGGGGTTTGTAGTTGGTTTGGAGGTTTCGCGTTTTGGAGGTTTAGGCGTCGCGTGGGGTTCGTATT
TTTGGGTAGTAGCGGTAGTCGAGATTTACGGTTAAGTTAAGGCGAAGAGTGGGTGAGTTTTTTGAATCGTTTT
TATTGGTCGTTTGTTTTTCGAGTTCGTTCGCGTGTTTTTGTAAGGTTGGGCGTTCGTTTTTAGTTTTGGGAGG
GGTGTAGCGGGTCGTAAGTTTTTTCGTGATTTGGATAGCGGTTTTTTTGGGCGAGCGTTGTTTAATTTTTCGT
TTATGCGCGGTTTTGGGGCGCGTTGTTTTTTAGTTTAGTTGGGTTGTGGTCGGGTCGGGGGTTGGGGAGAGTT
AGGGATTTATAGGAAGTTTTTTCGTTAGGGGTTTAAGGTTGTGGTAGATTATTAAGTTTGTGTTTAGGTTGGA
TTTTGATGGTAAGTTTTTTAACGTGGTAAAGTTGTTAGAGTTTGTTGTGTATTGACGAATGATTGGTGTATAG
TATTTAAAATCGTGTTATTTTTCGTTTTCGGGTTTTTTGGTAATAGAGGTTTTTATTTTTAGTTTTTAGAATA
TTGGTTTTATTTTTTTAGGATTTGGGTGGGGTTTAGGTTTTTTTTAAAATAGTTAATTAAGGTTTTAAATATG
TAATTTTGGTTAAATTTATTGGAAGGTGTTTGTAGATATAAATTTTTTGTATATTTTTAAATTTTTTATTTTG
TTATTTTTGTAAAATATATTTTTTTTTTATATTTTGACGTGTAATAAAAGGTTATTTATTTAATATGAATGTTA
AGTATAGAGGGAAAGTAATGTTTAAAAATAGTTTAGTGAAAAAAAAAAATAAAAAATTCGATTTTATTTAGTT
AAATGTAAAATAAGTGTGTATTTGAAAGTATAAAAGGTTGGAAATGTAATGTAATTTATAGTGTATTTTAGGT

ATTGGGTTTTTTTATATTTTTAATTGCGGATATAAATATGAGTAAGTTGGGTTGTGTTAATATTTAGGAATA
TTAGGGTAGTTATTTTGGTATAGTAAAATAGTAGTCGTATTAGAAATATGTTTTATTTTTTTTAAAAGGGGGT
GAAAGAGAATTTTGGGTCGAATTTTGGAGTTTTTAATATTTTAATATTAGGTTGTTTTTTGTTATTTTTTTTT
TTATGAAAAGATTTTATTGTAGAATTTAAATTTTATTTTGGTTAATGTTTGATGGAAATTAATAAATTTATTT
GATTTATAGTTTATTTTTTTGGTTTATGGGTATAGAGTTGATTGTGGGTTATATAGTTAATAAGTTTTTTTAT
TGTTATGGGTTTTTTTTTGGTTTTGTTTTTGAGTGTGGTTTTTTTATTATGTAGATAAATGGTTGGGTAATTT
TTTAGGGTTATTTTGGTTTTTTATTTAGATTTCGTTTGTAGTGGTTATTAGTTAGTAAATATTGTTAATTTGT
TAGTTATTTTAGGATTGGGTTTAGAAGATAGCGTTGAGTTTTTTTTTTTATTTTTTTTTTTATGTTATTATTT
GTTTGTAAAATTTTATTTTTTTTTAGGATATATCGTTTTATTATGTAGTTGAAATGTTTGTTTTTTTTTTTAG
ATTTTAAGGTTTTTTAGAGTAGAGGTTTTATTTTATTTAATTTTGTATTTTTTTTGTTTTATATTGTTGTTTA
TAATATAGTTAATGAATTTATATTGAATAAATGTAGGTGGATTTGTTAGTATTTTTACGATATTGTAATTTTT
AAAGTTAAAAGATTTTTTAAATATAGTGAATTATTTTTTATATTTGATTATAGTAGGTGATATTTTTATTTTT
AGTATTTTTTTTTTTTATTTTATAAATTATTATGTTGGTGATATGGTTTGGAGTTGTGTTTTTTTTTTAGATT
TTATGTTGAAATGTAATTTTTAGTGAGGGAGTTGGGGTTTGGTGGGAGGTGATTGGATTATGGGAGTAGTTTT
TTATGGTTTAATATTATTTTTTTTTGGTGTTGTTGTTACGTTAGTGTAGGGGTATTTAATTTCGTGGTCGTAG
ATTGGTATTAGTTAGTGTTTTTTTAGGAATTGGGTTGTATAGTAGGAGGGGAGTGGTAGGTTAGCGAGTATTA
TTGTTTGAGTTTTATTTTTTGTTAGATGAATAGTGGTATTAGATTTTTTTAAGTGTATGAATTTTATTGTAAA
TTGTATATGTAAGGGATTTAGGTTGTGTGTTTTTTATGAGAATTTAATTATTGTTTGATGATTTGAGGTGGAA
TAGTTTTATTTTAAAATTTTATTTTTTTTTTTTTATTTGGAAAAATTGTTTTTTACGAAATTAGTTTTTGGTG
TTAAAAAGGTTGGGGATTATTGCGATAATGTAAGACGTTTGTTTTTATTTTATTTTTCGTTATGATTATAAGT
TTTTTGAGTTTTTTTTAGAAGTAGATGTTGTTATGTTTTTTTTATAGTTTATGGAATTATGAGTTAATTAAAT
TTTTTTTTTTTATAATAATTATTTAGTTTTAGGTATTTTTTTATAGTAGTGCGAGAATAGCGTAATATAGTTG
GTTAAGTTATTAAATGATTTGGAATAATGTTTTTTAAGGAGATAGAGTGTTATTTTTTTTAGAGTAGGTATGA
GTGTTATTTTTATTTTTTTTTTTTTAATGTTGGTTATAGAGTTGGGTTTATAAATATTTGATGATTGGTATATG
TTTTATTTAGAATTTTTTTATATGTTAGTGGTGAATATTTAAATTATATTAATTTAAATTAAAAAAAAAGGGA
ATGTGTTGATTGTTTAATTGAAAAATTTAGGATTTAGTTTTAGGGGTTTTAAATATTGAAATATGTCGTTAGG
ATTTTTTTGTGTTTTTTTTTTGTTTTTTGTTTTGTTTTCGTGTTGGTTTTTTTTTTTTTTTAATGAAAATATGTT
TTTTTTTAGAGTATGGAGAAAAAAGTATGGTAGGTTTATATTGTGTTATTTTAATAAAATGTTAAAGATATTT
TATTTTTTTTTTTTTATTTTTATATATAAATATTAGAGAATGATTAGGTTTAAGTAGGGTTTTGTGTTATCGTT
AAGTTAATTAATGGGAAAATAGGAAATTGTTTAGGTTTGAGTTATATATTTATTTTTGTGATTAGGGAATGAG
CGTTATTATTTTATATAATTTTTTAAAATTATATTGGAGGGAGGAAGGGTATTTTTTTAAATGGAGGAAATGT
TTTAAATTAGAAAAGAATGTGGGTAAATTAAAATAATAGATATTTTTATTATGAATGTTTTGTAATTTTGAAA
TAGATAATATTTGGAAAATATTTTAAGAGTATTTTAATAAAGTAAATGTTGGTATATGTAAAAGTAATTCGTT
ATTTTTTGTTATAGTTAAATTTTTAAAATTTGGAGAATGTTTTAAGAATAGTTATGTTTTTTTATGATAGGAT
ATATTTTGAGAAATTTATTGTAAGGCGGATTTTATTGTTGAGGAAATATTATAGAGTGTATTTATATAAATT
TAGATGGTATAGTTTTTTATATATTTAGGTTAGATGGTATAGTTTATTGTTTTTAGGTTATTAATTTGTATAG
TATGTTATTGTGTTGAATATTGTGGGTAGTTGAAATATAATAGTAAGTATTTGTGTATTTGAATATAGAAAAG
GTATAGTAAATATATATATATCGTATAAAAGATAAAAAATTGTATATTTGTATAGGACGTTTATTATGAATAG
AATTTGTAGAATTGGAAGTTGTTTTGGGTGAGTTAGTGAGTGAGTAGTGAGTGAATGTAAAGGTTTGGGATAT
TATTGTATAGTATTATAGATTTTATAAATATTGTATATTAAGGTTATTTTATATTTATTGAAAAATTTTTTTT
TTTAATAATAAATTAATTTTAGTTTATTGTAATTTTATTTTATTAATTTATTGTTTTTATTTTTTTGTAATAA

TATTTAGTTTAAAATATAGTTATATTGTATAGTTATATAAAATATTTTGTTTATGTATTTATTTTTTAAGTTT
TTTTTTTATTTTTAATTTTTTTTTTTTTTTTTAGATTTTTTTGTTGAAATTAAGATATAAATATATATATTAGT
TTAGGTTTATTTAGGGTTAGGATTATTAGTATTATTGTTTTTTATTTTTATATTTTGTTTTATTGGAAGGTTT
TTAAGGGTAGTAATAGGTATGGAGTTGTTATTTTTTATGATAATAATGTTTTTTTTTGGAAGATTTTTTGAAG
GATTTGTTTAGGGTTGTTTATAGTTAGTTTTTTTTTTTGTAAGTAGAAGGAGTATATTTTATAATAATAATAAA
AAGTATAGTATTGTAAATATTAGGTAATAGGAATTTTTTTGTTTTATTATATTTTTATGGGATTATTGTTATA
TATGTAGTTAGTCGTTGATTAAAGTGTTATGTGATATATTATTGTATTTTAAATTATTTTTGGAAATAGTTTT
TTATTTAAAAAAGTATTATACGTGGTTTAAAATTTAGAAATATTTGGGATATTTTTTGTTAATTAAATTGTTG
TATATTTAATTTGTTTTTTTTATTGAATTTTATAAAGTGTAAATTGAAGATGTGTTTTTTTGGAAATAAATTT
AGAATTAATAAAAATAATGAATAGTATTGTGATATATGTTTTTTTTTTTTAAAAAATGTTATATTAATTTTGG
ATGAGGATTTGTGAGTTTGTTAAGGAAAAGATTTTTTTATTAAGTGAGAAATTTGAAAAGTTGTTTTTTATAG
GGTTGATTAGAAGATTGGGTATATTATTAATTCGGTATAATTTATTATTTTTATTGGAAATATATTAGTAATT
TATATTTTTTAAATAAGTATTAATTTTTTATTTTAAAAGAAAAATGGTATTTTGAAAATATTGTTTTAGATTT
TAGTGTTAATTTTAAAATTTTGTTAAATATAAATTTTATATTAGTTATTTTTAGTTTTTATTGATGATATTAT
TATTTTTAATATAATATATTTTTAATTATATTTGTTATTGAAAGGAGTTGGTTTTTTTTTAGATGGAAATTTG
AAATTAAATTTTTTTTTAAGTAAAAGGTAATATTTGATTTTTTGTAATGTATATAGGTATTTGGGTTATTTATT
GAATAAATAAGTAGATAAAGAAGAGTAGTTATTGTTGATAAGAGGGTATTGGTTTGAAATTTATAGATTATAA
TATGAAGTTAAATGTATAAATTAGTTATTATTTTTATTTTTATTGTATTTTATTTGTTATTTTAGTTTTTAAG
TAAGTAAATTTTTTATATTGTTATATTAAATATTTATATTATAAGTAGAATGGATATGAAAGTAATTATTTTT
ATTTTTATAGGTGGTTGAAGTTATATTATTTTATAGAATTAATGGAAAGTAGAAAAGATATTATAAATTAAGA
AGAATTTTGGAAAATGAAGTTTAGGAGAAATTTAGAAGAAGACGATTATTTG

Figure A3: Bissulfite modification STEAP1 gene sequence and location of the primers for methylation.

```
STEAP1B         CATGGGAACTTGTTAGAAATGAGAATCTCAGACCTCATCCCAGACATACTGAACCGGAAA 60
STEAP1          CATGGGAACTTGTTACAAATGAGAATCTCAGACCCCATCCCAGACATACTGAACCGGAAA 60
                ********** ********** ************************

STEAP1B         CTGTGGGTGGACAGCCCAACAATCGGTGTTTTAATATGCCCCCGGGTAATCTTATACACG 120
STEAP1          CTGTGGGCGGACAGCCCAACAATCGGTGTTTTAATAAGCCCCCGGGTAATCTTATACACG 120
                ******* ********** ********** **********

STEAP1B         CTGAAGTCTGAGAACCACTGCTCTAGAGAAATGCACAAACGTTCCTTAGTGGAGATTAAA 180
STEAP1          CTAAAGTCTGAGAACCACTGCTCTAGAGAAATGCACAAACGTTCCTTAGTGGAGATTAAA 180
                ** ********** ********** ********** ********** **********


STEAP1B         CAAAAGGCGAGGGGTTGGAGGGCGCGAAACGGACCTCAAGGGCAGGGCAGACGGCATTTG 240
STEAP1          CAAAAGGCGAGGGGTTGGAGGGCGCGAAACGGACCTCAGGTGCAGGGCAGACGGCATTTG 240
                ********** ********** ********** * **********

STEAP1B         AGAGGAAACTGGGAGAGGCCGGAGTGCGACTTGGGGAGTCTTAGCCTCCAAGGACGTTCC 300
STEAP1          AGAGGAAACTGGGAGAGGCCGGAGTGCGACTTGGGGAGTCTTAGCCTCCAAGGACGTTCC 300
                ********** ********** ********** ********** **********

STEAP1B          CGAACCCTGCGGACGCGGGGCGCCAGCAGGTGGCGCTGGACGCGCAACTGACAAGGAGGC 360
STEAP1           GGAACCCTGCGGACGCGGGGCGCCAGCAGGTGGCGCTGGACGCGCAACGGACAAGGAGGC 360
                 ********** ********** ********** ********** **********

STEAP1B         GGGGCCTGCAGCAGGCTTGGAGGCTTCGCGCTCTGGAGGCTCAGGCGCCGCGTGGGGCCG 420
STEAP1          GGGGCCTGCAGCTGGCTTGGAGGCTCCGCGCTCTGGAGGCTCAGGCGCCGCGTGGGGCCC 420
                ********** ********** ********** ********** **********

STEAP1B         GCACCTCCCGGCAGCAGCGGCAGCTGAGACTCACGGTCAAGCTACGGCGAGGAGTGGGTG 480
STEAP1          GCACCTCTGGGCAGCAGCGGCAGCCGAGACTCACGGTCAAGCTAAGGCGAAGAGTGGGTG 480
                ******* ********** ********** ********** ***** *********

STEAP1B         AGTCCCTTGAATCGTTCTCACTGGCCGTTTGCTCCTCGAGTCCGCCTGCGTGTCCCTGCA 540
STEAP1          AGTCCCTTGAATCGTTCTCACTGGCCGTTTGCTCCTCGAGTCCGCCCGCGTGTCCCTGCA 540
                ********** ********** ********** ********** **********
```

```
STEAP1B         AGGCTGGGCGCCCGCTCCCAGTCTTGGGAGGGGTGTAGCGGGTCGCAAGCCTTCTCGTGA 600
STEAP1          AGGCTGGGCGCCCGCTCCCAGTCTTGGGAGGGGTGTAGCGGGTCGCAAGCCTTCTCGTGA 600
                ************************************************************

STEAP1B         CCTGGACGGCGGCCCTCCTGGGCGAGCGGTGCTCAATTCTTCGCTTATGCGCGGCTCTGG 660
STEAP1           CCTGGACAGCGGCCCTCCTGGGCGAGCGCTGCTCAATTCTCCGCTTATGCGCGGCTCTGG 660
                ******* ******************** ********** ******************

STEAP1B         GGCGCCCTGTCTTCCAGCCCAGCTGGGTTGTGGCCGGGCCGGGGGCTGGGGGGAGCCAGG 720
STEAP1          GGCGCGCTGCCTTCCAGCCCAGCTGGGTTGTGGCCGGGCCGGGGGTTGGGGAGAGCCAGG 720
                ***** *** ********************************** ***** ********

STEAP1B         GACTCAGAGGAAGCCTTTTCGCTAGGGGCTTAAGGTTGTGGTAGATTATTAAGTTTGTGC 780
STEAP1          GACTCACAGGAAGCCTTTTCGCTAGGGGCTTAAGGTTGTGGTAGATTATTAAGTTTGTGC 780
                ****** ****************************************************

STEAP1B         TCAGGTTGGATTTTGATGGCAAGTTTTTTAACGTGGCAAAGTCGCCAGAGTTTGCTGTGC 840
STEAP1          TCAGGTTGGATTTTGATGGCAAGTTTTTTAACGTGGCAAAGTTGCCAGAGTTTGCTGTGC 840
                ****************************************** ****************

STEAP1B         ATTGACGAATGACTGGTGCACAGTACTTAAAATCGTGCTACTTCTCGTCTTCGGGTTTCC 900
STEAP1          ATTGACGAATGACTGGTGCACAGTACTTAAAATCGTGCTACTTCTCGTCTTCGGGTTTCC 900
                ************************************************************
```

Figure A4: Alignment of STEAP1 and STEAP1B sequences (promoter, first exon and first intron). Regions with high homology are depicted with an asterisk. CpG islands are highlighted with yellow colour.

With TMAs construction, we observed that the block is practically complete, with their proper holes filled with the appropriate tissue. It is necessary to take in consideration that this image corresponds to the block after alignment and following creation of cutting slides that justify an eventual complete lack of tissue in some holes (Figure A5).

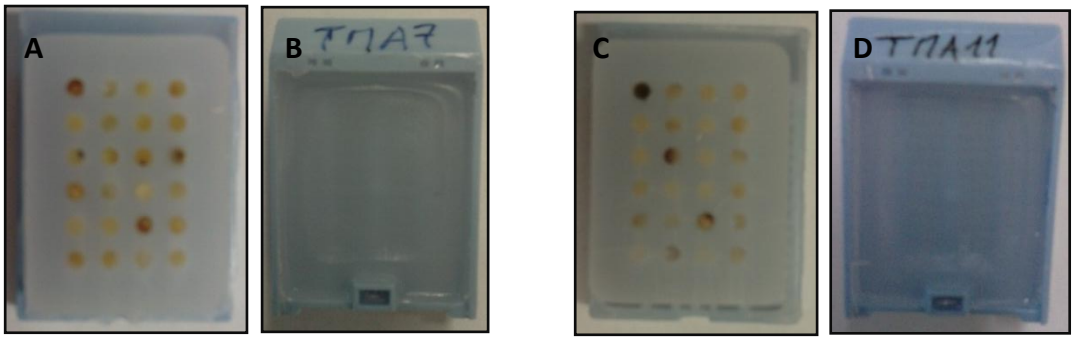

Figure A5: A – Image corresponding to a TMA block containing prostate cancer tissue, B – back TMA block itself appropriately subtitled. C – Image corresponding to a TMA block containing BPH, D – back TMA block itself appropriately subtitled.

In figure A6 was depicted the fragments from the corresponding block and it is clear the existence of all fragments after the immunohistochemistry, thereby infer the validation technique of “TMA”.

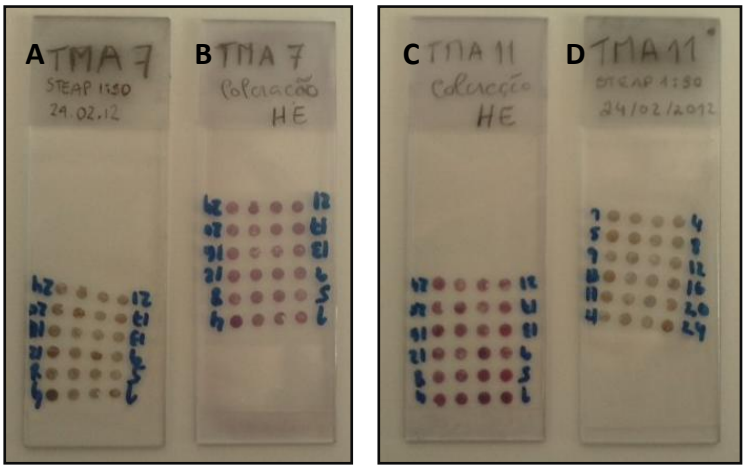

Figure A6: A - Slide corresponding to the block 7 above, suitably labeled (STEAP1 antibody), B – Slide stained with hematoxylin. C – Slide corresponding to the block 11 shown above, stained with hematoxylin, D – Slide suitably labeled (antibody STEAP1).

In figures A7 and A8, we can view a representation of a single fragment that allows a complete marking of the antibody used, which shows again the effectiveness of this technique.

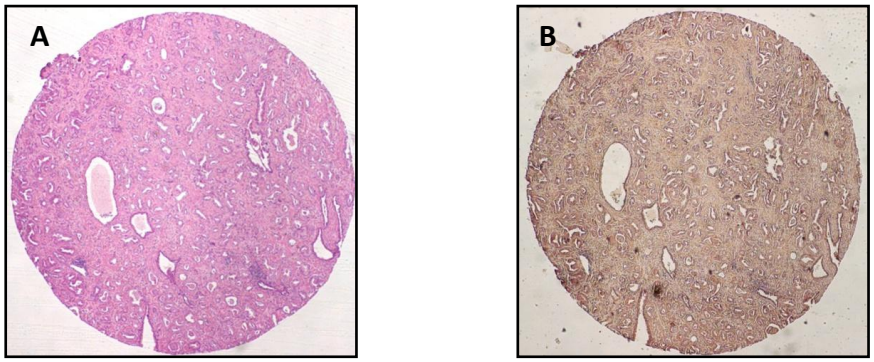

Figure A7: A – Image representative of a single fragment observed by conventional microscope with a magnification 4x, stained with haematoxylin in prostatic tumor tissue; B – Image representative of a single fragment observed by convencional microscope with a magnification 4x, corresponding to marking the antibody STEAP1 in prostate tumor tissue.

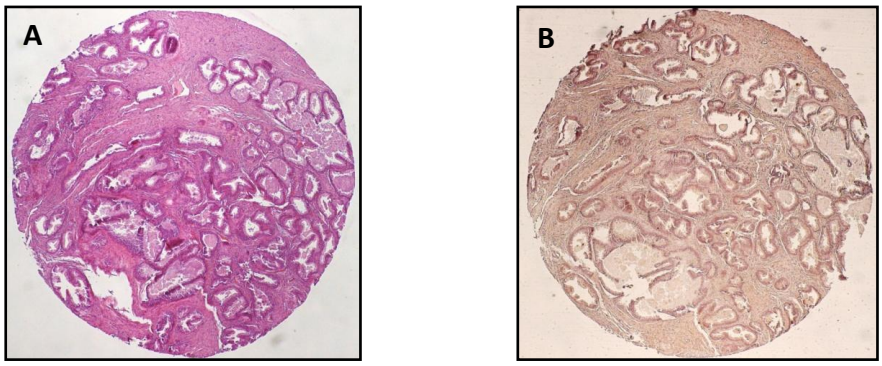

Figure A8: A – Image representative of a single fragment observed by conventional microscope with a magnification 4x, stained with haematoxylin, corresponding to a BPH; B – Image representative of a single fragment observed by convencional microscope with a magnification 4x, corresponding to marking STEAP 1 antibody, a tissue BPH.

Table A1: Patient characteristics according to the present pathologies.

Patient Number	Age	Histologic diagnosis	Total-PSA	Free-PSA	Gleason	TNM	Bone metastasis
H09/1577	62	Adenocarcinoma	7,43 µg/L	11,40%	7 (3+4)	pT2cNxMx R0	
H09/12198	59	Adenocarcinoma and ANT	11,67 µg/L	11,30%	7(4+3)	pT2cN0Mx	No
H09/947	64	Adenocarcinoma	6,94 µg/L	8,40%	7 (3+4)	pT2c	
H11/1568	61	Adenocarcinoma and ANT	6 µg/L		7 (3+4)	pT2aNxMx	No
H11/2741	58	Adenocarcinoma and ANT			6 (3+3)	pT2NxMx	
H10/14473	68	Adenocarcinoma and ANT	10,33 µg/L		7 (3+4)	pT2aNxMx	No
H11/6193	64	Adenocarcinoma and ANT	6,92 µg/L		7 (3+4)	pT3a	
H10/19013	64	Adenocarcinoma and ANT	6,28 µg/L	7,60%	10 (5+5)	pT3aN0Mx	No
H10/22274	62	Adenocarcinoma and ANT	3,91 µg/L	15,30%	6 (3+3)	pT2cNxMx R0	
H10/17824	62	Adenocarcinoma and ANT	5,10 µg/L	17,80%	6 (3+3)		No
H10/21981	62	Adenocarcinoma and ANT			7 (4+3)	pT3aNxMx R0	No
H10/18249	62	Adenocarcinoma and ANT	9,07 µg/L	14,40%	6 (3+3)	pT2cNxMx R0	No
H10/15893	62	Adenocarcinoma and ANT			7 (4+3)		No
H11/2441	60	Adenocarcinoma and ANT	4,77 µg/L	10,10%	6 (3+3)		
H10/19971	54	Adenocarcinoma and ANT	3,73 µg/L	22%	7 (3+4)		No
H11/53	64	Adenocarcinoma and ANT			7 (4+3)		
H10/23106	63	Adenocarcinoma and ANT	6,20 µg/L	6,60%	6 (3+3)	pT2a	
H10/14363	63	Adenocarcinoma	4,36 µg/L		7 (3+4)	pT3a	No
H10/20715	49	Adenocarcinoma and ANT			7 (3+4)	pT3aN0Mx	
H10/18633		Adenocarcinoma			7 (3+4)	pT2c	
H10/23770	70	Adenocarcinoma and ANT			7 (4+3)	pT2cNxMx	
H10/19919	70	Adenocarcinoma and PIN	7,98 µg/L	15,40%	6 (3+3)		
H10/17651	70	Adenocarcinoma	9,90 µg/L	7,90%	8 (4+4)	pT3aN0Mx R0	
H11/8473	64	Adenocarcinoma			7 (3+4)	pT2 c	
H10/16728	68	Adenocarcinoma			7 (3+4)	pT2cN0Mx	
H11/7107	56	Adenocarcinoma and ANT	8,92 µg/L	24,50%	6 (3+3)	pT2cNxMx	
H10/11794	65	Adenocarcinoma	7,15 µg/L	3,90%	7 (3+4)		

H11/486	67	Adenocarcinoma and ANT			6 (3+3)		No
H10/19716	62	Adenocarcinoma and ANT			9 (5+4)	pT3a N0	No
H10/21199	53	Adenocarcinoma, ANT and PIN	7,14 µg/L		7 (4+3)		
H10/13949	66	Adenocarcinoma, ANT and PIN	5,9 µg/L	20,20%	7 (3+4)		
H10/16852	59	Adenocarcinoma and ANT	9,22 µg/L	10,40%	7 (3+4)		No
H11/4956	41	Adenocarcinoma, ANT and PIN	5,75 µg/L		7 (3+4)	pT2cNxMx	No
H10/21008	71	Adenocarcinoma and ANT			7 (3+4)		
H10/7627	54	Adenocarcinoma, ANT and PIN			7 (4+3)		
H10/21764	55	Adenocarcinoma and ANT			6 (3+3)		
H10/5206	79	Adenocarcinoma	77,91 µg/L		9 (4+5)		Yes
H10/6746	59	Adenocarcinoma, ANT and PIN			7 (3+4)	pT2cNxMx R0	
H10/18197	83	Adenocarcinoma	8,13 µg/L	20,40%	8 (4+4)		
H10/17648	48	Adenocarcinoma and ANT			7 (3+4)	pT2cNxMx R0	
H10/11096	71	Adenocarcinoma and ANT			7 (3+4)	pT3aNxMx	
H10/11488	61	Adenocarcinoma and ANT			5 (2+3)		No
H10/14361	72	Adenocarcinoma and ANT	7,69 µg/L	15,10%	7 (3+4)	pT2c	No
H10/3408	64	Adenocarcinoma			7 (4+3)	pT2c No Mx	
H10/7559	77	Adenocarcinoma	3,65 µg/L	24,10%	6 (3+3)		
H10/5833	56	Adenocarcinoma, ANT and PIN	9,51 µg/L	7,80%	7 (4+3)	pT2cNxMx	
H10/6833	65	Adenocarcinoma and ANT			7 (3+4)	pT2bNoMx	
H10/4085	70	Adenocarcinoma	6,39 µg/L	4,10%	7 (4+3)		
H10/7179	62	Adenocarcinoma			7 (3+4)	pT2cNxMx	
H10/16784	65	Adenocarcinoma and ANT	3,24 µg/L	8,30%	7 (4+3)		No
H10/12719	68	Adenocarcinoma	7,88 µg/L	8,70%	7 (4+3)		No
H10/8642	69	Adenocarcinoma	3,84 µg/L	10,90%	9 (4+5)		Yes
H10/11642	67	Adenocarcinoma and ANT			7 (4+3)	pT3aN0MX	
H10/22762	65	Adenocarcinoma	12,13 µg/L		7 (3+4)		No
H10/13366	56	Adenocarcinoma and ANT			7 (3+4)	pT2bNxMx	No
H10/4207	60	Adenocarcinoma			7 (3+4)		No
H10/19844	69	Adenocarcinoma			6 (3+3)		

H10/4331	65	Adenocarcinoma	6,24 µg/L	11,20%	6 (3+3)		No
H10/16449	68	Adenocarcinoma, ANT and BPH	11,97 µg/L		6 (3+3)		
H10/12437	54	Adenocarcinoma	4,79 µg/L	11,90%	9 (4+5)		No
H10/18562	70	Adenocarcinoma and ANT			7 (3+4)	pT2c	
H10/9687	67	Adenocarcinoma, ANT and BPH	3,27 µg/L	7,30%	6 (3+3)		
H10/17709	59	Adenocarcinoma and ANT	3,96 µg/L				No
H10/8223	53	Adenocarcinoma				pT2c	
H10/9675	74	BPH					
H10/9517	85	BPH	2,96 µg/L	16,90%			
H10/9483	77	BPH	1,29 µg/L				
H10/17115	77	BPH	3,24 µg/L	22,50%			
H10/15289	72	BPH	3,06 µg/L	11,10%			
H10/16086	75	BPH	10,94 µg/L	15,50%			
H10/11458	73	BPH	11,57 µg/L				
H10/16285	75	BPH	3,277 µg/L	17,70%			
H10/16287	61	BPH	2,22 µg/L				
H10/11786	71	BPH					
H10/24105	59	BPH	14,80 µg/L				
H10/23010	77	BPH	0,17 µg/L	47,10%			
H10/19403	72	BPH	7,37 µg/L	39,30%			
H10/13365	73	BPH	1,26 µg/L				
H10/16479	80	BPH	5,65 µg/L	28,80%			
H10/11573	73	BPH	3,62 µg/L	19,10%			No
H10/12982	61	BPH	15,40 µg/L	17,40%			
H10/17503	73	BPH	11,31 µg/L				
H10/22495	87	BPH	11, 34 µg/L				
H10/19842	47	BPH	1,46 µg/L				
H10/11611	63	BPH	2,05 µg/L				
H10/10291	78	BPH	1,29 µg/L				
H10/16409	72	BPH	1,92 µg/L				
H10/11017	64	BPH	1,20 µg/L				
H10/15029	71	BPH	0,94 µg/L				
H10/13972	51	BPH					
H10/21700	66	BPH	3,01 µg/L	18,60%			
H10/19151	56	BPH	0,68 µg/L				
H10/17620	51	BPH	0,52 µg/L				
H10/23383	80	BPH	3,89 µg/L	21,10%			
H10/12196	76	BPH	5,50 µg/L	24,90%			

H10/19118	41	BPH					
H10/24042	88	BPH	0,72 µg/L				
H10/19628	56	BPH	3,38 µg/L	10,40%			
H10/10296	78	BPH					
H10/17156	72	BPH	0,88 µg/L	20,50%			
H10/17095	77	BPH	1,97 µg/L				
H10/17647	61	BPH	0,40 µg/L				
H10/17097	71	BPH	2,97 µg/L	16,80%			