UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Genetic and epigenetic mechanisms involved in regulation of *STEAP1* gene expression in LNCaP prostate cancer cells

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Resumo

O cancro da próstata é o segundo tipo de cancro mais frequentemente diagnosticado e a quinta principal causa de morte por cancro nos homens em todo o mundo. O desenvolvimento do cancro da próstata é caracterizado por alterações progressivas nos mecanismos genéticos e epigenéticos o que conduz a uma desregulação da expressão genética. O gene *Six transmembrane epithelial antigen of the prostate 1* (*STEAP1*) codifica uma proteína com seis domínios transmembranares. Nos tecidos normais, a expressão do *STEAP1* é muito baixa, no entanto é sobre-expresso em vários tipos de cancro nomeadamente no cancro da próstata. Vários estudos indicaram que a sobre-expressão do *STEAP1* parece promover o crescimento celular, sugerindo que este pode actuar como um oncogene. Estudos anteriores demonstraram também que o mRNA e a proteína STEAP1 apresentam uma maior estabilidade em linhas celulares de cancro da próstata LNCaP quando comparado com as linhas celulares da próstata não-neoplásicas PNT1A. Esta diferença pode ser devida a modificações pós-transcricionais e/ou pós-translacionais. No entanto, estas alterações não justificam a sobre-expressão do *STEAP1* em células tumorais, sugerindo assim o envolvimento de outros mecanismos de regulação. Portanto, o objectivo do presente trabalho foi explorar a hipótese de que alterações genéticas e/ou epigenéticas poderão estar envolvidas na sobre-expressão do *STEAP1*. A fim de avaliar a possível presença de alterações genéticas na sequência do gene *STEAP1*, foi sequenciada a região promotora do *STEAP1* em células LNCaP e PNT1A. Para estudar o envolvimento de mecanismos epigenéticos, foram comparados os padrões de metilação do *STEAP1* entre as linhas celulares PNT1A e LNCaP. Para além disso, foi ainda avaliado o efeito de um tratamento com inibidores das DNA metiltransferases (DNMT) e histonas desacetilases (HDAC) na expressão do gene *STEAP1* em células PNT1A. A análise da sequência da região promotora do *STEAP1* revelou algumas variantes tanto nas células LNCaP como PNT1A quando comparada com a sequência genómica disponível. A análise *in silico* das variantes mostrou diferenças nos fatores de transcrição que se podem ligar a cada variante alelica incluindo a ligação de activadores transcripcionais ao alelo alterado das variantes. A análise do padrão de metilação do *STEAP1* entre células PNT1A e LNCaP mostrou diferenças na região promotora próxima do local de início da transcrição. O tratamento com 5-Aza-2'-deoxicitidina (inibidor das DNMT) induziu um ligeiro aumento na expressão do *STEAP1* (três vezes em comparação com o grupo de controlo, $p<0.01$), enquanto que o tratamento com ambos os inibidores 5-Aza-2'-deoxicitidina e TSA (inibidor das HDAC) induziu um aumento acentuado na expressão do *STEAP1* (quinze vezes relativamente ao grupo de controlo, $p<0.001$). A diferença no padrão de metilação do *STEAP1* entre as células LNCaP e PNT1A, juntamente com o aumento da expressão do *STEAP1* em resposta ao tratamento com os inibidores de HDACs e DNMTs, indica que a expressão génica do *STEAP1* parece ser regulada por mecanismos epigenéticos.

Palavras-chave: Cancro da Próstata, STEAP1, Genética, Epigenética

Resumo Alargado

O crescimento e envelhecimento da população associado a um aumento da adopção de factores de risco tornaram o cancro um dos maiores problemas de saúde a nível mundial. O cancro da próstata é o segundo tipo de cancro mais frequentemente diagnosticado e a quinta principal causa de morte por cancro nos homens de todo o mundo. O processo de desenvolvimento do cancro da próstata é caracterizado por alterações progressivas nos mecanismos genéticos e epigenéticos que regulam a expressão genética. Uma das alterações genéticas mais frequente no cancro da próstata é a fusão do gene TMPRSS2, cuja expressão é regulada pelos androgénios com os genes da família de factores de transcrição ETS. Quanto às alterações epigenéticas, a alteração que é mais frequentemente encontrada em casos de cancro da próstata e lesões pré-neoplásicas é a hipermetilação da região promotora do gene que codifica a enzima Glutationa S-transfrease π.

O gene *Six transmembrane epitelial antigen of the protate 1* (*STEAP1*) foi o primeiro elemento da família de proteínas STEAP a ser identificado. O *STEAP1* codifica uma proteína com seis domínios transmembranares que se encontra localizada nas junções celulares das células epiteliais. Pensa-se que esta proteína possa actuar como um canal iónico ou proteína transportadora de pequenas moléculas tendo assim um papel na comunicação intercelular. Enquanto que nos tecidos normais a expressão do *STEAP1* é muito baixa ou mesmo nula, nos tecidos tumorais é sobre-expresso em vários tipos nomeadamente no cancro da próstata. Vários estudos indicaram que a sobre-expressão do *STEAP1* parece promover o crescimento celular, sugerindo que este pode actuar como um oncogene. Estudos anteriores demonstraram também que o mRNA e a proteína STEAP1 apresentam uma maior estabilidade em linhas celulares de cancro da próstata LNCaP quando comparado com as linhas celulares da próstata não-neoplásicas PNT1A. Esta diferença pode ser devida a modificações pós-transcricionais e/ou pós-translacionais. No entanto, estas alterações não justificam a sobre-expressão do *STEAP1* em células tumorais, sugerindo assim o envolvimento de outros mecanismos de regulação.

Portanto, o objectivo do presente trabalho foi explorar a hipótese de que alterações genéticas e/ou epigenéticas poderão estar envolvidas na sobre-expressão do *STEAP1* no cancro da próstata. Para testar esta hipótese foi delineado um conjunto de tarefas. A fim de avaliar a possível presença de alterações genéticas na sequência do gene *STEAP1*, nomeadamente mutações, foi sequenciada a região promotora do *STEAP1* em duas linhas celulares da próstata, uma neoplásica (LNCaP) e uma não-neoplásica (PNT1A). Foi também realizada uma análise *in silico* para avaliar se alguma das alterações encontradas está localizada numa região importante para a ligação de factores de transcrição. Quanto aos mecanismos epigenéticos foi avaliada a metilação do DNA e a acetilação de histonas. Para a análise de alterações ao nível da metilação do DNA foram comparados os padrões de metilação pelo método BSP do gene *STEAP1* entre as linhas celulares PNT1A e LNCaP. Para

avaliar ainda alterações na metilação do DNA e acetilação de histonas foi realizado um tratamento com inibidores de DNA metiltransferases (DNMT) e histonas desacetilases (HDAC) em células PNT1A. O efeito do tratamento na expressão do *STEAP1* foi avaliado através da técnica de PCR em tempo real.

A análise da sequência da região promotora do gene *STEAP1* revelou a presença de algumas alterações tanto nas células LNCaP como PNT1A quando comparada com a sequência genómica disponível. Algumas das variantes encontradas já se encontram identificadas na base de dados Ensembl. A análise *in silico* das variantes mostrou algumas diferenças entre os fatores de transcrição que se podem ligar a cada variante alelica nomeadamente a ligação de activadores transcripcionais como o C/EBPß e o LEF-1 ao alelo alterado das variantes. A análise do padrão de metilação do *STEAP1* entre as células PNT1A e LNCaP mostrou diferenças na região promotora próxima do local de início da transcrição. Enquanto que nas células PNT1A alguns dos dinucleótidos CG parecem estar metilados, nas células LNCaP parece haver uma desmetilação completa da região analisada. O tratamento com 5-Aza-2'-deoxicitidina (inibidor das DNMT) induziu um ligeiro aumento na expressão do *STEAP1* (três vezes em comparação com o grupo de controlo, $p<0.01$), enquanto que o tratamento com ambos os inibidores, 5-Aza-2'-deoxicitidina e TSA (inibidor das HDAC), induziu um aumento acentuado na expressão do *STEAP1* (quinze vezes relativamente ao grupo de controlo, $p<0.001$). Esta alteração na expressão do *STEAP1* provocada pelos inibidores das DNMTs e HDACs indica que tanto a metilação do DNA como a acetilação de histonas podem estar envolvidos na regulação da sua expressão.

Em suma a diferença no padrão de metilação do *STEAP1* entre as células LNCaP e PNT1A em conjunto com o aumento da expressão do mRNA do *STEAP1* em resposta ao tratamento com os inibidores de HDACs e DNMTs, são indicadores de que a expressão do *STEAP1* é regulada por mecanismos epigenéticos, nomeadamente a metilação do DNA e a acetilação de histonas.

Abstract

Prostate cancer is the second most frequently diagnosed type of cancer and the fifth leading cause of cancer death in men worldwide. Prostate carcinogenesis is characterized by progressive alterations in genetic and epigenetic mechanisms that deregulate gene expression. The Six Transmembrane Epithelial Antigen of the Prostate 1 (*STEAP1*) gene encodes a protein with six transmembrane domains. In normal tissues, *STEAP1* expression is very low but is overexpressed in several human cancers, mainly in prostate cancer. Some studies have indicated that *STEAP1* overexpression seems to promote cell growth, suggesting that *STEAP1* may act as an oncogene. Previous studies demonstrated that *STEAP1* mRNA and protein have higher stability in LNCaP prostate cancer cell lines when compared with PNT1A non-neoplastic prostate cell lines, possibly due to post-transcriptional and post-translational modifications. However, these alterations do not justify the overexpression of *STEAP1* in tumor cells, suggesting that other mechanisms may be involved. Therefore, the aim of this study was to explore the hypothesis that genetic and / or epigenetic alterations may be involved in overexpression of *STEAP1*. In order to evaluate genetic alterations in the *STEAP1* gene sequence, the promoter region of *STEAP1* in LNCaP and PNT1A cells was sequenced. To study the involvement of epigenetic mechanisms, the methylation patterns of *STEAP1* in PNT1A and LNCaP cells were compared. In addition, the effect of treatment with DNA methyltransferases (DNMT) and histone deacetylases (HDAC) inhibitors on *STEAP1* mRNA expression in PNT1A cells was evaluated. The sequence analysis of the promoter region of *STEAP1* revealed some differences in both PNT1A and LNCaP cells when compared with the available genomic sequence. *In silico* analysis of the identified variants revealed several alterations in the transcription factors (TF) that can bind to each allelic variant including the binding of transcriptional activators to the altered allele of the variants. The analysis of the methylation pattern of *STEAP1* gene in PNT1A and LNCaP cells showed differences in the promoter region near the transcription start site. The treatment with 5-Aza-2'-deoxycytidine (DNMT inhibitor) induced a slight increase in *STEAP1* mRNA expression (3 fold-variation in comparison with control group, $p<0.01$) while the treatment with both 5-Aza-2'-deoxycytidine and TSA (HDAC inhibitor) induced a marked increase in *STEAP1* mRNA expression (15 fold-variation relatively to control, $p<0.001$). The difference in the methylation pattern of *STEAP1* between PNT1A and LNCaP cells, along with the increased *STEAP1* mRNA expression in response to DNMT and HDAC inhibitors, indicates that *STEAP1* gene expression seems to be regulated by epigenetic mechanisms.

Keywords: Prostate cancer, STEAP1, Genetic, Epigenetic.

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List of abbreviations

aa	Amino acids
Akt	Protein kinase B
AR	Androgen receptor
AZA	5-Aza-2'-deoxycytidine
BPH	Benign prostate hyperplasia
BSP	Bisulfite sequencing PCR
C/EBPβ	CCAAT/enhancer-binding protein β
CDKN1B	Cyclin dependent kinase inhibitor 1B gene
DHT	Dihydrotestosterone
DNMT	DNA methyltransferases
DRE	Digital rectal exam
E. coli	*Escherichia coli*
GSTP1	Glutathione s-transferase π
H&E	Hematoxylin and eosin
HAT	Histone acetyl transferases
HDAC	Histone deacetylases
LEF-1	Lymphoid enhancing factor-1
LH	Luteinizing hormone
NKX3.1	NK3 homeobox 1 gene
PAP	Prostatic acid phosphatase
PCa	Prostate cancer
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PI_3K	Phosphatidylinositide 3-kinase

PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PIP_3	Phosphatidylinositol 3, 4, 5-triphosphate
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog gene
qPCR	Real-time quantitative PCR
ROS	Reactive oxygen species
SNP	Single-nucleotide polymorphism
STEAP1	Six transmembrane epithelial antigen of the prostate 1
TF	Transcription factor
TRUS	Transrectal ultrasound
TSA	Trichostatin A

1. Introduction

Partially submitted:

Barroca-Ferreira J, Pais JP, Santos MM, Gonçalves AM, Gomes IM, Sousa I, Rocha SM, Passarinha LA, Maia CJ. Targeting STEAP1 protein in human cancer: current trends and future challenges. Partial submitted to Current Cancer Drug Target

1.1. Anatomy and physiology of the prostate

The prostate is an accessory gland of the male reproductive system and is located in the pelvic region just below the bladder (1). This gland has the shape and size of a walnut and its main function is the production of an alkaline fluid containing acid phosphatase, proteases, sucrose and citric acid that allows sperm motility and protection (2, 3). The prostate gland is surrounded by a capsule of collagen and smooth muscle that gives rise to septa which extend to its interior dividing the gland in lobes (4). The prostate grows during puberty to full size due to increasing levels of androgens. After the age of 55 years, the growth is reinitiated due to the growth of nonmalignant cells in the periurethral zone (1).

In according to McNeal's description, the prostate can be divided into five different zones (Figure 1): the central zone, the peripheral zone, the preprostatic zone, the transition zone and the fibromuscular zone (5). The peripheral zone is the largest region, which comprises nearly 75% of the glandular tissue and appears to be more susceptible to develop cancer (1, 5, 6). The central zone comprises approximately 25% of the glandular tissue. The preprostatic zone or periurethral region is composed of glandular and non-glandular tissue that surrounds the urethra (5, 6). The transition zone represents less than 5% of the gland mass, but is the origin local of the benign prostatic hyperplasia (BPH), which is considered the most common disorder of the prostate (5). The anterior fibromuscular zone is a non-glandular structure that constitutes the anterior surface of the prostate (5, 6).

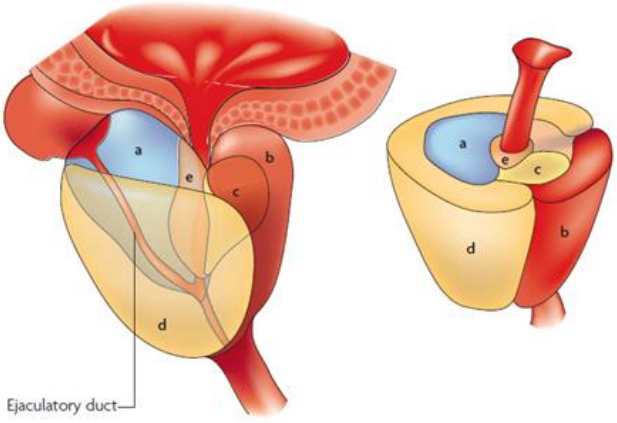

Figure 1: Human prostate anatomy according to McNeal's description. a. Central zone; b. Fibromuscular zone; c. Transitional zone; d. Peripheral zone; e. Periurethral gland zone (Adapted from (7)).

The prostate is dependent on androgens for the development and maintenance of its structural and functional integrity. Testosterone is the most abundant androgen in circulation and it is produced by Leydig cells in the testes. Others androgens, such as dehydroepiandrosterone (DHEA) and androstenedione (4-DIONE), are produced in the adrenal cortex and converted into testosterone in peripheral tissues. In target tissues like prostate, the testosterone is converted to dihydrotestosterone (DHT) due to high activity of 5-alpha-reductase (8, 9). Thus, DHT is considered the main androgen required for complete prostate morphogenesis. The main mechanism of action of DHT is mediated by its binding to the androgen receptor (AR), which in turn, bind to DNA to activate the transcription of genes involved in cell proliferation, survival, lipid metabolism and differentiation (10, 11).

The prostatic tissue is composed of stromal and epithelial cells (Figure 2). Within the epithelial cells, two different types can be distinguished morphologically: columnar luminal cells and basal cells (12). The columnar luminal cells express the AR and are dependent on androgens to survive. These cells constitute the exocrine compartment of the prostate epithelium, secreting prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) (12, 13). The basal cells do not have secretory activity and express very low levels of AR. Although these cells are androgen independent they are androgen responsive; they do not depend on androgens to survive but their growth and differentiation are stimulated by androgens. This basal layer lies beneath the columnar luminal cells layer and it is believed to have stem cells with the capacity to give rise to all types of prostatic epithelial cells (12–14). There is a third type of epithelial cells dispersed within the luminal and basal cells, the neuroendocrine cells. Although the function of this type of cells is still unknown, it is believed that they may be involved in the proliferation of the adjacent cells by paracrine secretion of neuropeptides. The neuroendocrine cells do not depend on androgens to survive and may play a role in prostate carcinogenesis (12, 13). The stromal cells contain fibroblasts and smooth muscle that provide structural and biochemical support to the prostate epithelium (3, 13, 15). These two types of cells produce the extracellular matrix that helps to generate a microenvironment that controls the growth of the adjacent epithelial cells (3, 15). Some studies have shown that AR is expressed in smooth muscle cells, but not in fibroblasts. Thus, it is believed that androgens act through paracrine signaling pathways on smooth muscle to maintain the fully differentiated growth-quiescent epithelium (3, 15). Ablation of androgens results in prostate involution and loss of epithelial cells by apoptosis. The re-administration of androgens reverse this process inducing the prostate return to normal size and function through rapid proliferation and differentiation of stem cells. The homeostasis between the epithelial and stromal compartments is regulated by a complex signaling pathway that involves the AR and other paracrine factors capable of maintaining the balance between proliferation and apoptosis (8, 16).

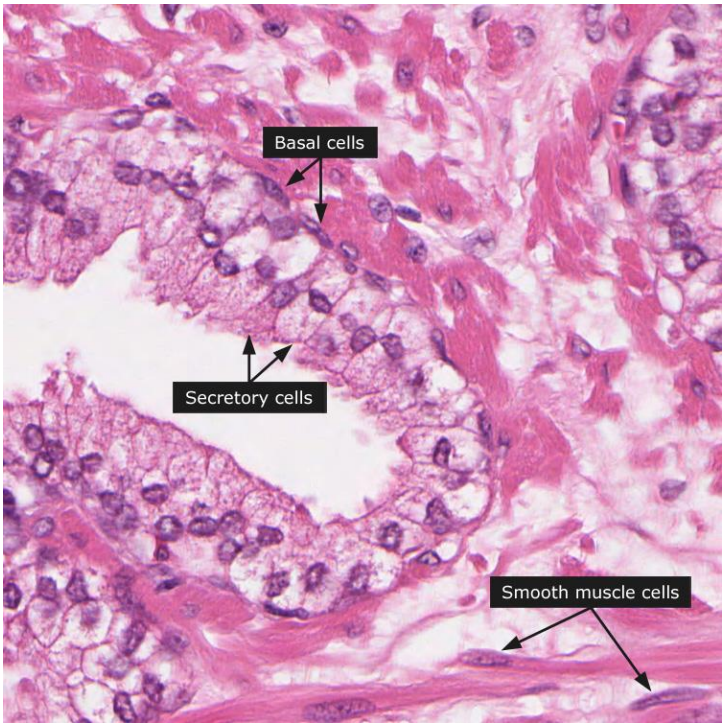

Figure 2: Histologic arrangement of the normal prostate (H&E stain) (Retrieved from http://www.proteinatlas.org/learn/dictionary/normal/prostate/detail+1/magnification+1 (30.08.2016)).

1.2. Prostate cancer

1.2.1. Epidemiology and Risk factors

Cancer is one of the leading causes of death in both more and less developed countries. The increasing incidence due to the growth and aging of the population associated with the increasing adoption of risk factors, such as smoking, overweight, physical inactivity and poor diet, made it a burden to the world society (17).

Prostate cancer (PCa) is the second type of cancer most frequently diagnosed in men, and it is the fifth leading cause of cancer death in men worldwide (18). In Portugal, PCa is the most frequently type of cancer diagnosed, representing more than 20% of the diagnosed cancers and is the fourth leading cause of cancer death in men (19, 20). The decrease trend in death rates should be mainly due to early detection and improvement of the available treatments (17).

Risk factors can be divided into two types: the non-modifiable factors and the external factors (modifiable factors). Non-modifiable factors are genetic susceptibility, age, ethnicity or family history, whereas external factors include lifestyle factors such as diet and physical activity (10, 21). Age is one of the most important risk factors for PCa. In men younger (until 50 years old), the incidence of PCa is very low (<0.1%). On the other hand, in men with more than 65 years old the incidence is much higher, representing approximately 85% of the cases (10, 21). Another important risk factor is ethnicity since the incidence of PCa among African-American men is approximately 60% higher than in white men. The lowest rates of PCa are found in Asian, but when these migrate to the US their risk of developing PCa increases, which indicates that external factors are also involved in the development of PCa (10, 22). Besides age and race, family history of the disease is another major risk factor for PCa. The relative risk of developing it increases with the number of family members affected and with the degree of relatedness, and is inversely related to the age at which family members were affected. For example, in men who have a first-degree relative (father or brother) with PCa, the relative risk of developing the disease increase two to threefold depending on the age at which the disease appeared (10, 21, 23). Several PCa susceptibility genes have been identified, such as the *RNAse L* gene in locus HPC1, the *ELAC2* gene in locus HPC2, the *MSR1* gene on chromosome 8 and the *BRCA* genes. One of the most important is the *RNAse L* gene, which encodes an endoribonuclease involved in the induction of apoptosis and regulation of cell cycle and cell differentiation among others. This gene has typically autosomal dominant hereditary with high penetration (22, 24).

Although several epidemiological studies of the possible association between external factors and PCa risk have been conducted, the results have been inconclusive. One of the external factors is the western lifestyle, which is characterized by diets rich in red meat, dairy products and high intake of fat that may lead to increased risk of developing PCa (10, 22, 25). Preparation of meat at high temperatures leads to the production of heterocyclic

amines and other carcinogens. This, together with the production of hydrogen peroxide from the fatty acids oxidation process, could induce damage in the prostate genome (10, 22, 26). Another factor that seems to be related to PCa risk is obesity. The increase of body mass index is associated with decreased risk of developing localized PCa and increased risk of developing aggressive PCa (21, 27, 28). Other external factors that may be associated with the increased PCa risk are infections, smoking and radiation exposure, but these associations remain unclear (10, 21). On the other hand, there are several protective factors such as vegetables and physical activity (22, 26). Vegetables, especially tomatoes have been associated with a lower risk of PCa due to the high content of antioxidants, such as carotenoids that seem to be associated with decreased oxidative DNA damage and reduction of serum PSA levels (26, 29, 30). Cruciferous vegetables are rich in phytochemicals like sulforaphane, and these phytochemical induces the expression of carcinogen detoxification enzymes that prevent DNA and cell damage from carcinogens (26, 31). Regarding physical activity, it is well known that it has many benefits including reduction of PCa risk, although the association between these remains unclear (21, 32).

1.2.2. Diagnosis and treatment

Screening of PCa is based on serum PSA (kallikrein-related peptidase 3; KLK3) levels, digital rectal examination (DRE) and the patient's symptoms. PSA, a kallikrein-related serine protease, which is responsible for the liquefaction of the seminal coagulum, is produced by both nonmalignant and malignant cells. Despite being prostate specific, the increase of PSA levels in serum are not cancer specific, and may result due to BPH or prostatitis. Therefore, a considerable number of false positives may occur, which decrease the specificity of PSA as a biomarker for PCa (1, 33). However, the predictive value of PSA screening can be improved by complementary tests, such as PSA velocity, PSA density, and free-PSA (34). PSA velocity is based on the rate of change in PSA levels over time, which increases the sensitivity and specificity of the PSA test. Even so, its predictive value is limited by intrasubject variability in PSA measurements (22, 34). PSA density correlates serum PSA levels with prostate volume. PCa cells release more PSA per volume unit than BPH tissue. The division of PSA levels by the volume of the prostate improves the specificity of PSA test because it allows the distinction between BPH and PCa (34, 35). PSA circulates in the blood in an inactive form, mainly aggregated with a protease inhibitor, while free PSA is quickly eliminated from the organism by glomerular filtration. A lower percentage of free-PSA is more associated with PCa than BPH, allowing an improvement of PSA test specificity (34–36).

Regarding DRE, it allows to assess whether there are alterations in prostate size and consistency. In most cases of patients with PCa the size of the prostate increase in the peripheral zone, which is coincident with the area examined in DRE (1).

The American Cancer Society recommends that men with 50 years or older should discuss with a health care about the risks and possible benefits of testing for PCa and make an informed decision. In the case of men at high risk, these should talk to a health care 5 to 10 years earlier. The test for PCa is based on serum PSA levels with or without DRE. When there is an increase in PSA levels associated with abnormal DRE, the patient is forwarded to a possible diagnosis of PCa. In order to confirm the diagnosis of PCa, the patients will be subjected to transrectal ultrasound (TRUS)-guided needle biopsy (1, 34). Histopathological diagnosis of PCa in needle biopsy specimens is performed by hematoxylin and eosin (H&E) staining and/or immunohistochemistry against cytokeratin, p63 and racemase (AMACR) proteins (37, 38).

After a diagnosis of PCa, it is necessary to classify the tumor in order to help choose a better treatment. Gleason score system, the most commonly used, is based on the histological pattern of PCa cells in prostatic tissue sections. This system is used to measure the histological aggressiveness, in which the histological pattern is classified according to its glandular pattern and differentiation degree. The dominant and secondary patterns are scored from 1 to 5, in which grade 1 represents a well-differentiated tumor and grade 5 an undifferentiated. The score of the dominant and the secondary patterns are summed to give a total score of 2 to 10. The biologic behavior is generally determined by the area with low differentiation, which is the area with the highest histologic grade (1, 39). The clinical stage of the tumor is one of the most important factors in the choice of treatment and it is generally classified using the tumor-nodes-metastasis (TNM) system. This system divides the tumors in three main stages: Primary tumor (T), Regional lymph nodes (N) and distant metastasis (M). T stage has 4 categories describing how the tumor has been identified, the size of the primary tumor and whether it has invaded nearby structures. N stage describes whether the cancer has spread to nearby lymph nodes while M stage describes whether the cancer has spread to distant parts of the body like bones and lymph nodes (34, 40).

Patients with localized PCa (those who do not appear to have metastasis after staging analyses) have three main treatment options: radical prostatectomy, radiation therapy and active surveillance (1, 34). Radical prostatectomy is usually performed in patients who have tumors confined to the prostate gland (stage T1 and T2) and can undergo surgery. The great advantages of radical prostatectomy is that it provides excellent control of the primary tumors without increasing morbidity (34, 41). For patients with PCa confined to the prostate or surrounding tissues that cannot perform surgery, radiation therapy is an alternative, which can be administered as external beam therapy, brachytherapy or a combination of both. The results of this type of treatment will depend on the stage and dosimetry of radiation. In brachytherapy, the radioactive source is implanted into the prostate or surrounding area. The patients that undergo brachytherapy generally have low Gleason score, low PSA level and tumors on stage T1 or T2 (34, 42). Active surveillance consists in regularly following the patient and initiating therapy if there are signs of tumor progression. In men with indolent or nonprogressive tumors, active surveillance prevents the morbidity of therapy (1, 43).

For patients with tumor extension to nearby structures or metastatic disease, other treatment options are hormonal therapy and chemotherapy in addition to the already mentioned above. The growth of PCa is essentially androgen-dependent since these stimulate proliferation and inhibit apoptosis. The main goal of hormonal therapy is to reduce androgen levels by surgery or therapy with anti-androgens or luteinizing hormone (LH) agonists (1, 9, 44). LH agonists lead to inhibition of testosterone secretion in the testis. Anti-androgens act mainly by inhibiting the signaling pathways triggered by AR activation. When PCa evolves to an androgen independent stage, the available treatment is essentially palliative (44).

1.2.3. Molecular pathways of carcinogenesis

PCa arise from precursor preneoplastic lesions that give rise to localized cancer, and then may progress rapidly until development of metastasis (45).

The main preneoplastic lesions are prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA), but it is not known if these lesions are part, or not, of the same pathway (Figure 3) (46).

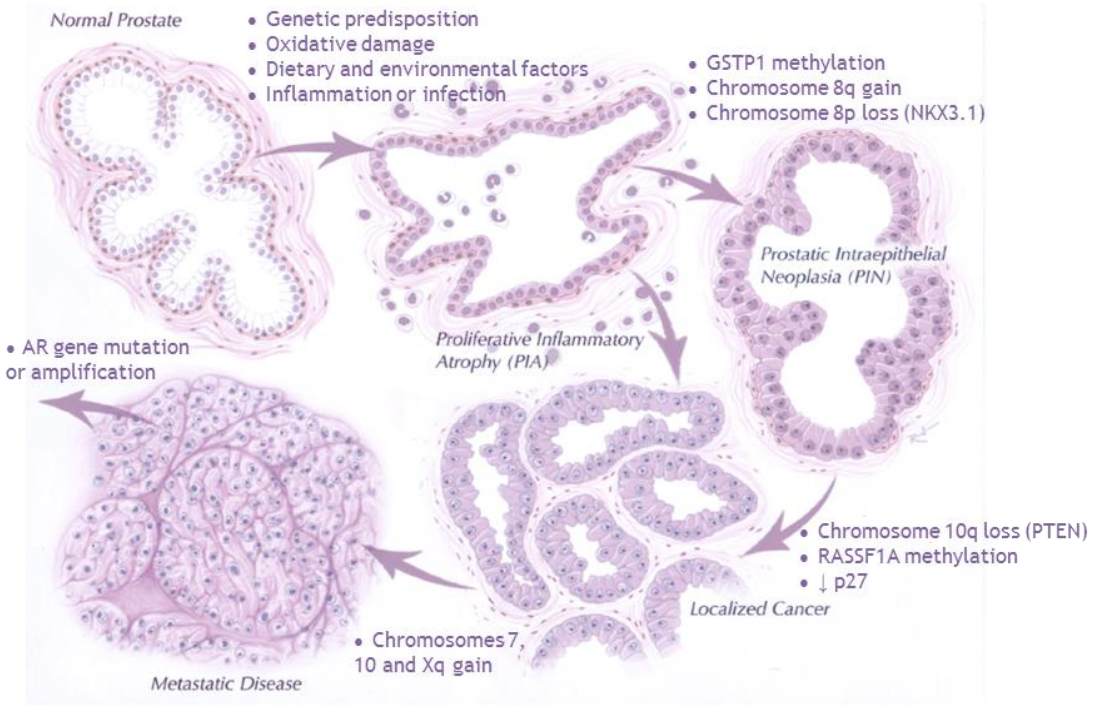

Figure 3: Model of PCa progression (adapted from (45)).

PIN is characterized by abnormal proliferation of the epithelium without stromal invasion, and can be classified into low grade or high grade (47, 48). In low-grade PIN, the nuclei of the cells are enlarged and the nucleoli are discreet or small, while in high-grade PIN the nuclei are large, the nucleoli are prominent and there is an increase of chromatin content similar to that found in carcinoma cells (49, 50). The normal orientation of epithelium proliferation is altered in PIN with increased proliferation in luminal surface instead of the basal cell compartment, which is also a characteristic of other preneoplastic lesions (48, 49).

There are several evidences that support high-grade PIN as a precursor of PCa, such as epidemiological studies, pathology and molecular alterations (50). The incidence of PIN, like PCa, also increases with age. In younger men, low-grade PIN is most frequently found, while high-grade PIN is more likely with advanced age. Also in concordance with PCa, PIN spreads through the prostate in multiple different patterns and its most common location is in the peripheral zone. In addition, PCa and PIN also have similar proliferative and apoptotic indices, are both multicentric and high-grade PIN is often present in areas that are in continuity with PCa (48–50). Some of the most frequent genetic alterations present in PCa are also present in high-grade PIN, particularly the loss of chromosome 8p and gain of chromosomes 8q, 7, 10 and Xq (48, 50, 51). Another characteristic of PIN is the higher microvessel density than in normal prostatic tissue, but smaller than in PCa, thus representing an intermediate state (48, 52). The progressive phenotypic and genetic abnormalities present in PIN represent an intermediate stage between normal prostatic epithelium and PCa. These progressive alterations are marked by the loss of secretory differentiation, including PSA, PAP and secretory proteins (48).

The detection of PIN can only be conclusive through biopsy. Serum PSA concentration, PSA density and the ratio of free to total PSA have a poor correlation with PIN, and due to its microscopic size cannot be detected by TRUS (48).

It has been proposed that PIA as a precursor of PIN and PCa since the cells present a similar phenotype (53). Prostate lesions with inflammation are often associated with atrophy of the epithelium and this type of lesion occurs preferentially in the peripheral zone (7). In a similar way as PIN and PCa, PIA is characterized by an increase of proliferation of epithelial cells in the luminal compartment instead of the basal cells (54). Some of the molecular pathways altered in PCa are also altered in PIA lesions, including downregulation of the tumor-suppressor genes such as NK3 homeobox 1 (*NKX3.1)*, cyclin dependent kinase inhibitor 1B (*CDKN1B)*, and phosphatase and tensin homolog (*PTEN)* (7, 26, 53).

PCa is a heterogeneous disease characterized by several alterations in key regulatory pathways involved in cell cycle regulation, DNA replication and DNA repair (55). Some of the most frequent altered molecular pathways involve the already mentioned tumor suppressor genes *NKX3.1*, *CDKN1B*, and *PTEN*, which are responsible for the regulation of prostate cells growth. However, they are downregulated not only in PCa but also in PIA and PIN lesions (26, 51). *NKX3.1* gene encodes a prostate restricted homeobox protein whose function seems to be essential for normal prostate development and can suppress the growth of prostate epithelial

cells. This gene appears to be a prostate specific tumor suppressor gene. Decreased *NKX3.1* expression seems to be more important for the initiation of the PCa than for the progression to an invasive status (7, 26, 51). *PTEN* gene encodes a phosphatase that has activity on lipids and proteins. This enzyme is responsible for the dephosphorylation and inactivation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a second messenger produced by active phosphatidylinositide 3-kinase (PI_3K) in response to activation of several receptors by growth factors. PIP_3 will recruit several proteins to the plasma membrane, such as protein kinase B (Akt). After Akt activation, several important signaling pathways will be activated, which are involved in inhibition of apoptosis and activation of cell proliferation (Figure 4) (51, 56). *CDKN1B* encodes the cyclin-dependent kinase inhibitor p27, which plays an important role in inhibition of cell cycle. The loss of PTEN expression leads to increased levels of PIP_3, and consequently, activation of the PI_3K-Akt signaling pathway. The continuous activation of this pathway inhibits the expression of *CDKN1B* gene, and consequently, the expression of p27 (26, 45, 51). On the other hand, Inhibition of PI_3K-Akt signaling pathway increases NKX3.1 expression, which in turn promotes p53 activation and inhibits AR promoter activity. Thus, the loss of PTEN expression leads to NKX3.1 downregulation which allows AR overexpression and activation of its target genes that may be involved in onset and progression of PCa (51, 57).

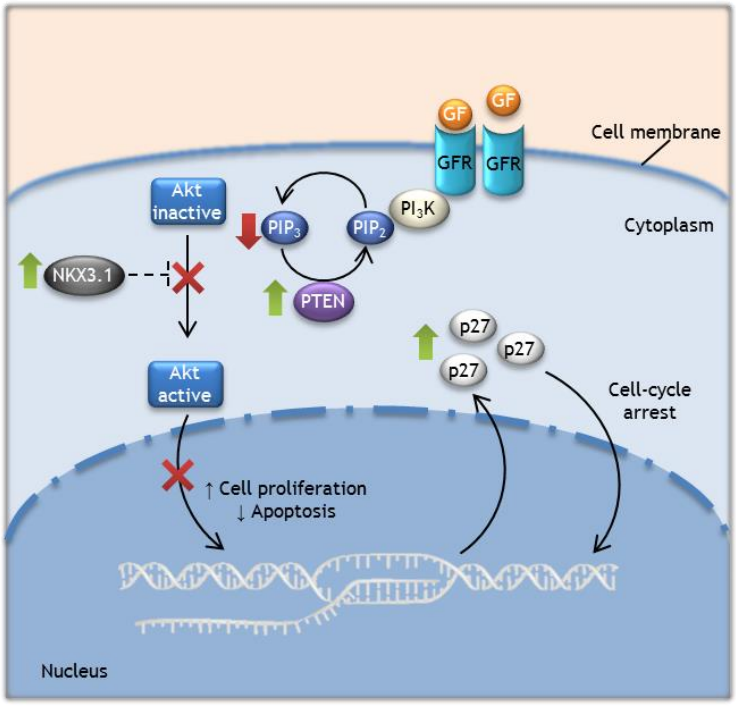

Figure 4: Molecular pathways in normal prostate cells. PTEN, NKX3.1, and p27 proteins regulate the proliferation and apoptosis of prostate epithelial cells. PTEN and possibly NKX3.1 inhibits the PI_3K-Akt signaling pathway, which leads to an increase in p27 levels and apoptosis and decreased proliferation.

Considering that PCa cells retain the AR signaling pathway, androgen ablation results in tumor regression. However, with the tumor progression and accumulation of molecular alterations, there is a gain of function in the AR signaling pathway. At this stage of PCa, which is androgen independent, the tumor cells are resistant to androgen ablation due to their acquired ability to activate the AR signaling pathways involved in cell proliferation and survival without requiring physiological levels of androgens (8). In androgen independent PCa, the AR signaling pathway remains essential for the growth and survival of the tumor. In fact, most of the tumors at this stage have high levels of AR expression and continue to express AR target genes (8). One of the alterations found in PCa is the AR gene amplification, which leads to increased sensitivity of PCa cells to low levels of androgens (58). Other mechanisms involved in androgen-independent phenotype include mutations that change the ligand specificity of the receptor, allowing the activation of the AR by other non-androgen molecules, such as steroid hormones, antiandrogens or even growth factors like insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF) (9, 59–63). Several studies have found that these alterations in the *AR* gene occur in androgen-independent PCa metastasis, but not in primary PCa. However, in primary PCa, the AR signaling pathway already shows alterations, including mutations in the genes that encode the nuclear receptor coactivators and corepressors NCOA2 and NCOR2, respectively (64, 65).

1.3. Genetic and epigenetic mechanisms involved in carcinogenesis of prostate cancer

The transformation of a normal cell into a neoplastic is a multistep process in which the cells gradually acquire alterations in proto-oncogenes, tumor suppressor genes and other genes involved in cellular functions. These alterations lead eventually to the disruption of the network that tightly regulates the homeostatic balance between cell death and proliferation. The molecular mechanisms involved in prostate carcinogenesis remain poorly understood, but it is clear that genetic and epigenetic alterations contribute to this process. Genetic alterations can lead to the expression of abnormal proteins, and consequently the disruption of the signaling pathways that may promote cancer onset and/or progression. Alterations in the cell epigenome may lead to changes in the transcriptional control that will deregulate the cellular mechanisms through inappropriate silencing or activation of cancer-related genes. Genetic, as well as epigenetic changes, are inheritable at the cellular level contributing to the growth of cancer cells. Alterations such as mutations, rearrangements, amplifications or hypomethylation often lead to overexpression or expression of constitutively activated proteins that will induce cellular transformation. On the other hand, alterations like mutations, deletions, allelic loss or hypermethylation are associated with silencing and/or loss of function of proteins. Transformation of normal prostate cells into PCa cells is characterized by a decreased expression or function of genes involved in cell-cycle control, cell adhesion, DNA damage repair and apoptosis, and by an increased expression or gain of function of genes related to cell proliferation, invasion, metastasis, and angiogenesis (reviewed by (51, 55, 66)).

1.3.1. Genetic mechanisms

Cancer development is characterized by the accumulation of genetic alterations that lead to aberrant gene expression. Genetic changes play an important role in tumourigenesis and also in intra- and inter-tumor heterogeneity. In accordance with other types of cancer, PCa is also characterized by the inactivation of tumor suppressor genes and activation of oncogenes, inhibiting apoptosis and allowing cell proliferation. Several genomic alterations have been identified in PCa, from single nucleotide mutations to chromosomal rearrangements, which lead to disruption of signaling pathways that control cellular functions. The most frequent genomic alterations present in PCa are point mutations, gene deletions, gene amplifications and chromosomal rearrangements (26, 55, 67).

One of the most common genetic alterations found in PCa cells is the fusion genes between the androgen-regulated gene *TMPRSS2* and the genes of the ETS transcription factors family *ERG* or *ETV1* (8, 68). TMPRSS2 is a type II transmembrane serine protease prostate specific, which is regulated by androgens. The ETS transcription factors family are involved in

cell proliferation and invasiveness, being then considered oncogenes (7, 68, 69). The TMPRSS2:ERG fusion appears to be an early event in prostate carcinogenesis that is sometimes already present in high-grade PIN, but is absent in PIA and BPH (69). AR activation induces chromosomal proximity between TMPRSS2 and ERG loci allowing the fusion between the 5' end of the *TMPRSS2* gene and the 3' end of the *ERG* or *ETV* genes. The region between these two genes is often deleted as a consequence. The resulting overexpression of the *TMPRS2* and *ERG* genes are thought to be important for the progression and invasiveness of PCa (8, 69).

Other common chromosomal abnormalities are the gains at 7p, 7q, 8q and Xq and the losses at 6q, 8p, 10q, 13q and 16q (46, 55, 70). One of the genes affected by these losses is the *NKX3.1* homeobox gene, which is located in chromosome 8p21 and has one of the two alleles frequently deleted in PCa (26). The loss of heterozygosity at chromosome 10q is associated with the loss of *PTEN* gene expression. The haploinsufficiency of *NKX3.1* and *PTEN* genes can be associated with abnormal proliferation of prostate cells (26, 45). The gains at chromosome Xq lead to aberrant AR activity due to *AR* gene amplification, increasing the sensitivity of PCa cells to very low levels of androgens. This alteration is more frequently found in androgen-independent tumors after hormonal therapy than in primary PCa. Although the cells with *AR* amplification have increased sensitivity, they still require androgens for proliferation (9, 26).

Another frequent genetic alteration found in the *AR* gene is somatic mutations in the ligand–binding domain. These mutations in the *AR* gene decrease the specificity of the AR to testosterone and DHT, allowing inappropriate activation by other steroid hormones, or even androgen antagonists. Therefore, the malignant cells can continue to activate the AR signaling pathway and promote proliferation when the levels of androgens are low by using other circulating steroid hormones. A common example is the missense mutation at position 877, which results in the exchange of an alanine for a threonine (T877A). This alteration in the ligand-binding domain allows the AR activation by antagonists. These mutations have a higher incidence in androgen-independent PCa previously treated with hormone therapy (9, 26, 55).

Some of the most frequent genetic alterations present in PCa are described in table 1.

Table 1: Genetic alterations found in human PCa.

Gene	Location	Alterations	Notes	References
Tumor suppressor genes				
NKX3.1	8p21.2	Allelic losses (↓ expression)	Encodes a prostate restricted homeobox protein	(7, 26, 71)
CDKN1B	12p13.1-p12	Allelic losses (↓ expression)	Encodes cyclin-dependent kinase inhibitor p27	(7, 26, 71)
PTEN	10q23.3	Allelic losses and mutations (↓ expression and/or function)	Encodes a phosphatase with activity on lipids and proteins	(7, 26, 71)
TP53	17p13.1	Mutations	Has many tumor suppressor functions like cell cycle arrest	(7, 26, 71)
Oncogenes				
AR	Xq12	Mutations and amplification (↑ expression or altered function)	Encodes the Androgen Receptor	(7, 26, 71)
MYC	8q24.21	Amplification	Transcription factor that regulates genes involved in cell proliferation, senescence, apoptosis, and cell metabolism	(7, 71)
ERG	21q22.3	Chromosomal rearrangement	ETS transcription factors family	(7, 71)
ETV1-4	7p21.3, 19q13.12, 1q21-q23, 17q21.31	Chromosomal rearrangement	Encodes ETS-like transcription factors 1–4	(7, 71)

1.3.2. Epigenetic mechanisms

In addition to the genetic alterations, the disruption of epigenetic mechanisms may conduct to deregulation of gene expression and is also part of the oncogenic process. Epigenetic mechanisms can be defined as heritable modifications that do not affect the DNA sequence (72). The main epigenetic mechanisms are DNA methylation and histone modifications, which have as main function to ensure proper regulation of gene expression by changing the chromatin structure (51, 73).

Histones are essential in the regulation of chromatin packaging by post-translational modifications that include acetylation and methylation among others. These alterations that occur in the N-terminal histone tails promote alterations in chromatin condensation and DNA accessibility. Histone acetylation is associated with a more relaxed chromatin state, which allows transcriptional activity by permitting the access of transcription factors. Histone acetylation is regulated by two enzymes: histone acetyltransferase (HAT) and histone deacetylases (HDACs) (55, 74, 75). Histone hyperacetylation is associated with transcriptionally active chromatin, while histone hypoacetylation is associated with transcriptionally inactive chromatin (76).

In PCa, increased expression of HDACs is frequent resulting in histone hypoacetylation (51, 55). A high expression of the histone deacetylase 1 (HDAC1) gene is associated with lower expression of its target genes (77). Some of the target genes of HDAC1 include Bax, p21, p27, maspin and p53 (51). Aberrant recruitment of HDACs to the promoter region of tumor suppressor genes could contribute to tumor development and progression. An example of epigenetic inactivation by hypoacetylation of the promoter is the cyclin-dependent kinase inhibitor p21, whose function is to inhibit cell-cycle progression. HDACs are also involved in deacetylation of non-histone proteins. Under stress conditions, p53 is phosphorylated and acetylated to promote protein stability and activation. HDAC1 is able to deacetylate p53, blocking its tumor suppressor activity and allowing tumor progression (75).

DNA methylation consists of the addition of a methyl group by covalent bonding at the 5' position of the cytosine that precedes a guanine. Usually, these CG dinucleotides are concentrated in large clusters, called CpG islands, which are mainly located in the promoter region and/or in the first exon (73). The methylation of these regions leads to gene silencing while unmethylation promotes active gene transcription. The methylation pattern is maintained by DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl group from S-adenosyl-methionine to cytosine (70, 73). Any abnormalities in DNA methylation, which is essential for normal cell function, may lead to the development of several cancers. (73)

Tumor cells are characterized by a methylation pattern that differs from normal cells. In tumor cells, hypermethylation is observed in promoters of specific genes, particularly tumor suppressor genes, and a global hypomethylation contributes to genomic instability and activation of oncogenes (73). In normal cells, DNA hypermethylation is generally observed in

satellite sequences and repetitive genomic sequences, being these regions silenced to ensure genomic stability and integrity. The disruption of this mechanism may lead to tumor development and progression (73). One of the genes found unmethylated in PCa was the retrotransposable element 1 (*LINE-1*). These repetitive sequences constitute approximately 5-10% of the human genome, which are hypermethylated in normal tissues. *LINE-1* hypomethylation was found in more than 50% of PCa cases and in more than 60% of PCa with lymph node metastases (70, 78). The most common example of hypermethylation in PCa is the Glutathione S-transferase π (*GSTP1*) gene a caretaker gene. This gene encodes a phase II detoxification enzyme that is hypermethylated in 90% of PCa, but also in PIA (5-10%) and PIN (70%) lesions. In normal prostate epithelium, the expression of this enzyme allows the detoxification of electrophilic compounds, including carcinogens. Therefore, it is believed that *GSTP1* silencing is involved as an earliest event on PCa development that will turn prostate cells more susceptible to mutations (9, 51, 70). DNA methylation alterations in PCa seems to have two phases, the first that will promote cell transformation, and a second that will promote malignant cancer progression. This is supported by the fact that hypermethylation of *GSTP1*, *APC*, *RASSF1*, *COX2*, and *MDR1* can be detected in localized and metastatic PCa while hypermethylation of estrogen receptor (*ER*), *MLH1* and *p14/INK4* only can be detected in a later phase of PCa progression (78, 79). It has been suggested that epigenetic changes in PCa are more common and arise earlier than genetic alterations and therefore may be future biomarkers for PCa (70, 80). An example of one of the most common alterations for which methods of detection are already being developed, in various types of samples, is the hypermethylation of the promoter region of the *GSTP1* gene (81–83)

DNA hypomethylation of specific genes in tumor cells is less common, and the majority of the hypomethylated promoters belong to tissue-specific genes (73). For example, the Urokinase plasminogen activator (*PLAU*) gene encodes a multifunctional protein that can promote tumor invasion and metastasis. In normal prostate cells, as well as in hormone responsive PCa cells, *PLAU* gene is weakly expressed while in hormone-independent and highly invasive PCa cells, it is highly expressed due to hypomethylation of its promoter region (66, 84).

Some of the most frequently epigenetic alterations present in PCa are described in table 2.

Table 2: Epigenetic alterations found in human PCa.

Gene	Location	Alterations	Notes	References
GSTP1	11q13	Hypermethylation (↓ expression)	Encodes an enzyme that catalyzes the conjugation of reduced glutathione to electrophilic substrates	(70, 74)
NKX3.1	8p21.2	Hypermethylation (↓ expression)	Encodes a prostate restricted homeobox protein	(55, 85)
APC	5q21-q22	Hypermethylation (↓ expression)	Adenomatous polyposis coli (APC) gene encodes a tumor suppressor protein	(70, 74)
RASSF1A	3p21.3	Hypermethylation (↓ expression)	Ras association domain family member 1 (RASSF1A) gene encodes a protein similar to the RAS effector proteins	(70, 74)
PTGS2	1q25.2-q25.3	Hypermethylation (↓ expression)	prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase, is a key enzyme in prostaglandin biosynthesis	(74)
LINE-1	22q11.1-q11.2	Hypomethylation	Retrotransposon element 1	(70)
PLAU	10q22.2	Hypomethylation (↑ expression)	Urokinase plasminogen activator gene encodes a secreted serine protease	(66)

1.4. Six transmembrane epithelial antigen of the prostate 1

1.4.1. General characteristics

The Six transmembrane epithelial antigen of the prostate 1 (*STEAP1*) gene was first identified by Hubert and colleagues as a gene overexpressed in PCa using a subtractive hybridization between benign prostatic tissue and PCa xenografts model (86). The *STEAP1* gene is one of the four members of the STEAP family, which includes the genes encoding the STEAP1-4 proteins. Also, a very similar gene to STEAP1 is encoded by the human genome, called *STEAP1B*.

STEAP1 is located at the long arm of chromosome 7q21 and has 10.4 kb, comprising 5 exons and 4 introns (Figure 5). This gene encodes two different mRNA transcripts of 4.0 kb and 1.4kb, but only the last is translated into a protein with 339 amino acids (aa) with approximately 40 kDa (86). STEAP proteins have in common a six-transmembrane domain, an intramembrane heme binding site and intracellular N- and C-termini. The role of the STEAP1 protein remains unclear due to the lack of FNO-like domain and Rossman fold, which are involved in oxidoreductase activity of iron and copper by STEAP2, STEAP3, and STEAP4 (87, 88). Although STEAP1 does not promote iron or copper reduction or uptake, its co-localization in endosomes with transferrin and transferrin receptor 1, specialized proteins in iron uptake, suggests that it may still play a role in metal homeostasis (87). STEAP1 protein is mainly localized on the plasma membrane of epithelial cells, particularly at cell-cell junctions, and its predicted structure supports the idea that this protein may act as an ion channel or transporter protein (86). In fact, it was reported that STEAP1 may allow the transport of small molecules between adjacent cells in culture, indicating that STEAP1 may be involved in intercellular communication (89, 90).

Regarding the *STEAP1B* gene, it is located on the same chromosome as *STEAP1*, but on the short arm (chr:7p15). *STEAP1B* gene shares high homology with *STEAP1* gene possibly due to gene duplication during genome evolution. This gene encodes two different mRNA transcripts, *STEAP1B1,* and *STEAP1B2*. *STEAP1B1* encodes a protein with 332 aa, while *STEAP1B2* encodes a protein with 245 aa. However, it remains to be demonstrated the expression of these proteins. Nevertheless, *in silico* analysis showed that both proteins have some similarity with STEAP1, but with fewer transmembrane domains. In concordance with STEAP1, STEAP1B also lacks the FNO-like domain and Rossman fold. (91, 92).

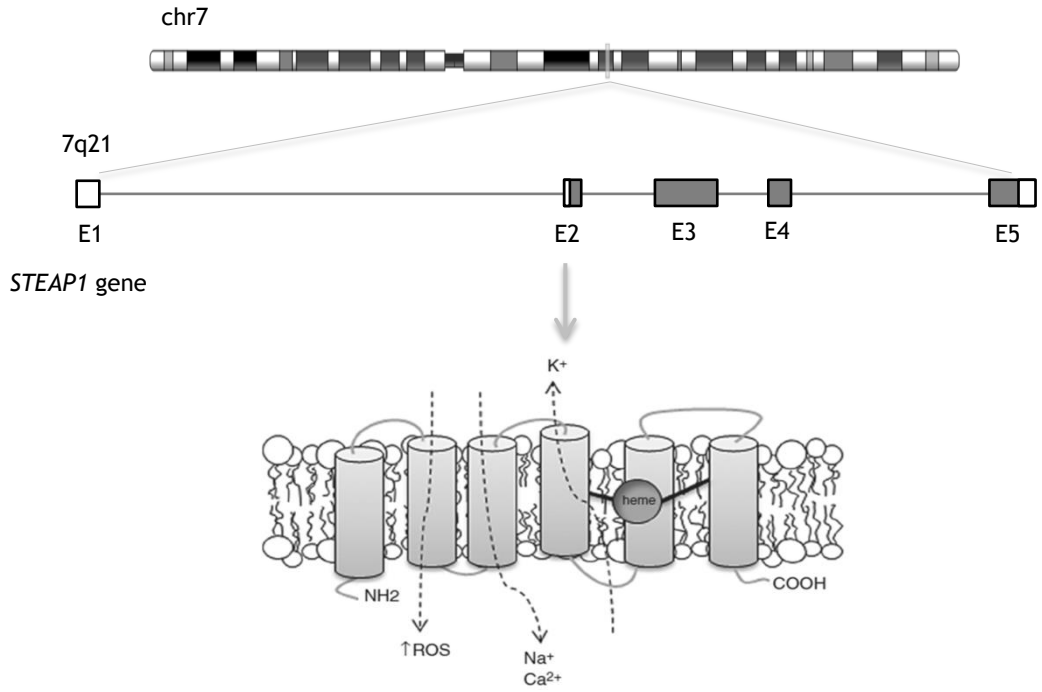

Figure 5: Location of *STEAP1* gene at the long arm of chromosome 7, *STEAP1* gene organization and schematic of STEAP1 protein structure (Adapted from (93)).

1.4.2. Expression in human tissues

STEAP1 is overexpressed in all stages of PCa, including metastasis, but its expression is most pronounced in androgen sensitive stages than in androgen-independent stages (86). STEAP1 is also overexpressed in other types of tumors, such as breast, lung, bladder, colon, pancreas, ovary and Ewing sarcoma, where the role of STEAP1 has also been studied (86, 89, 94). STEAP1 expression in normal tissues is almost restricted to prostate, but is also expressed at lower levels in other tissues such as bladder, fetal and adult liver, kidney, pancreas and skeletal muscle, as summarized in Table 3 (86, 87). Regarding PIN lesions, STEAP1 expression also shows high levels, suggesting that STEAP1 deregulation is an earlier event in PCa development (95). The STEAP1 levels found in BPH are very low and similar to those that are found in non-neoplastic adjacent tissue of PCa (95). The expression levels of STEAP1 protein in the different types of normal and cancer tissues are described in table 3.

Concerning *STEAP1B* gene, both mRNAs transcripts are expressed in prostate cell lines PNT1A, PNT2, LNCaP, and PC3. It was shown that *STEAP1B2* mRNA is overexpressed in neoplastic cells when compared to non-neoplastic cells, indicating that this gene may also be deregulated in cancer. On the other hand, *STEAP1B1* mRNA does not seem to be differentially expressed between neoplastic and non-neoplasic cells (91).

Table 3: Expression of STEAP1 protein in normal and cancer tissues.

	Protein		
Tissue	**Normal**	**Cancer**	**Reference**
Bladder	Low/Not detectable	Moderate/High	(89, 96)
Bone marrow	Not detectable	-	(86, 87)
Breast	Low	Moderate/High	(94)
Colon	Low	Low	(86, 87)
Heart	Not detectable	-	(86, 87)
Liver	Not detectable	-	(86, 87)
Lung	Not detectable	Moderate	(86, 87, 89)
Kidney	Low/Not detectable	Moderate/High	(86, 87, 96)
Pancreas	Low	-	(86, 87)
Placenta	Not detectable	-	(86, 87)
Prostate	Moderate	High	(86, 87)
Skeletal muscle	Not detectable	-	(86, 87)
Stomach	Low	-	(86, 87)
Thymus	Not detectable	-	(86, 87)

1.4.3. Biological functions and its regulation in normal and cancer cells

STEAP1 protein localization at cell junctions and its predicted secondary structure as a channel protein suggest that it may play a role in intercellular communication through the diffusion of ions and small molecules between cells (89, 90, 93). In fact, it has been demonstrated that ion channels contribute to the regulation of several biological processes, such as proliferation, differentiation, and apoptosis. In addition, the malignancy and invasiveness of PCa androgen-independent cells are associated with an altered expression of several ion channels in the plasma membrane, enhancing the apoptotic resistance (97).

In Ewing tumors, STEAP1 protein seems to promote cell growth by increasing intracellular reactive oxygen species (ROS) levels. In fact, STEAP1 overexpression is associated with increased proliferation and invasiveness through the increased cellular ROS levels. The oxidative stress, which may result from STEAP1 overexpression, may enhance tumor aggressiveness through the activation of genes involved in cell proliferation and invasiveness, such as *MMP-1*, *ADIPOR1*, and *DTX3L*. Also, high levels of ROS are associated with the activation of metastatic signaling pathways, which is associated with cancer aggressiveness (98).

The hypothesis that STEAP1 is involved in cancer cell proliferation is supported by the fact that the treatment of PCa cells with STEAP1 monoclonal antibodies is able to inhibit the

cell growth (89). Moreover, it was also verified that the knockdown of STEAP1 expression on tumor cells is associated with an antitumor effect due to the disruption of intercellular communication between tumor cells and adjacent tumor-associated stromal cells (90).

Regarding the regulation, it was already shown that STEAP1 expression is regulated by androgens, estrogens, and zoledronic acid. In respect to androgens and estrogens, it was demonstrated that *STEAP1* gene is down-regulated in rat prostate and mammary gland, as well as in LNCaP cells and MCF-7 breast cancer cells (94, 99). STEAP1 down-regulation by androgens is mediated by the AR signaling pathway and seems to be dependent on *de novo* protein synthesis (99). On the other hand, the STEAP1 down-regulation by estrogens does not seem to be mediated by the estrogen receptor signaling pathway (94, 99). Concerning the effect of zoledronic acid on STEAP1 expression in PCa cells, STEAP1 expression decrease in a dose-dependent manner (100). Zoledronic acid is one of the most commonly used bisphosphonates for the prevention and treatment of skeletal complications in PCa patients with bone metastasis (101).

The mechanisms that lead to overexpression of *STEAP1* in human tumors remain poorly understood. The regulation of *STEAP1* expression by post-transcriptional and post-translational mechanisms was already assessed through *STEAP1* mRNA and protein stability. In LNCaP cells, *STEAP1* mRNA and protein stability are higher when compared with PNT1A cells, suggesting that post-translational mechanisms may contribute for STEAP1 overexpression. *In silico* analysis of post-translational modifications in STEAP1 protein reveal some potential sites for N-glycosylation, glycation, phosphorylation and O-β-GlcNAcylation (91). These modifications play a role in common mechanisms of protein function regulation and may confer higher stability to proteins. Alterations in these mechanisms may lead to the development several diseases, including cancer (91, 102). However, these alterations still do not justify the overexpression of *STEAP1* in tumor cells, suggesting that other mechanisms may be involved.

2. Objectives

STEAP1 is overexpressed in several human tumors, particularly in PCa, and several investigators have pointed it out as a potential biomarker or therapeutic target. Regarding the regulation of *STEAP1* gene, some factors have been identified as involved in regulation of its expression, but the mechanisms that lead to *STEAP1* overexpression in PCa remain unknown. Therefore, the aim of this project was to identify molecular mechanisms involved in overexpression of the *STEAP1* gene in LNCaP PCa cells. To achieve this goal, it was hypothesized that genetic and/or epigenetic changes may be involved in *STEAP1* regulation in PCa. To test this hypothesis, several experimental approaches were delineated in order to:

- Identify mutations in the promoter region of the *STEAP1* gene in LNCaP cells.
- Compare the methylation pattern of the *STEAP1* gene between PNT1A (non-neoplastic cells) and LNCaP cells.
- Evaluate the effect of treatment with 5-Aza-2'-deoxycytidine (AZA) and Trichostatin A (TSA), DNMT and HDAC inhibitors respectively, on *STEAP1* mRNA expression in PNT1A cells.

3. Materials and Methods

3.1. Sequence analysis of the promoter region of the *STEAP1* gene

In order to evaluate if there is any genetic alteration in the *STEAP1* gene, which may lead to its overexpression, the promoter region and the first exon was sequenced according to Figure 6.

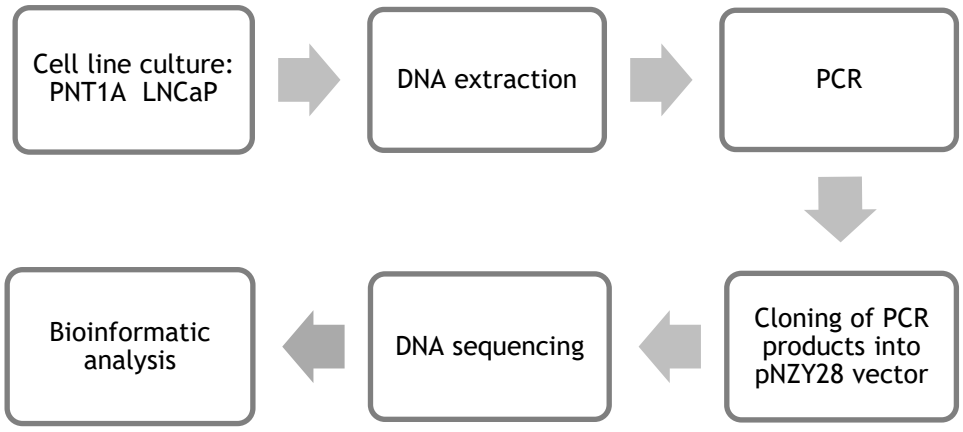

Figure 6: Diagram with the procedures used in the sequence analysis of the promoter region of the *STEAP1* gene.

3.1.1. Cell lines culture

PNT1A and LNCaP cell lines were cultured at 37ºC in a 5% CO_2 atmosphere with RPMI 1640 phenol-red medium (Sigma-Aldrich, Sintra, Portugal) supplemented with 10% FBS and 1% penicillin/streptomycin.

3.1.2. DNA extraction and quantification

DNA purification from PNT1A and LNCaP cells was performed using the Gentra Puregene Cell Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantification of the extracted DNA and its purity was assessed by measuring its absorbance at 260 and 280 nm in a nanophotometer (Implen NanoPhotometer UV/Vis spectrophotometer). DNA integrity was verified by agarose gel electrophoresis.

3.1.3. Polymerase chain reaction (PCR)

PCR reactions were carried out using 200 ng of genomic DNA from PNT1A or LNCaP cells in 25 µL reaction containing 1 U of TrueStart Hot Start Taq DNA Polymerase (Thermo Scientific, Massachusetts, EUA), 2.5 mM of $MgCl_2$, 2.5 µL of 10× TrueStart Hot Start Taq buffer (Thermo Scientific), 10 mM dNTPs and 300 nM of each primer. After initial denaturation at 95°C for 5 min, 35 cycles were carried out as follows: denaturation at 95°C for 1 min, annealing temperature (56°C, 58°C, 60°C and 62°C) for 45 sec and polymerization at 72°C for 30 sec, followed by a final extension at 72°C for 5 min. All steps of PCR reaction were carried out in a Thermocycler (BIO-RAD T100 Thermal Cycler). The four pairs of primers used are described in table 4 and the arrangement of the primers is shown in figure 7.

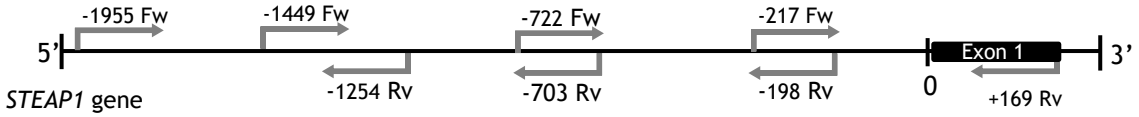

Figure 7: Arrangement of the primers used to amplify the promoter region and the first exon of the *STEAP1* gene

Table 4: Primers sequences and respective amplicon sizes used for amplification of the promoter region of *STEAP1* gene.

Primers	Sequence	Amplicon size (bp)
STEAP1_prom_-217fw STEAP1_ex1_169rv	5' taataagcccccgggtaatc 3' 5' CCACTCTTCGCCTTAGCTTG 3'	386
STEAP1_prom_-722fw STEAP1_prom_-198rv	5' aggaccggctgttaggtttt 3' 5' gattacccgggggcttatta 3'	525
STEAP1_prom_-1449fw STEAP1_prom_-703rv	5' aggcggcatgctagttaaga 3' 5' aaaacctaacagccggtcct 3'	747
STEAP1_prom_-1955fw STEAP1_prom_-1254rv	5' aaacaaaaatatttggggtttga 3' 5' tcattcttgggtgtttctcg 3'	702

3.1.4. Cloning of PCR products into pNZY28 vector

The PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, cloned into a pNZY28 vector (Nzytech) and amplified in competent *Escherichia coli* (E. coli) TOP10 cells. These were first plated in LB agar medium (CONDA, Madrid, Spain) with IPTG (0.5 mM), X-Gal (80 µg/mL) and Ampicillin (100 µg/mL) and incubated at 37ºC overnight. Then, at least 3 white colonies of each set of primers and cell line were selected and incubated at 37 ºC overnight in tubes with 3 mL of LB-Broth medium (1% Tryptone, 0.5% Yeast Extract, 1% NaCl; pH 7.5) with Ampicillin (100µg/mL). The plasmid DNA extraction was carried out using Miniprep kit (Nzytech) according to the manufacturer's instructions.

3.1.5. DNA sequencing

Sequencing of plasmid DNA (pDNA) was performed using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. For each sample of pDNA the DNA sequencing reaction was performed in both strands in forward and reverse directions with the standard sequencing primers M13rv and T7. The reagents and respective volumes used in the DNA sequencing reactions are described in table 5. Each DNA sequencing reaction was prepared in a PCR tube, where it was performed a pre-denaturation step at 96ºC for 5 min only with pDNA and H_2O. Then, Master Mix and sequencing primer was added to each PCR tube and the following thermal cycler program was performed: 96ºC for 8 min, followed by 30 cycles of 96ºC for 20 sec, 50ºC for 20 sec and 60ºC for 4 min, followed by a final step of extension at 60ºC for 8 min. After the DNA sequencing reaction, the DNA was precipitated with ethanol. After the pellet was dry, the sample was resuspended in 10 µL of Sample Loading Solution and incubated at room temperature for 10-15 min. The resuspended samples were transferred to wells of a sample plate and each well was overlaid with one drop of light mineral oil. The sequencing products were separated on an automated capillary DNA sequencer (GenomeLab™ GeXP, Genetic Analysis System; Beckman Coulter). The sequencing data analysis was performed using the programs Nucleotide BLAST and Clustal Omega.

Table 5: Reagents and volumes used in each DNA sequencing reaction.

Reagents	**Volume (µL)**
H_2O	3.75 µL
pDNA	2.0 µL
DTCS Quick Start Master Mix (Beckman Coulter)	4.0 µL
Primer M13RV or T7	0.25 L

3.1.6. Bioinformatic analysis

The prediction of putative transcription factors (TF) binding to the promoter region of *STEAP1* gene was carried out using the program Alggen Promo software 3.0. The maximum matrix dissimilarity rate was defined at 15%.

3.2. Analysis of methylation profile of the *STEAP1* promoter region

The procedures used to determine the methylation pattern of the *STEAP1* gene are described in figure 8. The cell line culture, DNA extraction, cloning of PCR products into PNZYA vector and DNA sequencing were performed in a similar manner to that described in section 3.1. The *STEAP1* gene sequence was first analyzed with the Methyl Primer Express software (Applied Biosystems) in order to assess if *STEAP1* had some relevant "CpG island".

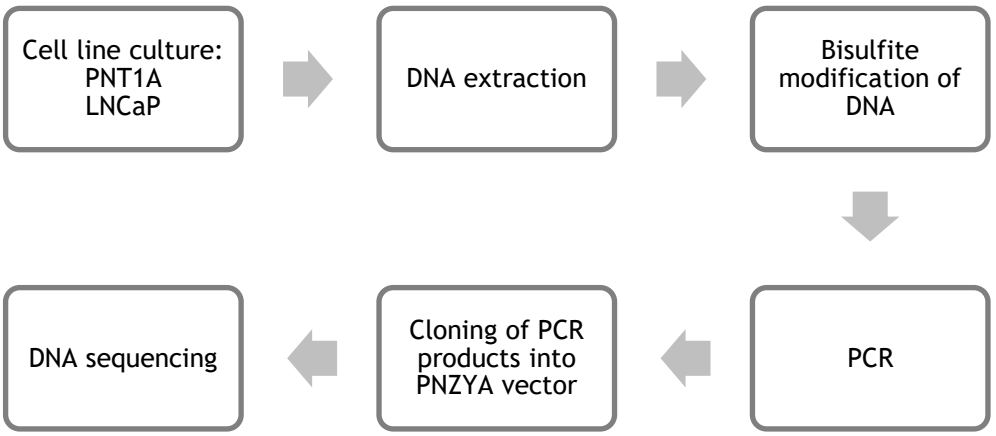

Figure 8: Diagram with the procedures used to determine the methylation pattern of the *STEAP1* gene.

3.2.1. Bisulfite modification of DNA

Bisulfite modification of DNA is one of the most used methods to determine the methylation pattern of a specific DNA sequence. The treatment of DNA with sodium bisulfite allows the detection of 5-methylcytosines since bisulfite converts unmethylated cytosines into uracils but spares the methylated cytosines that remain unchanged (Figure 9). This method consists of four main steps: denaturation of DNA, sulphonation (addition of bisulfite to cytosines and formation of a sulphonated cytosine derivate), deamination (hydrolytic deamination of the sulphonated cytosine derivate to a sulphonated uracil derivate) and desulphonation (removal of the sulphonate group by an alkali treatment to give uracil). To determine the methylation status, it is necessary to perform a subsequent PCR reaction with specific primers (103, 104).

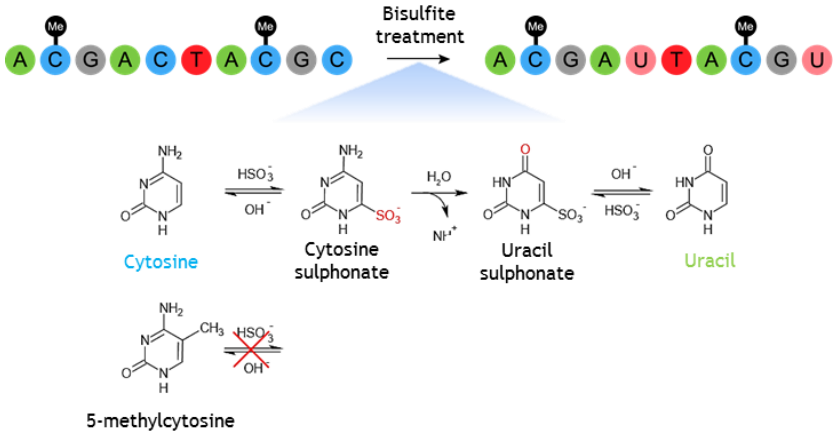

Figure 9: Bisulfite conversion of DNA. Bisulfite treatment converts cytosines to uracil. Methylation at position 5 of cytosine ring protects the compound from conversion.

In order to evaluate the methylation pattern, 1 µg of genomic DNA from PNT1A and LNCaP cells were converted using the EZ DNA Methylation-Gold kit (ZYMO RESEARCH, California, USA) according to the manufacturer's instructions. The modified DNA was eluted in 15 µl of M-Elution Buffer and stored at -20ºC.

3.2.2. PCR

PCR reactions were carried out in a similar manner to that described in section 3.1.3. using 200 ng of genomic DNA from PNT1A or LNCaP cells previously treated with sodium bisulfite. Three pairs of primers were designed using the program Methyl primer express v1.0 in order to not cover any CG-dinucleotides in their binding sites. The primer sequences and respective amplicon size are described in table 6 and the arrangement of the primers is shown in figure 10.

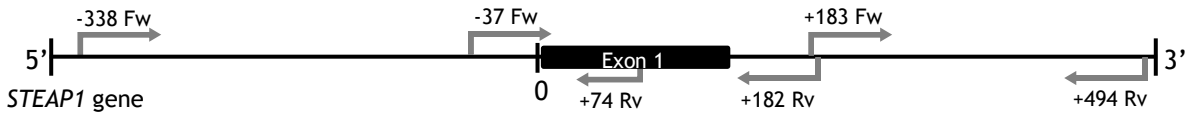

Figure 10: Arrangement of the primers used to amplify the bisulfite treated DNA.

Table 6: Primer sequences and respective amplicon size used for amplification of the bisulfite treated DNA.

Primer	Sequence	Amplicon size (bp)
STEAP1_-338 FW STEAP1_+74 RV	5' AAAGTGTGATTTGGGAATGTTTTT 3' 5' TTTTAAGTTAGTTGTAGGTTTT 3'	412
STEAP1_-37 FW STEAP1_+182 RV	5' TGGGGAGTTTTAGTTTTTAAGG 3' 5' TTAAGGGATTTATTTATTTTT 3'	219
STEAP1_+183 FW STEAP1_+494 RV	5' AAGAGTGGGTGAGTTTTTTGAA 3' 5' TTGTTATTAAAATTTAATTTGAG 3'	346

3.3. Treatment of PNT1A cells with DNMT and HDAC inhibitors and the effect on *STEAP1* mRNA expression

To evaluate the effect of treatment with DNMT and HDAC inhibitors on *STEAP1* mRNA expression in PNT1A cells, the procedures described in figure 11 were used.

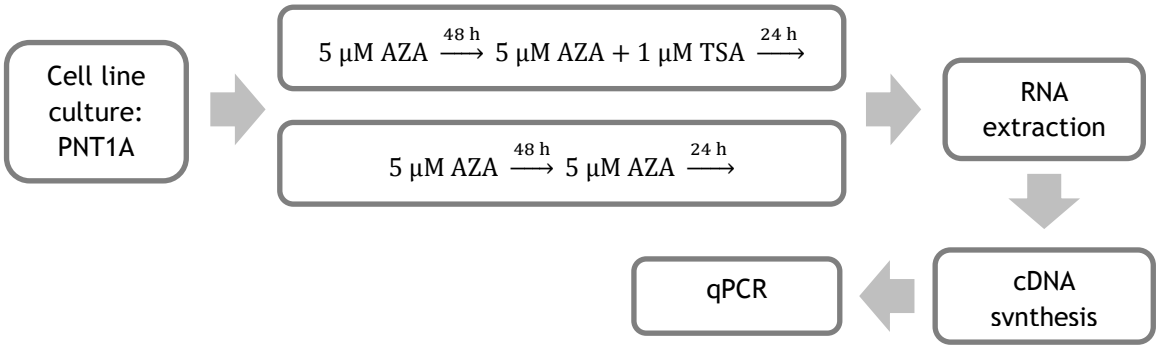

Figure 11: Diagram with the procedures used to evaluate the effect of treatment with DNMT and HDAC inhibitors on *STEAP1* mRNA expression in PNT1A cells.

3.3.1. Cell line culture and treatment

PNT1A cells were cultured as described in section 3.1.1. Approximately 3×10^5 cells were seeded in cell culture multiwell plates of 6 wells. When a growth confluence of 60% was achieved the RPMI medium was replaced by fresh medium supplemented with 5 µM of AZA (Sigma-Aldrich) for 72 hours, or 48 hours with 5 µM AZA followed by 24 hours with 5 µM AZA and 1 µM TSA (Sigma-Aldrich). In the control group, the medium was replaced by RPMI medium for 72 hours. The AZA and TSA reagents were dissolved in DMSO at 5mM and 1 mM, respectively, and stored in aliquots at -20ºC.

3.3.2. RNA extraction and cDNA synthesis

Total RNA extraction was performed using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA pellet was dried, resuspended in 20 µL of DEPC-treated water and storage at -80ºC. In order to assess the quantity of total RNA, its optical density was determined by measuring absorbance at 260 and 280 nm. Total RNA integrity was verified by agarose gel electrophoresis.

cDNA synthesis was performed in order to convert RNA in a single-stranded cDNA template. The NZY First-Strand cDNA Synthesis KIT (Nzytech) was used according to the manufacturer's instructions. For each sample, 1.0 ug of total RNA was converted.

3.3.3. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was carried out to evaluate the expression of *STEAP1* mRNA in PNT1A cells treated with AZA and AZA+TSA. To normalize the expression of *STEAP1*, human GAPDH (hGAPDH) and human beta-2-microglobulin (hß2M) primers were used as internal controls. qPCR reactions were carried out using 1 µl of cDNA synthesized in a 20 µl reaction containing 10 µl of Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific) and primers for each gene. After initial denaturation at 95°C for 5 min, 30 cycles were carried out as follows: denaturation at 95°C for 10 sec, annealing temperature 60°C for 30 sec and polymerization at 72°C for 20 sec. The specificity of the qPCR reactions was assessed by melting curves analysis. Samples were run in triplicate in each PCR assay. The primers efficiency was determined by a series of dilutions (1; 1:10; 1:100; 1:1000). All steps of qPCR reaction were carried out in a Real Time Thermocycler (BIO-RAD CFX Connect Real-Time PCR Detection System). Fold differences were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-(\Delta\Delta Ct)}$ (105). The primers sequence for each gene and respective amplicon sizes used in qPCR are described in Table 7.

Table 7: Primers sequences and respective amplicon size used in qPCR analysis.

Primers	Sequence	Amplicon size (bp)
hSTEAP_619fw hSTEAP_747rv	5' GGCGATCCTACAGATACAAGTTGC 3' 5' CCAATCCCACAATTCCCAGAGAC 3'	128
hGAPDH_74fw hGAPDH_149rv	5' CGCCCGCAGCCGACACAT C 3' 5' CGCCCAATACAATCCG 3'	75
hß2M_347fw hß2M_439rv	5' ATGAGTATGCCTGCCGTGTG 3' 5' CAAACCTCCATGATGCTGCTTAC 3'	92

3.3.4. Statistical analysis

Data analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, California, USA). The statistical significance of differences in *STEAP1* mRNA expression for the treatment with DNMT and HDAC inhibitors in PNT1A cells was assessed by Student's t-test. Significant differences were considered when $p<0.05$ compared to control values. All experimental data are shown as mean ± standard error of the mean (SEM).

4. Results and Discussion

4.1. Sequence analysis of the *STEAP1* promoter region

PCa is characterized by the accumulation of genetic alterations that contribute not only to tumor progression but also to the heterogeneity of the disease. Oncogene activation often occurs as a result of genetic alterations such as point mutations, chromosomal translocations or gene amplifications (26, 55, 67). Since several studies indicate that STEAP1 promotes cell growth, thus acting as an oncogene, it is important to understand the mechanisms that are behind its deregulation (89, 90, 98). In order to look for mutations that may cause STEAP1 overexpression, the sequence of the promoter region of *STEAP1* gene was sequenced using genomic DNA from PNT1A and LNCaP cells, which represent a non-neoplastic cell line of human prostate epithelium and an androgen dependent PCa cell line, respectively.

As already mention above, *STEAP1* and *STEAP1B* genes show a high homology to each other, which prevents the design of specific primers for the *STEAP1* gene and the direct sequencing of PCR products. Thus, it was necessary to clone the PCR product into a vector, which was then amplified in competent E. coli cells. The plasmid vector was extracted from several colonies of transformed E.coli cells and sequenced. The sequences obtained were analyzed using the program BLAST and Clustal Omega in order to identify the identity of the sequence. The sequences that matched to *STEAP1* were then compared with the genomic sequence, obtained at UCSC Genome Browser Home (CCDS5614.1), in order to verify if there was any difference in the nucleotide sequence.

The sequence analysis revealed some alterations when compared with the available genomic sequence of *STEAP1*. One of the alterations identified was the substitution of a guanine by a cytosine at position -1863 from the transcription start site (Figure 12). This variation was found in both PNT1A and LNCaP cells and was already identified in the Ensembl database (Variation: rs28164 SNP) (106). Population genetic studies for this variant indicate that the variant -1863 G/C is present in 98% of the world population and in 94% of the European population. The most frequent genotype for this variation is C|C. Another alteration found was the substitution of adenine for cytosine at position -1195 in LNCaP cells (Figure 13). Although this variation was already identified in the Ensembl database (Variation: rs112949159 SNP) there are no population genetic studies available. It was also found the deletion of the G at the position -640 in LNCaP cells (Figure 14). This variant, -640 G/-, was also identified in the Ensembl database (Variation: rs140486583 DELETION). Population genetic studies show that this variation has a frequency of 17% in the world population and 28% of the European population and that the most frequent genotype is G|G. The last alteration found was the substitution of an adenine for a guanine at the position -563 in LNCaP cells (Figure 15). So far, this variation has not yet been identified. All of the identified variants are upstream gene variants. Although it was possible to identify some variants in the *STEAP1* gene sequence with the sequencing method, it was not possible to determine whether these nucleotide changes were situations of heterozygosity or homozygosity.

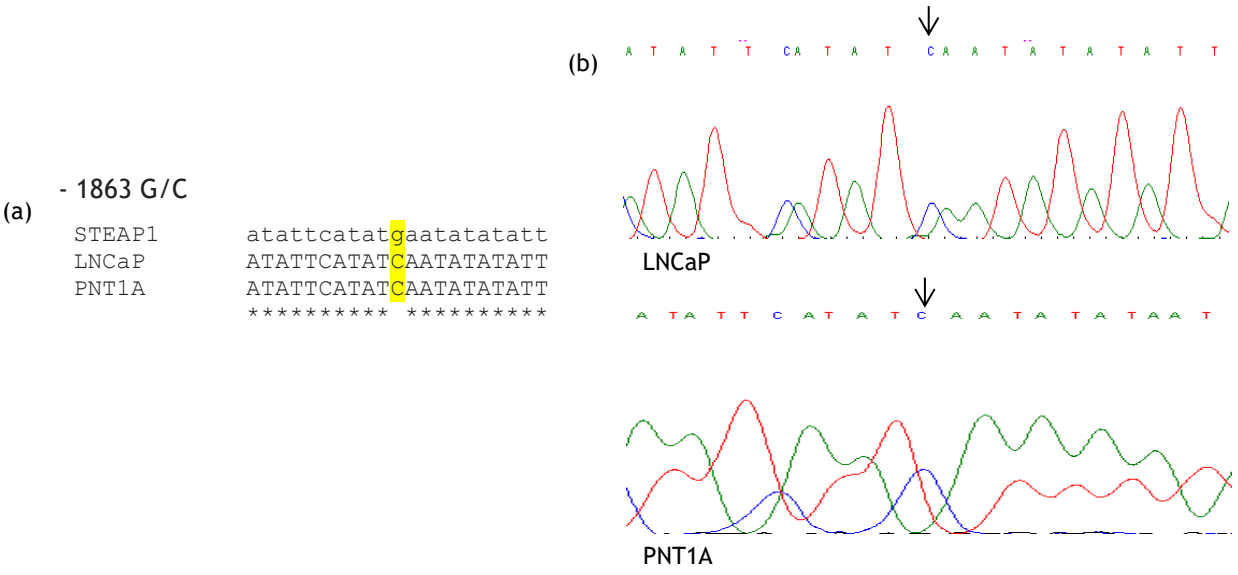

Figure 12: (a) Multiple sequences alignment of the *STEAP1* gene sequence with the sequences obtained from PNT1A and LNCaP cells sequencing. (b) Partial nucleotide sequence showing the variant -1863 G/C in LNCaP cells. The arrows indicated the position of the G to C substitution.

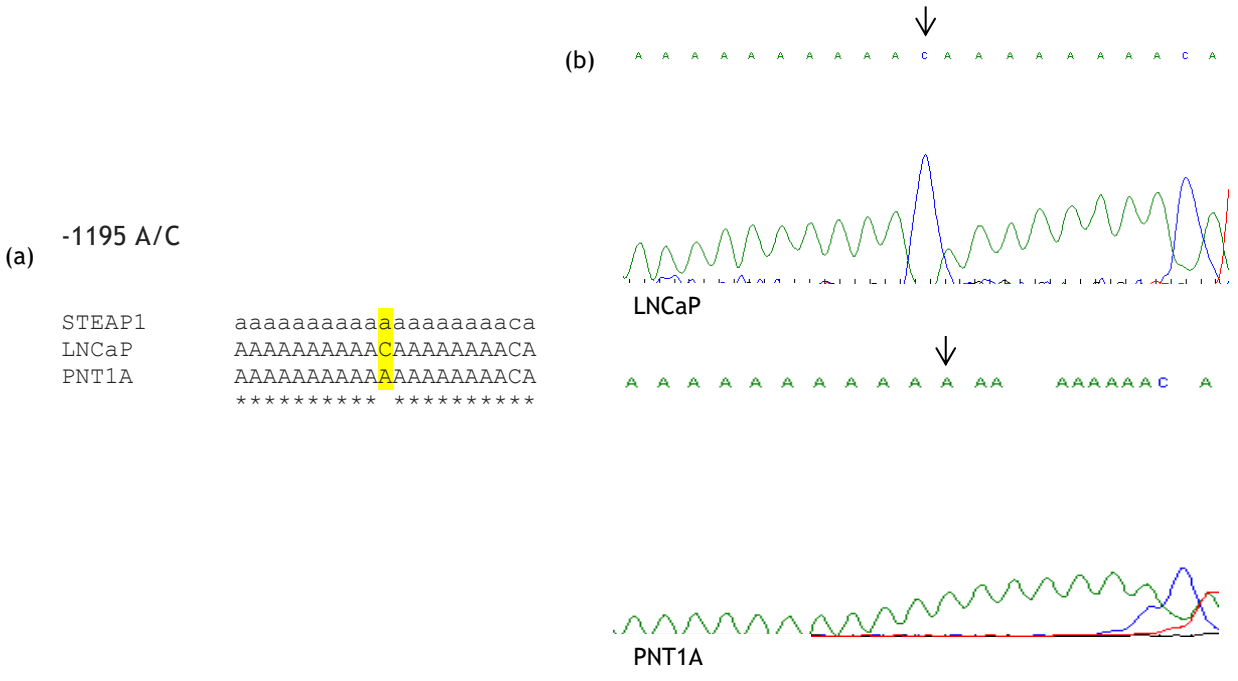

Figure 13: (a) Multiple sequences alignment of the *STEAP1* gene sequence with the sequences obtained from PNT1A and LNCaP cells sequencing. (b) Partial nucleotide sequence showing the variant -1195 A/C in LNCaP cells. The arrows indicated the position of the A to C substitution.

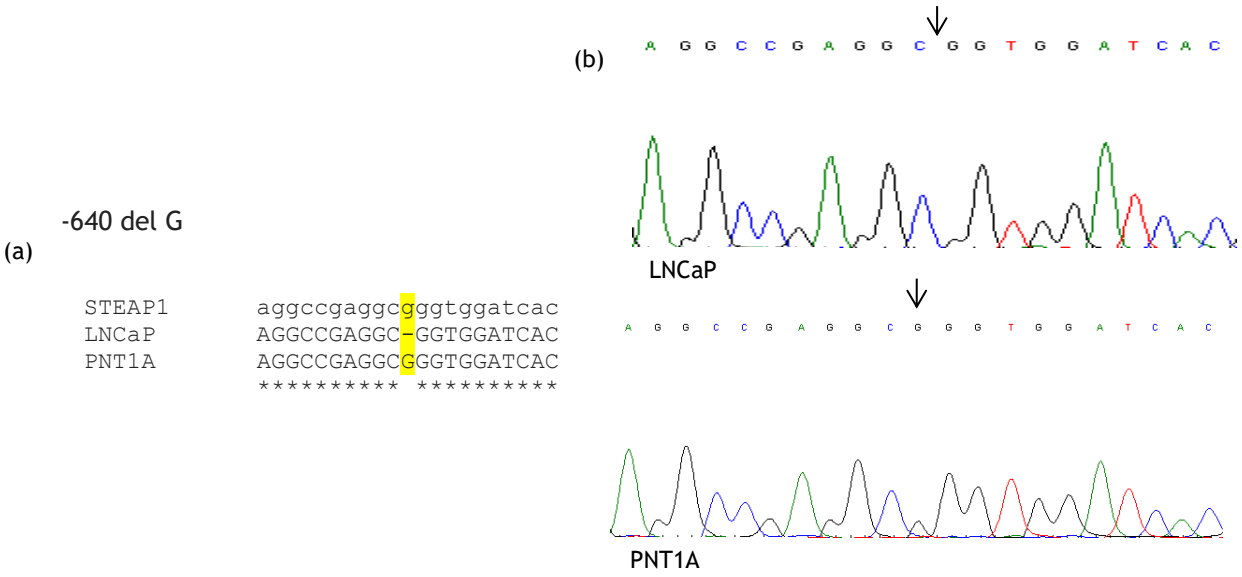

Figure 14: (a) Multiple sequences alignment of the *STEAP1* gene sequence with the sequences obtained from PNT1A and LNCaP cells sequencing. (b) Partial nucleotide sequence showing the variant -640 del G in LNCaP cells. The arrows indicated the position of the G deletion.

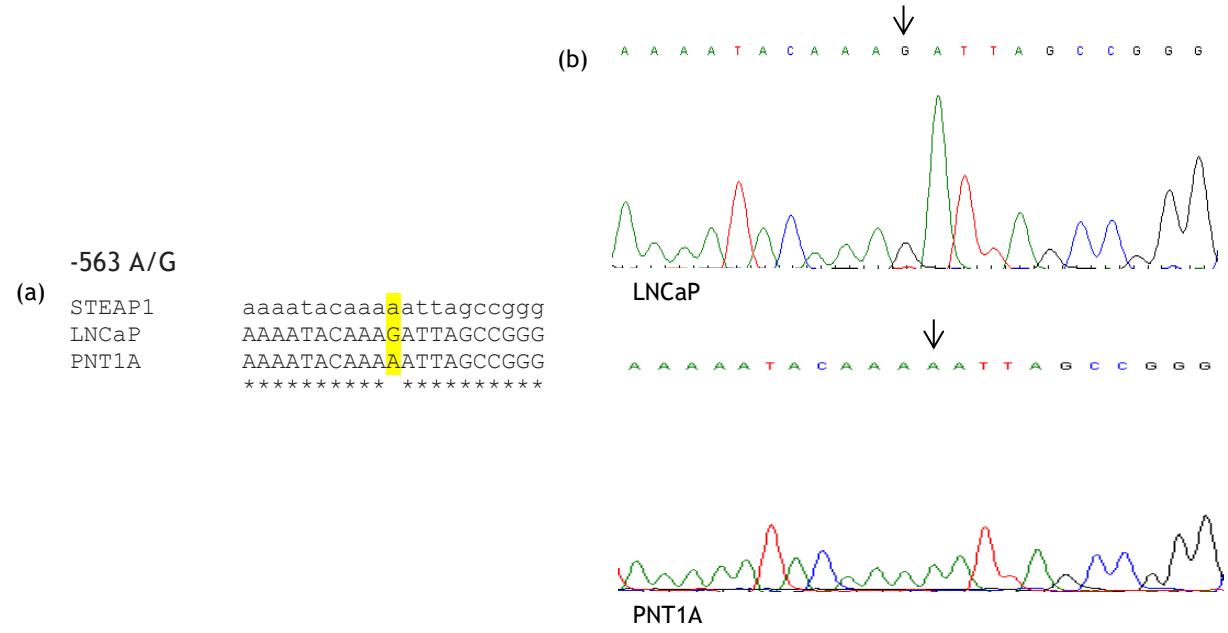

Figure 15: (a) Multiple sequences alignment of the *STEAP1* gene sequence with the sequences obtained from PNT1A and LNCaP cells sequencing. (b) Partial nucleotide sequence showing the variant -563 A/G in LNCaP cells. The arrows indicated the position of the A to G substitution.

In order to verify if some of the identified variants are located in important regions for the binding of TFs, an *in silico* analysis was carried out using the program Alggen Promo software 3.0 (107, 108). Table 8 summarize the putative TF binding to each allelic variant and the respective dissimilarity value for each one of the predicted bindings. In the table is also indicated the nucleotide sequence of the potential binding site. The dissimilarity value is defined as the rate of dissimilarity (%) between the putative and consensus sequences. The results for the putative TFs binding to each allelic variant showed some differences between the two alleles of each variant. In the case of the variant -1863 G/C, only the TF XBP-1 may bind to variant with "G", whereas the allele with "C" may bind to GATA-1 and two members of the TF family CCAAT/enhancer binding proteins (C/EBP). Although there are different TFs binding to each one of the alleles, this should not induce any alteration in *STEAP1* expression since the variant has a high frequency in the general population. For the variant -1195 A/C, there is no putative TF binding for the allele "A", but the change of "A" by "C" originates a putative target for several TFs, namely the FOXP3, two isoforms of the progesterone receptor (PR), the glucocorticoid receptor (GR) and the CCAAT/enhancer-binding protein ß (C/EBPß). Regarding the variant -640 G/-, although the TF E2F-1 binds to both allelic variants, the deletion of "G" results in an alteration of the TF RXR-alpha for the TF AP-2alphaA. Lastly, the variant -563 A/G was the variant that had the highest number of possible TFs binding. Although there are several TFs that can bind to both alleles, including the GR-beta and two isoforms of the TF HOXD, there were also some differences. While the allele "A" may allow the binding of TFIID and HNF-3alpha, the allele "G" is a putative target for the TCF-4E, SRY, and lymphoid enhancing factor-1 (LEF-1). The binding of different TFs to each allelic variant may lead to alterations in *STEAP1* gene expression. In fact, studies have demonstrated that some of these TFs may have a role in prostate carcinogenesis. For example, the C/EBPß is a leucine zipper TF that belongs to the family CCAAT/enhancer binding proteins (C/EBP). This TF regulates genes involved in cellular differentiation, proliferation, and inflammatory responses (109). C/EBPß gene gives rise to three isoforms, the C/EBPß A and B that function as transcriptional activators, and the isoform C/EBPß C that functions as a transcriptional repressor. Thus, its biological activity will depend on the ratio of C/EBPß isoforms. Although the role of C/EBPß in PCa is not yet completely understood, it was already associated with tumor progression in several types of cancer. However, it was demonstrated that C/EBPß regulates cell growth and is involved in TNF-α resistance in hormone-independent PCa cells (110). Also, C/EBPß expression seems to be associated with the induction of a senescence state, which may contribute to PCa cell survival (111). Another TF already associated with PCa is the LEF-1, an important TF of the Wnt signaling pathway that is involved in PCa development and progression. TMPRSS2–ERG fusion is one of the most frequent alterations present in PCa cells, which have as consequence the overexpression of the *ERG* gene that regulates several oncogenic pathways. *ERG* is able to activate the Wnt/LEF-1 signaling pathway promoting cell proliferation and invasiveness. LEF-1 knockdown results in complete silencing of the ERG- induced Wnt signaling pathway and its oncogenic properties (112). As

LEF-1 regulates positively the expression of the AR, its overexpression in PCa, mostly in the androgen-independent phase of the disease, is associated with increased growth and invasion ability of PCa cells (113). The alterations found in the promoter region of *STEAP1* may lead to changes in the binding of TFs allowing the binding of transcriptional activators that ultimately will contribute to STEAP1 overexpression in PCa. Further analysis will be required to assess if these changes in TFs binding occur in cells and if so whether these can alter STEAP1 expression.

Table 8: Summary of putative TF binding to each allelic variant identified using the TF binding site prediction program Alggen Promo software 3.0. On the nucleotide sequence of potential binding site is highlighted the nucleotide that is altered in each variant.

Variant	Allele	Transcription Factor	Sequence	Dissimilarity (%)
-1863 G/C	G	XBP-1	ATGAAT	7.172312
	C	GATA-1	TATCAA	1.038567
		C/EBPbeta	TCAA	1.366559
		C/EBPalpha	ATCAATA	4.235345
-1195 A/C	A	-	-	-
	C	FOXP3	AAAAAC	4.756447
		PR B	AACAAAA	11.148154
		PR A	AACAAAA	11.148154
		GR	CAAAAAA	0.000000
		C/EBPbeta	ACAA	0.000000
-640 G/-	G	RXR-alpha	GGGTGGA	4.423008
		E2F-1	GCGGGTGG	11.888116
	-	AP-2alphaA	CGAGGC	2.098119
		E2F-1	GCGGTGGA	10.026566
-563 A/G	A	GR-beta	AAATT	0.000000
		GR-beta	AATTA	0.840383
		TFIID	TACAAAA	1.537547
		HOXD9	AATACAAAAA	10.220007
		HOXD10	AATACAAAAA	10.220007
		GR	CAAAAAT	0.000000
		HNF-3alpha	CAAAAATT	10.500194
	G	GR-beta	AGATT	3.361531
		SRY	AATACAAAG	4.087393
		TCF-4E	TACAAAG	9.453578
		HOXD9	AATACAAAGA	10.220007
		HOXD10	AATACAAAGA	10.220007
		GR	CAAAGAT	3.763516
		LEF-1	ATACAAAG	8.759086

4.2. Methylation pattern of *STEAP1* in PNT1A and LNCaP cells

Since DNA methylation plays an important role in the regulation of gene expression and is one of the mechanisms involved in tumorigenesis, it is essential for decoding the human epigenome. The Bisulfite sequencing PCR (BSP) method is based on the bisulfite conversion of DNA followed by PCR and sequencing of the region to be analyzed. Bisulfite treatment of genomic DNA converts unmethylated cytosines into uracils while methylated cytosines remain unchanged. Thus, the treatment with bisulfite inserts alterations in the DNA sequence that are dependent on the methylation pattern of the region. After PCR reaction and sequencing, the methylated cytosines remain as cytosines, whereas unmethylated cytosines are altered to thymines. The comparison of the original sequence with the modified by bisulfite allows to know which cytosines are methylated or not. The BSP method allows the detection of the methylation status of multiple CG dinucleotides of a particular CpG island.

Due to the high homology between the *STEAP1* and *STEAP1B* genes, also here it was not possible to perform direct sequencing of PCR products. The sequences obtained were aligned with the sequences of the *STEAP1* and *STEAP1B* genes to identify the specific gene. The sequences of the *STEAP1* gene were then compared with the bisulfite modified genomic sequence in order to determine the methylation status of the CpG dinucleotides. In order to assess whether the *STEAP1* gene had some relevant "CpG island", the Methyl Primer Express software (Applied Biosystems) was used. The analysis showed a relevant “CpG island” covering part of the promoter region, the first exon and still part of the first intron, with a total of 84 CG dinucleotides (Figure 16).

Figure 16: Region of the *STEAP1* gene with a relevant CpG island. Parameters used to find CpG islands: minimum length of Island: 300 bp; maximum length of Island: 2000 bp; C+Gs/Total bases > 50%; CpG observed/CpG expected > 0.6.

The methylation pattern of the *STEAP1* gene was analyzed from position -388 (promoter region) to +494 (first intron) from the transcription start site in PNT1A and LNCaP cells, covering a total of 54 CG dinucleotides. The analysis of the methylation pattern of *STEAP1* revealed some differences between PNT1A and LNCaP cells near the transcription start site. In PNT1A cells, a group of CG dinucleotides located in the promoter region is methylated but not in LNCaP cells (Figure 17). The CG dinucleotides present in the first exon

and beginning of the first intron are demethylated in both PNT1A and LNCaP cells. This different profile of methylation near the transcription start site of *STEAP1* between PNT1A and LNCaP cells suggests that demethylation of *STEAP1* may occur in PCa cells and contribute to its overexpression. This hypothesis is also supported considering that *STEAP1* is over-expressed in LNCaP cells when compared to PNT1A cells (86, 91).

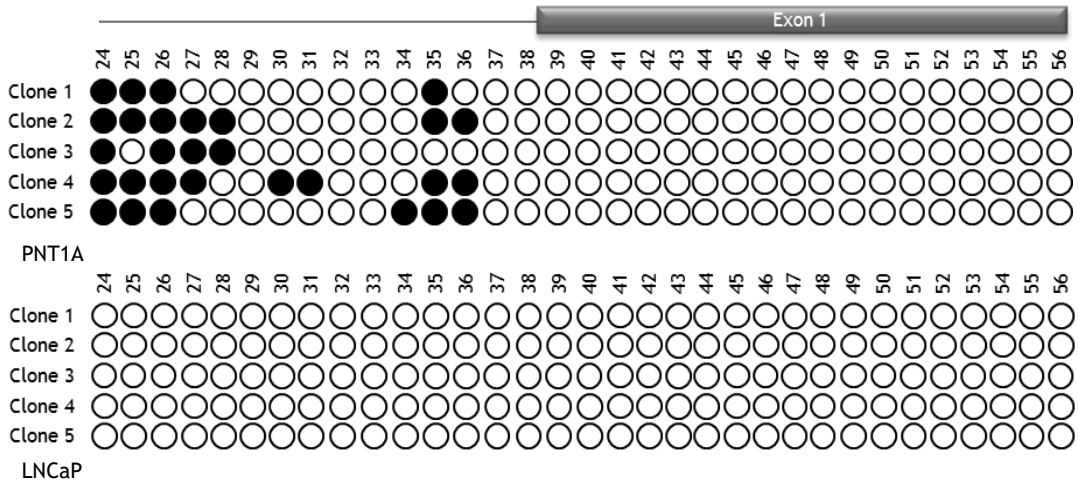

Figure 17: Bisulfite methylation analysis of *STEAP1* in PNT1A and LNCaP cells. Each circle represents a CpG site, the black circles represent the methylated nucleotides and the white circles represent the unmethylated ones. Each row represents a different clone and on the top is the position of each CpG site relative to the beginning of the CpG island identified.

4.3. Effect of treatment with DNMT and HDAC inhibitors on *STEAP1* mRNA expression in PNT1A cells by qPCR analyses

In order to support the results above suggesting that STEAP1 expression is regulated by epigenetic mechanisms, PNT1A cells were used to evaluate the effect of DNMT and HDAC inhibitors (AZA and TSA, respectively) on *STEAP1* mRNA expression. AZA is an epigenetic modifier that inhibits the activity of DNMTs, thereby causing the demethylation of DNA and subsequent overexpression of genes whose expression is repressed by methylation of its promoter region. TSA is also an epigenetic modifier that inhibits the activity of HDACs preventing these from removing the acetyl group added to histones by HATs. The resulting histone hyperacetylation, which causes a relaxation of chromatin structure, leads to activation of transcription of genes.

The expression levels of *STEAP1* gene in PNT1A cells after the treatment with AZA and TSA was analyzed by qPCR. As shown in figure 18, the qPCR results showed that the treatment with AZA induces a slight increase (3 fold-variation in comparison with control group, $p<0.01$) in *STEAP1* mRNA expression. Regarding the treatment with both AZA and TSA, a strong increase (15 fold-variation relatively to control, $p<0.001$) in *STEAP1* mRNA expression was observed. The increase in *STEAP1* mRNA expression induced by the treatment with the DNMT inhibitor indicates that *STEAP1* gene expression is in part regulated by its methylation pattern, which is consistent with the difference obtained in the methylation pattern between PNT1A and LNCaP cells. The marked increase in *STEAP1* mRNA expression induced by the treatment with the DNMT and HDAC inhibitors indicates that the *STEAP1* gene is synergistically activated by hypomethylation and histone hyperacetylation.

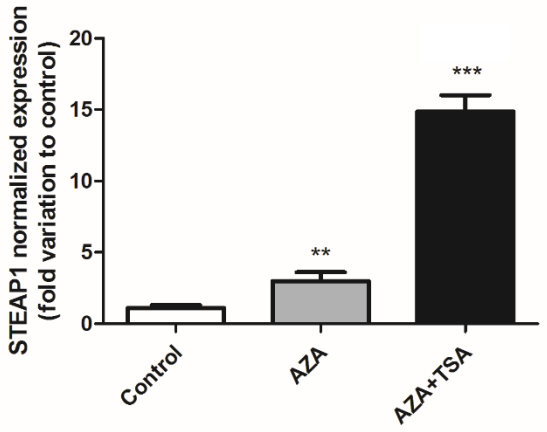

Figure 18: qPCR analysis of the effect of treatment with AZA and TSA (DNMT and HDAC inhibitors respectively) on *STEAP1* mRNA expression in PNT1A cells. (**$p<0.01$; ***$p<0.001$ relative to control).

5. Conclusions and Future Perspectives

The present work intended to evaluate possible genetic and epigenetic alterations in the *STEAP1* gene, which may be involved in its regulation. Regarding genetic alterations, the sequence analysis revealed the presence of the following variants in the promoter region of *STEAP1* gene: -1863 G/C in LNCaP and PNT1A cells and -1195 A/C, -640 G/-, -563 A/G only in LNCaP cells. Although some of these variants have already been identified as polymorphisms that occur in populations, it cannot be excluded that these alterations may affect the expression of the *STEAP1* gene. Thus, an *in silico* analysis was performed to evaluate if the variants are located in important regions for the binding of TFs. *In silico* analysis revealed several alterations in the TFs that bind to each allelic variant including the binding of transcriptional activators, such as C/EBPβ and LEF-1. However, it will be necessary to further assess if these changes in TFs binding occur in cells and if so whether these can alter STEAP1 expression. Also, and taking into account the experimental approach used in this work to sequence the *STEAP1* promoter region, it would be important to analyze the sequence using a sequencing strategy that allowed to sequence larger DNA fragments. Thus, it will be possible to determine the genotype (homozygous or heterozygous) for each variant.

In relation to epigenetic alterations, the promoter region of *STEAP1* gene is methylated in PNT1A cells but not in LNCaP cells, suggesting that demethylation of *STEAP1* gene may induce *STEAP1* overexpression in LNCaP cells. This hypothesis is supported by the treatment of PNT1A cells with the DNMT and HDAC inhibitors, which induced *STEAP1* gene overexpression. However, more studies are required in order to establish an association between these epigenetic modifications and PCa progression. In the future, it would be useful to evaluate the methylation pattern of the *STEAP1* gene in human prostate samples from patients with PCa, BPH, and/or PIN lesions, in order to establish if there is any correlation between these alterations and the clinical data of the patients.

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