THE ROLE OF MACROH2A1 IN PROSTATE CARCINOGENESIS

[Subtítulo do documento]

Síntese

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1. PROSTATE

1.1 PROSTATE ANATOMY, HISTOLOGY AND PHYSIOLOGY

Prostate, along with the seminal vesicles and the bulbourethral glands, constitute the male reproduction's accessory glands [1]. The prostate is a walnut-shaped organ, which the size grow with age, around 28 to 47cm2 and is localized under the bladder, near the rectum, surrounding the beginning of the urethra [2, 3]. The function of the prostate is to segregate an alkaline fluid, where one of the components is a serine protease of the Kallikrein family, the prostate-specific antigen (PSA) [4].

The prostate is composed by acini and ducts, organized in lobules, and delimited by fibromuscular stroma. Acinus per se consists of epithelial (secretory and basal) and neuroendocrine cells, surrounded by fibroblasts and smooth-muscle cells [5]. Stromal and epithelial cells express androgen receptors (ARs), depending on androgens (i.e. testosterone) to proliferate [5]. A thin layer of connective tissue surrounds the prostate, being connect with nerves and other tissues, constituting the prostatic capsule [1].

The model of prostate anatomy has been puzzlingly discussed through time and culminate divided into lobes, based on laboratory animal's analogy [6]. This concept was accepted until the decade of 1960s, when John E. McNeal start describing the most widely accepted anatomic divisions of the prostate: peripheral, central, transition and anterior fibromuscular stroma zones (Figure 1) [7, 8]. The peripheral zone is structured by a disc of tissue with radiated ducts laterally from the urethra lateral and distal, which constitutes 70% of the glandular prostate. The central zone is organized by ducts that follow the ejaculators ducts, constituting 25% of the prostate. The transition zone includes the prostatic urethra and arranges 5% of the glandular prostate. Lastly, the anterior fibromuscular forms the thick surface of the prostate and is responsible for sphincter functions [9, 10]. Cells of the transition zone proliferate dramatically throughout the puberty

and later, after the age of 55 years, leading to the increase of the main zone of the glandular prostate, the peripheral zone [5].

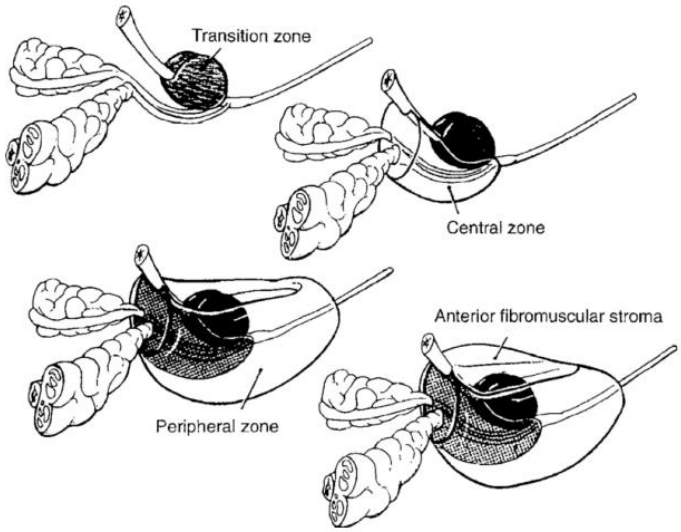

Figura 1 Anatomic zones of the prostate described by McNeal. Adapted from Hammerich et al, 2008 [8].

1.2. NON-CANCEROUS PROSTATE DISEASES

Prostate disorders are commonly being more frequent in men with advanced age [11]. The most common non-cancerous prostate diseases include benign prostatic hyperplasia (BPH), proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN).

BPH origins in the transition zone of the prostate and is described by an excesso of glands and stroma [12]. The possible risk factors for BPH are heredity, gene polymorphisms, diet, metabolic syndrome, exercise and cigarette smoking [13].

A PIN lesion take place in the peripheral zone and is commonly characterized by neoplastic resembles with undetectable abnormal changes phenotypically and not raising the PSA levels [14]. PIN lesions were firstly characterized by Bostwick and Brawer [15] in low and high grade PIN (LGPIN and HGPIN, respectively), which differ by architectural and cytological characteristics (Table 1) [16]. PIN spread through prostatic ducts and is

characterized by the conservation of the basal cell layer, while luminal cells are replaced by neoplastic cells [17]. Those neoplastic cells own a hyperchromatic nuclei and nucleoli enlargement [18].

	LGPIN	HGPIN
Architecture	Epithelial cells crowding and stratification, with irregular spacing	Similar to low-grade PIN; More crowding and stratification; four patterns: tufting, micro papillary, cribriform, and flat
Cytology Nuclei	Enlarged, with marked size variation	Enlarged; some size and shape variation
Chromatin	Normal	Increased density and clumping
Nucleoli	Rarely prominent*	Prominent
Basal cell layer	Intact	May show some disruption
Basement membrane	Intact	Intact

Tabela 1 Criteria for low and high PIN. Adapted from Bostwick and Cheng, 2012 [16].

PIA usually originates in the peripheral zone and is described by the rapidly epithelial cells division without full differentiation [16]. Proliferative cell regeneration is induced by inflammation or external factors, as chemicals or bacteria [16]. PIN and PIA share similar alterations at key molecular pathways and originates in the same prostate glandular zone, suggesting that PIA can be a precursor of PIN [19].

1.3. PROSTATE CANCER

The most prevalent malignant disease in prostate is adenocarcinoma corresponding to approximately 95% of the cases [8]. Prostate cancer (PCa) is characterized by a heterogeneous low proliferate carcinoma, asymptomatic when confined in the organ (latent tumors) [20]. The only recognized putative precursor of PCa is HGPIN, which pre-dates the onset of PCa by 5–10 years and, along with PCa, disrupt both cell layers (Figure 2) [21]. Other prostate diseases keep the basal cell layer intact [16].

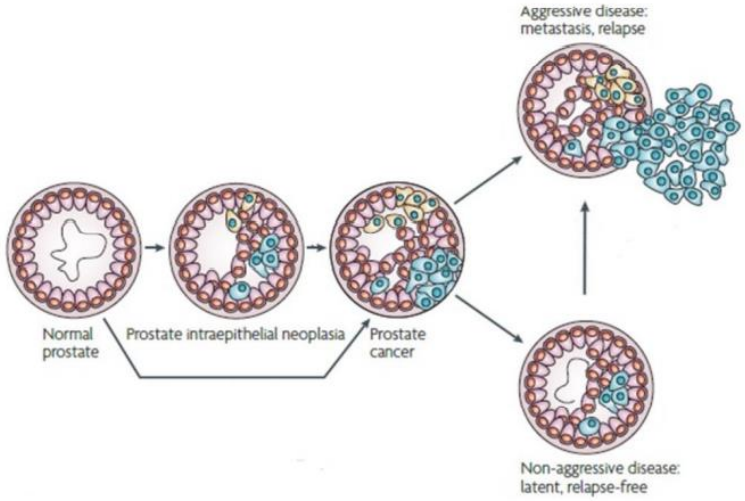

Figura 2 Cellular progression of prostate cancer. Adapted from Witte, 2009 [26].

Approximately 9% of isolated HGPIN is found in biopsies, although the prevalence of HGPIN with PCa in biopsies vary with the number of cores and race, not surpassing 45%. Irrefutably, HGPIN and PCa share the location [15] and have similar morphology, histology and chromosomal abnormalities [24]. Consequently, HGPIN diagnosis could be used as tool for patients with PCa predisposition [18]. The heterogeneity, slow-growing behavior and no symptoms in early phases, turns PCa in a real challenge for patient management, triggering late diagnosis, and consequently compromising prognosis and target therapies. Hence, it is important to comprehend underlying mechanisms and sequential pathways of PCa initiation and development.

1.4. EPIDEMIOLOGY OF PROSTATE CANCER: INCIDENCE AND MORTALITY

PCa is a major health concern due to growth and ageing of global population. PCa is the fourth more frequent cancer considering overall population and, after lung cancer, is the most common cancer in men. PCa incidence diverge drastically worldwide, thought could be related with the median-age and number of cases diagnosed per country. In the early 1990s, there was a dramatically

increase of PCa incidence worldwide, due to the introduction and largely use of transurethral resection of the prostate (TURP) and PSA screening for cancer detection in developed countries. Nonetheless, PCa incidence is higher in North America, Australia and Nordic countries, whereas lower incidence is found in Asia and Northern Africa (Figure 3A). In 2015, is expected that PCa would represent 25% of all new diagnosis cases in men.

Concerning PCa mortality, rates have been more constant through time. PCa mortality has been decreasing due to early diagnosis and therefore, the therapy is provided at earlier stages of the disease. Currently, PCa constitutes the fifth cancer related mortality worldwide, excluding non-melanoma skin cancer. PCa mortality rate is more prevalent in Africa and South America.

In Portugal, PCa is currently, the number one in incidence rates and second in mortality rate among men.

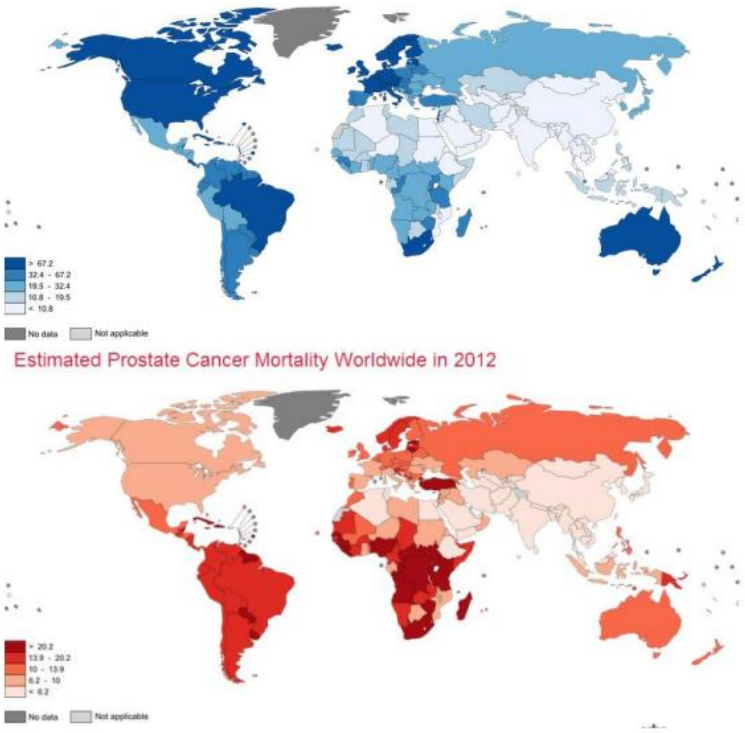

Figura 3 Estimated incidence (A) and mortality worldwide of prostate cancer in 2012. Adapted from Globocan [27].

1.5. RISK FACTORS

To date, there are established three risk factors which represent the furthermost main influences that could lead to PCa: age, family history and ethnicity [31].

PCa patients' average age is between 70 and 74 years, and it increases in older men [32]. Indeed, the likelihood of PCa development is 85% in men after 65 years old and higher than 90% in men with more than 90 years old [33]. It should be recalled that precursor lesions and PCa early phases are silence diseases, therefore, it may exist during years or decades before PCa is diagnosed [34].

Family history always represented a risk factor to develop cancer and PCa is no exception. Familial PCa represents 10-15% of all PCa diagnosed cases [35]. Additionally, it was observed that first-degree relatives of PCa patients have a higher risk to develop the disease. Furthermore, the number of affected members in a family and the early-onset of the disease increase even more the risk of prostate cancer [33]. Nevertheless, familial PCa and non-familial PCa are clinically and pathologically similar [31].

Lastly, ethnicity may justify PCa incidence divergence around the world. African-American men present 60% higher probabilities to develop PCa, even in younger ages, comparing to Caucasian American men [36]. However, immigration studies suggest that races with low PCa incidence, as Asians, increase dramatically the probabilities of develop PCa when immigrate to America [33, 34]. Hence, external factors, as environment, dietary habits, exercise, access to medical care and diagnosis tools and others [37, 38], might have an additional role in the likelihood of developing PCa [31].

1.6. DIAGNOSTIC TOOLS FOR PROSTATE CANCER

The efforts for development of tools for PCa detection is to effectively identify this disease while silently confined in the organ and, thus, curable. The

two complemental detection tools available nowadays are digital rectal examination (DRE) and PSA screening.

Since PCa develops in the peripheral zone of the prostate gland and knowing the prostate proximity to the distal rectum, about 18% of all PCa can be detected by DRE [5, 39]. However, DRE lacks in sensibility and depends on professional experience [39].

Alternatively to DRE, PCa can be detected by Transrectal ultrasound (TRUS) or Transrectal magnetic resonance imaging (MRI), though the last is more utilized to verify PCa invasion to nearby tissues [40, 41]. Alternatively, the glycoprotein PSA is segregated in epithelial cells of the prostate and release in the blood circulation. PSA quantification was introduced as a diagnosis tool in the 1980's, providing the identification of prostate diseases, with low levels of specificity and sensibility for PCa [42]. It is expected PSA levels between 0 to 4.0ng/ml in prostate-healthy men under 70 years old and slightly higher through age. Additionally, PSA levels can be influenced by obesity [43], cardiovascular disorders [44], type 2 diabetes [45] and other prostatic diseases besides PCa. This test demonstrates important limitations in specificity and sensibility but, to date, is the only available biomarker used for the detection and monitoring of treatment efficacy for prostate cancer [46].

Regardless limitations, the annual combination of DRE and PSA screening, in fact, diminish the number of advanced PCa patients [47]. If DRE and PSA screening results are PCa abnormal, is recommended a TRUS-guided systemic needle biopsies 3 to 6 months, with 12 or more small tissue cylinders (cores) removed each biopsy for analyzation [5, 48].

Moreover, it is important to take into account that PCa patients are, commonly, older than 50 years old and inaccurate regular diagnosis can lead to over diagnosis and over treatment of latent tumors and damage both physical and psychological. Therefore, it is advocated to avoid PSA screening in men over 75 years old [1]. All the points mentioned above strengthen the importance to develop specific non-invasive diagnosis methods for PCa [49].

1.7. PROGNOSTIC TOOLS FOR PROSTATE CANCER

Prognostics tools are designed to accurately distinguish clinically significant from indolent PCa. Currently, Gleason Score and the TNM systems assist clinicians in decision-making.

The heterogeneity of PCa is the main problem in prostate biopsies, since cores may not represent the complete tumor [50]. To decipher the glandular epitelial architectural patterns, ignoring cytologic details, in 1966, Donald F. Gleason elaborate a histological grading system based on the sum of the two more frequent glandular histological patterns present in each tumor: the Gleason Score [51]. This system scores well-differentiated pattern as 1 and as 5 the most undifferentiated. Therefore, Gleason grading system increases with the tumor aggressiveness, in a 2 (1+1) to 10 (5+5) combined score scale (Figure 4) [51]. Although limited by the pathologist proficiency and the cores removed, accurate Gleason Score is critical, once, can differ in malignancy based on the most frequent pattern, for example, a Gleason Score 5+3 (n=8) represent a worst prognosis than a Gleason Score 4+4 (n=8) or 3+5 (n=8) [50].

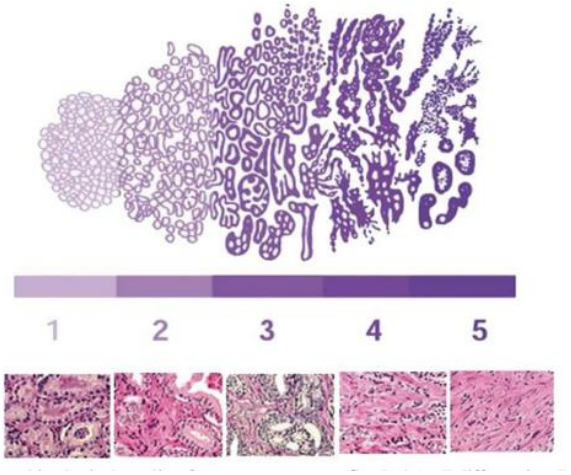

Figura 4 Gleason Score: histological grading for prostate cancer. Grade 1 (well differentiated): closely packed, uniform shaped glands. Grade 2 (well differentiated): infiltration into the surrounding stroma, more variation in gland size and spacing. Grade 3 (moderately differentiated): irregular size and shape, separation of the glands, less defined boundaries and less intervening stroma. Grade 4 (poorly differentiated): fusion of the glands with a ragged invasive edge. Grade 5 (undifferentiated): complete absence of gland formation with sheets or clusters of cells. Adapted from Harnden et al, 2007 [52].

To recognize the dimension of PCa and the level of extension, in 1950s was establish a clinical and pathological staging system for solid tumors: the TNM (Tumor Node Metastasis) classification system. The American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) systematically update the staging system that allows distinguish primary tumors clinically (T) and pathologically (pT), regional lymph nodes status clinically (N) and pathologically (pN) and distant metastases (M) (Table 2) [53]. Clinical staging is only associated to the evaluation of cancer spread, being firstly obtained during diagnosis, before treatment. Pathological staging is related to histological data and is firstly determined after radical prostatectomy (RP) thus there is no pT1 classification [54]. Concerning PCa metastasis, tumors frequently spread to bones, lymph nodes, lungs, liver and brain [55].

The TNM classification, along with Gleason Score and PSA screening results, provides a complete PCa stage, classified to I to IV, increasing with the PCa aggressiveness [54].

1.8. PROSTATE CANCER'S CLINICAL MANAGEMENT

The main goal of treatment of clinically localized PCa (stage I and II) is the cancer eradication, while no curative treatment is available for advanced PCa (stage III and IV) and treatment is only palliative support. Early-stages PCa present about 90% of progression-free survival after 5 to 10 years [56]. In fact, it is more frequent a man die with PCa than from PCa. Nevertheless, all aggressive therapies could lead to diferente side-effects, as urine or bowel dysfunction, fatigue, increased risk of diabetes or heart attacks and others [57]. Therefore, age, life expectancy, comorbidities and quality of life of the patients are taken in consideration to select the better treatment approach.

To avoid inadequate treatments, PCa patients can be monitored by watchful waiting (WW) or active surveillance (AS). It is suggested WW to patients who are not advised to undergo aggressive treatment. These patients are followed on 6 months and only are treated if PCa progress. AS is recommended for indolent tumors where therapies are pointless: low Gleason Score grade, low

PSA screening result and <50% presence of cancer in biopsies [58]. These patients are followed by systematically diagnosis procedures, evaluating the progression of PCa.

Clinically localized PCa can be treated with RP or radiotherapies. For early stage PCa patients with good general conditions for surgical intervention and with 10 or more years of life expectancy, the most adequate treatment is ablation of the prostate gland and the seminal vesicles by RP [59]. Radiotherapy may be an alternative to RP, showing high rates of disease-free survival, either by noninvasive external-beam radiation therapy or interstitial radiation therapy (brachytherapy), in which radioactive seeds with a life-time of 60 days are placed near the tumor [60, 61].

For advanced PCa patients, the treatment option is suppress the action or inhibit the production of testosterone, decreasing the prostate hormone-response. Androgen-deprivation therapy can be achieved by surgical castration (orchiectomy) or chemical castration, a combination of gonadotropin-releasing hormone analogues with antiandrogens (i.e. bicalutamide) [57]. These therapies may be used along with early-advanced PCa treatments. Unluckily, AR mutations lead to castration-resistance after 18-30 months of treatment [62]. The therapies available for metastatic castration-resistant PCa patients only provide supportive care.

2. EPIGENETICS

The nucleus of a human cell compacts the three billion base pair genome: DNA bonded to proteins, forming the chromatin [63]. When chromatin is strongly compacted is named heterochromatin and when is lesser condensed is designated euchromatin which is associated with transcription, DNA replication or repair and recombination processes [64]. Epigenetic mechanisms play a key role in chromatin dynamics and therefore in expression regulation.

The term “epigenetic” use the Greek prefix epi- which means over, beyond-genetics and was defined by Conrad Waddington, in the 1940s, as the branch of science of embryonic development studies, through experimental analysis. [65]. The “epigenetic landscape” was the explanation of cellular differentiation: how totipotent cells develops into all the different cells types in an organism with the same genome [66].

Epigenetic definition has been changing through time and currently, is defined as the heritable changes that occur in a gene regulation/function without alter the DNA sequence [67]. Epigenetics studies explain, for example, the differences among monozygotic twins or, in females, the silence of one X chromosome [66].

Epigenetic mechanisms are divided in four different main groups: DNA methylation, non-coding RNAs, post-translational modifications (PTMs) of histones and histone variants, which will be slightly described below. Alterations in epigenetic mechanisms affect innumerous cells processes, being implicated in several diseases, including cancer.

2.1. DNA METHYLATION

In mammals, DNA methylation refers to the addition of a methyl group, by DNA methyltransferases (DNMT), in a cytosine next to a guanine, known as CpG dinucleotides. CpG dinucleotides clusters are designed as “CpG islands” and are generally found in promoters, introns, repetitive sequences or untranslated sequences of the genome [66]. The latter are globally methylated in the genome being important to maintain DNA stability [68].

2.2. NON-CODING RNAS

Nearby 90% of all RNAs transcribed are non-coding RNAs that do not codifiy proteins [73]. Non-coding RNAs, as ribosomal RNAs, are grouped

according to size; microRNAs (miRNAs) are 18-30 nucleotides, 30–300nt are denominated small RNAs and non-coding RNAs with larger 300nt are considered long RNAs [73]. Non-coding RNAs are described as key players in gene regulation [73]. From these, miRNAs are the most well studied in cancer [73, 74].

MiRNAs are synthetized and processed in the nucleus and are transported to the cytoplasm to bind complementary mRNAs, repressing their function by degradation or by translation inhibition [74]. Interestingly, miRNAs could also be involved in the up-regulation of translation during the cell cycle [75].

Different mRNAs can be regulated by the same miRNA, the same way as different miRNA can target the same mRNAs [74]. About 30% of the human genes are regulated by time and tissue-specific miRNAs [76], interfering with several cellular pathways as differentiation, proliferation, apoptosis, and stress response [77].

In cancer, upregulated miRNAs target tumor suppressor genes and downregulated miRNAs target oncogenes [74]. Gene amplification, deletion, mutation and other epigenetic mechanisms can alter the miRNAs expression [74].

2.3. HISTONE POST-TRANSLATIONAL MODIFICATIONS

Eukaryotic DNA is packaged by histones, positively-charged proteins that easily bind with the negatively-charge DNA [78]. Eight histones, one pair of each H2A, H2B, H3 and H4, constitute a protein complex designed nucleosome that is wrapped by a core DNA 1.7 times and sealed by one H1 [79, 80], along with numerous hydrogen, electrostatic and hydrophobic bonds [81].

Histones are dynamic proteins responsible for DNA support and chromosomal remodel [82]. All histones share a similar structural architecture with α-helices bonded by short loops and a flexible undefined N-terminal tail where is more susceptible to occur covalent histone modifications (post-translational modifications), such as acetylation, methylation, phosphorylation or ubiquitination which impact on chromatin condensation and globally constitute the so-called histone code (Figure 5). [79, 82, 83]. These modifications are "written", "read" and "erased" by different histone modulating enzymes [84, 85].

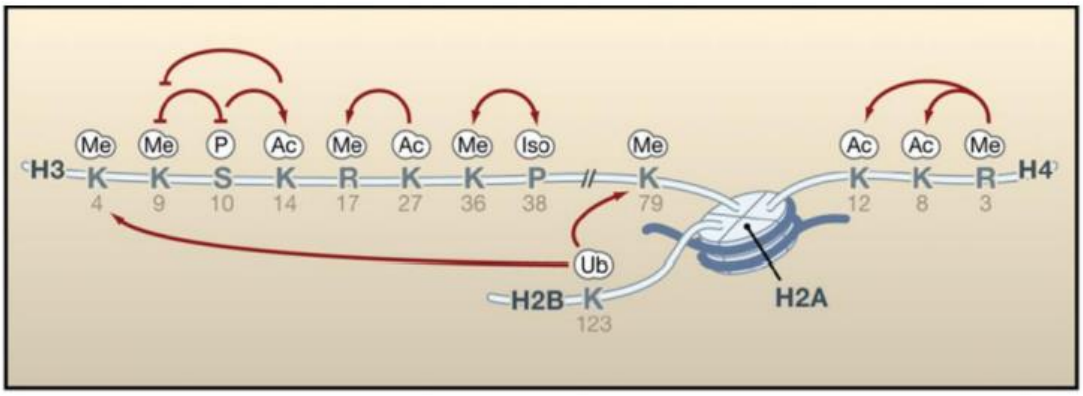

Figura 5 The negative and positive crosstalk between histone post-translational modifications. Adapted from Kouzadaries, 2007 [79].

Regarding histone acetylation, gene transcriptional activity is balanced due to alterations of electrostatic charge in the nucleosomes [86]. Therefore, hyperacetylation is characteristic of euchromatin by decreasing the histone-DNA affinity and allowing gene transcription, whereas hypoacetylation is related with heterochromatin [79].

Histone acetylation is "written" by histone acetyltransferases (HATs) and "erased" by histone deacetylases (HDACs) [84]. Histone methylation promotes transcription activation or repression depending on the residue and the number of methylation molecules added (mono-, di- or tri-) [87]. Indeed, tri-methylation of lysine 4 of H3 (H3K4me3) promotes active transcription while mono- and tri-methylation of lysine 27 of H3 (H3K27me and H3K27me3) inhibits gene transcription. The writers of histone methylation are histone methyltransferases

(HMT) and the erasers are histone demethylases (HDM) [79, 82].

Histone modifying enzymes expression are disrupted in cancer and the imbalance between writers and erasers affect the PTMs' profile [88]. Moreover, DNMTs are directly recruited by HMTs to inhibit genes' expression and recruit HDACs to increase the gene silencing. This interplay between DNA methylation and PTMs is also impaired in cancer [88].

2.4. HISTONES VARIANTS

The less studied epigenetic mechanism is the shift of canonical histones by sequential similar non-allelic histones variants [89]. Among species, histone variants are the mostly conversed proteins and have been considered functionally irreplaceable [90, 91].

On one side, canonical histones are genomically organized by clusters lacking introns [92]. The transcription is DNA replication-dependent and therefore, exclusive to the S phase of the cell cycle, and the mRNA obtained contains a unique 3′ stem loop [93]. On the other side, histone variants are orphan genes with introns and the mRNA translated holds a polyadenylated tail [81]. Although they are present throughout the cell cycle, variants are tissue and temporal-specific [94, 95]. Variants are named "replacement histones" because they substitute the canonical histones during development and differentiation, establishing cell identity [81].

The slightly sequential differences, along with unique PTMs of histone variants, result in nucleosome-DNA stability differences [96] and alters the efficiency of protein complexes responsible from histone deposition and displacement in the nucleosome. These adjustments change the accessibility of transcription factors into the chromatin, regulating the gene expression.

To date, histone variants have been described for all canonical histones, excluding H4 (Figure 6) [97]. H2A family is the largest histone family with the most

structurally diverse histone variants: H2A.X, H2A.Z, macroH2A, H2A.Bbd [98]. Variants of H2A are described by distinguish length, sequence and genome distribution [81]. Mis-regulation or mutations in these H2A histone atypical variants have been implicated in cancer initiation and progression [89].

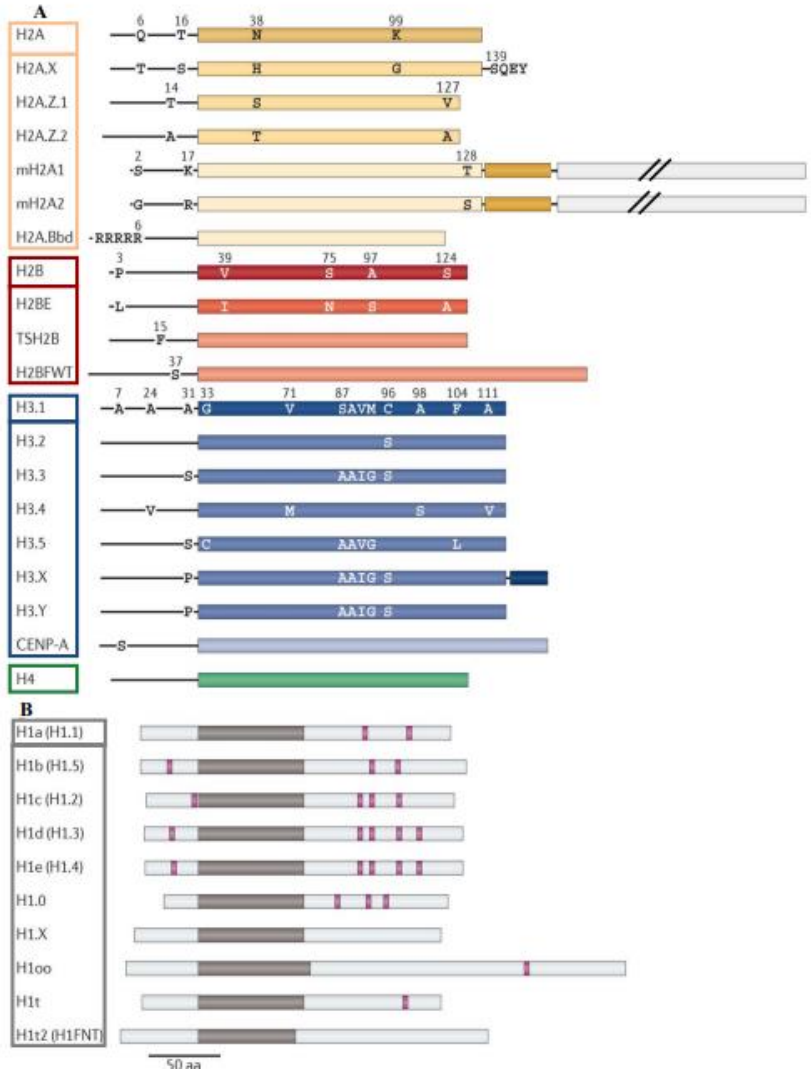

Figura 6 A. Human canonical and histone variants of H2A (yellow), H2B (red), H3 (blue), H4 (green). Unstructured amino- terminal tails are shown as black lines. Specific amino acid residues are depicted at key differences among variants of a common histone protein family. Different shades of color are used to indicate protein sequences that are highly divergent between canonical histones. B. Human canonical and histone variant linker H1. Unstructured amino- terminal tails are shown as light grey. Globular domains are shown in brown. Serine/threonine PXK phosphorylation sites targeted by cyclin-dependent kinases are indicated in magenta. Alternative names of variants are given in parentheses. aa, amino acid; mH2A1, macroH2A1. Adapted from Maze and al, 2014 [97].

3. CLINICAL SAMPLES

3.1 PATIENTS AND CLINICAL SAMPLES COLLECTION

Prostate samples of 197 primary tumors and 45 HGPIN (from here simply designated PIN) lesions were prospectively collected from patients diagnosed with the disease and primarily treated with RP, form 2001 and 2006, at the Portuguese Oncology Institute, Porto, Portugal. Samples of 15 morphological normal prostate tissues (MNPT), used as control, were collected from the peripheral zone of prostates not harboring PCa, obtained from radical cystoprostatectomy for bladder cancer. Immediately after surgery, all tissue specimens were frozen at -80ºC. Thick frozen sections were obtained from frozen tissues for stain identification and after, the tissue block was trimmed to maximize the yield of target cells (>70% of target cells). Histological slides from formalin-fixed, paraffin embedded (FFPE) tissue fragments were also obtained from the same surgical specimens for histopathological examination: Gleason Score and pathological staging evaluations. Relevant clinical data were acquired from clinical registers and these studies were approved by the institutional review board (Comissão de Ética para a Saúde – CES 019/2008) of Portuguese Oncology Institute - Porto, Portugal.

3.2 RNA EXTRACTION AND QUANTIFICATION

Samples were homogenized in Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and the total RNA were extracted from all 257 samples using PureLinkTM RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. All genomic DNA were eliminated with TURBO DNA-free (Ambion, Applied Biosystems), according to manufacturer's instructions. The concentration, purity ratios and quality of each sample were determined using a Nanodrop ND-1000 (ThermoScientific, Wilmington, DE, USA) and by an agarose gel electrophoresis. RNA samples were then stored at -80ºC

3.3 QUANTITATIVE REVERSE TRANSCRIPTION PCR (RT-qPCR)

For each tissue sample, first strand synthesis was performed using the TransPlex® Whole Transcriptome Amplification Kit (Sigma-Aldrich®, Schnelldorf, Germany) and QIAquick PCR Purification Kit (QIAGEN, Germany) for purification.

Expression of the target genes were quantified using Fast SYBR Green® Gene Expression Assay (Applied Biosystems®, Life TechnologiesTM, Foster City, CA, USA), and normalized to the expression of the endogenous control β-glucuronidase (GUSβ), a housekeeping gene (Table 3 and Figure 9). In each well, 0.1µL of cDNA samples were mixed with 5µL of 2x KAPA SYBR® FAST qPCR Master Mix Universal (Applied Biosystems®), 0.2µL of 50x ROX low and optimized for 0.2-0.4µL of 10µM primers (Sigma-Aldrich®), completed with sterile bidistilled water (B. Braun, Melsunger, Germany) for a total of 10µl. Each 96-well plate included 2 negative controls and, for standard curve, five sequential dilutions of a cDNA from human prostate RNA (Ambion®, Invitrogen, Carlsbad, CA, USA). PCR were programmed for 3 minutes at 95ºC, followed by 40 cycles of 3 seconds at 95ºC and 30 seconds at 60ºC. Relative expression was obtained by the ratio of the target mean quantity/reference gene mean quantity. All samples were analyzed in triplicate in 7500 Real-Time PCR system (Applied Biosystems®), and the mean value was used for data analysis.

Gene	*Forward Primer 5' -> 3'*	*Reverse Primer 5' -> 3'*
MacroH2A1.1	GGCTTCACAGTCCTCTCCAC	GGTGAACGACAGCATCACTG
MacroH2A1.2	GGCTTCACAGTCCTCTCCAC	GGATTGATTATGGCCTCCAC
MacroH2A1	TCCATTGCATTTCCATCCATCGGC	ACACGAAGTAACTGGAGATGGCCT
QKI	ATTAAACGGTCCCCTGAAGC	ATCAACAGCCCAAG TGTGAC
DDX5	GTAGCTCAGACTGGATCTGG	TCTCTAGGAATGGCTGGTGG
DDX17	AGAAGTAGCAAGACTGACTCC	CCCCCTCTCACTGTAATCTC
GUSβ	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA

Tabela 2 Primers sequences for macroH2A1 isoforms and total, splicing regulators and control primers [104, 105].

3.4 IMMUNOHISTOCHEMISTRY

Histological slides from FFPE tissue fragments were also obtained from the same surgical specimens and assessed for Gleason Score and TNM stage. Firstly, slides were deparaffinized in xylene (Sigma-Aldrich®, St. Louis, MO, USA) and then hydrated in a decreasing series of ethanol solutions (Merck, Darmstadt, Germany). Epitope retrieval was performed with ethylenediaminetetraacetic acid (EDTA) buffer (Thermo Scientific, Waltham, MA, USA) for 30 minutes, in a microwave at 700W. Endogenous peroxidase activity was neutralized for 20 minutes with 0.6% hydrogen peroxide (Merck). Protein detection was performed using the NovolinkTM Max Polymer Detection System (Leica Biosystems, Nussloch, Germany), according to manufacturer instructions. Slides were incubated with a rabbit monoclonal antibody specific for macroH2A1.1 (#12455; Cell Signaling Technology, Inc., Danvers, MA, USA) in a 1:1000 dilution at 4ºC, overnight inside a humid chamber. Subsequent washing steps were performed with tris-buffered saline with Tween® 20 (TBS-T) (Sigma-Aldrich®). Antigen-antibody binding reaction was revealed through the slides incubation for 7 minutes, in the dark, in a 0.05% (m/v) 3,3'-diaminobenzidine (DAB) solution (Sigma-Aldrich®) in phosphate-buffered saline (PBS) (Biochrom Ltd., Cambridge, United Kingdom) previously activated with a 0,1% hydrogen peroxide solution. Counterstaining of the slides was obtained with hematoxylin (Merck) for about 5 seconds and then slides were washed for 1 minute in a 0.25% ammonium solution (Merck). Lastly, the slides were dehydrated in an increasing series of ethanol content and diaphanized in xylene. After the coverslip was mounted, slides were dried. The FFPE tissues fragments that were not incubated with the antibody were used as negative control of the immunohistochemistry (IHC) reaction. For positive control, FFPE tissue from a normal testis was also included. Slides were observed at the optical microscope and evaluated for macroH2A1.1 immunoexpression by an experienced uro-pathologist. Scoring criteria were adapted from a previous publication of our research group [130]: samples with ≤10% of positive cells were considered "negative expression"; >10-

50% of positive cells were considered “intermediated expression”; >50% of positive cells, samples were categorized as “positive expression”.

3.5 PROSTATE CANCER CELL LINES

RWPE-1 were generously provided by Professor Margarida Fardilha from the University of Aveiro, Portugal; 22Rv1 cells were kindly provided by Dr. David Sidransky at the Johns Hopkins University School of Medicine, Baltimore, MD, USA;DU145 was obtained from the American Type Culture Collection (ATCC, Lockville, MD, USA) whereas LNCaP, PC-3 and VCaP cells were kindly ceded by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at the Institute for Cancer Research, Oslo, Norway. Regarding metastatic cell lines, LNCaP and VCaP are noninvasive and hormone-sensitive, while DU145 and PC-3 are invasive and hormonerefractory. For further in vitro studies for macroH2A1.1 overexpression, LNCaP were selected. The six cell lines used in this study were treated as optional growth medium and supplemented as recommended (Table 4) with 1% of Penicillin-Streptomycin (GIBCO®, Invitrogen, Carlsbad, CA, USA).

Cells were maintained in an incubator at 37ºC with 5% CO2. To harvest the cells for subculture, TrypLE™ Express (GIBCO®) dissociation reagent was used. All prostate cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories).

4. CONCLUSIONS

PCa is the most common tumor in men and a leading cause of mortality and morbidity, worldwide. Both genetic and epigenetic disruption have been implicated in its initiation and progression. Unravel the mechanisms underlying tumor development are key to provide a deeper knowledge of PCa biology, that

might be translated into diagnostic and prognostic, as well as provide novel therapeutic targets [20, 88].

Among epigenetic mechanisms, the shuffle of histones has been recently implicated in tumorigenesis [98]. This is apparent for the two macroH2A1 isoforms that have been the recent focus of several studies, attempting to unravel its role in cancer [126, 129]. MacroH2A1.1 is mostly considered a tumor suppressor, inhibiting stem-like properties, counteracting the functions of macroH2A1.2 [118, 123, 132]. Although its role has been previously tackled in breast and lung cancer, it has not been explored in prostate tumorigenesis, to the best of our knowledge. Thus, we aimed to determine the putative role of macroH2A1 isoforms in PCa and evaluate its biomarker performance.

Through the assessment of macroH2A1 isoforms transcript levels by qRT-PCR in prostate tissues, downregulation of macroH2A1.1 in PIN lesions and primary PCa, compared to normal prostate tissues was disclosed, when either GUSβ or macroH2A1 were used for normalization. This result is in line with previous observations on macroH2A1.1 expression in other primary cancers [103, 129]. Moreover, the intermediate expression levels depicted in PIN lesions is consistent with its putative PCa precursor role. However, macroH2A1.2 expression did not parallel that of macroH2A1.1, as only transcript levels in PIN were significantly lower than those of MNPT and PCa. Nevertheless, this result is in accordance with the lack of altered expression or slight upregulation of macroH2A1.2 in other tumor models [103, 128]. To further illuminate the biological variation of expression of each isoform, macroH2A1.1 transcript levels were normalized against macroH2A1.2. Although PIN displayed the lowest macroH2A1 levels compared to MNPT and PCa, this was mostly due to macroH2A1.2 downregulation. Compared to MNPT, macroH2A1.1 expression levels remained lower in PCa.

To the best of our knowledge, this study is the first to report variations in expression of macroH2A1 and its isoforms in prostate tissues, encompassing morphologically normal and neoplastic (pre-invasive and invasive)

lesions. Globally, macroH2A1.1 expression is gradually decreased along prostatic tumorigenesis, whereas macroH2A1 and macroH2A1.2 are downregulated in PIN. The variations in macroH2A1 are mostly affected by macroH2A1.2 isoform. These alterations are associated with altered expression of splicing regulators, specifically QKI and macroH2A1.1, as well as DDX5 and macroH2A1 and macroH2A1.2.

Interestingly, less differentiated and more aggressive PCa displays lower QKI and macroH2A1.1 expression, as expected for putative tumor suppressors. Although no significant correlation was observed between macroH2A1.1 transcript and protein expression, the percentage of immunostained cells globally reflected the variations observed in transcript levels.

In vitro, stable macroH2A1.1 overexpression attenuates the malignant phenotype, by decreasing cell viability, probably due to increase of cell differentiation.