Universidade de Aveiro

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Departamento de Ciências Médicas

Oriana de Jesus Ribeiro

Resistência à Radioterapia do Cancro da Próstata: o papel das enzimas epigenéticas

Prostate Cancer Radiotherapy Resistance: role of epigenetic enzymes

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Prostate Cancer Radiotherapy Resistance: role of epigenetic enzymes

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Carmen de Lurdes Fonseca Jerónimo, Coordenadora do Grupo de Epigenética e Biologia do Cancro do Centro de Investigação do IPO Porto & Professora Catedrática Convidada do Departamento de Patologia e Imunologia Molecular do Instituto de Ciências Abel Salazar, da Universidade do Porto, e da Doutora Luisa Alejandra Helguero, Professora Auxiliar da Universidade de Aveiro e coorientação do Professor Doutor João Santos, Coordenador do Grupo de Física Médica, Radiobiologia e Proteção contra Radiação do Centro de Investigação do IPO Porto.

“It is not the most intellectual of the species that survives,
It is not the strongest that survives,
but is the one that is able best to adapt and
adjust to the changing environment in which it finds itself”

Charles Darwin

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O júri

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Palavras-chave Cancro da Próstata; Radioterapia, Radioresistência, Modificações Epigenéticas; Reparação dos danos de DNA

Resumo

O cancro da próstata (PCa) apresenta uma das maiores taxas de incidência nos homens em todo o mundo. Apesar da baixa taxa de mortalidade, alguns cancros são heterogéneos e adquirem um fenótipo agressivo, podendo disseminar, tornando-se resistentes à terapia. No que diz respeito às abordagens terapêuticas, a radioterapia é eficaz como tratamento primário, no tratamento do cancro da próstata localizado. No entanto, a eficácia da radiação diminui nos estadios mais avançados, e durante 5 anos de acompanhamento, cerca de 20-40% dos pacientes com cancro da próstata de elevado risco podem ter recorrência do cancro ou metástases à distância após a radioterapia.

De forma a obter um tratamento eficaz, é necessário estabelecer um equilíbrio funcional entre os 5'Rs da Radiobiologia que definem o resultado dos pacientes em resposta à exposição da radiação ionizante.

Após a radioterapia, alterações epigenéticas ao nível celular, como a remodelação da cromatina, afetam a expressão de vários genes-alvo relacionados com o crescimento celular, reparação de danos de DNA e desregulação do ciclo celular.

Existe uma necessidade urgente de compreender os mecanismos moleculares, bem como o comportamento divergente do cancro da próstata, superando assim as principais limitações das terapias atuais para melhorar os resultados clínicos finais do doente.

Keywords Prostate Cancer; Radiotherapy; Radioresistance; Epigenetic Modifications; DNA damage repair

Abstract

Prostate Cancer (PCa) displays one of the major incident rates among men worldwide. Despite low mortality rates, some of these tumours are heterogeneous, acquire aggressive phenotype and might disseminate, becoming resistant to therapy. With regard to therapeutic approaches, radiotherapy is effective as a primary treatment, in the treatment of localized PCa. However, radiation effectiveness decreases in more advanced stages, and within 5 years of follow-up, about 20-40% of patients with high-risk PCa may present tumour recurrence or distant metastasis after radiotherapy.

In order to obtain an effective treatment, it is necessary to establish a functional balance between the 5'Rs of Radiobiology that define the patients' outcome in response to ionizing radiation exposure.

After radiotherapy, epigenetic changes at the cellular level, such as chromatin remodeling, affect the expression of several target genes related to cell growth, DNA damage repair and dysregulation of the cell cycle.

There is an urgent need to further understand the molecular mechanisms as well as the divergent behaviour of PCa, overcoming the main limitations of current therapies to improve the final clinical patient' outcomes.

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LIST OF ABBREVIATIONS

ADT - androgen deprivation therapy

ATCC - American Type Culture Collection

ATM - ataxia-telangiectasia-mutated

AR - androgen receptor

BER - base excision repair

BSA - bovine serum albumin

CPG - Cambridge Prognostic Group

CSCs - cancer stem cells

CT - computed tomography

D - dose

DAPI - 6-diamidino-2 phenylindol

DDR – DNA damage repair

DNA-PKcs - DNA-dependent protein kinase catalytic subunit

DNMTs - DNA methyltransferases

DRE - digital rectal examination

DSBs – Double-strand breaks

EBRT - external beam radiation therapy

EDTA - Ethylenediamine tetraacetic acid

FBS - fetal bovine serum

Gy - Gray

HR - Homologous recombination

IF - immunofluorescence

IgG - immunoglobulin G

IR - ionizing radiation

KDMs - histone lysine demethylases

KMTs - lysine methyl transferases

LDR - low dose rate

LMA – low melting point agarose

LQ - linear quadratic

mCRPC - metastatic castration-resistant prostate cancer

MMR - mismatch repair

mpMRI - multiparametric magnetic resonance imaging

MRI - magnetic resonance imaging

MU - monitoring units

NED - neuroendocrine differentiation

NER - nucleotide excision repair

NHEJ - non-homologous terminal junction

NMA - normal melting point agarose

PARP1 - poly (ADP-ribose) polymerase 1

PBS - phosphate buffered saline

PCa - Prostate Cancer

PET - positron emission tomography

PFA - paraformaldehyde

PIC - protease inhibitor cocktail

PSA - prostate specific antigen

ROS - reactive oxygen species

RR – Radioresistance

SDS-PAGE - polyacrylamide dodecyl-sodium sulfate gels

SSBR - single-strand breaks repair

SSBs - single-strand breaks

TBS-T - Tris-buffer saline-tween 20

TRUS - transrectal ultrasound-guided

WB – Western Blot

WHO - World Health Organization

γ-H2AX - Phosphorylated H2A histone family member X

I INTRODUCTION

1.1. Epidemiology of prostate cancer

According to the World Health Organization (WHO), cancer remains the major leading cause of death before the age of 70 in 112 out of 183 countries (1).

Additionally, prostate cancer (PCa) present extremely high incidence rates among men worldwide (2). According to GLOBOCAN 2020, PCa is the second cancer with higher incidence in men. Specifically, 1.4 million of men were diagnosed with (Figure 1), and 375.000 men died with PCa. The incidence rates in Europe in 2020 reached 470.000 people (3). In 2020, in Portugal, PCa had the highest incidence rates in men. As incidence rates remain high, there is a need to improve the clinical outcomes of these patients (4). Moreover, the mortality rate of PCa rises with age (3). Despite low relative mortality rates in comparison to other cancer models, PCa are generally defined as a highly heterogeneous and aggressive malignancy (5). Generally, when diagnosed at more advanced stages or due to a consequence of disease progression, standard treatment options are not effective at long-term (6). Overall, there is an urgent need to deeply understand the molecular mechanisms and the behaviour of PCa, overcoming the main limitations of current therapies to improve the final clinical patient' outcomes.

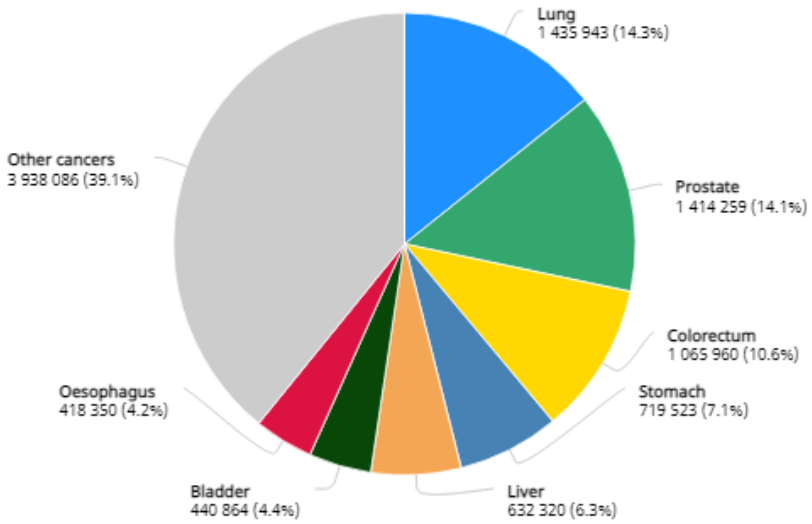

Figure 1 - *Cancer incidence in men in 2020 (Globocan adapted)*

1.2. Diagnosis and Staging

The blood serum biomarker, Prostate Specific Antigen (PSA) is the primary detection tool for PCa screening. Despite its low specificity, PSA is the most sensitive biochemical marker for monitoring PCa. In fact, both epithelial normal and tumour cells express PSA and androgen receptor (AR), characteristic markers of prostate gland, although the specific mechanisms of action, the alternative splicing and the intensity of expression are different among them (7, 8). In addition to the PSA screening, digital rectal examination (DRE) is another detection approach for PCa (9-12). DRE is recommended when PSA levels are high and there are any suspicious of malignancy. Also multiparametric magnetic resonance imaging (mpMRI) is done for local staging. When the PSA levels are high or a DRE is abnormal, a transrectal ultrasound-guided (TRUS) biopsy is performed (13). Moreover, in an initial diagnosis, computed tomography (CT) and magnetic resonance imaging (MRI) are used to determine the PCa stage and establish an adequate treatment (14). Positron emission tomography (PET) imaging in PCa provides more functional information and can detect metastasis. It is also used to analyse the extension of cancer and its localization (15).

Remarkably, patient' risk stratification allows a better clinical discrimination between patients. Recently, there are five risk stratification levels revised and recommended to follow as global guidelines for PCa treatment. Each level is defined according Gleason score, which is a score given to PCa based on its microscopic appearance, PSA levels and T stage (table 1) (16). According with tumour length and other pathological parameters, tumours can be classified using TNM staging system into four stages. Specifically, stage I, corresponding a reduced tumour size not detectable by conventional imaging techniques and, even, not palpable (17). Stage II, tumour that is detectable by DRE screening, however still confined to the prostate gland (17). Stage III, in which tumour evade prostate often in adjacent regions, such as, seminal vesicles (17). Lastly, stage IV, representing the most advanced and aggressive clinical form, in which tumours invade adjacent structures and organs often spreading to distant sites, with the appearance of metastatic disease (17). Thus, with properly tumour classification by the pathologist, different treatment approaches might be chosen (18).

Table 1 *- Cambridge Prognostic Group (CPG) classification according to Parry M., et al, 2020*

CPG	Criteria
1	Gleason score 6 (grade group 1) AND PSA < 10ng/ml AND stages T1-T2
2	Gleason score 3+4=7 (grade group 2) OR PSA 10-20ng/ml AND stages T1-T2
3	Gleason score 3+4=7 (grade group 2) AND PSA 10-20ng/ml AND stages T1-T2 OR Gleason score 4+3=7 (grade group 3) AND stages T1-T2
4	One of the following: Gleason score 8 (grade group 4) OR PSA > 20ng/ml OR stage T3
5	Any combination of Gleason score 8 (grade group 4), PSA > 20 ng/ml or stage T3 OR Gleason score 9-10 (grade group 5) OR stage T4

1.3. Clinical management of PCa: The outstanding of radiotherapy

Treatment modalities for low-risk disease often include active surveillance, and/or radiotherapy with low dose rate (LDR) brachytherapy, or moderate hypofractionated external beam radiation therapy (EBRT) without androgen deprivation therapy (ADT) (18-20). Additionally, first-line treatment options for intermediate-risk patients include either radical prostatectomy, a surgical procedure for partial or complete removal of the prostate gland, or moderate hypofractionation irradiation schemes along with short-term ADT (4-6 months) (18-20).

Concerning high-risk localised PCa and locally advanced disease, radiotherapy (EBRT with 76-80Gy) plus long-term ADT, for, at least, 2 to 3 years, should be considered as a therapeutic decision by the clinician (18-23).

Overall, either radiotherapy or prostatectomy are the well-stablished active therapies for PCa care. Nevertheless, surgical resection often induces side effects with extremely negative impact for patient's quality of life. Specifically, erectile dysfunction or urinary incontinence are common post-surgery effects (24). Nonetheless, the radiation of prostate gland might induce erectile problems, although less frequent, as well as lymph node damage (25).

Additionally, adjuvant ADT are routinely used, in particular, for advanced stages, as previously discussed. Briefly, ADT consists in the testosterone production inhibition by directly blocking the binding of androgen to the AR (26). The blockage of AR signalling prevents the growth and progression of PCa, since it is the main driver for the evolution of this type of cancer (19).

1.4. DNA damage repair pathways

Radiotherapy induces several forms of DNA damage (27). Approximately, in a single cell 10 000 damaged bases, 1 000 single-strand breaks (SSBs) and 40 double-strand breaks (DSBs) are produced, per gray (Gy) (28). DSBs are considered the most lethal and toxic lesions induced by ionizing radiation (IR) (29). Despite their low proportion, if only a single DSB is unrepaired, this event might result in genomic instability and subsequent cell death (29). The DSBs result can directly trigger cell death or activate the response to DNA damage repair (DDR), which allows cell to survive (29).There are two main well-known mechanisms of DDR - homologous recombination (HR) and non-homologous end joining (NHEJ) - allowing for an efficient repair (30). HR takes place during the S phase of cell cycle and promotes the removal of a part of the DNA. NHEJ is triggered in G0/G1 phase as well as G2/M, and promotes DSBs repair by joining the ends of the lesion together throughout the cell cycle (31, 32).

When damage is limited to one of the DNA strands, SSBs, different repair mechanisms as BER (base excision repair), SSBR (single-strand breaks repair), NER (nucleotide excision repair) and MMR (mismatch repair) can be activated.

According to other studies in PCa, in addition to the resistance mechanisms that lead to the repair of SSBs and DSBs, protection from paralyzed replication bifurcations caused by BRCA1 / 2 inactivation may occur. BRCA1 and BRCA2 are among the most frequently mutated genes in metastatic castration-resistant prostate cancer (mCRPC).

It is important to emphasize that tumours with impaired DNA damage repair might response better to cell killing therapies, such as radiotherapy (28, 33, 34).

A response based on DDR is essential to maintain the integrity of the genome in regions exposed to IR (32). The DDR is a complex network involving DNA damage sensor proteins, such as the poly (ADP-ribose) polymerase 1 (PARP1), the ataxia-telangiectasia-mutated (ATM), and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), (Figure 2).

The DNA-PKcs is recruited and activated by the Ku-70/80 heterodimer bound to DSBs allowing NHEJ repair (32). Increased DNA-PKcs expression is observed in a large fraction of late-stage tumours, including prostate, and it is associated with poor outcome and resistance to radiation treatment (32).

ATM has a central function in DSBs repair and is recruited by the MRN complex. It phosphorylates numerous downstream substrates, including the histone H2AX (32). ATM is activated by phosphorylation of BRCA1 to phosphorylate p53 at the Ser15 site (31).

PARP detects SSBs, specifically, BER, inducing post-translational poly (ADP-ribosylation (PARylation)) to modulate chromatin structure, and guided the repair pathway by recruiting numerous factors to the damage site (35). According to Wengner, *et al.*, PARP-1 inhibitors represent a novel treatment option for PCa patients with certain DDR alterations.

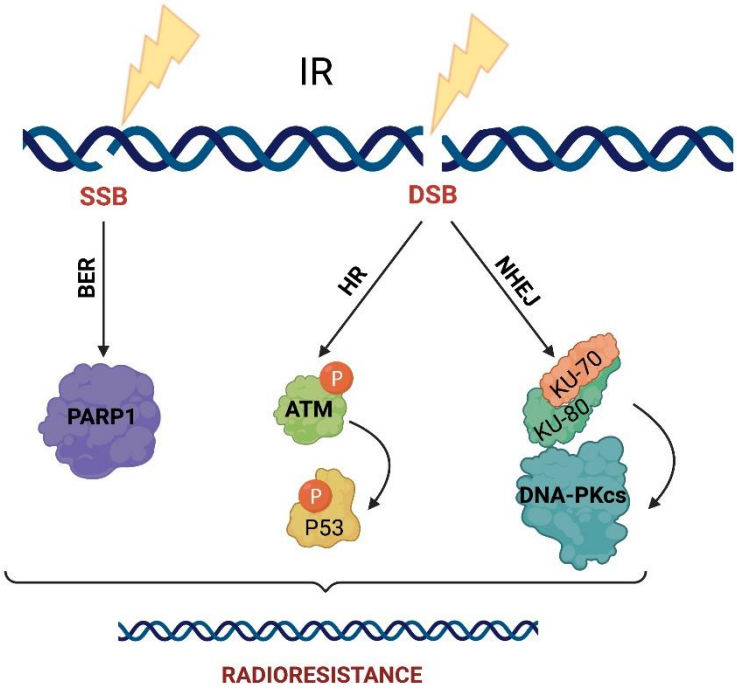

Figure 2 *- Schematic representation of DNA damage repair network. Created with BioRender.com. Abbreviations: IR, ionizing radiation; SSB, single strand breaks; DSB, double strand breaks; BER, base excision repair; HR, homologous recombination; NHEJ, non-homologous end-joining; PARP1, poly (ADP-ribose) polymerase 1; ATM, ataxia-telangiectasia-mutated; DNA-PKcs, DNA-dependent protein kinase catalytic subunit*

1.5. Mechanisms of resistance to radiotherapy

Indeed, radiotherapy is a key cytotoxic first-line therapy for PCa (36). Hence, it has a fundamental role in increasing the survival and quality of life of patients with PCa. Despite the successful rates for locally confined disease, 20-40% of high-risk PCa patients experience long-term recurrences, within 5 years follow-up (27, 37, 38).

Overall, there are several molecular and cellular mechanisms being major drivers of therapy failure (22). Particularly, cancer stem cells (CSCs) percentage, neuroendocrine differentiation (NED) and hypoxic foci became over the years key predictive factors of radioresistance (RR) (27). The concept of 5'Rs which conduct patients' outcome in radiotherapy response allows for better understand the radiobiology of cancer cells. Indeed, DNA damage Repair, Repopulation of the tumour cells, intrinsic Radiosensitivity, Reoxygenation of the deepest tumour cell layers and Redistribution of cells to different phases of cell cycle are the main known R's of radiobiology (36, 38-42).

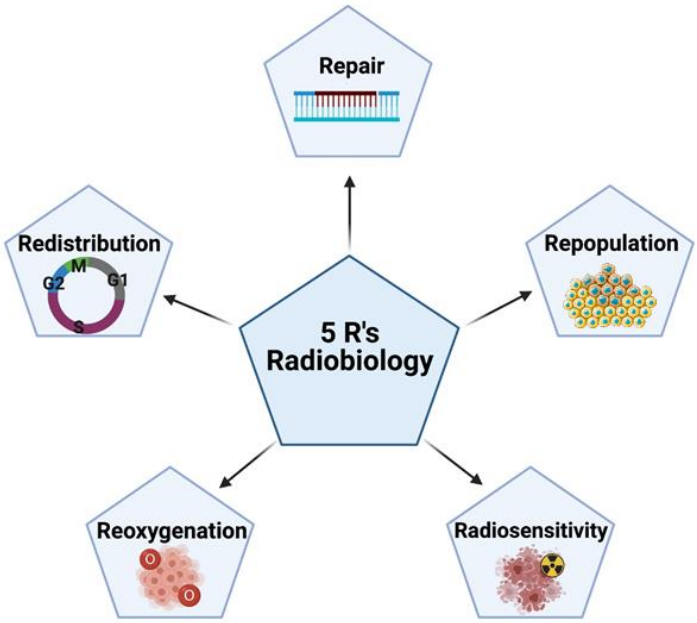

Figure 3 - 5R's of Radiobiology: *Repair of DNA damage; Repopulation of the tumor; intrinsic Radiosensitivity; Reoxygenation of hypoxic tumor; Redistribution of cells. Created with BioRender.com*

Radiation arbitrarily interacts with DNA molecule, causing wide range of lesions including DSBs, as discussed previously (39, 43). In redistribution, radiosensitivity is dependent on the cell cycle: the highest RR of cells occurs in the S phase, compared to the G2 / M phases. Moreover, the effectiveness of radiotherapy is measured according to the level of tumour oxygenation, since it depends on predictive factors such as hypoxia, lack of nutrients, reduced pH and abnormal development of the vasculature. Therefore, during reoxygenation, it is possible that the surviving hypoxic tumour cells become more radiosensitive due to the oxygen supply (35, 40, 41).

During fractionation spares in radiotherapy, redistribution and reoxygenation facilitate increased overall cell kill by redistributing the resistant cell population into more sensitive states over time. However, repair and repopulation produce increased cell survival by allowing for recovery of cells after individual radiation doses as well as by allowing proliferation between radiation doses (44, 45).

According to a critical review by Chaiswing, *et al*., the maintenance of a balance of reactive oxygen species (ROS) in PCa is an important mechanism of RR. PCa cells have a higher level of ROS, including oxidative stress markers, compared with normal prostate

cells and the PCa recurrence and progression is associated with the level of oxidative stress (22, 46).

Resistance to radiotherapy can be intrinsic or treatment-induced. Given the heterogeneity of PCa cells, it is likely that certain cells have intrinsic RR, whereas others have the ability to acquire RR over the course of radiotherapy (20, 27). The intrinsic RR is present within the cell even before the treatment has started and it can be attributed to several factors. On the other hand, acquired RR leads to the development of adaptive responses induced by the irradiation itself (20).

An equilibrium between previous mentioned radioresistant associated factors must be achieved in order to ensure therapeutic efficacy, always taking in consideration the limitation of normal surrounding tissues. Properly adjustment should be done to obtain the highest patients benefit with serious late complications. (44, 45).

Linear quadratic (LQ) is a model that can be used to assess cell survival curves (45). This model assumes that there are two components to evaluate radiation-induced cell killing (45). One that it is directly proportional to the irradiated dose, alpha component and the other one, the beta component that it is proportional to the square of the dose (45, 47). This model assumes the following equation to determine cell survival:

$$S = e^{-\alpha D - \beta D^2}$$

S is the cell surviving fraction for a specific single dose (D), and α and β are constants (45). The components of cell killing that are proportional to dose and to the square of the dose are equal if:

$$\alpha D = \beta D^2$$

or

$$D = \frac{\alpha}{\beta}$$

The ratio of α to β is often used to describe the response of cells to radiation exposure (45). Lower α/β ratios are commonly associated with late responding tumours, such as PCa (45). Indeed, this type of cells often behave like normal tissue, due to a longer cell cycle periods and slowly proliferative rates comparing to other highly proliferative tumour models (45). For PCa cells often there is the need to apply hypofractionation schemes, with relatively higher doses per fraction than those used in the conventional treatment (46).

The need to find and explore new predictive biomarkers of response and understand the molecular mechanisms that lead to the acquisition of resistant behaviours leads us to a study at the epigenetic alterations level in PCa.

1.6. Epigenetics and cancer

Epigenetics is a stably heritable phenotype resulting from changes in chromatin organization and transcriptional genes regulation passing through generations without alterations in the DNA sequence (48, 49). Therefore, the human DNA content is exactly the same in somatic cells, while gene expression patterns have distinct differences between cell types that can be clonally inherited (49, 50). Epigenetics is a fundamental component to the normal organism development, as well as its responsiveness to environmental signals (51). Nowadays, there are extended knowledge concerning epigenetic changes and the association with diseases or external factors (environment) (51). Therefore, differences in epigenetic states in normal or in tumour tissues are crucial for the identification of disease biomarkers, as well as associating them with the corresponding stage (diagnosis and prognosis) (48). There are also changes in the expression levels of several epigenetic players, commonly used as discriminators and targeted biomarkers in cancer (48). Specifically, the progression of cancer associates with the gain or loss of epigenetic enzymes (49, 52).

Consequently, epigenetic mechanisms can influence the gene activity at the transcriptional and post-transcriptional levels and/or at post-translational modifications (53), contributing to the development and progression of most human cancers (54).

According to Goering, *et al*., in PCa, events as DNA repair, apoptosis, cell cycle control, steroid hormone response and metastasis are driven by gene silencing through hypermethylation phenomenon (55).

Of note, DNA methylation, histone post-translational modifications, histone variants and chromatin remodeling complexes are the commonly reported epigenetic mechanisms in cancer **(Figure 4)**. Changes at the epigenetic level are considered an important hallmark for disease management (56).

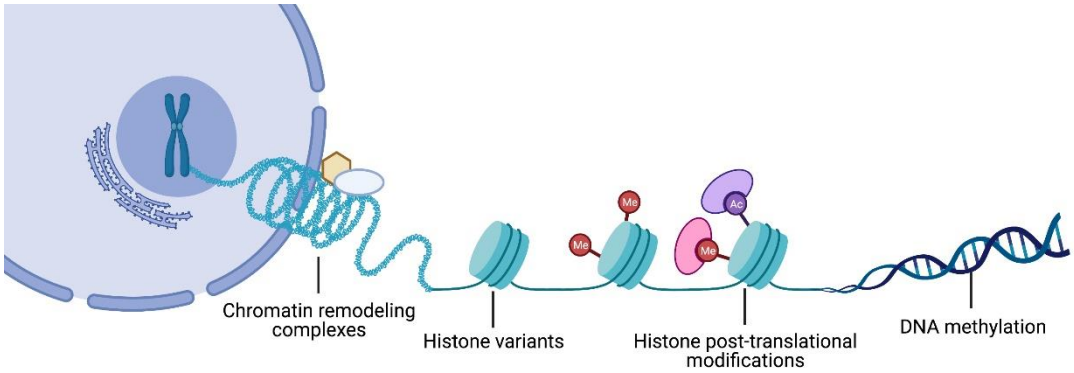

Figure 4 - Epigenetic regulatory mechanisms in gene expression. *Epigenetic mechanisms comprising DNA methylation, histone post-translational modifications, histone variants and chromatin remodeling complexes. Created with BioRender.com*

DNA methylation is an epigenetic event that affects cell function by altering gene expression and refers to the covalent addition of a methyl group, catalyzed by DNA methyltransferase (DNMT) to the 5-carbon of cytosine in a CpG dinucleotide. In PCa, modifications in this epigenetic mechanism are highly prevalent and constitute a crucial factor in the disease development and progression (54, 55, 57).

In normal cells, most CpG islands, commonly known as preferential methylation sites, are unmethylated allowing specific gene transcription activation (57). Conversely, in tumours, many CpG islands exhibit aberrant hypermethylation resulting in inappropriate transcriptional repression and gene inactivation (57).

DNA methylation is the most widely studied and common epigenetic modification. It plays important roles in the carcinogenesis, and has been extensively associated with radiotherapy resistance (58). The use of demethylating agents as 5-Azacytidine, improved therapeutic efficacy of tumour cells (58, 59).

This type of epigenetic modification is typically regulated by three widely studied DNA methyltransferases (DNMTs) in mammalian cells (59). DNMT1 is responsible for maintaining methylation pattern (59). Conversely, DNMT3a and DNMT3b are responsible for the unmethylated DNA modification and the *novo* methylation, respectively (59).

Moreover, human chromatin is composed by DNA chain coupled with histone octamer proteins organized in four cores, H2A, H2B, H3 and H4 (60). These specific proteins can acquire several post-translational covalent alterations in N-terminal tails, such as

methylation of arginine's or lysine's residues, as well as acetylation, phosphorylation or ubiquitination (61, 62). Histones are proteins that are critical in the packing of DNA into the cell and into chromatin and, then in chromosomes (61). Histones are the most important structural components of nucleosome, the fundamental chromatin unit. Due to the high proximity between these proteins and the DNA chain, it is of great importance to understand how histone epigenetic changes occur and influence gene transcription. Furthermore, these modifications influence all mechanisms associated with DNA, such as recombination, replication, repair, regulation, transcription and packaging (63).

The change in electrostatic charges, as well as, the recruitment of binding proteins that are part of the chromatin remodeling complexes, resulting from histone modifications, led to changes in the chromatin structure, making it more or less accessible to other proteins, such as transcription factors, influencing the transcription process (36).

Histone acetylation and methylation represent the most reviewed histone post-translational modifications (61). Histone acetylation is frequently associated with open structure of chromatin allowing generally transcription activation (64). Conversely, the effect of histone methylation can be mostly pleiotropic, since it may result in both gene transcription-repression/activation, through chromatin compression and decompression, according with the specific modification on lysine residues (64). This role of regulating the transcription can influence not only the cell cycle, but also the metabolism and tumour aggressiveness skills (62). In fact, H3K9me2,3 and H3K27me3 are well-known methylated residues associated with global transcription repression (65), while gene transcriptional activation is commonly driven by trimethylation of H3K4 and H3K36 (66).

As methylation has a pleiotropic effect, it becomes a focus of study with a particular interest in order to understand its dynamics. So, there are enzymes that are responsible for the demethylation and methylation of histones. Accordingly, these epigenetic enzymes that regulate this process are KDMs, histone lysine demethylases, and KMTs, lysine methyl transferases (67). Thus, KDMs modified histones through the removal of methyl groups, whereas KMTs through the insertion of methyl groups (68).

According to several authors, some studies have shown that KDMs are dynamically located in the cellular and tissue microenvironment, and its deregulation is often associated with human diseases, such as development and progression of PCa (69-72). The determination of expression levels of these enzymes in prostate biopsies might be able to convey relevant prognostic information in a pre-therapeutic setting (70).

Another study evaluated the methylation of H3K4 and H3K9 in PCa cells, without exposure to IR. H3K9me2,3 were significantly reduced in PCa tissues, comparing with the normal ones. On the other hand, all methylation states of H3K4 (histone associated with the activating marker) were positively regulated in androgen-independent tumours and correlated with clinical-pathological parameters (63), suggesting that these changes are possibly associated with cancer and are predictive of clinical outcomes.

Also Liao, *et al*., investigated other histone marks in prostate tumorigenesis, as H3K18 acetylation and H3K27 methylation (63). In a cohort of PCa cases, there was a significant association between the levels of acetylated H3K18 and the increased risk of tumour recurrence (63). H3K27me1,3 were reported to positively correlate with aggressive tumour characteristics. Specifically, in H3K27me3, the concentrations were lower in men with metastatic disease, comparing with localized and advanced local tumours (63, 73).

Furthermore, these studies led to the association of histone changes as events associated with increased risk of PCa recurrence, as well as, with shorter survival.

1.7. Radioresistance and epigenetic regulation in PCa

As previously referred, according to recent studies, radiation can induce genetic and epigenetic changes in PCa cells that might confer RR (74).

Histone modifications are not so commonly studied in this field, thus making it possible to deepen the knowledge of these proteins.

Nonetheless, according to Peitzsch, *et al*., in a PCa study, cells' irradiation induced changes in trimethylation of H3K4 and H3K36, which are associated with transcriptional activation (75). Conversely, a significant decrease in H3K27me3 (gene silencing-associated) was observed over the time after IR (75). Moreover, increased KDM3A expression was implicated in RR of PCa cell lines (76).

In esophageal squamous cell carcinoma, KDM3A upregulation in cell lines and tumours tissues, played a critical role in esophageal cancer aggressiveness and RR, comparing with the normal esophagus (77). Also in Hepatocellular carcinoma, KDM3A was involved in RR, increasing the malignant potential of the carcinoma (78).

Moreover, Katagi, *et al*., studied the inhibition of KDM6B in demethylation of H3K27me3 at near sites of DNA damage and after IR exposure in diffuse intrinsic pontine glioma. This inhibition resulted in a restored K27 methylation in these tumour cells, defective

recruitment of repair factors, leading to lower efficacy of DNA damage repair, and consequently radiosensitization (79).

Hence, IR was suggested to induce genomic instability, and to the phenotypic features evolution of the tumour-initiating populations during the course of IR exposure.

In addition to these specific modifications of enzymes and histone markers caused by IR exposure, the DSBs are commonly found in DNA chain leading with H2AX phosphorylation (γ-H2AX), Phosphorylated H2A histone family member X, a DNA damage marker (80).

According to Oorschot *et al*., γ-H2AX foci is used as a radiosensitivity parameter, and the decay of these foci is associated with the cell survival, such as repair of DNA damage after radiation treatment (81). This was corroborated with a study that concluded that after 2 Gy, higher surviving fractions correlated with a lower number of residual γ-H2AX foci in PCa cell lines (81).

Regarding the modifications in the histone regulatory enzymes, according with other study, in different types of cancer, including PCa, levels of KDM4B increased after 6H of exposure to IR. These levels were associated with decreased expression of γ-H2AX foci, as well as increased cell survival (82).

Consequently, radiotherapy induces changes in the activity of KDMs with specific enzyme activity for histone marks associated with repression, leading to the opening of chromatin. In PCa, these changes that occur at the level of lysine demethylases are not yet clear (77). The greater the ability to repair DNA damage induced by IR, more radioresistant cells are, leading to the deregulation of certain genes, such as silencing those related to DNA repair or advancing the cell cycle, which may lead to the understanding of radiobiological behaviour of these radioresistant cells (75). Changes in histones, as well as changes in the chromatin structure are the basic changes driven by RR in PCa (78).

II AIMS

Prostate adenocarcinoma is one of the most incident cancer in men worldwide. Remarkably, it remains a fundamental public health problem. Over the years, have been a significant progress to improving patient early detection and prognosis. Despite the advances in PCa treatment with innovative and accurate techniques, many patients remain vulnerable to tumour recurrence and progression of the disease. Radiotherapy represents one of the main active treatment approaches for PCa. Early stage PCa detection is treatable and often associated with a good prognosis. However, when diagnosed in later stages, with locally advanced disease, the effectiveness of radiotherapy may be compromised, due to tumour RR.

Thus, the main goal of this dissertation is to determine the effect of epigenetic regulation underlying PCa radiotherapy resistance, using 2D cell cultures exposed to ionizing radiation.

Accordingly, the specific aims are:

1. Assess cell survival curves for the different prostate cell lines with different IR (Gy).
2. Determine protein expression of repressive marks histones (H3K9me2, H3K9me3, H3K27me3), comparing wild-type cells before any treatment and after exposure to radiation.
3. Assess the presence of DNA damage foci (γ-H2AX) and downstream DNA repair markers (γ-ATM, DNA-KPcs and BRCA1).

III MATERIALS ANS METHODS

1. Cell culture

In order to study the role of epigenetic regulation in PCa RR, *in vitro* studies were performed using six American Type Culture Collection (ATCC) PCa cell lines comprising LNCaP, 22Rv1, C4-2, C4-2B, PC-3 and DU145 available in the laboratory. All PCa cells growth in RPMI-1640 (PAN-Biotec, Germany) complete medium supplemented with 10% fetal bovine serum FBS (Biochrom, Germany) and 1% penicillin/streptomycin (GRiSP, Portugal), and were maintained at 37ºC with 5% CO_2 and 74% N_2.

Mycoplasma test detection was periodically performed through TaKara PCR Mycoplasma Detection assay (Clontech Laboratories, EUA) using the primers: GPO1: ACTCCTACGGGAGGCAGCAGTA and MGSO: TGCACCATGTGTCACTCTGTTAACCTC in order to ensure the optimal and reliable cell growth before all experiments.

Table 2 - PCa cell lines characterization. *Cell growth conditions*

Cell lines	Origin	Culture properties	Growth medium	Supplement
LNCaP	Lymph node metastatic	Adherent	RPMI-1640	10% FBS + 1% penicillin/streptomycin
C4-2	Mouse vertebral metastasis	Adherent	RPMI-1640	
C4-2B	LNCaP cell xenograft	Adherent	RPMI-1640	
22Rv1	Mouse CWR22R xenograft	Adherent	RPMI-1640	
PC-3	Lumbar vertebral metastasis	Adherent	RPMI-1640	
DU145	Brain metastasis	Adherent	RPMI-1640	

RPMI, Rapid Prototyping & Manufacturing Institute; FBS, fetal bovine serum

2. Total and histone protein extraction

The extraction of total and histone protein was performed in order to determine protein levels under specific conditions of PCa cell lines. The initial process consisted in removing the cells from the culture flasks, using the enzyme trypsin. The flasks with the cells were placed in the incubator at 37ºC. Subsequently, were washed with cold phosphate buffered saline 1X (PBS 1X).

For total protein extraction, cells were scraped in lysis buffer, RIPA (Kinexus Bioinformatics Corporation), supplemented with protease inhibitor cocktail (PIC) (Roche, Switzerland). After 15 minutes in ice, samples were centrifuged at 13,300 rpm for 30 minutes at 4ºC and, then, the supernatant was collected.

In histone extraction, cells were resuspended in Triton X-100 Extraction (Sigma-Aldrich, Germany) Buffer at 0.5% in PBS 1x supplemented with PIC 1x. Then, cell lysis occurred on ice for 10 min with gentle agitation, followed by centrifugation at 2000 rpm for 10 min to remove and discard the supernatant, two times. Finally, cells were incubated overnight with the HCl 0.2 under agitation, followed by a centrifugation at 2000 rpm for 10 minutes, 4ºC. For longer storage periods cells were maintained at -80ºC.

Finally, using the Pierce BCA Protein Assay kit (Thermo Scientific Inc.), the protein concentration in the samples was determined.

3. Western Blot

A total of 30µg protein from each cell line was resuspended in loading buffer, denatured at 95ºC for 5 minutes, and loaded in polyacrylamide dodecyl-sodium sulfate gels (SDS-PAGE). For total and histone protein extracts, 8% and 12.5% running gel was used, respectively. Next, polyacrylamide gels with total protein and histones were transferred into a nitrocellulose membrane (BioRad Laboratories). This procedure was done using a Tris-Glycine buffer 1X buffer on Trans-Blot Turbo Transfer system (Bio-Rad, USA), semidry transfer system at 25V and 1.3 mA during 10 minutes for histones, whereas with a Trans-Blot Turbo System (Bio-Rad) at 50V during 1 hour at 4ºC for total protein. Before the immunoblotting with antibody at appropriate dilution, membranes were incubated in blocking solution (5% BSA (Fisher Scientific, USA) /TBS-0.1% Tween (GRiSP), for 1h at room temperature. Afterwards, membranes were incubated overnight at 4ºC with primary antibodies (table 2). After incubation, membranes were washed three times with TBS-T (Tris-buffer saline-tween 20) and incubated with specific horseradish peroxidase secondary

antibody, diluted 1:5000, for 1h, with agitation at room temperature. Finally, using the chemiluminescence technique (Clarity WB ECL substrate, Bio-Rad), the results of the immunoblotting were pictured.

In this technique, β-Actin and H3 housekeepings (load control) were used, in order to quantify the results by the optical densitometry method, using the ImageJ analysis software (version 1.6.1, National Institutes of Health).

4. Immunofluorescence

Immunofluorescence (IF) is a technique that allows to detect and locate the expression of the protein of interest.

For the PCa cell lines, immunofluorescence was performed for the DNA repair marker (H2AX), as well as for the markers of the KDM3A and KDM6B demethylase enzymes, and methyltransferases G9a and EZH2, and the respective markers of histone, H3K9me2 and H3K27me3. For this technique, about 3 x 10^4 cells / mL were inserted into 96-well black plates. Then cells were placed at 37ºC and 5% CO2, to allow them to adhere. Then, cells were exposed to 2Gy of IR, and the IF technique was performed after 2h and 24h. Therefore, initially the cells were washed in 5 minutes with PBS 1X, followed by fixation with 4% paraformaldehyde (PFA) (ChemCruz, USA) for 10 minutes. Permeabilization was performed with the 0.25% Triton X-00 solution in PBS 1X for 15 minutes. Blocking was done with 5% BSA for 30 minutes, and finally the primary antibody was incubated. The dilutions are described in table 2. The incubation time of the antibodies was overnight, at room temperature, except for the damage marker (1 hour), at room temperature.

Subsequently to the primary antibody, 1 hour of incubation was performed with secondary antibodies: anti-rabbit immunoglobulin G (IgG) Alexa Fluor™ 448 goat (A11008, Invitrogene) and anti-mouse immunoglobulin G (IgG) Alexa Fluor™ 594 goat (A11032, Invitrogene) for KDM3A, KDM6B, EZH2, H2AX, H3K9me2 and H3K27me3 and for G9a, respectively. Finally, the cells were stained with 4 ', 6-diamidino-2 phenylindol (DAPI) (AR1176, BOSTER Biological Technologies) and Olympus IX51 fluorescence microscope with an Olympus XM10 digital camera, was used for taking pictures, through CellSens software.

Table 3 *- Primary antibodies used in WB and IF*

Primary antibody	Company, clone	WB Dilution	Second antibody specie	IF Dilution
y-H2AX	Cell Signaling, 2577S	1:500	Anti-rabbit	1:500
EZH2	Novocastra_NCL-L	1:1000	Anti-mouse	1:250
KDM3A	Abcam, ab91252	1:500	Anti-rabbit	1:250
KDM6A	Cell Signaling UTX_D3Q1I	1:1000	Anti-rabbit	-
KDM6B	Abcam, ab38113	1:500	Anti-rabbit	1:250
H3K9me2	Cell Signaling, D85B4	1:250	Anti-rabbit	1:250
H3K27me3	Millipore, 07-449	1:250	Anti-rabbit	1:250
G9a	Perseus Proteomics, PP-A8620A-00	1:500	Anti-mouse	1:250
B-actin	Sigma-Aldrich, A1978	1:10,000	Anti-mouse	-
H3	Abcam, ab1791	1:3000	Anti-rabbit	-

WB, Western Blot; IF, immunofluorescence

5. Colony formation assay: cell survival curves

Colony formation assay is an *in vitro* cell survival assay that evaluates the ability of a single cell to form a colony (considered valid with ≥50 cells) (83). Colony formation assay was performed to evaluate the cellular response under a spectrum of radiation doses (84). The test conditions used to this *in vitro* assay were control group, and treatment groups with 2Gy, 4Gy, 6Gy or 8Gy of radiation.

PCa cell lines were placed in 6-well plates with an optimized concentration, (ranged from 1000 to 2000 per well) with 2mL of complete culture medium. After a 24 hours' incubation at 37ºC and 5% CO_2, the cells were exposed to the different radiation described above, and remained in the incubator at 37ºC and 5%CO_2 for 7 days. The linear accelerator

photon Truebeam (Varian Medical Systems, California, USA) was used to irradiate the different lines. The IR measurement field was 25 x 25 cm^2, and the dose rate in the monitoring units (MU) applied was 600 MU / min. After 7 days in culture, the cells were washed in PBS 1X, and fixed under agitation in methanol for 10 minutes. Next, cells were stained with Hemacolor solution 2 (Sigma-Aldrich/Merck) for 1 minute, following Hemacolor solution 3 (Sigma-Aldrich/Merck) for 1 minute, and then, washed with PBS 1X. To finish this technique, distilled water was used to wash the cells, and the 6-well plates were left inverted overnight to dry. The Olympus IX51 microscope made possible to count the colonies, and the statistical cell survival curves analysis were done by linear quadratic equation, that assumes that there are two components to cell killing by radiation, one that is proportional to dose and one that is proportional to the square of the dose.

$$LQ: S = e^{-\alpha D - \beta D^2}$$

6. Comet Assay

The repair of DNA damage influences the effectiveness of radiotherapy. Therefore, in addition to an analysis of the damage marker (γ-H2AX), the comet assay technique is used to determine these damages. Briefly, after exposure to radiation (2 and 8Gy), at different time points, 3,000 cells from 4 PCa cell lines were harvested by trypsinization, using Trypsin (Pan Biotech, Germany), washed in PBS 1X, resuspended in 0.5% low melting point agarose (LMA) (w / v), (Invitrogen / Fisher Scientific, USA) and then placed on a sheet. This sheet was previously covered with 1% of the normal melting point agarose (NMA) layer (w / v) (GRiSP, Portugal), covered with a coverslip and incubated for 30 minutes at 4ºC for polymerization. Then, in order to proceed with the evaluation of the DNA molecule, the cells were immersed in lysis buffer, pH 10 (2.5 M NaCl, 100 mM ethylenediamine tetra-acetic acid (Na_2EDTA), 10 mM Tris base, Triton X -100 1%) at 4ºC for 2 hours, without lighting. During 40 minutes at 4ºC, cells were incubated with alkaline electrophoresis buffer, pH = 13 (300 mM NaOH, 1 mM Na_2EDTA) at 4ºC to allow the DNA to open. Single-cell gel electrophoresis was performed for 30 minutes at 21 V, 300 mA, on a horizontal electrophoresis platform. Finally, the 10 minutes' incubation process of the sheets in the neutralization buffer (0.4 M Tris-Base, pH 7.5) and the fixation in 5 minutes in methanol (Supelco/ Sigma-Aldrich) was done twice. Following the colouring with DAPI, the slides were assembled. Using the Olympus IX51 fluorescence microscope with an Olympus XM10 digital camera and CellSens software, the comets were visualized, Figure1. Through the OpenComet v.1.3.1, plugin for the ImageJ software (version 1.6.1, National Institutes of

Health), the analysis of the formed comets was made. The assessment of global DNA damage (SSB and DSB) was determined by measuring the moment of the tail that assesses the % of DNA fragmentation, by multiplying the % of the DNA tail and averages of the head and tail distance. To the analysis be viable, a sample must have contained at least 50 comets.

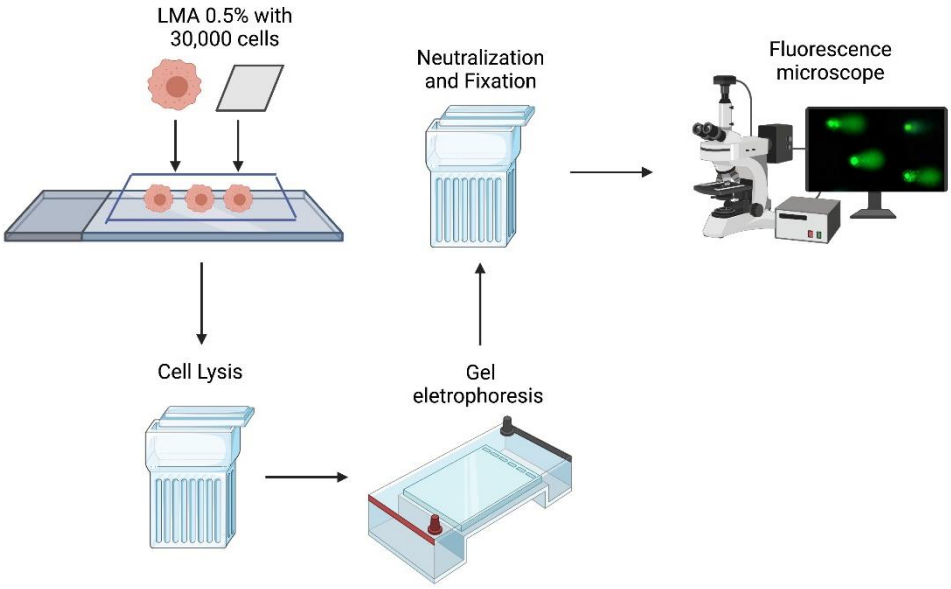

Figure 5 *- Single-cell gel electrophoresis (Comet Assay). Abbreviations: LMA, low melting point agarose*

7. Statistical analysis

Statistical analysis was performed using the GraphPad Prim 7.0 software (GraphPad Software Inc., Chicago IL, USA). For multiple comparison between groups (all conditions versus the vehicle), non-parametric Kruskal-Wallis test was used.

P-values were considered statistically significant when inferior to 0.05. Significance is shown vs. the respective control and depicted as follows: *p≤0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ^{ns}p>0.05 (non-significant).

IV RESULTS

1. Differential radiobiologic response to ionizing radiation among PCa cell lines

Colony formation assay was performed to evaluate the cellular response under a spectrum of radiation doses.

A differential radiobiological behaviour was observed among PCa cell lines. Of note less responsiveness was observed for PC-3 and DU145 cell lines over the radiation exposure, compared with the other cell lines (Figure 6A). Taking together, lines with greater response to radiation were more radiosensitive such as LNCaP, C4-2, C-2B and 22Rv1, whereas cells with less radiation response were more radioresistant, such as PC-3 and DU145 (Figure 6A).

Likewise, greatly differences were found between the 3 cell lines C4-2, 22Rv1 and DU145 with increasing resistant behaviour among them accordingly (Figure 6B). Once again it was possible to confirm that DU145 line was the most radioresistant one, since it remains with a larger number of colonies at 6 Gy of IR (Figure 6B). Meanwhile, no colonies were observed with this radiation dose for C4-2 and 22Rv1 (Figure 6B). Of note the same trend was observed for the other cell lines (Supplemented Figure 1).

The same trend of response was observed with 2Gy (SF2) of exposed radiation. Because 2Gy is the fraction commonly used in patient fractionation schemes, it is crucial to assess the response to this type of low radiation in the different lines (Figure 6C). Through this response, a threshold of the survival curve that influences the radiosensitivity parameters (α and β) was obtained (Table 4).

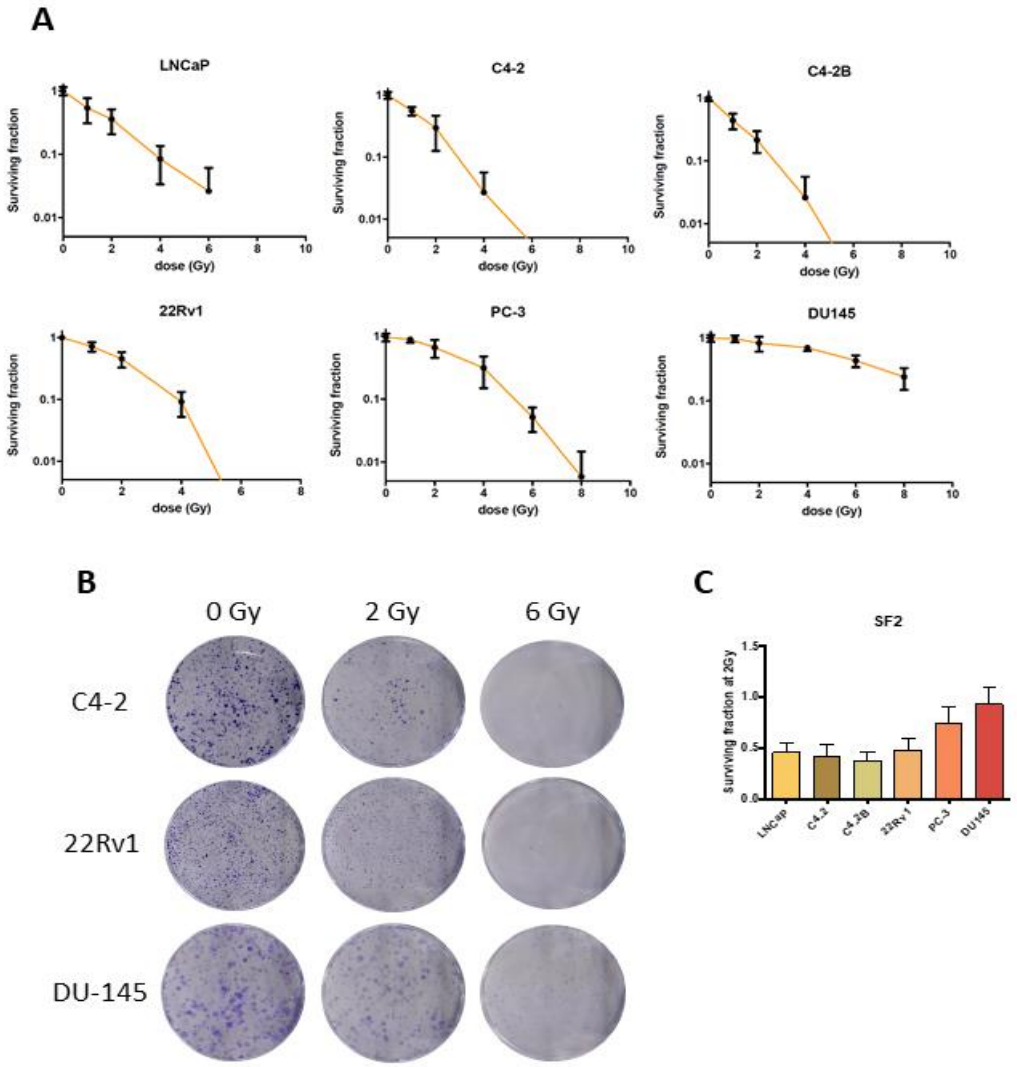

Figure 6 - Characterization of PCa cell lines to radiotherapy treatment. A, *Cell survival curves for LNCaP, C4-2, C4-2B, 22Rv1, PC-3 and DU145 with irradiation doses raging from 0 to 8Gy.* ***B,*** *Representative picture of stained colonies with 0, 2 and 6 Gy for C4-2, 22Rv1 and DU145 cell lines.* ***C****, Surviving fraction at lower doses, represented by standard fractionation dose at 2Gy. Abbreviations: Gy, Gray; SF2, Surviving Fraction at 2Gy*

α and β are constants and its ratio describe bendiness of cell survival curve and are often used to quantify fractionation sensitivity of tissues to ionizing radiation (45). Accordingly, less responsive cell lines have a larger cell cycle division and slow proliferation rates, showing lower ratio α / β.

Herein, the RR behaviour of PC-3 and DU145 PCa cells in comparison with the other cell lines was due to their extremely lower α/β ratios, 1.56 and 1.57, respectively (Table 4).

Table 4 *- Summary values of α / β ratios for PCa cell lines*

	LNCAP	C4-2	C4-2B	22RV1	PC-3	DU145
α	0.5528	0.4853	0.7962	0.2327	0.078	0.02824
β	0.0068	0.076	0.007	0.08691	0.057	0.01795
α/β	81.29	6.385	113.74	2.68	1.36	1.57

2. Epigenetic landscape in PCa cell lines: The role of histone methylation

Western Blot (WB) was performed in order to evaluated the expression of histone repressive and activate markers at basal levels. In Figure 7A, the most aggressive cell lines (radioresistant-like) displayed lower expression levels of histone repressive-associated marks, such as H3K9me2 and H3K27me3, while no differences were apparent concerning active-associated histone marks, such as H3K4me3.

In accordance, lower nuclear protein expression levels of histone repressive markers were found in the most radioresistant cell lines, PC-3 and DU145 (Figure 7B).

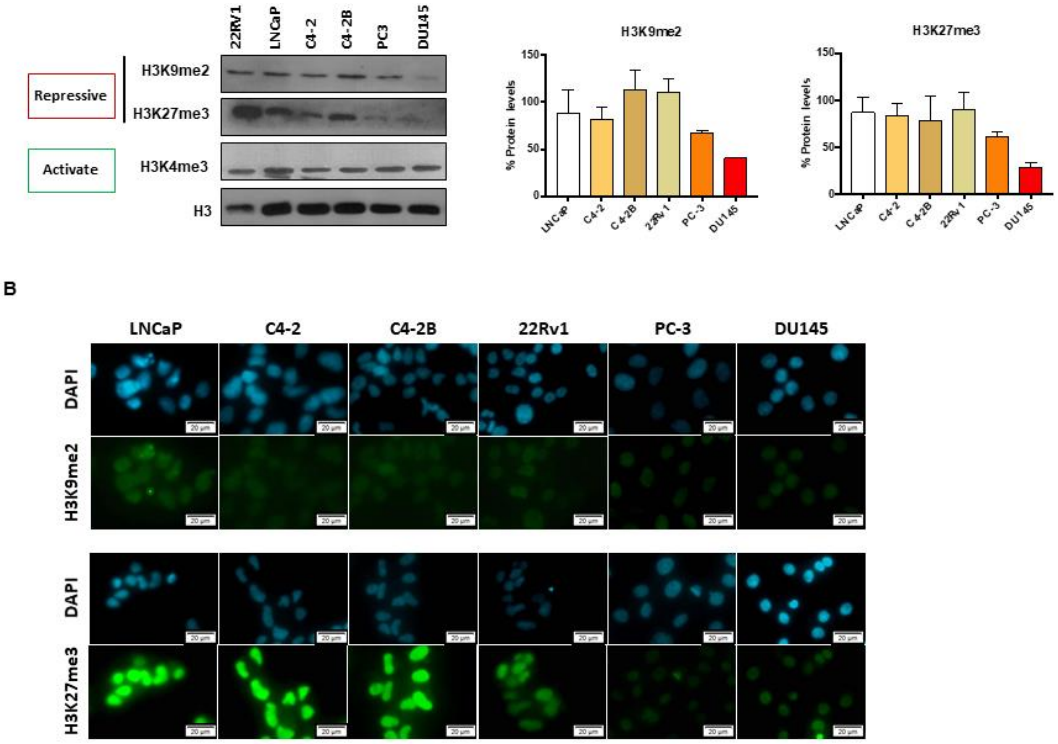

Figure 7 - Characterization of repressive and activate-associated histone marks in WT PCa cell lines. A*, H3K9me2, H3K27me3, H3K4me3 (17kDa) protein expression by representative Western blot pictures and % of protein levels of repressive markers using optical density values.* ***B,*** *Nuclear expression of H3K9me2 and H3K27me3 (green staining) and DAPI (blue staining) by immunofluorescence at 400x magnification (scale bar 20 µm). H3 was used as a loading control.*

3. Low repressive histone methylation markers might be associated with PCa radioresistance

Western Blot was accomplished in order to evaluate the KDM's, KMT's and histones expression in cell lines comparing vehicle cells with cells exposed to 2Gy of IR at different time points (2h, 24h and after two consecutive fractionations, 2+2Gy). Herein, KDM3A and KDM6B expression was higher in DU145 cell line compared to the radiosensitive cell lines, C4-2 and C4-2B, even in vehicle samples (Figure 8A). However, over irradiation time, slightly differences in lysine demethylases expression were observed for all the cell lines. Nonetheless, in C4-2B cells, a tendency of KDMs expression decrease was found (KDM3A and KDM6A/B) upon 48h of IR or either, after two fractions of 2Gy (Figure 8A). Conversely, regarding histone lysine methyltransferases (KMTs), a slight decrease was detected for DU145 in both G9a and EZH2, being more pronounced in EZH2 (Figure 8A).

For the corresponding repressive histone marks, both of them showed lower expression levels in vehicle, as well as over the treatment time for DU145 cell lines. Concerning the remaining cell lines, C4-2 and C4-2B, a slight decrease in the expression of both histone methylation markers was found, in particular upon two irradiation fraction (2+2Gy), however not so reduced as for DU145 (Figure 8B).

Furthermore, the same aforementioned trend was recognized in IF images for C4-2 and DU145 cells lines at vehicle and 24h after 2Gy exposure, for both KDMs/KMTs (Figure 8C) and histones (Figure 8D).

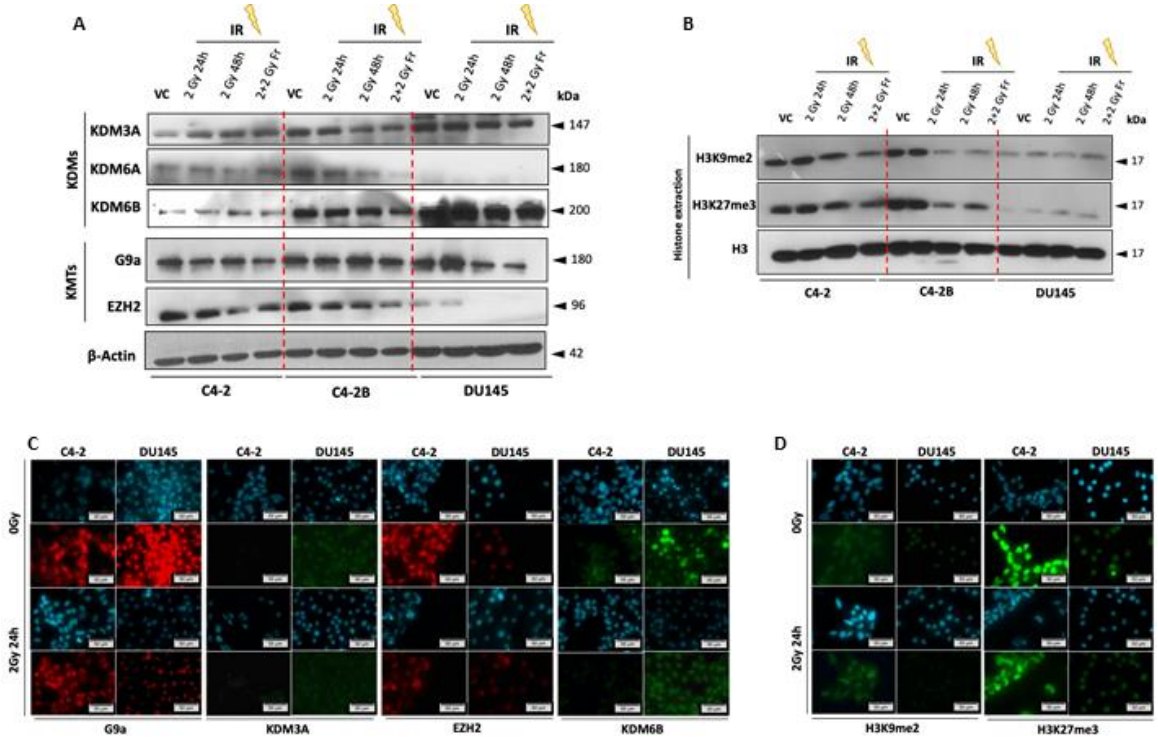

Figure 8 - Characterization of KDMs, KMTs and repressive marks histones in PCa cell lines. A, *KDM3A (147 kDa), KDM6A (180kDa), KDM6B (200kDa), G9a (180 kDa), and EZH2 (96 kDa) expression in vehicle, treatment of 24h and 48h after 2Gy and 2+2Gy by Western blot.* ***B,*** *H3K9me2 (17 kDa) and H3K27me3 (17 kDa) expression in WT, treatment of 24h and 48h after 2Gy and 2+2Gy by Western blot.* ***C,*** *Representative images of immunofluorescence in vehicle (0Gy) and 24h after 2Gy conditions, in which G9a and EZH2 are stained with red IgG antibody, KDMs stained with green IgG antibody and DAPI (blue staining), at 400x magnification (scale bar 50 µm).* ***D,*** *Representative images of immunofluorescence in WT and 24h after 2Gy conditions, which histones are stained with green IgG antibody and DAPI (blue staining), at 400x magnification (scale bar 50 µm). Abbreviations: Gy, gray; KDMs, lysine demethylases; KMTs, lysine methyltransferases; IR, ionizing radiation; Fr, fraction; VC, vehicle*

4. DNA damage repair in PCa cell lines upon IR exposure

After exposure to IR, the generation of DSB with consequent DNA damage, leads to an increase in H2AX phosphorylation (γ-H2AX). After 2h of IR, higher γ-H2AX marker was observed in all cell lines, comparing with the vehicle, although less impressive in DU145 (Figure 9A). Remarkably, after 24h, PC-3 and DU145 appeared to partially recover DNA damage as a decrease in γ-H2AX levels was verified, while LNCaP, C4-2, C4-2B and 22Rv1 cell lines remained with high levels of this damage-associated marker (Figure 9A).

Comet assay is a technique used to determine DNA damage after IR. Therefore, the amount of DNA that left the cell nucleus (tail) correspond to the amount of DNA damage. Four selected cell lines, C4-2, C4-2B, 22Rv1, DU145, with differential radiosensitivity were exposed to different IR doses and evaluated at different time-points. Vehicle cells showed

almost no damage (Figure 9B). Radiation with 8Gy induced more DNA damage, and consequently the tail had a larger size. The most radiosensitive cells, like C4-2, C4-2B and 22Rv1, showed higher DNA damage in the different time-points, both with 2 or 8Gy, comparing with DU145 cell line (Figure 9B). Remarkably, C4-2 displayed significant DNA damage levels comparing with vehicle, both y-H2AX at 24h (Figure 9A) and comet assay in all tested conditions (Figure 9B). Of note, higher DNA damage differences were achieved immediately after IR exposure, at 30min or 2h for the remaining cells (Figure 9B).

The bar graphs in Figure 9C represent the %DNA damage through the size of the tail summarized by tail moment values at 24h after IR exposure. Herein, cut-off values allowed a categorization of %DNA damage, in which tail moment between 15 and 30 means intermediate damage, whereas tail moment higher than 30 means high damage. Then, the most radioresistant cell line, DU145 exhibited reduced damage, while the most radiosensitive line, C4-2 presented the most impressive damage. Lower percentage and intensity damage after 24h of 2Gy in C4-2B and 22Rv1 cell lines, previously characterized as radioresponsive cells, might be explained by the notorious reduction in cell viability upon radiation exposure in these particular cells. Thus, the remaining cells after 24h, were able to partially recovery the damage. Nonetheless, C4-2B and 22Rv1 still presented higher levels and higher intensity of DNA damage in comparison with DU145 (Figure 9C).

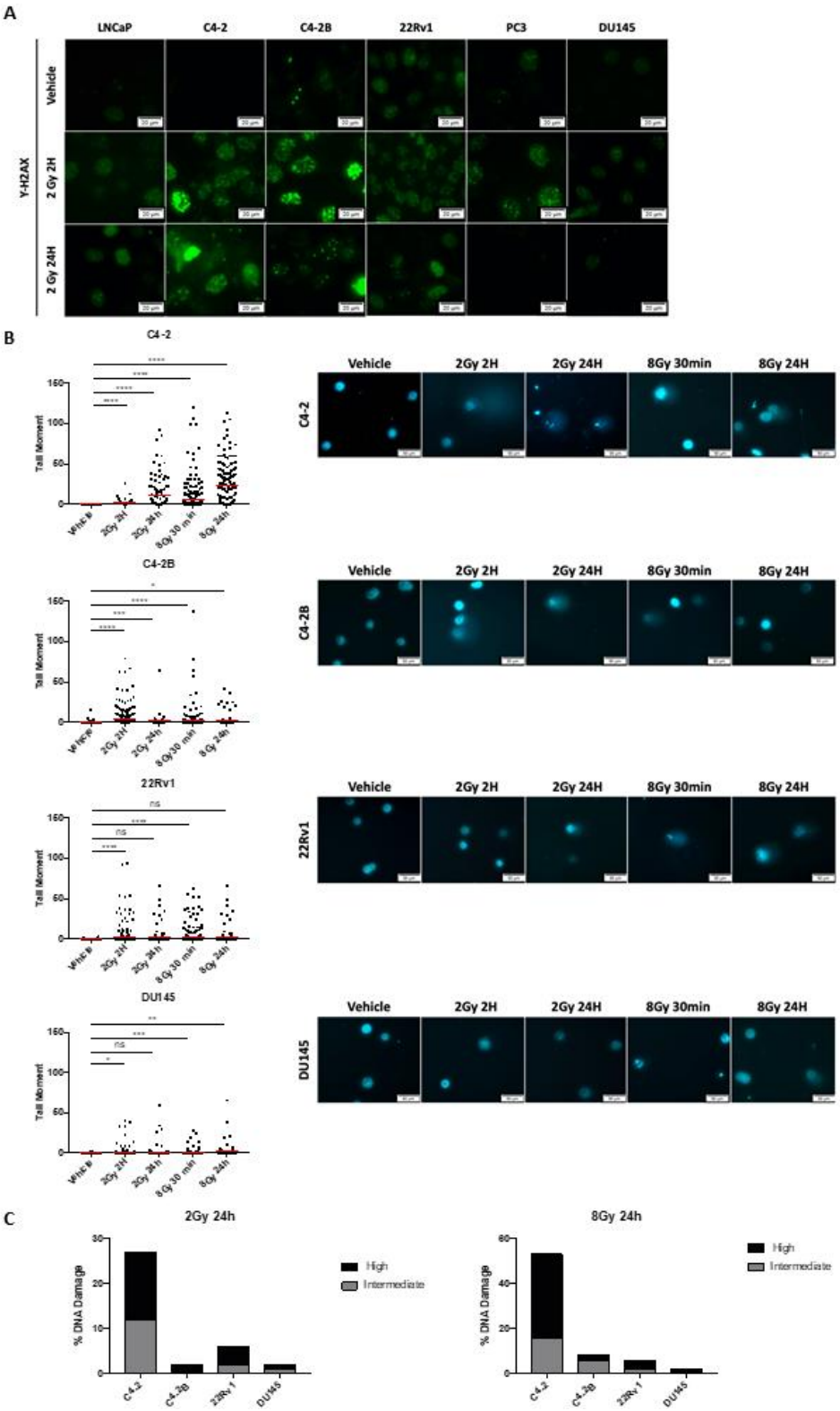

Figure 9 - Assessment of DNA damage response after exposure to IR in PCa cell lines. A, *Representative images of nuclear γ-H2AX expression in vehicle, 2Gy 2h and 2Gy 24h by immunofluorescence at 400x magnification (scale bar 20 μm).* ***B,*** *Tail moment values and representative microscopic images at 200x of magnification (scale bar 50 μm), at differents points after 2Gy (2H and 24H) and 8Gy (30min and 24H) of IR. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns, non-significant* ***C,*** *characterization of DNA damage intensity (intermediate or high) according a defined cut-off of tail moment after 24h of 2Gy and 8Gy exposure in PCa cell lines. Abbreviations: Gy, gray*

5. DNA damage repair-related genes in PCa cell lines upon IR exposure

Furthermore, the most radioresistant cell line also presented the higher levels of γ-ATM, cleaved PARP and γ-p53 protein expression comparing with the radiosensitive cells (Figure 5A). Regarding the DNA-PKcs repair marker, no significant differences were observed among PCa cell lines (Figure 10). Overall these results indicate different activation levels of the most common DNA damage repair pathways, HR and NHEJ.

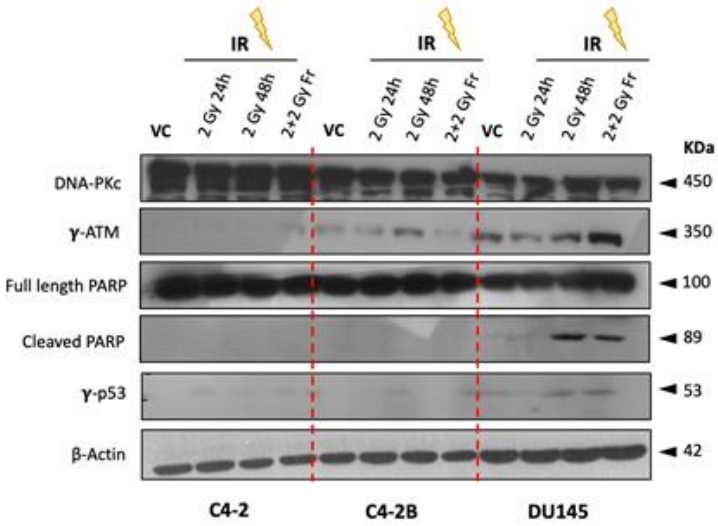

Figure 10 - Characterization of repair-associated markers in PCa cell lines in response to DNA damage. A, *DNA damage repair markers expression, DNA-PKcs (450kDa), γ-ATM (350kDa), PARP (89kDa) and γ-p53 (53kDa) in vehicle, treatment of 24h and 48h after 2Gy and 2+2Gy by Western blot. Abbreviations: Gy, gray; IR, ionizing irradiation; Fr, fraction; VC, vehicle*

V DISCUSSION

Prostate adenocarcinoma is one of the most incident cancer in men worldwide and treatment failure of advanced stages of the disease remains one of the major public health problems (2). Indeed, despite the advances in PCa treatment, namely with radiotherapy, many patients remain vulnerable to tumour recurrence and progression of the disease (5). Therefore, it would be of major relevance to determine the effect of epigenetic regulation in PCa resistance and to discover predictive markers of response to these treatment approach.

PCa cell lines have different responses to radiation. However, there is a tendency for the rate of resistance to increase after radiotherapy, being necessary to understand the behaviour of the different lines.

The DU145 and PC-3 cell lines showed a more aggressive behaviour (radioresistant), compared to the other cell lines. These results are in the same line of previous studies in which cells subjected to IR showed survival fractions similar to the ones obtained by us (85, 86). Furthermore, our *in vitro* results are also in accordance with the results obtained by others on a radiosensitive cell line, LNCaP (86). Conversely, aggressive cells exposed to IR suffered significant changes in the levels of histone repressive marks. In fact, a similar result was obtained by other researchers, in which a significant decrease of H3K9me2 and H3K9me3 was demonstrated in PCa tissues at basal conditions (87). Another study, revealed that KDM3A, KDM4A/B, KDM6A/B were often overexpressed in PCa, being the latter associated with a specific reduction of H3K27me3. According to Ngollo, *et al.,* these higher levels were detected in a more advanced stage of the disease, suggesting that the levels of KDM6A/B expression increased with the severity of the PCa (88). Remarkably, these cells already showed a slight decrease in histones' repressive marks under baseline conditions, suggesting a predisposition for RR.

Furthermore PCa cells exposed to IR, over the time, were shown to have a significant decrease in H3K27me3 in metastatic disease comparing with locally-confined PCa (75), even when KDM3A was upregulated (76).

Remarkably, reduced γ-H2AX expression, lower DNA fragmentation and increased cell survival fraction was more evident in radioresistant cells comparing with the radiosensitive ones, after IR exposure. Accordingly, γ-H2AX foci are the first event at the cellular level after DNA strand breaks being commonly used as a valid measure for radiosensitivity, whereas foci decay is associated with DNA damage repair after radiation treatment (81). As expected, over the time after IR, an evident decrease in the expression of the γ-H2AX marker was observed for PC-3 and DU145 cell lines, indicating less DNA

damage and, consequently, high RR. Importantly, similar results were previously reported in different tumour models, which revealed a notable decrease in this damage-associated marker upon IR (77, 89). In PCa model, γ-H2AX foci was already found to fade after 24h of 2Gy IR (76). Therefore, resistant cell lines, after exposure to IR seemed to have a capacity to recover DNA damage.

Moreover, DNA damage repair (DDR) markers were assessed to characterize the modifications induced by RR that lead to DNA damage recovery.

The DDR-markers linked to the homologous pathway (γ-ATM, γ-p53) as well as cleaved PARP-1, were upregulated in the radioresistant cell lines, in particular 48h after exposure to IR and after 2 consecutive fractions of 2Gy. Conversely, the DDR-marker of the non-homologous pathway (DNA-PKcs) almost did not differ among cell lines and different treatments. Thus, we postulate that the homologous pathway might be the most prevalent in the regulation of damage repair markers in our tumour model. Additionally, findings from *in vitro* assays using different PCa cell lines and other tumour models, suggested that RR allows DDR-markers deregulation, more specifically with the activation of ATM/p53 signalling pathway (38, 90, 91). Moreover, according to Stark *et al*., in PCa, PARP-1 not only transcriptionally regulates the genes that contribute to tumour growth, but also is implicated in metastasis and RR (92). Many studies using PARP inhibitors showed positive results in PCa treatment (93). However, only clinical trials with Olaparib, Niraparib, Veliparib or Rucaparib in monotherapy or in combination with anti-hormonal therapies were carried out (94, 95). Nonetheless, no data is available concerning the use of PARP inhibitors to improve radiotherapy successfully rates in PCa. Herein, as PARP overexpression was consistently shown in radioresistant DU145 cell lines upon IR exposure, we may suggest this player as a promising therapeutic target to increase PCa radiosensitivity (38).

According to our hypothesis, more aggressive cells (radioresistant) such as PC-3 and DU145 that overexpressed KDMs, while presented reduced KMTs levels led to a stronger decrease in the expression of histone repressive markers, allowing for chromatin opening. Consequently, it might lead to transcription activation of DDR-related genes, and γ-H2AX foci reduction.

VI CONCLUSION AND FUTURE PERSPECTIVES

Overall, in our study, after exposure to IR, KDMs were upregulated and histone repressive marks was decreased, which allowed chromatin to open and activate the transcription of specific genes. Moreover, the ability of DNA damage recovery might be due to the DDR-markers overloading.

Indeed, our results suggest that IR can induce resistance to radiotherapy inducing epigenetic modifications that sustain tumour growth, recurrence and eventually metastasis. Therefore, the understanding of these modifications is essential to allow the reversion of this process.

As future perspectives, to further support our experimental results, we intend to extend this study to PCa clinical samples, tissue or liquid biopsies, in order to improve the clinical applicability of our findings. Additionally, to better understand the dynamics of ionizing radiation and their interplay with epigenetic alterations in PCa, we intend to perform *in vitro* fractionation assays, exposing the PCa cells to consecutive doses of radiation to induce selection of radioresistant cells populations, to further understand morphological changes acquired in that process.

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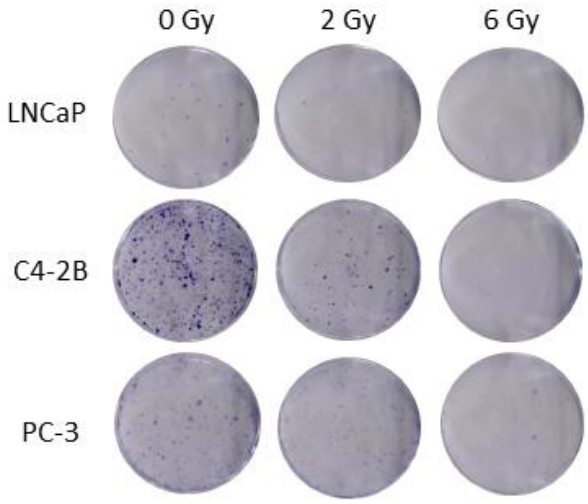
VIII APPENDIX



Supplementary Figure 1 - Characterization of PCa cell lines to radiotherapy treatment. *Representative picture of stained colonies with 0, 2 and 6 Gy for LNCaP, C4-2B and PC-3 cell lines*