Combination of Novel Androgen Receptor Signalling Inhibitors with Radiation Therapy in Prostate Cancer

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Abstract

Prostate cancer (PCa) is the fifth leading cause of cancer-related deaths amongst men worldwide. Androgen deprivation therapy (ADT) in combination with radiation therapy (XRT) is the standard of care for high-risk localized PCa. Unfortunately, most patients become resistant to ADT due to continued androgen receptor (AR) signalling pathway. The goal of this thesis was to investigate whether enzalutamide, a second-generation AR antagonist, enhances the effect of radiation in PCa cells.

Enzalutamide is an AR signalling inhibitor that not only blocks binding of androgens to the AR but is also shown prevents its translocation to the nucleus, DNA binding, and subsequent transcriptional activation of target genes in PCa cells. We have shown that enzalutamide increased the radiosensitivity of PCa cells significantly more than ADT. Enzalutamide sensitized hormone-sensitive PCa cells to XRT through increased levels of DNA damage and impaired the DNA repair process. Furthermore, concurrent administration of enzalutamide and radiation led to a maximal radiosensitivity when compared to either drug administration prior or after XRT.

Thoroughly understanding the mechanism of radiosensitivity could assist in finding novel prognostic biomarkers and/or potential drug targets of radiosensitivity. These biomarkers may be used to predict the treatment outcomes and to identify radiation-resistant PCa patients. To identify radiosensitivity gene signatures induced by enzalutamide, we performed gene expression profiling following treatment of hormone-sensitive (LNCaP) and hormone-resistant (C4-2) PCa cell lines. We identified that enzalutamide alone or in combination with ADT enhanced the effect of XRT through immune and inflammation-related pathways in LNCaP cells and metabolic-related pathways in C4-2 cells. Moreover, the Kaplan–Meier survival curves generated from the cancer genome atlas prostate adenocarcinoma (TCGA PRAD) dataset showed that the low expression of the candidate genes in patients with PCa correlated with disease recurrence and poor patient prognosis.

Taken together, our pre-clinical results suggest that the combination of enzalutamide and XRT may be a new treatment option at an early-stage of PCa. The possibility of this treatment option is supported by ongoing clinical trials for patients with intermediate-risk disease. Furthermore, we identified potential predictive and/or prognostic biomarkers for response to combined AR inhibitor and XRT therapy. These biomarkers require further validation in clinical trials before they can be incorporated into clinical practice.

Résumé

Le cancer de la prostate (PCa) est la cinquième plus importante cause de décès liés au cancer chez les hommes à l'échelle planétaire. La norme de soins pour les PCa à hauts risques est la thérapie de privation d'androgène (ADT) en combinaison avec la radiothérapie (XRT). Malheureusement, la majorité des patients deviennent résistants à l'ADT à cause de la voie de signalisation des récepteurs d'androgènes (AR) qui continuent de s'exprimer. L'objectif de cette thèse était de vérifier si un antagoniste d'AR de nouvelle génération, l'enzalutamide, augmente l'effet de la radiation sur les cellules de PCa.

L'enzalutamide est un inhibiteur synthétique de AR, qui en plus de bloquer la liaison des androgènes, empêche la translocation de AR dans le noyau, sa liaison avec l'ADN ainsi que l'activation de la transcription de ses gènes cibles dans les cellules de PCa. Nous avons montré que l'enzalutamide augmente la sensibilité à la XRT des cellules de PCa de manière beaucoup plus efficace que l'ADT. L'enzalutamide rend sensibles à la XRT les cellules de PCa dépendantes aux androgènes grâce à une augmentation du niveau de dommage à l'ADN et en altérant le processus de réparation de l'ADN. De plus, l'administration simultanée d'enzalutamide et de radiation mène à une radiosensibilité maximale lorsque comparée à l'administration de médicament avant ou après la XRT.

Une compréhension complète des mécanismes de radiosensibilité permettrait d'aider la recherche de nouveaux bios marqueurs pronostiques et/ou de médicaments qui pourraient potentiellement augmenter la radiosensibilité. De plus, ces biomarqueurs pourront peut-être prédire les résultats d'un traitement et identifier les patients résistants à la radiation. Afin d'identifier la signature des gènes radiosensibles suite à l'action de l'enzalutamide, nous avons fait un profil des gènes exprimés après le traitement (enzalutamide et XRT) par les lignes de cellules de PCa sensibles aux hormones (LNCaP) et résistantes aux hormones (C4-2). Nous avons identifié que l'enzalutamide seul ou en combinaison avec la ADT augmente les effets de la XRT par les chemins immunitaires et inflammatoires chez les cellules LNCaP et les voies métaboliques chez

les cellules C4-2. De plus, les courbes de survie Kaplan-Meier générées avec les ensembles de données de l'atlas du génome du cancer des adénocarcinomes de la prostate (TCGA PRAD) montrent que la régulation à la baisse de tous les gènes candidats était associée avec des progressions et des récurrences dans la cohorte de PCa.

Dans l'ensemble, nos résultats précliniques suggèrent que la combinaison d'enzalutamide et de XRT peut être une nouvelle option de traitement pour les cancers de la prostate à un stade précoce. Cette possibilité est supportée par des essais cliniques en cours chez les patients avec une maladie de risque intermédiaire. De plus, nous avons potentiellement identifié des bios marqueurs prédictifs et/ou pronostiques de la réponse à une combinaison de XRT et thérapie d'inhibiteur d'AR. Ces biomarqueurs nécessitent une validation lors d'essais cliniques avant de pouvoir les ajouter dans un traitement.

Dedication

This thesis is dedicated to my parents for their love, patience, endless support and encouragement during all my life.

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I would like to express my special appreciation and thanks to my supervisor, Dr. Thierry Muanza for accepting me as a Ph.D. student and encouraging and giving me the opportunity to grow in his laboratory. His invaluable insight and broad clinical vision helped me grow as a student, researcher and as a person.

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Thank you, Lord, for always being there for me.

Preface & Contribution of Authors

This thesis is presented in a manuscript-based format. It consists of five chapters. The contributions of each author are described below.

Chapter 1 includes introduction and literature review:

- Maryam Ghashghaei wrote the introduction and my supervisor, Dr. Muanza revised it.

Chapter 2 includes published paper in the Prostate journal:

- Maryam Ghashghaei: Performed the experiments; Analyzed the data; Contributed reagents/materials/analysis tools; Wrote the manuscript; Reviewed the manuscript.
- Miltiadis Paliouras: Contributed reagents/materials/analysis tools; Reviewed the manuscript.
- Mitra Heravi: Analyzed the data; Reviewed the manuscript.
- Hamed Bekerat: Designed the setting and performed radiation for in vitro study.
- Mark Trifiro: Provided study material; Reviewed the manuscript.
- Tamim M. Niazi: Reviewed the manuscript; Grant support.
- Thierry Muanza: Conceived and designed the experiments; Analyzed the data; Wrote the manuscript; Reviewed the manuscript; Gave final approval of the manuscript.

Chapter3 includes published paper in Scientific Reports/Nature journal:

- Maryam Ghashghaei: Performed the experiments; Analyzed the data; Contributed reagents/materials/analysis tools; Wrote the manuscript; Reviewed the manuscript.
- Tamim M. Niazi: Reviewed the manuscript; Grant support.
- Celia M.T. Greenwood: Analyzed the data; Reviewed the manuscript; Performed statistical analysis.
- Kathleen Oros Klein: Analyzed the data; Reviewed the manuscript; Performed statistical analysis.

- Thierry Muanza: Conceived and designed the experiments; Analyzed the data; Wrote the manuscript; Reviewed the manuscript; Gave final approval of the manuscript; Grant support.

Chapter 4 includes published paper in Current Oncology journal:

- Maryam Ghashghaei: Provided the summary, classification, comparison and evaluation of all data; Wrote the manuscript.
- Sara Elakshar: Reviewed and revised the manuscript.
- Thierry Muanza: Reviewed the manuscript; Gave final approval of the manuscript.

Chapter 5 includes discussion and conclusion:

- Maryam Ghashghaei wrote the discussion and Dr. Muanza revised it.

Contribution to original knowledge

In this original study, we have shown that combination of new androgen receptor inhibitors with radiation therapy in localized prostate cancer (PCa) treatment is a feasible strategy and should be studied further. Ultimately, clinical trials could be designed to evaluate the efficacy of these combination modalities in the clinic.

In this thesis, we have identified for the first time that:

- Androgen receptor inhibitors such as enzalutamide can enhance radiation response more than ADT (androgen deprivation therapy) in hormone-sensitive and hormone-resistant PCa cell lines.
- The radiosensitivity effect of enzalutamide is schedule dependent.
- Enzalutamide enhances the effect of radiation via impairment of DNA repair process.
- Several radiosensitivity gene signatures induced by enzalutamide in the PCa cell lines.
- The identified biomarker signatures correlate with clinical outcome of prostate cancer.

This thesis provides strong evidence supporting the importance of simultaneous administration of enzalutamide and ionizing radiation which might extremely change the efficacy of radiation treatment at an early-stage of PCa progression and provide concrete justification for assessing the predictive power of the biomarkers in clinical trials.

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List of Abbreviations

AA	Abiraterone acetate
AD	Androgen dependent
ADT	Androgen deprivation therapy
AF1	Activation function-1
AFFIRM	Atrial Fibrillation Follow-up Investigation of Rhythm Management
AI	Androgen independent
AIF	Apoptosis inducing factor
ALP	Alkaline phosphatase
APAF1	Apoptotic protease activating factor 1
AP-sites	Apurinic/apyrimidinic sites
AR	Androgen receptor
ARE	Androgen response elements
AR-Vs	AR splice variants
AS	Active surveillance
ATG	Autophagy-related genes
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
AWS	Anti-androgen withdrawal syndrome
BCR	Biochemical recurrence
BER	Base excision repair
BF	Biochemical failure
BID	BH3-interacting domain death
bPFS	Biochemical progression-free survival
BPH	Benign prostatic hyperplasia
BRCA1	Breast cancer gene 1
bRFS	Biochemical relapse-free survival

CAB	Combined androgen blockade
CDK	Cyclin-dependent kinase
chk1	Checkpoint kinase 1
CI	Combination index
CPD	Cyclobutane pyrimidine dimers
CRPC	Castration-resistant prostate cancer
CSC	Cancer stem cells
csFBS	Charcoal dextran-treated fetal bovine serum
CSS	Cancer-specific survival
CTC	Circulating tumour cells
CTCAE	Common terminology criteria for adverse events
CYP17A1	Cytochrome P450 17A1
DBD	DNA-binding domain
DDR	DNA damage response
DE	Differentially expression
DE-EBRT	Dose-escalated external beam radiation therapy
DEF	Dose enhancement factor
DER	Dose enhancement ratio
DFS	Disease-free survival
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signalling complex
DM	Distant metastasis
DMFS	Distant metastasis-free survival
DNA-PKcs	DNA-dependent kinase, catalytic subunit
DRE	Digital rectal exam
DSB	Double-strand break
DSS	Disease-specific survival

EBRT	External beam radiotherapy
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ENZA	Enzalutamide
EORTC	European organization for research and treatment of cancer
ER	Endoplasmic reticulum
ERG	Erythroblast transformation-specific related gene
ETS	Erythroblast transformation-specific
EZH2	Enhancer of zeste homolog 2
f/t PSA	Free/total prostate specific antigen
FA	Fanconi anemia
FADD	Fas-associating protein with a novel death domain
FDA	Food and Drug Administration
FDHT	Fluoro-5α-dihydrotestosterone
AR ^{FL}	Full-length androgen receptor
FPKM	Fragments per Kilobase million
FSH	Follicle-stimulating hormone
G	Gap2
G1	Gap1
GEO	Gene Expression Omnibus
GG-NER	Global genome nucleotide excision repair
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GS	Gleason score
Gy	Gray
HDR	High-dose rate
HIF-1a	Hypoxia-inducible factor-1 alpha
hK2	Human glandular kallikrein 2

Hk3	Human kallikrein-related peptidase 3
HPG	Hypothalamic–pituitary–gonadal
HR	Homologous recombination
HRs	Hazard ratios
HSP	Heat shock protein
ICLs	Interstrand DNA cross-links
IFBS	Inactivated fetal bovine serum
IGF	Insulin-like growth factor
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGFPBs	Insulin-like growth factor binding proteins
IL-6	Interleukin-6
ILS	Interleukins
IMRT	Intensity-modulated radiation therapy
Int	Intermediate
KEGG	Kyoto Encyclopedia of Genes and Genomes
KGF	Keratinocyte growth factor
KLK3	Kallikrein-related peptidase 3
LBD	Ligand-binding domain
LDR	Low-dose rate
LET	Linear energy transfer
LF	Local failure
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
LT-ADT	Long-term androgen deprivation therapy
M	Metastasis
M	Mitosis
MAP	Mitogen-activated protein
mCRPC	Metastatic castration-resistant prostate cancer

mCSPC	metastatic castration-sensitive prostate cancer
MDC1	Mediator of DNA-damage checkpoint 1
MET	Mesenchymal-epithelial transition
MFS	Metastasis-free survival
miRNA	MicroRNA
MOMP	Mitochondrial outer membrane permeabilization
MRI	Magnetic resonance imaging
MRN	The Mre11-Rad50-Nbs1
mTOR	Mammalian target of rapamycin
MTT	3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide
N	Node
NAD	Nicotinamide adenine dinucleotide
NCCD	Nomenclature committee on cell death
NCT	National clinical trial
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
NTD	N-terminal domain
O6MeGua	O6-methylguanine
OFH	Oxygen fixation hypothesis
OS	Overall survival
PAP	Prostatic acid phosphatase
PARP1	Poly (ADP-Ribose) Polymerase 1
PCa	Prostate cancer
PCNA	Proliferating cell nuclear antigen
PCSM	Prostate cancer-specific mortality
PE	Plating efficiency
PFS	Progression-free survival

PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIKKs	Phosphoinositide 3-kinase-related protein kinases
PIN	Intraepithelial neoplasia
pol β	Polymerase β
PR	Progesterone receptor
PRAD	Prostate adenocarcinoma
PSA	Prostate-specific antigen
PSA-CR	Prostate specific antigen-complete response
PSAD	Prostate-specific antigen density
PSA-RFS	Prostate-specific antigen relapse-free survival
PSA-TZ	Prostate-specific antigen transition zone density
PSAV	Prostate-specific antigen velocity
PSMA	Prostate-specific membrane antigen
PTEN	Phosphatase and tensin homolog
QOL	Quality of life
Q-Q plot	Quantile-quantile plot
RBE	Relative biological effectiveness
RFS	Relapse-free survival
RP	Radical prostatectomy
RPA	Replication protein A
RTK	Receptor tyrosine kinase
RTOG	Radiation therapy oncology group
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
S	Synthesis
sa-β-gal	Senescence β-Galactosidase
SBRT	Stereotactic body radiation therapy
SF	Surviving fraction
Smac	Second mitochondria-derived activator of caspases

SPINK1	Serine peptidase inhibitor Kazal type 1
SPOP	Speckle-type POZ protein
SRC	Steroid receptor coactivator
SRE	Skeletal-related event
SRT	Salvage radiation therapy
SSB	Single-strand break
ssDNA	Single-stranded DNA
ST-ADT	short-term androgen deprivation therapy
T	Testosterone
Tau-1	Transcription activation units 1
tBID	Truncated BID
TCGA	The Cancer Genome Atlas
TC-NER	Transcription-coupled nucleotide excision repair
TCR	T-cell antigen receptor
TGF-β1	Transforming growth factor-β1
TMPRSS2	Transmembrane protease serine 2
TNF	Tumour necrosis factor
TNM	Tumour-node-metastasis
TOP2B	Topoisomerase II beta
TRAIL	TNF-related apoptosis-inducing ligand
TROG	Trans-Tasman Radiation Oncology Group
US	Ultrasound
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein
XRT	Radiation therapy
3D-CRT	Three-dimensional conformal radiation therapy
53BP1	P53-binding protein 1

Chapter 1

Introduction

1.1. Prostate gland

The prostate is an exocrine gland, about the size and shape of a walnut, and is part of the male reproductive system. It is located in the pelvic region, in front of the rectum, and surrounds the part of the urethra just below the bladder (Figure 1-1). The main function of the prostate gland is to secrete an alkaline fluid containing various enzymes including zinc and citric acid. These enzymes mix with sperm during ejaculation and function to protect the sperm from acidic denaturation. Initially, prostatic buds appear at approximately 10 weeks of gestation as solid cords which branch and form lumens in response to androgen [1].

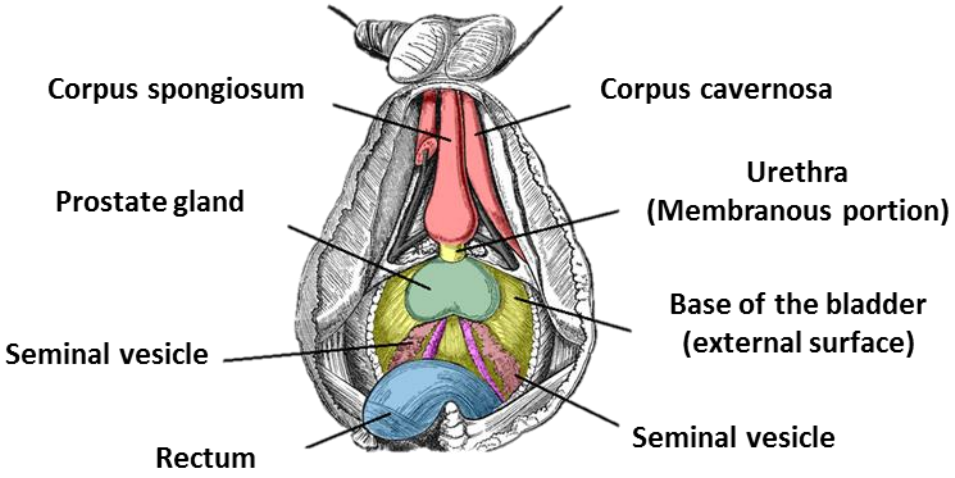

Figure 1-1. Inferior view of the structures in the male reproductive system. Modified from the prostate gland-TeachMeAnatomy.info. (https://teachmeanatomy.info/pelvis/the-male-reproductive-system/prostate-gland/).

The prostate gland is divided into three distinct zones (Figure 1-2): peripheral, central, and transition. The peripheral zone comprises about 70% of the prostate and makes up the bulk of the gland with longer ducts. About 70–80% of prostate cancers

(PCa) originate from this zone. The central zone accounts for 25% of the prostate gland. It is posterior to the transition zone and surrounds the ejaculatory ducts. Very few PCa originate from the central zone; and these cancers tend to be more aggressive and more likely to invade the seminal vesicles. The transition zone occupies about 5% of the prostate volume and surrounds the part of the urethra that passes through the prostate (i.e. the prostatic urethra). Benign prostatic hyperplasia (BPH) originates from the transition zone. Approximately 20% of PCa develop in this zone [2]. The prostatic ducts can be divided into two compartments, including the epithelial and stromal compartments. The epithelial compartment of the prostate is composed of basal, secretory luminal, neuroendocrine, and transit-amplifying cells with different morphology, location, and distinct marker expression. The stromal tissue consists of smooth muscle cells, endothelial cells, and fibroblast cells [3].

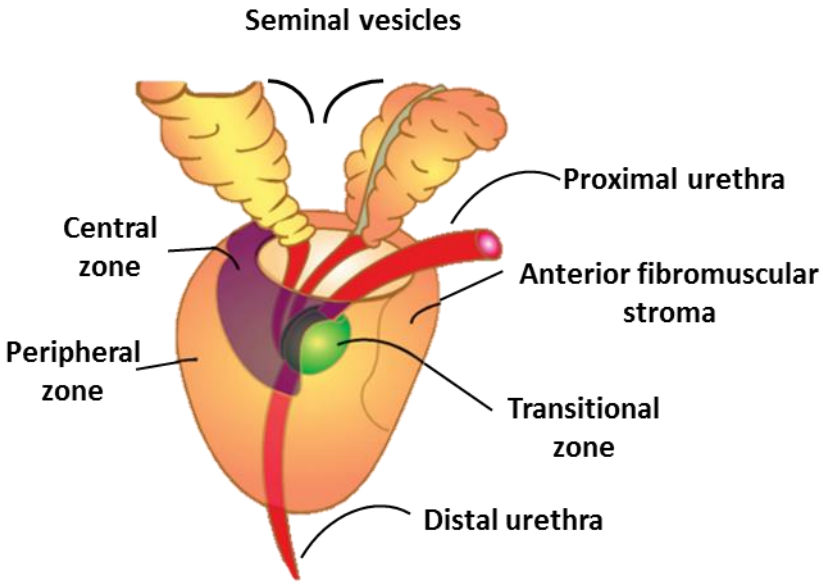

Figure 1-2. Zones of the prostate. Modified from Campbell-Walsh Urology 9th edn (Saunders Elsevier, Philadelphia, 2007).

1.2. Androgens

Androgens (male sex hormones) are involved in the growth, development, and function of the prostate. Testosterone is the main androgen which is synthesized from cholesterol in Leydig cells in the testicle and circulates throughout the body. Testosterone is converted to the more potent androgen, dihydrotestosterone (DHT), via enzyme5α-reductase in certain tissues including the prostate gland, seminal vesicles, skin, hair follicles, the liver, and brain [4]. The hypothalamic–pituitary–gonadal axis (HPG) regulates the production of testosterone in the Leydig cells in the testicle (Figure 1-3). Low testosterone levels in the blood stimulate the hypothalamus to release gonadotropin-releasing hormone (GnRH). GnRH binds to secretory cells of the anterior pituitary and stimulates the synthesis and secretion of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulates testosterone synthesis in Leydig cells. The testosterone then acts via a negative feedback loop on the hypothalamus and pituitary to inhibit the release of GnRH, LHA and FSH [5]. The Adrenal glands produces small amounts of androgens like dehydroepiandrosterone (DHEA) and androstenedione which can be converted to testosterone, a process that is clinically relevant for men undergoing hormonal therapy for PCa [6].

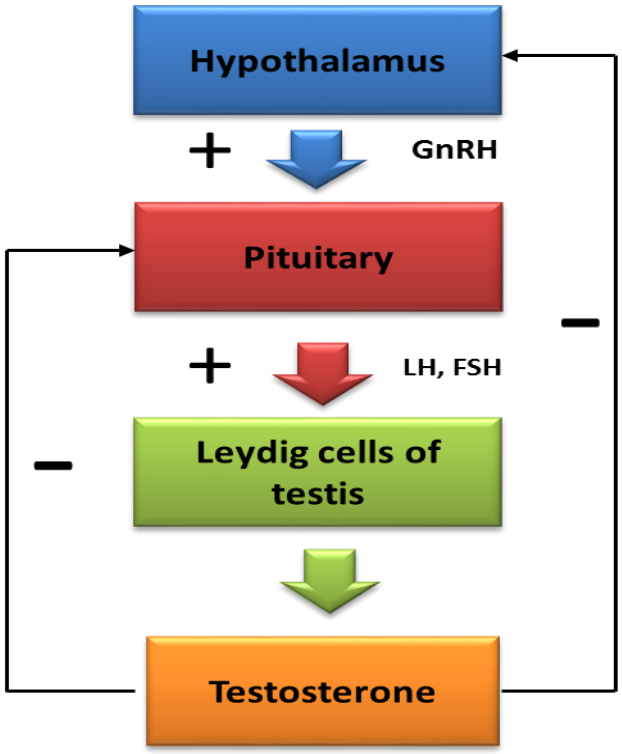

Figure 1-3. Hypothalamic-pituitary-testicular axis. FSH=Follicle-stimulating hormone; GnRH=Gonadotropin-releasing hormone; LH= Luteinizing hormone. Modified from M. Iftekhar Ullah et al. (2014). Drug Design, Development and Therapy. DOI:10.2147/DDDT.S43475.

1.2.1. Androgen signalling through the androgen receptor (AR)

Androgens exert their biological effects throughout the body by binding to the androgen receptor (AR), a steroid-inducible transcription factor that is critical not only for the development and maintenance of the normal prostate but also the development and progression of PCa [7].

1.3. Androgen receptor (AR)

AR is a member of the steroid and nuclear receptor superfamily that shares a similar three-dimensional structure to other steroid hormone receptors including estrogen,

progesterone, glucocorticoid, and mineralocorticoid receptors [8,9]. The AR gene is located on the X chromosome at Xq11-12 and is made up of eight exons which code a protein of 919 amino acids with a molecular weight of 110-kDa [10]. Like other members of the nuclear receptor family, the AR consists of three major functional domains [11] (Figure 1-4):

1.3.1. N-terminal domain (NTD)

The AR-NTD (residues 1–537, encoded by exon 1) is critical for the transactivation of the AR. This highly variable domain contains a ligand-independent transactivation domain termed activation function 1 (AF1), which is constitutively active. The AF1 domain (residues 142-485) contains two transcription activation units [Tau-1 (residues 101–360] and Tau-5 (residues 360–528)] that are essential for the full activity of the AR. There are two motifs in AR-NTD (FXXLF motif at residues 23–27 and a WXXLF motif at residues 433–437) which facilitate direct interactions with the AF-2 in the ligand binding domain (LBD) of AR. This interaction (termed N/C interaction) stabilizes the androgen in the LBD pocket and is critical for all AR functions. It has been shown that the AR NTD contains specific sequences which mediate protein-protein interactions with the carboxyl-terminal LBD and the general transcription factors TFIIF and TFIIH [12].

1.3.2. DNA-binding domain (DBD)

The DBD (residues 538-625, encoded by exons 2 and 3) is highly conserved among steroid hormone receptors. Each DBD monomer (66 amino acids) contains two zinc finger motifs, each of which contains four cysteine residues that coordinate a zinc ion. The first zinc finger motif interacts with a DNA major groove, while the second zinc finger plays a role in DBD-mediated AR dimerization. The classical androgen response elements (AREs) on the DNA are recognized by the AR-DBD [12,13].

1.3.3. Hinge region

The flexible and nonconserved hinge region, located between the DBD and the LBD (residues 626-669, encoded by part of exon 4), is involved in the DNA binding and

nuclear translocation of the AR. AR nuclear translocation is mediated by a bipartite nuclear localization signal (NLS) that usually contains either one or two clusters of basic residues. NLS is located between the DBD and the hinge region of the AR. AR acetylation has been found on three residues (K630, K632, and K633) within the hinge region of the AR. The three residues are important for the ligand-dependent activation of AR. The study by Thomas and colleagues suggested that acetylation of these residues regulates transcriptional activity, subcellular distribution, and the folding of the AR, coactivator, and corepressor binding [14].

1.3.4. Ligand-binding domain (LBD)

The carboxy-terminal LBD is the second most conserved region of the AR (residue 670-919, encoded by exons 4–8). Its ligand binding pocket is composed of helices 3, 4, 5, 7, 11, 12, and the AF-2 helix, as well as residues from the β strands 1 and 2. There are hydrogen bonds and/or hydrophobic interactions between these residues and steroid ligands (T and DHT) [15]. In the LBD domain, helices 3, 4 and 12 form a hydrophobic cleft, known as the activation function 2 (AF-2) region [12]. Upon ligand binding, there is a conformational change in helix 12 which completes the structure of this pocket. This pocket is the binding site for numerous AR coactivators, including steroid receptor coactivator-1 (SRC1), TIF2/GRIP1/SRC2 and SRC3 (members of the p160 SRC family) [16].

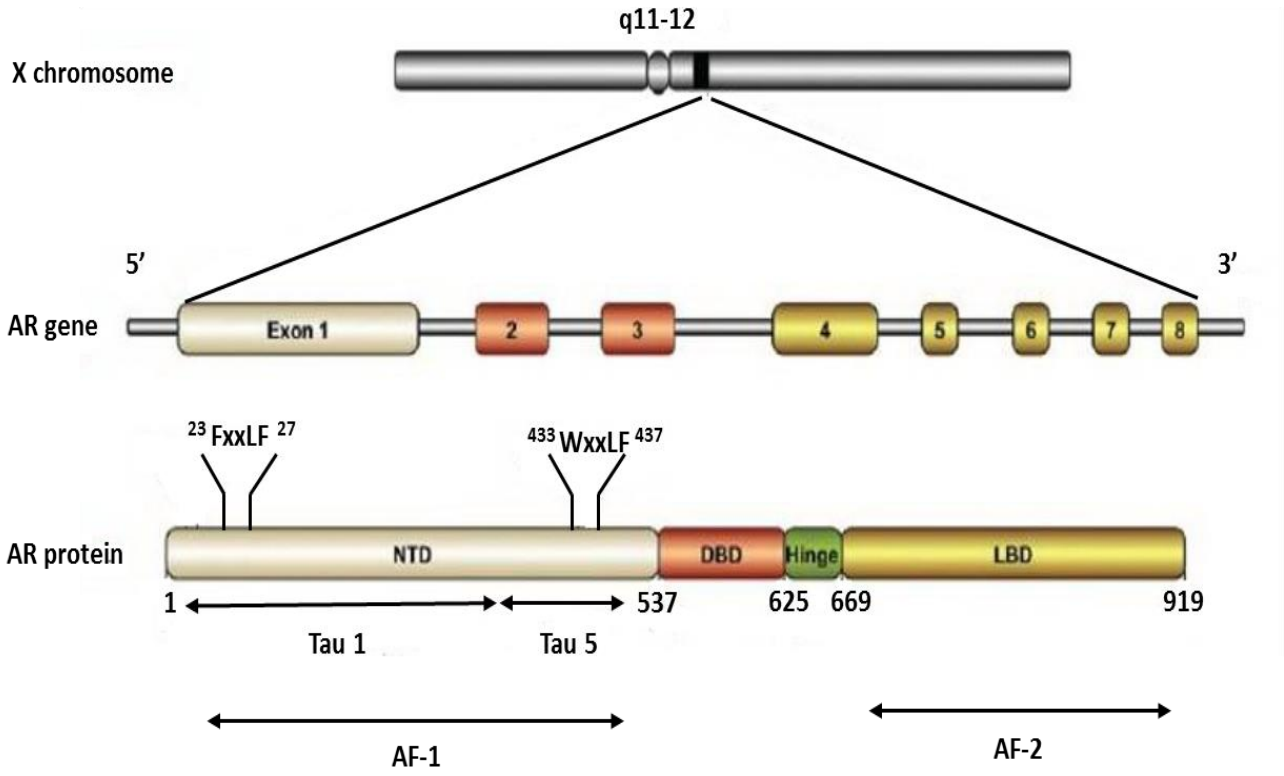

Figure1-4. Androgen receptor structure. Modified from Peter E Lonergan and Donald J Tindall. (2011). Androgen receptor signalling in prostate cancer development and progression. J Carcinogen 2011, 10:20. DOI**:** 10.4103/1477-3163.83937.

1.3.5. Androgen signalling in the prostate

The AR is a ligand-inducible transcription factor. It regulates the expression of target genes including prostate specific antigen (PSA) that promote the growth, survival, and differentiation of both the normal and malignant prostate [10]. Prior to ligand binding, the AR resides primarily in the cytoplasm, where it remains in a complex with heat shock proteins (HSP-90, HSP-70, and HSP-56), cytoskeletal proteins and other chaperone proteins. These molecular chaperones induce the correct folding of the AR for its ligand binding (DHT) and maintain its structural integrity [17]. Upon stimulation of the AR by DHT, the AR dissociates from the heat shock protein, is dimerized through DBD, translocates to the nucleus, and binds to specific DNA sequences called androgen

response elements (AREs) [18-20]. The ARE is located in the promoter regions of androgen-target genes. AR binding regulates the expression of specific genes which leads to the growth and survival of the prostate. AF1 presents in its N-terminus region and controls the majority of AR transcription activity through the recruitment of various factors, including RNA polymerase II and other transcription-initiation proteins [12]. Chromatin-associated AR can be released from the DNA and move back to the cytoplasm, where the AR can be rebound by the ligand and mediate many cycles of gene regulation (Figure 1-5).

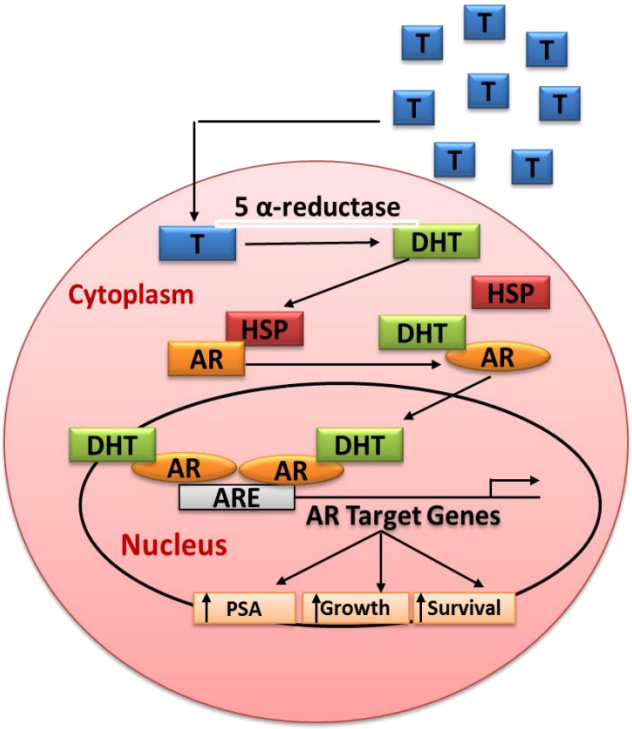

Figure 1-5. Mechanisms of the androgen receptor signalling in prostate cells. T=Testosterone; DHT=Dihydrotestosterone; AR=Androgen receptor; HSP=Heat shock protein. Modified from Punit Saraon et al. (2011). Molecular Alterations during Progression of Prostate Cancer to Androgen Independence. DOI: 10.1373/clinchem.2011.165977.

1.4. Prostate Cancer

1.4.1. The molecular biology of prostate cancer

PCa is a multifocal, heterogeneous disease with different stages of progression including prostatic intraepithelial neoplasia (PIN), latent and clinical adenocarcinoma, distant metastasis, and castration-resistant PCa (CRPC) (Figure 1-6). Every stage of its progression is correlated with genetic alterations and the loss of specific chromosome regions involved in the process. The contribution of genetic factors was found to constitute 58% of the risk of developing PCa. Genomic abnormalities include chromosomal loss or gain, gene amplification, mutations, and fusions. About 12% of metastatic PCa cases are caused primarily by inherited genetic mutations mainly in DNA repair genes such as BRCA1, BRCA2, and ATM. The molecular etiology of the disease will be described by several molecular subtypes as follows [21]:

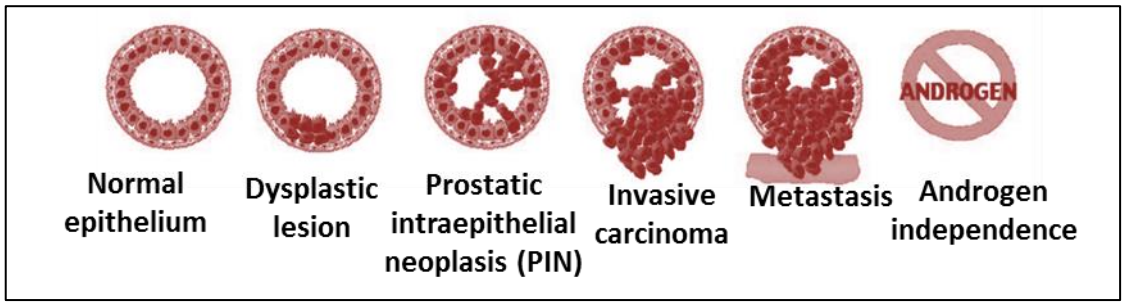

Figure 1-6. Schematic representation of prostate cancer progression. Modified from Andrea Vecchione et al. (2007). Molecular genetics of prostate cancer: Clinical translational opportunities. Journal of experimental & clinical cancer research: CR 26(1):25-37.

1.4.1.1. Castration-resistant prostate cancer (CRPC)

CRPC, also known as hormone-refractory PCa, is the progression of PCa despite hormonal ablation. The major underlying mechanisms causing CRPC are mediated by AR or the androgen axis. They can be categorized into seven distinct subsets including AR amplification, AR mutations, other oncogenic signalling pathways, and alterations in

AR coregulators, increased expression of enzymes involved in steroidogenesis, AR splice variants, and glucocorticoid receptor (Figure 1-7).

1.4.1.1.1. AR amplification

Gene amplification and increased AR expression enhance the AR's sensitivity to low androgen levels [22]. This occurs in about 20-30% of CRPC patients [23] and less than 2% in primary tumours [24]. Furthermore, AR overexpression converts bicalutamide and other AR antagonists into an AR agonist [25]. AR gene amplification accounts for most instances of increased AR protein expression, but increased AR expression can be found without any association with gene amplification [26]. In other words, the increased levels of AR expression may be due to the increased protein half-life that occurs in CRPC. In brief, AR overexpression can allow for greater sensitivity to very low level of androgens and facilitate AR signalling throughout androgen deprivation therapy (ADT).

1.4.1.1.2. AR mutations

Mutations in the AR can result in defective AR function including loss-of-function AR alterations and gain-of-function AR alterations [27]. The four different types of AR mutations that generate a defective AR are: (I) single point mutations resulting in amino acid substitutions or premature stop codons; (II) nucleotide insertions or deletions most often leading to a frameshift and premature rumination; (III) complete or partial gene deletions; and (IV) intronic mutations causing alternative splicing. Although AR mutations are very rare in the early stages of PCa, approximately 10-30% of CRPC patients carry AR mutations. There are over 100 point mutations in the AR gene, most of them located in distinct domains (49% in LBD, 40% in NTD, 7% in DBD and 2% in hinge) in CRPC patients [28]. The most significant AR mutations occur in the LBD [29]. The T877A mutation remains the most frequently occurring point mutation in the LBD, which allows for the activation of the AR with abnormal ligands such as estrogens, progestins, and even anti-androgen hydroxyflutamide [30]. This mutation was initially detected in the LNCaP PCa cell line [27]. Additionally, the F877L mutation (LBD-AR) can turn AR antagonists such as enzalutamide into potential agonists [31]. These mutations not only increase the AR's sensitivity to classical ligands, but also decrease the specificity of

ligand binding, allowing AR activation through alternative steroidal molecules, including estrogens, corticosteroids, and progesterone. Mutations in the NTD of the AR, the critical domain for the interaction of the AR and the co-regulator, can reduce AR transactivation activity [32]. The mutations in the hinge regions of the AR gene increases AR transactivation activity and decreases ligand specificity [28]. In the setting of AR overexpression, about 15%–30% of patients treated with conventional anti-androgens develop the W741 AR mutation that allows for tumour growth [33].

1.4.1.1.3. Alterations in AR coregulators

Coregulators are proteins that interact with transcription factors to either enhance (coactivator) or suppress (corepressor) the transcription of specific genes [34]. Altered expression of AR coregulators can cause aberrant AR signalling [35]. For example, increased levels of the AR coactivator not only increase the sensitivity of AR to a lower level of androgens [36] but also make it responsive to a wider range of androgens [37]. Overexpression of the AR coactivator can also permit the activation of the AR by non-classical ligands such as estradiol and hydroxyflutamide [38]. Conversely, downregulation of the AR corepressor enhances AR signalling, as the coactivator and the corepressor competitively bind with the AR and facilitate or suppress AR transcription activity. Furthermore, AR antagonists inhibit AR signalling through the recruitment of corepressors, which suggests that the loss of corepressors promotes AR activity even in the presence of AR antagonists [39,40] . The relative transcriptional activity of the AR depends on the ratio of coactivators to corepressor, and perturbation of this ratio may increase AR activity independently of androgen levels.

1.4.1.1.4. Increased expression of enzymes involved in steroidogenesis

An alternative mechanism to activate AR signalling in CRPC is via the increased intracrine biosynthesis of androgen. Despite the low level of testosterone in the blood, a high level of testosterone and DHT in PCa activates the AR-signalling pathway [41]. Increased expression of the key enzymes involved in androgen biosynthesis causes high intratumoural levels of DHT and facilitates the reactivation of this synthetic pathway [42]. Abiraterone acetate blocks the production of androgen precursors, such as

dehydroepiandrosterone (DHEA) and androstenedione, through inhibition of the CYP17A1 enzyme.

1.4.1.1.5. Other oncogenic signalling pathways

Numerous growth factors, cytokines, and hormones have been involved in the activation of AR in the absence of an AR ligand. Growth factor kinase signalling pathways such as phosphoinositide-3-kinase (PI3K), protein kinase B (AKT), extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) have been shown to stimulate AR target gene expression in castrate state. The insulin-like growth factor 1 (IGF-1) as well as the keratinocyte growth factor (KGF) and the epidermal growth factor (EGF) were shown to independently induce AR activity in the absence of androgen through downstream phosphorylation of either AR or its associated proteins [43]. Interleukin-6 (IL-6) was shown to be involved in ligand-independent activation of the AR in LNCaP and DU-145 PCa cells. IL-6 phosphorylates the steroid receptor coactivator (SRC-1) which increases the interaction between AR-NTD and SRC1 [44,45]. Finally, the overexpression of tyrosine kinase receptors such as Her2/neu (ErbB2) activates AR in both the absence and presence of androgen through AR phosphorylation [46].

1.4.1.1.6. Expression of AR splice variants (AR-Vs)

Another mechanism of castration-resistant growth of PCa is the production of constitutively active splice variants of the AR (AR-Vs) that lack the AR ligand-binding domain [47]. AR-Vs (AR-V1, AR-V7, AR-V4, and AR-V6) activate AR target genes and develop disease progression in a ligand-independent manner [48]. AR-V1, AR-V4, and AR-V6 primarily localize in the cytoplasm while AR-V7 mainly localizes in the nucleus. Among various AR-Vs identified in PCa, AR-V7 is one of the most abundant and best-characterized variants in CRPC patients [49,50]. It was reported that increased transcription of AR-V7 is associated with more advanced malignancy and a shorter survival rate. Antonarakis et al. reported that the expression of AR-V7 in circulating tumour cells (CTCs) predicts poor treatment responses such as reduced progression-free-survival (PFS) and reduced overall survival (OS) in metastatic castration-resistant PCa (mCRPC)

patients treated with enzalutamide and abiraterone [51]. AR antagonists or castration induce two of the most common AR splice variants, AR-V7 and ARv567es, the expression of which is associated with CRPC progression and treatment resistance [47,52].

1.4.1.1.7. AR by-pass signalling: Glucocorticoid receptor

Steroid receptors, including glucocorticoid receptor (GR) and progesterone receptor (PR), share a conserved sequence homology of their DBD with the AR [53]. Almost all PCa expresses the AR, whereas GR is only present in 30% of PCa cases [54]. Increased expression of GR following ADT may be a resistance mechanism driving CRPC [55]. GR can also act as a substitute for AR signalling. GR or other nuclear steroid receptors can bypass AR pathways and promote the development of CRPC.

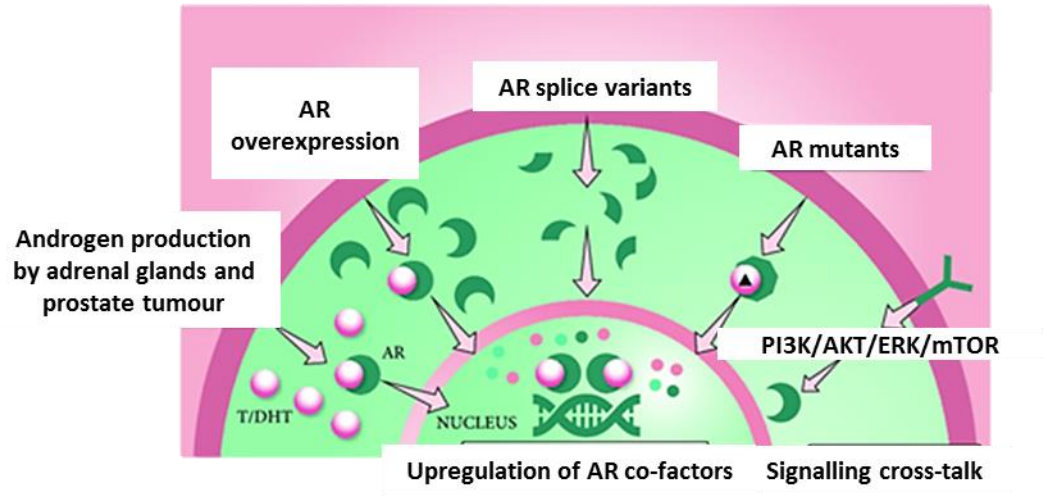

Figure 1-7. Continued androgen receptor signaling in castration-resistant prostate cancer. Modified form Jack Schalken and John M. Fitzpatrick. (2015) Enzalutamide: Targeting the androgen signalling pathway in metastatic castration-resistant prostate cancer. BJU International 117(2) · March 2015

1.4.1.2. ETS gene fusions

ERG (ETS related gene) is a member of the ETS (erythroblast transformation-specific) transcription factors family. These transcription factors play important roles in cellular proliferation, differentiation, migration, invasion, and angiogenesis. TMPRSS2 (transmembrane protease serine 2) is highly expressed in human prostate epithelium and induced by the androgen in androgen-sensitive PCa. The gene fusion between TMPRSS2 and ERG commonly occurs in PCa and results in the overexpression of oncogenic transcription factors [56].The frequency of these TMPRSS2-ERG fusions is 15% in high-grade PIN lesions and 50% in localized PCa. The overexpression of TMPRSS2-ERG remains a novel therapeutic target because of its PCa specificity and overexpression in many stages of tumour development.

1.4.1.3. SPINK1 and SPOP

SPINK1 (Serine peptidase inhibitor Kazal type 1) is the prognostic biomarker in ductal adenocarcinoma of PCa. SPINK1 is overexpressed in almost 6% of all PCa and 10% of TMPRSS2:ETS fusion genes-negative PCa [57]. Speckle-type POZ protein (SPOP) is the most commonly mutated gene in PCa and acts as an E3 ubiquitin ligase adaptor that directly binds target proteins and promotes their ubiquitination and proteolysis [58].

1.4.1.4. MYC

MYC gene (proto-oncogene) encodes a nuclear phosphoprotein that participates in a variety of cellular functions, including cell cycle, cell growth, apoptosis, cellular metabolism and biosynthesis, adhesion, and mitochondrial biogenesis. The Myc family consists of three major members: c-myc, l-myc, and n-myc. MYC is known to directly and indirectly regulate the transcription of numerous genes including *L*-MYC, NKX3-1, PIM1, TMPRSS2, SPARC, EGF, and prostate stem cell antigen (PSCA) family genes, including *Ly6* [59]. De Marzo and colleagues reported that in early stages of PCa, an 8q24 copy number gain results in c-MYC (also sometimes referred to as MYC) activation [60].

1.4.1.5. NKX3.1

NK3 homeobox 1 (NKX3.1) is a prostatic tumour suppressor gene located on chromosome 8p21. It is an androgen-regulated homeobox gene whose expression is predominantly localized in the prostate epithelium. NKX3-1 acts as a transcription factor that has a critical function in prostate development and tumour suppression. Furthermore, NKX3-1 is a negative regulator of epithelial cell growth in prostate tissue. NKX3.1 is also found in testis, ureter, and pulmonary bronchial mucous glands. The expression of NKX3.1 decreases in almost 50 % of PIN lesions of primary prostate tumours and 80% of metastatic tumours [61]. NKX3.1 is a highly sensitive and specific marker of prostate adenocarcinoma. It is particularly useful in poorly differentiated high-grade neoplasms involving the prostate and adjacent organs where PSA and/or prostatic-specific acid phosphatase (PSAP) may be weakly expressed or lost [62] . In the Hi-MYC mice (overexpression of MYC), reduced NKX3.1 expression was seen in the PIN and NKX3.1 was almost completely lost in invasive adenocarcinomas [63].

1.4.1.6. PTEN

PTEN (the phosphatase and tensin homolog), a tumour suppressor gene, regulates the cell cycle, cell proliferation, and apoptosis. PTEN somatic mutation increases the activity of the PI3K/AKT signalling pathway in 42 % of primary and 100 % of metastatic PCa [64]. The study by Carver and colleagues suggested that PTEN deletion is associated with reduced AR transcriptional output in human and murine PCa tumours [65]. Moreover, $PTEN^{+/-}$heterozygous mice develop prostatic epithelial hyperplasia and dysplasia consistent with the growth suppressive activities of PTEN in prostate carcinoma cell lines [66].

1.4.2. Epithelial-mesenchymal transition (EMT) in prostate cancer progression

Accumulating evidence suggests that during disease progression to mCRPC, androgens/AR are key players driving epithelial-mesenchymal transition (EMT)/mesenchymal-epithelial transition (MET) [67]. Mechanistically, there are some pathways known to activate EMT such as transforming growth factor β (TGF-β) and receptor tyrosine kinase (RTK) / Ras signalling, in addition to the well-known canonical

Wnt-B-catenin, Notch, Hedgehog, and NFκB dependent pathways [68]. Androgens induce EMT-related changes in PCa progression, irrespective of their androgen sensitivity and AR status, via activation of the Snail transcription factor [69]. In addition, an inverse relationship between AR expression and EMT induction suggests that after long-term ADT, downregulation of AR expression may be contributing to PCa metastasis [69]. As reported, ADT induces EMT transition via suppression of E-cadherin and induction of N-cadherin and Vimentin in PCa cells expressing AR [70]. Liu and colleagues reported that EMT can be induced by AR through repression of the E- cadherin factor or via activation of Snail transcription [71]. Enhancer of zeste homolog2 (EZH2), a histone-lysine N-methyltransferase, is overexpressed in mCRPC [72]. EZH2 targets the genes involved in EMT, including E-cadherin and DAB2IP genes. Additionally, EZH2 inhibits SLIT2 tumour suppressor gene under the drive of AR-dependent TMPRSS2-ERG fusion in aggressive PCa disease [73].

1.4.3. Diagnostic, prognostic, and monitoring markers

1.4.3.1. Prostate-specific antigen (PSA)

Human kallikrein-related peptidase 3 (KLK3), known as PSA, is a 33-kDa glycoprotein secreted by prostatic epithelial cells. Its function is to liquefy seminal coagulum in the human ejaculate. PSA also expressed by multiple non-prostatic tissues both in men and women. Mashkoor and colleagues reported that PSA expression is highly related to breast and colon cancer in women [74]. Initially identified by Albin and his group in 1970, PSA was purified from prostatic tissue by Wang and colleagues in 1979 and detected in sera obtained from PCa patients in 1980 [75]. It is noted that PSA is a known critical downstream target gene of the AR and an important biomarker of disease onset and progression. PSA accumulates in nuclei of androgen-stimulated PCa cells and is required for AR mRNA and protein expression. PSA has a key function in promoting PCa cell growth in vivo and in vitro. Since the late 1980s, PSA has been the most widely used and important tumour marker for PCa in the clinical setting. In serum, most PSA is bound to protease inhibitors such as a1-antichymotrypsin and a2-macroglobulin, while the remaining PSA exists freely. The normal PSA levels are between 0 and 4.0 ng/ml. The serum half-life of PSA is 2-3 days. To enhance the specificity of PCa detection,

various PSA-based diagnostic parameters have been suggested to make this task easier. These parameters include age-specific PSA, total PSA, PSA density (PSAD), PSA transition zone density (PSA-TZ), free/total PSA ratio (f/t PSA), and PSA velocity (PSAV) [76]. Prostatic diseases and urologic manipulations can temporarily change the serum level of PSA. Following radical prostatectomy (RP), PSA rapidly declines to undetectable levels in most patients. After surgery, PSA persistence at a high level is an indicator of independent persistent disease [77]. Furthermore, PSA is used as a surrogate endpoint to monitor disease progression following radiation therapy. After radiation therapy, PSA levels however decline more slowly and may not reach their lowest level due to the persistence of normal prostate tissue [78].

1.4.3.2. Prostate-specific membrane antigen (PSMA)

Prostate-specific membrane antigen (PSMA), with a molecular weight of 100 kDa, is an integral membrane glycoprotein. PSMA is expressed in normal, benign and malignant prostate tissues including intraepithelial neoplasia and metastatic carcinoma [79]. PSMA plays a key role in prostate carcinogenesis and disease progression. PSMA expression is significantly higher in lymph node and distant metastases as compared to primary tumours [80]. PSMA ^{68}Ga-PET/CT can be performed to detect loco-regional and distant disease in PCa patients with biochemical recurrence [81]. PSMA overexpression on RP specimens is significantly associated with an unfavorable biochemical recurrence free survival rate [82]. Hupe et al. reported that PSMA is an independent prognostic marker for disease recurrence following curative surgery at time of initial diagnosis on biopsy [80].

1.4.3.3. Prostatic acid phosphatase (PAP)

PAP, a non-specific phosphomonoester, is produced by the lysosomes of prostate's epithelial cells. PAP level (diagnostic biomarker) proportionally increases with PCa progression [83]. PAP was the first biochemical marker used for the diagnosis and staging of PCa. Although PAP is identified in many organs like the liver, brain, and lungs, the highest concentration of PAP is in the prostate [84]. An elevated level of PAP is not sensitive enough for PCa screening. The value of acid phosphates measurement in the

diagnosis of metastatic disease has been reduced by the increased sensitivity and specificity of PSA screening [85].

1.4.3.4. Alkaline phosphatase (ALP)

Serum ALP, a nonspecific bone turnover marker, evaluates treatment efficacy and predicts OS in men with CRPC [86]. The results of meta-analysis revealed that high serum ALP (prognostic biomarker) is significantly associated with poor OS and PFS of PCa. It is reported that PSA and ALP are important predictors in patients with bone metastasis [87,88]. After widespread bone metastasis, the serum level of ALP increased significantly.

1.4.3.5. Human glandular kallikrein 2 (hK2)

Human glandular kallikrein 2 (KLK2, also known as hK2) is a secreted serine protease from the same gene family as PSA. It exhibits ~80% homology in the amino acid sequence with PSA. Dihydrotestosterone increases the expression levels of KLK2. KLK2 is relatively elevated during PCa progression and thus may be a useful biomarker of PCa. Like PSA, the highest level of KLK2 is in prostatic tissue, although it is expressed in other tissues. Unlike PSA, KLK2 exists mostly in a free, unbound state in the serum. The sensitivity and specificity of KLK2 in detecting organ-confined disease were 37% and 100%, respectively, compared to a sensitivity and specificity of 14% and 100%, respectively, for total PSA [89]. Many studies indicate that the measurement of serum hK2 with PSA can improve the diagnosis of PCa.

1.4.3.6. Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding proteins (IGFPBs)

The effects of IGF-1, a member of the insulin-like growth factor (IGF) family, are mediated principally through the IGF-1 receptor (IGF-1R). Access to the IGF-1 receptor is regulated by the insulin-like growth factor binding proteins (IGFBPs) such as IGFBP1-6. IGF-1 and IGFBPs have been reported to be associated with tumour progression and prognosis in prostate, breast, lung, and colon cancer [90]. Plasma IGFBP-2 and IGFBP-3 levels were correlated with disease progression. While IGFBP-3 was found to be highest in healthy subjects, followed by localized disease, the IGFBP-3 levels were lowest in

patients with bone metastasis [91]. The study by Gregory and colleagues revealed that IGFBP-5 mRNA reduced by 90% following castration of tumour-bearing mice compared to non-castrate androgen-stimulated mice [92].

1.4.3.7. Transforming growth factor-β1 (TGF-β1)

The cytokines in the TGF-β1 family of polypeptides have been implicated in different steps of cancer development. When compared to normal prostate tissue, elevated levels of TGF-β1 have been found in neoplastic prostatic epithelium [93]. There is a positive correlation between elevated plasma levels of TGF-β1 and PCa progression [94]. Pre- and postoperative plasma levels of TGF-β1 were significantly increased in patients with extraprostatic extension, seminal vesicle invasion, and lymph nodes metastasis [95].

1.4.3.8. Interleukin-6 (IL-6)

IL6, a pro-inflammatory cytokine, is expressed in prostate tumours and in the stromal tumour microenvironment. IL-6 plays an important role in the regulation of immune function and bone metabolism. Twillie et al. reported that IL-6, a prostate exocrine gene product, is a candidate mediator of PCa morbidity and a candidate marker of disease activity [96]. IL-6 protein concentrations are increased by approximately 18-fold in human PCa in comparison to benign tissue. Moreover, IL-6 receptor concentrations are increased 8-fold in PCa tissue in comparison with normal tissue. The level of IL-6 soluble receptor was highest in bone metastasis patients, followed by those in patients with regional lymph node metastasis. The serum level of IL-6 may be associated with the prognosis of patients with PCa.

1.5. Current treatment options for prostate cancer

1.5.1. The incidence of prostate cancer

PCa is the second most common cancer and the fifth leading cause of cancer death worldwide, and the third leading cause of cancer deaths among men in Canada. The Prostate Cancer Canada estimates that about 1 in 7 men will be diagnosed with PCa in his lifetime and 1 in 29 will die from it. They also reported that there are 21,300 cases and 4,100 deaths in 2017 [97]. The PCa incidence also increases with aging. It is most

frequently diagnosed in Canadian men aged between 60-69 years. Whereas, the incidence of PCa is high, the progression of PCa is relatively slow. There are several risk factors associated with PCa, including age, family history, genetics, race, infection or inflammation, diet, and lifestyle [98]

1.5.2. Diagnosis of prostate cancer

The clinical course of localized prostate is highly variable and difficult to predict; many PCa patients show indolent cancers (well-differentiated tumour) while others suffer from high-grade cancer (poorly differentiated tumour) leading to metastases, morbidity, and death [99]. There are several methods to diagnose PCa. The decided method is based on the type of cancer, signs and symptoms, age and medical condition. According to the Canadian guideline, PSA blood test and a physical DRE (digital rectal exam) are the initial screening tools for PCa [100]. Due to their different sensitivities and specificities, both of the tests are used in conjunction.

PCa incidence is increasing globally, especially in western countries, due to aging population and more intensive PSA screening [100,101]. Although PSA is a prostate-specific antigen, it is not a cancer-specific marker [100]. There are many factors that affect the PSA level, including inflammation (benign prostatic hyperplasia and prostatitis), urologic manipulation, and trauma [102]. While DRE has high specificity for PCa, it has a low sensitivity profile and is not considered an effective detection tool on its own. Despite the well-known findings related to PCa growth on DRE, such as the asymmetry of prostatic lobes and the presence of hard nodules, DRE alone cannot be reliable [103,104]. If a DRE or PSA test detects an abnormality, magnetic resonance imaging **(**MRI**)/**ultrasound (US)fusion-guided prostate biopsy are used to confirm the diagnosis of PCa [105]. Imaging techniques like PSMA PET/CT and choline PET/CTare used to identify if the PCa has spread outside of the prostate. The diagnosis of PCa is confirmed by histopathology of tissue samples obtained through ultrasound-guided prostate biopsy. Predicting PCa biology is based on Gleason grade, PSA density, clinical stage, and tumour volume.

1.5.3. Prostate cancer staging

Cancer staging is the process of determining the extent of cancer in the body in terms of growing and spreading [106]. There are two types of PCa staging: clinical and pathologic staging. In clinical staging, the grade and volume of the prostate are estimated based on the results of DRE, biopsy, x-rays, and CT and/or MRI scans. The pathologic staging is based on pathological examination of the RP specimen and any lymph nodes removed at the time of surgery. This staging is used to determine tumour burden, surgical margins status, extracapsular extension, and seminal vesicle and pelvic lymph node invasion. In making prognosis and indicating the need for further treatment, pathologic staging is more accurate than clinical staging.

The tumour-node-metastasis (TNM) staging system is an internationally accepted system for the assessment of the extent of disease [107] (Figure 1-8). The letter "T" plus a number (0 to 4) describes the size and the extend of the tumour. T1 tumour confined to the prostate, not palpable by DRE or visible by imaging. T2 tumours are palpable but confined to the prostate. T3 tumours extend through the prostatic capsule. T4 tumours invade adjacent structures.The letter "N" plus a number (0 to 3) stands for lymph nodes. The N category may be N0 if cancer was not found in the regional lymph nodes, N1 if cancer has spread to 1 to 3 regional lymph nodes, N2 if cancer has spread to 4 or more regional lymph nodes or Nx if the regional lymph nodes cannot be evaluated. The M category shows whether there are metastases (M1) or not (M0).

Once the T, N, and M are determined, the TNM combinations are grouped into four stages (I, II, III, IV), with stage I being an early and stage IV being an advanced disease. In stage I, the tumour is localized in the prostate and the cells look similar to normal cells and tend to be slow-growing. In stages IIA and IIB, the tumour is felt using DRE. Although there is no spread of the tumour outside the prostate, the cells look abnormal and tend to grow faster. Cancer has spread into nearby tissue in stage III. In stage IV, the tumour has spread to other parts of the body, such as the bladder, rectum, bone, liver, lungs, or lymph nodes.

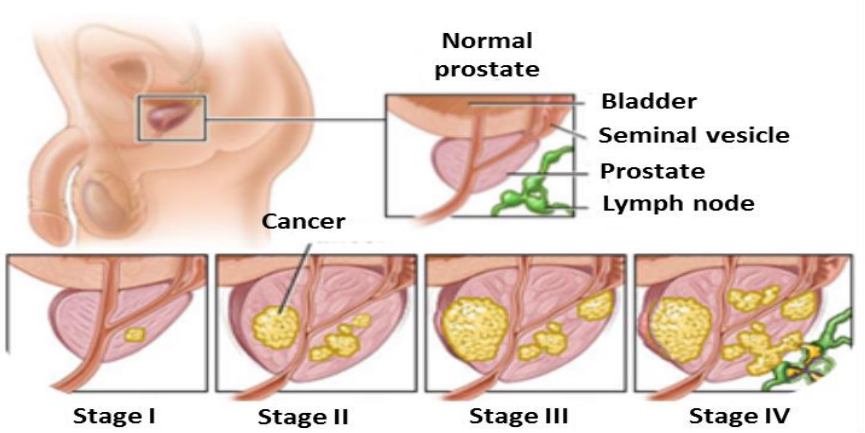

Figure 1-8. Different stages of prostate cancer. Modified form Treatments Related to Various Stages of Prostate Cancer. http://www.mpuh.org/centreforroboticsurgery/treatments-related-to-various-stages-of-prostate-cancer/.

1.5.4. Prostate cancer grading

The Gleason score (GS) is the most common grading system for PCa [108]. The Gleason score ranges from 1-5 and describes how much cancer cells look like healthy cells when viewed under a microscope (Figure 1-9). Grades 1 cancer cells (well-differentiated) look and act like normal cells. Grade 2 and Grade 3 cancer cells (moderately-differentiated) look different than normal cells but aren't as abnormal as poorly differentiated or undifferentiated cells. Grade 4 cancer cells (poorly differentiated) look, act and are arranged very differently than normal prostate cells. Grade 3 and Grade 4 tumours tend to grow rapidly and spread faster than tumours with a lower grade. Grade 5 cancer cells (undifferentiated) are very abnormal. They look, act and are arranged very differently than normal prostate cells and are also more aggressive. Since prostate tumours in a single patient often have areas with different grades, two grades are assigned for each patient. The GS consists of the sum of the two most common grade patterns observed under the microscope, which are ranked based on the scale of 1 (non-aggressive) to 5 (very aggressive). The majority of the patients have a GS of ≥ 6. GS 2–4

carcinoma is considered well-differentiated, GS 5–7 is moderately differentiated, and GS 8–10 is poorly differentiated to undifferentiated (highly aggressive).

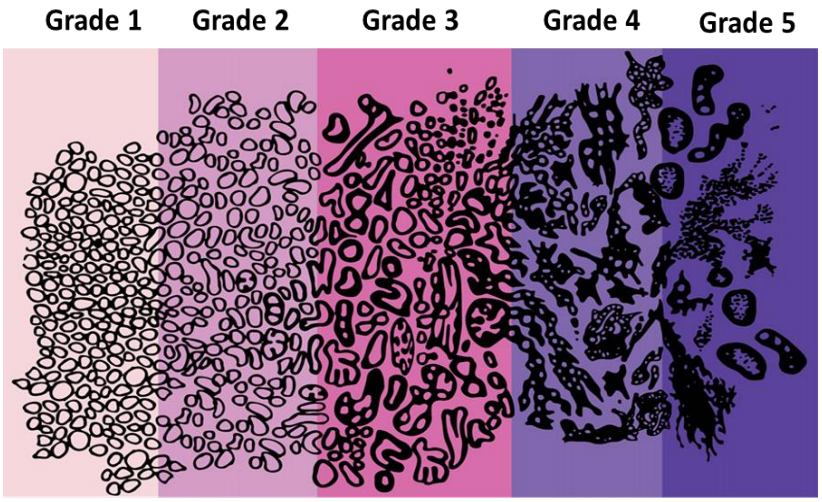

Figure 1-9. Gleason's Pattern. Modified form National Institutes of Health. https://training.seer.cancer.gov/prostate/abstract-code-stage/morphology.html.

1.5.5. Prostate cancer risk stratification

PCa risk group is based on the PSA level, the stage of cancer, and the GS [109]. The aim of this stratification is to decide which treatment options are the best and design clinical trials based on this option. According to D'Amico et al., there are three risk stratification groups: low-risk (T1/T2a, and PSA ≤10 ng/ ml, and GS ≤6), intermediate-risk (T2b, and/or PSA 10–20 ng/mL, and/or GS=7), and high-risk groups (≥T2c or PSA >20 ng/mL or GS= 8–10).

1.5.6. Localized prostate cancer: Treatment options

PCa treatment decisions are based on the stage and grade of cancer, blood levels of PSA, probable side effects, the preferences of the patient, and their overall health. Active surveillance (AS) has become an important alternative for patients with "low-risk"

or “favorable-risk” disease (GS=6). The purpose of AS is to delay definitive therapy in these patients to avoid long-term side effects including erectile dysfunction and incontinence. Based on ASCO (American Society of Clinical Oncology) guidelines, ASCO protocols should include a PSA test every 3 to 6 months, a DRE at least yearly, a prostate biopsy within 6 to 12 months and then a biopsy at least every 2 to 5 years [110]. Watchful waiting is a treatment choice for a man with low-risk localized PCa and a life expectancy of fewer than 10 years [111]. For these patients, the PSA test, DRE and biopsy are not recommended. For intermediate and high-risk PCa patients (GS ≥ of 7), active treatment is recommended. The treatment options for intermediate-risk PCa patients are RP or radiation therapy [brachytherapy and external beam radiotherapy (EBRT)] with or without short-term ADT (4-6 months). RP or RT with long-term ADT (2-3 years) is typical options for high-risk PCa patients. Boylu et al. reported that almost two-thirds of high-risk PCa patients benefit from RP without additional RT or ADT [112].

1.5.6.1. Radiation therapy in prostate cancer

Radiation treatment modalities for PCa include different types of EBRT and brachytherapy. EBRT techniques include three dimensional conformal radiation therapy (3D-CRT), intensity-modulated radiation therapy (IMRT) and hypofractionated, image-guided stereotactic body radiation therapy (SBRT). Over the last several decades, 3D-CRT and IMRT have evolved to deliver higher doses of radiation to the prostate while reducing doses to surrounding organs. The IMRT technique has become the standard of care to deliver EBRT. SBRT techniques deliver conformal, high-dose radiation in 5 or fewer treatment fractions.

1.5.6.1.1. Hypofractionated radiation therapy

Traditionally, EBRT is given in equal daily fractions, five days a week, to allow for repair of injured normal tissue and to permit tumours to re-oxygenate between the treatments. A radiotherapy prescription, therefore, consists of a total dose, a fraction number and an overall treatment time (i.e. 76 Gy in 38 fractions over 7.5 weeks). Radiation prescription can be either standard (fraction size=1.8 or 2 Gy) or hypofractionated (fraction size>2 Gy and given in a smaller number of daily fractions).

Fraction size of 2 Gy offers the best balance between desired tumour kill and unwanted normal tissue injury for most tumours. Evidence suggests that prostate carcinoma does not behave like other cancers in its radiation responsiveness. The sensitivity of the tissue to radiation fraction size is described by an alpha/beta component of the linear quadratic equation (where α and β are the linear and quadratic components of the cell kill respectively). Most cancers, and all rapidly dividing normal tissues (acute reacting tissues), have an alpha/beta ratio of approximately 10 Gy [113]. Slowly dividing late-reacting normal tissues (e.g. fibroblasts and muscles,) have an alpha/beta of between 3 to 5 Gy. Wang and colleagues reported that the alpha/beta ratio for PCa is 0.9-1.5 Gy [114]. A low alpha/beta ratio for PCa means that hypofractionated radiotherapy is more efficient at tumour killing than standard fractionation and will produce equivalent tumour control with a lower total dose and a shorter overall treatment time. One of the suggested hypofractionation schedules for PCa is 68.13 Gy in 25 fractions. This dose is biologically equivalent to about 82.2 Gy when using a standard 2 Gy/fraction regimen (α/β =1.5 Gy) [115,116]. This biological dose escalation is expected to improve the biochemical control rate with acceptable rectal toxicity. The conventional fractionation (74-78 Gy in 37-39 fractions) or hypofractionation (60-68 Gy in 20-25 fractions) is the most common radiation dose fractionation for high-risk PCa patients. Improved conformal radiation therapy technology has allowed for dose escalation and hypofractionated radiation delivery.

1.5.6.1.2. Brachytherapy

Brachytherapy treatment involves placing radioactive source(s) into the prostate gland. There are currently two methods of PCa brachytherapy: (1) permanent low-dose-rate (LDR) radioactive seed implantation using iodine 125 (^{125}I) or palladium 103 (^{103}Pd) or (2) temporary high-dose-rate (HDR) catheter-based implantation using iridium 192 (^{192}Ir) [117]. The dose prescribed for monotherapy is usually 145 Gy and 125 Gy for ^{125}I and ^{103}Pd, respectively. HDR brachytherapy delivers the dose at 10-15 Gy in single fraction in addition to 40-50 Gy delivered using EBRT and ADT.

Prostate brachytherapy can be used as a monotherapy for patients with very low, low, or favorable intermediate-risk PCa, depending on life expectancy. For low-risk PCa,

the 2-years biochemical recurrence- free survival (bRFS) rates were similar between the LDR brachytherapy (96.1%) and the RP (97.4%) [118]. After 6 months' follow up, continence was better in the brachytherapy group while potency was better in the RP group. Furthermore, a randomized control trial revealed that LDR brachytherapy alone is safe and effective for the management of favorable intermediate-risk PCa [119]. LDR or HDR brachytherapy boost, in combination with EBRT and ADT, has debatably become the standard of care for managing high-risk localized PCa. The results of some trials have shown that brachytherapy in combination with EBRT and ADT leads to improved biochemical control over EBRT plus ADT alone, but with higher toxicity [120,121]. The ASCENDE RT (Androgen Suppression Combined with Elective Nodal and Dose-Escalated Radiation Therapy) trial found that intermediate and high-risk PCa patients treated with EBRT plus LDR boost have a significantly better biochemical-progression free survival (bPFS) compared to EBRT alone (78Gy) (83% vs. 62% bPFS at 9 years in favor of LDR boost) [122].

Bittner and colleagues reported in patients who received tri-modality therapy (brachytherapy+EBRT+ADT), the 9-year progression-free and disease-specific survival (DSS) rates were 87% and 91%, respectively [123]. The advantages of brachytherapy are quick recovery, a lower risk of some side effects and short term hospitalization (1-2 day), and the disadvantages are side effects such as acute urinary retention, sexual and bowel problems, a general or spinal anesthetic, and infertility.

1.5.7. Relapse and metastatic prostate cancer: Treatment options

Metastatic cells break off from their primary tumour and spread through lymph or blood to the other parts of the body. The most common sites of PCa metastasis are bone and lymph nodes, and less frequently the liver, lung, and brain. ADT is the standard treatment for metastatic PCa patients [124]. ADT aims to reduce the levels of androgens or stop them from affecting PCa cells**.**

1.5.7.1. Androgen deprivation therapy (ADT)

ADT can be achieved by either bilateral orchiectomy or medical castration using GnRH agonists or GnRH antagonists to reduce testosterone to castrate levels <1.7 nmol/ l [125]. 60-75% of intraprostatic androgens are made from testosterone of testicular origin and the remaining androgen is synthesized from the adrenal precursor by the prostate itself [126]. Combined androgen blockade (CAB) is the combination of medical or surgical castration and a nonsteroidal AR antagonist. CAB reduces the level of circulating androgens and blocks the action of any remaining non-testicular androgen [127]. Although ADT is still the standard treatment for metastatic castration-sensitive PCa (mCSPC), superior outcomes may be achieved if ADT is combined with docetaxel chemotherapy or abiraterone acetate [128]. Despite the initial response to ADT, most of the patients ultimately experience disease recurrence and progress to CRPC [129].

1.5.7.1.1. Surgical castration

Surgical castration **(**orchiectomy) is the surgical removal of one or both testicles to prevent testosterone production in the male body [130]. Following the surgery, the blood testosterone level falls very quickly with favorable responses such as tumour shrinkage and pain relief in the patients. Some men with metastatic cancer prefer the single treatment of surgical castration due to the lower risk of complications and side effects compared to ongoing hormone therapy (medical castration).

1.5.7.1.2. Medical castration

Medical castration (chemical castration) involves drugs that completely suppress the production of androgens by manipulating the HPG axis. Pituitary stimulation with GnRH agonists such as leuprolide, goserelin, and buserelin induces both FSH and LH, thereby promoting testicular androgen synthesis [131]. This action causes an initial surge in serum testosterone due to pituitary overstimulation. This is followed by a downregulation of LH production that leads to a castrate level of testosterone during the second and third week of treatment [132]. Degarelix is the third generation of GnRH antagonist for the first-line treatment of androgen-dependent advanced PCa. Degarelix binds directly to LHRH

receptors in the pituitary gland, which blocks the action of endogenous LHRH on the pituitary receptors with no initial surge in gonadotropin or testosterone levels [133].

1.5.7.1.3. Androgen receptor antagonists (anti-androgens)

Anti-androgens, including nonsteroidal agents (bicalutamide, flutamide, nilutamide, and more recently enzalutamide, apalutamide and darolutamide), and steroidal agents (cyproterone acetate, and spironolactone) competitively inhibit AR action by binding to and blocking access to the LBD of the AR [134]. Unlike the steroidal anti-androgens, the nonsteroidal agents do not interact with the other steroid receptors and they function as relatively pure AR antagonists [135]. Compared to flutamide and nilutamide, bicalutamide has a higher affinity for AR, a longer half-life and less hepatotoxicity [136]. Despite these characteristics, the affinity of bicalutamide for AR is 30-100 times less than DHT, which means a more potent AR antagonist is required [137] . Enzalutamide has 5- to 8-fold greater binding affinity for the AR compared to bicalutamide [138]. Compared to bicalutamide, apalutamide binds to the same ligand-binding pocket but has a 7-to 10-fold greater binding affinity for the AR receptor [139]. Darolutamide has a higher binding affinity for the AR compared with enzalutamide and apalutamide [140]. CAB can result in anti-androgen withdrawal syndrome (AWS) following the discontinuation of an anti-androgen [141]. About 30% of patients will benefit from AWS, exhibiting clinical benefits such as decreasing PSA values and an improvement in pain caused by tumour growth [142]. AWS is generally associated with a switch from antagonistic to the agonistic activity of these anti-androgens due to mutations in AR [143] .

1.5.7.2. Hormonal therapy failure

ADT is initially effective as observed by a marked decrease in serum PSA levels and a reduction in the tumour volume. Despite the relative success of hormonal therapy, nearly all patients will eventually progress to CRPC [144]. CRPC is defined as PCa progression despite the castrate level of serum testosterone (<50ng/dL) and may present as biochemical progression or radiologic progression [145]. Biochemical progression is an increased expression of PSA level for more than 3 consecutive measurements following PSA nadir [146]. Radiologic progression is the appearance of either 2 or more new lesions

on bone scan or soft tissue lesions [147]. Further hormonal treatment with anti-androgens or chemotherapies does not considerably prolong the life expectancy of most patients with CRPC. Docetaxel (a member of the taxane family of microtubule stabilizer/inhibitors) plus prednisone became the standard treatment for patients with mCRPC in 2004 [148]. Although, some patients can experience a significant response to docetaxel therapy, on average there are minimal benefits.

Recently, a new generation of AR axis-targeted agents has been developed to extend life expectancy. Enzalutamide has demonstrated clinical activity in patients who have failed both ADT [149] and docetaxel-based chemotherapy [150]. The other agent is abiraterone which inhibits cytochrome P450-C17A1 (CYP17) enzymes required for adrenal and intratumoural androgen biosynthesis [151]. Both abiraterone [152] and enzalutamide [153] improved OS in patients with mCRPC who previously received chemotherapy (abiraterone: 3.9-month survival benefit, enzalutamide: 4.8-month survival benefit).

1.5.8. Novel anti-AR signalling agents in castration-resistant prostate cancer

1.5.8.1. CYP17A1 inhibitor: Abiraterone acetate

Abiraterone acetate (Zytiga), a prodrug of abiraterone, is a potent, selective, and irreversible inhibitor of cytochrome P450 17A1 (CYP17A1). It can block androgen synthesis in adrenal, testicular, and prostate tissues [154]. Abiraterone acetate is co-administered with prednisone to manage adverse events related to mineralocorticoid excesses, such as hypokalemia, hypertension, and congestive heart failure [155].

1.5.8.2. AR LBD inhibitors

1.5.8.2.1 Enzalutamide

1.5.8.2.1.1 Enzalutamide: Mechanism of action

Enzalutamide (previously known as MDV3100 and available commercially as Xtandi) is a second-generation anti-androgen that inhibits multiple steps of AR signalling. The mechanism of action is to: (1) inhibit binding of androgens to AR LBD;

(2) prevent nuclear translocation of AR; (3) prevent AR binding to DNA; (4) prevent coactivator recruitment and AR-mediated DNA transcription [156]. In contrast to bicalutamide (first-generation anti-androgen), enzalutamide is a pure antagonist, with no detectable agonist effects in LNCaP cells overexpressing wild-type AR (LNCaP/AR). Enzalutamide induces LNCAP/AR xenograft tumour regression in castrated mice through apoptosis, whereas bicalutamide treatment only slows tumour growth. The results of 16β-[^{18}F] fluoro-5α-dihydrotestosterone [^{18}F] FDHT in LNCaP/AR showed that the half maximal inhibitory concentration (IC50) of enzalutamide, 18-FDHT natural ligand and bicalutamide were 21.4±4.4 nM, 11.5±2.0 nM, and 160±29 nM respectively [156]. Enzalutamide inhibits the expression of PSA/KLK3 genes (AR target genes) in LNCaP/AR cells. Furthermore, enzalutamide inhibits the in vitro growth of VCaP cells which is characterized by endogenous AR gene amplification.

1.5.8.2.1.2. Enzalutamide treatment in castration-resistant prostate cancer

These promising preclinical results led to a phase 1/2 trial in order to assess the pharmacokinetics, safety, tolerability, and a maximum tolerated dose of enzalutamide in CRPC patients [157]. In this trial, 140 patients with progressive mCRPC were given oral doses of enzalutamide from 30 mg to 600 mg daily in order to assess the efficacy and safety of the drug. Antitumour effects were observed at all dosage levels, including serum PSA declines ≥ 50% in 56% of patients, and time to PSA progression was greater in the chemotherapy-naïve group (41 weeks) compared to chemotherapy-pretreated group (20 weeks). The terminal half-life of 1 week was reported in patients. The maximum tolerated dose was 240 mg/day with main side effects being headaches, hot flashes, and fatigue. Three patients developed seizures at higher doses, which was concerning, so the dosage was reduced to 160 mg daily for phase 3 trials. This study also assessed the tumour response based on changes in serum PSA levels, imaging studies, circulating tumour cell (CTC) counts, and time to disease progression in castration-resistant disease. The results of this study supported that AR signalling plays a critical role in the progression of CRPC. Some evidence suggests that enzalutamide provides significant benefit for men with CRPC who had previously failed chemotherapy or hormonal treatments.

Based on these promising phases 1/2 results, the phase 3 AFFIRM (Atrial Fibrillation Follow-up Investigation of Rhythm Management) trial evaluated the efficacy of enzalutamide versus placebo in men with mCRPC who had received previous docetaxel-based chemotherapy [158]. Patients treated with enzalutamide had a median OS rate of 18.4 months versus 13.6 months for the placebo-controlled treated patients. Furthermore, patients treated with enzalutamide had a ≥50% reduction in PSA levels in 54% of cases compared with only 1.5% in the placebo group and had a reduction in PSA level of ≥90% in 25% and 1% of patients respectively [159]. The other secondary endpoints, including radiographic progression-free survival (rPFS), time to PSA progression, soft tissue response rate, quality of life, pain palliation and time to the first skeletal-related event, were improved in enzalutamide treated arm versus placebo. The treatment was well-tolerated, with common side effects including fatigue, diarrhea, and hot flashes. The seizure occurred at a rate of less than 1% in patients treated with enzalutamide. Based on the positive results in terms of efficacy, toxicity profile and quality of life in the AFFIRM study, enzalutamide was approved by the FDA (Food and Drug Administration) in 2012 for the treatment of mCRPC patients who have previously received docetaxel. Phase 3 PREVAIL study demonstrated that enzalutamide improved OS rates (32.4 months vs. 30.2 months, HR = 0.71; P <.0001) in chemotherapy-naïve men with mCRPC [160]. In PREVAIL data, the benefit of enzalutamide was shown with respect to all secondary endpoints, including the time to the first skeletal-related event (SRE), soft-tissue response rate, time to PSA progression, and PSA decline ≥50%. Based on the results of the PREVAIL trial, enzalutamide was approved by the FDA in 2015 for treating mCRPC patients who did not receive chemotherapy. STRIVE [161] and TERRAIN [162] are two double-blind phase II randomized trials that compared enzalutamide with bicalutamide. The TERRAIN study demonstrated significant improvements in PFS and health-related quality of life with enzalutamide versus bicalutamide in mCRPC. In the STRIVE trial, enzalutamide significantly reduced the risk of PCa progression or death versus bicalutamide in non-metastatic and metastatic CRPC.

1.5.8.2.1.3. Mechanisms of resistance to enzalutamide treatment

Enzalutamide demonstrated improved clinical activity in CRPC. However, resistance to enzalutamide develops over a period of time and the molecular mechanisms of the acquired resistance include AR splice variants (AR-Vs), novel F876L mutation, glucocorticoid receptor, and cytokines.

1.5.8.2.1.3.1. AR splice variants (AR-Vs) and AR mutation

One mechanism of enzalutamide resistance is the presence of constitutively active AR variants (AR-Vs) including AR-V7, AR-V1 and, AR-V567es in CRPC patients [163,164]. The AR target genes regulated by AR variants are distinct to those regulated by full-length AR (AR^{FL}) [165]; Genes affected are related to the M phase of the cell cycle. Li et al. demonstrated that bicalutamide or enzalutamide could not inhibit the growth of CWR-R1 and 22Rv1 cells due to the enriched expression of truncated AR (AR-Vs) in the cells [166]. High levels of AR-V7 mRNA in CRPC tumours have been associated with poor patient survival [167]. 22Rv1, VCaP, and LNCaP95 (androgen-resistant PCa cell lines) contain AR-V7 and AR-V567es transcripts which are also found at low levels in LNCaP cells (androgen-sensitive PCa cell lines) [164]. EPI-002 inhibits the expression and transcriptional activity of AR-Vs and reduce AR-V7 expression [168]. The transcriptional activity of AR-V7 can also be inhibited with niclosamide [169].

Another mechanism of enzalutamide resistance is the emergence of AR F876L mutation (substitution of phenylalanine for leucine at the 876 positions) in LBD. This mutation is adjacent to the homozygous T877A mutation in LNCaP cells [170]. Korpal et al. reported there is an antagonist-to-agonist switch in enzalutamide resistant LNCaP due to a missense AR F876L mutant [170].

1.5.8.2.1.3.2. Glucocorticoid receptor (GR)

Importantly, it has been proposed that the upregulation of the GR is the other mechanism of resistance to enzalutamide [171]. Charles Sawyer's group demonstrated that GR overexpression reactivates AR signalling and increases AR target genes expression in patients treated with enzalutamide. The DNA-binding domain of AR and GR are 77%

identical and both AR and GR bind AREs with high affinity [172]. Furthermore, glucocorticoid drugs such as prednisone are used to suppress the levels of pituitary adrenocorticotropic hormone and decrease the production of adrenal androgens [173]. GR agonists may thus contribute to therapy resistance and the potential of receptor antagonists has to be further examined [174].

1.5.8.2.1.3.3. Cytokines

Nuclear factor kappa B (NF-κB) controls the expression of several cytokines including interleukins (ILs) in normal and malignant tissue [175]. IL-6 is highly expressed in CRPC tumours and increases transcriptional activity of the AR in a ligand-independent manner. Increased level of IL-6 is associated with metastatic and advanced PCa [176]. NF-κB2/p52 subunits contribute to the development of enzalutamide resistance via activation of AR and induction of AR-V7 [177].

1.5.8.2.2. Apalutamide

Apalutamide (also known as ARN-509), a selective and irreversible inhibitor of AR, has a similar mechanism of action to enzalutamide [178]. Compared to enzalutamide, apalutamide has the same in vitro activity, but better in vivo activity in CRPC xenograft mouse models [156,179]. It is fully antagonist to AR overexpression and does not induce AR nuclear translocation or DNA binding [180].

1.5.8.2.3. Darolutamide

Darolutamide, a high-affinity AR antagonist, has a novel chemical molecular structure. This structure is distinct from the structure of other AR antagonists which may result in differences in biology and resistant mechanisms [140]. Darolutamide has inhibitory activity against known AR mutants including F876L, W741L, and T878A which confers resistance to both enzalutamide and apalutamide [140].

1.5.8.3. AR degrader: ASC-J9®

ASC-J9®, also known as dimethyl curcumin, is a recently-developed AR-degradation enhancer that effectively inhibits invasion of CRPC cells. The study by Yamashita and colleagues suggested that ASC-J9 is able to degrade both AR ^{FL} and AR3, a major AR splice variant that lacks the AR ligand-binding domain [181]. The consequences of such degradation of both AR^{FL} and AR3 might then result in the suppression of AR-targeted genes *(PSA, TMPRSS2,* and *FKBP5*) expression and cell growth in different CRPC cells in vitro and in vivo [182]. It has been shown that bicalutamide or enzalutamide could enhance PCa metastasis through modulation of the TGF-β1/Smad3/MMP9 pathway, but ASC-J9® could simultaneously suppress PCa cell growth and invasion via downregulation of MMP9 expression [183].

1.5.9. Immunotherapy in prostate cancer

The standard treatments for PCa patients include surgery, radiation therapy and hormone therapy. In recent years, immunotherapy has become an important cancer treatment modality in PCa. Immunotherapies fall into three categories such as checkpoint inhibitors, cytokines, and cancer vaccines.

1.5.9.1. Prostate cancer vaccines

1.5.9.1.1. Sipuleucel-T

Sipuleucel-T (Provenge) is an autologous immune cell–based vaccine used in the treatment of men with asymptomatic or minimally symptomatic mCRPC[184,185]. Vaccines' target is PAP which is elevated in patients with bone metastasis and correlates with poor prognosis[186]. According to the phase III clinical trial known as Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT), in the sipuleucel-T group, there was a 4.1 month improvement in median survival as compared with the placebo group (25.8 months vs. 21.7 months)[187].

1.5.9.1.2. Prostvac-VF

Prostvac-V/F (PRO) is a prostate cancer vaccine that is comprised of two viral vectors, vaccinia that is a potent immunologic priming agent and fowlpox that is used as a boosting agent[188]. The results of the PROSPECT trial revealed that Prostvac-V/F did not improve OS for mCRPC patients compared with placebo[189].

1.5.9.1.3. DNA-based vaccines

DNA-based vaccines consist of genetically engineered DNA containing the coding sequence of a targeted antigen, producing a protective immunological response. The results of a phase I trial showed that DNA vaccines could elicit immune responses in patients with advanced prostate cancer[190].

1.5.9.2. Checkpoint inhibitors

1.5.9.2.1. Anti-CTLA-4 therapy in prostate cancer

Cytotoxic T lymphocyte antigen-4 (CTLA-4) represents a potent inhibitor of T cell-mediated immunity. The anti-CTLA-4 blocking antibody ipilimumab, the first immune checkpoint inhibitor, enhances antitumor immunity. Ipilimumab has yielded modest clinical activity based on PSA response rate and PFS in mCRPC patients[191].

1.5.9.2.2. Anti-PD-1 therapy in prostate cancer

Programmed cell death protein 1 (PD-1) is an inhibitory molecule expressed on the surface of T cells. Programmed death-1 ligand (PD-L1), a ligand for PD-1, is upregulated by interferons. PD-1 has a role in regulating immune responses and programmed cell death. The key role of this immune checkpoint receptor in the inflammatory process is reducing T cell activity in peripheral tissue, preventing autoimmunity[192,193]. In September 2014, FDA approved pembrolizumab, an anti-PD-1 antibody, for the treatment of patients with advanced melanoma. Pembrolizumab shows antitumor activity and disease control with acceptable safety in mCRPC previously treated with next-generation hormonal agents and docetaxel [194]. Furthermore, Pembrolizumab is being investigated in a phase II study in mCRPC after ADT (NCT02312557)[195] .

1.5.9.3. Cytokines

Stimulation of the immune system by affecting the cytokines may result in a strong antitumor immune response. Some studies revealed that the elevated levels of several interleukins such as IL-1α, IL-2, IL-4, IL-6, IL-7, and IL-11 leads to PCa progression [196,197]. For example, increased levels of IL-6 influence growth and survival pathways in PCa [198]. Mackiewicz et al. demonstrated that vaccination with TRAMP-H6 (vaccines modified with hyper-IL-6) and TRAMP-H11 (vaccines modified with hyper-IL-11) extended OS of treated mice in the orthotopic prostate cancer model compared to untreated controls [199].

1.5.9.4. Different treatment combinations for prostate cancer

There are some ongoing studies on combination of vaccines with ADT and radiotherapy. Radiation therapy stimulates T cell priming by facilitating immunogenic cell death resulting in antigen release and inflammatory signals [200]. ADT, on the other hand, stimulates immune cell trafficking and tumor penetration [201]. The synergistic effect of ADT and immunotherapy has been evaluated in several clinical trials. Furthermore, ENZA induce immunogenic modulation in PCa. There are some ongoing clinical trials evaluating the efficacy of ENZA in combination with PROSTAC-VF in patients with CRPC (NCT01867333) and non-metastatic castration-sensitive prostate cancer (NCT01875250) [202]. These treatment strategies may be used in combination with immunotherapy. The immune modulation depends on many factors such as ADT type, radiation therapy strategy (type, dose, and duration), and administered immunotherapy agent.

1.6. Radiation Therapy

Radiation therapy has been used to treat cancer and other diseases since the late 19th century. Since then, there has been enormous progress in improving the effectiveness of radiation therapy and reducing side effects. Recently, radiation therapy has been used in combination with other modalities for a wide range of malignancies in order to downstage locally advanced cancers, increase organ preservation, minimize toxicity and consequently improve patients' overall survival rates. At least two-thirds of all patients with localized malignant tumours receive radiation therapy during their course of the disease [203]. The primary goal of radiation therapy is to kill cancer cells while minimizing damage to surrounding healthy tissues. Radiotherapy can serve as a definitive treatment, neoadjuvant or adjuvant therapy, prophylactic or palliative therapy. Definitive radiation therapy is when radiation is used as the primary treatment modality with or without chemotherapy. The neoadjuvant approach is when radiation therapy is given before surgery, with or without chemotherapy, while adjuvant therapy is used after definitive therapy which usually consists of surgery in order to improve local control. The goal of palliative radiation therapy is to control the symptoms of bleeding, pain, airway obstruction.

1.6.1. Type of radiation therapy

Radiation therapy is a therapy using ionizing radiation, generally as part of cancer treatment to control or kill malignant cells. Radiation therapy is delivered using a machine called linear accelerator. Ionizing radiation is any type of particle or electromagnetic wave that has enough energy to remove one or more orbital electrons from an atom (ionize) [204]. These unstable atoms undergo fast chemical changes. There are two main types of ionizing radiation: electromagnetic radiation and particulate radiation. X-rays and gamma-rays are two types of electromagnetic waves that can ionize atoms [204]. X-rays (photons) are produced by a linear accelerator, while gamma rays are emitted from a radiation source such as cobalt 60 or cesium-137. Particulate radiation includes alpha, beta, electrons, protons, and heavy ion particles. Upon interaction of ionizing radiation with the nucleus, two energetic particles are produced: alpha and beta particles. An alpha particle consists of two protons and two neutrons. The beta particle is made of

either a position or an electron. While electromagnetic radiations are indirectly ionizing radiations, charged particles are directly ionizing.

1.6.2. Mechanism of action of radiation therapy

DNA is the biological target of radiation therapy, and radiation-induced DNA damage leads to cell death [205]. In mammalian cells, an exposure of 1 Gy of γ-rays will produce different types of DNA lesions, including 850 pyrimidine lesions, 450 purine lesions, 1000 single-strand break (SSB), and 20-40 double-strand breaks (DSB) per cell [206]. DSB is difficult to repair and, therefore, is considered the most lethal form of DNA lesions within the cells. These breaks are prone to develop chromosomal abnormalities and to increase the second tumour formation due to inaccurate repair [207]. The effect of radiation on cells appears to be most intense during the G2 phase and mitosis while the S phase is the most radioresistant [208]. This suggests that the maximum effect of radiation should occur before or during cell division. Bergonie and Tribondeau reported that the sensitivity of cells to irradiation is directly proportional to their reproductive activity and inversely proportional to their degree of differentiation [209]. Furthermore, other biological and physical factors affect the cellular response to radiation such as linear energy transfer (LET), relative biological effectiveness (RBE), oxygen effect, and fractionation.

1.6.2.1. Linear energy transfer

Linear energy transfer **(**LET) refers to the energy transferred per unit length of the track of a charged particle [204]. There are two types of ionizing radiation: high-LET (α and β particles) and low-LET (X-ray and γ -ray). At atomic scale, low-LET radiation produces sparse ionizing events far apart. Instead, high-LET radiation instead produces ionizing events densely spaced at the atomic scale [210].

1.6.2.2. Relative biological effectiveness

Absorbed dose is a measure of the energy deposited in a mass of some material by ionizing radiation. The gray (Gy) is a derived unit of ionizing radiation dose in the

International System of Units (SI). It is defined as one Joule of energy absorbed per kilogram of matter. Equal doses of different types of radiation have different biological effects, consequently it is common to use X-rays as the standard to compare different types of radiation. Therefore, the RBE of some test radiation (r) is defined by the ratio D250/Dr, where the doses of X-ray (D250) and the test radiation (Dr) required for the same biological effect [204]. Since high-LET radiation deposits most of their energy in a small part of the cell, high-LET are more destructive to the biologic matter than low-LET radiation which induces more sparsely radicals within a cell. Compared with low-LET radiation, high-LET radiation produces clustered DNA damage such as DSB [211]. Generally, BRE depends on radiation quality (LET), radiation dose, number of dose per fraction, dose rate, and biological endpoints.

1.6.2.3. Effects of oxygen on radiation response

It has been recognized that tumour oxygenation may modulate the efficacy of various treatment modalities, particularly radiation therapy. It is reported that the oxygen effect occurs only if oxygen is present either during or within a few milliseconds after radiation [212]. The oxygen-fixation hypothesis (OFH) is widely regarded as the mechanism responsible for the enhancement of radiation damage by oxygen [213]. The tumours' absorption of radiation leads to the production of highly reactive free radicals. These radicals, in turn, produce biological damages either directly in the target molecule (such as DNA) or indirectly with other atoms (or molecules) in the cell as well as diffuse far enough to reach and damage DNA. In the indirect damage, the radiation hits the water molecules, the major (~80%) constituent of mammalian cells, and produces free radicals (R•). In the presence of molecular oxygen, oxygen fixes these unstable molecules (R•) in DNA in a form that cannot be easily repaired (RO2•) while the damage is said to be chemically "fixed". Thus, this damage is recognized by DDR pathways to induce enzymatic processing of the lesions and perhaps their successful repair. In the absence of oxygen or in the presence of reducing species, the unstable R• molecules can react with H+ and chemically restore its original form without the need for biological and enzymatic intervention [204].

1.6.2.4. Fractionation

The interest in fractionated radiation therapy began in the first decade of the 20th century. Modern radiation therapy was based on a fractionated dose instead of single-dose radiation. A rationale for changing single-dose radiation into fractionated doses was based on the presence of a correlation between the cell proliferative activity and susceptibility to radiation-induced damage. In 1901, Robert Kienbock first reported cells with high mitotic activity are highly radiosensitive [214]. Fractionated radiotherapy began in order to increase the damage to the tumour (by reoxygenation of hypoxic tumour areas and redistribution of cells in the cell cycle) and also to spare normal tissue (by repair of sublethal damage and repopulation from surviving cells). Distribution and reoxygenation increase the sensitivity of the tissue cells to a subsequent radiation dose, while repair and repopulation increase the resistance of the irradiated tissue between two radiation doses. The 4 R's (repair, redistribution, reoxygenation, and repopulation) along with radiosensitivity, represent the foundation of radiation fractionation under the 5R's of radiobiology [215]. Radiosensitivity is the fifth member of the R's. Intrinsic radiosensitivity or radioresistance in different cell types affects the radiation response [216]. Hematological cells, epithelial stem cells, and gametes are radiosensitive cells and myocytes, neurons, melanoma, and sarcoma are radioresistant cells [217].

1.7. DNA damage response pathways (DDR)

DNA is the main repository of genetic information of all living organisms. The integrity and stability of genetic information need to be protected in order to be delivered to the next generation. There are some factors that can induce DNA lesions including exogenous sources like UV radiation, ionizing radiation, chemical compounds, genotoxic drugs, and endogenous events occurring during replication processes through DNA replication and cell division (Figure 1-10). DNA damage response pathways (DDR) pathway is activated to regulate and coordinate the cellular response to DNA damage. The DDR consists of signal sensors, transducers, and effectors. In this pathway, sensors directly recognize DNA damage and activate the most upstream DDR kinases such as ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related) and DNA-PK

(DNA-dependent protein kinase). The mediator proteins facilitate the phosphorylation events within DDR network. DDR effectors are substrates of DDR kinases that participate in many cellular processes important for genomic stability, such as DNA repair, DNA damage checkpoints, and cell death [218](See section 1.7.3. for more details).

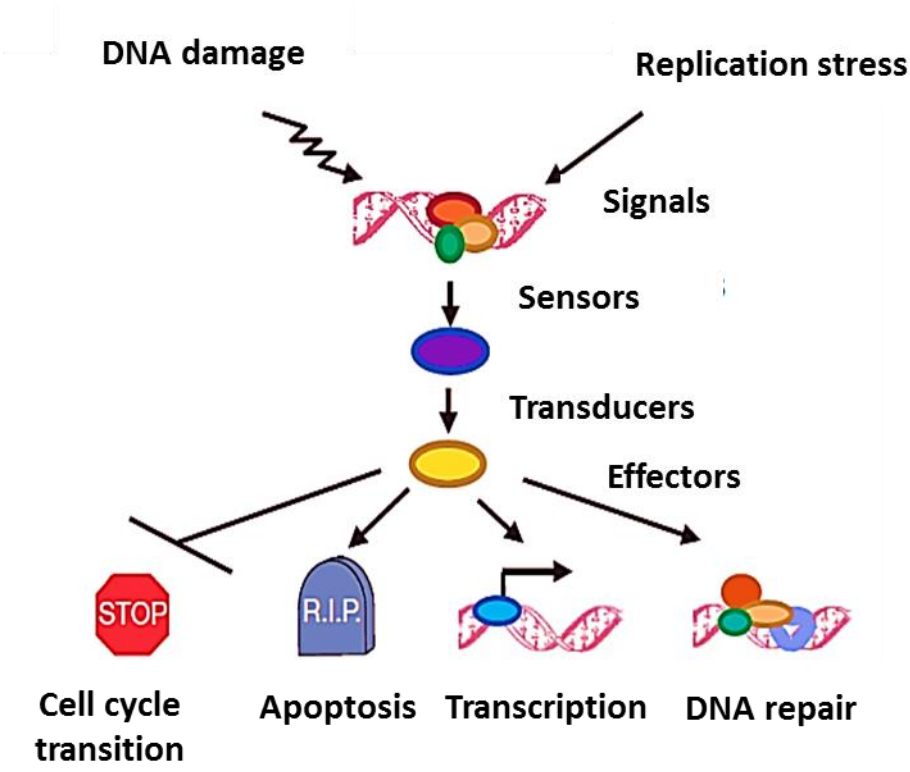

Figure 1-10. DNA damage response signal transduction pathway. Modified from Bin-Bing S. Zhoua1 & Stephen J. Elledge. (2000). The DNA damage response: putting checkpoints in perspective. Nature volume 408, pages 433–439.

1.7.1. DNA damage mechanisms

It is estimated that environmental factors, such as ultraviolet, can result in around 10^5 DNA lesions per cell per day [219] . Active compounds, including reactive oxygen and nitrogen compounds, can form DNA adducts. These adducts impair base-pairing and/or block DNA replication and transcription, base loss, or DNA single-strand breaks (SSBs). In addition, double-strand breaks (DSBs) are formed when two SSBs are located in close proximity or when the DNA-replication apparatus encounters the SSBs. DSBs are highly toxic for the cells and can cause genome rearrangements and cell death [220]. Moreover, radiation induces different type of DNA damages such as alteration or loss of one or more bases, destruction of hydrogen bond between base pairs, SSB, DSB, and cross-linking the strand (Figure 1-11).

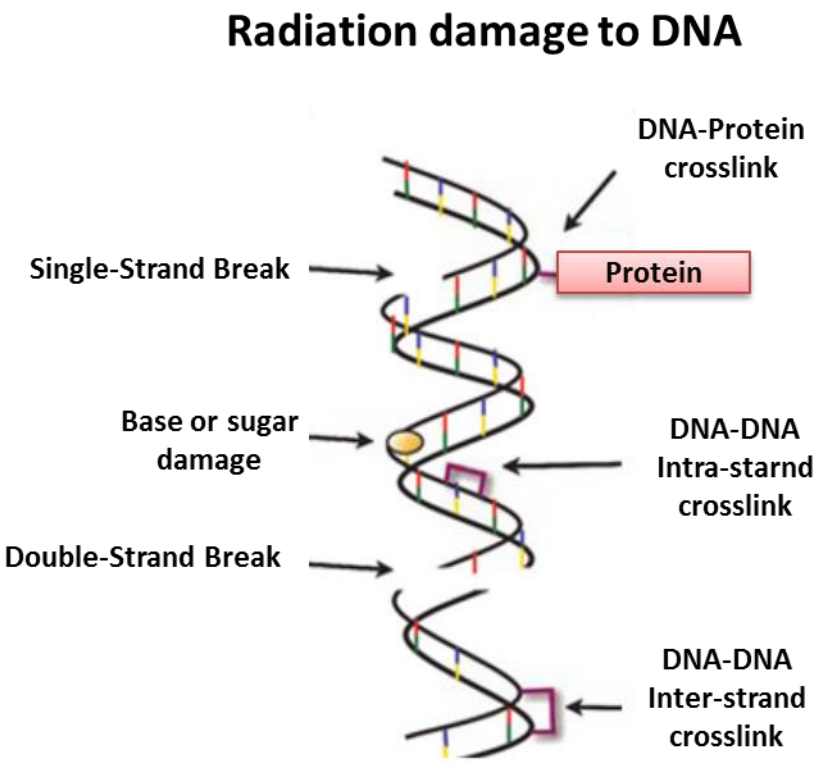

Figure 1-11. Types of DNA damage induced by radiation. Modified from Joy N. Kavanagh et al. (2013). DSB Repair - A radiation perspective. Antioxidants & Redox Signalling. DOI: 10.1089/ars.2012.5151.

1.7.1.1. DNA base damage

DNA base damages, such as O6 -methyl guanine, thymine glycols, and other reduced, oxidized, or fragmented bases in DNA, are produced by reactive oxygen species or by ionizing radiation. UV radiation can also indirectly induce the DNA base damages by generating reactive oxygen species as well as by producing specific products such as cyclobutane pyrimidine dimers (CPD) and photoproducts [221] . Chemical produces various base adducts such as bulky adducts by large polycyclic hydrocarbons or simple alkyl adducts by alkylating agents. Over half of chemotherapy drugs such as cisplatin, mitomycin C, nitrogen mustard, and adriamycin produce base adducts [222,223].

1.7.1.2. DNA backbone damage

Oxidative stress produces DNA backbone damage such as AP or abasic sites, single-and double-strand DNA breaks [224]. AP sites (apurinic/apyrimidinic site) can be formed by spontaneous depurination or by base excision repair (BER) of modified bases [225,226]. Damaging agents, such as ionizing radiation, directly produce SSB. Ionizing radiation or other DNA-damaging agents such as topoisomerase I / II or bleomycin (BLM) forms DSB [227].

1.7.1.3. Cross-Links

Drugs that form interstrand cross-links include bifunctional alkylating agents such as cisplatin, nitrogen mustard, and mitomycin C. Wilson et al. reported that the reaction of cellular DNA with unsaturated aldehydes such as crotonaldehyde or dialdehyde such as malondialdehyde can produce interstrand cross-links in cells.

1.7.2. DNA repair mechanism

In each of our cells, the DNA accumulates thousands of lesions every day. Cells contain multiple DNA repair mechanisms including direct repair, base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-

homologous end joining (NHEJ) and inter-strand cross-links repair. While some of these repair mechanisms can function independently to repair simple lesions, the DDR including multiple DNA processing steps regulates the repair of more complex lesions (See section 1.7.3 for details).

1.7.2.1. Direct repair

The cell attempts to repair DNA molecule through two direct repair mechanisms: the photoreversal of UV-induced pyrimidine dimers by photolyase as well as the removal of the O6-methyl group from O6-methylguanine (O6MeGua) in DNA by methylguanine DNA methyltransferase. The photolyase does not exist in many species such as humans, whereas methylguanine DNA methyltransferase is abundant in nature.

1.7.2.2. Base-excision repair (BER)

BER is the predominant mechanism responsible for the detection and repair of damaged DNA bases. Such damaged bases are recognized and removed by DNA-glycosylase, which results in a so-called apurinic/apyrimidinic sites (AP-sites) in DNA. The AP-sites are recognized by apurinic/apyrimidinic endonucleases, APE1. DNA polymerase β (pol β) completes DNA synthesis and fills the single nucleotide gap induced by APE1 through its polymerase activity [228,229]. Then, either the presence of the DNA ligase I or a complex of DNA ligase III and XRCC1 seals the nicks [230]. Poly (ADP-ribose) polymerase-1 (PARP-1), which is activated by strand breaks, participates in gap sealing with DNA ligase III and XRCC1[231].

1.7.2.3. Nucleotide excision repair (NER)

NER is the main repair pathway responsible for the removal of the bulky DNA lesions formed by UV light and environmental mutagens. NER has two distinct sub-pathways: 1) Transcription-coupled NER (TC-NER) which specifically recognizes and repairs the lesions in the transcribed strand of active genes, and 2) Global genome NER (GG-NER) which recognizes and repairs the lesions in the whole genome as well as the non-transcribed strands of an active gene [232]. These sub-pathways differ only in recognizing the damaged sites. In TC-NER, RNA polymerase II is stopped at the DNA

damage site on transcribed strand, while in GG-NER, a specialized protein called XPC controls the pathway and identifies the DNA damage. In the NER system, the excision nuclease enzyme, a multisubunit enzyme, removes the damaged bases with 22–30 base oligonucleotides. This results in single-stranded DNA (ssDNA) which is acted upon by DNA polymerases, and associated factors before proceeding to ligation. The excision enzyme can also remove all simple single-base damages [233]. The backbone conformational changes due to DNA damage is recognized by the excision nuclease [222]. There are 5 basic steps to NER are: (1) damage recognition and lesion verification by low specified recognition factors such as RPA, XPA and XPC-TFIIH (ATP independent); (2) DNA unwinding and formation of a long-lasting DNA protein complex (ATP dependent); (3) dual incisions and release of the excised oligomer by XPG and XPF-ERCC1; (4) gap filling by POLδ/ε with the help of replication accessory proteins, proliferating nuclear cellular antigen (PCNA) and replication factor C (RFC); and (5) ligation by DNA ligase III [234,235].

1.7.2.4. Double-strand break (DSB) repair pathway

DSB can be caused by exposure to exogenous agents such as ionizing radiation and certain chemicals, as well as through endogenous processes, including DNA replication and repair. DSBs are repaired by two major mechanisms: homologous recombination (HR) or non-homologous end joining (NHEJ). HR, a highly conserved pathway, is the main repair mechanism during the late S and G2 phases of the cell cycle because of the presence of sister chromatid. NHEJ is active in all cell cycle stages [236,237]. HR is generally considered to be error-free because it utilizes an intact homologous DNA sequence as a template for the repair of DSBs, whereas NHEJ is considered to be error-prone [238]. It has been noted that HR plays a major role in the recovery of collapsed replication forks while NHEJ is essential for V(D)J recombination and is thought to be the major pathway for repair of radiation-induced DSB [222,239].

1.7.2.4.1. Homologous recombination (HR) pathway

The Mre11-Rad50-Nbs1 (MRN) complex plays an important role in the sensing and processing of DSBs, as well as facilitating repair through NHEJ and HR pathways

[240]. The RAD50/MRe11 complexin is one of the first factors recruited to sites of DSBs is order to keep DNA molecules in close proximity before DNA repair starts. The endonuclease activity of Rad50-MRe11 is important for processing the several different structures induced by ionizing radiation or chemicals that create single strand ends. Rad52 is also recruited to the site of the DNA break [241,242]. In the next step of HR, recombinase Rad51, along with BRCA 1/ 2, cover the processed DNA ends [243,244]. After the recombination step, the XRCC3 and Rad51C nucleases cut the crossed DNA strands to complete the repair process [245].

1.7.2.4.2. Non-homologous end joining (NHEJ) pathway

NHEJ, as one of the important pathways in eukaryotic cells, is responsible for the repair of DSBs. The initial step in the NHEJ pathway is the recognition and binding of the Ku70/Ku80 heterodimer to the DSB ends [246,247]. This complex protects DNA from degradation and recruits the catalytic subunit of the DNA-dependent kinase (DNA-PKcs) [248]. In response to DNA damage, DAN-PKcs is one of the first molecules to phosphorylate (most importantly at S2609) [249]. It has been suggested that p-DNA-PKcs also recruits phosphorylated Artemis to the site of DNA damage [250]. The Artemis: DNA-PKcs complex generates DNA ends that can be ligated with minimal nucleotides loss. The final step in the repair of a DSB is mediated by DNA ligase IV, which is in contact with XRCC4 dimers. XRCC4 dimers stabilize DNA ligase IV and stimulate adenylation and the ligation activity of Ligase IV [248]. The Mre11-Rad50-Nbs1 (MRN) complex may also participate in NHEJ, particularly when this pathway is utilized for V (D)J recombination. The MRN complex also protects the degradation of the DNA end [251].

1.7.2.5. Cross-link repair

Interstrand DNA cross-links (ICLs) are formed by cellular metabolic processes and by chemotherapeutic reagents [252]. NER and HR repair pathways have also been implicated in the repair of interstrand cross-links (ICLs). NER has been proposed to play an important role in the repair of ICLs of non-replicating cells (G0/1) through incisions made by XPF-ERCC1 [253]. The excision is followed by translesion synthesis and excision of the 'flipped out' ICL. HR has been involved in the repair of ICLs specifically during S

phases, which are replicating phases [252,254]. The collision of a replication fork at the ICL site leads to the activity of the FA (Fanconi Anemia) pathway via a group of proteins (FANCP, FANCD2, RAD51, RAD51C, BRCA2, FANCN, and BRCA1).The unhooking of cross-linked DNA and the formation of DNA strand breaks results as a function of NER components. These breaks are substrates for RAD51-dependent HR. BRCA2 and FANCN promote RAD51 nucleoprotein filament formation and strand invasion. HR is then completed and replication can be re-established in the replication fork [255].

1.7.3. Cell-cycle checkpoint signalling pathway

In the DDR pathway, ATM-chk2 and ATR-chk1 are two key components which are activated by DSBs and replication protein A (RPA)-coated ssDNA respectively to coordinate DNA repair, cell cycle progression, transcription, apoptosis, and senescence [256]. The activity of cyclin-dependent kinase (CDK) is reduced by ATM-Chk2 and ATR-Chk1 proteins [257]. CDK inhibitors prevent cell cycle progression during different phases in order to increase the time available for DNA repair before replication or mitosis phases [258]. In ATM/ATR pathways, DNA repair is done by activating DNA repair proteins and also recruiting repair factors to DNA sites. DDR signalling induces cell deaths, apoptosis or cellular senescence if the DNA damage cannot be repaired. In the DDR pathway, several phosphoinositide 3-kinase-related protein kinases (PIKKs) such as ATM, ATR, and DNA-PK phosphorylate the serine-139 of the histone H2A variant, producing γH2AX. H2AX at DNA damage sites, ubiquitin-adduct formation, and recruitment of DDR factors promote DSB repair and enhance DSB signalling.

1.7.3.1. DNA damage checkpoints

The DNA damage checkpoint provides the cell with an opportunity to repair DNA damage before the crucial processes of DNA replication and chromosomal segregation. In eukaryotes, the cell cycle consists of four distinct phases: G1 (Gap1) phase, S (synthesis) phase, G2 (Gap2) phase, and M (mitosis) phase. The transition from one phase to the next is regulated by cell cycle checkpoint proteins in both normal conditions and in the case of induced DNA damage. Cell cycle checkpoints are associated with

biochemical pathways that delay or arrest the cell-cycle progression in the case of DNA damage, which allows time for the repair to take place.

1.7.3.1.1. The G1/S checkpoint

In the presence of DNA damage, the G1/S cell cycle checkpoint prevents the progression of cells from the G1 phase to enter into the DNA synthesis S phase. Current evidence suggests that the main targets of ATM and ATR, such as P53, Chk1 and Chk2, are phosphorylated following their activation by DSB or SSB respectively. Phosphorylation, inactivation, and degradation of Cdc25A by the Chk2 protein kinases leads to G1 arrest [259]. Loss of active Cdc25A results in phosphorylation and deactivation of Cdk2, which is incapable of phosphorylation of Cdc45. Cdc45 phosphorylation is required to initiate replication at sites of replication origin complexes.

Similarly, in the case of UV-induced DNA damage, ATR would sense the damage and phosphorylate Chk1 through the formation of RAD17-RFC and RAD9-RAD1-HUS1 [260]. Once Chk1 is activated, it phosphorylates Cdc25A leading to G1 arrest. While Chk1 and 2 initiates G1 arrest, P53 maintain G1/S arrest [261]. Phosphorylation of P53 at Ser15 by ATM/ATR or at Ser 20 by Chk1/2 induces the transcription of its target gene p21. P21 binds and deactivates the S-phase-promoting Cdk2-CyclinE complex and maintains G1/S arrest [262].

1.7.3.1.2. The S checkpoint

In response to DSB induction during the S phase and/ or replication stress, cells induce the S-phase checkpoint to arrest DNA replication and cell cycle progression [263]. Such inhibition is critical for DNA repair and to preserve genomic integrity from one generation to the next. The inhibition of late-firing replication origins and replication forks lead to S phase arrest [264]. In response to direct DSB or a DSB formed due to a nicked or gapped DNA, the replication is inhibited by ATM through two different mechanisms. ATM activates Chk2 through phosphorylation of Thr68 to induce degradation of Cdc25A [265]. Cdc25A degradation keeps Cyclin E/Cdk2 in an inactive/phosphorylated form and blocks the Cdc45 loading on the origin of DNA replication. ATM also inhibits DNA synthesis by phosphorylation of NBS1, SMC1,

BRCA1 and FANCD2. ATR-ATRIP heterodimer is the main sensor of DNA damage induced by UV or the chemicals that create bulky base lesions [266]. After the binding of ATR to DNA damage sites and the phosphorylation of Chk1 by ATR, the Cdc25A is downregulated through phosphorylation and finally inhibits the firing of DNA replication origins [267].

1.7.3.1.3. The G2/M checkpoint

The G2/M checkpoint**,** also known as the DNA damage checkpoint, ensures that cells don't initiate mitosis until damaged DNA or incompletely replicated DNA is sufficiently repaired after replication. Like the other checkpoints, ATM and ATR are activated by DSBs and SSBs respectively. In both cases, mitosis is inhibited by Cdc25A downregulation and Wee1 upregulation, both of which controls cdc2/Cyclin B activity [268]. Unlike the G1/S checkpoint, the maintenance of G2/M arrest may have been independent of p53. Several studies have shown that p53-nonfunctional tumour cells accumulate in the G2 phase in response to DNA damage [269]. This could be due to the fact that p73 can activate the p53-target gene, p21 [270] .

1.8. Cell death

The cellular DNA damage response is regulated and coordinated by the DDR signalling pathway. The DNA damage response will be different depending on the cell type and the extent of the DNA damage. Mild DNA damage can be repaired via the upregulation of CDK inhibitors (CKIs), cell cycle arrest and several repair mechanisms. When the DNA damage is more severe and irreparable, cells can undergo permanent cell-cycle arrest (senescence) or cell death (apoptosis/necrosis). In addition to DNA damage, the cell death process can be caused by other signals/stimuli factors. Chemotherapies, growth factor withdrawals and endoplasmic reticulum (ER) stress can induce apoptosis through mitochondria-dependent signalling, while death receptor ligands such as tumour necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and Fas can induce apoptosis through the death receptors signalling pathway [271].

1.8.1. Mode of cell death

According to the nomenclature committee on cell death (NCCD), "a cell should be regarded as ‘dead' when the cell has lost the integrity of its plasma membrane and/or the cell, including its nucleus, has undergone complete fragmentation into discrete bodies and/or its corpse (or its fragments) has been engulfed by an adjacent cell in vivo". In particular, senescent cells, cells that are arrested in the cell cycle, should be considered alive [272]. In this section, apoptosis, autophagy, necrosis, senescence, and also mitotic catastrophe will be briefly discussed as it is specifically relevant to radiation.

1.8.1.1. Apoptosis

The term apoptosis (programmed cell death) was first used by Kerr and colleagues in 1972 [273]. Apoptosis occurs during various physiological events including embryonic development, normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, wound repair and tissue renewal [274,275]. The process of apoptosis is an energy-dependent process that might or might not involve the activation of caspases [275]. Caspases are a family of proteases that cause disassembly of the cell by cleaving a set of proteins. Dysregulation of apoptosis can result in pathological disorders such as developmental defects, autoimmune diseases, neurodegeneration or cancer. Some of the major morphological changes that occur with apoptosis include cell rounding up, reduction in cellular and nuclear volume (pyknosis), chromatin condensation, nuclear fragmentation, a minor modification of cytoplasmic organelles, and plasma membrane blebbing [272]. Guerrero and colleagues reported that enzalutamide induces apoptosis as determined by an increase in activated caspase-3 levels inLNCaP-AR cells [276]. Radiation induces mostly the intrinsic apoptotic pathway (death receptor pathway) but, depending on dose and cell type, the extrinsic apoptotic pathway (mitochondrial pathway) might be the consequence of irradiation.

1.8.1.1.1. Intrinsic pathway

The intrinsic apoptotic pathway is activated by non-receptor stimuli, such as DNA damage, ER stress, metabolic stress, UV radiation or growth-factor removal. The central event in the 'intrinsic' pathway is the mitochondrial outer membrane permeabilization

(MOMP) which needs to be regulated tightly otherwise it leads to cancer development. MOMP leads to the release of apoptotic proteins such as cytochrome c and activation of caspase-9. Binding of cytochrome c to apoptotic protease activating factor 1 (APAF1) induces the formation of a heptameric apoptosome complex. Apoptosome then recruits and activates initiator caspase-9 which in turn activates the effector caspase-3 and 7, resulting in the completion of apoptosis [271]. The X-linked inhibitor of apoptosis protein (XIAP) is the most potent inhibitor of apoptosis. In addition to cytochrome c, other proteins such as the second mitochondria-derived activator of caspases (Smac)/direct IAP binding protein with low isoelectric point, pI (DIABLO), can bind into the same pockets in XIAP which are used to bind caspases and eliminate the caspase inhibitory activity of XIAP during apoptosis [277].

1.8.1.1.2. Extrinsic pathway

With regard to the extrinsic pathway, an adaptor protein such as Fas-associating protein with a death domain (FADD) binds to Fas or other death receptors and recruits caspase-8 into the death-inducing signalling complex (DISC) for apoptosis induction. By assembling with FAS through FADD adapter molecules, caspase-8 becomes enzymatically activated, thereby cleaving caspase-3 and 7, which leads to cell apoptosis [278]. The extrinsic pathways can crosstalk to the intrinsic pathway following the DISC assembly. Activated caspase 8 is able to cleave and activate BH3-interacting domain death agonist (BID), a pro-apoptotic Bcl-2 in the BH3-only subfamily. Truncated BID (tBID) translocates to mitochondria and interacts with BAX. Activation of BAK and BAX by tBID leads to apoptotic state [271]. Apoptosis-inducing factor (AIF) is a pro-apoptotic protein which is released from the mitochondria upon apoptotic stimuli and induces caspase-independent apoptosis in the cell. Following the release from mitochondria, AIF translocates to the nucleus, binds to the DNA randomly and causes DNA fragmentation by the recruitment of nucleases [279].

1.8.1.2. Autophagy

Macroautophagy (which refers to autophagy) is morphologically defined as a type of cell death with the accumulation of autophagosome (double-membrane enclosed

vesicles) in the dying cells [272]. The fusion of autophagosomes and the cellular lysosome system initiates the degradation of the phagocytized material. In general, autophagy acts as a pro-survival mechanism to protect cells at the time of cellular stress including starvation. In this recycling process, cellular components are degraded to increase nutrient availability and eliminate toxic wastes [280].

Autophagy is highly regulated by autophagy-related genes (ATG) which is expressed through the modulation of inhibitory signals of the mammalian target of rapamycin (mTOR). Under stress conditions, such as nutrient starvation, a core complex of the catalytic subunit VPS34, the adaptor VPS15 (p150), and Beclin 1 (ATG6) activates downstream ATG factors that are involved in the initiation, elongation and maturation of autophagy. ATG 12 and LC3, (microtubule-associated protein light chain 3) control the elongation step of autophagy and these proteins are degraded during the last step of maturation into the autolysosome [281].

1.8.1.3. Necrosis

Necrosis is a type of cell death characterized by a gain in cell volume (oncosis), rupture of the plasma membrane, swelling of cytoplasmic organelles and subsequent loss of intracellular contents [272]. Previously, necrosis was considered to be an accidental or uncontrolled type of cell death. Recent studies have revealed that necrosis is tightly regulated by a set of signal transduction pathways and catabolic mechanisms [282]. For instance, necrotic cell death can be induced by death domain receptors such as TNFR1, and Toll-like receptors such as TLR3 in the presence of caspase inhibitors. Necroptosis is a regulated form of necrosis that is initiated by receptor-interacting protein 1 (RIP1) and RIP3 kinases. In the presence of extensive DNA damage or unrepaired DNA damage, excessive PARP activation resulted in NAD+ (nicotinamide adenine dinucleotide) depletion and further ATP consumption in order to resynthesize NAD. This, in turn, results in programmed necrosis. Furthermore, necrosis can also occur in physiologically relevant situations (e.g., ovulation, immune defense, the death of chondrocytes controlling the longitudinal growth of bones and cellular turnover in the intestine) and in pathological conditions, such as epilepsy or Alzheimer's disease.

1.8.1.4. Mitotic catastrophe

Mitotic catastrophe (also known as mitotic death) results from premature induction of mitosis before completion of the S and G2 phase. Mitotic catastrophe is characterized by unique nuclear alterations such as micronucleation (the appearance of chromosomes or chromosomal material outside of the two daughter nuclei) and multinucleation (the appearance of two or more nuclei similar or heterogeneous in size, resulting from deficient separation during cytokinesis and abnormal mitoses) [272]. Studies have also shown that mitotic catastrophe can occur through apoptosis or necrosis, which suggests that it is a process preceding other modes of cell death rather than a specific mode of cell death [283]. Chumduri et al. reported that there is a reverse relationship between apoptosis sensitivity and ploidy control [284]. Therefore, cells resistant to paclitaxel- or nocodazole-induced apoptosis undergo mitotic catastrophe and develop polyploidy. Failure to activate caspase is associated with resistance to apoptosis upon mitotic catastrophe in the cells lacking the pro-apoptotic multidomain Bcl-2 homologs Bax and Bak. This, in turn, leads to oncogenic polyploidization [285].

1.8.1.5. Senescence

In response to a variety of stress, mammalian cells undergo a persistent proliferative arrest known as cellular senescence. In contrast to apoptosis, senescent cells remain viable, metabolically active, and are able to secrete factors that may promote tumour growth and progression. Radiation induces senescence by activation of p53 and subsequent expression of p21 followed by permanent G1-arrest. It is reported that senescent tumour cells are dormant and might be reawakened by factors secreted from tumour stroma cells, months or years after radiotherapy [286]. The cells without functional p53 will not undergo a permanent cell cycle arrest and senescence but will die by apoptosis, necrosis, autophagy or mitotic catastrophe.

1.9. Mechanisms of radioresistance and strategies for radiosensitization of cancers

1.9.1. Radioresistance mechanisms of cancer

Radiotherapy is the standard curative treatment for different types of tumours. However, despite the improvement of therapeutic techniques, many patients subsequently experience recurrence due to the intrinsic resistance of cancer cells to radiation. Radioresistance remains a major clinical problem, leading to a poor prognosis in cancer patients. It is important to identify the underlying mechanisms and develop novel strategies to solve this issue. It is reported that the radioresistance mechanisms of cancers are mediated through the deregulation of several biological processes such as PI3K/AKT signalling pathways, NF-κB signalling pathway**,** DNA damage repair mechanisms, and miRNAs regulation [287].

1.9.1.1. PI3K/AKT signalling pathway

PI3K-AKT activation is one of the most common events in various tumour types and regulates many cellular processes, including proliferation, invasion, apoptosis, and radioresistance [288,289]. Akt promotes cancer progression through phosphorylation of several downstream targets. These targets include negative regulation of the Bcl-2-associated death promoter (BAD), (a pro-apoptotic protein) [290] and positive regulation of hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) (which both are pro-survival proteins) [291]. Therefore, AKT target genes prevent cell death in order to promote cancer growth and metastasis. Moreover, studies have shown that the synergism between radiation and BKM120 (a PI3K inhibitor) inhibits the activation of Akt by radiation, leading to enhanced cell apoptosis and reduced levels of DSB repair in hepatocellular carcinoma cells [292].

1.9.1.2. NF-κB signalling pathway

The NF-κB family of transcription factors is involved in the regulation of different biological responses. The NF-kB complex is activated in response to different stimuli, such as cytokines, growth factors, and stress-inducing agents like reactive oxygen species (ROS) [293]. NF-κB plays a crucial role in cancer progression by inducing cell invasion, migration, and proliferation. Furthermore, NF-κB is a stress sensitive heterodimeric transcription factor that regulates many stress-responsive factors, including radiation damage response. Once NF-κB initiates pro-survival signalling pathways post-radiation,

it not only stimulates radio resistance but also enhances the malignant potential of repopulation tumours.

1.9.1.3. Double-strand break repair

Evidence suggests that there is a correlation between DSB repair and the radioresistance of cancer cells. HR and NHEJ are two repair mechanisms which are involved in DSB repair.

1.9.1.3.1. Homologous recombination

In response to radiation-induced DSB, different damage sensors (such as ATM and BRCA1) are activated and recruit DNA repair complexes to facilitate damage recovery [294]. For example, hyperactivation of ATM induces the radioresistance of breast cancer cell subpopulations. Moreover, upregulation of phosphorylated DNA damage response proteins after radiation therapy has been reported in glioblastoma cancer stem cells (CSCs). KU-55933 (ATM kinase inhibitor) enhances the effects of radiation in glioblastoma CSCs via impairment of the DSB repair [295]. In brief, these findings suggest that the combination of DSB repair molecular inhibitors with radiation may be a novel approach to improve radiation therapy.

1.9.1.3.2. Non-homologous end joining

Hyperactivation of NHEJ increases radioresistance in several cancer types, including esophageal squamous cell carcinoma, PCa, glioblastoma, lung cancer, cervical cancer, and oral squamous cell carcinoma [296,297]. Studies have shown that a higher expression of NHEJ factors (such as DNA-PKcs, Ku70, and Ku86) leads to radioresistance of cervical cancer [298].

1.9.1.4. MicroRNAs regulation

MicroRNAs (miRNAs) are small (containing about 22 nucleotides) noncoding RNA molecules that play an important role in physiological processes such as cell proliferation, development, differentiation, and metabolism [299]. miRNAs have been shown to be involved in tumourigenesis, acting as oncogenes or tumour suppressors

depending on their cancer-related target genes [300]. Several studies have shown that some miRNAs could act as "radio sensitizers" to enhance the radiation response of cancer cells and may serve as therapeutic targets [301]. Those miRNAs are involved in some radiobiological mechanisms, such as DDR, autophagy, and survival pathway alterations. It is reported that following radiation therapy, miR-205 inhibited DNA damage repair by targeting ZEB1 and Ubc13. Loss of miR-205 was associated with poor survival of breast cancer patients [302].

Taken together, advances in our understanding of the mechanisms of radiosensitivity will help identify novel diagnostic markers and therapeutic targets to improve the efficacy of radiotherapy.

1.9.2. Sensitization of prostate cancer to radiation therapy

Radiation therapy is a common treatment modality for localized PCa. However, cancer recurrences are still frequent and stratification of patients into appropriate risk groups is essential to improve treatment outcome. Radiation therapy is used to treat cancer through initiating DNA damage in cancer cells, most notably DSBs, directly or indirectly through the accumulation of ROS. Some cancer cells are capable of utilizing DSB repair pathways to abrogate the cytotoxicity induced by radiation. This, in turn, increases cancer progression or recurrence. There are some pathways and representative molecules that have been successfully targeted to improve radiosensitivity in pre-clinical studies of PCa. These pathways include DNA repair pathways, AR/PI3K/Akt/PTEN/mTOR signalling pathways, and cell cycle checkpoints and regulators.

1.9.2.1. DNA repair pathways: Ataxia telangiectasia mutated (ATM)

DSBs are detected by the MRN complex, which eventually activates ATM. ATM then initiates a cascade of DNA damage responses, resulting in the activation of cell cycle checkpoints. The consequent arrest in cell cycle progression enables the radiated cells to perform DNA repair. Fan et al. demonstrated that downregulation of ATM could enhance the radiosensitivity of PCa cells [303]. It is reported that silibinin, a natural polyphenolic flavonoid, can inhibit radiation-induced DNA repair involving ATM and

downstream Chk1/2 [304]. Furthermore, silibinin increases the sensitivity of PC-3 and DU145 cells to radiation by reduced clonogenic formation, enhanced radiation-induced G2/M arrest, apoptosis, and ROS formation in vivo. Silibinin also inhibits radiation-induced nuclear translocation of DNA-PK, leading to a delayed resolution of gamma-H2AX. In vivo, a combination of silibinin and radiation lead to a greater inhibitory effect on DU145 cell xenograft growth compared to radiation alone. Thus, silibinin can enhance the effect of radiation by decreasing radiation-induced DSB repair.

1.9.2.2. DNA repair pathways: Poly (ADP-ribose) Polymerases (PARP)

PARP1 is the most highly expressed member of the PARP family. PARP is involved in DNA SSB repair that usually takes place during DNA synthesis [305]. Studies have shown that inhibition of PARP1 leads to conversion of unrepaired SSBs to DSBs, a process which requires HR. Therefore, cancer cells with BRCA mutations and HR deficiency are sensitive to PARP1 inhibition because the resultant DSBs are left unrepaired, leading to cytotoxicity [306]. The combination index (CI) for clonogenic survival following rucaparib (PARP inhibitor) and radiation treatments revealed synergistic interactions in a panel of PCa cell lines, which is strongest for VCaP (ETS gene fusion proteins) and LNCaP (PTEN loss) cells [307]. These results were correlated with persistent DNA breaks as determined by phospho-H2AX, p53BP1, and Rad51 foci as well as senescence indicated by b-galactosidase activation. In sum, PARP inhibitor increases the effect of radiation in PCa cells, and it is worthwhile to determine the clinical efficacy of this combined modality treatment.

1.9.2.3. Androgen receptor and DNA repair

Radiation therapy remains one of the mainstay treatments for localized PCa. However, radiation therapy increases the expression and activity of AR and renders subsequent disease recurrence. Radiation induced-AR activity correlates with increased DNA repair and cancer cell survival. Clinically, patients with higher levels of AR-regulated hK2 protein after radiation have a higher risk of disease recurrence [308]. Polkinghorn et al. demonstrated that ARN-509, an AR antagonist, downregulated transcription of DNA repair genes in a xenograft model of CRPC [309]. They also identified

that 32 DNA repair genes were direct targets of AR. Combining ARN 509 with radiation increased DNA damage and decreased clonogenic survival fraction in PCa cells. Studies have shown that ARN-509 suppresses the NHEJ pathway in cancer cells. The proteins Ku70 (69.8 kDa) and Ku80 (82.7 kDa) form a heterodimeric complex that is an essential component of the NHEJ pathway. Knockdown of XRCC6 coding for Ku70 reduces DNA-PKcs activity in PCa cells. ADT reduces the level of Ku70 and increases DNA damage signals in clinical sample [310].

Following radiation therapy, both AR ^{FL} and AR-Vs recruited to the DNA damage site in PCa cells. Genetic silencing of the AR-VS enhances the effect of radiation in cancer cells. Following androgen stimulation of androgen-deprived prostate cells, AR and topoisomerase II beta (TOP2B) can form a complex and bind to the regulatory regions of AR-targeted genes. In turn, the genomic DNA-cleaving activity of TOP2B results in a transient DNA DSBs [311]. Taken together, AR plays a critical role in determining the outcome of radiation therapy for the treatment of PCa. Hence, a combination of radiation with a more potent AR inhibitor in the treatment of PCa is of clinical significance and warrants further investigation.

1.9.2.4. DNA checkpoint kinase: Checkpoint kinase 1 (Chk1)

Following detection of DSBs by ATR kinase, activation of Chk1 inhibits CDK1/2 and CDC25 phosphatases via phosphorylation [312]. The signalling cascade allows cells to repair the DNA damage before the cell progresses through the cell cycle. Therefore, Chk1 inhibition provides an opportunity to increase the radiosensitivity of cancer cells [313].

1.9.2.5. PI3K/Akt/mTOR pathway

mTOR, the mammalian target of rapamycin, plays the main role in controlling the growth and metabolism of cells in response to mitogens. mTOR is a downstream target of Akt (protein kinase B) [314]. The PI3K/Akt/mTOR signalling pathway has a critical role in cancer metastasis and radiotherapy. This pathway is commonly dysregulated in cancer due to mutations, methylation, deletion, and post-translational modifications. Chang et al. reported that BEZ235 (dual PI3K/mTOR inhibitor) enhances the effect of radiation by

increasing apoptosis and limiting colony formation [315]. Although AEE788 (EGFR inhibitor) decreased Akt phosphorylation in PC-3 and DU145 cells, no radiosensitivity effect was detected in a colony forming assay [316]. The authors reported AEE788 increased the radiosensitivity of DU145 tumours by destroying tumour blood vessels in vivo.

1.9.2.6. Hypoxia-inducible-factor 1-a (HIF-1a)

Hypoxia is one of the main features of solid tumours and is associated with increased radiation resistance [317]. HIF-1a is a key transcriptional regulator of the hypoxic response and plays a critical role in cellular adaptions to hypoxic conditions. High expression of HIF-1a is correlated with adverse clinical outcomes and mortality [318]. Hypoxic PCa cells exhibit a survival advantage compared to cells fully oxygenated after radiation therapy. This survival advantage was associated with induction of a G2/M cell cycle arrest and reduction of both apoptosis and senescence [318].

Generally, understanding the mechanisms of radioresistance will help overcome the radioresistance of PCa and prevent metastasis. Targeting these molecules or their corresponding pathways by specific inhibitors may enhance the radiosensitivity of PCa. Combined radiosensitizer and radiation therapy is a promising therapeutic approach in PCa cell lines both in vitro and in vivo. These combined approaches should be further explored in clinical trials to validate their clinical effects in the treatment of PCa.

1.10. Combination therapy

Combination therapy is a treatment modality that combines two or more therapeutic agents, and it is a cornerstone of cancer therapy. Compared to monotherapy, the combination of anti-cancer drugs enhances treatment efficacy because it targets key pathways in a synergistic or an additive manner. Combined modality provides therapeutic anti-cancer benefits, such as reducing tumour growth and metastatic potential, arresting mitotically active cells, reducing cancer stem cell populations, and inducing apoptosis. The rationale for combination therapy is to overcome drug resistance. In personalized medicine, while cancer-causing mutations are targeted by specific drugs, the treatment

responses are short-lived due to the diverse genetics of cancer cells. In other words, a small population of cancer cells do not response to the treatment and they survive and multiply, which causes treatment resistance. Therefore, combined modalities are much more efficient in eradicating tumour cells than monotherapy.

1.10.1. Goals of combination therapy

Combined modality therapies enhance clinical benefit rates compared to standard treatment. They also minimize toxicity with the same level of efficiency by using lower doses of drugs with non-overlapping toxicity. Combination therapy aims to increase the sensitivity of cancer cells through the combination of two agents, to reduce treatment resistance and to target the tumour microenvironment.

1.10.2. Synergy/additivity and antagonism

In oncology, the goal of different combined modalities is to enhance the cell cytotoxicity through beneficial pharmacodynamics interaction. While an additive effect occurs when the final effect is equal to the sum of the effects of the two drugs, a synergistic effect refers to the interaction between two or more agents that produces a greater effect than the sum of their individual effects. On the other hand, an antagonistic effect is defined as less than the expected outcome from an additive interaction [319,320].

1.10.3. Evaluation of combination therapy

There has been some controversy about the definition of the above-mentioned terms and also whether these terms are transferable into a clinical setting. MTT or SRB assays are short-term in vitro colorimetric assays to determine the synergistic cytotoxic effect of drugs [319,321]. There has never been a commonly accepted standard definition for synergism or additivism; therefore, in 1984, Chou and Talalay introduced the scientific term “combination index” (CI) for the quantitative definition of synergism (CI<1), additive effect (CI=1), and antagonism (CI>1) [322-324]. They proposed the following formula for the calculation of the combination index [324]:

$$CI = D1/Dx1 + D2/Dx2$$

Dx1 and Dx2 are the concentrations of drugs that produce x % inhibition when used alone, and D1 and D2 are the concentrations of drugs that produce the same x % inhibition when used in combination. The clinical investigators expect to achieve a better clinical outcome with the combined modality compared to single monotherapy. Synergy against cancer cells, as determined by in vitro assay, is the only component to be considered when assessing the clinical benefit [319].

1.10.4. Combination with radiation

In 1979, Steel and Packham introduced for the first time a framework to combine cytotoxic chemotherapy and radiation. In their proposed framework, four mechanisms were described by which drugs in combination with radiation could improve the treatment outcome: spatial co-operation; toxicity independence; protection of normal cells; and enhancement of tumour response. In 2007, Bentzen et al. suggested the new frameworks for combining drugs with radiation therapy which consists of five mechanisms: spatial co-operation, cytotoxic enhancement, biological co-operation, temporal modulation, and normal tissue protection [325].

1.10.4.1. Spatial cooperation

Spatial Cooperation refers to the concept that different treatment approaches affect distinct anatomical sites of disease (i.e. radiation therapy act locoregionally while chemotherapy acts systemically) [326]. Because these modalities function independently (independent players), the desired outcome can be achieved by combining a full dose of each agent with non-overlapping toxicities (toxicity independence). These modalities are usually administered concurrently in order to avoid or reduce the cytotoxicity.

1.10.4.2. Cytotoxic enhancement

Cytotoxic enhancement means increased cell killing via inhibition of DNA repair mechanisms. The purpose of combination therapy is to enhance the cytotoxic effect on tumour cells relative to either therapy alone. For enhanced cytotoxicity, the drug should be present at the time of radiation as drugs exploiting this mechanism are directly modifying the initial stage of radiation-induced DNA damage. In this case, the drug will

enhance the effect of radiation and the main clinical endpoint is, therefore, locoregional control [325].

1.10.4.3. Biological cooperation

This term refers to the strategies that target a subpopulation of cells within the tumour itself and use different mechanisms of cell death or delayed tumour growth. Biologic cooperation implies that some portion of the actual radiation target is resistant to radiation and that this portion becomes the target of the concomitant drug. Tirapazamine, a hypoxic cell sensitizer, is the most prominent drug used in biologic cooperation. Hypoxic cancer cells are resistant to radiation therapy. Tirapazamine is most potent in anoxic conditions, so it targets the hypoxic subpopulations of cells.

1.10.4.4. Temporal modulation

The four R's of classical radiobiology (re-oxygenation, repair, redistribution, and repopulation) refers to factors that enhance the tumour response to fractionated radiation therapy. For instance, anti-proliferative treatments could inhibit accelerated repopulation between fractions that might be undetectable using single-fraction doses in vitro. Conversely, although inhibition of DNA damage repair may enhance the radiosensitivity of the tumour, if the DNA repair inhibition occurs in normal tissue, outcomes may be worse in fractionated therapy. Depending on which approach is most important in normal and tumour cells, the therapeutic index can be shifted in either a beneficial or a detrimental direction. Therefore, temporal modulation refers to treatments that optimize the four R’s between fractionated radiation therapies [327].

1.10.4.5. Normal tissue protection

Normal tissue protection refers to a drug acting on normal tissue to prevent it from the toxic effects of radiation therapy [328]. Toxicity independence is the use of drug and radiation whose antitumour effectiveness is additive but whose toxicities to normal tissue are at least somewhat independent. The clinical objective of combined drug-radiation treatment is to improve survival rates without compromising the quality of life [325]. For this purpose, the risk factors for local and distant recurrences as well as the early

and late adverse effects should be carefully considered in parallel. It seems that combined-modality therapy might be of great help to reach the ultimate objective. Future treatment strategies are more likely to offer best-fit targeted therapy to each patient's disease. These strategies include developing future regimens exploiting spatial co-operation and temporal modulation mechanisms to optimize the balance between the risks and benefits of treatments [325].

1.10.5. Measuring radiation sensitivity

Dose enhancement ratio (DER) is the term used to measure the cellular radiosensitivity via clonogenic cell survival assay [329]. DER is defined as the ratio of surviving cells with radiation alone compared to drug-radiation combinations. DER=1 suggests an additive radiation effect and DER>1, a supra-additive effect as against a sub-additive effect in the case of DER<1. Furthermore, the dose enhancement factor (DEF) is defined as the ratio of the radiation dose of nondrug-treated cells and the drug-treated ones at the surviving fraction of 0.1 or 0.01 [330].

1.11. Hypothesis and the Rationale for the Project

For treatment purposes, PCa is divided into three risk strata: low, intermediate and high-risk PCa. The first two groups are treated with radiation therapy or surgery alone, whereas the high-risk patients are mostly treated with XRT and ADT. Unfortunately, the majority of the high-risk PCa patients become resistant to ADT due to continued AR signalling. AR has also been implicated in radiation failure in PCa. AR acts as a transcriptional regulator for a variety of DNA repair proteins including DNA-PKcs, Ku70, PARP-1, and RAD51. Enzalutamide, an inhibitor of the AR signalling pathway, was approved by the FDA and Health Canada for the treatment of mCRPC. Furthermore, PCa cells are capable of extremely "devious" behavior when faced with attempts to shut down AR activity and resistance to enzalutamide has become a serious medical problem. Therefore, the emphasis needs to be on preventing the development of metastatic disease. The aim of this thesis was to evaluate the efficacy of enzalutamide and its combination with radiation in hormone-sensitive and hormone-resistant PCa cells. In this project, we

hypothesize that enzalutamide enhances the effect of radiation by the inhibition of AR signalling and the alteration of the DNA repair process.

Chapter 2

Enhanced Radiosensitization of Enzalutamide via Schedule Dependent Administration in Androgen-Sensitive Prostate Cancer Cells

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Running Title: Enhanced radiosensitization by enzalutamide

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2.1. Preface

The study presented in the second chapter focuses on the interaction of enzalutamide, an androgen receptor antagonist, with radiation in androgen-dependent and androgen-independent prostate cancer cell lines. In this chapter, we have investigated the effect of enzalutamide in combination with radiation on cancer cells' proliferation, colony survival, cell cycle arrest, DNA damage response and repair, apoptosis, and senescence. Additionally, we aimed to investigate the optimal timing of combination of enzalutamide and radiation in PCa cell lines.

2.2. Abstract

Background: Prostate cancer (PCa) is a progressive disease and the most diagnosed cancer in men. The current standard of care for high-risk localized PCa is a combination of androgen deprivation therapy (ADT) and radiation (XRT). The majority of these patients however become resistant to the combined treatment due to incomplete responses to ADT as a result of selective cells maintaining androgen receptor (AR) activity. Improvement can be made if increasing radiosensitivity is realized. Therefore, the aim of this study is to investigate the efficacy of the next-generation PCa drug, enzalutamide (ENZA), as a radiosensitizer in XRT therapy.

Methods: Using a number of androgen-dependent (LNCaP, PC3-T877A) and androgen-independent (C4-2, 22RV1, PC3, PC3-AR-V7) PCa cell lines, the effect of ENZA as a radiosensitizer was studied alone or in combination with ADT and XRT. Cell viability and cell survival were assessed, along with determination of cell cycle arrest, DNA damage response and repair, apoptosis, and senescence.

Results: Our results indicate that either ENZA alone (in AR positive, androgen-dependent PCa cells) or in combination with ADT (in AR positive, androgen-independent PCa cells) potentiates XRT response [Dose enhancement factor (DEF) of 1.75 in LNCAP cells and 1.30 in C4-2 cells] stronger than ADT+XRT conditions. Additionally, ENZA sensitized androgen-dependent PCa cells to XRT in a schedule-dependent manner; concurrent administration of ENZA with XRT led to a maximal radiosensitization when compared to either drug administration prior or after XRT. In LNCaP cells, ENZA treatment significantly prolonged the presence of XRT-induced γH2AX up to 24 hours after treatment; suggesting enhanced DNA damage in LNCaP cells. ENZA also significantly increased XRT-induced apoptosis and senescence.

Conclusions: Our data indicates that ENZA acts as a stronger radiosensitizer compared to ADT. We have also observed that its efficacy is schedule-dependent and related to increased levels of DNA damage and a delay of DNA repair processes. The initial abrogation of DNA-PKcs activity by AR inhibition and its subsequent recovery might represent an important mechanism by which PCa cells acquire resistance to combined anti-androgen and XRT treatment. This work suggests a new use of ENZA in combination with XRT that could be applicable in clinical trial settings for patients with intermediate-risk PCa.

2.3. Introduction

Prostate cancer (PCa) is the most frequently diagnosed neoplastic malignancy and the second leading cause of cancer-related deaths amongst men in the united states [1]. One of the treatment options for locally advanced PCa is radiation therapy (XRT). Unfortunately, there is a 20%-30% failure rate, prompting disease recurrence [2]. The combination of radiotherapy with hormonal therapies, new treatment options for these patients, has demonstrated improved overall survival (OS) outcomes [3-5]. However, the majority of the patients become resistant to ADT either due to the incomplete blockade of androgen receptor (AR)-ligand signalling, AR amplifications, AR mutations, aberrant AR co-regulator activities, or AR splice variants expression [6,7] with disease recurrence potentially progressing toward more advanced hormone-resistant PCa.

The AR is a member of the nuclear receptor that is activated by binding the androgen hormones and in turn regulating the transcriptional activation of specific androgen-dependent genes [8,9]. Increased AR activity has been implicated in XRT failure in PCa [10]. Following XRT, the AR becomes activated as a result of DNA damage leading to enhanced transcription of a large subset of DNA repair genes, including DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), KU70, PARP-1 and RAD51 [3,11], thereby enhancing the DNA repair capacity and hence promoting radioresistance. As such, DNA-PKcs and PARP-1 generate a positive feedback loop that enhances activity of AR and facilitates AR-dependent transcriptional transactivation [3,11,12]. AR also directly interacts with KU70 and facilitates the binding of DNA-PKcs to DNA [13,14]. Additionally, the decrease of KU70 levels after castration suggests the existence of a direct association between the inhibition of AR activity and the impaired repair of DNA double-strand breaks (DSBs) [15]. Spratt et al. reported that following XRT, there is an increase in the serum level of Hk2 proteins (AR target gene) in about 20% of the patients. They also mention that KU70, KU80, NF-κB, and the STAT family are potential candidates to increase AR transcription and expression after XRT. These data demonstrate that an upregulation of the AR expression post XRT would be the proposed mechanism by which adjuvant ADT post XRT increases survival benefits. The investigators also suggest that upregulation of AR post XRT might be prevented by a more potent AR inhibitor [16].

Enzalutamide (ENZA), formerly MDV3100, is a next-generation potent antagonist of AR activity and signaling pathway, and was approved by the FDA and Health Canada for the treatment of metastatic castrate resistant PCa (CRPC) [17]. Castrate resistant PCa (CRPC) is

defined by disease progression and the elevation of serum PSA despite ADT and represents the most aggressive type of PCa with a median survival rate of less than two years [18-20]. ENZA binds to the ligand binding domain (LBD) of the AR and then blocks AR activity by inhibiting the nuclear translocation of AR and subsequently impairing AR DNA binding, co-activator recruitment and receptor-mediated gene transactivation [21]. It also displays much higher affinity for the AR than other anti-androgens such as bicalutamide [22].

In an effort to improve PCa patient survival, we investigated the ability of ENZA to potentiate the effect of XRT in androgen-dependent (AD) (LNCaP, PC3-T877A) and androgen-independent (AI) (C4-2, 22RV1, expressing full length AR; and PC3-AR-V7, expressing AR splice variant AR-V7, PC3) PCa cells. Notably, PC3-AR-V7 cells, lacking an LBD, is expressed in approximately 3-10% of all PCa cases [23], and is insensitive to the ENZA treatment [24]. We show that ENZA enhances the effect of XRT via the inhibition of androgen-dependent signalling and the alteration of DNA repair process.

2.4. Material and methods

2.4.1. Reagents

Cell culture reagents were obtained from Gibco, Invitrogen (Burlington, Ontario, Canada). Fetal bovine serum (FBS) and Charcoal Stripped Fetal Bovine Serum (CS-FBS) were obtained from Wisent Inc., (St-Bruno, Canada). 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma-Aldrich, (Oakville, Canada). ENZA was purchased from Selleckchem Com, (Cedarlane, Paletta Court, Burlington, Ontario, Canada) and reconstituted in dimethyl sulfoxide (DMSO).

2.4.2. Cell Culture

LNCaP and PC3 cell lines were obtained from ATCC (Manassas, VA), C4-2 cells were provided by Dr. N. Zoubeidi (The prostate center, Vancouver General Hospital, University of British Columbia) [25], and 22RV1 cells were obtained from ATCC (CRL-2505™). The cells were cultured in RPMI-1640 supplemented with 10% (vol/vol) heated-inactivated fetal bovine serum (iFBS), 50U/mL of penicillin, and 50 μg/mL of streptomycin. The cell expressing AR mutant (PC3-T877A) and the AR splice variant (PC3-AR-V7) were created by the stable transfection of PC3 cells, with either AR-T877A or AR-V7 constructs. The AR-T877A variant is the same full length AR variant found in LNCaP cells. They were maintained in RPMI-1640 supplemented with 10% (vol/vol) iFBS and 50 mg/ml Geneticin (G418). The cells were incubated at 37°C in 95% air/5% CO_2 and were tested for mycoplasma contamination using the Mycoplasma PCR Detection kit (Richmond, Canada) and found to be mycoplasma free**.** For hormone deficient treatment (androgen deprivation therapy (ADT)), we have used phenol red-free media supplemented with 10% charcoal dextran-treated serum (csFBS).

2.4.3. Irradiation

Irradiation was carried out at room temperature using a Varian Clinic EX Linear Accelerator (Winnipeg, Manitoba, Canada) at a dose rate of 600 cGy/min as previously described [26]. The experimental verification of dose delivered in all experiments was conducted by the radiochromic film dosimetry protocol developed by Tomic et al. [27].

2.4.4. Transient transfection, RNA Isolation and RT-PCR

PC3 and PC3-T877A cells were transfected with 300 ng per well of hormone sensitive MMTV-CD44 reporter construct using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Twenty-four hours post transfection, PC3 (CD44 +ve) and PC3-T877A (CD44 +ve) cells were treated with or without DHT (10nM) or ENZA (10 µM). Total RNA was isolated 24 hours after treatment using Trizol according to the manufacturer's instructions (Invitrogen). The RNA concentration was determined by an ND-1000 Spectrophotometer (Nanodrop, Rockland, DE, USA) and the RNA purity was confirmed by 260/280 nm optical density value of 1.8–2.0. The isolated RNA was then converted into cDNA with Oligo dT and PrimeScript Reverse Transcriptase (TAKARA, Dalian, China) regents. The primers used in the PCR reactions are: *CD44* (5'-GAGGGATCCGCTTCCTGCCC-3'; 5'-CTCCCGGGCCACCTCCA-3'), *β-ACTIN* (5'-ATCTGGCACCACACCTTCTA-3'; 5'-CGTCATACTCCTGCTTGCTG-3'), *PSA* (5'-CCCACTGCATCAGGAACAAAAGCG-3; 5'-GGTGCTCAGGGGTGGCCAC-3'). The PCR amplified products were subjected to electrophoresis on a 1% agarose gel containing ethidium bromide.

2.4.5. Cell growth assay

Growth inhibition was measured using the MTT assay as previously described [28]. Briefly, cells were plated at the density of 3000 cells/well in 96-well plates and incubated overnight. The next day, they were treated for 24 hours with various concentrations of ENZA (0-50 µM) or the same volumes of dimethyl sulfoxide (vehicle controls). Cells were washed with a drug-free media, incubated for 72 hours at room temperature, MTT solution (Sigma-Aldrich, Oakville, Canada) was added to each well, and incubated for 4 hours at 37°C. The assay was stopped at room temperature and the optical density was measured at 560nm on a BIO-TEC micro-plate reader.

2.4.6. Clonogenic assay

Colony forming assay was performed as previously described [29]. Cells were plated at specific cell numbers in 6-well plates, treated for 24 hours with ENZA (10 µM) or ADT and irradiated (2, 4, 6, and 8 Gy). We defined ADT condition of growing cells in csFBS. After XRT, cells were washed with drug free media and incubated for 12-14 days. After this period, the

colonies were fixed with 70% EtOH, stained with methylene blue and then counted; only colonies containing more than 50 cells were considered. The plating efficiency (PE) was measured by dividing the number of colonies formed in the untreated control groups by the number of cells seeded ×100. Surviving fraction (SF) was determined by the number of colonies at the specific radiation dose divided by the number of cell plated at the same dose multiplied by PE. To plot the cell survival curve, the SFs were normalized to the plating efficiency of the not irradiated controls. Radiosensitivity was measured by the dose enhancement factor (DEF), the ratio of the radiation doses at SF of 0.1 or 0.01 of nondrug-treated cells to the drug-treated ones.

2.4.7. Cell cycle and apoptosis analysis

As per our previously published methods [30], cells were treated with ENZA (10 μM) 2 hours before XRT (4Gy), harvested 12 hours (PC3, PC3-AR-T877A, PC3-AR-V7, C4-2, 22RV1) or 24 hours (LNCaP) later and fixed with ice-cold 70% ethanol. After fixation, cells were incubated for 30 minutes at 37°C in Propidium Iodide (PI) (50 μg/ml)/ RNase (20 μg/ml) staining solution (both Sigma) in PBS and their DNA content was measured by flow cytometry on FACS Calibur (BD Biosciences, San Jose, CA). An analysis was performed by Flow Jo software (Tree Star Inc., Ashland, OR) and the number of cells in each phase of the cell cycle was determined and calculated as a percentage of the total cell population.

Analysis of the apoptosis was performed by Annexin-V–fluorescein isothiocyanate and a PI staining kit (Annexin V apoptosis detection kit (sc-4252 AK, Dallas, TX). Hormone-sensitive and hormone-insensitive PCa cells were treated with ENZA and/or XRT as above, harvested 24, 48 and 72 hours later, and stained according to the manufacturer's instructions. Cells were analyzed by flow cytometry (BD Bioscience) and characterized as follows: cells appearing at the lower right quadrant (Annexin V+/PI−) and upper right quadrant (Annexin V+/PI+) were identified as early and late apoptotic cells, respectively. The cells at the lower left quadrant were identified as viable cells (FL1-H (PI), FL2-A (A-V FITC)).

2.4.8. DNA damage analysis

DNA damage analysis was performed as previously described [31]. Briefly, cells were treated with ENZA (10 μM) and / or XRT (4Gy), harvested 1 and 24 hours post treatment and fixed with 1% formaldehyde. Cells were permeabilized with 0.2 % Triton-X/1% BSA solution

and incubated overnight at 4˚C with anti-phospho-γH2AX (S139, 1:600, Abcam, Cambridge, UK), phospho-ATM (S1981, 1:300, Abcam) or phospho-DNA-PKcs (S2056, 1:300, Abcam) antibodies. The following day, cells were washed, incubated for 1 hour at room temperature with the appropriate fluorochrome conjugated secondary antibody, re-suspended as above in PI/RNase staining solution and analyzed by BD FACS Calibur (BD Biosciences). Analysis was performed by FlowJo software and the level of γH2AX, phospho-ATM and phospho-DNA-PKcs positive cells was calculated as a percentage of the total cell population.

2.4.9. Immunofluorescence analysis

LNCaP and C4-2 cells were seeded on CC2-coated, four-chamber slides (Nunc Lab-Tek II; Thermo Scientific), approximately 25,000 cells/well in 0.5ml total volume. After treatment, cells were fixed with 1% paraformaldehyde, permeabilized in 0.2% Triton X-100, blocked in BSA 1%, then immunostained with the primary antibody (γ-H2AX; 1:400, Millipore), and followed by secondary antibody (Alexa Fluor 488 Dye; 1:500, Life Technologies). Cells were also stained with DAPI. Images were captured using confocal microscopy (Wave FX SD) with a 63X objective. Foci were counted manually for at least 100 cells per treatment condition.

2.4.10. Senescence detection

The detection and analysis of senescence was performed using a Senescence β-Galactosidase Staining (SA-β-gal) Kit, (Cell Signalling Technology, Whitby, Canada) as per manufacturer instructions [32]. Broadly, 1000 cells were seeded in 6-well plates and treated for 24 hours with ENZA and /or XRT as above. SA-β-gal staining was performed six days after XRT of subconfluent cultures and the percentage of b-galactosidase-positive cells was determined by counting through inverted microscope.

2.4.11. Statistical analysis

All the experiments were repeated three times, and statistical analysis was conducted using a student's t-test. Data are presented as a mean and SEM from multiple independent experiments. A probability level of $P \leq 0.05$ was considered significant. *, ** and *** was labeled for $P<0.05$, $P<0.01$ and $p<0.001$ respectively.

2.5. Results

2.5.1. Specific effect of ENZA on AR activity and cell viability

To assess the androgen sensitivity and inhibition by ENZA of AR expressing LNCaP cells, cells were stimulated with 10 nM DHT and treated with different doses of ENZA (2.5 µM, 5 µM, or 10 µM). The inhibition by ENZA of AR activity was then assessed by *PSA* RT-PCRs (Figure 2-1A). 10 µM ENZA was able to completely inhibit PSA expression in LNCaP cells, whereas at lower concentrations it could not. Similarly, we also wanted to confirm the AR activity of our stable AR variant expressing in the cell lines when stimulated with 10 nM DHT (Figure 2-1B). This experiment was carried out by transiently transfecting PC3 and PC3-stable cell lines with the androgen sensitive MMTV-CD44 reporting construct and performing RT-PCRs. As expected, the PC3 cells, null for AR, did not show any hormone-dependent CD44 expression. PC3-T887A cells clearly showed androgen-dependent expression of the CD44 construct, while PC3-AR-V7 showed constitutive transactivation regardless of the stimulation condition. This data verifies that the cell lines selected for this study show the appropriate androgen-dependent response, and we used these cell lines for further analysis.

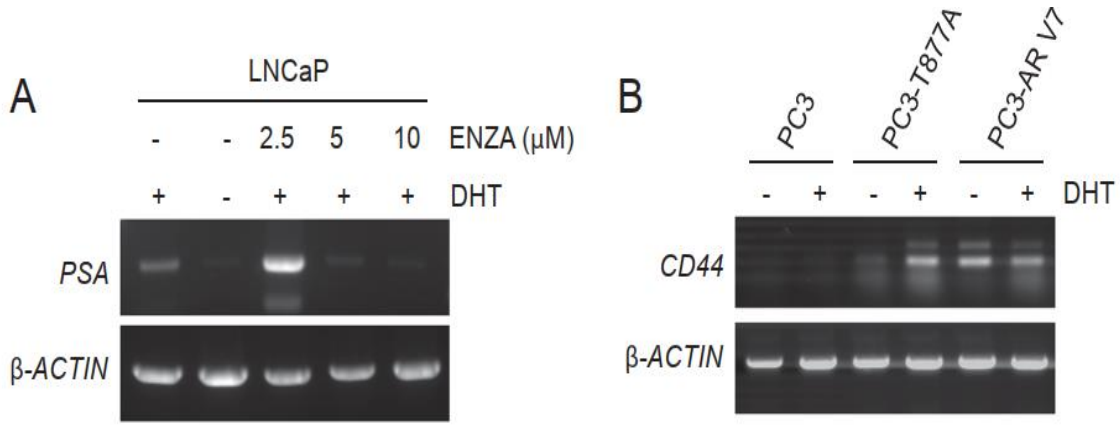


Figure 2-1. RT-PCR analysis of mRNA expression of PSA and CD44 in PCa cell lines. (A) LNCaP cells were treated with ENZA (2.5, 5, 10 µM) with or without DHT and the expression of PSA mRNA was detected by RT-PCR. (B) PC3, PC3-AR-V7 and PC3-T877A cells were treated with or without DHT and the change in mRNA expression of CD44 was identified by RT-PCR. Ethidium bromide-stained agarose gels are shown.

We next determined the effect of ENZA on the cell survival clonogenic and MTT assays in our panel of AD and AI PCa cell lines. The clonogenic assay showed that ENZA reduced PCa cell survival in a dose-dependent manner in the AD cell lines, LNCaP and PC3-T877A (Figure 2-2A). The MTT assay revealed that AI PCa cells (C4-2, 22RV1, PC3 and PC3-AR-V7) demonstrated less sensitivity for ENZA than AD cell lines (Figure 2-2B). At higher concentrations of ENZA, off-target effects were observed for AI PCa cell lines; a similar effect was also seen with high concentrations of bicalutamide (80 μM) in AR-null PC3 and DU145 cell lines [33]. From these results, we selected to use 10 μM ENZA concentrations for all subsequent experiments.

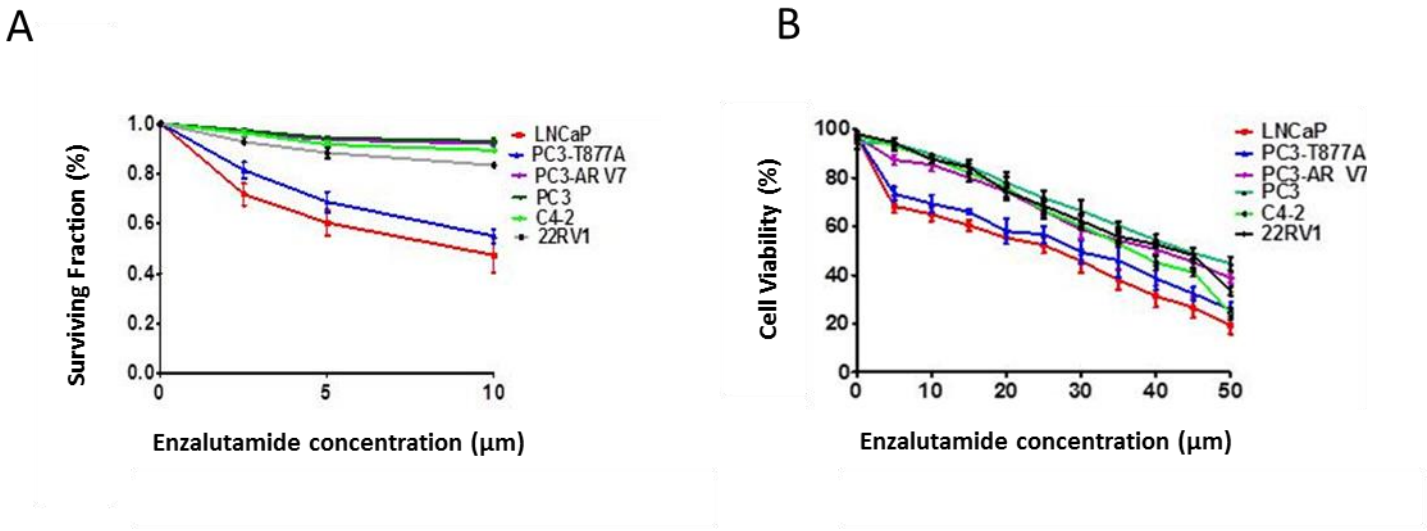
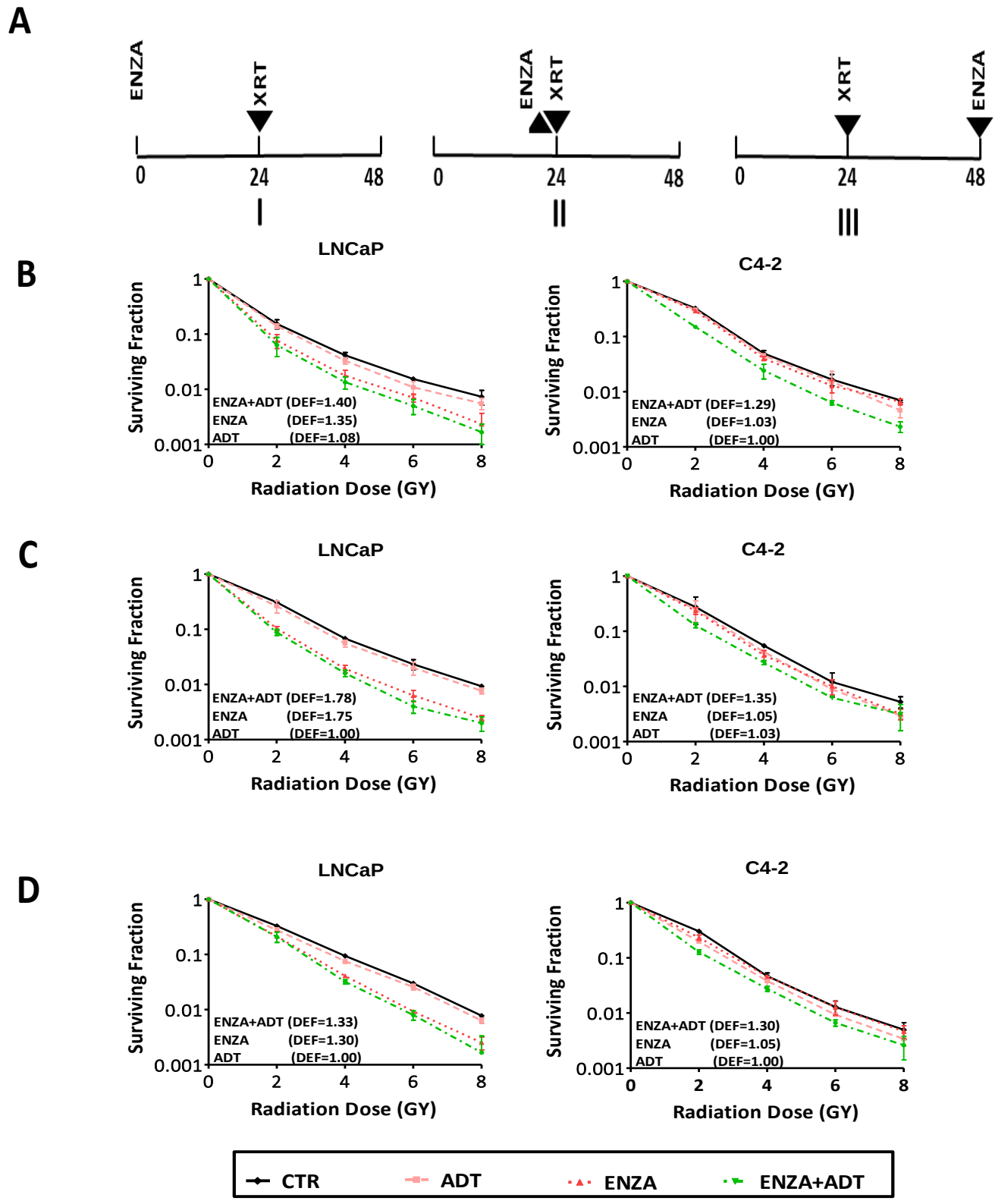


Figure 2-2. Clonogenic and MTT assays to determine the effective dose of ENZA in PCa cell lines. (A) Clonogenic assays were performed with different concentrations of ENZA (0, 5, and 10 μM) and (B) MTT assay to analyze the growth and survival of hormone–sensitive (LNCaP, PC3-T877A) and hormone-insensitive (22RV1, C4-2, PC3, PC3-AR-V7) PCa cell lines.

2.5.2. Enzalutamide increases the sensitivity of hormone-sensitive prostate cancer cell lines to radiation.

To determine the radiosensitivity effects of ENZA with or without ADT in the PCa cells, the clonogenic assay was performed. We also evaluated alternative scheduling regimens in AD and AI PCa cells. The following scheduling regimes were defined and evaluated; I- ENZA added 24 hours before XRT, II- XRT followed by 24 hours later ENZA, or III- ENZA added 2 hours before XRT (Figure 2-3A). AD and AI PCa cells were treated with 10 μM ENZA for each scheduling event, irradiated, and then colony formation was evaluated 10-12 days later. As shown in Figure 2-3B, the combination of ENZA and XRT yielded independent enhanced cell killing with a dose enhancement factor (DEF) values at SF = 0.1 of 1.35 ± 0.02 for LNCaP and 1.29 ± 0.05 for the isogenic AI C4-2 cells. We also observed that PC3-T887A transgenic cells (DEF = 1.30±02) behave as LNCaP cells and the AI 22RV1 (1.25±0.05) is comparable to the C4-2 cell; this suggests that enzalutamide radiosensitization is a result of direct action of a functional AR protein. No radiosensitization was observed when the PC3 or PC3-AR-V7 cells were pre-treated with ENZA (in all cases DEF=1.00, at SF=0.1) (Table 2-1, Supplementary Figure 2-1& 2-2). In cells treated with ENZA 2 hours prior to XRT, a supra-additive dose enhancement with DEF= 1.75±0.08 for LNCaP was documented (Figure 2-3C). Subsequently, as ENZA treatment 2 hours prior to XRT was more effective than other scheduling regimes, it was selected for all further experiments. Furthermore, the addition of ADT culture milieu to ENZA 2 hours before XRT enhanced the effect of XRT in C4-2 cells (DEF=1.35±0.01) while there was no radiosensitivity effect of ENZA or ADT alone in these cell lines (in all cases, DEF=1.00). The scheduling phenomena that we identified with ENZA treatment was not observed with ADT scheduling; the lack of ADT radiosensitization and scheduling has been observed by other investigators [34]. Moreover, we found that the combination of ENZA with ADT (DEF =1.78±0.01) in our scheduling conditions did not appear to further enhance the effects of ENZA alone in AD PCa cells. These results indicate that ENZA alone can significantly enhance XRT response regardless of deprivation of androgens and can be potentiated by the defined scheduling protocol we employed.


A
ENZA
XRT
0
24
48
I
ENZA
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II
XRT
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0
24
48
III
B
LNCaP
Surviving Fraction
1
0.1
0.01
0.001
ENZA+ADT (DEF=1.40)
ENZA (DEF=1.35)
ADT (DEF=1.08)
0
2
4
6
8
Radiation Dose (GY)
C4-2
Surviving Fraction
ENZA+ADT (DEF=1.29)
ENZA (DEF=1.03)
ADT (DEF=1.00)
Radiation Dose (GY)
C
LNCaP
Surviving Fraction
ENZA+ADT (DEF=1.78)
ENZA (DEF=1.75
ADT (DEF=1.00)
Radiation Dose (GY)
C4-2
Surviving Fraction
ENZA+ADT (DEF=1.35)
ENZA (DEF=1.05)
ADT (DEF=1.03)
Radiation Dose (GY)
D
LNCaP
Surviving Fraction
ENZA+ADT (DEF=1.33)
ENZA (DEF=1.30)
ADT (DEF=1.00)
Radiation Dose (GY)
C4-2
Surviving Fraction
ENZA+ADT (DEF=1.30)
ENZA (DEF=1.05)
ADT (DEF=1.00)
Radiation Dose (GY)
CTR
ADT
ENZA
ENZA+ADT

Figure 2-3. The effect of ENZA and /or ADT in combination with XRT on clonogenic survival of PCa cells. (A) Scheduling protocol of ENZA+XRT treatment, I – Rx 24 hours before XRT; II – Rx 2 hours before XRT; III – Rx 24 hours post XRT. For ADT treatment, csFBS media was substituted prior to initiation of scheduling protocol. (B) LNCaP and C4-2 cells have been treated with ENZA (10 μM) or ADT, with scheduling protocol I. (C) Scheduling protocol II. (D) Scheduling protocol III. RX=Drug; XRT=Radiation.

		Scheduling Protocol [a]								
		I			II			III		
		ENZA	ADT	ENZA+ADT	ENZA	ADT	ENZA+ADT	ENZA	ADT	ENZA+ADT
AD	LNCaP	**1.35±0.02 ***	1.08±0.01 NS	**1.40±0.03 ****	**1.75±0.08 *****	1.00 NS	**1.78±0.01 ****	**1.30±0.05 ***	1.00	**1.33±0.04 ***
	PC3-T877A	**1.30±0.03 ****	1.00 NS	**1.28±0.02 ***	**1.65±0.01 ***	1.03 NS	**1.59±0.03 ***	**1.35±0.06 ***	1.02	**1.32±0.05 ***
AI	C4-2	1.03 NS	1.00 NS	**1.29±0.05 ***	1.05 NS	1.03 NS	**1.35±0.03 ****	1.05 NS	1.00	**1.30±0.03 ***
	22RV1	1.00 NS	1.03 NS	**1.25±0.05 ***	1.00 NS	1.00 NS	**1.30±0.03 ****	1.00 NS	1.00	**1.27±0.04 ***
CTR	PC3	1.00 NS	N/A	N/A	1.00 NS	N/A	N/A	1.00 NS	N/A	N/A
	PC3-AR V7	1.00 NS	N/A	N/A	1.00 NS	N/A	N/A	1.00 NS	N/A	N/A

Table 2-1. Calculation of dose enhancement factors for different scheduling protocols. Scheduling Protocol**:** I – Rx 24 hours before XRT; II – Rx 2 hours before XRT; III – Rx 24 hours post XRT. N/A=not applicable, experiment not need to be performed; AD=Androgen dependent; AI=Androgen independent; CTR= Control; * $p<0.05$; **$p<0.001$; ***<0.001; NS-not significant; RX=Drug; XRT=Radiation.

2.5.3. Inhibition of AR by ENZA and combination of ENZA with XRT does not influence PCa cell cycle distribution, but apoptosis and cell senescence are affected

Multiple pathways are involved in maintaining the genetic integrity of a cell after its exposure to ionizing radiation and cell cycle regulation is perhaps one of the most important determinants of cell radiosensitivity [35]. Therefore, cell cycle analysis was performed in order to determine the influence of ENZA, XRT and their combination on the cell cycle distribution. As shown in Figure 2-4A and Supplementary Figure 2-3, ENZA alone does not alter cell cycle distribution of either AD or AI PCa cells. However, XRT alone is able to significantly induce a G2/M cell cycle arrest in these cells. The G2/M distribution from the combination of ENZA and XRT was almost identical to that of XRT alone. The same results were obtained for C4-2 and 22RV1 cells following treatment with XRT with or without ENZA+ADT treatment. Therefore, we can conclude that the inhibition of AR by ENZA and its combination with XRT has no influence on cell cycle distribution of PCa cells.

Guerrero et al. reported that ENZA induces apoptotic cell death and tumour shrinkage in mouse xenograft models [36]. Therefore, to determine the contribution of early apoptotic event to ENZA -mediated radiosensitization, annexin V staining was performed 48 hours after XRT in AD and AI PCa cells (4Gy). As shown in Figure 2-4B and Supplementary Figure 2-4A, the combination of ENZA with XRT induces 55% apoptosis 48 hours post-treatment compared to 5% apoptosis in the control cells, 18% apoptosis in ENZA-treated cells, and 37% apoptosis in XRT-treated cells. Similarly, a significant increase in an early apoptotic event of ENZA+XRT treatment was observed in PC3-T877A; suggesting that the radiosensitizing effect of ENZA may involve augmentation of XRT-induced apoptosis. In C4-2 cells, the percentage of apoptotic cells was 18% in the ENZA+ADT+XRT group, 8% in the ENZA group, and 3.4 % in the XRT group. We did not observe enhancement of apoptosis in the AR-null PC3 cell line or in the constitutively active PC3-AR-V7 cells treated either with ENZA alone or in combination with XRT.

Overall, our analysis has demonstrated that a significant fraction of ENZA+XRT-treated cells undergoes apoptosis through the contribution of ENZA action on AR responsive cells. As PC3 cell death is not apoptosis dependent, we also explored whether combined ENZA+XRT treatment may trigger senescence in this cell line. To this end, senescence-associated beta-galactosidase (SA-β-gal) staining was performed to detect senescent cells in irradiated AD

(LNCaP and PC3-T877A) and AI (PC3 and PC3-AR-V7) PCa cells with or without ENZA treatment. The results show that ENZA+XRT combined treatment induces more SA-β-gal positive senescent cells than either ENZA or XRT treatment alone in AD PCa cells, while AI PCa cells do not significantly induce SA-B-gal expression under ENZA+XRT treatment (Figure 2-4C). These results suggest that ENZA may radiosensitize PCa cancer cells by enhancing XRT-induced senescence (Supplementary Figure 2-4B).

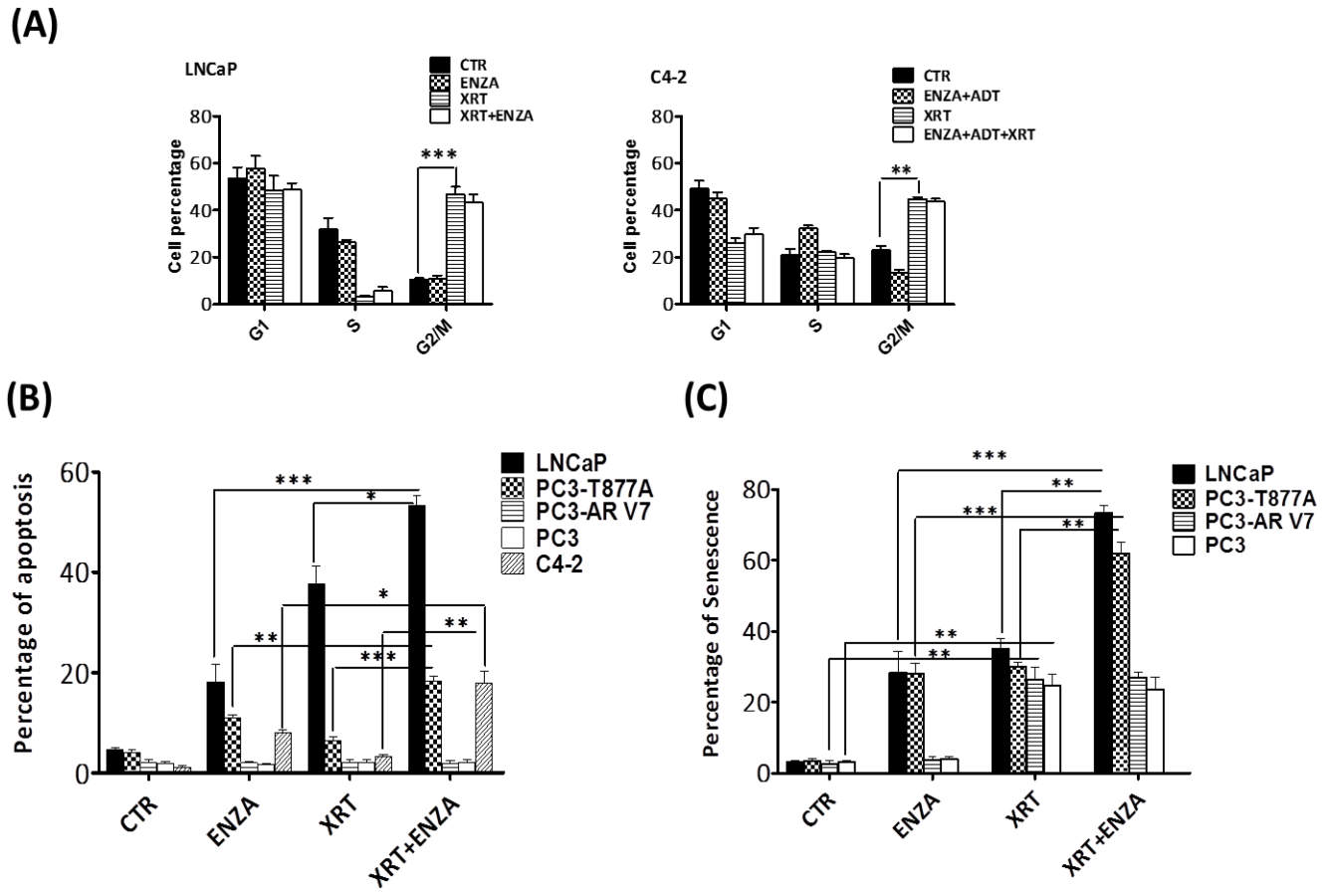


Figure 2-4. Effect of ENZA and/or XRT on cell cycle distribution, apoptosis and senescence in PCa cell lines. (A) Cells were treated with ENZA (10 μM) alone and in combination with XRT (4 Gy) for 12 hours (LNCaP) and 24 hours (C4-2), stained with propidium iodide (PI) and then analyzed by flow cytometry to determine the distribution of cells in each phase of the cell cycle. The graphs show the percentage of the cells following ENZA and/or XRT treatment. (B) The cells have been treated with ENZA (10 μM) and/or XRT (4Gy), harvested 48 hours after treatment, stained with Annexin V/PI and analysed by flow cytometric analysis. The bar graph shows the percentage of all apoptotic events following ENZA and/or XRT treatment. (C) Senescence-associated β-galactosidase (SA-β-gal) staining of hormone-sensitive and hormone-insensitive PCa cells after ENZA and/or XRT treatment for 24 hours. Data represent as mean and SEM from three independent experiments.

2.5.4. ENZA combined with XRT increases the level of DNA double-strand breaks

DNA damage through DNA double-strand breaks (DSBs) is the major mechanism by which XRT induces cell lethality. We therefore examined whether the increased sensitivity to XRT conferred by ENZA might be caused by enhanced DSBs induction and/or inhibition of their repair. As a measure of DNA damage, we evaluated the level of phosphorylated histone H2AX (γH2AX), established as a sensitive indicator of DSBs and a response to DNA repair. LNCaP cells were exposed to ENZA in combination with XRT treatment and the level of γH2AX was determined 1 hour and 24 hours later by flow cytometry. As shown in Figure 2-5A1, exposure of cells to ENZA alone did not increase γH2AX levels. Irradiation of cells with 4Gy significantly increased the level of γH2AX (as detected at 1 hour post treatment), progressively decreasing 24 hours post treatment. Exposure of PCa cells to ENZA 2 hours prior to XRT however resulted in a greater level of γH2AX than either of the individual treatments alone at 1 hour and 24 hours post treatment. Increased synergistic effects were noted in the combined treated group (48±4.3 for 1 hour and 25±2.2 for 24 hours) in comparison to γH2AX levels in cells treated with either ENZA or XRT alone (5 ±0.6 for 1 hour and 6 ±0.7 for 24 hours; 27 ±2.5 for 1 hour and 10 ±2.1 for 24 hours, respectively). Furthermore, immunofluorescent analysis for γH2AX foci revealed that LNCaP and C4-2 cells exposed to ENZA and XRT show more γH2AX foci (42±1.5 in LNCaP, 40±1.8 in C4-2) compared to ENZA (5±0.9 in LNCaP , 3±0.5 in C4-2) or XRT (22±1.2 in LNCaP and 20±1.5 in C4-2) (Figure 2-5A2 and Supplementary Figure 2-6). Since ENZA with ADT potentiates the radiation effect in C4-2 and 22RV1 cells, the effect of this combination therapy was also assessed on the activity of γH2AX and p-ATM while the results was the similar to the AD PCa cells. In addition, we have observed almost identical responses to combined ENZA+XRT treatment in PC3-T877A hormone-sensitive PCa cells as well as no response in hormone-insensitive PC3 and PC3-AR-V7 cells (Supplementary Figure 2-5B). Analysis of phosphorylation of ATM at serine 1981, a known marker of DNA damage response (DDR) activity, also revealed increased activity of ATM in response to ENZA+XRT treatment both at 1 and 24 hours in AD (LNCaP and PC3-T877A) and AI (C4-2 and 22RV1) PCa cells (Figure 2-5 B and Supplementary Figure 2-5B).

Following radiation therapy and induction of DNA damage, DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) is phosphorylated at multiple sites including auto-phosphorylation at Ser2056. This is a prerequisite for the activation of non-homologous end

joining (NHEJ) pathway, the main pathway for repair of DNA DSBs. We therefore analyzed the activity of NHEJ pathway by measuring the level of DNA-PKcs phosphorylation. We observed significantly decreased activity of the DNA-PKcs 1 hour post-treatment in AD LNCaP and PC3-T877A PCa cells treated with ENZA and XRT when compared to the cells treated with XRT or ENZA alone. Interestingly, 24 hours later, the activity of DNA-PKcs in these cell lines had not only been restored but also augmented when compared to the cells treated with ENZA or XRT alone (Figure 2-5C and Supplementary Figure 2-5C). Additionally, ENZA+ADT+XRT led to a similar phenomenon of DNA-PKcs response in AI C4-2 and 22RV1 cells. In conclusion, our results suggest that ENZA inhibits XRT-induced DNA DSB repair.

Our data indicates ENZA acts as a stronger radiosensitizer when added to the cells 2 hours before XRT rather than 24 hours before or after XRT. We next evaluated whether the observed differences could be attributed to the differences in the level of DNA damage or differences in the radiosensitization's mode of action. To this aim, the activity of γH2AX, p-ATM and p-DNA-PKcs were measured as previously in cells treated with ENZA 24 hours before irradiation and we did not observe any difference in the level of either γH2AX or activity of p-ATM and p-DNA-PKcs between cells treated with XRT or its combination with ENZA. Similarly, activity of all proteins declined after 24 hours with none showing significant differences from untreated cells.

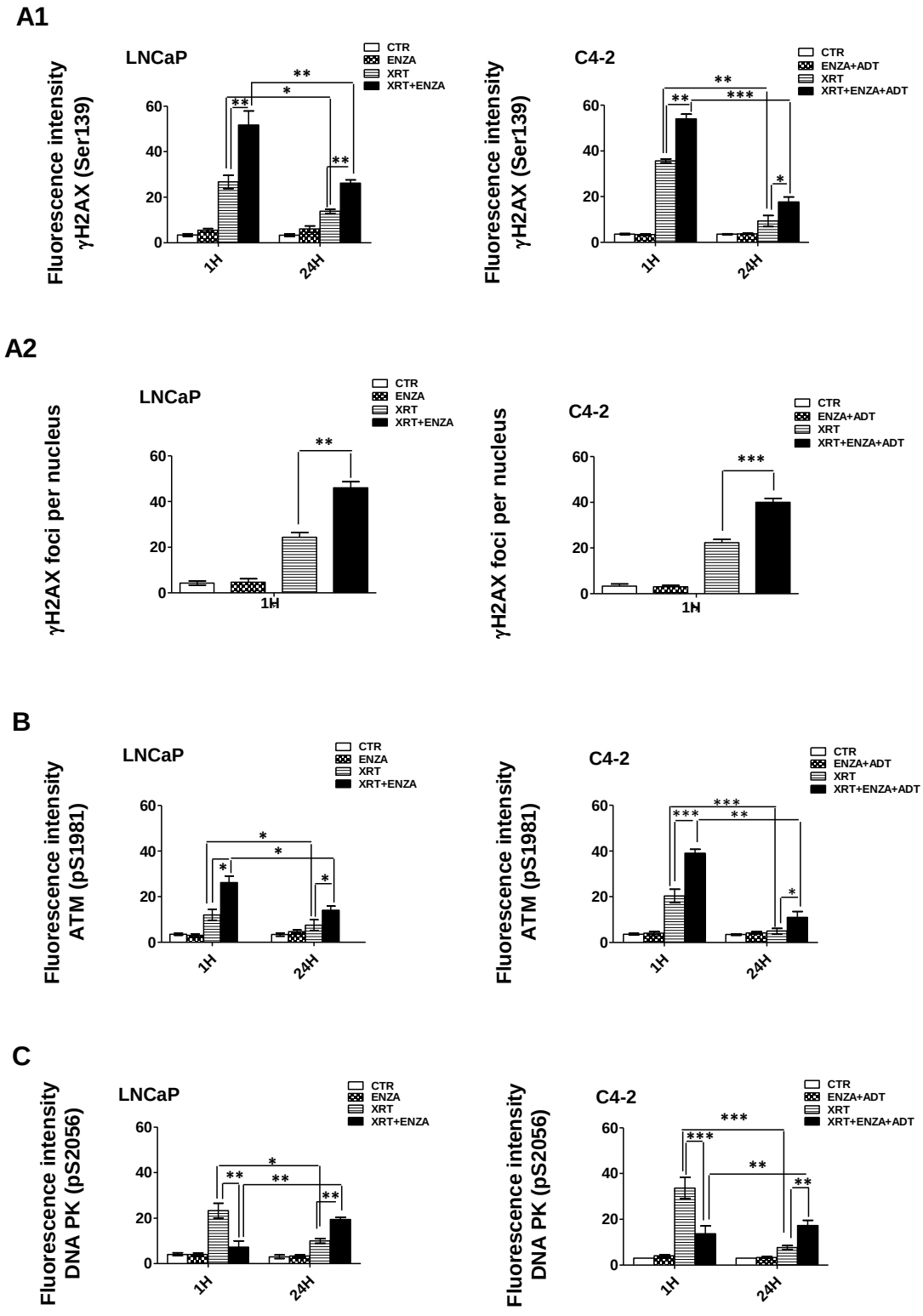

A1
LNCaP
CTR
ENZA
XRT
XRT+ENZA
Fluorescence intensity γH2AX (Ser139)
C4-2
CTR
ENZA+ADT
XRT
XRT+ENZA+ADT
Fluorescence intensity γH2AX (Ser139)
1H
24H
A2
LNCaP
γH2AX foci per nucleus
C4-2
γH2AX foci per nucleus
B
LNCaP
Fluorescence intensity ATM (pS1981)
C4-2
Fluorescence intensity ATM (pS1981)
C
LNCaP
Fluorescence intensity DNA PK (pS2056)
C4-2
Fluorescence intensity DNA PK (pS2056)

Figure 2-5. DNA double-strand break repair analysis in PCa cell lines. LNCaP and C4-2 PCa cells were treated with ENZA and /or ADT 2 hours before XRT and the cells were harvested 1 hour and 24 hours post treatment and analyzed by flow cytometry to determine the level of γH2AX (Ser 139), ATM (Ser1981) and DNA-PKcs (Ser 2056). The graphs show the percentage of (A) γH2AX as analyzed by flow cytometry; (B) Represents the number of γH2AX foci per nucleus, as assessed by immunofluorescence; and (C) p-ATM, and (D) DNA-PKcs positive cells which was determined by flow cytometry analysis.

2.6. Discussion

Following radiation therapy, there is an increase in AR activity due to DNA damage of cells. This AR activity augments the expression and activity of many repair proteins, which thereby enhances the radioresistance of human PCa cells [3,11,12,37]. In this study, we demonstrate for the first time that ENZA acts as a stronger radiosensitizer than ADT in hormone-sensitive human PCa cells. The mechanism of this radiosensitivity may entail two processes which are critical for tumour growth and radioresistance. ENZA inhibits the activity of proteins like γH2AX, p-ATM and p-DNA-PKcs, which are involved in DNA damage response and repair processing. ENZA enhances XRT–induced apoptosis and senescence through the inhibition of DNA repair. Together, these results suggest that ENZA may be used as a potent radiosensitizer in hormone-sensitive PCa.

Pre-treatment of LNCaP cells with ENZA 2 hours before XRT strongly radiosensitizes them with a dose enhancement factor (DEF) of =1.75±0.08 at a surviving fraction of 0.10 (SF=0.1) (Figure 2-3C). This robust effect was not visible in LNCaP cells pre-treated with ADT (DEF=1.00) nor in PC3 cells pre-treated with ENZA or ADT (in all cases DEF=1.00 at SF=0.1). Our results thus suggest that a more effective and profound blockade of AR activity and signalling with ENZA significantly potentiates the effect of radiation treatment, when compared to ADT in hormone-sensitive PCa cells. Previous reports have shown ADT modestly potentiates radiation response in AR-positive cells, and that ADT has no effect in AR-negative PCa cells (PC3) [12]. Although ENZA alone did not enhance (DEF=1.05±0.006) the radiation response in AI C4-2 and 22RV1 cells, the addition of ADT condition with ENZA enhanced their radiation response.

More importantly, we observed the extent of radiosensitization by ENZA is greatly dependent on the administration schedule. In the first administration schedule (ENZA added 2 hours before XRT), we noted a significantly higher DEF value when compared to the second and third one (ENZA added 24 hours before XRT or 24 hours after XRT). This suggests that ENZA´s efficacy and molecular mechanism of radiosensitization relates to the drug administration schedule. Because of ENZA's long half-life (7 days) and its long term efficacy [17], our drug scheduling phenomena (2 hours vs. 24 hours) could not be explained by significant loss of the drug's activity. Additionally, initial inhibition of phosphorylation of DNA-PKcs in "ENZA added 2 hours before XRT group" and the lack of this inhibition in "ENZA added 24

hours before XRT group" suggests two different radiosensitization mechanisms. It has been reported that ENZA administration 24 hours before XRT potentiates the effect of XRT through downregulation of platelet-derived growth factor receptor (PDGFR) pathway [38]. Furthermore, our data shows that administration of ENZA 2 hours before XRT is the optimal treatment schedule of all three schedules tested. On the other hand, we did note that Quero et al. evaluated the use of bicalutamide (Casodex) as a radiosensitizer, actually elicited an antagonistic, and less pronounced interaction upon radiation administration in LNCaP cells [33]. Specifically, with respect to neoadjuvant and XRT scheduling, the investigators observed that bicalutamide treatment 48 hours prior to radiation had the least radiomodulating effect in AD cell line.

It has been shown that following radiation therapy, genomic stability is preserved through several stress pathways like cell cycle arrest, DNA repair, apoptosis and cellular senescence which are activated by TP53 [39]. Therefore, it is critical to evaluate the TP53-dependent cell fate decisions within a common cell population following ENZA and/or XRT treatment [40]. In this study, two modes of cell deaths (apoptosis and senescence) were assessed in AR functional LNCaP cells and AR null PC3 cells; ENZA+XRT induced 55% apoptosis in LNCaP cells while there was no apoptosis in PC3 cells. Our data also demonstrated enhanced apoptosis by ENZA in AR-positive PC3-T877A and C4-2 cells. Moreover, efficient processing and repair of DNA damage are major contributors to the suppression of apoptosis as well as clonogenic cell death, following genomic insult by radiation or other DNA damage-inducing agents. We expect that apoptosis and clonogenic deaths can be induced by any agent that affects DNA repair processes. The combination of ENZA and radiation enhances the level of apoptosis in hormone-sensitive PCa cells while there is no effect in hormone-insensitive PCa cells; suggesting that the sensitizing effect of ENZA may involve augmentation of XRT-induced apoptosis in AD PCa cells.

Following DNA damage, senescence inhibits tumour growth through irreversible growth arrest and by restricting the length of time in which cells can replicate [41]. We also observed that ENZA enhanced XRT-induced senescence only in hormone-sensitive PCa cells whereas radiation induces senescence in both hormone-sensitive and-insensitive PCa cells. Senescence can be activated through the absence of PTEN and the presence of ETS gene function in hormone-sensitive PCa cells [42].

It has also been reported that AR enhances DNA repair resolution through regulating the expression and activity of DDR proteins independent of effects on cell cycling [12]. Consistent with this, our cell cycle analysis confirmed the radiosensitizing effect of ENZA was independent of effects on cell cycling, as more hormone-sensitive and hormone-insensitive PCa cells were blocked in the G2/M phase 12 hours (LNCaP) and 24 hours (22RV1, C4-2, PC3, PC3-T877A, PC3-AR-V7) post XRT treatment. Consequently, the percentage of cells in G1 phase was decreased significantly. The percentage of the cells in the G2/M phase was the same for ENZA+XRT group and the XRT alone group. Cancer cells are most radiosensitive in the G2/M phases and less sensitive in the S phase of the cell cycle [29].

The most lethal form of DNA damage generated by XRT on DNA are DSBs, with subsequent unrepaired DSB inducing cell death [43]. ADT in combination with XRT also increases γH2AX activity more than XRT-treated group (up to 24 hours) due to the inability to repair DSB caused by XRT [12]. It is possible that ENZA, through blocked AR signalling, interferes with the repair of XRT-induced DNA DSBs. Consistence with this data, we found that administration of ENZA 2 hours before XRT induced γH2AX activity two-fold greater than monotherapy in hormone-sensitive PCa cells and this effect was remained constant for 24 hours (Figure 2-5A). Additionally, p-ATM activity was increased following combined treatment compared to ENZA or XRT. Importantly, we have observed no differences between cells treated with XRT and ENZA+XRT when ENZA was added 24 hours before XRT. These results indicate that ENZA (2 hours before XRT) can enhance the effect of radiation through inhibition of DNA repair processes.

DNA-PKcs plays a key role in DNA DSB repair pathways and is the main target of AR in response to XRT [3,11,12]. Previous studies have indicated that DNA damage causes AR activity, increasing the expression and activation of DNA repair genes, and resulting in therapeutic resistance [12]. Consistent with this, we found for the first time that in combined treatment group (ENZA plus XRT), the level of DNA-PKcs activity was significantly less than the XRT treated group 1 hour after treatment. However, 24 hours post treatment, the activity of DNA-PKcs in the combined treatment group increased, compared to XRT-treated cells (Figure 2-5C). This suggests that initial abrogation of DNA-PKcs activity by AR inhibition and its subsequent recovery might represent an important mechanism by which PCa cells acquire resistance to the ENZA+XRT treatment. DNA-PKcs have been shown to be one of the main factors mediating the

progression and metastasis of PCa [34]. It has also been demonstrated that there is a cross regulation between AR and AKT pathway [44]. Additionally, enhanced activity of AKT due to AR inhibition induces tumour cell survival through the accumulation of DNA-PKcs on the DSB site [45]. Therefore, a possible molecular mechanism for ENZA resistance 24 hours after treatment is enhanced activity of DNA-PKcs in the AKT pathway. Taken together, our findings indicate that ENZA and XRT impair the DNA damage repair process through enhanced activity of γH2AX and P-ATM, and decreased activity of DNA-PKcs.

Attempts to clinically evaluate the anti-androgen bicalutamide as a potential radiosensitizer in radiation therapy for localized PCa [4,46,47] indicate that only the inclusion of bicalutamide to existing the ADT regime with radiotherapy can significantly improve OS in these men, vs. men solely on ADT prior to XRT. Therefore, the work by Quero et al. suggests that the beneficial clinical outcomes of bicalutamide and radiation on increased patient survival may be a result of the combination of an ADT environment, rather than the independent action we see with ENZA [33]. However, one of the outstanding issues related to the prolonged prescription of both ADT and the anti-androgen is the development of treatment resistance and the androgen independence, as a direct result of genetic selection of subversive or promiscuous AR somatic mutations such as the AR-W741L bicalutamide resistant mutations or AR splice variants [48-52]. Similarly, an ENZA resistant AR-F876L mutation was found almost immediately after the approval of the drug for the treatment of localized CRPC [53]. Currently, there are several ongoing clinical trials to assess the efficacy of ENZA with XRT (NCT02203695, NCT02064582, NCT02023463, NCT02028988, and NCT0244644).

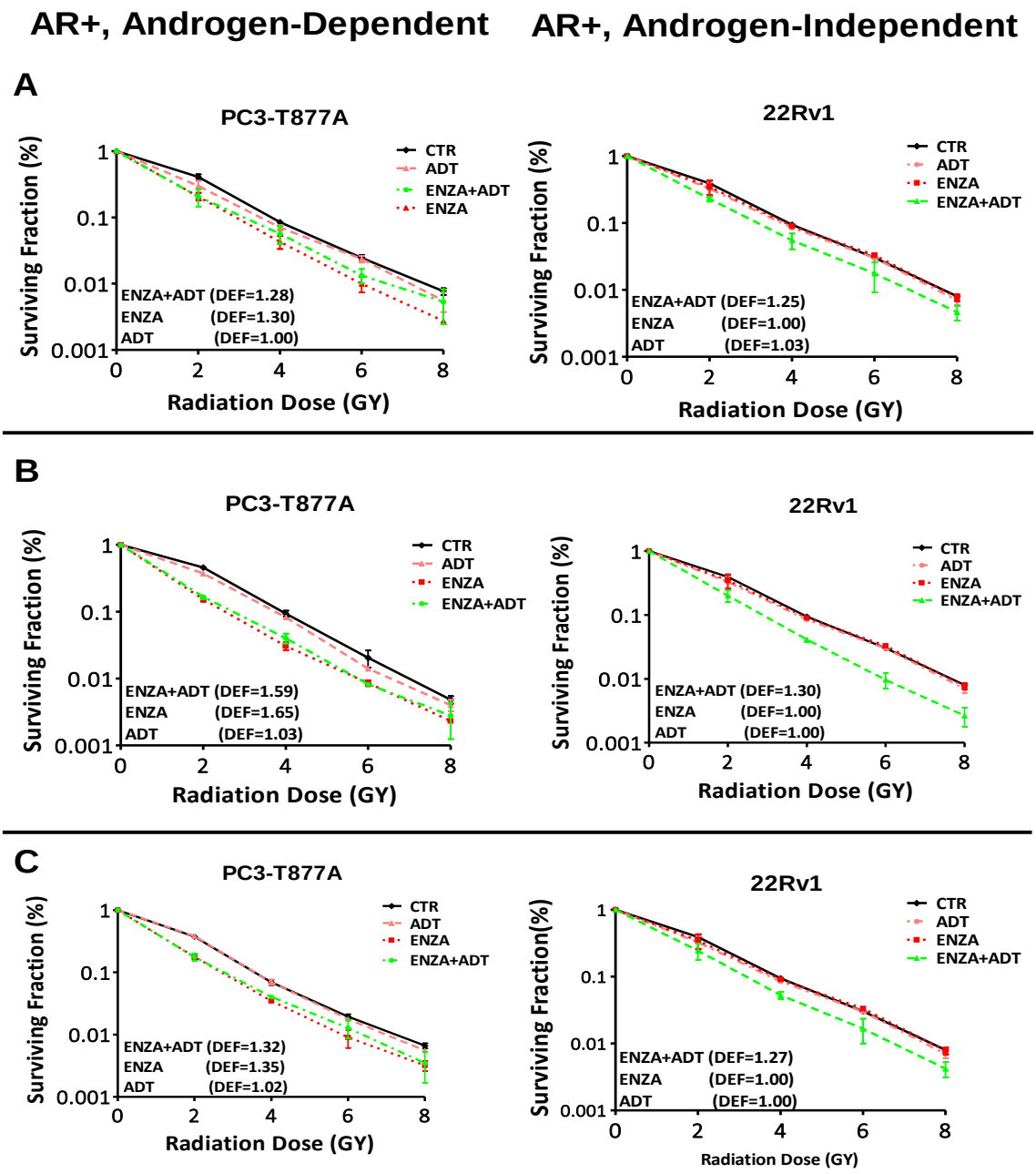
Our present study demonstrates that ENZA acts as a stronger radiosensitizer than ADT. This suggests that the radiosensitivity may be due to an inhibition of the induction of DNA repair because of the blocked AR function, leading to enhanced XRT-induced apoptosis and senescence [54]. Thus, the combination of ENZA and XRT may be a new treatment option, and support ongoing clinical trials for PCa at an early- stages of disease, rather than the current limited use of ENZA in advanced metastatic disease.

2.7. Acknowledgments

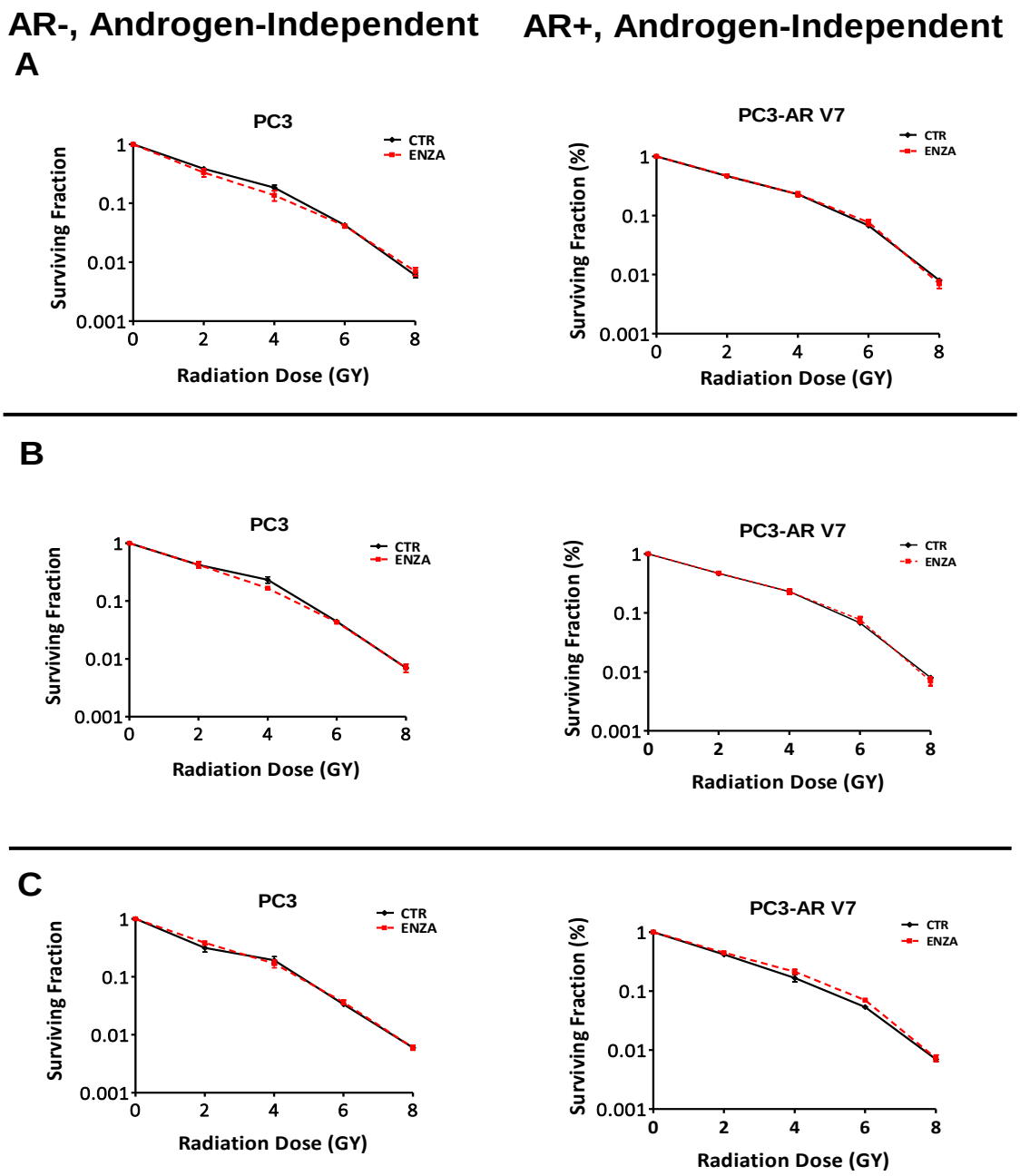
The authors would like to recognize Seung S. Lee's contribution in the revision of this manuscript.

2.8. Supplementary Data

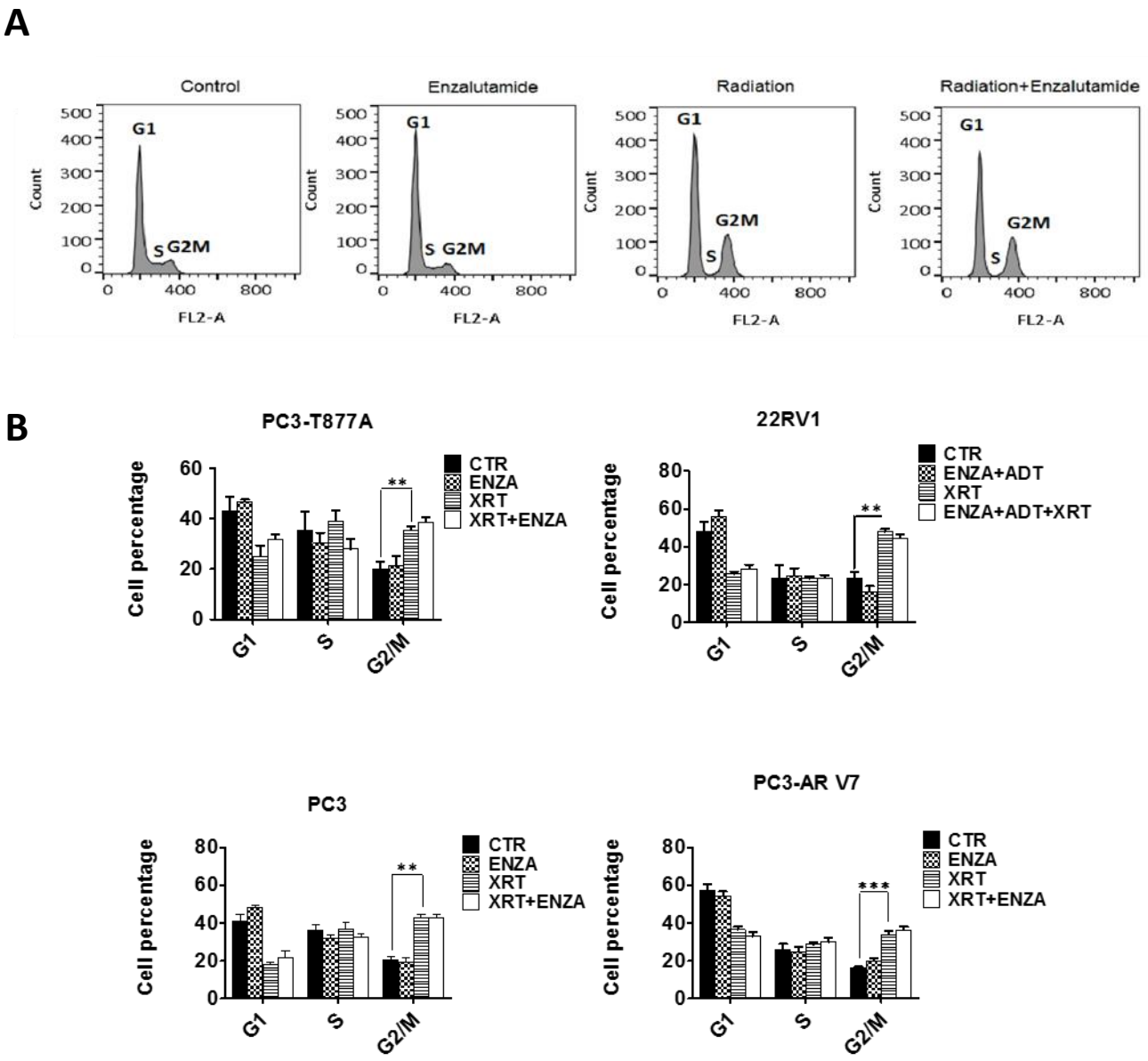
2.8.1. Supplementary Figures



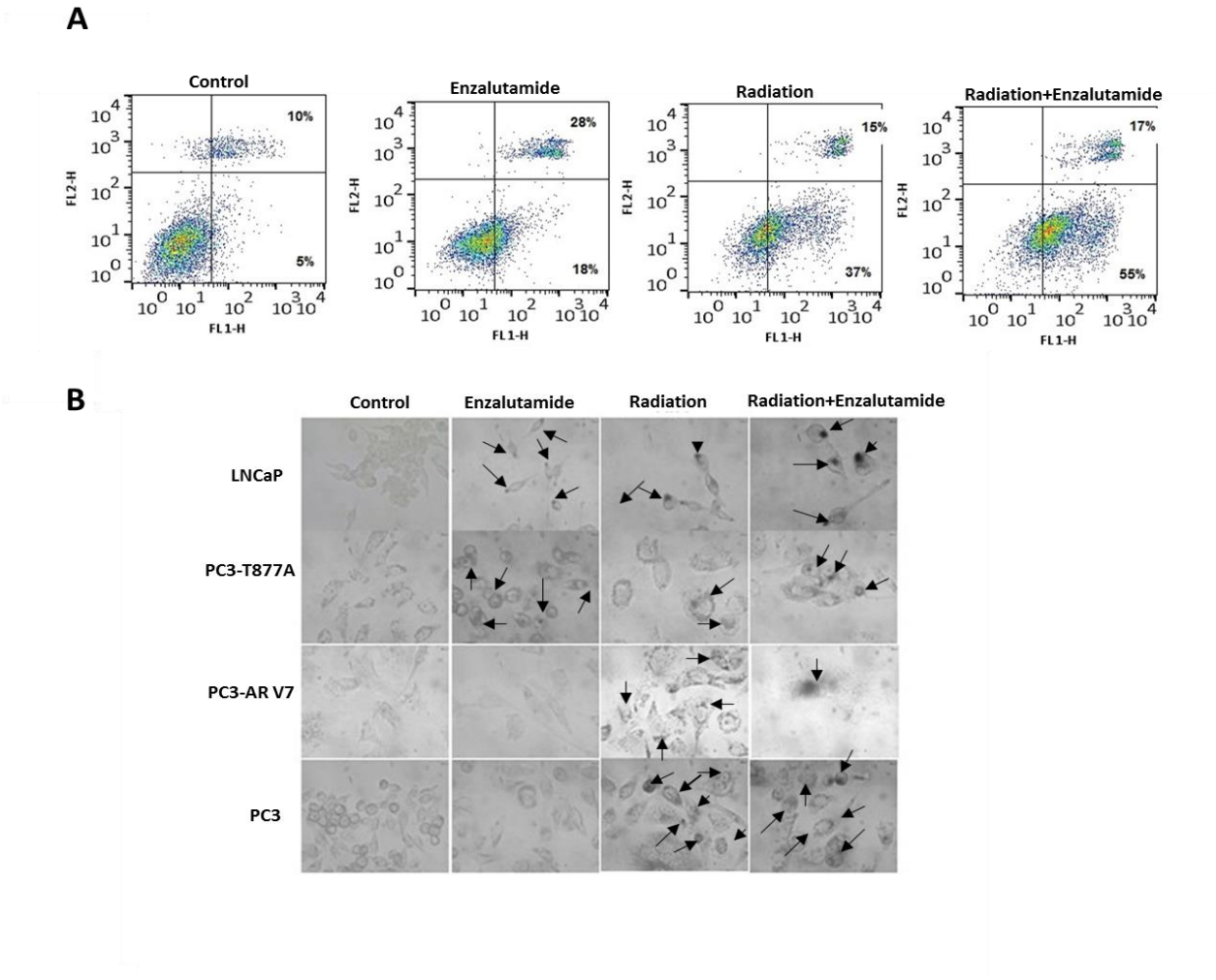
Supplementary Figure 2-1. The effect of ENZA and/or ADT in combination with XRT on clonogenic survival of PC3-T877A and 22RV1 cell lines under each scheduling protocol. PC3-T877A and 22RV1 cells have been treated with ENZA (10 μM) or ADT 24 hours before XRT (A), 2 hours before XRT (B), and 24 hours after XRT (C). ENZA=Enzalutamide, XRT=Radiation, ADT=Androgen deprivation therapy.



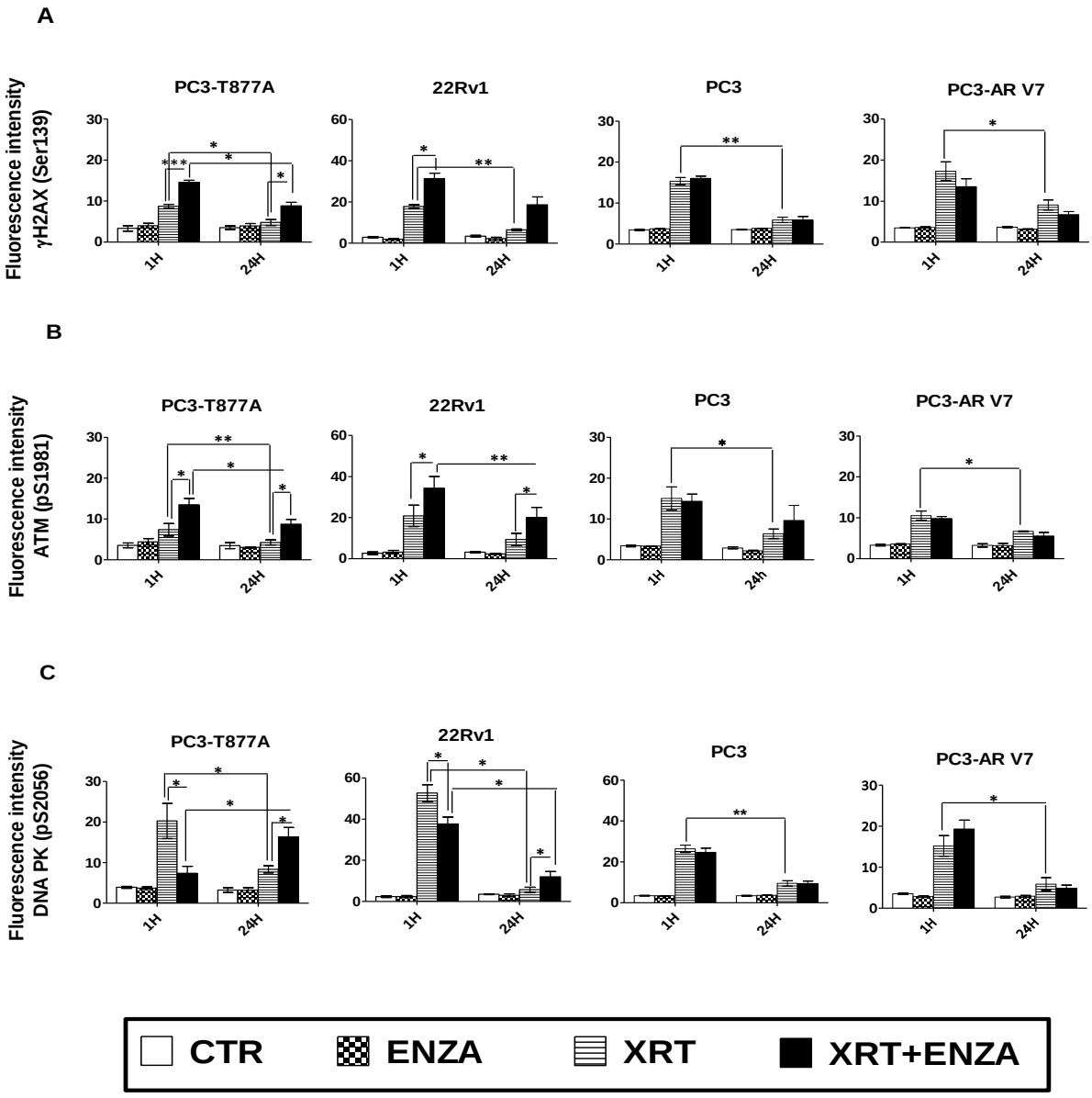
Supplementary Figure 2-2. The effect of ENZA and/or ADT in combination with XRT on clonogenic survival of PC3 and PC3-AR-V7 cell lines, under each scheduling protocol. PC3 and PC3-AR-V7 cells have been treated with ENZA (10 µM) 24 hours before XRT (A), 2 hours before XRT (B), and 24 hours after XRT (C). ENZA=Enzalutamide, XRT=Radiation.



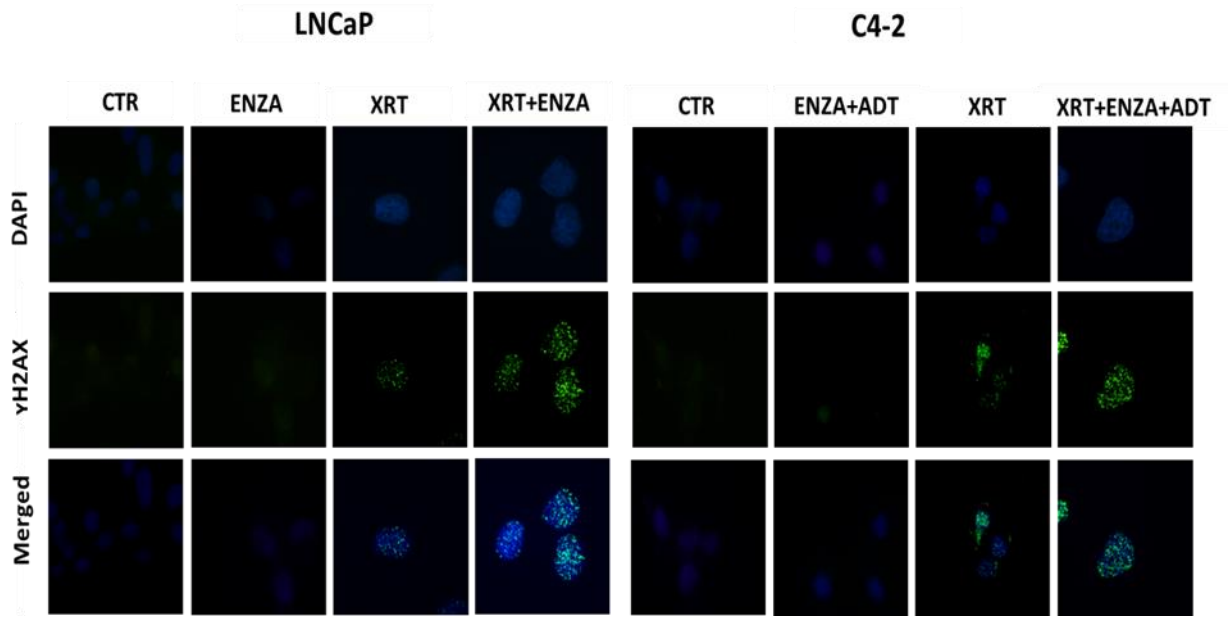
Supplementary Figure 2-3. (**A) Representative histograms for the cell cycle analysis of LNCaP cells untreated (Control) or treated with ENZA and/or XRT. (B) Cell cycle arrest of PC3-T877A, 22RV1, PC3 and PC3-AR-V7 cell lines untreated (control) or treated with ENZA and/or XRT.** Cells were treated with ENZA (10 μM) alone and in combination with XRT (4 Gy) for 12 hours (LNCaP) and 24 hours (PC3-T877A, 22RV1, PC3 and PC3-AR-V7), stained with propidium iodide (PI) and then analyzed by flow cytometry to determine the distribution of cells in each phase of the cell cycle. The graphs show the percentage of the cells following ENZA and/or XRT treatment. ENZA=Enzalutamide, XRT=Radiation, ADT=Androgen deprivation therapy.



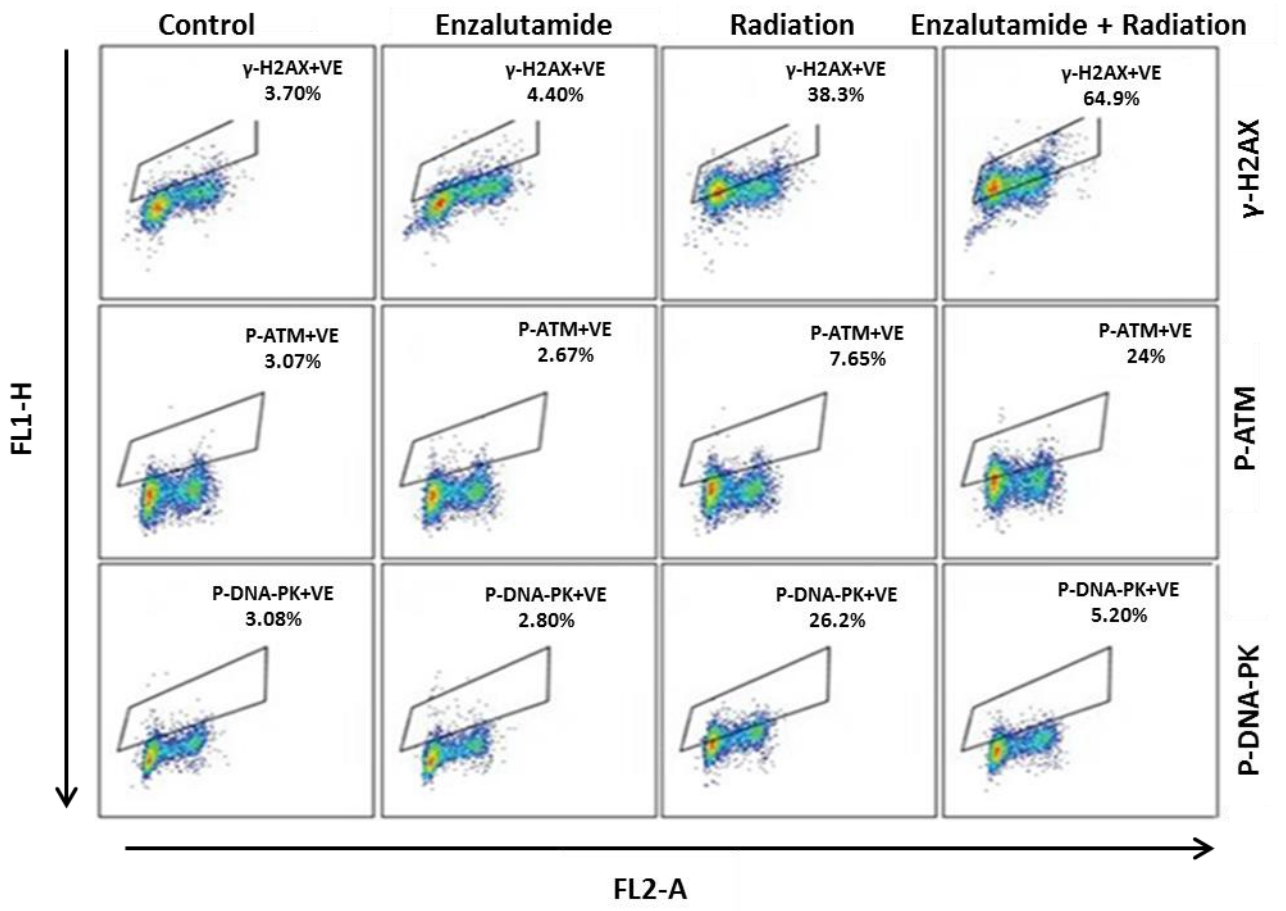
Supplementary Figure 2-4. (A) Apoptotic rate determined by Annexin V-FITC/PI staining in LNCaP cells untreated (control) or treated with ENZA and/or XRT. (B) Senescence-associated β-galactosidase activity in PCa cell lines untreated (control) or treated with ENZA and/or XRT. (A) LNCaP cells have been treated with ENZA (10 µM) and/or XRT (4Gy), harvested 48 hours after treatment, stained with Annexin V/PI and analysed by flow cytometry. (B) Senescence-associated β-galactosidase (SA-β-gal) staining of hormone-sensitive and hormone-insensitive PCa cells after ENZA and/or XRT treatment for 24 hours. Data represent as mean and SEM from three independent experiments.



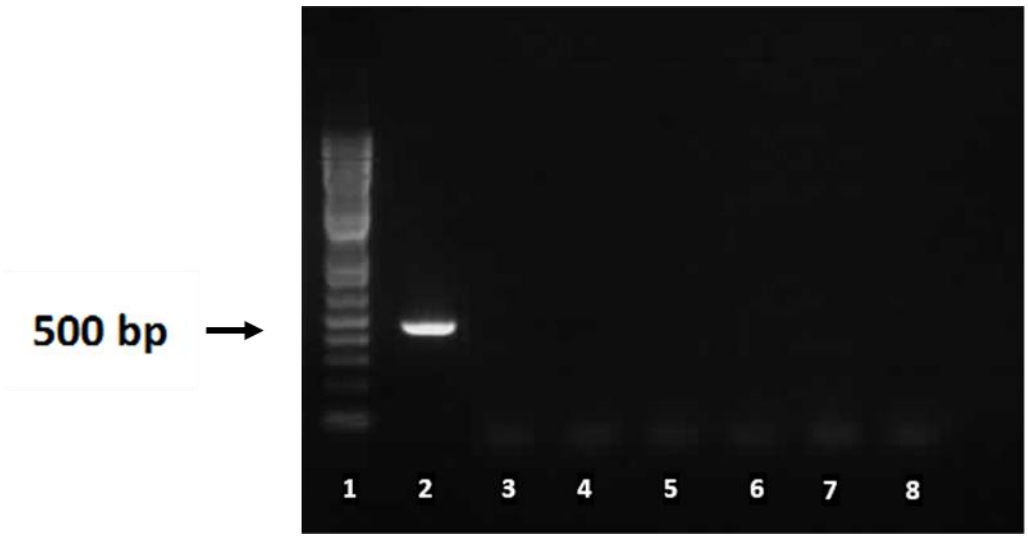
Supplementary Figure 2-5. Assessment of γ-H2AX (A), p-ATM (B), and p-DNA-PKcs (C) in PC3-T877A, 22RV1, PC3 and PC3-AR-V7 cell lines untreated (control) or treated with ENZA and/or XRT. The cells were treated with ENZA 2 hours before XRT, harvested 1 hour and 24 hours post treatment, and analyzed by flow cytometry to determine the level of γH2AX (Ser 139), ATM (Ser1981) and DNA-PKcs (Ser 2056). ENZA=Enzalutamide, XRT=Radiation.



Supplementary Figure 2-6. Immunofluorescent visualization of γH2AX foci (green) in LNCaP and C4-2 cells 1 hour after treatment with ENZA and/or ADT. Nuclei was stained with DAPI (blue). ENZA=Enzalutamide, XRT=Radiation, ADT=Androgen deprivation therapy.



Supplementary Figure 2-7. Distribution of γ-H2AX (Ser139), ATM (Ser1981), and DNA-PKcs (Ser2056) 1 hour after treatment with ENZA and/or XRT in LNCaP cells. LNCaP cells were treated with ENZA and /or ADT 2 hours before XRT, harvested 1hour after treatment and analyzed by flow cytometry to determine the level of γH2AX (Ser 139), ATM (Ser1981) and DNA-PKcs (Ser 2056).



Supplementary Figure 2-8. PCR analysis for detection of mycoplasma contamination in PCa cells. Lane1: DNA Marker Lane2: Positive control Lane3: LNCaP Lane4: PC3-T877A Lane5: PC3 Lane6: PC3-AR-V7 Lane7: C4-2 Lane8: 22RV1

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Chapter 3

Identification of a Radiosensitivity Molecular Signature Induced by Enzalutamide in Hormone-sensitive and Hormone-resistant Prostate Cancer Cell lines

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3.1. Preface

In the second chapter, we have shown that enzalutamide enhances the effect of XRT through impaired DNA damage repair process. In the next chapter, our aim is to identify the radiosensitivity gene signature(s) induced by enzalutamide in PCa cell lines and to clarify the biological pathways which play important roles in the regulation of radiosensitivity. In this study, in order to identify a common radiosensitivity gene signature and relevant biological pathways, we performed gene expression profiling following treatment of hormone-sensitive (LNCaP) and hormone-resistant (C4-2) PCa cell lines. We hypothesized that these gene signatures play a role in the radiosensitivity of PCa cell lines in different combination therapies.

3.2. Abstract

Prostate cancer (PCa) is the second most common cancer amongst men worldwide. A novel androgen receptor (AR) antagonist, enzalutamide (ENZA) has recently been demonstrated to enhance the effect of radiation (XRT) by impairing the DNA damage repair process. This study aimed to identify a radiosensitive gene signature induced by ENZA in the PCa cells and to elucidate the biological pathways which influence this radiosensitivity. We treated LNCaP (AR-positive, hormone-sensitive PCa cells) and C4-2 (AR-positive, hormone-resistant PCa cells) cells with ENZA alone and in combination with androgen deprivation therapy (ADT) and XRT. Using one-way ANOVA on the gene expression profiling, we observed significantly differentially expressed (DE) genes in inflammation-and metabolism-related genes in hormone-sensitive and hormone-resistant PCa cell lines respectively. Survival analysis in both the TCGA PRAD and GSE25136 datasets suggested an association between the expression of these genes and time to recurrence. These results indicated that ENZA alone or in combination with ADT enhanced the effect of XRT through immune and inflammation-related pathways in LNCaP cells and metabolic-related pathways in C4-2 cells. Kaplan–Meier analysis and Cox proportional hazard models showed that low expression of all the candidate genes except for PTPRN2 were associated with tumour progression and recurrence in a PCa cohort.

3.3. Introduction

Prostate cancer (PCa) is the most frequently diagnosed malignancy and is among the top three cancer-related deaths in American and Canadian men [1-3]. Radiation therapy (XRT) is one of the treatment options for locally advanced PCa. Unfortunately, 30–50% of patients undergoing XRT will experience a biochemical recurrence within 10 years [4]. Although the combination of XRT with androgen deprivation therapy (ADT) for high-risk localized PCa patients has demonstrated an improved overall survival (OS) rate, almost half of these patients develop a resistance to ADT. This resistance is caused by the incomplete blockade of AR-ligand signalling, AR amplifications, AR mutations, aberrant AR co-regulator activities, or AR splice variants expression with the development of castration-resistant tumours [5]. The AR is a type of nuclear receptor that is activated when bound by androgenic hormones. AR activation regulates the transcription of specific genes [6]. In 2012, ENZA, an AR antagonist, was approved by the FDA and Health Canada for the treatment of metastatic castration-resistant PCa (mCRPC) [7]. Castration-resistant PCa (CRPC) is defined by disease progression and a rise in serum prostate-specific antigen (PSA) levels despite ADT. CRPC represents the most aggressive type of PCa with a median survival rate of fewer than two years [8]. ENZA targets the AR signalling pathway at three key stages: (1) It blocks binding of androgens to the AR; (2) It prevents AR nuclear translocation within the nucleus; (3) It inhibits binding of activated AR to chromosomal DNA, which prevents transcription of target genes [9]. We have shown that ENZA enhances the effect of XRT through an impaired DNA damage repair process [10]. The aim of our research is to identify the radiosensitivity gene signature(s) induced by ENZA in PCa cells and to clarify the biological pathways that play important roles in the regulation of radiosensitivity.

From a clinical standpoint, the ability to predict tumour response to XRT therapy is a necessary avenue to improve treatment outcomes. Gene expression profiling is a major tool used to stratify which patients will benefit from radiosensitivity. These genomic data will also increase the understanding of the cellular mechanisms of intrinsic radioresistance in cancer. It is promising to integrate the biomarkers of radiosensitivity to develop personalized radiation therapy. Following radiation therapy, changes in gene expression have been detected in multiple cancer cell lines including PCa. Although p53, ATM mutations, and the loss of PTEN are

associated with human PCa radiosensitivity, they do not accurately predict which individual tumour will eventually fail definitive radiation therapy [11]. Given the complexity of radiation-induced responses, comprehensive gene expression microarray analysis enables the identification of a wider range of genes and signalling pathways involved in the response to radiation [12]. There are many factors that influence the transcriptional profile seen after exposure to irradiation such as genetic background, cell type, XRT dose, dose rate, and time after XRT [13]. While radiation induces cell death through DNA damage, there are no clinically predictive markers available to indicate the likelihood of an effective treatment outcome [11]. Clinicopathologic factors and PSA levels both aid in decision making when selecting treatment for the individual patient, yet there is conflicting evidence as to the predictive and prognostic value of these markers [14]. In this study, in order to identify a common radiosensitivity gene signature and relevant biological pathways, we carried out gene expression profiling following treatment of LNCaP and C4-2 cell lines. We hypothesized that these gene signatures play a role in the radiosensitivity of PCa cell lines in different combined treatment modalities.

3.4. Results

3.4.1. Differentially expressed genes by one-way ANOVA in hormone-sensitive and hormone-resistant prostate cancer cell lines

We have demonstrated that ENZA with or without ADT enhances the effect of XRT in both hormone-sensitive (LNCaP) and hormone-resistant (C4-2) PCa cell lines [10]. To identify radiosensitivity gene signatures and elucidate related signalling pathways, we performed gene expression analysis on the cells treated with XRT or ENZA±ADT in combination with XRT. By performing a one-way ANOVA with contrasts, we determined the most significant (*p*-value <0.0001) differentially expressed (DE) genes among the three experimental groups (ENZA+XRT vs. XRT, ADT+XRT vs. XRT, ENZA+ADT+XRT vs. XRT) in LNCaP (Table 3-1) and C4-2 (Table 3-2) cell lines, 13 and 11 genes were identified, respectively. The *NKX3-1* was the only common gene for all three comparisons and it appeared in both LNCaP and C4-2 cells groups.

Gene	ENZA+XRT	ADT+XRT	ENZA+ADT+XRT	AveExpr	P-Value	Adj. P-Value
NKX3-1	-0.8163	-1.085	-1.277	10.75	2.062e-06	0.05607
LAT	1.041	1.051	0.5554	5.165	1.124e-05	0.0882
ZMIZ1	-0.7282	-0.1722	-0.6085	8.726	1.251e-05	0.0882
INSL6	-0.7812	-0.83	-0.6041	3.513	1.314e-05	0.0882
CDH10	0.3829	1.172	0.8857	2.843	1.692e-05	0.0882
SLC39A5	0.02344	-0.3479	-0.9146	4.706	2.049e-05	0.0882
PTPRN2	0.6226	1.143	0.9196	4.765	2.271e-05	0.0882
LRFN1	-0.2878	-0.6021	-0.8624	6.478	3.009e-05	0.09189
SPDEF	-0.2894	-0.4062	-0.5095	10.28	3.042e-05	0.09189
TEX44	-0.4086	0.1263	-0.5077	5.724	4.161e-05	0.1131
23069178	-0.7734	-0.264	-0.6781	3.846	5.748e-05	0.1249
PDE9A	-0.7457	-0.5213	-0.9291	8.719	6.206e-05	0.1249
MMEL1	-0.3271	-0.7629	-0.262	6.291	6.437e-05	0.1249
OSBPL10	1.073	0.7663	1.023	5.868	6.79e-05	0.1249
TC1800006649.hg.1	-0.4451	0.354	-0.4623	3.297	6.891e-05	0.1249

Table 3-1. The most significant DE genes from one-way ANOVA for each treatment condition (ENZA+XRT vs. XRT, ADT+XRT vs. XRT, ENZA+ADT+XRT vs. XRT) in LNCaP cells. XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy. DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

Gene	ENZA+XRT	ADT+XRT	ENZA+ADT+XRT	Ave Expr	P-value	Adj. P-value
CYP1A1	0.5771	2.417	2.498	7.441	9.098e-08	0.002474
TRIB2	-1.052	-0.837	-1.336	5.552	6.11e-07	0.006839
CYP1A2	0.4795	3.093	2.894	7.047	7.546e-07	0.006839
FBXL5	0.01488	0.6239	0.5458	8.664	1.349e-06	0.009166
CYP1B1	-0.5141	0.6484	0.7994	5.068	1.762e-05	0.09561
HTA2-neg-47421856_st	0.1306	0.0377	2.3520	2.059	2.11E-05	0.09561
SEMA6A	0.152	-0.5594	-0.6021	7.675	2.8e-05	0.1088
TIPARP	0.02464	0.7372	0.8546	7.523	4.115e-05	0.1354
NKX3-1	-0.6767	-0.497	-1.145	10.75	4.483e-05	0.1354
PFKFB3	-0.1228	-0.6118	-0.6407	6.928	6.377e-05	0.163
PMEPA1	-1.045	-0.4885	-1.259	8.374	6.594e-05	0.163
C1orf116	-0.8854	-0.03169	-0.6941	10.96	7.653e-05	0.1734

Table 3-2. The most significant DE genes from one-way ANOVA for each treatment condition (ENZA+XRT vs. XRT, ADT+XRT vs. XRT, ENZA+ADT+XRT vs. XRT) in C4-2 cells. XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy. DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

Comparing the expression of genes observed in each experimental group to the gene expression of the non-radiated control group, we identified two differently expressed genes. The results obtained from the comparative analysis of ENZA vs. control [CTR], ADT vs. CTR, ENZA+ADT vs. CTR in LNCaP are shown in Table 3-3, whereas the results obtained from the comparative analysis of the three C4-2 experimental groups are shown in Table 3-4. Using LNCaP cells we have identified 13 genes which were modulated following radiation, while *CYP1A1*, *CYP1A2*, and *FBXL5* were found to be deferentially expressed in both radiated and non-radiated conditions in C4-2 cells, therefore, these genes are unlikely to be associated with regulation of radiation sensitivity.

SYMBOL	ENZA	ADT	ENZA+ADT	AveExpr	P-Value	Adj. P-Value
HTA2-pos 2978683_st	-1.789	-1.74	-1.725	2.104	2.359e-09	6.414e-05
CYP1A1	0.6475	2.191	2.089	7.441	2.675e-06	0.0314
HTA2-neg-47419193_st	-0.5908	-0.9197	-0.8388	2.45	3.464e-06	0.0314
CYP1A2	-0.1485	2.119	2.198	7.047	4.064e-05	0.2762
MUC7	0.3477	-0.5006	0.1016	4.032	9.765e-05	0.4557

Table 3-3. The most significant DE genes from one-way ANOVA for each treatment condition (ENZA vs. CTR, ADT vs. CTR, ENZA+ADT vs. CTR) in LNCaP cells. CTR=Control, ENZA=Enzalutamide, ADT=Androgen deprivation therapy. DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

SYMBOL	ENZA	ADT	ENZA+ADT	AveExpr	P-Value	Adj. P-Value
FBXL5	0.1701	0.8815	0.7326	8.664	6.511e-09	0.000177
CYP1A1	0.6429	2.501	2.357	7.441	1.498e-07	0.001899
CYP1A2	0.4665	3.208	3.128	7.047	2.096e-07	0.001899
CYP1B1	-0.2779	0.8443	0.9887	5.068	1.133e-05	0.07704
CCNG2	0.0294	0.6596	0.6017	10.93	2.234e-05	0.1215
TIPARP	0.1094	0.7962	0.8448	7.523	5.932e-05	0.2588
VSTM4	0.8132	-0.06463	0.403	3.244	6.664e-05	0.2588

Table 3-4. The most significant DE genes from one-way ANOVA for each treatment condition (ENZA vs. CTR, ADT vs. CTR, ENZA+ADT vs. CTR) in C4-2 cells. CTR=Control, ENZA=Enzalutamide, ADT=Androgen deprivation therapy. DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

Three genes (*LAT*, *PTPRN2*, and *PDE9A*) out of 13 genes identified to be differently expressed in the LNCaP had previously annotated functions in KEGG pathways. *LAT* as a gene involved in KEGG immune and inflammatory pathways, whereas *PTPRN2* and *PDE9A* were previously shown to be associated with type I diabetes mellitus pathway and purine metabolism, respectively. In C4-2 cells, three genes (*CYP1A1*, *CYP1A2*, and *CYP1B1)* out of 11 genes identified to be differently expressed had previously annotated functions in the KEGG metabolic pathways as genes involved in Tryptophan metabolism, steroid hormone biosynthesis, metabolism of xenobiotics by cytochrome P450, ovarian steroidogenesis, retinol metabolism,

and in general as metabolic pathway regulators. Comparisons of gene expression in the experimental groups to control non-radiated groups in LNCaP cells, resulted in identification of differences in the expression of *MUC7* and *CCNG2* genes, which were annotated in KEGG as the genes involved in salivary secretion pathway, p53 signalling pathway, and foxo signalling pathways, respectively.

In brief, our results suggest that either ENZA alone, or in combination with ADT, may potentiate radiation response through immune and inflammation-related pathways in LNCaP cells and in metabolic-related pathways in C4-2 cells.

3.4.2. Heatmap of differentially expressed genes based on the one-way ANOVA analysis of expressed genes in hormone-sensitive and hormone-resistant prostate cancer cell lines

Hierarchical clustering based on the DE genes identified from one-way ANOVA results in a clear separation between XRT alone and ENZA with or without ADT in combination with XRT in LNCaP cells (Supplementary Figure 3-1). While there is minor heterogeneity between the biological triplicates, the overall changes in gene expression due to this treatment are consistent in all triplicates, allowing the formation of the two distinct clusters. Interestingly, in relationship to the XRT groups, the ENZA ± ADT in combination with the XRT group showed higher expression levels of *LAT* (involved in immune response pathways), *OSBPL10*, and *PTPRN2* genes. Conversely, ENZA with or without ADT and XRT treated cells showed lower expression levels of *NKX3-1*, *ZMIZ1*, *PDE9A* and *SPDEF* genes which interact with AR in the same pathway.

The data generated from the gene expression analysis of C4-2 cells is illustrated as a gene expression heatmap of the DE genes graphically illustrating comparisons using a one-way ANOVA. We demonstrated that there are two gene clusters (ADT+XRT with XRT and ENZA+XRT with ENZA+ADT+XRT). Decreased levels of expression of *C1orf116, NKX3-1*, and *PMEPA1* genes were identified in ENZA ±ADT in combination with XRT condition compared to XRT± ADT. Furthermore, ENZA+ ADT treatment resulted an increase in the radiation response through downregulation of *PFKFB3, TRIB2,* and *SEMA6A* as well as the upregulation of *CYP1B1* and *TIPARP* (Supplementary Figure 3-2) in C4-2 cells.

3.4.3. Differentially expressed genes and KEGG annotation analysis with the addition of ENZA with or without ADT to XRT in hormone-sensitive and hormone-resistant prostate cancer cell lines

The microarrays expression of ~27,000 genes after a cut off *p*-value<0.0001 was applied. The most significant DE genes when the treatment included ENZA in addition to XRT relative to XRT alone were *LAT, ZMIZ1*, *INSL6* and *OSBPL10* in LNCaP cells as well as *TRIB2, AAK1, FEZF2, OR4P4* and *ADRB2* in C4-2 cells. Furthermore, we also identified genes that were significantly differentially expressed relative to XRT in ADT+ XRT (Supplementary Table 3 (1-2)) and ENZA+ADT+XRT in both LNCaP and C4-2 cells (Supplementary Table 3 (3-4)). In the non-radiated group, the most significantly DE genes following treatment with ENZA, ADT, and ENZA+ADT in both LNCaP and C4-2 cells are listed in Supplementary Table 3 (5-10).

Regarding the cells treated with ENZA, ADT and XRT, 4 out of the 16 genes (*NAPE-PLD, ZBTB16, LBH,* and *TANK*) are associated with known KEGG pathways in LNCaP cells. *TANK* is involved in KEGG immune-related pathways and *NAPE-PLD, ZBTB16, LBH* are known to be part of metabolic pathways. In addition, the 2 most upregulated genes (*DUSP19* and *OSBPL10*) have immune and metabolic functions. Analysis of gene expression in C4-2 cells revealed that the *CYP1A1* and *CYP1A2* are the most upregulated genes. These two genes were previously reported to be induced by ENZA treatment [15]. Our results demonstrated that their expression is upregulated by ENZA under no irradiated conditions as well.

3.4.4. Venn diagram illustrating the overlap between the three conditions in hormone-sensitive and hormone-resistant prostate cancer cell lines in comparison to radiation

The total number of genes in each condition was 27189. The Venn diagram demonstrated that the number of significantly different DE genes in LNCaP cells (166 genes: 80 upregulated and 86 downregulated) was much greater than C4-2 cells (80 genes: 45 upregulated and 35 downregulated). The Venn diagram also showed 17, 41, 60 genes in LNCaP cells and 21, 6, 23 genes in C4-2 cells that were deregulated (cut off *p*-value<0.01) by ENZA+XRT, ADT+XRT, and ENZA+ADT+XRT treatments, respectively. Only 0.016% (2/118: *NKX3-1* (downregulated), *INSL6* (downregulated)) of the genes in LNCaP and 0.04% (2/50: *NOD1* (upregulated), *TRIB2*

(downregulated)) of the genes in C4-2 cells were common among all three treatment conditions (Supplementary Figure 3-3). Furthermore, the number of common genes between different treatment conditions in LNCaP and C4-2 cell lines is as follow: ENZA+XRT/ADT+XRT (LNCaP= 4, C4-2= 0), ENZA+XRT/ENZA+ADT+XRT (LNCaP= 8, C4-2= 5), and ADT+XRT/ENZA+ADT+XRT (LNCaP= 9, C4-2= 7).

3.4.5. Validation of microarray data by real-time quantitative RT-PCR

To confirm the microarray data, we selected 13 DE (the genes involved in immune-related pathways such as *LAT* [16], *NKX3-1* [17], *ZMIZ1* [18], *TANK* [19], *SPDEF* [20], and *TRAF5* [21], and the genes involved in metabolic-related pathways such as *CYP1A1* [22], *CYP1A2*, *PTPRN2* [23], *OSBPL10* [24], *KBTBD2* [25], *SLC39A5* [26], and *PDE9A* genes from different treatment conditions and tested their expression using quantitative RT-PCR (qRT-PCR). The qRT-PCR analysis confirmed the differential expression of 9 out of 13 (69%) selected genes in the same direction predicted by the microarray analysis. These genes include *LAT*, *PTPRN2*, *NKX3-1*, *PDE9A*, *ZMIZ1*, *CYP1A1*, *CYP1A2*, *TANK* and *TRAF5* (Figure 3-1).

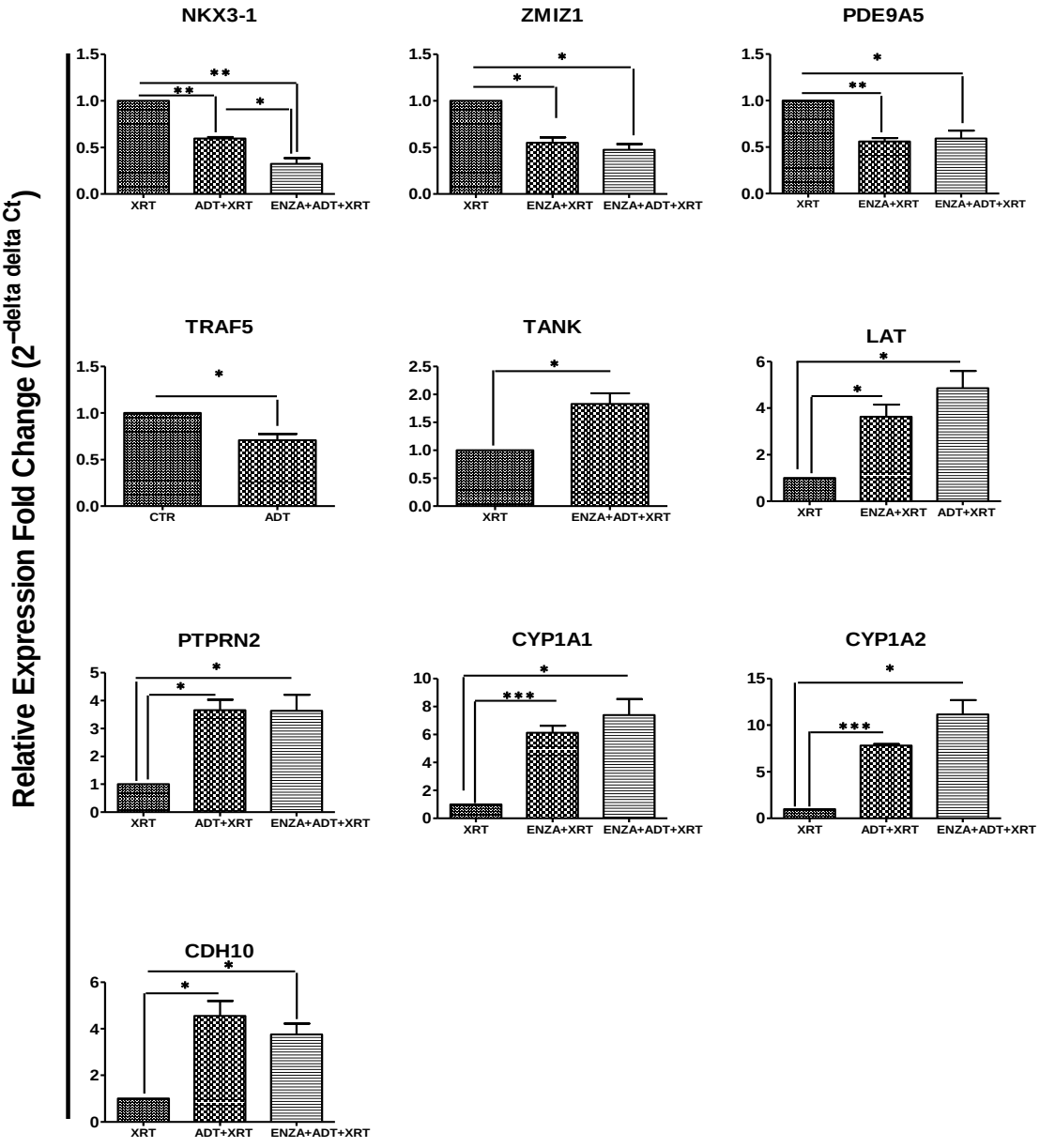


Figure 3-1. Validation by RT-qPCR of the microarray data. A set of upregulated and downregulated genes in LNCaP and C4-2 cells was analysed by RT-qPCR to validate the microarray data. All the RT-qPCR results were normalized to the expression level of GAPDH in each sample. Results are presented as the mean ± SE. The level of significance in the statistical analysis is indicated as (*) *p*-value <0.05, (**) *p*-value <0.01, (***) *p*-value <0.001 using two-tailed test.

3.4.6. Analysis of the associations between candidate genes and risk of recurrence

To evaluate the clinical significance of the candidate genes detected by one-way ANOVA analysis, we used The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA PRAD) (n=438) as our primary source of clinical information. In this database, 12.6% of patients received radiation therapy and 87.4% did not receive radiation therapy. We performed a univariate Cox proportional hazards survival model and found that the expression of a set of 10 genes was associated with time to recurrence ($p<0.05$ without any correction for multiple testing). Figure 3-2 shows forest plot of the hazard ratio (HR) and confidence interval (CI) for each significant gene for patients treated with and without radiation. The radiation group did not reach the significance, perhaps due to low power (56 patients). The expression of the *KBTBD2* genes was associated with a HR for recurrence of 1.50 ($P = 3.1\times10^{-4}$) and for *PTPRN2* with an HR of 0.7 ($P = 3.5\times10^{-2}$) (Supplementary Table 3-11). The density plot showed the same distribution of all the genes in radiated and non-radiated groups (Supplementary Figure 3-4). We also performed a multivariate Coxph model with ten significant genes and radiation status as a covariate. In the multivariate model, we found that only three of the genes that showed univariate significance (*SLC39A5, OSBPL10, and SLC16A6*) remained associated with survival which implies that only these three genes have independent effects on survival. Moreover, we identified that the interaction amongst the most significant genes (*SLC39A5, OSBPL10,* and *SLC16A6)* from the multivariate model with radiation status was not significant. Furthermore, the Kaplan-Meier curves showed a statistically significant difference between high-and low-gene expression groups in rates of disease-free survival (DFS) (Figure 3-3, Supplementary Figures 3 (5-6). DFS analysis revealed that patients bearing tumours with the lower expression of the genes (except *PTPRN2*) led to a significantly shorter time until recurrence ($P<0.05$). Five-year DFS rate of all the candidate genes was shown in Supplementary Table 3-12. The 10-year survival rate was found to be higher in patients with a higher expression of these genes (Figure 3-3).

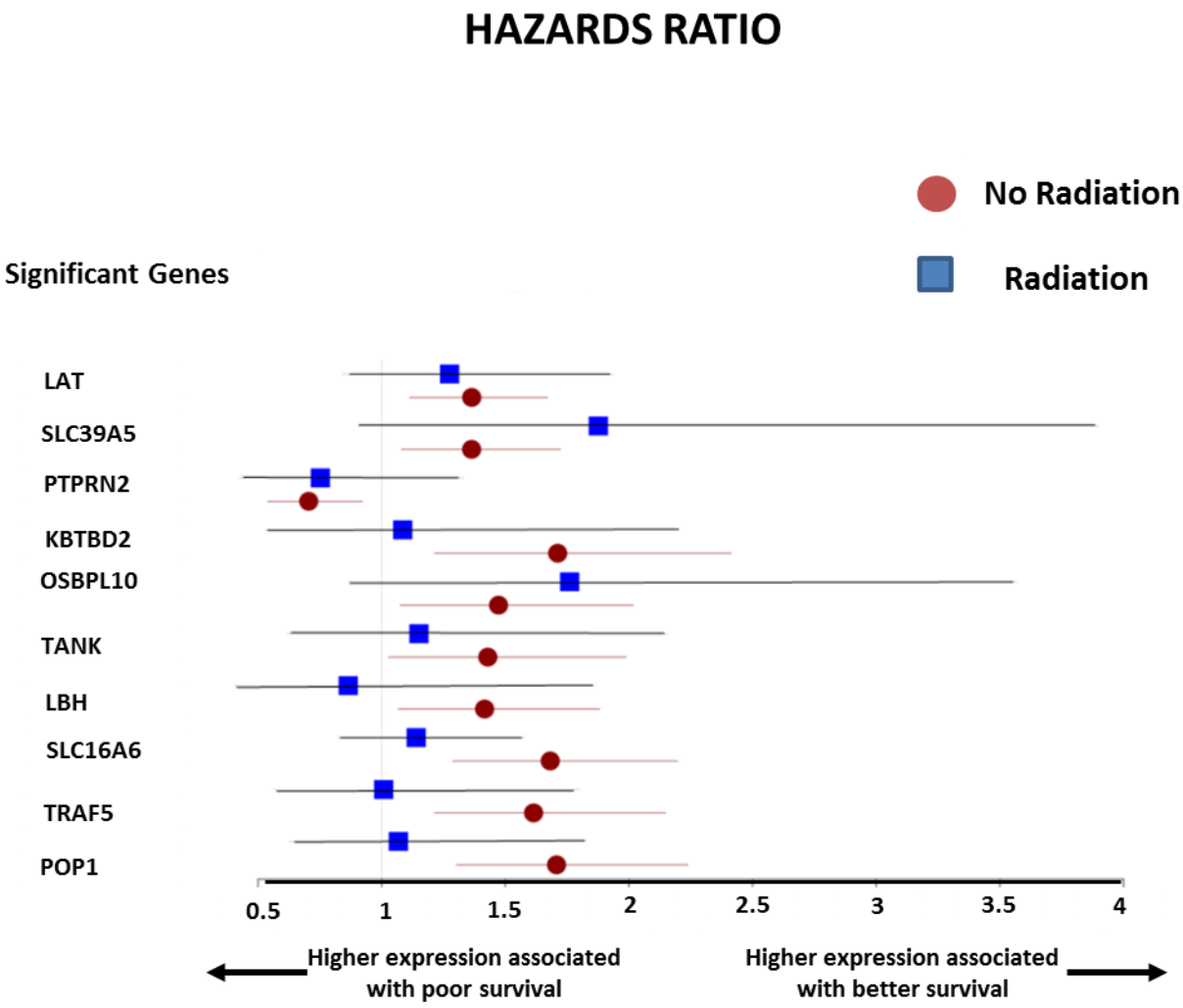
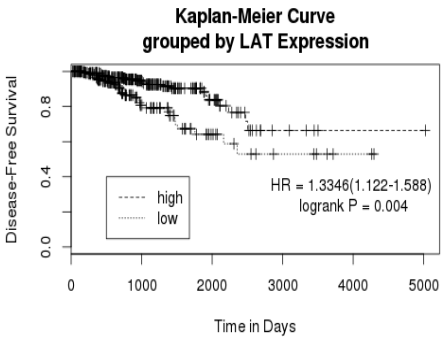
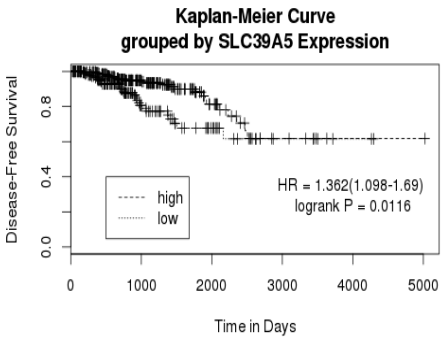


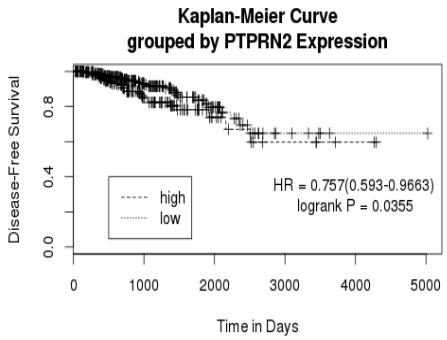
Figure 3-2. Forest plot of the hazard ratio and confidence interval for each significant gene for patients treated with or without radiation.

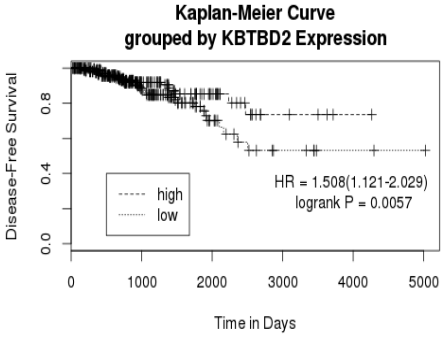
3.4.7. Expression levels of the ten candidate genes in prostate cancer patients

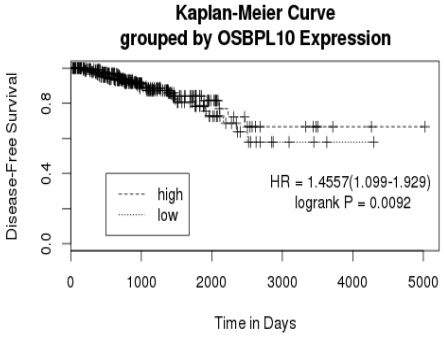
To examine the expression level of the candidate genes in human PCa tumours, we analysed RNA-Seq data from the TCGA PRAD dataset, and microarray data from GSE25136 dataset. Both data sets contain log2-transformed FPKM (fragments per Kilobase million) values of all the genes. The expression of the top 10 candidate genes is summarized by two box plots from TCGA-PRAD and GSE25136 databases (Supplementary Figure 3-7). The expression profile for the 10 genes selected based on the univariate Cox model results is different in the RNA sequencing data from the expression data derived from the Affymetrix array. The TCGA database revealed *LAT* and *SLC39A5* mRNA had the lowest FPKM value among all the candidate genes while *PTPRN2* expression had the highest of the other candidate genes with a median log2 FPKM value of about 6. The *PTPRN2* gene had a smaller effect size of recurrence compared to *LAT* and *SCLC39A5* genes. These results revealed that there is a reverse relationship between the expression level of *PTPRN2*, *LAT* and *SLC39A5* genes and hazard ratio of tumour recurrence.

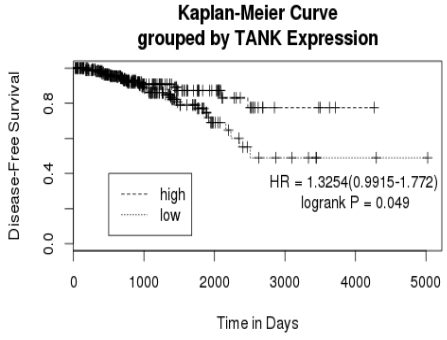

Kaplan-Meier Curve
grouped by LAT Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.3346(1.122-1.588)
logrank P = 0.004

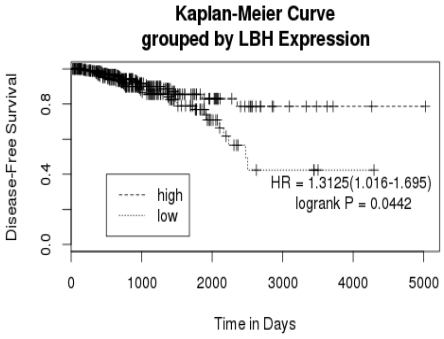

Kaplan-Meier Curve
grouped by SLC39A5 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.362(1.098-1.69)
logrank P = 0.0116

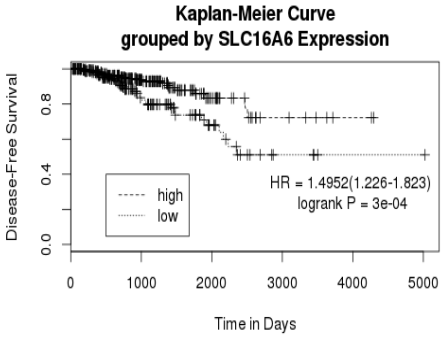

Kaplan-Meier Curve
grouped by PTPRN2 Expression
Disease-Free Survival
Time in Days
high
low
HR = 0.757(0.593-0.9663)
logrank P = 0.0355

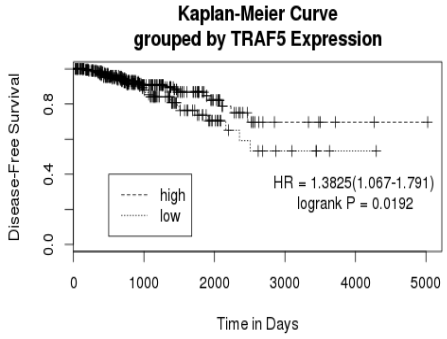

Kaplan-Meier Curve
grouped by KBTBD2 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.508(1.121-2.029)
logrank P = 0.0057


Kaplan-Meier Curve
grouped by OSBPL10 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.4557(1.099-1.929)
logrank P = 0.0092


Kaplan-Meier Curve
grouped by TANK Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.3254(0.9915-1.772)
logrank P = 0.049


Kaplan-Meier Curve
grouped by LBH Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.3125(1.016-1.695)
logrank P = 0.0442


Kaplan-Meier Curve
grouped by SLC16A6 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.4952(1.226-1.823)
logrank P = 3e-04


Kaplan-Meier Curve
grouped by TRAF5 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.3825(1.067-1.791)
logrank P = 0.0192

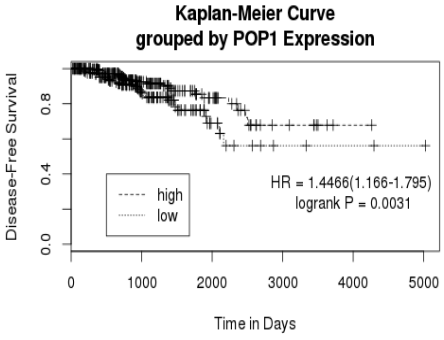

Kaplan-Meier Curve
grouped by POP1 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.4466(1.166-1.795)
logrank P = 0.0031

Figure 3-3. Kaplan–Meier disease free survival (DFS) curves. Kaplan-Meier curve compares the DFS in patients with high (above mean) and low (below mean) expression of the candidate genes, analysed from TCGA PRAD database (n=438). The survival curves were compared using the log-rank test.

3.4.8. The effects of the expression of candidate genes on recurrence of prostate cancer

In the second PCa dataset (GSE25136), time to recurrence was not available; however a yes/no variable was available for recurrence. We tested whether the expression was different between these 2 datasets (TCGA PRAD, GSE25136). Linear regression analysis was used to test the effects of expression of the candidate genes on recurrence. According to the GSE25136 dataset, genes including *AAK1*, *CYP1B1*, *NKX3-1*, *PTPRN2*, *TGF1* and *TRIB2* showed a different effect size of recurrence ($p < 0.05$ without any correction for multiple testing) (Table 3-5). The *PTPRN2* gene was common to both datasets with HR of 0.75 and -0.345 in the TCGA PRAD and GSE25136 datasets respectively. These results highlight the importance of the *PTPRN2* gene in evaluating the recurrence risk.

Gene	Estimate	P-Value
AAK1	-0.4066	0.008166
CYP1B1	0.1428	0.04047
NKX3-1	0.9468	0.001266
PTPRN2	-0.345	0.01773
TGIF1	0.2587	0.01104
TRIB2	0.2482	0.005419

Table 3-5. Simple linear regression models showing the association of the variables with recurrence statues using GSE25136 data.

3.5. Discussion

For decades, XRT has been used therapeutically to treat localized PCa. However, some patients treated with XRT experience biochemical recurrence within 10 years. Although ADT in combination with XRT increases OS in localized PCa, the disease progresses to CRPC due to the continued AR signalling pathway. We demonstrated that a profound AR inhibitor, ENZA, enhances the effect of XRT in both hormone-sensitive and hormone-resistant PCa disease [10]. Gene expression profiling is a valuable tool to elucidate the biomarkers of radiosensitivity in order to predict treatment response. These biomarkers would predict tumour response to radiotherapy and identify subgroup(s) of patients who do not respond, thereby offering different treatment options [11].

In this study, we demonstrate that ENZA alone or in combination with ADT increases radiosensitivity through immune and inflammation-related pathways in LNCaP cells, and through metabolic-related pathways in C4-2 cells. The Kaplan–Meier curve reveals that the low expression of the candidate genes in patients with PCa exhibits an earlier onset of tumour progression and recurrence compared to patients with a high expression of the genes.

One-way ANOVA and KEGG annotations were performed to identify a radiosensitive gene signature induced by ENZA±ADT in the PCa cells, and to clarify the biological pathways that play important roles in the regulation of radiosensitivity. The gene expression data were subjected to a one-way ANOVA analysis for each treatment condition (ENZA+XRT, ADT+XRT, ENZA+ADT+XRT).

We identified the most significant DE genes: *NKX3-1*, *ZMIZ1*, *SPDEF*, *PDE9A*, *LAT*, *PTPRN2*, and *OSBPL10* in LNCaP, and *CYP1A1* and *CYP1A2* in C4-2 cell lines.

ZMIZ1 is an AR co-activator that enhances AR-mediated transcription [27,28]. The study by Peng and colleagues suggested that the inhibition of *ZMIZ1* reduced the growth of a human PCa cell line [29]. *ZMIZ1* is also a candidate oncogene in non-melanoma skin cancer. *ZMIZ1* is overexpressed in breast, ovarian, and colon cancers, and in human cutaneous squamous cell carcinoma [30]. It was reported previously that *ZMIZ1*, in collaboration with NOTCH1, induces T-cell acute lymphoblastic leukemia (T-ALL) in mice. *ZMIZ1* inhibition slows the growth of leukemic cells and increases their sensitivity to corticosteroids and NOTCH inhibitors [31].

Zhang and colleagues reported that the androgen analog R1881 increased *NKX3.1* mRNA (a transcription factor expressed in epithelial cells of the prostate), and that the AR antagonist flutamide decreased *NKX3.1* mRNA in LNCaP cells [32]. *NKX3.1* was shown to inhibit estrogen receptor signalling in murine models of breast cancer [33]. *SPDEF*, a SAM pointed domain containing the ETS transcription factor, is upregulated by androgen treatment and downregulated by either the knockdown of AR or by treatment with bicalutamide [34,35]. Furthermore, the overexpression of *SPDEF* was show to increase breast cancer progression [36].

Dihydrotestosterone (DHT) increases the expression of *PDE9A*, a member of the cyclic nucleotide phosphodiesterase (PDE) family, in LNCaP cells [37]. *PDE9A* is also the main regulator of basal cyclic guanosine monophosphate (cGMP) levels in human breast cancer cells [38]. *PTPRN2*, an EMT-related (epithelial-mesenchymal transition) gene, is significantly higher in circulating tumour cells of CRPC patients than those of castration-sensitive patients [39]. Moreover, *PTPRN2* induces metastatic breast cancer cell migration through PI (4,5) P_2-dependent actin remodeling [40]. Dmitriev and colleagues identified *OSBPL10* as a biomarker of PCa. *OSBPL10* plays a key role in the maintenance of the body's cholesterol balance [41]. Dobashi and colleagues reported a point mutation in the *OSBPL10* gene as a prognostic indicator for diffuse large B-cell lymphoma [42]. *LAT*, a linker for the activation of T-cells, is a 36-Kd transmembrane protein that becomes rapidly tyrosine-phosphorylated by ZAP-70/Syk protein tyrosine kinases following activation of the T-cell antigen receptor (TCR) signal transduction pathway [16,43-45]. Sanada and colleagues reported DHT suppressed 3-methylcholanthrene (3MC)-induced transcription of the CYP1 family (*CYP1A1*, *CYP1A2*, *CYP1B1*) in LNCaP cells [46]. Our data confirm that the treatment of PCa cells with ENZA enhances the effect of radiation through downregulation of *NKX3-1*, *ZMIZ1*, *SPDEF*, and *PDE9A* genes and upregulation of *LAT*, *PTPRN2*, and *OSBPL10* genes in LNCaP cells, as well as upregulation of *CYP1B1* gene in C4-2 cells.

Furthermore, the KEGG annotation for the DE genes from the one-way ANOVA revealed that overexpression of *LAT* following combination therapy induced radiosensitization of LNCaP cells though enhanced inflammation and immunity pathways. Such pathways include the Fc epsilon RI signalling pathway, Fc gamma R-mediated phagocytosis, Th1 and Th2 cell differentiation, NF-kappa B signalling pathway, T cell receptor signalling pathway, and Th17

cell differentiation pathways. Androgen/AR exerts suppressive effects on T cell proliferation and modulates the balance of Th1 and Th2 responses [47-49]. In adaptive immunity, androgen/AR suppresses the development and activation of T and B cells [47] and also inhibits Th17 differentiation [50-52]. Removal of this suppression by AR inhibition causes an enlarged thymus gland and extreme export of immature B cells [47]. The immunogenic modulation property of ENZA increases the sensitivity of PCa cells to T cell-mediated lysis, and this immunogenic modulation is dependent on AR [53,54]. ADT enhances Th1 differentiation of CD4 T cells and CD4-mediated immune responses by down-regulating the Ptpn1 gene, a direct target of AR, in PCa patients [51]. Moreover, ADT increases the infiltration of T cells into benign glands and tumour sites in human prostates [55].

All these observations, taken together, strongly support the idea that AR inhibition by ENZA enhances the effect of radiation through immunity-related genes in hormone-sensitive PCa cells. In C4-2 cells, the most upregulated genes (*CYP1A1, CYP1A2* and *CYP1B1*) are involved in steroid hormone biosynthesis and metabolism of xenobiotics by cytochrome P450. Weiss et al. reported that ENZA induced the mRNA expression level of *CYP1A1* and *CYP1A2* [15]. These two genes were also found to be upregulated in non-radiated controls, thus suggesting that the modulation of expression of these two genes is not specific to the radiation response.

Importantly, we were able to validate most of the DE expressed genes (9 out of 13 genes) derived from microarray analysis by performing quantitative qRT-PCR. These DE genes are involved in immune-related pathways (e.g. *LAT, TANK*, and *TRAF5*) and metabolic-related pathways (e.g. *CYP1A1, CYP1A2*). Overall agreement of the qRT-PCR data with the microarray data was almost 69% (Figure 3-1). The four (4/13) other genes showed a trend to significant differential expression with qRT-PCR (Supplementary Figure 3-8).

As recurrence of localized PCa following treatment can lead to mCRPC, there is a critical need for the identification of reliable prognostic biomarkers to predict cancer recurrence following treatment of localized PCa. This led us to perform a univariate Cox proportional hazards survival model using the R package called 'survival,' and searching for an association between expressions of each of the identified 55 candidate genes and the time to recurrence. We found a correlation between the expression of ten genes (10 out of 55 genes) and time to recurrence.

Furthermore, the effect size of recurrence in the non-radiated group compared to the radiated group was larger, which resulted in a stronger effect on DFS in these cohorts. This may be due to the small sample size in the radiated group (n=56) versus the non-radiated group (n=382). Furthermore, our density plot revealed a similar expression of all the genes in both groups. However, a multivariate Cox regression model of the both cohorts revealed that *SLC39A5, OSBPL10, SLC16A6* have a significant independent effect on survival. In conclusion, our results demonstrate that these three genes separately or together correlate with OS in patients with PCa.

Furthermore, by using the gene expression data and clinical data from patient cohorts (TCGA PRAD database) we have shown that the low expression of candidate genes (including *LAT, SLC39A5, KBTBD2, OSBPL10, LBH, SLC16A6, TANK, TRAF5,* and *POP1*) correlates with disease recurrence and poor patient prognosis. To confirm this, we separated patients into two groups according to high and low gene expression level and then subjected them to Kaplan-Meier analysis.

As shown in Figure 3-3, the two groups of patients had significantly different times to recurrence. We found that low expressed genes (except *PTPRN2*) were associated with worse DFS, as indicated by the presence of biochemical recurrence (rising PSA levels following local therapy) or radiological tumour recurrence/metastasis [56]. The 5-Year DFS in the low expression gene group was less than in the highly expressed gene group (Supplementary Table 3-12). Kaplan–Meier analysis demonstrated a complete separation of the curves between high and low expression of the *LAT, KBTBD2, TANK, LBH, SLC16A6,* and *TRAF5* genes. Furthermore, the GSE25136 database revealed the effect of seven candidate genes, including *AAK1, CYP1B1, NKX3-1, PTPRN2, TGF1* and *TRIB2,* on the risk of recurrence. Among these candidate genes, the *PTPRN2* was common between the two clinical datasets (Table 3-5). As defined by the Gene Ontology Consortium, *PTPRN2* gene is involved in AR activity in PCa patients. Lastly, we observed an inverse relationship between the expression of *PTPRN2, LAT* and *SLC39A5* and HR of tumour recurrence from the TCGA PRAD dataset. The significance of these weakly expressed genes and PCa recurrence warrants further investigation from the clinical trials biobank.

Although the present study revealed potential predictive and/or prognostic biomarkers for response to combined AR inhibitor and XRT therapy, it has a limitation. It would have been

ideal to compare the effect of the candidate genes on recurrence between irradiated versus non-irradiated PCa patients. Unfortunately, the sample size of irradiated patients (12.6%) was smaller than the non-irradiated, but the density plot analysis of each of the genes revealed similar expression in both groups.

The identification and validation of biomarkers for clinical applications remains an important issue for the purposes of improved diagnostics and therapeutics in many diseases, including PCa. Via gene expression profiles, we identified potential predictive biomarkers that correlate with clinical outcomes. Currently, ENZA is a FDA approved drug for metastatic CRPC. Our previously published data demonstrated its efficacy in modulating response to radiotherapy in both hormone-sensitive and hormone-resistant PCa cell lines [10]. These results and the radiosensitivity gene profile documented in this study provide a good justification for the pre-clinical rationale for clinical trials assessing the combination of ENZA with XRT at an early-stage of PCa rather than mCRPC. Our study documented strong evidence for the importance of simultaneous treatment with ENZA and XRT, which might dramatically change the efficacy of XRT treatment and provide strong justification for assessing the predictive power of the identified markers in a clinical trial.

Overall, the gene signature described above predicted an enhanced radiation response to the combination therapy in our initial experiments [10]. Additionally, these genes are shown to be associated with an improvement in recurrence-free survival in the PCa patients' cohort of the TCGA. Therefore, these findings deserve further validation of predictive potential of identified markers in a clinical trial that would examine long-term survival and efficacy of combined ENZA and XRT treatment in PCa patients displaying an identified molecular signature who would be enrolled into the study at an early-stage of PCa progression.

3.6. Materials and Methods

3.6.1. Reagents and prostate cancer cell lines

Cell culture reagents were obtained from Gibco, Invitrogen (Burlington, Ontario, Canada). Fetal bovine serum (FBS) and charcoal stripped fetal bovine serum (CS-FBS) were obtained from Wisent Inc. (St-Bruno, Canada). ENZA was purchased from Selleckchem Com, (Cedarlane, and Paletta Court, Burlington, Ontario, Canada) and reconstituted in dimethyl sulfoxide (DMSO). LNCaP cells were obtained from ATCC (Manassas, VA) and C4-2 cells were provided by Dr. N. Zoubeidi (The Prostate Center, Vancouver General Hospital, University of British Columbia) [57]. The cells were cultured in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (iFBS), 50U/mL of penicillin, and 50 μg/mL of streptomycin. The cells were incubated at 37°C in 95% air/5% CO_2 and were tested for mycoplasma contamination using the mycoplasma PCR detection kit (Richmond, Canada) and found to be mycoplasma free. For hormone deficient treatment, ADT, we have used phenol red-free media supplemented with 10% charcoal dextran-treated serum (csFBS).

3.6.2. Irradiation

LNCaP and C4-2 cell lines were irradiated to 4.0 Gy in a solid water equivalent phantom (sun nuclear corporation, Florida, USA) at the depth of 3 cm using 6 MV energy from a Varian Clinac EX machine **(**Palo Alto, California, United States). The source to phantom distance was 100 cm and the field size was 25 cm x 25 cm at the phantom surface. The dose rate of the machine was verified prior to cell irradiation using a calibrated ionization chamber, and the dose rate at the depth of the irradiated cells was verified independently using Eternal Beam Therapy films (EBT3 films, Ashland, Kentucky, USA) following the film dosimetry protocol established by Devic et al. [58].

3.6.3. RNA extraction

To study the differential gene expression, LNCaP and C4-2 cells were treated with ENZA (10 μM) ± ADT 2 hours before radiation (4Gy), harvested 4 hours later and the total RNA was isolated by the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Experiments were repeated 3 times with new plated cells for each replicate. RNA concentration and purity for each sample was verified with a Nanodrop ND-1000

spectrophotometer (Nanodrop, Rockland, DE, USA). The ratios of absorbance 260/280 nm and 260/230 nm were ~2 for all the samples, and the RNA concentration was ~500 ng/μL per sample. RNA integrity was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA Integrity Number (RIN) values close to 10 were obtained, indicative of high quality RNA samples. Pure RNA samples were stored at −80 °C until cDNA libraries were prepared.

3.6.4. Expression Microarray analysis

Sense-strand cDNA was synthesized from 100 ng of total RNA, and fragmentation and labelling were performed to produce ss-cDNA with the GeneChip reagent kit according to manufacturer's instructions (Thermofisher Scientific-Affymetrix, Waltham, MA USA). After fragmentation and labelling, a 2.8 μg DNA target was hybridized on Clariom S assay HT, human (Thermofisher Scientific -Affymetrix, Waltham, MA USA) and processed on a Gene Titan instrument (Thermofisher Scientific-Affymetrix, Waltham, MA USA) for hybridization-Wash-Scan automated workflow. For the analysis of the resulting data, several R packages from the bioconductor project (www.bioconductor.org) were used. Affymetrix gene expression microarray data was first background-corrected and normalized via the 'rma' method in the bioconductor package 'oligo'. The RMA method includes background subtraction, normalization with the RMA algorithm and summarization using an approach called median-polish. The expression values are then transformed to the log2 scale. We performed a hierarchical clustering of the samples using the full panel of genes. The samples clustered well according to treatment conditions. Annotation for the Affymetrix Clariom S Assay HT for human was provided using the 'clariomshumanhttranscriptcluster.db' database provided by bioconductor. We applied two models for identifying genes that were differentially expressed. In model 1, we restricted the analysis to the radiated LNCaP cells. We compared gene expression between three conditions: ENZA±ADT in combination with XRT vs. XRT. In model 2 we repeated the same analyses in C4-2 cells. For all models we used the bioconductor package 'limma' to identify DE genes. A gene was regarded as being DE if p-value<0.0001.The heatmap of genes from One-way ANOVA differential expression analyses was drawn using the function heatmap.2 from the rpackage 'gplots'. By default the clustering uses the 'hclust' function with the Euclidean

distance. The microarray data have been submitted to the public functional genomics data repository Gene Expression Omnibus (GEO) under the accession number GSE126881.

3.6.5. Quantile-Quantile plot analysis

We mapped all the *p*-values on quantile-quantile (Q-Q) plot (Supplementary Figures 3 (9-10) and we compared what we have seen in our data (observed *p*-values) to what we expect (expected *p*-values) [59]. From our Q-Q plot, there is an elbow or an upward deviation of the observed *p* (black dots) values from the expected *p*-values (diagonal line) especially at the top right corner of the plot, so these genes are behaving differently than what we expect, that why we choose that threshold and that cut off *p*-value <0.0001. Our sample size was very small, and there were ~27000 genes analyzed. *P*-values reported for each gene were corrected for multiple comparisons using Benjamini & Hochberg (B&H) false discovery rate (FDR) [60]. Due to the small sample size, the adjusted *p*-value was not significant for many of the genes identified, however we have adopted a highly stringent cut-off *p*-value ($p< 0.0001$) for significant changes.

3.6.6. Real-time PCR

The DNase I (Qiagen, Valencia, CA) treated mRNA samples (1 µg) extracted from three independent biological replicates was reverse transcribed to cDNA using Super-Script III reverse transcriptase (Thermo Fisher scientific, Waltham, MA USA). Gene-specific primer pairs were designed using the NCBI Primer-BLAST tool (Supplementary Table 3-13). Amplification of the selected genes was performed using Applied Biosystems 7500 SYBR Green detection chemistry according to the manufacturer's instructions. PCR amplification was preceded by incubation of the mixture for 60 s at 95˚C, and the amplification step consisted of 40 - 45 cycles. Denaturation was performed for 15 s at 95˚C, annealing was performed at 60˚C for 10 s, and extension was performed at 60˚C for 30 s, with fluorescence detection at 72˚C after each cycle. After the final cycle, a melting point analysis of all samples was performed within the range of 60˚C - 95˚C with continuous fluorescence detection. qRT-PCR data were obtained from three independent experiments. The relative ratio of the threshold cycle (Ct) values between the endogenous controls (*GAPDH, ACTB*) and the specific gene was calculated for each sample. The validation procedure was performed with the same experimental design as for microarray analysis using the following genes (HGNC ID are in brackets): *LAT* (HGNC:18874), *OSBPL10* (HGNC:16395),

PTPRN2 (HGNC:9677), *SLC39A5* (HGNC:20502), *NKX3-1* (HGNC:7838), *SPDEF* (HGNC:17257), *ZMIZ1* (HGNC:16493), *PDE9A* (HGNC:8795), *CYP1A1* (HGNC:2595), *CYP1A2* (HGNC:2596), *TANK* (HGNC:11562), *TRAF5* (HGNC:12035), *KBTBD2* (HGNC:21751), *CDH10*(HGNC:1749), *ACTB* (HGNC: 132), *GAPDH* (HGNC:4141).

3.6.7. Association with recurrence

Two publicly available databases, TCGA PRAD (https://portal.gdc.cancer.gov/) and GSE25136 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25136), were used to assess the association between the expression of 10 candidate genes and time to recurrence. TCGA data for PRAD projects were downloaded from the TCGA portal maintained by GDC. Although the genes were identified in a microarray study, for the current project we use RNA sequencing data, specifically FPKM normalized expression values (fragments per kilobase million). Days to recurrence (RFS) and recurrence indicator (RFS_ind) were also obtained using the UCSC Zena Browser. Of the 498 samples, recurrence information was missing for 60 samples and therefore these samples were removed. In addition, data were downloaded from 79 prostate tumours from the GEO portal, project GSE25136. The data were normalized with Microarray Suite version 5.0 (MAS 5.0) with global scaling as the normalization method. A recurrence indicator variable was provided for each patient by the GSE25136 dataset. Clinical and pathologic data (patient age, Gleason score, pathological T, pathological M, clinical-M, clinical-T, radiation therapy) for TCGA PRAD patients have been shown in Supplementary Table 3-14. We applied a univariate and multivariate cox proportional hazards survival model and Kaplan Meier plot, using the coxph function in the R package 'survival'. Time to recurrence was estimated with the Kaplan–Meier product limit estimator. Hazard ratios and *p*-values were estimated with univariate Cox proportion hazards models. Results were expressed as hazard ratios (HRs) with 95% confidence intervals (CIs). DFS curves were plotted according to the Kaplan-Meier method, and their *p*-values were calculated by the log-rank test for patients with the low (below mean) and high (above mean) expression of the genes. All differences were considered statistically significant at the level of $P < 0.05$. Statistical analyses were conducted in the R platform. The level of significance in the statistical analyses is indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3.7. Supplementary Data

3.7.1. Supplementary Tables

LNCaP

Gene	log FC	P-Value	Adj. P-Value
PTPRN2	1.143	3.433e-06	0.03458
CDH10	1.172	3.947e-06	0.03458
MMEL1	-0.7629	4.743e-06	0.03458
INSL6	-0.83	5.398e-06	0.03458
NKX3-1	-1.085	7.445e-06	0.03458
LAT	1.051	7.632e-06	0.03458
KBTBD2	0.7891	2.587e-05	0.09145
CSDC2	-0.7017	2.691e-05	0.09145
TC0700013330.hg.1	0.7684	3.307e-05	0.09991
ZNF669	0.5804	5.724e-05	0.1556
MOCS1	-0.5972	9.925e-05	0.2366

Supplementary Table 3-1. The most significant DE genes in LNCaP cells treated with ADT+XRT vs. XRT. XRT=Radiation, ADT=Androgen deprivation therapy, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

C4-2

Gene	Log FC	P-Value	Adj. P-Value
CYP1A1	2.417	7.505e-07	0.02041
CYP1A2	3.093	3.241e-06	0.04406
FBXL5	0.6239	9.19e-06	0.08329
CC2D2A	0.7147	8.507e-05	0.4707

Supplementary Table 3-2. The most significant DE genes in C4-2 cells treated with ADT+XRT vs. XRT. XRT=Radiation, ADT=Androgen deprivation therapy, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

LNCaP

Gene	Log FC	P-Value	Adj. P-Value
NKX3-1	-1.277	4.275e-07	0.01162
SPDEF	-0.5095	4.289e-06	0.04264
LRFN1	-0.8624	4.705e-06	0.04264
PDE9A	-0.9291	9.066e-06	0.06162
SLC39A5	-0.9146	1.509e-05	0.08203
NAPEPLD	0.6952	1.935e-05	0.08561
SLC25A37	-0.7242	2.204e-05	0.08561
TANK	0.5541	2.771e-05	0.09416
ZBTB16	-1.5	5.048e-05	0.1422
OSBPL10	1.023	5.229e-05	0.1422
FAM57A	-0.7497	5.967e-05	0.1475
23072083	0.9159	7.366e-05	0.1608
DUSP19	1.021	7.689e-05	0.1608
LBH	-0.6985	8.414e-05	0.1619
PTPRN2	0.9196	9.003e-05	0.1619
SLC16A6	-0.7354	9.786e-05	0.1619

Supplementary Table 3-3. The most significant DE genes in LNCaP cells treated with XRT+ENZA+ADT vs. XRT. XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

C4-2

Gene	Log FC	P-Value	Adj. P-Value
TRIB2	-1.336	8.25e-08	0.002243
CYP1A1	2.498	4.065e-07	0.005527
NKX3-1	-1.145	3.036e-06	0.02752
CYP1A2	2.894	9.636e-06	0.0655
PMEPA1	-1.259	1.804e-05	0.09812
HTA2-neg-47421856_st	2.352	2.535e-05	0.1149
TGIF1	0.5261	4.256e-05	0.1653
FBXL5	0.5458	6.558e-05	0.2229

Supplementary Table 3-4. **The most significant DE genes in C4-2 cells treated with XRT+ ENZA+ADT vs. XRT.** XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

LNCaP

Gene	Log FC	P-Value	Adj. P-Value
HTA2-pos-2978683_st	-1.789	6.89e-09	0.0001873
FANCD2OS	-0.7816	4.601e-05	0.3744
LRP4	-0.5396	8.313e-05	0.3744
ZBTB16	-1.435	9.258e-05	0.3744
SYT8	0.8242	9.51e-05	0.3744

Supplementary Table 3-5. The most significant DE genes in LNCaP cells treated with ENZA vs. CTR. CTR=Control, ENZA=Enzalutamide, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

C4-2

Gene	Log FC	P-Value	Adj. P-Value
VSTM4	0.8132	7.962e-05	0.9998

Supplementary Table 3-6. The most significant DE genes in C4-2 cells treated with ENZA vs. CTR. CTR=Control, ENZA=Enzalutamide, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

LNCaP

Gene	Log FC	P-Value	Adj. P-Value
HTA2-pos-2978683_st	-1.74	1.276e-08	0.000347
HTA2-neg-47419193_st	-0.9197	1.146e-06	0.01558
CYP1A1	2.191	4.193e-06	0.038
OLFM4	0.9543	2.188e-05	0.1487
TC0600014205.hg.1	1.029	4.253e-05	0.2313
HTA2-pos-47421979_st	1.386	5.965e-05	0.242
ALPP	0.7721	7.238e-05	0.242
ZNF391	0.726	7.951e-05	0.242
TRAF5	-0.9546	8.295e-05	0.242
KCTD8	0.7943	8.901e-05	0.242

Supplementary Table 3-7. The most significant DE genes in LNCaP cells treated with ADT vs. CTR. CTR=Control, ADT=Androgen deprivation therapy, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

C4-2

SYMBOL	Log FC	P-Value	Adj. P-Value
FBXL5	0.8815	1.356e-08	0.0003686
CYP1A1	2.501	3.978e-07	0.005408
CYP1A2	3.208	1.726e-06	0.01564
HTA2-neg-47422186_st	0.8041	2.205e-05	0.1499
ADAMTS3	-0.7052	5.788e-05	0.3147
SLC5A1	0.8682	7.697e-05	0.3488
CCNG2	0.6596	9.242e-05	0.359

Supplementary Table 3-8. The most significant DE genes in C4-2 cells treated with ADT vs. CTR. CTR=Control, ADT=Androgen deprivation therapy, DE=differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

LNCaP

SYMBOL	Log FC	P-Value	Adj. P-Value
HTA2-pos-2978683_st	-1.725	1.542e-08	0.0004193
HTA2-neg-47419193_st	-0.8388	5.567e-06	0.07568
CYP1A1	2.089	9.123e-06	0.08268
SYT8	0.8408	7.301e-05	0.4123
POP1	-0.6949	7.864e-05	0.4123
TC0900010900.hg.1	-1.004	9.325e-05	0.4123

Supplementary Table 3-9. The most significant DE genes in LNCaP cells treated with ENZA+ADT vs. CTR. CTR=Control, ENZA=Enzalutamide, ADT=Androgen deprivation therapy, DE= differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

C4-2

SYMBOL	Log FC	P-Value	Adj. P-Value
FBXL5	0.7326	5.774e-07	0.0157
CYP1A1	2.357	1.191e-06	0.01618
CYP1A2	3.128	2.682e-06	0.02431

Supplementary Table 3-10. The most significant DE genes in C4-2 cells treated with ENZA+ ADT vs. CTR. CTR=Control, ENZA=Enzalutamide, ADT=Androgen deprivation therapy, DE= differentially expressed. *P*-values were corrected for multiple comparisons using FDR (B&H).

Gene	P-value	HR	95% CI	HR (Radiation)	HR (No Radiation)
SLC16A6	0.000318	1.495	1.226-1.823	1.139	1.682
POP1	0.003125	1.447	1.166-1.795	1.067	1.707
LAT	0.00401	1.335	1.122-1.588	1.275	1.364
KBTBD2	0.005673	1.508	1.121-2.029	1.085	1.712
OSBPL10	0.009153	1.456	1.099-1.929	1.76	1.473
SLC39A5	0.01157	1.362	1.098-1.69	1.876	1.364
TRAF5	0.0192	1.383	1.067-1.791	1.008	1.614
PTPRN2	0.03551	0.757	0.593-0.9663	0.7521	0.7049
LBH	0.04417	1.313	1.016-1.695	0.8646	1.416
TANK	0.04896	1.325	0.9915-1.772	1.151	1.429

Supplementary Table 3-11. List of hazards ratio and *p*-value for 10 significant candidate genes. Significance was defined at p<0.05 without any correction for multiple testing. CI=Confidence interval, HR=Hazard ratio.

	5-year DFS	
	Low gene expression	High gene expression
LAT	64%	85%
SLC39A5	67%	85%
PTPRN2	72%	80%
KBTBD2	72%	85%
TANK	84%	88%
OSBPL10	72%	82%
LBH	72%	82%
SLC16A6	70%	82%
TRAF5	70%	82%
POP1	70%	85%

Supplementary Table 3-12. DFS rates of 5 -year for all the candidate genes. DFS= Disease free survival. Yrs. =Year.

Gene	Sequence	TM
LAT-F	GAGCTACGAGAACGAGGGTG	64.5
LAT-R	CTGGTTCTGGGGGTAACGAC	64.5
OSBPL10-F	CATCGACACAACCACACTGC	62.4
OSBPL10-R	AAGTATACCCAGCCATCGCC	62.4
PTPRN2-F	GCCTGTGTGAACGATGGAGT	62.4
PTPRN2-R	TTCGGGAGGTCTGCAAGTTC	62.4
SLC39A5-F	GGAAGAGTCAAAGGCCCCTC	64.5
SLC39A5-R	CTGGGAGCCGTTCAGACAAT	62.4
NKX3-1-F	CAGAGACCGAGCCAGAAAGG	64.5
NKX3-1-R	CTGAGTGTGGGAGAAGGCAG	64.5
SPDEF-F	AAAGAGCGGACTTCACCTGG	62.4
SPDEF-R	CTGTGGGGCTTGAGTAGCAA	62.4
ZMIZ1-F	GGTCCCACCCAGGCGTAT	64.5
ZMIZ1-R	GAATAGGAAGGGACTGGGGC	64.5
TANK-F	GAGGTGCACCATCCATCACA	62.4
TANK-R	ACTAAGGATGCCGGGTCTCT	62.4

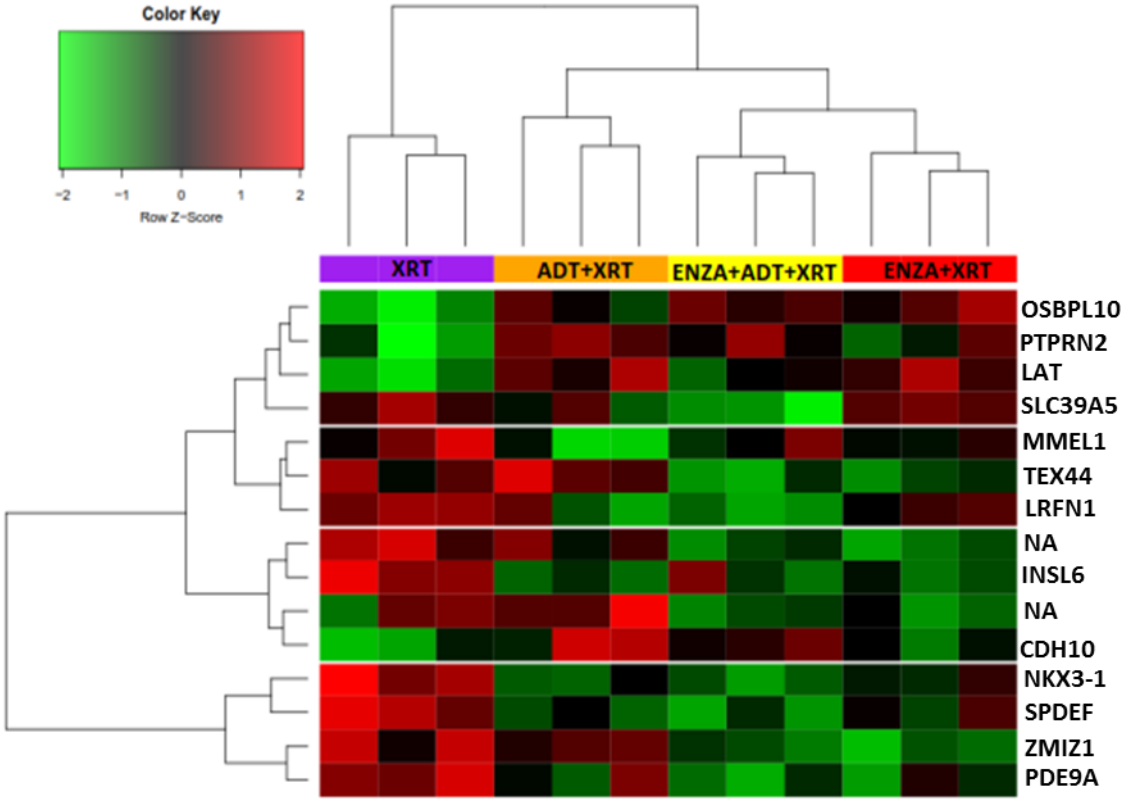
Gene	Sequence	TM
PDE9A-F	GGCCATCTACCTGGACATCG	64.5
PDH9A-R	TTGATGGCCACAGGTCTCAC	62.4
CDH10-F	GTGTGCTTGTGACAGCCAAG	62.4
CDH10-R	GAGGAGGATGGCGATCAAGG	64.5
ACTB-F	AGAGAGGCATCCTCACCCTG	60.69
ACTB-R	GATAGCACAGCCTGGATAGCA	59.65
TRAF5-F	AACCTGACCCCAATAGCAGC	62.4
TRAF5-R	TCAGTTAAGTCCACGGCCAC	62.4
GAPDH-F	GAGAAGGCTGGGGCTCATTT	62.4
GAPDH-R	AGTGATGGCATGGACTGTGG	62.4
KBTBD2-F	AGCCCCCAAGCAGAAAAAGT	60.4
KBTBD2-R	TGCGGACAAAAAGCATTGGG	60.4
CYP1A1-F	CACGGAGTTTCTTCTGGCCT	62.4
CYP1A1-R	CAATTCGGATCTGCAGCACG	62.4
CYP1A2-F	TGTCAGTGGCCAACGTCATT	60.4
CYP1A2-R	GAAGAAGTCCAGGGGGTTCC	64.5

Supplementary Table 3-13. Primer sequences used in the qRT-PCR analysis.

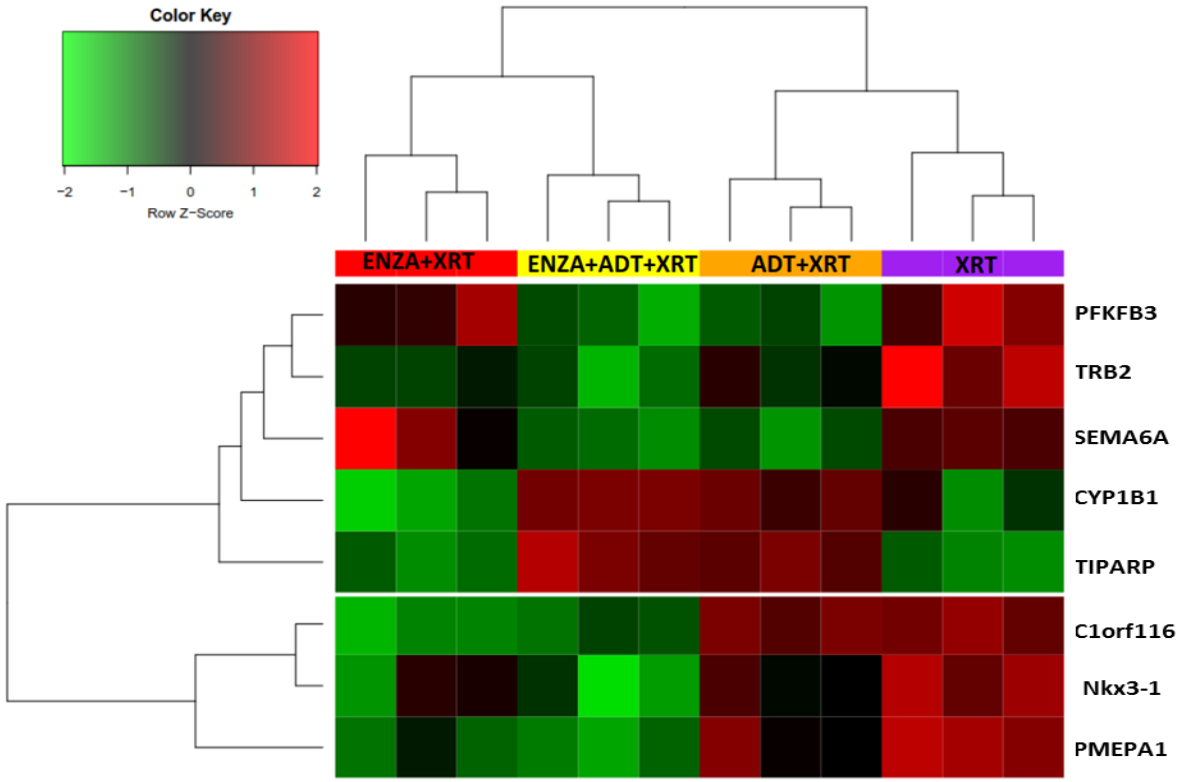
	Patients (n=438)
Age (Year)	60 (41-80)
GS	
≤6	35 (7.98%)
=7	229 (52.24%)
≥8	170 (38.81%)
Pathological T	
T4	10 (2.27%)
T3a	145 (33.0%)
T3b	115 (26.3%)
T2a	10 (2.27%)
T2b	9 (1.95%)
T2c	150 (34.3%)
Pathological N	
N0	360 (82.2%)
N1	78 (17.8%)
Biochemical Recurrence	
Yes	56 (12.8%)
No	382 (87.2%)
Clinical-M	
M0	436 (99.5%)
M1a	1 (0.175%)
M1b	1 (0.175%)
M1c	1 (0.175%)
Clinical-T	
T4	2 (0.398%)
T3a	42 (9.54%)
T3b	17 (3.78%)
T2a	63 (14.3%)
T2b	63 (14.3%)
T2c	54 (12.3%)
T1a	1 (0.199%)
T1b	2 (0.398%)
T1c	185 (42.1%)
Radiation Therapy	
Yes	55 (12.6%)
No	383 (87.4%)

Supplementary Table 3-14. Pathological and clinical data for prostate cancer patients

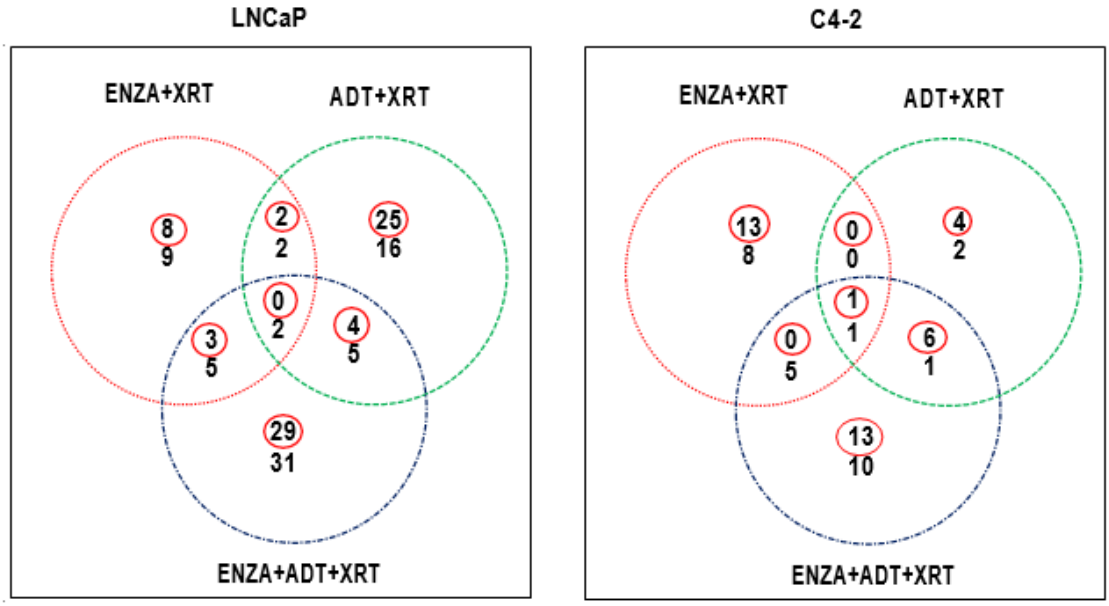
3.7.2. Supplementary Figures



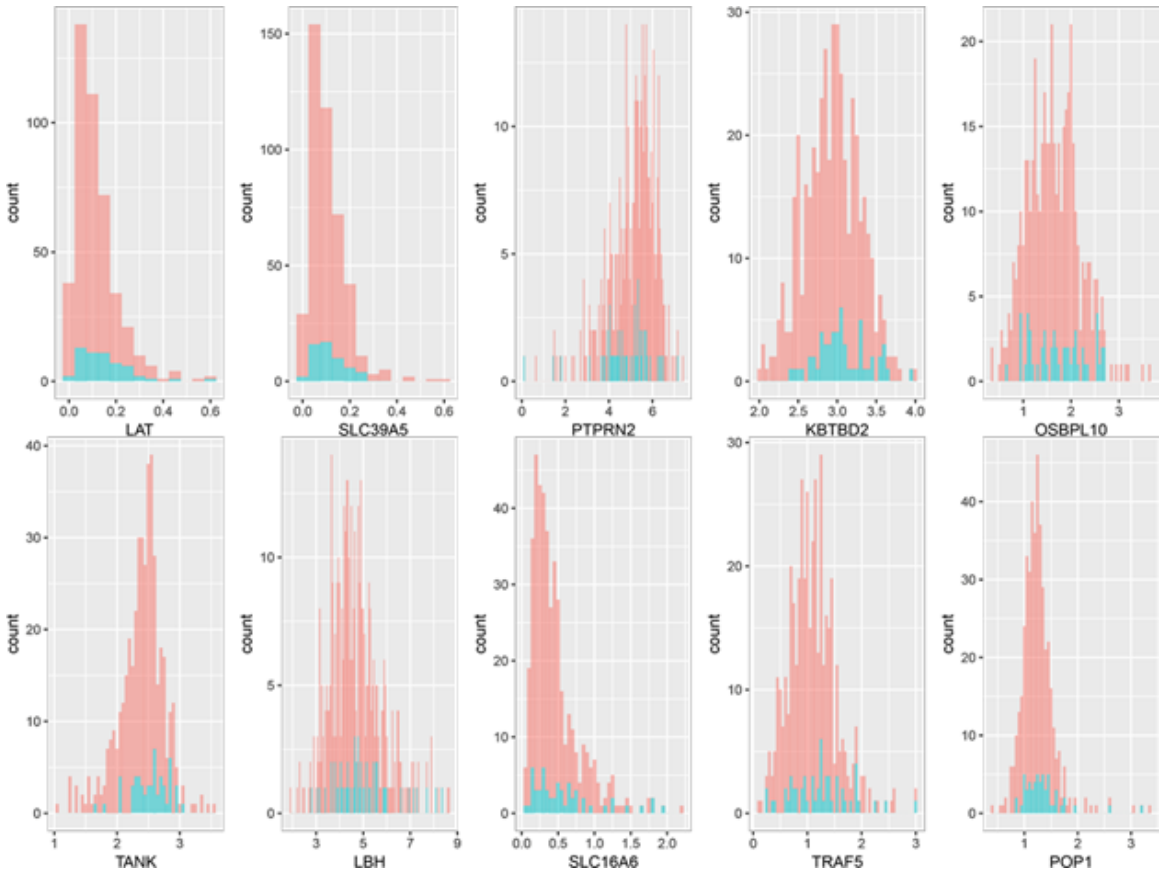
Supplementary Figure 3-1. Heatmap of DE genes from one-way ANOVA in LNCaP cells treated with ENZA and/or ADT in combination with XRT. Purple, red, orange and yellow represent the four conditions, respectively: XRT, ENZA+XRT, ADT+XRT, and ENZA+ADT+XRT. XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy, DE=differentially expressed.



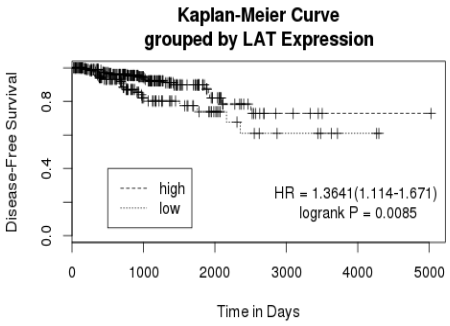
Supplementary Figure 3-2. Heatmap of DE genes from one-way ANOVA in C4-2 cells treated with ENZA and/or ADT in combination with XRT. Purple, red, orange and yellow represent the four conditions, respectively: XRT, ENZA+XRT, ADT+XRT, and ENZA+ADT+XRT. XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy, DE= differentially expressed.

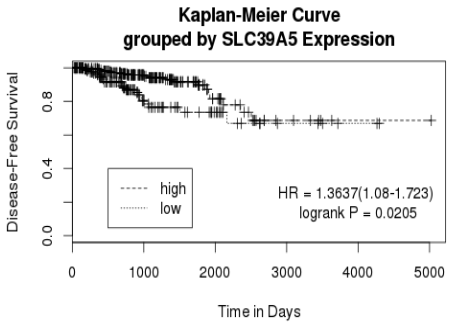


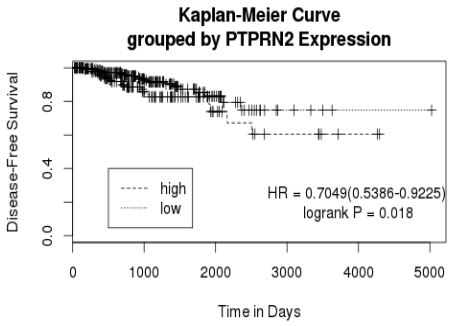
Supplementary Figure 3-3. Venn diagrams of upregulated and downregulated genes in LNCaP and C4-2 cell lines treated with ENZA and/or ADT in combination with XRT. The diagram shows deregulated genes (cutoff *p*-value<0.01) after treatment with ENZA+XRT, ADT+XRT and ENZA+ADT+XRT in LNCaP and C4 - 2 cell lines. The numbers in the oval represents the number of upregulated genes in each treatment conditions and the numbers down the oval represents downregulated genes. XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy. *P*-values were corrected for multiple comparisons using FDR.

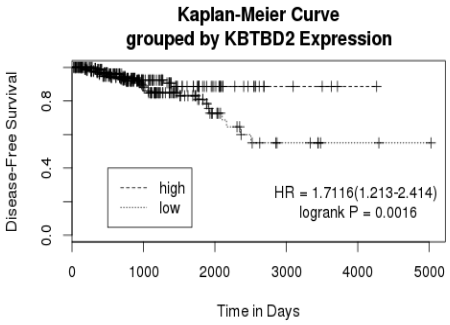


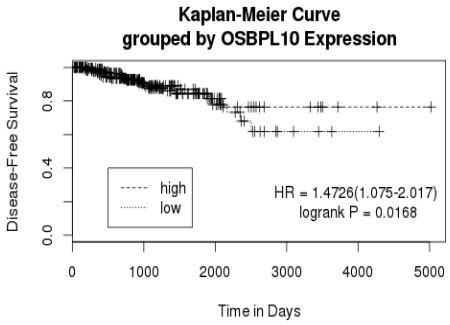
Supplementary Figure 3-4. The density plot of 10 candidate genes using TCGA PRAD dataset.

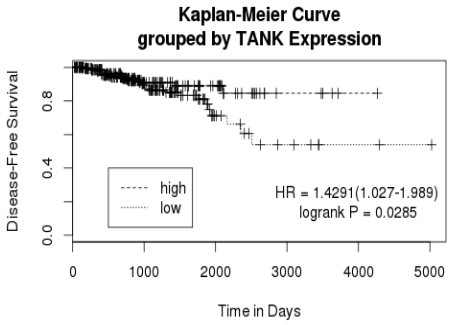

Kaplan-Meier Curve
grouped by LAT Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.3641(1.114-1.671)
logrank P = 0.0085

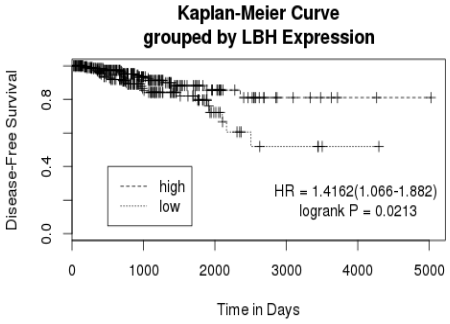

Kaplan-Meier Curve
grouped by SLC39A5 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.3637(1.08-1.723)
logrank P = 0.0205

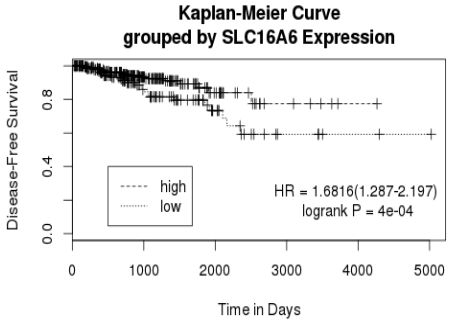

Kaplan-Meier Curve
grouped by PTPRN2 Expression
Disease-Free Survival
Time in Days
high
low
HR = 0.7049(0.5386-0.9225)
logrank P = 0.018

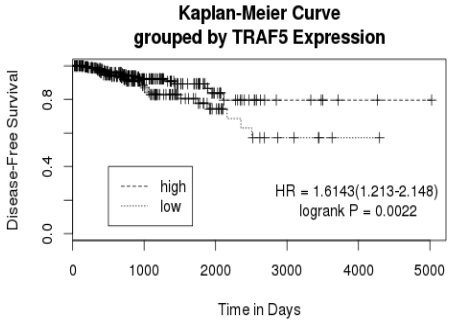

Kaplan-Meier Curve
grouped by KBTBD2 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.7116(1.213-2.414)
logrank P = 0.0016

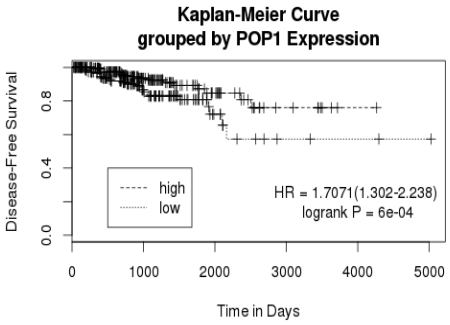

Kaplan-Meier Curve
grouped by OSBPL10 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.4726(1.075-2.017)
logrank P = 0.0168


Kaplan-Meier Curve
grouped by TANK Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.4291(1.027-1.989)
logrank P = 0.0285

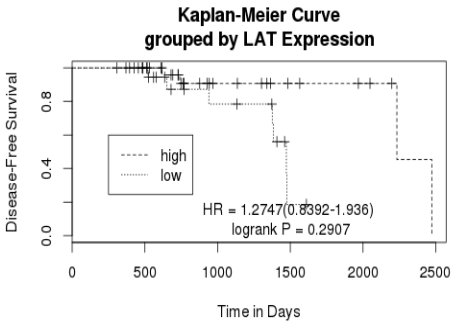

Kaplan-Meier Curve
grouped by LBH Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.4162(1.066-1.882)
logrank P = 0.0213

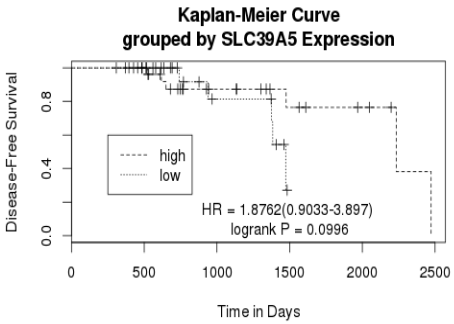

Kaplan-Meier Curve
grouped by SLC16A6 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.6816(1.287-2.197)
logrank P = 4e-04

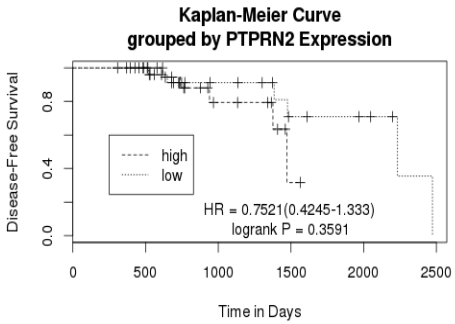

Kaplan-Meier Curve
grouped by TRAF5 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.6143(1.213-2.148)
logrank P = 0.0022

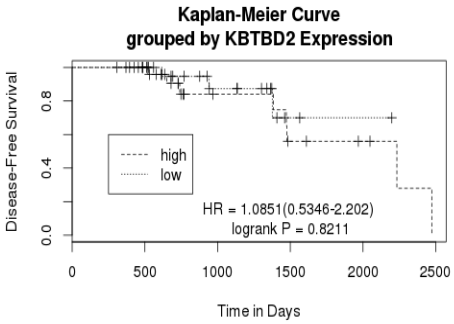

Kaplan-Meier Curve
grouped by POP1 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.7071(1.302-2.238)
logrank P = 6e-04

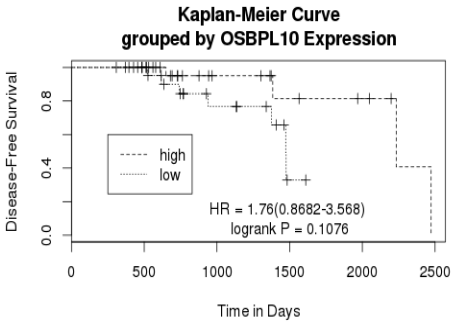
Supplementary Figure 3-5. Kaplan–Meier disease free survival (DFS) curves for non-radiated groups. Kaplan- Meier curve compares the DFS in patients with high (above mean) and low (below mean) expression of the candidate genes, analyzed from TCGA PRAD database in non-radiated group. The survival curves were compared using the log-rank test.

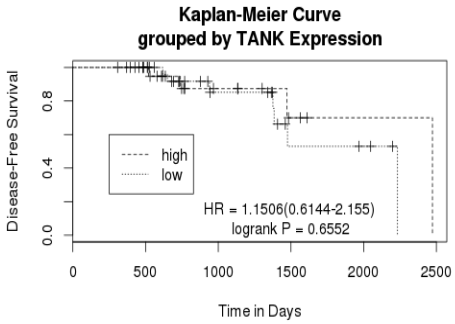

Kaplan-Meier Curve
grouped by LAT Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.2747(0.8392-1.936)
logrank P = 0.2907

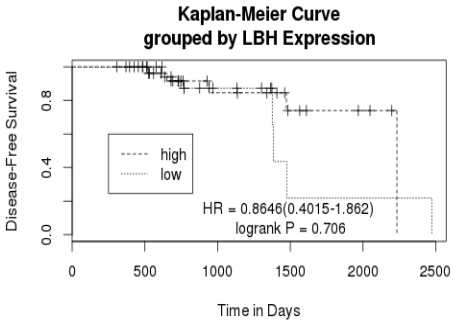

Kaplan-Meier Curve
grouped by SLC39A5 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.8762(0.9033-3.897)
logrank P = 0.0996

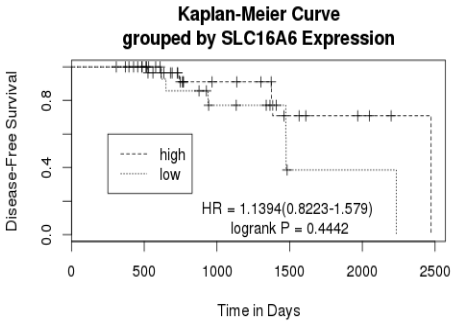

Kaplan-Meier Curve
grouped by PTPRN2 Expression
Disease-Free Survival
Time in Days
high
low
HR = 0.7521(0.4245-1.333)
logrank P = 0.3591

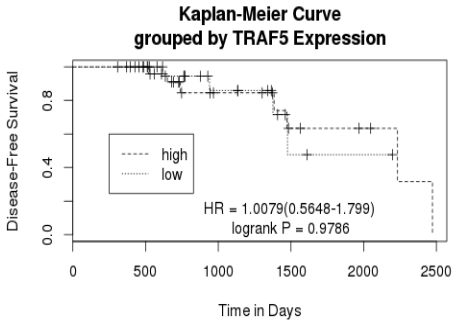

Kaplan-Meier Curve
grouped by KBTBD2 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.0851(0.5346-2.202)
logrank P = 0.8211

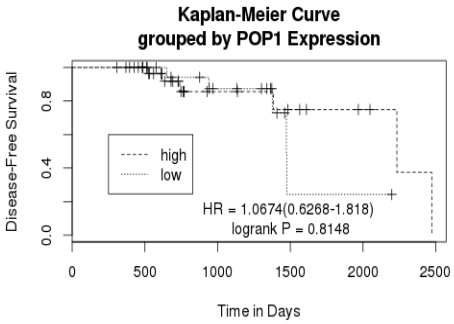

Kaplan-Meier Curve
grouped by OSBPL10 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.76(0.8682-3.568)
logrank P = 0.1076


Kaplan-Meier Curve
grouped by TANK Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.1506(0.6144-2.155)
logrank P = 0.6552

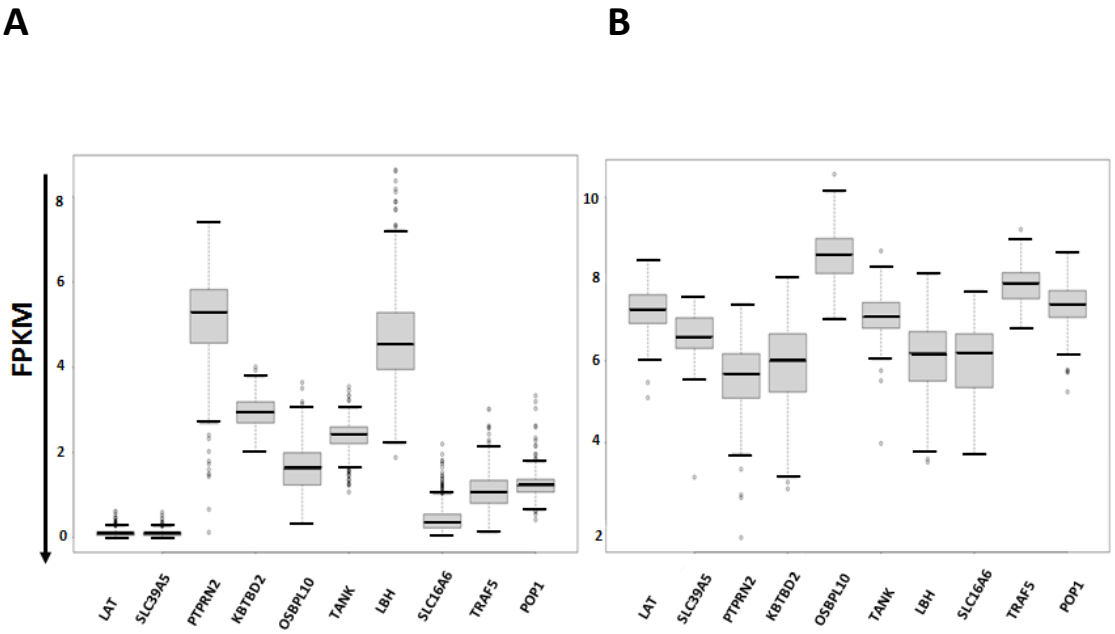

Kaplan-Meier Curve
grouped by LBH Expression
Disease-Free Survival
Time in Days
high
low
HR = 0.8646(0.4015-1.862)
logrank P = 0.706


Kaplan-Meier Curve
grouped by SLC16A6 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.1394(0.8223-1.579)
logrank P = 0.4442

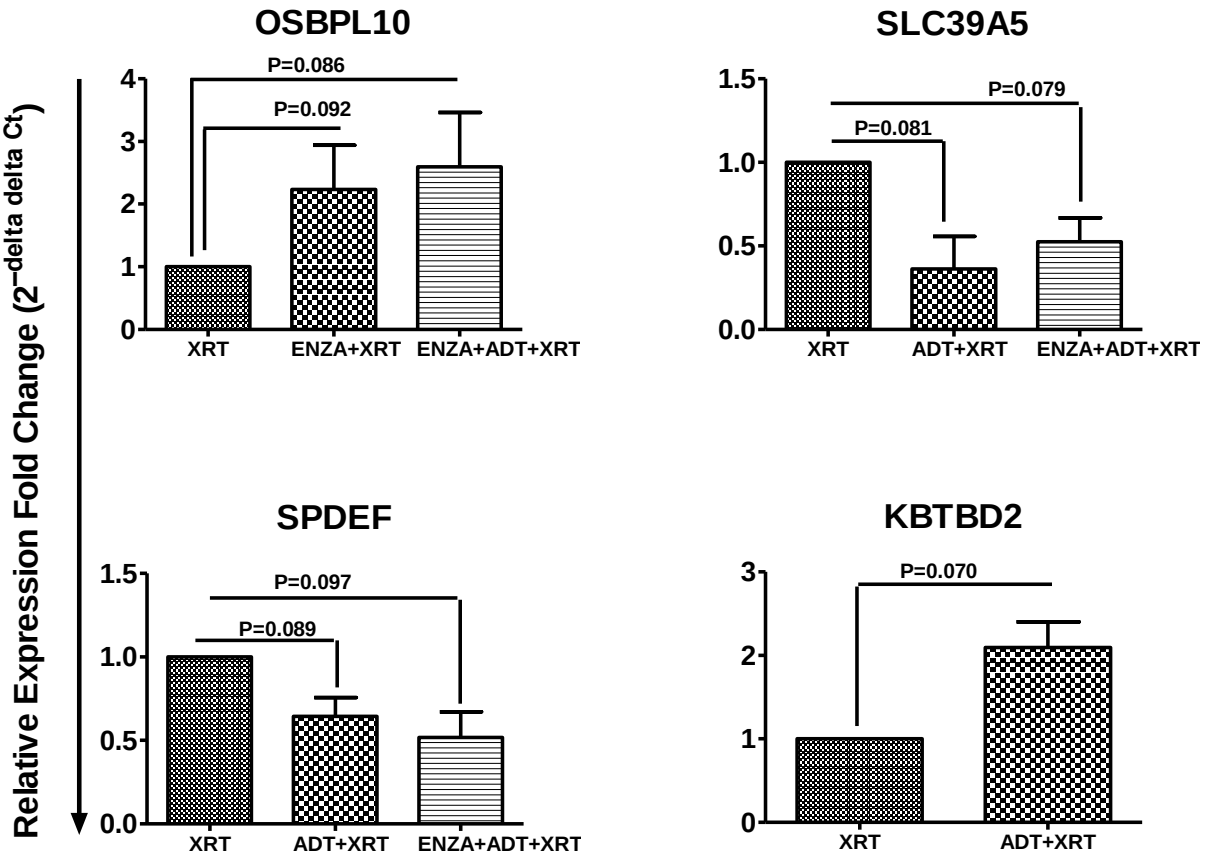

Kaplan-Meier Curve
grouped by TRAF5 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.0079(0.5648-1.799)
logrank P = 0.9786


Kaplan-Meier Curve
grouped by POP1 Expression
Disease-Free Survival
Time in Days
high
low
HR = 1.0674(0.6268-1.818)
logrank P = 0.8148

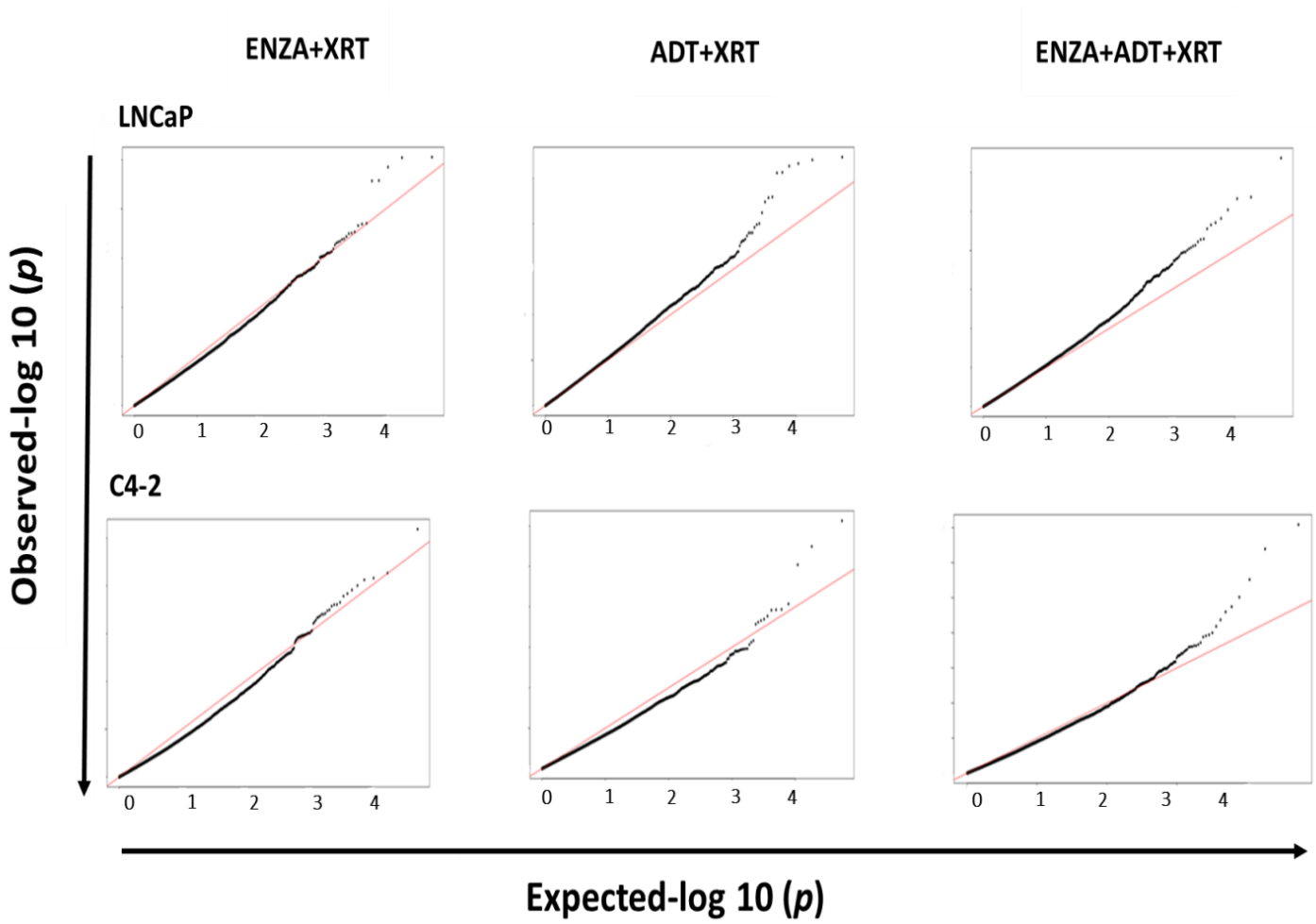
Supplementary Figure 3-6. Kaplan–Meier disease free survival (DFS) curves for radiated groups. Kaplan- Meier curve compares the DFS in patients with high (above mean) and low (below mean) expression of the candidate genes, analyzed from TCGA PRAD database in radiated group. The survival curves were compared using the log-rank test.



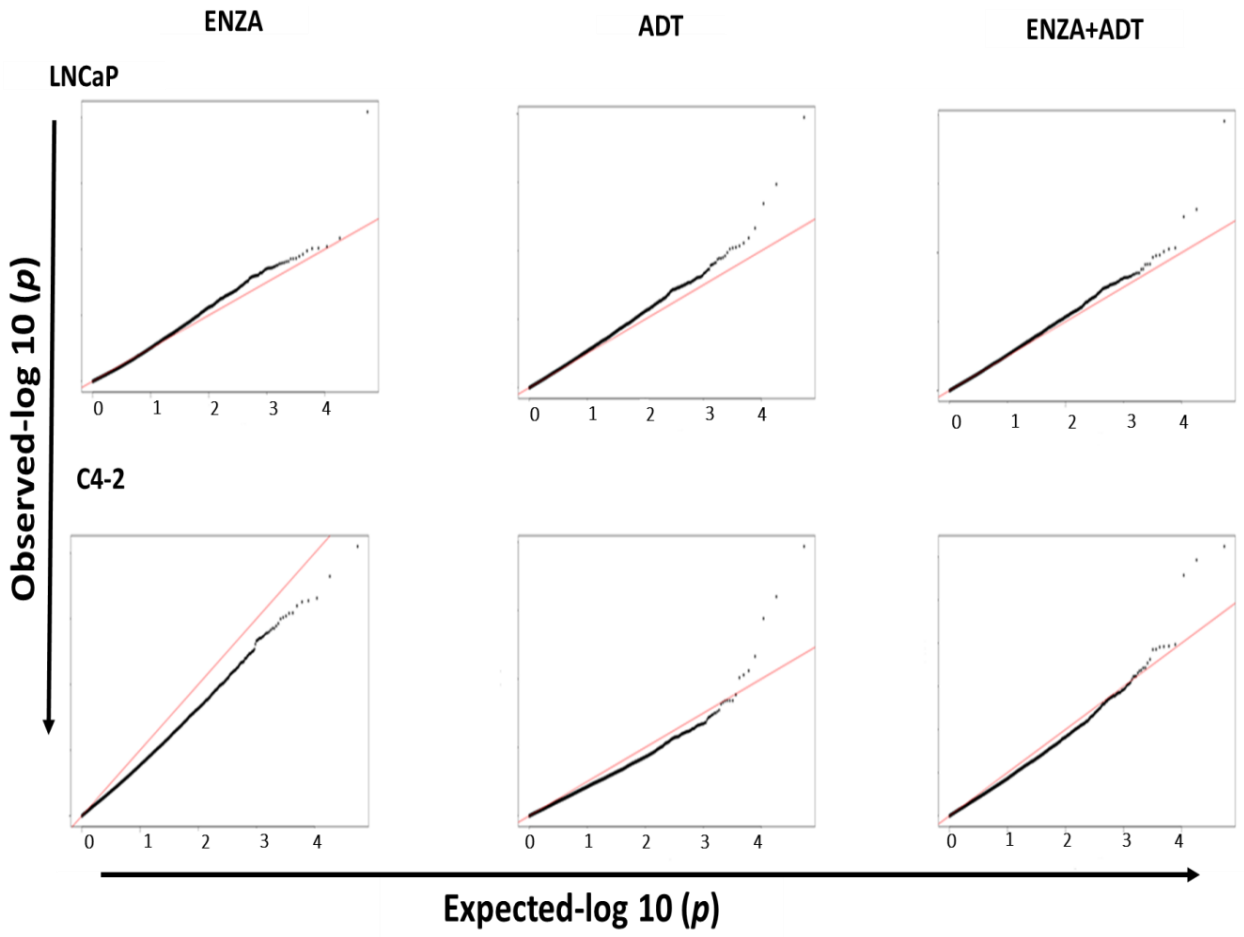
Supplementary Figure 3-7. Box plot of log2-transformed FPKM values for selected candidate genes from TCGA-PRAD (A) and GSE25136 (B) datasets. Box plots represent the differences in transcript levels of each candidate gene in two datasets. The median value is represented by the middle line in the boxes.



Supplementary Figure 3-8. Validation of the microarray data by RT-qPCR. A set of up regulated and downregulated genes in LNCaP cells was analyzed by RT-qPCR to validate the microarray data. All the RT-qPCR results were normalized to the expression level of GAPDH in each sample. Results are presented as the mean ± SE. The level of significance in the statistical analysis is indicated as (*) *p*-value <0.05, (**) *p*-value <0.01, (***) *p*- value <0.001 using two-tailed test.



Supplementary Figure 3-9. Quantile-quantile plot of observed-log 10 (*p*) values vs. expected-log 10 (*p*) values in LNCaP and C4-2 cells in radiated groups (ENZA+XRT, ADT+XRT, ENZA+ADT+XRT). Diagonal line (expected *p*-values) indicates a *p*-value distribution. Upward deviations indicate *p*-value more significant than expected. XRT=Radiation, ENZA=Enzalutamide, ADT=Androgen deprivation therapy.



Supplementary Figure 3-10. Quantile-quantile plot of observed-log 10 (*p*) values vs. expected-log 10 (*p*) values in LNCaP and C4-2 cells in non-radiated groups (ENZA, ADT, ENZA+ADT). Diagonal line (expected *p*-values) indicates a *p*-value distribution. Upward deviations indicate *p*-values more significant than expected. CTR=Control, ENZA=Enzalutamide, ADT=Androgen deprivation therapy.

3.8. References

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Chapter 4

Combining Prostate Cancer Radiotherapy with Androgen Receptor Axis Targeted Therapies

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Running title: Combining novel androgen receptor with radiation therapy

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4.1. Preface

The narrative review presented in the next chapter discusses the relevant history of the combination of ADT and XRT, explores the potential mechanisms for their synergy, and considers strategies for improved therapeutic combinations. Furthermore, we aimed to discuss ongoing trials investigating the topic.

4.2. Abstract

Background: Prostate cancer (PCa) is the most common non-dermatological cancer and third leading cause male cancer mortality in Canada. In high risk patients with localized or recurrent PCa, management typically includes the combination of long-term androgen deprivation therapy (ADT) and radiotherapy (XRT). New androgen receptor axis targeted therapies (ARATs) offer an option to intensify therapy that awaits validation.

Methods: In this narrative review, we report upon the relevant history that has supported combining ADT with XRT. A search of PubMed collected literature published between 1995 and 2019 involving prostate cancer and novel ARATs (abiraterone acetate, enzalutamide, apalutamide, darolutamide). Literature discussing clinical trials of the combination of these modalities were extracted then synthesized into a combined molecular and clinical discussion. Potential treatment intensification mechanisms and rationales are explored.

Results: Early results from three phase I/II trials demonstrated that concurrent abiraterone acetate, ADT, and XRT is safe, improves the extent of chemical castration, and has limited treatment failures. A single in vitro study has implied synergy for radiosensitization beyond what is facilitated by conventional ADT. Studies investigating the combination of other ARATs with XRT are underway, including multiple phase III trials, but short term results are not yet available.

Conclusion: This review outlined the rationale for combining XRT with the novel ARATs and summarised the ongoing trials awaited to report on the subject.

Keywords: Androgen deprivation therapy, Abiraterone acetate, Apalutamide, Darolutamide, Enzalutamide, Prostate cancer, Radiation therapy, Treatment intensification

4.3. Introduction

Prostate cancer (PCa) is the most common non-dermatological malignancy to afflict men in both Europe and North America [1]. Given the broad prognosis among localized PCa patients, they are typically stratified into either low, intermediate, or high-risk (Table 4-1). In high-risk PCa, a common treatment approach is combining radiotherapy (XRT) with androgen deprivation therapy (ADT). Extensive evidence from randomized control trials and meta-analyses have demonstrated that ADT with XRT, generally external beam radiotherapy (EBRT), further benefitted the full spectrum of pertinent oncological outcomes in localized PCa: overall survival (OS), metastasis-free survival (MFS), biochemical progression free survival (bPFS), and local failure (LF) [2-10].

Even though dose escalated EBRT (DE-EBRT) has improved multiple relevant endpoints, the highest risk groups of PCa patients are still being considered for treatment intensification. Without such intensification, by 6 years almost 50% of these patients will have failed treatment with DE-EBRT and long-term ADT (LT-ADT) [11]. To refine our ability to design trials that further intensity therapy we must better understanding ADT's mechanisms for sensitizing PCa to XRT. This review walks through the relevant history of combining these therapies, explores the potential mechanisms for their synergy, and considers strategies for improved therapeutic combinations.

Risk group	Low-risk	Intermediate-risk	High-risk
NCCN			
Tumour stage	cT1c–cT2a and	cT2b–2c and/or	cT3 or
PSA value	<10.0 ng/mL and	>0.0–20.0 ng/mL and/or	>20.0 ng/mL or
Gleason score	<7	= 7	8–10
D'Amico			
Tumour stage	cT1c–2a and	cT2b and/or	cT2c–cT3 or
PSA value	<10.0 ng/mL and	>10.0–20.0 ng/mL and/or	>20.0 ng/mL or
Gleason score	<7	= 7	8–10

Table 4-1. Risk group according to the National Comprehensive Cancer Network and D'Amico. NCCN: National Comprehensive Cancer Network, **PSA**: Prostate specific antigen.

4.4. Evidence supporting neoadjuvant or adjuvant ADT in combination with EBRT

Several phase III randomized trials have proven the benefit of combining neoadjuvant and adjuvant ADT with EBRT for high-risk PCa patients [2-6] (Table 4-2). Though meta-analysis identified that the addition of ADT significantly decreased the risk of biochemical failure (BF), LF, distant metastases (DMs), DSS, and OS, these studies were in the era of conventional EBRT dosing (≤70 Gy) [12].

Once DE-EBRT (≥74Gy) was noted to improve biochemical outcomes relative to conventional doses [13], it was necessary to demonstrate that still ADT improved outcomes, in order to justify its toxicity [14]. Two trials in combined populations of high & intermediate risk patients, EORTC 22991 and MRC RT01, have long terms results that demonstrated combination short-term ADT (ST-ADT) improved upon DE-EBRT alone in regards to 5-year and 10-year bPFS, respectively, while survival data matures [7,8]. Preliminary results from phase III DE-EBRT trials (PCS III, GETUG 14) implied that only a bPFS benefit may exist for adding ST-ADT to DE-EBRT in intermediate-risk prostate patient [9,10] .

Trials	Population	Management arms	5-yrs outcome	10-yrs outcome
Conventional dose XRT				
D'Amico [2]	Int/high-risk PCa	Arm 1= XRT Arm 2= XRT + ADT (6 mos.)	OS = 77/88 bPFS=55/79	OS = 61/74 (8 yrs.) DSS= 84/78 (8 yrs.) OS = 28/35^{NS} (15 yrs.)
RTOG 85-31[3]	High-risk PCa	Arm 1= XRT Arm 2= XRT+ADT (Indefinite ADT)	bRFS = 21/55 DM = 29/15 OS=71/76	DM = 39/24 OS = 39/49 bPFS =9/31 LF =38/23 DSS=78/84
EORTC 22863 [4]	High-risk PCa	Arm 1= XRT Arm 2= XRT+ADT (3yrs.)	bPFS =45/76 DM = 29/10 OS = 62/78 LF=1/7	DFS = 23/48 OS = 40/58 DSS= 10/30
RTOG 8610 [5]	High-risk PCa	Arm 1= XRT Arm 2= XRT+ ADT (4 mos.)	bRFS = 10/28 DM = 39/29 OS = 68/72^{NS}	OS= 34/43 ^{NS} bRFS=80/65 DM=27/35 DSS=23/36
RTOG 9408 [6]	Low/Int/high-risk	Arm 1= XRT Arm 2= XRT+ ADT (4 mos.)	LF=39/21 (2 yrs.)	bRFS =59/47 OS=57/62 CSS=92/96 DM=8/6

Dose-escalated XRT				
EORTC 22991 [7]	Int/high-risk PCa	Arm 1 = XRT (70, 74, 78 Gy) Arm 2 = XRT (70, 74, 78 Gy) + ADT (6 mos.)	bRFS = 70/83 DM = 4/8[NS] OS = 88/91[NS]	NR
MRC RT01 [8]	Low/Int/high-risk	Arm 1 = XRT (64 Gy) + ADT (3-6 mos.) Arm 2 = XRT (74 Gy) + ADT (3-6 mos.)	-	bPFS=43/55 OS=71/71* *=NS btw arm 1-2
PCS III [9]	Int-risk PCa	Arm 1= XRT (70 Gy) + ADT (6 mos.) Arm 2= XRT (76 Gy) + ADT (6 mos.) Arm 3 = XRT (76 Gy)	BF= 7/2/14 [*] DFS = 93/97/86 [*] OS = 90/94/91[NS] *=NS btw arm 1-2	BF= 22/22/33 [*] DFS = 78/78/67 [*] OS = 63/72/75[NS] *=NS btw arm 1-2
GETUG 14 **EU20503/NCT00104741** [10]	Int-risk PCa	Arm 1= XRT (80 Gy) Arm 2 = XRT (80 Gy) + ADT (4 mos.)	bRFS =76/84 OS = 94/93 [NS]	NR

Table 4-2. Phase III trials comparing ADT plus XRT to XRT. ADT= Androgen deprivation therapy; **BF**= Biochemical failure; **bRFS=** Biochemical relapse-free survival; **DSS**= Disease-specific survival; **DFS**= Disease-free survival; **DM**= Distant metastases; **EORTC=** European organization for research and treatment of cancer; **Gy**= Gray; **Int**= Intermediate; **LF=** Local failure; **Mos**= Months; **NS**= Not significant; **NR**= Not reported; **OS**= Overall survival; **RTOG**= Radiation therapy oncology group; **XRT=** Radiation therapy; **Yrs.** = Years.

4.5. The Optimal duration of ADT in combination with EBRT

Among four randomized trials, it was observed that at least 4-6 months of ADT were required for clinical benefit (Table 4-3). In these heterogeneous trials of intermediate and high risk patients, biochemical control rates with ST-ADT were compared to EBRT alone. TROG 96.01 demonstrated that both 3 months and 6 months of ADT were superior to no ADT [15]. Quebec L-101 and L-200 instead compared 3-5 or 10 of months ADT, appreciating improved biochemical control with both regimes [16]. However, only RTOG 9910 has intercompared ST-ADT durations. They did not find that extending ST-ADT from 4 to 9 months further improved any biochemical or survival outcomes [17].

The extension of ADT to its present standard 24-36 months in high risk PCa was largely based on EORTC 22961 and RTOG 9202 [18-20]. They compared ST- and LT-ADT with conventional dose EBRT in predominantly high-risk PCa patients. Both studies showed improved DSS and OS with LT-ADT (28–36 months), as opposed to ST-ADT (4–6 months).

Two trials evaluated if this length of ADT was still necessary for high risk PCa patients in the era of DE-EBRT. DART01/05 found that extending ADT from 4 months to 28 months improved biochemical control, MFS and OS [21]. PCS IV was designed to and showed that intermediate-term ADT (18 months) was not inferior to LT-ADT (36 months) in regards to OS [22]. Given the gains in quality of life with a shorter course of ADT, these results have prompted consideration for intermediate-term ADT as a new standard of care for select high risk PCa patients treated with DE-EBRT.

Trials	Population	Management arms	5-yrs outcome	10-yrs outcome
Conventional dose XRT				
TROG 96.01 [15]	Int-risk PCa	Arm 1=XRT Arm 2= XRT + ADT (3 mos.) Arm 3= XRT + ADT (6 mos.)	bRFS=32/49/52 DM=19/22/13* *NS btw arm1-2	bRFS=70/60/53 OS=57/63/71* *NS btw arm1-2
Quebec L-101 [16]	Int/High-risk PCa	Arm 1= XRT alone Arm 2= XRT + ADT (3 mos.) Arm 3= XRT + ADT (10 mos.)	bRFS (7 yrs.) 42, 66, 69 [S]	NR
Quebec L-200 [16]	Low/Int/high-risk PCa	Arm 1= XRT + ADT (5 mos.) Arm 2= XRT + ADT (10 mos.)	bRFS (4 yrs.) 70, 70 [NS]	NR
RTOG 9910 [17]	Int-risk PCa	Arm 1= XRT+ADT (9 mos.) Arm 2= XRT+ADT (4 mos.)	NR	bRFS=73/73[NS] DM = 6/6N[S] OS = 67/66[Ns]
EORTC 22961[18]	High-risk or locally advanced disease	Arm 1= XRT+ADT (6 mos.) Arm 2= XRT+ADT (36 mos.)	OS=81/85 DSS= 95/97	NR
RTOG 9202 [19]	High-risk or locally advanced disease	Arm 1= XRT+ADT (4 mos.) Arm 2= XRT+ADT (28 mos.)	bRFS=44/72 DM=17/12 OS=79/80 [NS]	bRFS=32/48 DM=23/15 OS=51/54 [NS]
Dose-escalated XRT				
DART01/05 [21]	Int/high-risk PCa	Arm 1 = XRT (70, 74, 78 Gy) + ADT (4 mos.) Arm 2 = XRT (70, 74, 78 Gy) + ADT (28 mos.)	bRFS = 81/90 DMFS = 83/94	NR
PCS IV [22]	High-risk PCa	Arm 1= XRT+ADT (36 mos.) Arm 2= XRT+ADT (18 mos.)	OS=91/86 [NS]	OS=62/59 [NS]

Table 4-3. Clinical trials comparing different durations of ADT with conventional or dose-escalated XRT. ADT= Androgen deprivation therapy; **bRFS=** Biochemical relapse-free survival; **DSS=** Disease-specific survival; **DM**= Distant metastases; **EORTC=** European organization for research and treatment of cancer; **Gy**= Gray; Int= Intermediate; **LF=** Local failure; **Mos=** months; **NR**= Not reported; **NS**= Not significant; **OS=** Overall survival; **RTOG**= Radiation therapy oncology group; **TROG=** Trans-Tasman Radiation Oncology Group; **XRT=** Radiation therapy; **Yrs.** = years.

4.6. ADT plus XRT: Intracellular Mechanisms for Interaction

There are numerous preclinical studies, in vitro and in vivo, that evaluated the interactions between ADT and XRT. From patient specimens, ADT has been observed to induce apoptosis in epithelial cells and inhibit proliferation [23]. In vitro studies of LNCaP cells (an androgen receptor (AR)-positive hormone-sensitive human PCa cell line) treated with ADT and XRT produced more apoptosis than either monotherapy [24]. Combination treatment with goserelin and XRT of both LNCaP and PC3 (AR-negative hormone-insensitive human PCa cell line) cultures inhibited cell proliferation via epidermal growth factor receptor inhibition [25]. However, neither study demonstrated the statistically significant reduction in survival required to demonstrate radiosensitivity.

Zietman et al. were the first to show that ADT reduces the TCD50, the dose of XRT necessary to the control 50% of the cultured tumors [26]. They hypothesized two non-exclusive molecular mechanisms for this radiosensitization - suppression of tumor neovascularization would improve blood flow through the more competent vasculature and/or apoptosis-induced cytoreduction would facilitate vascular access to hypoxic tissues.

Tumor hypoxia has since been associated with impaired outcomes in PCa [27]. Mechanistically, hypoxia's stimulation of angiogenic factors (ex. vascular endothelial growth factor (VEGF)) impairs tumor oxygenation secondary to the formation of incompetent vasculature [28]. As ADT suppresses hypoxia-induced AR activity, the subsequent inhibition of hypoxia-inducible factor 1α transcription reduces VEGF expression and limits neovascularization of hormone-sensitive PCa cells, providing in vitro support for this hypothesis [29,30].

It is important to appreciate that radiotherapy initially upregulates AR expression, with preclinical studies showing that radiotherapy induces the expression of AR regulated proteins [31]. Goodwin et al.'s work outlines how castration-resistant PCa (CRPC)'s addiction to the AR influences DNA repair and thus radioresistance. Following XRT, DNA double strand breaks (DSBs) activated the AR to enhance the expression of numerous DNA repair genes: DNA-dependent protein kinase catalytic subunit (DNA-PKcs), KU70, Poly-ADP ribose polymerase 1 and DNA repair protein RAD5 [32]. More than 32 DNA repair genes contain AR binding sites in their enhancer sequences [33].

The induction of these proteins can induce a positive feedback loop that causes radioresistance [32]. Initially, XRT recruited DNA-PKcs to a DSB. Subsequent DNA-PKcs activation also increased the transcription of the AR. The AR then induced the expression and activity of additional DNA-PKcs via AR-mediated DNA repair. Ultimately, the DNA-PKcs and AR upregulated each other in a process that expedited repair of XRT's DSBs. Importantly, intervention can interrupt the process. Following castration, the decreased KU70 expression observed in prostate tissue implied the tumor became more radiosensitive [32].

4.7. Strategies for improved therapeutic combination of ADT and EBRT

Relative to older anti-androgens, which were efficacious despite only achieving partial antagonism of the AR [34,35], modern AR-antagonists (ex. apalutamide, enzalutamide) have significantly improved binding affinity, can penetrate the cell for intracytosolic binding, and inhibit the nuclear translocation of the AR [36,37]. Preclinically, our group demonstrated that a modern AR-antagonist induced radiosensitisation beyond what was seen with ADT alone [38]. Enzalutamide alone potentiated the response to radiation in LNCaP cells and in combination with ADT in C4-2 (hormone-resistant human PCa cells) with dose enhancement factors of 1.75 and 1.30, respectively. Maximal radiosensitisation was achieved when enzalutamide was provided concurrently – as opposed to prior or post XRT – and increased γH2AX expression was consistent with enhanced DNA damage.

The other new agents in this class of modern AR axis-targeted therapies (ARATs) are abiraterone and darolutamide. Darolutamide is an AR-antagonist as well, but maintains efficacy against the AR F876L, AR W741L and AR T877A resistance mutations which limit apalutamide and enzalutamide efficacy [39]. Also, its limited access to cerebral circulation has produced a modest neurocognitive adverse effect profile [40].

Abiraterone's mechanism differs significantly. Despite castrate levels of serum testosterone being achieved by ADT in most patients, intraprostatic androgen or adrenal androgens (dehydroepiandrosterone and androstenedione) production are sufficient to maintain the expression of androgen-responsive genes [41,42]. Abiraterone selectively and irreversibly reduced both of these androgen biosynthesis pathways by potent inhibition of CYP17A1,

suppressing the predominant remaining pathway for androgen biosynthesis [43]. Theoretically, this could better potentiate the synergistic benefits seen with less complete suppression of the androgen axis [26].

The most recent clinical trial investigating DE-EBRT and LT-ADT in a high risk in need of further intensification showed worrisome rates of relapse, approaching 50% at 6 years post-treatment [11]. While treatment intensification with chemotherapy can improve survival, the benefit came at the cost of increased toxicity, including treatment related deaths. Implementing next-generation ARATs offers a more tolerable route to treatment intensification the localized setting, preserving docetaxel as an effective avenue for metastatic PCa [44-46]. As these ARATs have demonstrated clinical efficacy in more advanced clinical settings [47-51] and the preclinical evidence that these agents are radiosensitizers [38], their combination with XRT is the next logical step for treatment intensification in high-risk PCa patients.

4.8. Material and methods

A literature search of PubMed was conducted and relevant articles were reviewed. The following search terms and phrases were used individually and in combination: “localized PCa”, “androgen deprivation therapy”, “radiation therapy”, “randomized trial”, “review”, “high-risk PCa, “intensification”, “enzalutamide”, “abiraterone acetate”, “apalutamide”, and “darolutamide”, “clinical trials”. The time frame was from 1995 to 2019. All published, presented, or registered trials addressing the concurrent use of novel ARATs with XRT, were extracted for further review. Extracted works that combined XRT with a novel ARAT had their populations, interventions, and outcomes extracted and summarized.

4.9. Results

4.9.1. Novel molecules targeting AR pathway in prostate cancer

4.9.1.1. Clinical Trials combining abiraterone acetate with XRT

Three trials combined abiraterone with XRT were found (Table 4-4). A single phase I study investigated the safety of combining abiraterone with salvage radiotherapy [52] and two phase II trials evaluated efficacy as evaluated by the extent of castration, as assessed by testosterone levels [53,54]. Among the two phase II trials, they varied in their populations and ADT duration. At a median follow-up at 21 and 23 months, there has been a single treatment failure in the phase II studies. Of note, in the one two arm study, comparing abiraterone monotherapy to combined therapy with ADT, only 78% of men achieved castration levels of testosterone with monotherapy compared to 100% with combination therapy. Toxicity data noted a 64% cumulative incidence in grade 3 lymphopenia while undergoing DE-EBRT [54].

NCT Identifier	Phase	#Pts.	Eligibility	Management Arms	Status	Primary endpoints	Results/end points
NCT01780220 (CARLHA)[52]	I, II	43	BCR after PR	Arm 1 = AA+ P + ADT+ XRT Arm 2 = AA + P + XRT	Completed	Determine MTD	MTD was 750 mg daily when provided with XRT; Complete castration – 100% in Arm 1; 78% in Arm 2
NCT01717053[53]	II	37	High-risk PCa	Arm 1 = AA+ P + ADT+ XRT	Completed	PSA < 0.1 ng/mL at one year	Median follow-up of 23 Mos; PSA <0.1 ng/mL in 52%; PSA < 0.5 ng/mL in 95%; no failures by Phoenix definition to date 32% Grade 3 toxicities, no Grade 4+ toxicity; 62% had testosterone recovery at 12 months
NCT01023061 (RAD1)[54]	II	24	Int/high-risk PCa	Arm 1 =AA+ P+ ADT+ XRT	Completed	Toxicity evaluation	No Grade 4 toxicity and no treatment was stopped prematurely by protocol. Grade 3 toxicities included lymphopenia (64%), hypertension (9%), elevated LFTs (9%), fatigue (5%), and hypokalaemia (5%). Single incidence of BF(1/22), median follow-up 21 Mos

Table 4-4. Clinical trials: Abiraterone acetate plus XRT for prostate cancer. AA= Abiraterone acetate; **ADT**= Androgen deprivation therapy; **BCR**= Biochemical recurrence; **BF=** biochemical failure; **Int**= Intermediate; **LFTs**= liver function tests; **Mos**= Months; **MTD**= Maximum Tolerated Dose; **NCT**= National clinical trial; **P**= Prednisolone; **PSA=** prostate specific antigen; **RP**= Radical prostatectomy; **XRT**= Radiation therapy.

4.9.1.2 Clinical trials combining enzalutamide with XRT

Nine ongoing clinical trials assessing the combination of enzalutamide and XRT were found (Table 4-5), seven of which are phase II [55-62]. The patient populations evaluated are predominantly intermediate and high-risk PCa, with two trials evaluating patients undergoing salvage radiotherapy [59]. The primary endpoints in most of these phase II trials were acute and late toxicities and biochemical endpoints.

Two studies are randomized control trials – ENZARAD [60] (n=802; accrual finished) and NCT02203695 [62] (target n=122). NCT02203696, a multicentre trial randomizing patients undergoing salvage radiotherapy and ST-ADT to receive either enzalutamide or placebo has a primary endpoint in biochemical control. ENZARAD is a fully accrued phase III trial that randomized high-risk PCa patients to receive either enzalutamide or placebo for 24 months in addition to DE-EBRT and LT-ADT. Its primary outcome is overall survival and based on the timing of its accrual it is expected to be the first phase III trial to provide insight as to whether an ARAT can safely and effectively intensify DE-EBRT and ADT.

NCT Identifier	Phase	Eligibility	#Pts.	Management Arms	Primary endpoint	Secondary endpoint
NCT02028988 [55]	II	Int-risk PCa	64	Arm 1 = ENZA+XRT	PSA level after 6 months of ENZA therapy	QOL, hormonal levels, and body fat
NCT02508636 [56]	II	High-risk PCa	11	Arm 1 = ENZA+ ADT+ XRT	Rate of acute toxicity Rate of late toxicity PSA-CR Rate	bPFS, DFS, time to clinical progression and QOL.
NCT03196388 [57] (ENZART)	II	Int-risk PCa	70	Arm 1 = ENZA+XRT	80% reduction of baseline PSA	-
NCT02064582 [58]	II	High-risk PCa	9	Arm 1 = ENZA +ADT+XRT	Assess the safety and tolerability of combining ENZA+ ADT + XRT	Assessing intra-tumoural androgen regulated gene expression pre and post combination therapy
NCT02057939 (STREAM)[59]	II	BCR following PR	38	Arm 1= ENZA+ADT+SRT	PFS	bPFS, PFS, PSA less than 0.1, PSA nadir and time to testosterone recovery
NCT02446444 [60] (ENZARAD) (ANZUP 1303)	II I	High-risk PCa	802	Arm 1 = ENZA+ ADT +XRT Arm 2 = NSAA + ADT+XRT	OS	CSS, bPFS, PFS, and MFS

NCT02023463 [61]	I	Int/high-risk PCa	25	Arm1 = ENZA+ ADT+XRT	Acute toxicities	PSA levels and QOL
NCT02203695 [62]	II	BCR following PR	122	Arm 1 = SRT + PBO Arm 2 = SRT + ENZA	Freedom of PSA progression	MFS rate and local recurrence

Table 4-5. Ongoing clinical trials: Enzalutamide plus XRT for prostate cancer. ADT= Androgen deprivation therapy; **bPFS**= Biochemical progression-free Survival; **BCR**= Biochemical recurrence; **CRPC**= Castration resistant prostate cancer; **DFS**= Disease-free survival; **ENZA**= Enzalutamide; **MFS**= Metastatic-free survival; **NSAA**= Non-steroidal Anti-androgen; **NCT**= National clinical trial; **OS=** Overall survival; **PCa**= Prostate cancer; **PFS**= Progression-free survival; **PSA=** Prostate Specific Antigen; **PBO=** Placebo; **PSA-CR**= Prostate Specific Antigen-Complete Response; **Int**= Intermediate; **RP**= Radical prostatectomy; **SRT=** Salvage radiation therapy; **XRT**= Radiation therapy.

4.9.1.3. Clinical trials combining apalutamide with XRT

Seven ongoing trials, all either phase II or III, were found to evaluate the combination of apalutamide with XRT [63-69] (Table 4-6). Three of seven trials combined apalutamide with DE-EBRT. The exceptions were the only identified trials to combine stereotactic body radiotherapy with a novel ARAT [68,69] and two trials of salvage radiotherapy with an ARAT [65,66]. Notably, there were two phase III randomized control trials including high risk PCa patients. The fully accrued ATLAS has a primary outcome of MFS, an established surrogate for OS [70]. ATLAS intensified the accepted standard of DE-EBRT and LT-ADT, randomizing to either apalutamide or placebo bicalutamide. In contrast, the EORTC's upcoming phase III randomized control trial limits ADT to the neoadjuvant and concurrent period with a primary outcome of DFS, but will *not* consider biochemical failure as disease progression. Intermediate and high-risk PCa patients will receive ADT and DE-EBRT, randomized to receive either apalutamide or placebo while on ADT.

NCT Identifier	Phase	Eligibility	#Pts.	Management Arms	Status	Primary endpoint	Secondary endpoint
NCT02531516 (ATLAS) [63]	III	high-risk & locally advanced PCa	1500	Arm 1 = Placebo + bicalutamide + ADT +XRT Arm 2 = Apalutamide + bicalutamide + ADT +XRT	Active, not recruiting	MFS	time to local-regional recurrence, time to castration-resistant disease, time to DM, and OS
NCT03488810 [64]	III	Int/High risk PCa	990	Arm 1 = ADT+ XRT Arm 2 = Apalutamide +ADT + XRT	Not yet recruiting	DFS	PFS, OS and PSA value and prostate cancer specific survival
NCT03311555 (STARTAR) [65]	II	BCR after RP	42	Arm 1 = Apalutamide + ADT + Docetaxel + SRT	Recruiting	PFS	Proportion of subjects with a PSA of <0.1 ng/mL and testosterone recovery, time to testosterone recovery, bPFS and median PSA nadir value
NCT03141671 (FORMULA-509) [66]	II	BCR after RP	190	Arm 1 = ADT+ bicalutamide + SRT Arm 2 =ADT +AA + apalutamide + P + SRT	Recruiting	PFS	PSA PFS, MFS, CSS, OS, time to testosterone recovery, grade 1-5 toxicities, time to reinitiation of ADT, QOL and cardiovascular events consisting of myocardial infarction
NCT03371719 [67]	II	Locally advance PCa	324	Arm 1 = PBO + XRT Arm 2 = Apalutamide + XRT	Recruiting	bPFS	DM, MFS, DFS, and cancer-specific mortality
NCT02772588 (AASUR) [68]	II	Very high-Risk PCa	58	Arm 1 = Apalutamide + AA+ P + ADT + SBRT	Recruiting	Proportion of pts. with biochemical failure	-
NCT03503344 (PILLAR) [69]	II	CRPC	60	Arm 1 = SBRT Arm 2 = Apalutamide + SBRT	Not yet recruiting	Proportion of pts. with undetectable serum PSA	Time to PSA progression, PFS and incidence of adverse events

Table 4-6. Ongoing clinical trials: Apalutamide plus XRT for prostate cancer. ADT= Androgen deprivation therapy; **bPFS**= Biochemical progression free survival; **BCR**= Biochemical recurrence; **CRPC**= Castration resistant prostate cancer; **DFS**= Disease-free survival; **Int**= Intermediate; **MFS**= Metastasis-free survival; **NCT**=National clinical trial; **PBO**= Placebo; **PFS**= Progression-free survival; **Pts.** = Patients; **P**= Prednisone; **RP**= Radical prostatectomy; **SBRT**= Stereotactic body radiotherapy; **XRT**= Radiation therapy.

4.9.1.4. Clinical trials combining darolutamide with XRT

The employed search methodology did not identify any clinical trials combining darolutamide with radiotherapy directly. Outside of the established search methodology, abstracts made reference to the upcoming Darolutamide Augments Standard Therapy for Localized High-Risk Cancer of the Prostate (DSAL-HiCaP), a randomized phase III trial in high-risk PCa patients receiving radiotherapy. Participants are randomized to receive concurrent darolutamide or placebo with XRT and LT-ADT [71] .

4.10. Discussion

This review of the clinical and preclinical evidence highlighted radiotherapy's influence on AR-mediated protein expression as well as the AR's role in enhancing DNA repair and radioresistance [31,32]. Such data outlines how the combination of ADT and XRT can disrupt this interaction to facilitate the survival benefits seen in PCa patients[33]. Despite this combination, key trials still show that an unacceptable amount of men with high-risk PCa will not achieve long-term disease control [11,19,72].

There has been limited demonstration in preclinical work than an ARAT can provide further synergy beyond ADT's known potentiation of XRT's mediated DNA damage. Combined with known clinical efficacy of these agents, there is a strong rationale to combine these agents with XRT to facilitate treatment intensification. This present review of the literature demonstrated that a multitude of studies exploring this concept are underway. While studies combining abiraterone have been completed and are not followed with phase III trials, there are randomized phase III evaluations combining XRT with apalutamide (ATLAS; NCT03488810 [63]), darolutamide (DSAL-HiCaP [71]), and enzalutamide (ENZARAD [60]). Of these, both ATLAS and ENZARAD have fully accrued though even early results are still awaited. Another incidental observation was that upcoming assessments in oligometastatic PCa may also produce clinical data about subgroups that received an ARAT in combination with radiotherapy [73]. A notable absence was that no phase III trial is exclusively accruing patients in the salvage setting, though four randomized phase II trials explore this setting [59,62,65,66] .

Reflecting on past preclinical data can direct the field in its next steps to intelligently fill this rapidly crowding clinical trials space. In consideration of ARAT and XRT scheduling, the timing of bicalutamide treatment relative to XRT impacted the radiosensitivity of hormone-sensitive cell lines [34]. Furthermore, our group's preclinical work demonstrated that concurrent enzalutamide - compared to neoadjuvant enzalutamide, adjuvant enzalutamide, or ADT – provided the most potent radiosensitization [38].

Such observations direct the field to a few key areas that should be considered for preclinical investigation prior to clinical trials. This includes: (1) Establishing the most effective radiosensitizer(s) amongst novel ARATs and (2) the scheduling of these agents to optimise

radiosensitization. With these studies completed, the duration and timing of a novel ARAT could be optimized and then be compared to the different available agents. It would be reasonable to expect that maximally suppressing the androgen axis with the combination of abiraterone and a modern AR-antagonist could further potential radiosensitization. Such preclinical studies could have adequate signal generated to support a clinical trial.

The ongoing clinical work and the opportunities for preclinical studies hold great promise for to direct and establish novel strategies to enhance outcomes for high-risk PCa patients.

4.11. Conclusion

Suppressing the function of the AR remains an essential component in treating advanced PCa, historically via ADT. While ADT works synergistically with XRT to provide further benefit, it is unknown if the implementation novel ARATs can further potentiate this interaction. Early preclinical experiments and phase I/II studies have implied that these combinations may be efficacious. Multiple phase III trials in high risk prostate cancer patients are ongoing and will more firmly address these hypotheses.

4.12. Conflict of interest disclosures

The authors have read and understood Current Oncology's policy on disclosing conflicts of interest, and the authors declare no conflict of interest.

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Chapter 5

General Discussion

5.1. General Discussion

Following radiation therapy, DNA damage-activated AR promotes expression of many DNA repair genes, thereby enhancing the DNA repair capacity and promoting the radioresistance of human PCa cells [1,2]. This concept offers a rationale base for the clinically observed synergy between ADT and radiation therapy.

Despite the use of ADT and radiation therapy in the clinic, some evidence suggests that many men with high-risk PCa will not achieve long-term disease control [3]. A rational strategy to improve the treatment outcome for high-risk PCa would be to take new AR-targeted drugs that are proven to be effective in mCRPC and combine them with radiation to obtain synergistic anticancer activity. These agents that are currently used in the clinic are abiraterone, enzalutamide, apalutamide, and darolutamide; and all have been shown to be highly effective at prolonging cancer control and decreasing tumour progression even in men with CRPC [4]. Thus, these agents could potentiate the effect of radiation through further suppression of the AR in a population in need of effective treatment intensification. Indeed, there are several ongoing phase II trials evaluating the clinical benefit of this combination therapy in localized PCa cohorts (Table 4-5, 4-6).

With the above hypothesis in mind, our group investigated the fundamental mechanism underlying the theory and can provide many supporting data. We found that enzalutamide alone (in hormone-sensitive PCa cells) or in combination with ADT (in hormone-resistant PCa cells) potentiates radiation response stronger than ADT [5]. These results suggest that a more effective blockade of AR signalling with enzalutamide significantly increases the effect of radiation when compared to ADT in hormone-sensitive and hormone-resistant PCa cells. Evidence suggests that ADT modestly potentiates the effect of radiation in AR-positive PCa cells (LNCaP) and has no effect in AR-negative PCa cells (PC3) [6].

Any agent that affects DNA repair processes would be expected to potentiate the induction of apoptosis and clonogenic cell death following DNA damage induction. We identified that enzalutamide enhances the effect of radiation through the impaired DNA repair process, leading to enhanced radiation-induced apoptosis and senescence. Studies have shown that ADT in combination with radiation inhibits AR-mediated DSB repair, and increases radiation-induced cell death in PCa cells both in vitro and in vivo [6].

DNA-PKcs plays a key role in NHEJ pathway, the main pathway for the repair of DSBs in human. Following radiation-induced DNA DSB, DNA-PKcs was reported as a main target of AR, controlling AR-mediated DNA repair and radioresistance in PCa cells [7]. DNA-PKcs expression and activity is induced by AR, and AR activity is enhanced by DNA-PKcs, thus creating a positive feedback loop controlling DSB repair after radiation. The interaction between AR and DNA-PKcs, therefore, indicates that targeting AR may enhance the effect of radiation in PCa cells. In our study, we found that enzalutamide indeed enhanced the effect of radiation through inhibition of DNA-PKcs in PCa cells [5]. Furthermore, the initial inhibition of phosphorylation of DNA-PKcs 2 hours after radiation, and the lack of this inhibition 24 hours after radiation, suggests a possible mechanism by which the PCa cells acquire resistance to the combination of enzalutamide and radiation treatment. It is reported that AR inhibition by enzalutamide increases the Akt phosphorylation and tumour cell survival through the accumulation of DNA-PKcs on DSB site for efficient NHEJ DNA-DSB repair [8]. Perturbation of DNA-PKcs activity in AKT pathway may be one of the molecular mechanisms for enzalutamide resistance 24h after radiation.

Furthermore, the radiosensitization induced by enzalutamide is clearly schedule dependent. Concurrent administration of enzalutamide and radiation (enzalutamide added 2 hours before radiation) leads to a maximal radiosensitization when compared to the other schedules (enzalutamide added 24 hours before radiation or 24 hours after radiation) [5]. One explanation for this effect could be that enzalutamide needs to be administered at the same time with radiation as the DNA repair will be inhibited most effectively by enzalutamide at this time point compared with the other schedule time. These results suggest that the timing of enzalutamide in relationship to radiation is perhaps more critical to evaluate than just a random drug delivery.

Radiation therapy is one of the treatment options for localized PCa. However, for half of the patients, radiotherapy fails to adequately provide tumour control [3]. Hence, PCa treatment needs the identification of specific biomarkers to predict treatment outcome and to identify PCa patients who can benefit from radiation therapy. Our aim was to identify radiosensitive gene signatures induced by enzalutamide and elucidate their related signalling pathways in HS and HR PCa cells.

Using One-way ANOVA statistical analysis together with the KEGG annotations, we found that upregulation of *LAT* following combination of enzalutamide and radiation increased radiosensitization of LNCaP cells through enhanced immune and inflammation–related pathways. Furthermore, upregulation of *CYP1A1, CYP1A2, and CYP1B1* genes following combination of enzalutamide, ADT and radiation increased the radiosensitivity of C4-2 cells through the KEGG metabolic pathway. *CYP1A1* and *CYP1A2* were also found to be upregulated in non-radiated controls, thus suggesting the expression of these two genes is not specific to the radiation response. Some evidence suggests that androgen/AR exerts suppressive effects on the development and activation of T and B cells and also inhibits Th17 differentiation [9]. Sofia et al. reported that the immunogenic modulation induced by enzalutamide increases the sensitivity of PCa cells to T cell-mediated lysis [10]. Enzalutamide induces immunogenic modulation in an AR-dependent manner. Weiss et al. reported that enzalutamide at concentrations of ≥ 4 μM increases CYP1A1 and CY1A2 mRNA expression [11]. Taken together, these results strongly support the idea that AR inhibition by enzalutamide enhances the effect of radiation through immune-related pathways in hormone-sensitive PCa cells and metabolic related pathways in hormone-resistant PCa cell lines.

Locally recurrent PCa following treatment can lead to mCRPC, so there is a critical need to identify reliable prognostic biomarkers to predict cancer recurrence following treatment. Univariate Cox proportional hazards survival model revealed that there is a correlation between our candidate genes including *LAT*, *SLC39A5*, *KBTBD2*, *OSBPL10*, *LBH*, *SLC16A6*, *TANK*, *TRAF5*, and *POP1* and time to recurrence. Furthermore, a multivariate Cox regression model for the complete cohort of patients (radiated and non-radiated groups) indicated that *SLC39A5*, *OSBPL10*, *SLC16A6* have a significant independent effect on survival. These results showed that, separately or together, these three genes are associated with PCa patients' overall survival rate. Moreover, the Kaplan–Meier curve revealed that the low expression of these genes in PCa patients show an earlier onset of tumour recurrence compared to patients with a high expression of the genes.

Overall, this study's results provide the pre-clinical rational for clinical trials in order to assess the combination of enzalutamide and radiation for early-stage PCa. Our study revealed significant evidence regarding the importance of concurrent administration of enzalutamide with

radiation which might intensely alter the efficacy of radiation treatment and provide strong justification to assess the predictive potential of the identified biomarkers in clinical trials.

5.2. Clinical research proposal for validation of our biomarker in localized prostate cancer

Identifying and validating biomarkers for clinical usages is a predominant issue in the improvement of diagnostic and therapeutic methods in many diseases such as PCa. Via gene expression profiles, we identified potential prognostic and predictive biomarkers such as SLC39A5 that correlate with clinical outcome. For clinical validation of SLC39A5 biomarker in localized PCa, we propose a phase II randomized clinical trial based on our in vitro results as follow:

Title: The prognostic value of SLC39A5 biomarker in intermediate-risk localized PCa patients

Background and Rational

Currently, there are some ongoing clinical trials assessing the effect of sequential enzalutamide with radiation therapy in intermediate-risk PCa [12,13]. In these trials, the primary outcomes are PSA level and toxicity, while the secondary outcomes are PFS and QOL. Our pre-clinical data demonstrated that concurrent administration of enzalutamide and radiation (enzalutamide added 2h before radiation) lead to a maximal radiosensitization when compared to either drug administration prior (enzalutamide added 24h before radiation) or after (enzalutamide added 24h before radiation) radiation [5]. The synergistic effect between enzalutamide and radiation corresponded to impaired DNA damage repair. Patients can reasonably complete hormonal therapy and radiation in a concurrent fashion to limit the risk of toxicity associated with long term use of neoadjuvant and adjuvant enzalutamide.

Furthermore, we have found that our radiosensitive gene signatures (such as *SLC39A5*) induced by enzalutamide were associated with an improvement in recurrence-free survival (RFS) as reported in the PCa patients' cohort of the TCGA. The Zinc Transporter *Zip5* (*Slc39a5*), a member of solute carrier family 39, regulates cellular zinc homeostasis which serves as a catalytic and/or structural cofactor for a variety of proteins [14].

To date, there are no publications assessing the expression and predictive/prognostic importance of SLC39A5 biomarker in PCa patients treated with radical radiation therapy. We hypothesize that the overexpression of SLC39A5 gene will lead to better bPFS.

Objectives

A-To validate if the expression of SLC39A5 gene correlate with clinical outcome in intermediate- risk PCa patients treated with XRT with or without ENZA.

B-To evaluate if concurrent administration of ENZA with XRT results in a change in adverse events.

Endpoints

Primary Endpoints

A-To determine the predictive value of SLC39A5 gene in bPFS in patients treated with ENZA and XRT.

Secondary Endpoints

A-To determine the acute and delayed toxicity of concurrent combination of ENZA with XRT.

B-To determine whether concurrent administration of ENZA with XRT affects local failure and distant metastasis compared to radiation treated group.

Methods

This study is a double-blind controlled randomized phase II trial. In this study, our clinical team will provide paraffin-embedded tissue blocks of intermediate-risk PCa patients treated with radiation with or without enzalutamide. The tissue microarray (TMA) construction will be performed in the Jewish General Hospital's research pathology core facility. Slides of the finished blocks will be used for immunohistochemistry analysis of SLC39A5 gene.

In addition, blood samples of our target population will be obtained for evaluation of correlation between the expression of SLC39A5 gene and the clinical outcomes. For this purpose, total RNA will be isolated from peripheral blood lymphocyte using total-RNA isolation reagent PAX gene Blood RNA Kit (Qiagen, Hilden, Germany) and qPCR will be performed according to the methodology described in the previously published papers.

Study design

- Study Population

Unfavorable intermediate-risk PCa patients who meet one of these criteria:
CT2b and/or PSA>10.0–20.0 ng/mL and/or GS= 7

- Proposed intervention

1. Experimental Arm: Enzalutamide+ADT+ Radiation

A- Enzalutamide: Xtandi, oral, 160mg/ daily for a total of 2 months, given concurrent with radiation

B- ADT: Leuprolide, 22.5 mg intramuscular injection every 3 month for a total of 6 months.

C- XRT: Conventional fractionation: 78 Gy in 39 fractions; Hypofractionation: 66 Gy in 22 fractions; EBRT + Brachytherpy boost (BB): 46 Gy in 23 fractions + BB (15 Gy in single fraction)

2. Control Arm: ADT+ Radiation

A- ADT: Leuprolide, 22.5 mg intramuscular injection every 3 month for a total of 6 months.

B- XRT: Conventional fractionation: 78 Gy in 39 fractions; Hypofractionation: 66 Gy in 22 fractions; EBRT + Brachytherpy boost (BB): 46 Gy in 23 fractions + BB (15 Gy in single fraction)

Statistics

The primary outcome is the acute and delayed toxicity differences will be measured by Common Terminology Criteria for Adverse Events (CTCAE) version 4. The predictive value of the SLC39A5 marker will be based on the RFS and OS in each group.

Expectation

We expect that an improved clinical outcome will be correlated with a higher expression of the SLC39A5 gene expression in PCa patient's cohorts.

Limitations

A clinical outcome of value in PCa patients will be difficult to see and will require a big study size. This is because these patients do relatively well (ie. Their PSA's rarely progress during their two years of hormone therapy).

We might encounter some outliers among tested. If this is the case, we will verify the quality of mRNA used in the experiments and investigate other possible reasons why the mRNA expression of our candidate genes might be different from the expected results. If necessary, we will perform RNAseq analysis to explore it in depth.

5.3. Conclusion

Concurrent administration of enzalutamide with radiation may be a new treatment option for intermediate-risk localized PCa. Moreover, our radiosensitive gene signatures may be a valuable predictive and/or prognostic biomarker for identifying subgroup(s) of patients that would not respond to radiation therapy, thereby offering different treatment options. In brief, these candidates may be useful for more reliable identification of relapses or therapy failures prior to the recurrence of local or distant metastasis.

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Chapter 1

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