

CHAPTER - 1

INTRODUCTION AND REVIEW

OF LITERATURE

ABSTRACT:

Prostate cancer (1.46 million cases) is ranked as the second most common cancer following lung cancer (1.57 million cases). Various treatments have been devised for prostate cancer, including androgen blockers, stimulators, and other broad-spectrum chemotherapy drugs. Which may lead to drug resistance later. Drug resistance and metastasis contribute to over 90% of cancer deaths. Identifying novel therapies for drug-acquired resistance cancer is challenging due to the different physiological conditions, origins, metastasis, and reoccurrence of prostate cancer. Androgens play a crucial role in prostate cancer, as they bind to and activate the androgen receptor (AR) signaling, leading to cancer. Mutation in AR makes cancer androgen independent and leads to cancer relapses even though the anti-androgen therapy is being given. Cells can also develop sensitivity from AR mutations that lead to activation by molecules other than androgens. AR and tumour microenvironment playing an influential role in prostate cancer progression. Energy sensitive AMPK pathway and AR signalling pathway are interlinked so the drug which are targeting both the way and fulfilling the CRPC therapy constrains was our goal.

Metformin, marketed under the trade name Glucophage, is the first-line medication for the treatment of type 2 diabetes but AMPK pathway activation by metformin leading to mTOR inhibition can cause autophagy and thus showed anticancer activity. Hence, we tried to explore the metformin anticancer activity for prostate cancer in the *in vitro* models to find out novel therapeutic approaches for CRPC. We observed that AR and ARV7 were translationally inhibited by treatment. Moreover, Cell survival and invasion efficiency were also inhibited by metformin. The efficacy of metformin is altered by AR induction, leading to the hypothesis that AR induction, low energy, and ROS imbalance, (inherent properties of the tumour microenvironment) may alter its behaviour. Ambiguity in metformin's effects has been reported in other cancers, so we also aimed to understand the mechanism of its cell death pathway under AR heterogeneity and stress conditions. We found that metformin can inhibit prostate cancer depending on AR and stress status. Metformin is effective up to a certain level of stress; beyond that, pro survival behaviour was observed.

Further, We evaluated anticancer property of Swertiamarin, one of the bioactive from *Enicostemma littorale* plant. This compound shares many properties with metformin and is being studied in our lab for its multifunctional properties related to glucose and lipid lowering efficacy. In few research papers its anticancer activity has been reported. So we analysed to find out its efficacy on different prostate cancer cell lines and its potential was compared with respect to metformin. Our results revealed that metformin failed to inhibit CRPC under excessive stress conditions, Whereas Swertiamarin showed better efficacy in androgen independent and AR induced CRPC cell lines.

1. Introduction and Review Literature

Cancer is a disease where cells start to grow uncontrollably, which means there is no regulation over cell multiplication by any means of the body's regulatory mechanisms that are used to control cell growth. Cancer is a pathological state characterized by the process of cellular differentiation in which normal cells transform into various specialized cell types. This transformation leads to pathologically heterogeneous microenvironments, typically localized within the initial organ. This early stage of cancer is often referred to as benign cancer. Cancer cells possess the capability to invade neighbouring tissues and, if unchecked, can disseminate to distant anatomical sites. This phenomenon, known as metastasis, results in the widespread dissemination of cancerous cells throughout the organism. Various types of cancer are prevalent in the contemporary era, with multifactorial etiologies contributing to their development, although their precise origins remain elusive. Lifestyle, habits, pollution, and genetic mutation emerge as few of the major contributors. Furthermore, HPV (Herpes simplex virus) and hepatitis are also the major contributors.

1.1 Epidemiology of cancers

In the vast realm of global health data, GLOBOCAN, an online database, having comprehensive cancer statistics, chronicled a staggering 244 million cancer cases in the year 2016[(Sung et al., 2021)]. As of 2022, the database unveiled Age-Standardized Rates (ASR) per 100,000 for Incidence and Mortality across Oceania and various continents. The numerical narrative reveals 409,364,280,164 no of incidences and mortality rates of 93.4, 83.9, 106.3, and 88.3 percent for Oceania, North America, Europe, and Asia, respectively. Each figure told a story of the relentless impact of cancer on diverse corners of the globe. According to the CDC (Centers for Disease Control and Prevention),

Cancer is the second-leading cause of death in the United States, first being heart disease. One out of every four deaths in the U.S. are from cancer. Among them approximately 15 types of cancer as the most prevalent tumors. In world ranking lung cancer claimed the top spot meanwhile, breast cancer secured the second position in incidence and mortality while for second in incidence is breast cancer and in mortality colorectum. In 2022, GLOBOCAN reported approximately 15 types of

cancer as the most prevalent tumors. Among males, prostate cancer (1.46 million cases) ranked as the second most common cancer following lung cancer (1.57 million cases)[(Bray et al., 2024)].

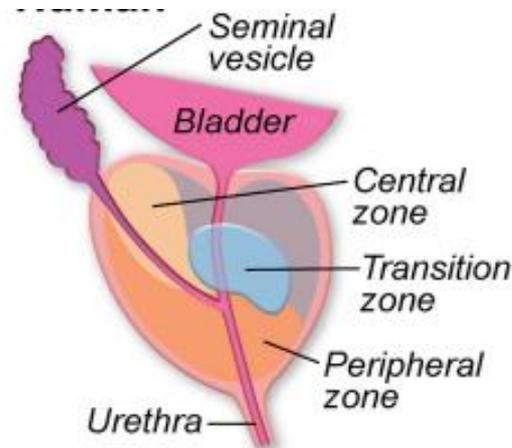


Figure 1.1 Anatomy of Prostate gland (G. Wang et al., 2018)

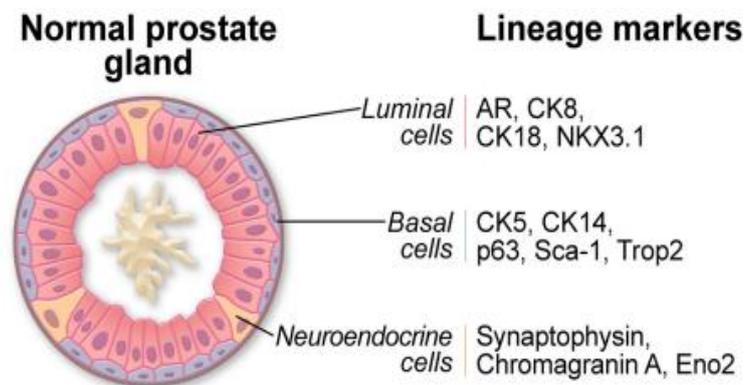


Figure 1.2 Cellular type of Prostate and its markers (G. Wang et al., 2018)

1.2 Anatomy and role of the prostate gland

Prostate gland in human urogenital system is required to form a seminal fluid of semen. Prostate is located beneath the bladder and comprises three major zones central, peripheral, and transitional. The central zone (CZ) encompasses the ductal tube extending from the seminal vesicle to its junction with the descending urethra. The peripheral zone (PZ) is located at the posterior of the gland. The Transition zone (TZ) is just below the bladder and surrounded to the transitional urethra (Figure 1.1)

[(G. Wang et al., 2018)].

Prostate cancer can originate in any of the aforementioned zones but is most commonly found in the peripheral zone. On a cellular level, the prostate contains three main types of epithelial cells: luminal, basal, and neuroendocrine (Fig 1.2). The prostate gland also possesses other cell types, such as fibroblasts, smooth muscle cells, endothelial cells, immune cells, autonomic nerve fibers, and associated ganglia cells. Cells of this type can influence both the biology and the clinical behavior of the prostate gland. These three main types of cells can be identified by their protein expressions, as mentioned in Fig 1.2. Luminal cells show high expression of AR (androgen receptor), CK8(cytokeratin-8), and CK18 (cytokeratin-18), whereas in the case of basal cells, AR expression is less pronounced but shows high expression of p63, SC-1, and Trop2. Similarly, neuroendocrine cells can be identified by their neuroendocrine markers such as Synaptophysin, Chromogranin A, and Eno2, and such cells are androgen-insensitive cells which secrete neuropeptides such as bombesin, calcitonin, and neurotensin. These cells would help the epithelial cells to proliferate and differentiate [(Abrahamsson, 1999), (Shen & Abate-Shen, 2010); (Amorino et al., 2007)]. Due to the negligible AR expression, basal cells become androgen-independent for their survival [(Bonkhoff & Remberger, 1993); (Prins, 2008), (Long et al., 2005)]. Luminal cells form columnar-shaped cells above the basal cells constituting an exocrine compartment that secretes a Prostate Specific Antigen (PSA) and Prostatic Acid Phosphatase (PAP) into the lumen. NE cells are very few and scattered in the prostate. The primary function of the prostate as a male reproductive organ is the production of prostatic fluid, which contributes to approximately 30% of the semen volume Figure 1.3 (Table 1.1). The constituents within prostatic fluid play a crucial role in enhancing sperm motility and providing nourishment. Prostatic fluid is alkaline (pH 7.3-7.5) milky fluid that contains citric acid, calcium, zinc, acid phosphatase, and fibrinolysin (Table 1.2) [(Donkervoort et al., 1977)].

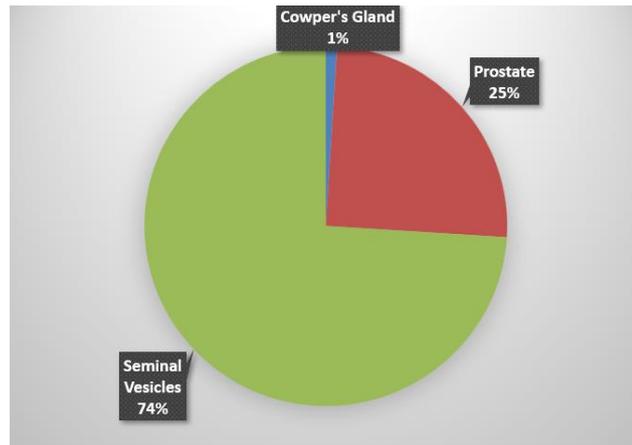


Figure 1.3 Semen volume portion distribution

Sources	% Volume	Components
Cowper's gland	<1%	mucin
Prostate	25-30%	Citric acid, Proteolytic Enzymes (PSA, amylase, Pepsinogen etc.), Prostatic fluid, seminal plasmin, prostaglandins
Seminal Vesicles	65-75%	Fructose, semenogelin, prostaglandin

Table 1.1 Source of semen volume and its components

Component	Significance
Citric Acid	ATP production via the Krebs Cycle
Proteolytic enzymes (PSA, Pepsinogen, amylase, etc.)	Break down the clotting proteins from the seminal vesicles
Prostatic Acid Phosphatase	Hydrolyze a broad variety of small organic phosphomonoesters under acidic condition
Seminalplasmin	An antibiotic that destroys bacteria
Prostaglandins	Smooth muscle contraction for sperm transport

Table 1.2 Composition and significance of Prostatic fluid

1.3 Prostate Cancer Epidemiology and Classification:

Prostate cancer ranked as the Fourth most common cancer following lung cancer in 2022. On the global stage, prostate cancer held eighth rank in mortality [(Bray et al., 2024)]. Delving into the regional nuances, Population-Based Cancer Registries (PBCR) of India reported 34,540 new cases and 16,783 deaths which representing a significant portion, over 60%, of the prostate cancer burden in the South-Central Asia region [(Budukh et al., 2023)]. Commendable Average percent change (33.7%) increase in the 5-year age-standardized net survival for prostate cancer for India during 2010-2014. While this marked progress was noteworthy, a sharp contrast emerged when compared to the United States, with survival rates of 44.3% and a staggering 97.4% [(Zhu et al., 2021)]. A perplexing trend was unfolded in United States where cancer incidence increased for prostate cancer by 3% annually from 2014 through 2019 after two decades of decline. In 2023 estimated new cases and deaths for the prostate cancer was 28,830 and 34,700 respectively which is 29% of cancer diagnosed in male. [(Siegel et al., 2023)]. On the global stage, prostate cancer held a formidable position, ranking fourth in incidence and eighth in mortality.

However, a stark disparity emerged among ethnicities, where black men faced a two-fold higher mortality rate compared to their counterparts. Amidst the concerning statistics, Asia is traditionally considered a low-incidence area, but the incidence and mortality of prostate cancer have rapidly increased across the continent [(Zhu et al., 2021)] Studies have found that the outcome of ADT might be more favorable in Asian than in North American patients. In older age (~70 years), the possibility of cancer is high without any identifiable etiological factors [(Packer & Maitland, 2016)]. Besides genetic factors, few other factors like lifestyle, dietary habits, race, and age also play a role in the development of prostate cancer. In the present era, Digital Rectal Examination (DRE) and blood tests for Prostate-Specific Antigen (PSA) are used to detect abnormal prostate growth. A DRE examination can only assess one side of the prostate, whereas a blood test for PSA can comprehensively evaluate the entire prostate. PSA levels can indicate prostate pathology even in the absence of symptoms. PSA would be <4ng/mL in normal humans; while 4-10ng/mL level is considered borderline PSA range for risk for individuals for prostate cancer. If the PSA level is detected above 10 ng/mL, the chances of having prostate cancer is

more than 50%.

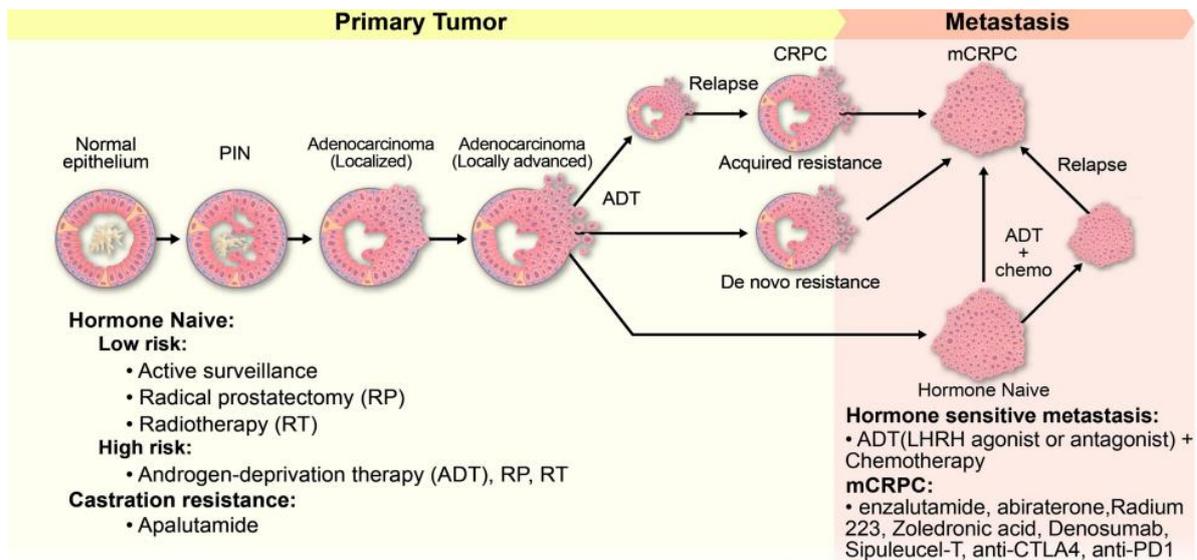


Figure 1.4 Progression of Prostate Cancer (G. Wang et al., 2018)

An elevated PSA detection level leads to an assessment of tumor grade, which can be determined through a biopsy. This procedure involves the removal of a small portion of the prostate for pathological examination of the prostate cells. After the biopsy, tumor grade can be concluded by evaluating the Gleason score and grade groups (Table 1.3). Group Grades system [(Kweldam et al., 2019)] is used to show pathological staging on how quickly cancer can spread. Malignant transformation of the cells starts from prostatic intraepithelial neoplasia (PIN). PIN is further subdivided into Low-grade PIN and High-Grade PIN of which only High-Grade PIN is considered as high chance of cancer cell transformation. The pathologist uses the TNM Staging System to describe how far the prostate cancer has spread. This system describes the tumor (T), lymph node (N) and metastasis (M) to lymph nodes and/or bones or other organs. The results are combined to determine the stage of cancer I to IV. Based on PSA level, Gleason score and TNM stages prostate cancer can be classified that whether low, intermediate or high-risk prostate cancer and the treatment approach can be tailored respectively the analysis of the genetic profile of prostate cancer plays a role in identifying markers, assisting in the planning of treatment and forecasting potential outcomes.

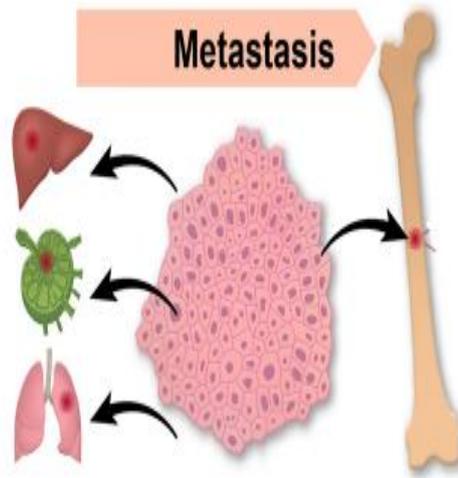


Figure 1.5 A schematic depiction of metastasis of Prostate cancer cells to the body organs(G. Wang et al., 2018)

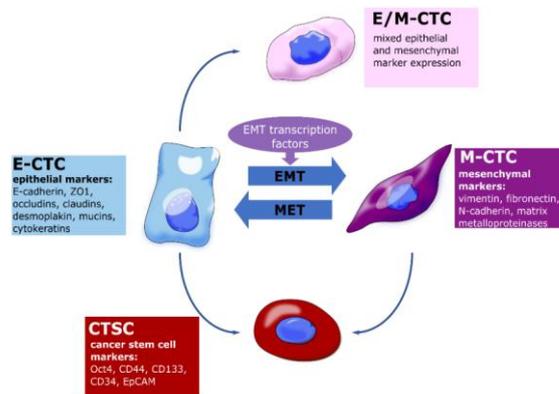


Figure 1.6 EMT-dependent formation of CTCs (Noubissi Nzeteu et al., 2022)

Gleason score	Grade
Gleason score of 6 or less	Low grade (well-differentiated)
Gleason's score of 7	Intermediate Grade (moderately-differentiated)
Gleason scores of 8 to 10	high-grade (poorly differentiated)

Table 1.3 Gleason score and grade

Approximately 60%–75% of prostate cancers arise in the peripheral zone of the prostate [(McNeal, 1969),(Haffner et al., 2009)]. The starting of luminal cell dysplasia called PIN may start to invade through basal lamina and metastasis firstly to

the lymph nodes near the prostate causing an advanced adenocarcinoma which can also disseminate to the liver, lungs, and bones by generating Circulating Tumor cells (CTC).

CTCs with stem-cell-like properties, termed circulating tumor stem-like cells (CTSCs). CTSCs are irregular cells whose phenotype may vary between cancer subtypes and even within the same type of cancer [(Noubissi Nzeteu et al., 2022)]. Before tumor cells are released, the primary tumor has the capability to create a favorable and accommodating microenvironment in surrounding tissue by releasing specific factors such as VEGF-A, tumor necrosis factor α (TNF- α) and TGF- β . Thus, EMT increases mortality by creating a favorable environment to spread cancer cells and create cell heterogeneity. In metastases, EMT (epithelial–mesenchymal transition) plays a critical role because of losing the cell-cell adhesion, polarity, and reorganization of the cytoskeleton causing a generation of CTCs. Several factors that promote oncogenesis can trigger EMT. Some of these factors are the transforming growth factor beta (TGF- β), as well as the Wnt and Notch signaling pathways. This process involves the activation of multiple transcription factors, such as ZEB, TWIST, and Snail proteins, which serve as transcriptional repressors of epithelial characteristics like E-cadherin while promoting the expression of mesenchymal features like vimentin. Cell adhesion molecules, especially E-cadherin, play a crucial role in maintaining epithelial cell junctions and apicobasal polarization, and their expression is reduced. In contrast, there is an increase in the levels of vimentin, fibronectin, and N-cadherin, which promotes the shift towards a more mobile state. Thus, prevalence of prostate cancer metastasis can be evaluated by examining the presence of EMT markers.

Despite successful treatment, some prostate cancer cases may experience a recurrence, which can manifest in various ways like biochemical recurrence, local recurrence, regional recurrence and castrate resistant prostate cancer. Biochemical recurrence shows minor higher level of PSA (2ng/ml) after prostatectomy (RP) or radiation for prostate cancer without showing any disease symptoms. Local recurrence in which increase in size of a suspicious lesion in a months after treatment and regional prostate cancer refers to prostate cancer that is identified to have grown outside the prostate organ and into nearby structures such as seminal vesicles, soft

tissues,organs, and lymph nodes. Regionally identified disease can be associated with more higher risk grade prostate cancer. In 1941 Huggins and Hodges identified that the Hormone Responsiveness of cancer cells played a central role in the castration treatment which is reported to cause tumor regression. Hence, androgen deprivation therapy (ADT) was established and drugs and treatments to stop the synthesis or binding of hormones to the receptor were used as the main course of treatment previously. Primary tumor gives a good response to ADT treatment but eventually becomes resistant to ADT and transform into resistant to ADT therapy which is called Castration-Resistant Prostate Cancer (CRPC) which leads to metastasis Castrate Resistant Prostate Cancer (mCRPC).

1.4 Mechanism of Prostate Cancer

1.4.1 Role of AR signaling pathway:

Testosterone, the main androgen produced by the body, is predominantly released by the Leydig cells of the testes (90%). Small amounts are also produced in the adrenal glands (10%). The vast majority of testosterone is synthesized in the testes, with a minimal quantity generated by the adrenal glands. While it's uncommon for prostate cells to naturally produce testosterone, certain prostate cancer cells can develop the capability to produce this hormone [(Dillard et al., 2008)]. Free circulating testosterone, which is not bound to steroid hormone-binding protein, can enter prostate cells, where it can be converted to its more potent metabolite, dihydrotestosterone (DHT) [(Radmayr et al., 2008)], which in turn binds to the AR protein. Normally low level of androgen sensitizes the release of LHRH of the hypothalamus which stimulates the pituitary gland to produce Luteinizing hormone (LH) which can stimulate testicles to produce androgens. The AR is a nuclear transcription factor that can activate and regulate the expression of many genes involved in growth and proliferation. The androgen receptor (AR), is also known as NR3C4 (nuclear receptor subfamily 3, group C, member 4). Androgen receptors belong to the steroid hormone receptors like Estrogen progesterone. The gene of the androgen receptor is located at Xq11-12. Androgen receptors consist of a total length of 919 amino acids and a total molecular weight of 110kDa. The full-length androgen receptor gene transcript includes eight exons: exon 1 codes for the terminal N, exons 2 and 3 codes for the DBD (DNA binding domain, and exons 4 to 8 codes for the LBD. The AR protein consists of 3 domains: DNA-binding domain, ligand-binding domain, and N-terminal domain (Figure 1.8)[(Crona & Whang, 2017)]. The NTD is generally considered to be constitutively active, harbors transcriptional activation function-1 (AF-1), and is critical for engaging the cellular transcription complex which comprises transactivation units 1 & 5. Among the two, TAU-5 is responsible for the majority of constitutive transcriptional activity and has been associated with aberrant AR activation in CRPC cells [(Crona & Whang, 2017)] . Inhibitory studies of the N-terminal domain resulted in decreased AR transcriptional activity, demonstrating that this domain plays a crucial role in the transcriptional transactivation activity of the AR. The ligand-binding domain, where androgens bind,

results in a conformational change in the AR, where it dissociates from heat shock proteins in the cytoplasm and localizes to the nucleus. In the nucleus, the AR binds to specific DNA sequences, called androgen-responsive elements, via the DNA-binding domain, promoting further association of factors into a complex, which leads to gene transcription. Various genes are regulated by the AR, including kallikrein-related peptidase 3 (KLK3), which encodes kallikrein-3 also known as PSA. AR amplification has not been found in any untreated prostate cancer samples, suggesting that AR amplification is one byproduct of hormone therapy leading to AIPC. Increased AR expression sensitized prostate cancer cells to lower-than-normal concentrations of androgens [(Saraon et al., 2011)]. Moreover, low AR persists in CRPC despite androgen blockade by chemical castration or surgical castration. Thus, Prostate cancer cells lead to castrate resistance mediated by AR or the androgen axis. Cells can also develop sensitivity from AR mutations that lead to activation by molecules other than androgens (decrease ligand specificity) [(Y. Chen et al., 2008)]. Serum PSA levels will again rise despite anti-androgen therapy, suggesting that the agent has begun functioning as an AR agonist; this is validated by the PSA decrease noted with anti-androgen withdrawal [(Chandrasekar et al., 2015)]. Due to such cases, it is becoming necessary to understand the canonical and noncanonical pathways of AIPC (Figure 1.7). Mutation in AR ligand binding site plays a major role in AIPC. Mostly in CRPC, AR ligand binding sites have H877 mutation broadened specificity, there are H874Y, V715M, L701H+T877A, and Y741C also found. AR splice variants have also been identified and found overexpressed in AIPC. Since AR is a nuclear receptor, it necessitates the interaction of numerous interactive proteins to become a transcriptional complex these interactive proteins are either coactivators or coregulators. AR coactivators can be categorized into different groups. SRC/p160 coactivators, like TIF2, GRIP1, and SRC1, have common structural components and the capability to recruit other transcription factors, thereby initiating the transactivation of genes regulated by AR. AR corepressor proteins are capable of establishing complexes with AR to suppress the transcription of genes regulated by AR. Any changes in the expression of these corepressor proteins can also have a significant impact on the progression of AIPC. Two extensively studied AR corepressors are NCoR (nuclear receptor corepressor) and its counterpart, SMRT (silencing mediator for retinoid and thyroid hormone receptors). Both of these corepressors have the ability to recruit histone deacetylases. The AR can be triggered

via ligand-independent pathways, often referred to as outlaw pathways. Additionally, the AR plays nongenomic roles by engaging with different signaling pathways and modifying their function. Numerous growth factors, cytokines, kinases, and various proteins have been shown to activate the AR even when androgen concentrations are low or nearly absent. Growth factors like insulin-like growth factor-1 (IGF1), keratinocyte growth factor, and epidermal growth factor (EGF) possess the capability to trigger the AR enabling the induction of AR target gene transactivation even in situations of low androgen availability [(Culig et al., 1996)]. IGF1 cannot activate the AR when antiandrogen treatment is given which suggests a direct interaction between these proteins. AR coactivators such as TIF2 and insulin-degrading enzyme expression increased by IGF1 which promotes the AR. EGF also activates AR by induction of TAT1 expression, a ligand for RTKs which illustrates the importance of RTKs. Key receptor tyrosine kinases (RTKs) known to exhibit elevated expression in various cancers, including prostate cancer, include HER2 signaling, which remains unaffected even when antiandrogen treatment is applied. Various cytokines are shown to activate the AR like the nuclear factor (NF)- κ B which is upregulated in many AIDC. IL-6 and IL-8 expressions are shown to increase in prostate cancer [(Malinowska et al., 2009)]. Likewise, IGF-1 IL-6 and IL8 both are shown to be decreased when antiandrogen treatment is administered and it proves direct connection with the AR signaling pathway. Neonatal morphogenesis of the prostate is directed by androgens and its growth in puberty. Most of the cells of the prostate express detectable levels of Androgen Receptor (AR) except stem cells of so as AR is important in cancer development. Estrogens also exert profound direct and indirect effects on the prostate. The natural role of estrogens during prostatic development is uncertain, but excessive estrogenization during prostatic development can lead to benign prostatic hyperplasia (BPH) as well as prostate cancer in older males. The changes in ratio of testosterone and estradiol in older males occur as testosterone levels tend to decrease and estrogen level remain constant or elevated during senescence that coincides observed in prostate cancer patients [Brander et.al 1983, Hayes et al 1992].

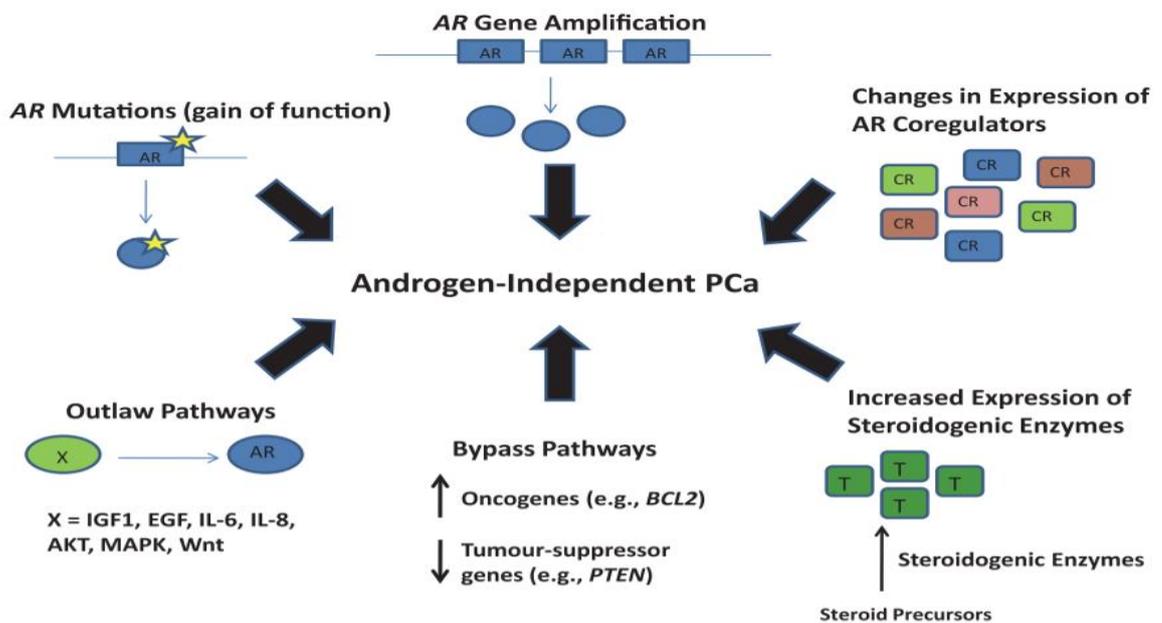


Figure 1.7 Pathways of Androgen-independent Prostate Cancer(Saraon et al.

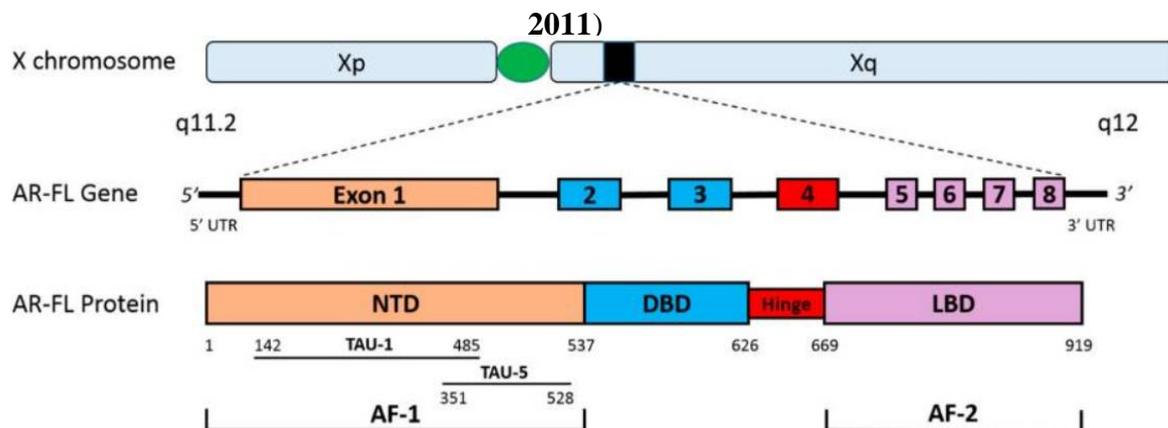


Figure 1.8 Androgen Receptor Structure(Crona and Whang 2017)

1.4.3 Apoptosis

Apoptosis, or programmed cell death, is a fundamental biological process that plays a significant role in the development and progression of prostate cancer. In normal prostate tissue, apoptosis helps control cell growth and prevents the accumulation of potentially harmful mutations. Dysregulation of Apoptosis in Prostate Cancer often arises due to genetic mutations and alterations that disrupt the balance between cell survival and cell death. Cancer cells can acquire changes that promote their resistance to apoptosis, allowing them to evade cell death and continue proliferation.

Intrinsic Pathway:

In prostate cancer cells, the intrinsic pathway is often initiated due to various stressors or damage, such as DNA damage, oxidative stress, or anti-cancer treatments. Mitochondria release pro-apoptotic factors, such as cytochrome c, into the cytoplasm. Cytochrome c binds to Apaf-1, which forms the apoptosome complex, ultimately leading to the activation of caspase-3 (figure 1.11). Activated caspase-3 cleaves a variety of cellular proteins, leading to the characteristic morphological changes of apoptosis, including cell shrinkage, chromatin condensation, and DNA fragmentation.

Extrinsic Pathway:

The extrinsic pathway involves the activation of death receptors, such as Fas (CD95) and tumor necrosis factor receptor 1 (TNFR1), on the cell surface. Binding of death ligands, such as Fas ligand (FasL) or tumor necrosis factor (TNF), to their respective receptors triggers a signaling cascade [(Department of Cell Biology, Histology and Pharmacology, University of Valladolid, Soria, Spain et al., 2018)]. Ultimately, this cascade activates caspase-8, which can directly activate caspase-3 (figure 1.9), or it can lead to bid cleavage, which can, in turn, activate the intrinsic pathway and caspase-3. Once caspase-3 is activated, it cleaves multiple cellular substrates, including structural and regulatory proteins, resulting in the hallmark features of apoptosis. These features include DNA fragmentation, nuclear condensation, cell membrane blebbing, and the formation of apoptotic bodies. The activation of caspase-3 is just one aspect of the complex process of apoptosis, but it is a critical step in controlling cell death and preventing the survival and proliferation of cancer cells [(Liu et al., 2017)]. Inducing caspase-3 activation is a therapeutic goal in many cancer treatments, including prostate cancer therapy. Agents that activate caspase-3 or promote apoptosis in cancer cells are areas of active research and drug development

Apoptosis Pathways in Prostate Cancer: In prostate cancer, apoptosis can be triggered by both intrinsic (mitochondrial-mediated) and extrinsic (receptor-mediated) pathways. Caspase-3 plays a central role in the common downstream execution phase, regardless of which pathway is activated. There are several key apoptotic pathways and proteins involved in prostate cancer, including Bcl-2 Family

Proteins. Dysregulation of anti-apoptotic Bcl-2 family proteins can inhibit apoptosis in prostate cancer cells. BCL2 is regulated by transcription factors including p53, WT1, NF- κ B and by promoter methylation. Phosphorylation of BCL2 increased interaction to BAX and BAK, while reducing the association between BCL2 and the pro-apoptotic BH3 proteins BIM, PUMA, and BAD[(Ranjan & Iwakuma, 2016, p. 53),(Ali & Kulik, 2021)](Figure 1.10)

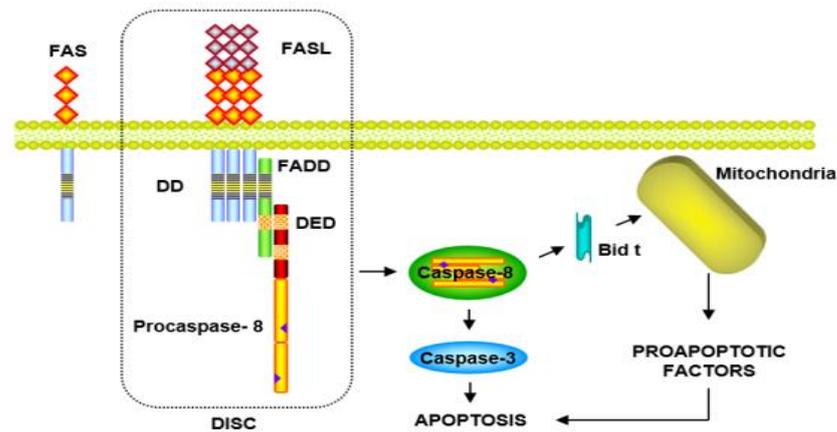


Figure 1.9 Caspase-dependent Extrinsic apoptotic pathway(Soria, Spain et al. 2018)

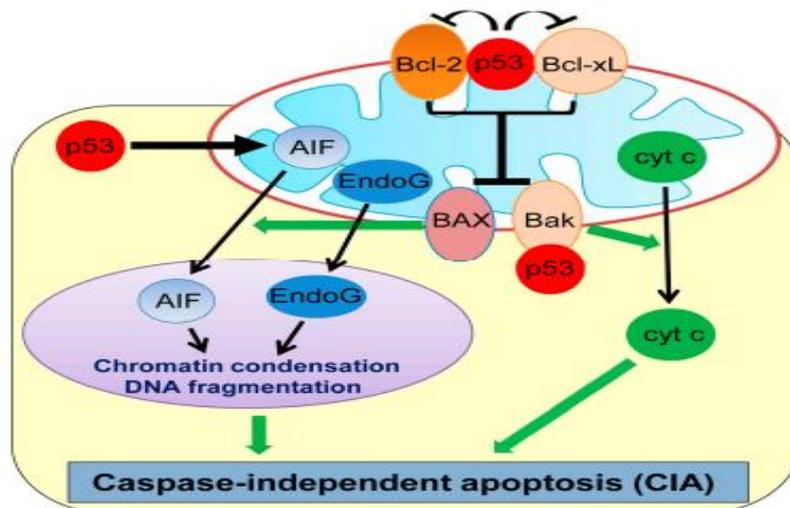


Figure 1.10 Caspase-independent apoptotic pathway(Ranjan and Iwakuma 2016:53)

The induction of apoptosis in prostate epithelial cells through androgen ablation involves the BCL2 protein. Elevated expression of BCL2 was observed in secretory epithelial cells and basal cells following castration. In prostate cancer, a reduction in

BCL2 mRNA levels and an increase in BCL2 promoter methylation were reported compared to adjacent normal tissue [(Ali & Kulik, 2021)]

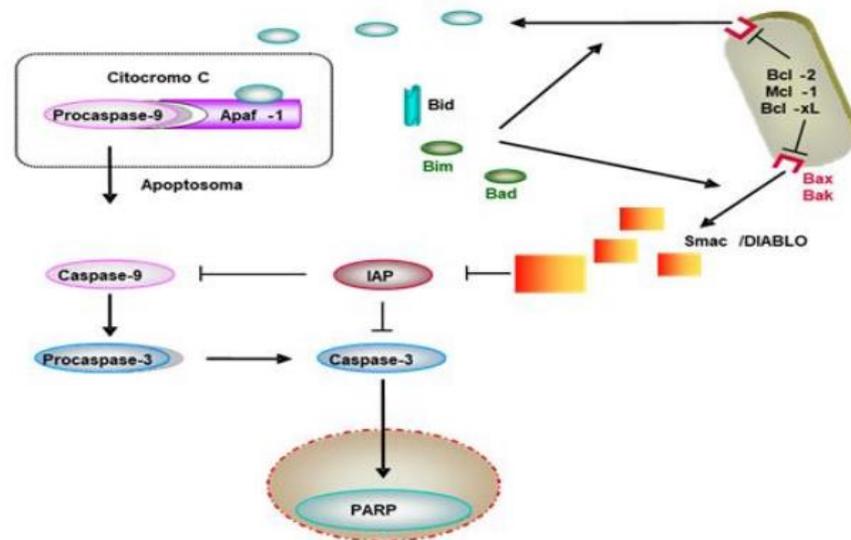


Figure 1.11 Caspase dependent intrinsic apoptotic pathway (Soria, Spain et al. 2018)

prostate cancer. Understanding the apoptotic dysregulation in prostate cancer has led to the development of targeted therapies.

Drugs and treatments are being designed to induce apoptosis in cancer cells or to overcome their resistance to apoptosis. For example, PARP inhibitors can sensitize prostate cancer cells to apoptosis-inducing agents. PARP-1 is an enzyme involved in DNA repair and genomic stability maintenance, and its role in inducing apoptosis, or programmed cell death, has significant implications for the development and progression of prostate cancer.

PARP-1 is pivotal in DNA damage repair processes. It detects DNA strand breaks and initiates the repair machinery, ensuring the integrity of the genetic material. In healthy cells, this process helps maintain genomic stability and prevent the formation of malignancies. However, in prostate cancer, the fine balance between apoptosis and survival is disrupted. Caspase-3 is a key enzyme involved in the execution phase of apoptosis, a process in which cells self-destruct in a controlled manner. Caspase-3 activation is a critical step in the apoptosis pathway in various cells, including prostate cancer cells.

1.4.4 Autophagy

Autophagy and apoptosis occur when cells are under stress. Macroautophagy/autophagy is a “self-degradation” mechanism capable of facilitating the turnover of long-lived and toxic macromolecules and organelles. There are three primary types of autophagy, namely chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. Microautophagy is the least characterized, but describes a general term for a non-selective pathway that leads to the sequestration of cytoplasmic cargos directly at the limiting membrane of the lysosome through membrane invagination. CMA is a selective form of autophagy which marks individual proteins for lysosomal degradation. The major form of autophagy is macroautophagy in which transient double-membrane compartments known as phagophores are produced to engulf cargoes, resulting in their subsequent containment within autophagosomes, and degradation following fusion with lysosomes. This kind of autophagy plays a significant role in maintaining cellular homeostasis. Overall, autophagy is divided into six steps including initiation, expansion, closure, fusion, degradation and recycling. The ULK1 (unc-51 like autophagy activating kinase 1) complex containing ATG13 (autophagy related gene13), ATG101 and RB1CC1 are involved in autophagy activation. The serine/threonine kinase ULK1, specifically, participates in the phosphorylation of phosphatidylinositol 3-kinase (PtdIns3K) complex I components including BECN1 and PIK3C3/VPS34 and mediates phagophore production at the endoplasmic reticulum (ER). In the expansion step, the ATG12–ATG5 complex is formed through action of the ATG7 and ATG10 enzymes and is recruited to the phagophore membrane. After translation as precursor forms, MAP1LC3/LC3 (microtubule associated protein 1 light chain 3) and GABARAP proteins (representing two subfamilies referred to as Atg8-family proteins due to homology with yeast Atg8) are cleaved by ATG4. These proteolytically processed proteins are then covalently attached to phosphatidylethanolamine at the phagophore membrane in an ATG3- and ATG7dependent process like, and involving, the generation of the ATG12–ATG5 conjugate. The next step is expansion of the phagophore to engulf the cargo, followed by maturation of the autophagosome. At this step, LC3-II is separated from the surface of the autophagosome to complete the maturation step and allow fusion directly with a lysosome or after first fusing with an endosome. Fusion occurs with

the help of molecular components that include tethering factors such as RAB7 and soluble N-ethylmaleimide-sensitive factor-activating membrane fusion protein (SNARE) proteins. Finally, upon fusion with a lysosome, the contents are degraded by lysosomal enzymes, and the breakdown products are released back into the cytosol for reuse (figure 1.12) [(Ashrafizadeh et al., 2022)].

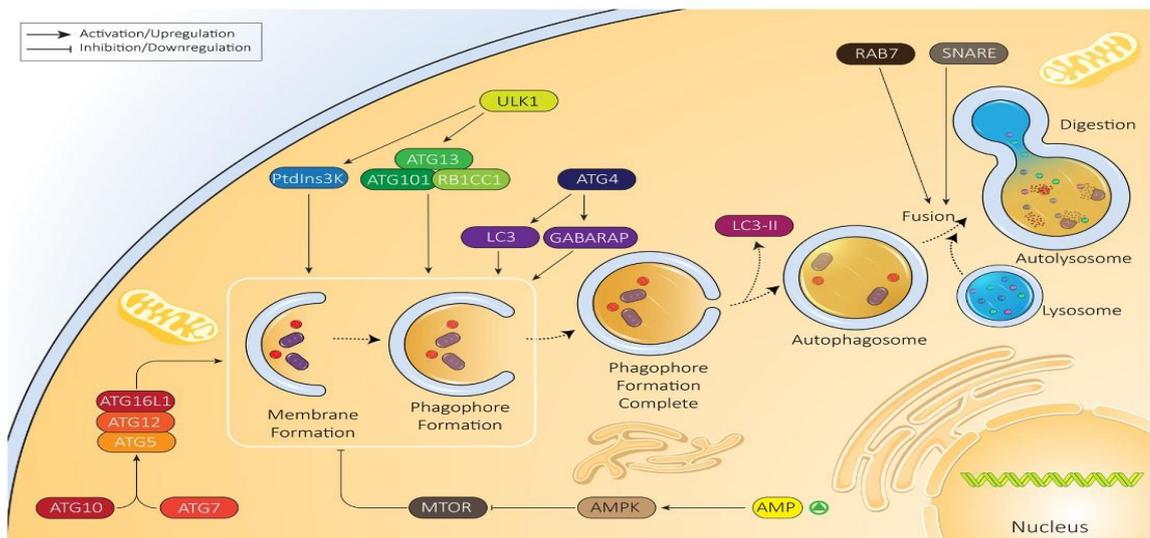


Figure 1.12 Autophagy mechanism and its regulation (Ashrafizadeh et al. 2022)

Autophagy interrelated with apoptosis

Normally, autophagy precedes apoptosis and maintains cell homeostasis. Apoptosis or other types of programmed cell death, is activated once stress is prolonged for a critical duration or exceeds the intensity threshold. ACD (autophagic Cell Death) characterized by the absence of chromatin condensation, accumulated cytoplasmic vacuolization, LC3 lipidation and caspase-independent apoptosis. Autophagy may thus function as a guardian or executioner of apoptosis depending on the surrounding microenvironment, therapeutic intervention and the stage of carcinoma. Various factors function in both apoptosis and autophagy like Beclin-1. Beclin-1 promotes autophagy by activation of core autophagic components, such as lipid kinase VPS34 and Beclin-1/VPS34/vps15 core complex and binds to Bcl-2 and inhibits both autophagy and apoptosis at basal levels. Nutrient starvation activates JNK1 (C-Jun N-terminal protein kinase 1), which phosphorylates the regulatory loop of Bcl-2 and then severs the interaction between Bcl-2 and Beclin-1. Starvation (low energy) leads

to increase AMP which cause the activation of AMP-activated protein kinase (AMPK) leads to the inhibition of mTOR. C-Jun N-terminal protein kinase 1 (AMPK) is a multimeric serine/threonine protein kinase. AMPK acts as a fine-tuned sensor of the overall cellular energy charge that regulates cellular metabolism to maintain energy homeostasis. In liver cells, AMPK suppresses autophagy whereas it appears to stimulate autophagy in many other cell types, including fibroblasts, colon carcinoma cells and skeletal muscle [(Klionsky et al., 2016)]. In homeostasis condition mTOR inhibited the autophagy.

1.4.5 Oxidative stress

Differences in oxidative stress and antioxidant capacities in different populations may cause different risk level of prostate cancer. Indeed, clinical evidence of faster PCa disease progression in obese individuals further corroborates the hypothesis that oxidative stress and inflammation, are key players in the initiation, progression and therapeutic resistance of PCa [(Mondal et al., 2020)]. Furthermore, ADT enhances the vulnerability of PC cells to toxic oxidative stress induced by radiation. Ionizing radiation which generates multiple highly reactive oxygen species (ROS) from cellular water, which oxidize DNA, proteins and lipids and may cause, disruption of plasma, mitochondrial, nuclear, endoplasmic reticulum membranes and as well as alteration of cellular protein functions and their degradation[(Lu et al., 2010)] A dysfunctional or overwhelmed ETC can result in the leakage of electrons, which are captured by molecular oxygen [O₂] to ultimately generate free oxygen radicals such as the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH[·]). Chronic exposure to high levels of these ROS can cause significant damage to DNA, proteins and lipids, and ultimately result in decreased antioxidant defense mechanisms. The ataxia-telangiectasia-mutated (ATM) protein is a key regulator of the DNA damage response following oxidative stress. Interestingly, exposure to AR antagonists induces both telomere DNA damage and damage response inhibition in the CRPC cell line 22Rv1(Splice variant ARV7 +Ve cell line) [(Reddy et al., 2019).]. Changes in the behavior of the PCa cells undergoing ADT is likely due to an adaptive response to a combination of stresses from the hypoxic tumor environment, which results in the activation of multiple alternate second messenger signaling that increase both AR gene expression and ligand-independent AR activation. Interleukins

(ILs) like IL-6 and its receptor IL-6Ra signalling multiple downstream signaling and many of these utilize ROS secondary messengers. NF- κ B, STAT and C/EBP, which are activated during IL-6 signaling, can directly interact with AR and regulate its function.

1.4.6 Genetic Mutations:

Genetic mutations that drive the initiation, progression, and aggressiveness of prostate cancer. Genomic alterations occurred in PCa like the early stage of PCa, frequent genomic alterations include TMPRSS2-ERG fusions in 40–60% of patients and SPOP mutations in 5–15% of patients. Interestingly, Asian patients with PCa have fewer PTEN deletion, TP53 mutations, and RB1 loss are correlated with lineage plasticity and neuroendocrine prostate cancer [(Y. He et al., 2022)]

1.4.7 Epigenetic Changes:

Epigenetics is the heritable control of gene expression in the absence of changes in the DNA coding sequence. Alterations of epigenetic modifications, such as histone methylation and acetylation, as well as DNA methylation, are ubiquitous in PCa. Lysine methyltransferase (KMT), histone lysine demethylase (KDM), histone acetyltransferase (HAT), bromodomain and extra terminal (BET), histone deacetylase (HDAC) or DNA methyltransferase (DNMT), alter the gene expression of oncogene or or repress the tumor suppressor gene expression in prostate cancer e.g. a molecular interplay between TMPRSS2-ERG fusions and DNA methylation in specific prostate tumors, normalized methylation levels at 15 frequently methylated loci showed large differences between cancer and benign samples[(Kumaraswamy et al., 2021)]. Notably, GSTP1, APC, RASSF1A, PTGS2, and ABCB1 loci were hypermethylated in more than 85% of cancers. Some of loci hypermethylation exhibits biochemical recurrence like HAPLN3, KLF8 etc. [(P. G. Patel et al., 2019)]. Aberrations in DNA damage response genes, such as BRCA1, BRCA2, ATM, CHEK2, and CDK12, occur in approximately 20% of metastatic PCa.

TMPRSS2-ERG fusions, whereas genomic alterations in FOXA1, ZNF292, and CHD1 are observed in more than 40% of these patients. Genomic alteration of PTEN and TP53 often occurs across different stages of PCa. Oncogene MYC amplification

or WNT signaling activation via APC loss and CTNNB1 amplification are also frequent, occurring in approximately 10–30% of all mCRPC cases. RB1 loss is seen in approximately 10% of case of cancer reoccurrence

1.5 Prostate Cancer Therapy:

Anticancer therapy either becomes irresponsive or cells become resistant. As shown in Figure 1.4[(Kweldam et al., 2019)] Primary tumors are responsive to different therapy. Whereas High-risk patients are treated with aggressive therapy like surgery and radiation-based therapies. A major challenging decision of therapies lies in intermediate-risk (e.g., Gleason 3 + 4) cancer due to patients showing considerable differences in outcomes. Numerous newly proposed classification systems have emerged with the aim of more precisely categorizing these intermediate-risk cases into subgroups distinguished as either favorable or unfavorable [Serrano and Anscher2016]. Furthermore, significant endeavors are directed towards the advancement of biomarkers e.g., transcriptome-based gene signatures in order to predict disease aggressiveness and prognosis more precisely For individuals who undergo treatment for localized prostate cancer and encounter disease recurrence, characterized by an increase in PSA levels, ADT is frequently employed alongside surgical procedures or radiation therapy. In cases of metastatic disease, the initial treatment strategy typically encompasses ADT, often with chemotherapy. ADT can be implemented through two primary methods: surgical castration, specifically orchiectomy, or, more commonly, chemical castration utilizing medications that target the androgen receptor (AR) signaling pathways regulated by the hypothalamic-pituitary-testicular axis, such as gonadotropin-releasing hormone agonists, AR antagonists, and CYP17A1 inhibitor. While most patients exhibit an initial positive response to ADT, recurrence ultimately develops in most cases, resulting in the development of mCRPC (figure 1.4). Till 2010 treatment for CRPC was docetaxel chemotherapy as the gold standard and other chemotherapy like cabazitaxel got approval. Apart from employing taxane-based chemotherapy, the treatment landscape for mCRPC has seen substantial expansion over the past decade. The FDA has sanctioned potent second-generation anti-androgen therapies, which currently comprise enzalutamide, abiraterone, and apalutamide, alongside innovative agents like EPI-506. The potent AR antagonists, enzalutamide and apalutamide, have been

shown to increase the survival of patients with mCRPC [Scher et al. 2012; Beer et al. 2014]. By initiating an inflammatory insult normal cells are converted into proliferative inflammatory atrophy (PIA) which is usually shown to merge with PIN and cancerous cells and both develop the progression of cancer. PIA does not harbor genetic defect however BCL2(anti-apoptotic gene) and GSTP1 both seems to upregulate in PIA. Tumor suppressor genes PTEN, CDKN1B, and NKX3.1 are downregulated which activates the epigenetics of state prior to carcinogenesis. These genes' downregulation also targets heterozygous deletion of adenocarcinoma [(De Marzo et al., 2007)]. Several large-scale genomic studies in both primary prostate tumors and metastatic castration-resistant prostate cancer (mCRPC) has identified recurrent DNA copy number changes, mutations, rearrangements, and gene fusions [Taylor et al. 2010; Barbieri et al. 2012]. These signature genetic alterations target the pathways of AR, PI3K–PTEN, Wnt, DNA repair, and components of the cell cycle in nearly all metastatic prostate cancers and a significant proportion of primary prostate cancers [(The Cancer Genome Atlas Research Network et al., 2013); (Robinson et al., 2015)]. Such as Tmprss2: ERG fusion is found in approximately 50% of localized prostate cancers [(Clark & Cooper, 2009)]. This genetic rearrangement or fusion occurred due to the shortening of telomer causing after every cell division. Telomeres serve as a buffer mechanism against the end-replication challenge in mammalian cells, where chromosome ends are trimmed by approximately 100-200 base pairs after each cell division, resulting in the gradual shortening of telomeres as individuals age Normal prostate epithelia and stem cells lacking a telomerase activity yet levels of enzyme increase during onset and progression into CRPC [(O'Sullivan & Karlseder, 2010)]. Basal cells do not contain telomerase thus it is removed in cancer while luminal cells possess so it causes cancer in patients.

As per some studies, androgenic AR is a key player for tumor progression than intratumoral AR suggests that targeting CYP17 blockade could be an intriguing therapeutic approach for AIPC patients [(Hofland et al., 2010)].

1.6 Metformin as an anticancer drug

Metformin, marketed under the trade name Glucophage among others, is the first-line medication for the treatment of type 2 diabetes and PCOS (Polycystic ovary syndrome). The initial indication of metformin's potential as a therapeutic treatment

date back to 1922, when chemists Emile Werner and James Bell noted its capability to lower glucose levels in rabbits while leaving heart rate and blood pressure unaffected [(Guarnaccia et al., 2021)]. Subsequently, it was introduced as a medication in France in 1957 and later in the United States in 1995. FDA (Food and Drug Administration) granted approval in 1994 for the management of type 2 diabetes mellitus in the United States [(L. He & Wondisford, 2015)]. In 1998, the UK Prospective Diabetes Study analyses had reported a lower incidence of certain types of cancer among diabetic populations taking metformin, despite data indicating that these diabetic populations were overall more prone to developing cancer. This has led to a deeper investigation into the role of metformin in cancer [(Skuli et al., 2022)] Metformin received considerable attention as a potential anti-cancer agent as it exhibits a strong and consistent anti-proliferative action on several cancer cell lines, including breast, colon, ovarian, pancreatic, lung, and prostate cancer cells. [(Zou et al., 2019),(Ikhlas & Ahmad, 2017)]. For example, from 2005, there have been reports suggesting that metformin may reduce the risk of cancer globally by as much as 23%. For instance, findings from the Taiwan National Health Insurance Data Survey, encompassing a cohort of 12,005 individuals prescribed metformin between 2000 and 2007 compared to a population of 4,597 patients receiving alternative oral medications, suggest that metformin intake is associated with an up to 88% decreased likelihood of developing any form of cancer. The deregulation of energetic and cellular metabolism is a signature of cancer cells its high energy demand and redox imbalance is an inherent property of them which may be regulated by AMPK activation and by mitochondrial glycerophosphate dehydrogenase (mitoGPD). Mammalian contains at least two pools of NADH and NAD⁺, nucleocytosolic and mitochondrial. Hepatic lactate and pyruvate reach equilibrium with cytosolic NADH/NAD⁺ through the action of lactate dehydrogenase, while β -hydroxybutyrate and acetoacetate equilibrate with mitochondrial NADH/NAD⁺ via β -hydroxybutyrate dehydrogenase. Metformin is a hydrophilic molecule and therefore cannot pass through the cell's membrane by passive diffusion. Metformin uptake done by PMAT in the intestine and oct1/3 in the liver (Figure 1. 13)[(L. He & Wondisford, 2015)]. Metformin undergoes no metabolic breakdown in either animals or humans and exits the body unchanged via renal excretion. Following oral ingestion, metformin permeates into enterocytes via the plasma monoamine transporter (PMAT) and organic cation transporter 3 (OCT3) located on the apical membrane. Subsequently, it

exits the enterocytes through OCT1 on the basolateral membrane. Metformin is when delivered to the liver via portal vein, achieved concentrations in the portal vein are between 40 and 70 μM and this uptake is done by Oct 1/3 while its exit from hepatocytes is done by MATE1(multidrug and toxin extrusion 1). Following hepatic uptake, the systemic plasma concentration of metformin decreases to a range of 10–40 μM in animals. Within the renal system, metformin is absorbed from the bloodstream into renal epithelial cells through OCT2 transporters and subsequently excreted into the urine via MATE1/2k transporters [(L. He & Wondisford, 2015)]. In tumor cells also organic cation transporter 1 (OCT1), is responsible for the uptake of metformin [(Ikhlas & Ahmad, 2017)]. Metformin drug half-life is 6.2hr and elimination life is 17.6hrs. It can cross the blood-brain barrier. The standard clinical dose of metformin is (1500–2250 mg/day).

Its lowering glucose effect requires activation of AMP-activated protein kinase (AMPK) to inhibit hepatic gluconeogenesis [(Y. Zhou et al., 2015)]., increase peripheral uptake of glucose in muscle cells, and delay gastrointestinal glucose absorption in the intestine. The gluconeogenesis inhibition happens mainly in the liver due to OCT1 high expression.

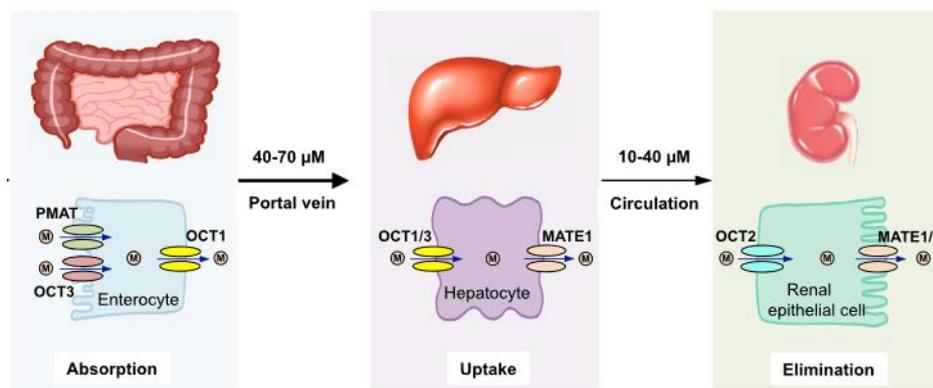


Figure 1. 13 Metformin uptake and elimination(He and Wondisford 2015)

AMPK activation by metformin may happen by indirect upstream activation of LKB1 kinases which is known as tumor suppressor gene [Carling D, Mayer FV et al.]. Metformin impedes mitochondrial complex I, disrupting adenosine triphosphate (ATP) synthesis and leading to an accumulation of adenosine monophosphate (AMP). This AMP accumulation subsequently triggers the activation of the glycolytic

pathway. The primary Cytosolic NADH/NAD⁺ ratio increased in the livers by metformin and NADH/NAD⁺ ratio decreased as well in mitochondria. A higher NADH/NAD⁺ ratio impairs glucose production from lactate. Additionally, metformin suppresses mitochondrial glycerophosphate dehydrogenase (mitoGPD) in hepatic cells, altering both mitochondrial and cytosolic redox states and diminishing reactive oxygen species production. These effects are associated with inhibition of gluconeogenesis. Cancer cells primarily generate ATP through oxidative phosphorylation (OXPHOS) but also utilize glycolysis for ATP up to some degree. Cancer cell sensitivity is observed towards metformin which have glucose utilization deficiencies or complex I impairments, while cancer cells without those deficiencies are not sensitive to Metformin [(Song et al., 2015)]. Combining metformin with glycolysis inhibitors effectively hampers such kind of cancer cell growth. (Key components in Figure 1.14 include adenosine diphosphate (ADP), lactic dehydrogenase (LDHA), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide hydride (NADH), pyruvate dehydrogenase (PDH), cyclic AMP-dependent protein kinase (PKA), and TCA cycle.

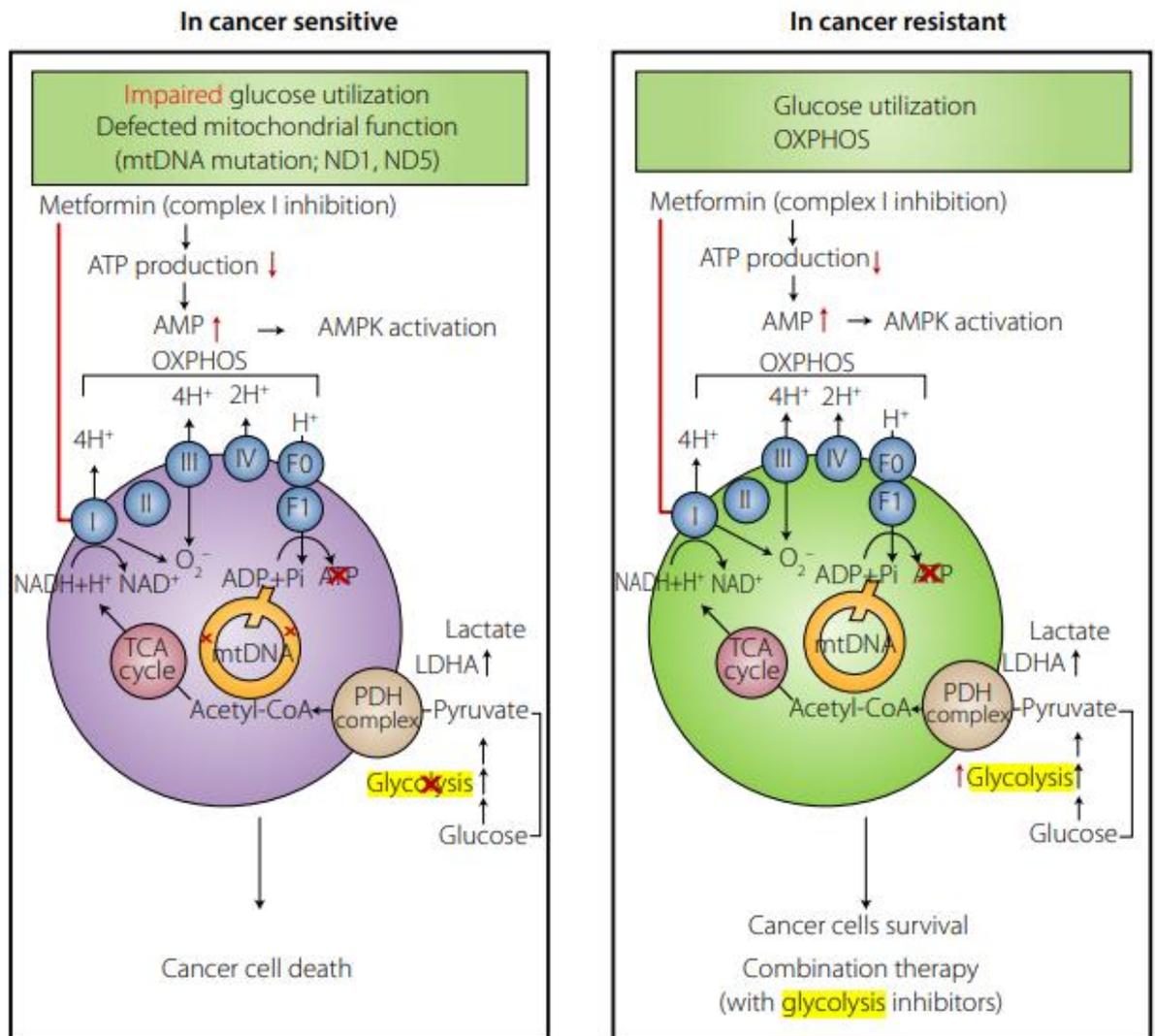


Figure 1.14 Metformin metabolism in cancer cells(Song et al. 2015)

Metformin exhibits a strong and consistent anti-proliferative action on several cancer cell lines, including breast, colon, ovarian, pancreatic, lung, and prostate cancer cells. Its lowering glucose effect requires activation of AMP-activated protein kinase (AMPK) to inhibit hepatic gluconeogenesis [(Zhou, 2001).], increase peripheral uptake of glucose, and delay gastrointestinal glucose absorption. AMPK activation by metformin may happen by indirect upstream activation of LKB1 kinases which is known as tumor suppressor gene [Carling D, Mayer FV et al.]. Metformin led activation of AMPK lead mTOR inhibition a prime regulator of Autophagy Figure 1.5[(Marjanovic et al., n.d.)] Metformin is generally well-tolerated and has a low risk of adverse effects. Combining it with specialized anticancer drug may not only enhance its anticancer effects but also reduce the side effects associated with more

aggressive cancer treatments [(Skuli et al., 2022)].

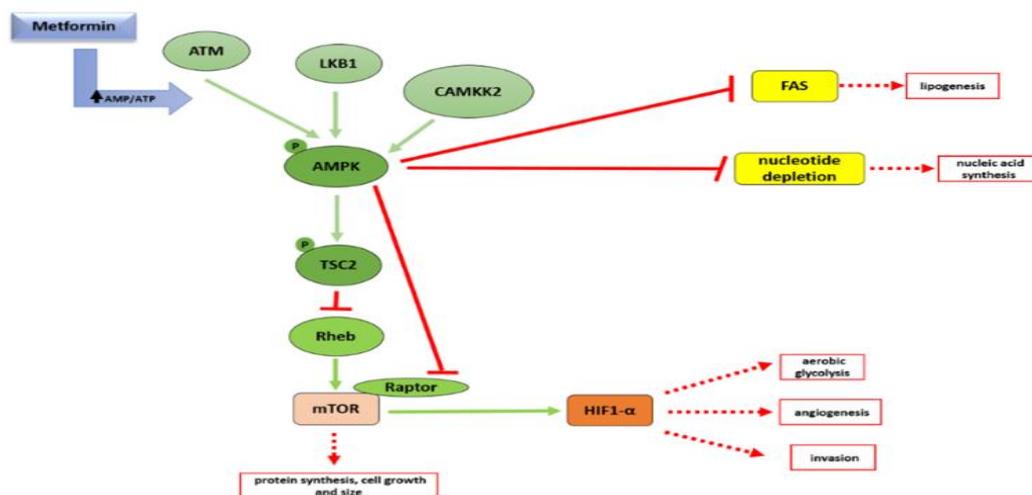


Figure 1.5 Metformin on cancer metabolism(Marjanovic et al. n.d.)

Moreover, metformin lead inhibition of the mTOR downregulates expression of multidrug resistance protein 1/P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) [(Ling et al., 2014),].

1.7 Scope of Swertamarine

Swertiamarin is one of the bioactive in Qing Ye Dan & *Enicostemma littorale* plant which is a seco-iridoid glycoside. It possesses multifunctional properties, including analgesic, anti-inflammatory, antiarthritis, hepatoprotective, antidiabetic, antioxidant, antimicrobial, anticancer, neuroprotective and gastroprotective properties. Swertiamarin has been extensively studied in our lab for its antidiabetic, hepatoprotective and antiadipogenic activity It modulates a wide range of molecular targets, including IR, PI3Kinase inflammatory cytokines, transcription factors, growth factors, apoptosis-related proteins, receptors, in various pathological conditions. [(Muhamad Fadzil et al., 2021), (T. P. Patel et al., 2016)]. Swertiamarin suppressed the expression of proinflammatory cytokines, including tumor necrosis alpha (TNF- α), interleukin (IL)-6 (IL-6), and IL-8, in cells (BEAS-2B cells, RAW264.7 cells and HEK 293T cells) and in an acute lung injury animal model by targeting the AKTpleckstrin homology (AKT-PH) domain. Swertiamarin thus can act as a natural AKT inhibitor to regulate inflammatory conditions [(Muhamad Fadzil et al., 2021)] Swertiamarin exerts anticancer effects on human cervical cancer cells via induction of

apoptosis, inhibition of cell migration and targeting of MEK-ERK pathway [wang et.al 2018]. In light of this combinatorial efficacy of SM it can be a potential prostate cancer therapy

1.8 Prostate cancer In Vitro Models:

Since prostate cancer's reliance on androgens, considerable efforts have been dedicated to gaining a more comprehensive insight into the signaling of the androgen receptor (AR) with our current knowledge being largely derived from experimental cell culture and animal models. In vitro cell cultures have the advantage of being relatively cheap and typically have a high replicative capacity ensuring sufficient material for long-term use. Various in vitro and in vivo models have been developed to facilitate research on prostatic conditions, including benign prostatic hyperplasia (BPH) and different phases of prostate cancer (PCa), such as castration-resistant (CR) and metastatic disease. Each model system exhibits distinct features related to androgen responsiveness and the expression of the androgen receptor (AR). In vitro cell models exhibiting characteristics of androgen receptor (AR)-dependent and AR-independent mechanisms that promote prostate cancer (PCa) progression to castration-resistance. Several pathways have been identified by which PCa cells can overcome androgen depletion and thereby facilitate tumor progression to CR-PCa and can be divided into: i) ligand-dependent mechanisms, which promote AR activation despite castrate levels of androgens; ii) ligand-independent mechanisms, which facilitate AR activation by nonandrogenic factors and/or altering the intrinsic behavior/sensitivity of AR; and iii) indirect mechanisms that act downstream of AR activation (e.g. chromatin remodeling via histone deacetylases, re-emergence of tumors via CSCs and AR-dependent expression of oncogenic ETS transcription factors). Cell lines that have been used to study these different mechanisms are indicated in Figure 1.16[(Noubissi Nzeteu et al., 2022)]/

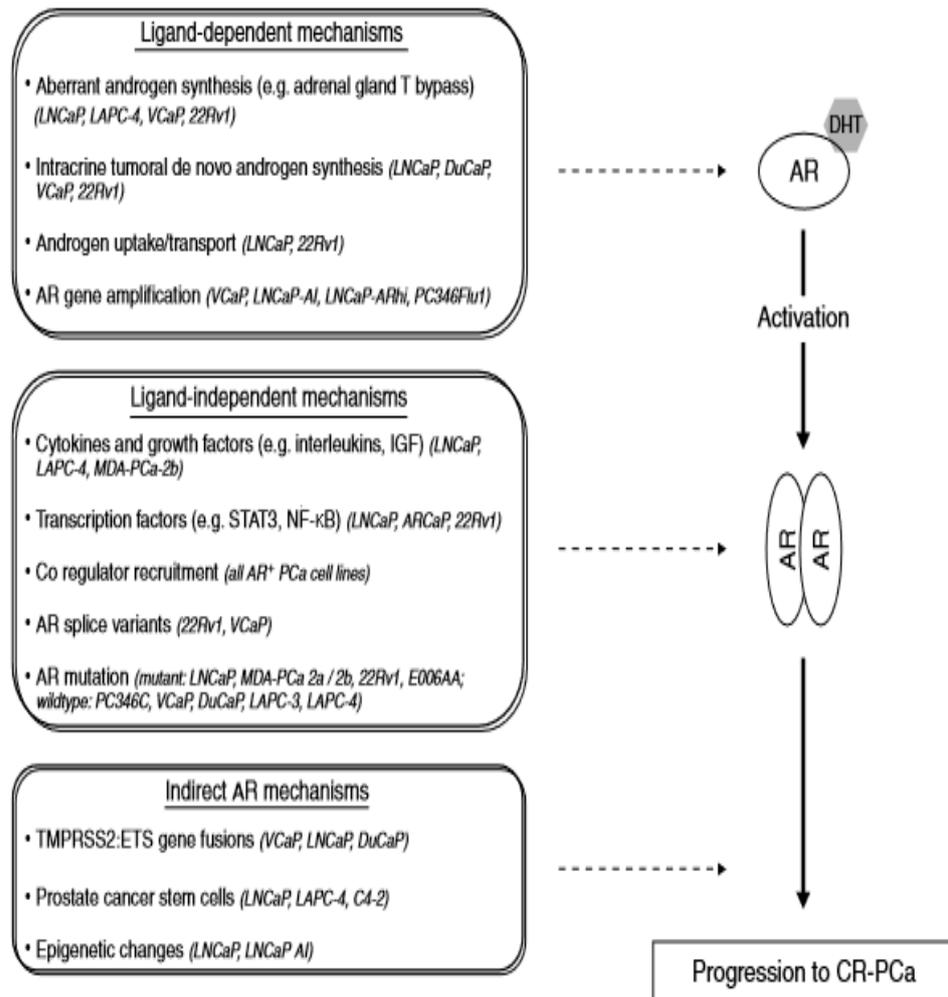


Figure 1.16 Different PCa In Vito models based on AR heterogeneity(Zoubeidi et al. 2007)