



Androgen Receptor Signaling in the Development of Castration-Resistant Prostate Cancer

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Most prostate cancers are androgen-sensitive malignancies whose growths depend on the transcriptional activity of the androgen receptor (AR). In the 1940s, Charles Huggins demonstrated that the surgical removal of testes in men can result in a dramatic improvement in symptoms and can induce prostate cancer regression. Since then, androgen deprivation therapies have been the standard first-line treatment for advanced prostate cancer, including: surgical castration, medical castration, antiandrogens, and androgen biosynthesis inhibitors. These therapies relieve symptoms, reduce tumor burden, and prolong patient survival, while having relatively modest side effects. Unfortunately, hormone deprivation therapy rarely cures the cancer itself. Prostate cancer almost always recurs, resulting in deadly castration-resistant prostate cancer. The underlying escape mechanisms include androgen receptor gene/enhancer amplification, androgen receptor mutations, androgen receptor variants, coactivator overexpression, intratumoral *de novo* androgen synthesis, etc. Whereas, the majority of the castration-resistant prostate cancers continuously rely on the androgen axis, a subset of recurrent cancers have completely lost androgen receptor expression, undergone divergent clonal evolution or de-differentiation, and become truly androgen receptor-independent small-cell prostate cancers. There is an urgent need for the development of novel targeted and immune therapies for this subtype of prostate cancer, when more deadly small-cell prostate cancers are induced by thorough androgen deprivation and androgen receptor ablation.

Keywords: prostate cancer (PCa), androgen receptor (AR), androgen deprivation therapy (ADT), castration-resistant prostate cancer (CRPC), small-cell prostate cancer (SCPC), antiandrogen

ANDROGENS AND THE ANDROGEN RECEPTOR IN THE PROSTATE GLAND

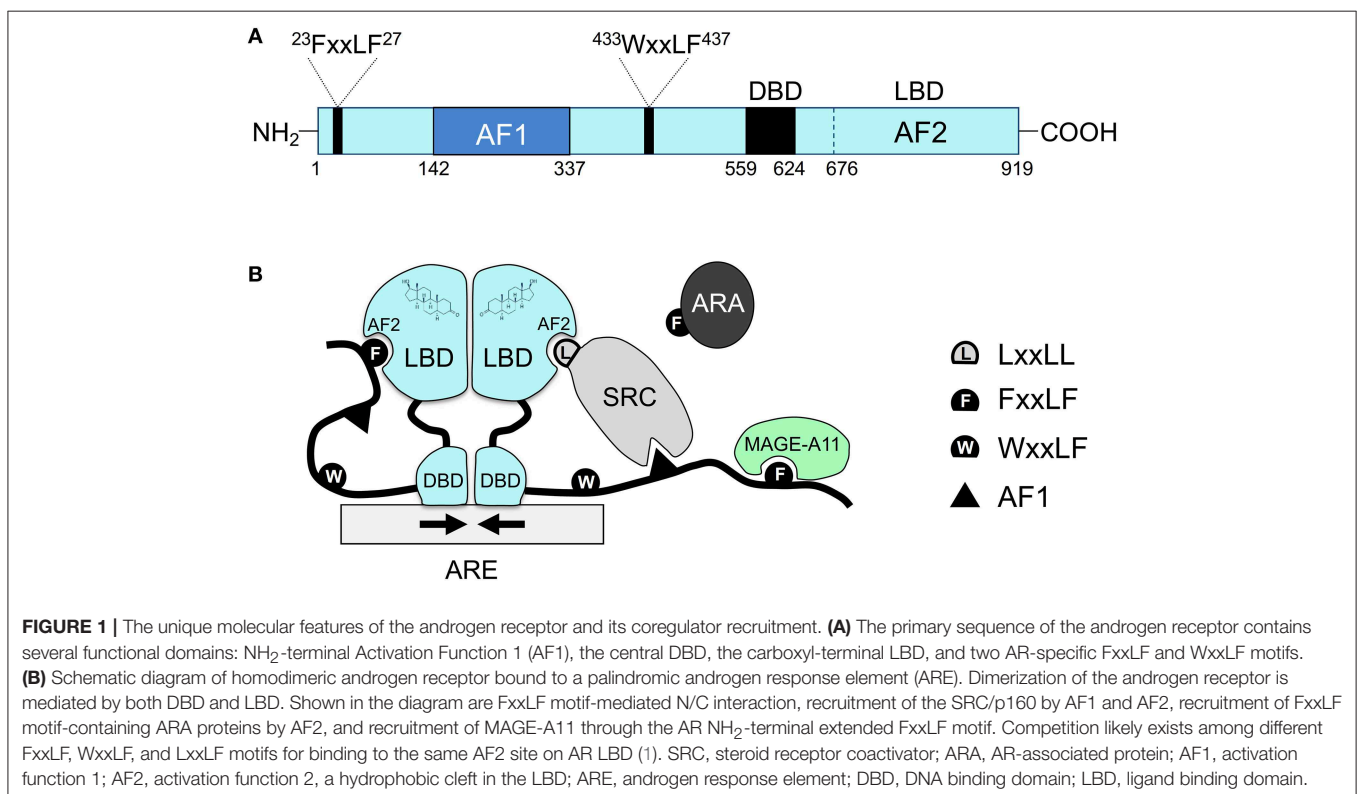
The prostate is a walnut sized male reproductive gland located between the bladder and the penis. It secretes the prostatic fluid that helps to nourish and transport sperm. Androgen signaling plays a pivotal role in the development and function of a normal prostate gland. There are two native androgens in humans, testosterone (T), and 5 α -dihydrotestosterone (DHT). Testosterone is produced mainly in the testis, with a small amount being produced in the adrenal glands in men.

Testosterone is converted to the more potent androgen dihydrotestosterone by the enzyme 5 alpha-reductase located in the prostate, skin, scalp, etc. Both testosterone and dihydrotestosterone can bind to a single nuclear receptor protein, the androgen receptor, which is an androgen-dependent transcriptional activator and a member of nuclear receptor superfamily.

Similar to other nuclear hormone receptors, the androgen receptor protein contains three main functional domains: the NH₂-terminal unstructured transcriptional activation domain, the central DNA binding domain (DBD), and the carboxyl-terminal ligand binding domain (LBD) (**Figure 1A**). Between DBD and LBD, there is a flexible hinge region (amino acid 624–676), which harbors a bipartite nuclear localization signal (NLS). In the classical model, the androgen receptor binds to androgen response elements (AREs) as a homodimer, and dimerization is mediated by both DBD and LBD (2, 3) (**Figure 1B**). Whereas, other nuclear receptors recruit LxxLL motif-containing coactivators such as the steroid receptor coactivator (SRC)/p160 family coactivators through their ligand binding domains, the androgen receptor ligand binding domain preferentially engages in the FxxLF motif-mediated NH₂-terminal and carboxyl-terminal (N/C) interaction (4–7) or recruits FxxLF motif-containing coregulators (5, 8) (**Figure 1B**). Nevertheless, the androgen receptor can still recruit the SRC/p160 family of coactivators mainly through its unstructured NH₂-terminus and LBD (9). Moreover, the androgen receptor can also recruit an AR-specific MAGE-A11 coactivator through its extended NH₂-terminal FxxLF motif (10).

In the absence of hormones, the androgen receptor is associated with heat shock proteins and located in the cytoplasm in an inactive conformation. Upon androgen binding, the androgen receptor quickly undergoes conformational change, nuclear translocation, recognition of androgen responsive elements in the genomic DNA, and recruitment of coactivator machineries, resulting in transcription of target genes, such as prostate-specific antigen (PSA) and transmembrane protease serine 2 (TMPRSS2).

Dihydrotestosterone is a significantly more potent androgen than testosterone both *in vitro* and *in vivo*. While this variance in potency was commonly attributed to their different binding affinities, dihydrotestosterone actually binds to the androgen receptor with similar or somewhat higher affinity compared with testosterone (11, 12). In contrast, these two androgens bind to the androgen receptor with very differing kinetics (11, 12). The rate of dissociation for dihydrotestosterone from the androgen receptor is about three to five times slower than testosterone (11, 12). Therefore, it is largely their binding kinetics, rather than affinity, which accounts for the differential androgenic activities of these two hormones. As we know, in the field of drug discovery, the notion that drug-receptor binding kinetics could be as important as affinity in determining drug efficacy is becoming more widely accepted (13). In further support of the importance of androgen binding kinetics, the unique androgen receptor inter-domain N/C interaction slows the rate of androgen dissociation without affecting androgen binding affinity and is required for optimal target gene transcription (4).



CO-EVOLUTION OF ANDROGEN DEPRIVATION THERAPY (ADT) AND PROSTATE CANCER

Surgical and Medical Castration

Prostate cancer occurs in the prostate gland. It is the most commonly diagnosed non-skin cancer and the second leading cause of cancer death in men in the United States. Based on his finding that the growth of prostate glands in dogs depended on testosterone, Charles Huggins demonstrated that surgical removal of testes in men, which produces more than 90 percent of testosterone, can result in a dramatic improvement in symptoms and can induce regressions of prostate cancers at any site (14). Since then, androgen deprivation therapy has been the standard first-line treatment for advanced prostate cancer (15). In addition to surgical castration, gonadotropin-releasing hormone (GnRH) analogs such as leuprolide, goserelin, and buserelin can suppress gonadotropin secretion and thus block the production of testicular androgens. As a result of its cosmetic and psychological concerns, medical castration via GnRH analogs has been the mainstay treatment for advanced prostate cancer.

First-Generation Antiandrogens

Although surgical and medical castration can suppress testosterone production in the testes, the adrenal glands can still produce small amounts of androgens. To neutralize the activity of these residual androgens, antiandrogens were used to block androgen receptor signaling in prostate cancer cells (Figure 2). For example, cyproterone acetate (CPA), a synthetic steroid, was used as a prototypical antiandrogen (16). However, due to its relative ineffectiveness, CPA was replaced by more potent non-steroidal pure antiandrogens, such as Flutamide (Eulexin), bicalutamide (Casodex), and nilutamide (Nilandron). Unlike GnRH analogs, these antiandrogens do not prevent androgen production in the body. Instead, the antiandrogens bind to the androgen receptor with a relatively high affinity but lack the ability to activate transcriptional activity of the androgen receptor. Therefore, the antiandrogens function by competitively blocking testosterone and dihydrotestosterone from binding to the androgen receptor. For instance, flutamide and its active metabolite hydroxyflutamide bind to androgen receptors with a K_i of $\sim 3,395$ and ~ 134 nM, respectively (17). Bicalutamide is a more potent non-steroidal antiandrogen; its affinity for androgen receptors is two to four times more potent than hydroxyflutamide and nilutamide (18). Bicalutamide was thus modestly effective in prostate cancer patients who developed resistance after flutamide treatment (19). While effective on their own, antiandrogens are not usually used in monotherapy. Instead, they have proven to be used in conjunction with medical or surgical castration (20–22).

“Androgen-Independence” to Castration-Resistance

The combination therapy of GnRH analogs and antiandrogens has promoted the survival of prostate cancer patients (21, 23). Unfortunately, most prostate cancers develop resistance to the combined androgen deprivation therapy after several years,

becoming so-called “androgen-independent” prostate cancer. Surprisingly, it was found that, even after castration, the testosterone and dihydrotestosterone levels in locally recurrent prostate cancer tissues remain high enough to activate androgen receptors (24, 25). In support of this observation, the androgen receptor target gene PSA remains expressed in recurrent prostate cancer tissues, despite the castrate levels of androgens in serum (24, 25). Moreover, it has been reported that in the recurrent metastatic prostate cancers, intratumoral *de novo* androgen synthesis by overexpressed steroidogenic enzymes may contribute to elevated testosterone levels (26). Taken together, it becomes evident that recurrent cancers after medical or surgical castration are not truly androgen-independent (27), as they continuously depend on androgens and the androgen receptor to survive and grow. These recurrent cancers have been more appropriately classified as castration-resistant prostate cancer (28).

Mechanisms of Castration Resistance

Subsequent studies have revealed multiple mechanisms which may contribute to the androgen receptor-dependence in castration-resistant prostate cancer. Firstly, increased androgen receptor expression can be caused by androgen receptor gene amplification (29–33) or androgen receptor enhancer amplification (34, 35). Secondly, increased expression of androgen receptor coactivators SRC1 and TIF2 stimulates androgen receptor activity in the presence of the weaker androgen androstenedione (36). The expression of the MAGE-A11 coactivator, which is recruited through androgen receptor NH₂-terminal FxxLF motif, is increased in castration-resistant prostate cancer (37, 38). Thirdly, mutations in the androgen receptor ligand binding domain enable the androgen receptor to be activated by antiandrogens or other steroid hormones (39). For instance, the androgen receptor with the LNCaP mutation T877A can be activated by flutamide, estrogen, and progesterone (40, 41). The androgen receptor with L701H/T877A double mutations can be activated by glucocorticoids (42). Fourthly, constitutively active androgen receptor variants which lack ligand binding domains are another underlying mechanism of the castration resistance (43–45). Additionally, growth hormones such as epidermal growth factor (EGF) increase TIF2/GRIP1 coactivation of androgen receptor activity in recurrent cancer cells (46). Insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), and EGF can all activate androgen receptor activity in the absence of androgens (47).

Antiandrogen Withdrawal Syndrome

Interestingly, in some patients, when an antiandrogen is no longer working, simply stopping the antiandrogen treatment can stop cancer growth for a short period of time. This phenomenon is known as antiandrogen withdrawal syndrome. Decreases in PSA levels and/or clinical improvement after discontinuation of antiandrogens upon disease progression have been shown by flutamide, bicalutamide, and nilutamide withdrawal (48–50). One mechanism of antiandrogen withdrawal syndrome is acquired mutations in the androgen receptor ligand binding domain including mutation T877A and H874Y. Not surprisingly,

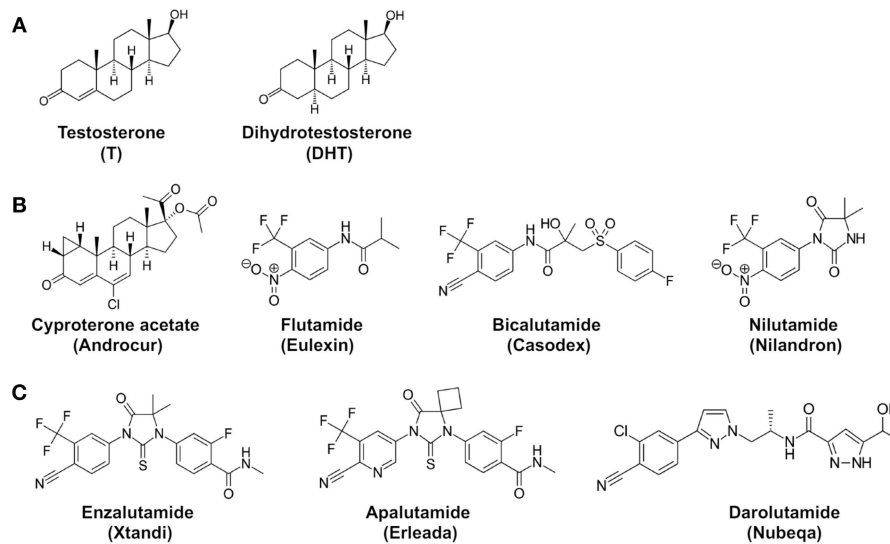


FIGURE 2 | Chemical structures of androgens and antiandrogens. **(A)** Two main androgens, testosterone (T), and dihydrotestosterone (DHT). **(B)** First-generation antiandrogens, cyproterone acetate (Androcur), Flutamide (Eulexin), Bicalutamide (Casodex), Nilutamide (Nilandron). 2-hydroxyflutamide is the major active metabolite of flutamide in the body. **(C)** Representative second-generation antiandrogens, Enzalutamide (Xtandi), Apalutamide (Erleada), and Darolutamide (Nubeqa). Structures are adopted from Wikipedia.

these mutations have converted antiandrogens to androgen receptor agonists (51).

Second-Generation Antiandrogens

To overcome castration resistance, more potent antiandrogens, known as second-generation antiandrogens, have been designed to achieve maximal androgen blockade (52). These second-generation antiandrogens include Enzalutamide (Xtandi), Apalutamide (Erleada), and Darolutamide (Nubeqa) (Figure 2). Enzalutamide and apalutamide are structurally similar to each other, having 5- to 8-fold higher binding affinities for androgen receptors in comparison to first-generation antiandrogens. Importantly, these antiandrogens function as pure antagonists for the androgen receptor in the presence of mutations such as T877A. Darolutamide is structurally distinct and shows 8- to 10-fold higher affinity for the androgen receptor than enzalutamide and apalutamide, and can inhibit the enzalutamide-resistant mutant androgen receptor (53). Therefore, darolutamide appears to be an even more potent second-generation antiandrogen. In addition to these three FDA-approved second-generation antiandrogens, other antiandrogens are also being developed. For instance, a potent AR inhibitor JNJ-73576253 (TRC253), developed by Janssen Pharmaceuticals, is a pan-inhibitor of AR, even in the presence of certain activating mutations, and is currently in Phase 1/2A clinical trial (54).

These more potent second-generation antiandrogens have been successful in prolonging the survival of men with castration-resistant prostate cancer. For instance, in men with metastatic castration-resistant prostate cancer after chemotherapy, enzalutamide produced an overall survival benefit of 4.8 months compared to the placebo (55). For patients with metastatic prostate cancer who have not received chemotherapy,

enzalutamide also significantly increased progression-free survival and overall survival (56). Moreover, enzalutamide, apalutamide, and darolutamide all had significantly prolonged metastasis-free survival in men with high-risk non-metastatic castration-resistant prostate cancer (57–59). As shown by the latest phase III trials, both enzalutamide and apalutamide could significantly increase the progression-free survival and overall survival for men with metastatic hormone-sensitive prostate cancer (60–62).

In addition to the development of second-generation antiandrogens, Abiraterone (Zytiga) was developed as an irreversible steroid inhibitor of CYP17, a key enzyme in androgen synthesis. Abiraterone acetate inhibits the production of androgens in the testes, adrenal glands, and prostate tumors. In patients with metastatic castration-resistant and chemotherapy-resistant prostate cancer, Abiraterone produced an overall survival benefit of 3.9 months in comparison to the placebo (63). More recently, the phase III LATITUDE trial has shown that the combination of Abiraterone plus prednisone with ADT conferred significant progression-free and overall survival benefits for patients with newly diagnosed high-risk metastatic castration-sensitive prostate cancer (64, 65).

Repeated Resistance and Underlying Mechanisms

Unfortunately, while second-generation antigens can prolong the survival of castration-resistant prostate cancer patients, the relief is temporary. Once again, castration-resistant cancers become resistant to the newest inhibitors. The novel mutation F876L, which is evolved in the androgen receptor ligand binding domain during the treatment of enzalutamide, converts enzalutamide

to an agonist (66–68). Enzalutamide-resistant prostate cancer can also bypass androgen receptor blockade by glucocorticoid receptor activation (69). Because the DNA binding domains of glucocorticoid receptor and androgen receptor are highly homologous and recognize identical DNA response elements, the glucocorticoid receptor can substitute for the androgen receptor to activate a subset of androgen receptor target genes which are required for prostate cancer survival and growth. In addition, the androgen receptor variant AR-V7 is associated with resistance to enzalutamide and Abiraterone (70, 71). Niclosamide, a novel inhibitor of AR-V7, may be able to overcome enzalutamide resistance (72). The crucial steroidogenic enzyme AKR1C3 is found to be overexpressed in enzalutamide-resistant prostate cancer cells and mediates enzalutamide resistance (73). The chemokine receptor CXCR7 is found to be overexpressed in enzalutamide-resistant prostate cancer cells and can activate MAPK to confer enzalutamide resistance (74). Up-regulation of coactivator GREB1 may also contribute to enzalutamide resistance (75).

One interesting observation is a reciprocal negative feedback regulation between AR and PI3K/Akt signaling pathways in prostate cancer. Pten loss contributed to the development of castration-resistant prostate cancer in mouse models (76, 77). It was thus postulated that combined inhibition of AR and PI3K pathways may achieve more potent inhibition of tumor growth. Indeed, in a phase Ib/II clinical trial, combination of abiraterone with Ipatasertib, an Akt inhibitor, showed more potent anticancer activity than abiraterone alone in metastatic castration-resistant prostate cancer patients (78). Moreover, the combination of Akt inhibitor AZD5363 and enzalutamide showed synergistic anti-prostate cancer effects in preclinical models (79) and has been tested in a phase I clinical trial (80). However, in another phase II clinical trial, a pan-class I PI3 kinase inhibitor BKM120 (buparlisib), with or without enzalutamide co-treatment, had only limited efficacy in men with metastatic castration-resistant prostate cancer (81).

AR CO-FACTORS IN PROSTATE CANCER

Eukaryotic DNA wraps around histone proteins and forms an inhibitory chromatin structure. Gene activation by the androgen receptor requires assistance from other transcription factors. Among these factors, GATA2 and FoxA1 play particularly essential roles in androgen receptor signaling in prostate cancer cells. GATA2 belongs to the GATA family of transcription factors which contains six members in mammals. GATA2 factors bind to a consensus DNA sequence (A/T)GATA(A/G) and regulate gene expression. GATA factors are expressed in a tissue-specific manner and play fundamental roles in cell-fate specification (82). The role of GATA2 in androgen signaling was first implicated by the involvement of GATA2 in androgen regulation of the PSA gene (83). Binding motifs for GATA factors and Oct1 are enriched on AR binding regions in LNCaP cells, suggesting that these transcription factors cooperate with AR in mediating the androgen response (84). In addition to its co-factor function, GATA2 might directly regulate androgen receptor mRNA and

protein expression in prostate cancer cells (85–87). Inhibition of GATA2 by small-molecule compounds is a potential strategy in blocking AR expression and signaling in castration-resistant prostate cancer (86).

FoxA1 is member of the forkhead family of DNA binding factors and plays a key role in androgen receptor-induced gene transcription. FoxA1 functions as a pioneer factor because it can bind to highly compacted chromatin and allows these genomic regions to be more accessible to other transcription factors. Therefore, FoxA1 functions to guide androgen receptor binding to the genomic sites in prostate cancer cells (88, 89). In normal prostate luminal epithelial cells, it plays an important role in maintaining the differentiation status. FoxA1 mutations occur frequently in primary and metastatic prostate cancers and may contribute to prostate tumorigenesis and cancer progression (90, 91). Loss of FoxA1 promotes prostate cancer progression to neuroendocrine small-cell prostate cancer (92). FoxA1 also has androgen receptor-independent function in prostate cancer (93).

NOVEL STRATEGIES IN CASTRATION-RESISTANT PROSTATE CANCER TREATMENTS

Even with the latest androgen deprivation therapies, castration-resistant prostate cancers are rarely cured. They simply become resistant again. Strikingly, a substantial subset of these resistant cancers still express androgen receptors and/or their variants; their growth and survival are still dependent on androgen receptor signaling. Scientists in the field of prostate cancer research are relentless in pursuing novel strategies for more complete ablation of androgen receptor signaling.

Prompted by the clinical success of selective estrogen receptor downregulator (SERD) Faslodex (ICI 182,780 or Fulvestrant) (94), selective androgen receptor downregulators (SARDs) have been developed. For instance, a SARD compound AZD3514 (95) had undergone phase I clinical trial (96). Binding of SERD or SARD causes severe receptor conformational change, resulting in receptor degradation. Another strategy is to specifically degrade the androgen receptor protein through Proteolysis Targeting Chimeras (PROTACs). Briefly, a PROTAC molecule consists of two covalently linked ligands: one ligand binds to the target protein whereas the second ligand binds to an E3 ligase system. Several AR targeting PROTACs have been reported, including enzalutamide-derived ARCC-4 (97) and aryloxy tetramethylcyclobutane-derived ARD-69 (98, 99). ARCC-4 and ARD-69 represent a novel class of drugs which directly targets the androgen receptor protein for degradation, but their *in vivo* anti-prostate cancer activities remains to be established in mouse models. Similarly, small-molecule degraders of the Bromodomain and Extra-Terminal (BET) family of epigenetic regulators, which are essential for prostate cancer growth, showed *in vivo* anti-cancer efficacy in a castration-resistant VCaP xenograft mouse model (100).

Another strategy is to silence androgen receptor gene expression at the transcriptional level. Androgen receptor

gene expression is driven by an orphan nuclear receptor ROR γ in metastatic castration-resistant prostate cancer (101). ROR γ antagonists XY018 and SR2211 potently suppressed the expression of the full length androgen receptor and truncated androgen receptor variants at the transcriptional level, consequently inhibiting prostate cancer growth in xenograft mouse model (101). It has been shown that enzalutamide-resistant prostate tumors are sensitive to ROR γ antagonists, suggesting that such a strategy may be able to overcome resistance to second-generation antiandrogens. In comparison to older strategies, this treatment can silence the expression of both full length and truncated variant androgen receptors.

One more exciting area for cancer drug development is the use of synthetic lethality. Because a subset of cancers contains defects in their DNA repair system, they become particularly vulnerable to inhibition of DNA repair enzymes. Olaparib, an inhibitor of poly(ADP-ribose) polymerase (PARP) 1 and 2, two key enzymes involved in DNA repair, has been approved by FDA for germline BRCA-mutated metastatic breast cancer (102). In a phase II trial, olaparib produced a high response rate in castration-resistant prostate cancers with DNA-repair defects including BRCA2 loss and ATM aberrations (103). A phase II trial further shows that olaparib in combination with abiraterone increased progression-free survival in men with metastatic castration-resistant prostate cancer (104).

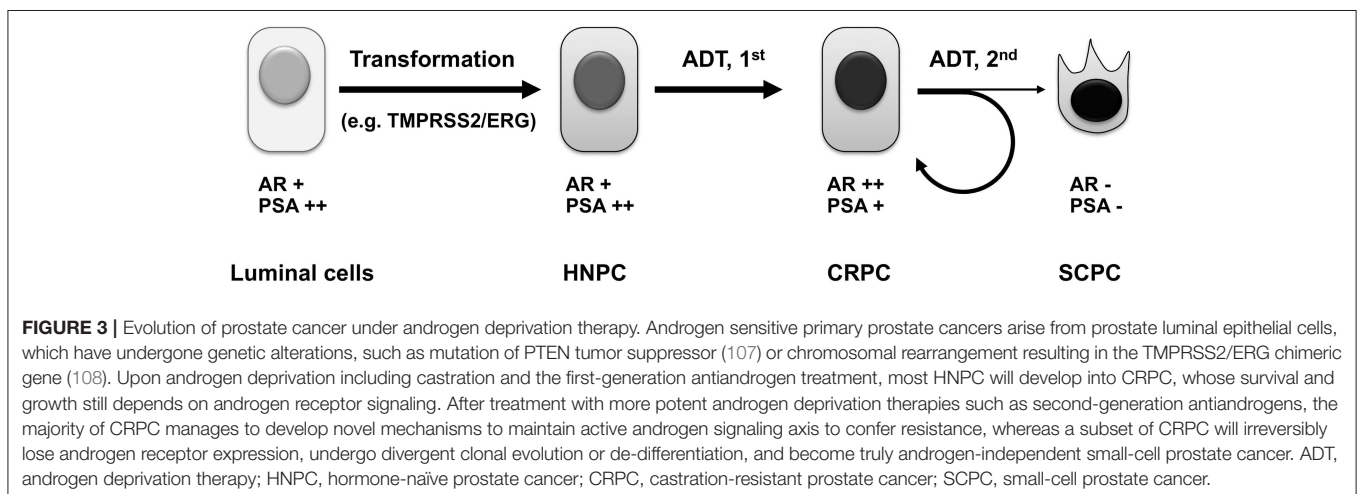
FUTURE PERSPECTIVE

Although androgen deprivation therapy prolongs the survival and improves the quality of life of prostate cancer patients, it does not cure the disease. With more complete androgen deprivation therapies and androgen receptor ablation in the near future, we hypothesize that more castration-resistant prostate cancers will undergo de-differentiation, eventually lose androgen receptor expression, and become truly androgen-independent androgen receptor-negative small-cell prostate cancers (105, 106) (Figure 3). These small-cell prostate cancers have neuroendocrine markers or basal stem cell gene signatures

(109), and they will no longer respond to hormone therapy or androgen receptor targeting therapy. There will be an urgent need to develop novel targeted therapies for this subtype of prostate cancer, when more small-cell prostate cancers are induced by complete androgen deprivation and androgen receptor ablation. It has been reported that these cancers contain gene amplification of AURKA and MYCN, which are promising therapeutic targets for this subtype of cancer (106).

Recent advances in immunotherapy are revolutionizing the treatment of cancer. For example, Sipuleucel-T (Provenge) for CRPC is the first FDA-approved therapeutic cancer vaccine (110). However, while the use of Sipuleucel-T prolonged overall survival, it did not lead to PSA reduction, tumor shrinkage, or improve disease free survival. The checkpoint blockade therapies using antibodies to block CTLA-4 or PD-1 have achieved long-term clinical benefits, and even cures a subset of cancers (111). Tumor infiltrating lymphocytes (TIL) have also shown huge promise in treating cancers (112). The success of checkpoint blockade and TIL therapies are dependent on the tumor mutational burden (113, 114). With more somatic mutations, cancer cells are more likely to be recognized by T lymphocytes as “non-self” foreigners and thereby likely to be eliminated by the immune system. Prostate cancer cells are known to have low mutation rates (115, 116) and therefore the vast majority of prostate cancers are insensitive to current single checkpoint blockade immunotherapies. Only a small subset of prostate cancers with mismatch repair defects or CDK12 mutations are likely to respond to checkpoint blockade (117, 118). Nevertheless, the combination of PD-1 and CTLA4 inhibitors in a phase II CheckMate 650 trial elicited durable clinical responses in metastatic castration-resistant prostate cancers (119). It is also possible that continuous androgen deprivation therapies will cause more mutations and genomic alternations, and render prostate cancer cells more vulnerable to immunotherapy (117).

In addition, the recently emerging chimeric antigen receptor (CAR) T cell therapy is a promising strategy for treatment of castration-resistant prostate cancer. The CAR T cell



immunotherapy has recently been approved by FDA for treatment of refractory pre-B cell acute lymphoblastic leukemia and diffuse large B cell lymphoma (120). Because CAR-engineered T lymphocytes recognize cancer cells through cancer cell surface antigens, their anti-cancer activity is not dependent on mutations in cancer cells. This is particularly important for prostate cancers which harbor low amount of somatic mutations. In the literature, there are several reports of PSMA-specific CAR T-cell therapies which have shown anti-prostate cancer activity *in vitro* and in mouse models (121, 122). Additionally, CAR-engineered natural killer (NK) cell therapy is another promising treatment for castration-resistant prostate cancer. Taken together, with these new targeted and immune therapies in sight, scientists and patients can be optimistic about eventually winning the battle against castration-resistant prostate cancer.

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QF and BH conceived and wrote the manuscript.

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