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Review

Targeting androgen receptor-independent pathways in therapy-resistant prostate cancer

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Abstract Since androgen receptor (AR) signaling is critically required for the development of prostate cancer (PCa), targeting AR axis has been the standard treatment of choice for advanced and metastatic PCa. Unfortunately, although the tumor initially responds to the therapy, treatment resistance eventually develops and the disease will progress. It is therefore imperative to identify the mechanisms of therapeutic resistance and novel molecular targets that are independent of AR signaling. Recent advances in pathology, molecular biology, genetics and genomics research have revealed novel AR-independent pathways that contribute to PCa carcinogenesis and progression. They include neuroendocrine differentiation, cell metabolism, DNA damage repair pathways and immune-mediated mechanisms. The development of novel agents targeting the non-AR mechanisms holds great promise to treat PCa that does not respond to AR-targeted therapies.
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1. Introduction

Prostate cancer (PCa) is the most frequently diagnosed non-cutaneous malignancy and a leading cause of cancer death in men in the United States [1]. Its incidence is also increasing in Asian countries including China where PCa had been considered relatively uncommon [2].

Localized PCa is curable by prostatectomy or radiation therapy. For patients who miss local therapeutic opportunities such as those with advanced or metastatic PCa, hormonal therapy becomes the standard treatment. Targeting androgen receptor (AR) signaling pathway is the molecular basis for hormonal therapy which either inhibits androgen production or blocks AR function. Although hormonal therapy is initially effective, patients eventually experience therapy resistance and develop castration resistant PCa (CRPC). Two new agents, abiraterone which prevents intratumoral androgen synthesis and enzalutamide which more effectively antagonizes androgen binding to AR, have proven to be effective in treating CRPC by inhibition of continued AR signaling. Unfortunately, despite their clinical benefits, resistance to these drugs is inevitable and the disease will progress to a lethal stage which remains incurable [2].

AR signaling is central to the therapeutic resistance development and disease progression. However, in addition to AR-dependent mechanisms, numerous other factors have been shown to contribute to the progression of therapy-resistant PCa including neuroendocrine (NE) differentiation, the alterations of cell metabolism, DNA damage repair pathways and immune-mediated mechanisms. This review focuses on AR-independent mechanisms and potential treatment strategies to overcome these clinical barriers.

2. AR-independent therapeutic strategies

Although AR signaling is a distinctive feature of PCa and represents a major therapeutic target, it is well established that tumors can bypass a functional requirement for AR by either *de novo* or therapy-induced mechanisms [3].

2.1. NE differentiation-related therapeutic strategies

NE differentiation has been demonstrated to be a major resistant mechanism as exemplified by the emergence of small cell neuroendocrine carcinoma (SCNC) after AR-targeted therapies for prostatic adenocarcinoma [4,5].

De novo SCNC is extremely rare, accounting for less than 1% of primary PCa. However, SCNC is estimated to be present in up to 20% of patients who fail conventional ADT or the newer AR-targeted therapies, indicating that an androgen-deprived environment favors NE tumor cells [6,7]. Loss or inactivation of *retinoblastoma 1* (*RB1*) and tumor suppressor *p53* (*TP53*) plays an important role in the development of SCNC. Clinically, treatment-induced SCNC (t-SCNC) has much more frequent alterations of *RB1* and *TP53* genes compared to prostate adenocarcinomas (70%—90% *vs*. 30%—50%) [8—10]. Mechanically, loss of function of *RB1* and *TP53* could induce NE differentiation by

upregulating SOX2, a transcription factor which is implicated in lineage plasticity [11] as well as cooperating with EZH2, a methyltransferase enzyme [12—14]. We have demonstrated that IL8-CXCR2-p53 pathway keeps the rare NE cells of adenocarcinoma in a quiescent state normally. *TP53* mutations inactivate this pathway leading to hyper-proliferation and aggressive behavior of NE cells and the development of SCNC [9]. Genomic studies demonstrated a co-occurrence of *MYCN* and *AURKA* amplification in 40% of SCNC samples [7]. In addition, *AURKA*, which encodes the Auroka kinase A (Aurka) in humans, also plays an important role in stabilizing N-Myc by forming a complex [15]. The amplification of *AURKA* also suggests that SCNC cells may need to gain additional clonal expansion abilities in order to repopulate the tumor since *AURKA* is a key player to promote cell cycle and proliferation [16,17].

A recent study by Bluemn et al. [3] identified three tumor types including ARPC (AR$^+$/NE$^-$), NEPC (AR$^-$/NE$^+$) and double negative PCa (DNPC, AR$^-$/NE$^-$), largely based on immunohistochemical studies. Their study demonstrated that the emergence of this DNPC is due to the elevated fibroblast growth factor (FGF) and mitogen-activated protein kinase (MAPK) pathway activity.

Although N-Myc, RB1 and TP53 play essential roles in the pathogenesis of SCNC, they are not easily druggable. Because N-Myc is stabilized by AURKA, an Aurka inhibitor, Alisertib (formerly as MLN8237), underwent a phase II clinical trial (NCT01799278) for CRPC/SCNC which had a modest response in the 59 patients enrolled [18]. Furthermore, N-Myc can transcriptionally activate DNA damage response proteins including poly ADP ribose polymerase (PARP)1 and PARP2. Combination treatment of SCNC cells with Aurka inhibitor alisertib and PARP inhibitor olaparib resulted in a significant suppression of tumor growth in preclinical models [15]. Two EZH2 inhibitors, GSK126 and EPZ6438, were able to sensitize RB1- and TP53-negative cells and mice to enzalutamide. EZH2 inhibitor combined with enzalutamide also had a greater effect than either drug alone [12]. For the DNPC, based on the finding that FGF and MAPK signaling pathways are markedly activated, the FGF receptor (FGFR) inhibitor CH-5183284 was tested and showed a significant anti-tumor efficacy both *in vitro* and *in vivo* [3].

Although targeting multiple NE factors may be able to turn off "proliferation switch" of SCNC, practical challenges exist. For instances, small molecules that directly inhibit Myc are difficult to develop because of a lack of hydrophobic invaginations and noncontiguous contacts [19]. SRRM4-specific inhibitors are currently unavailable due to a lack of crystal structure [20]. Tumor suppressors such as RB and TP53 are also difficult to target [18]. Therefore, effective therapies against SCNC remain elusive at the moment.

2.2. Metabolism-related therapeutic strategies

The alteration in the metabolism of cancer cells is a hallmark of malignant transformation. The details of metabolic reprogramming differ in different cancer types. Significant efforts have been made to study cellular metabolism of PCa in order to better understand its

pathogenesis as well as to develop potential metabolically targeted treatments [21−26].

The proliferation of normal cells is dependent on the presence of many important factors in the microenvironment including oxygen and nutrients provided by the blood. The fast proliferation rate and a hostile environment often force cancer cells to undergo a profound reprogramming of metabolism affecting key metabolic pathways. One of the metabolic pathways affected is glucose metabolism, the consequence of which is known as Warburg effect [27]. It describes a distinct propensity of cancer cell that converts glucose primarily to lactate instead of entering the tricarboxylic acid (TCA) cycle [27]. It has been hypothesized that this pathway is used to produce metabolic intermediates to increase the rate of biomass production and to speed up cell cycle. An important consequence of Warburg effect is that a very small amount of energy is generated in comparison to the normally used TCA cycle [28].

Another metabolic hallmark of cancer cells is an elevated uptake and consumption of glutamine which is the most abundant amino acid in human body [29]. In certain tumors, glutamine becomes an essential nutrient for maintaining proliferation although it is defined as a non-essential amino acid [30]. The increased glutaminolysis provides tumor cells with carbon backbone, nitrogen and needed building blocks. More importantly, glutamine is able to enter TCA cycle to replenish the glucose-derived TCA intermediates whose levels are low due to the Warburg effect [31].

The prostate epithelial cells undergo unique metabolic changes during tumor onset and progression from the prostate intraepithelial neoplasia (PIN) to metastasis [32]. Studying metabolism in PCa has the potential to discover novel therapeutic targets.

2.3. Glucose metabolism

Clinical experience indicates that unlike other tumor types, glucose is not an important nutrient for PCa because ^{18}F-fluorodeoxyglucose-positron emission tomography (^{18}F-FDG-PET) is not sensitive for the diagnosis of primary PCa due to its limited uptake by PCa [33]. However, glucose utilization varies and depends on the stage of the disease [24]. The degree of glycolysis differs between androgen-dependent and androgen-independent PCa [34]. Therefore, the impression that ^{18}F-FDG-PET is not useful for the detection of PCa because PCa cells are not glycolytic should be reconsidered [33].

Since cancer cells have a higher nutrient need for proliferation, the glucose uptake rate needs to be higher hence there are higher levels of glucose transporters. There are two different types of glucose transporters: Sodium-glucose linked transporters (SGLTs encoded by *SLC5A* gene) and facilitative glucose transporters (GLUTs encoded by *SCL2A* gene). GLUTs is a family of glucose transporters which perform the glucose uptake function by a mechanism of facilitated diffusion. Among the 14 members, GLUT1 has been the most studied in PCa due to its significant overexpression [35]. Expression of GLUT1 is strongly correlated with pathological grade and clinical stage. Its mRNA levels are higher in poorly differentiated primary PCa (high Gleason score) compared to those in low grade tumors [36].

Tris (hydroxymethyl) amonomethane (THAM or Tris), a primary amine with no counter ion, inhibits metastasis and progression in PCa by down-regulating GLUT1 expression [37]. Because GLUT1 expression is regulated by AR signaling, its expression may be low in AR negative tumor cells such as SCNC. Therefore, a strategy to target GLUT1 may be dependent on the tumor cells' AR status.

2.4. Glutamine metabolism

Due to the Warburg effect in advanced and therapy-resistant PCa, it is believed that PCa cells rely on the enhanced glutamine metabolism to maintain a functional TCA cycle as a compensatory mechanism. After being taken up into the cells by transporters such as ASCT2 which is encoded by *SLC1A5* gene, glutamine is converted to glutamate via glutaminase and further to α-ketoglutarate (α-KG). As a TCA cycle intermediate, α-KG is able to either synthesize lipids through reductive carboxylation pathway or produce ATP and anabolic carbons along the cycle process. Glutamine metabolism not only provides a major substrate for respiration but also for the synthesis of other macromolecules such as proteins, nucleotides and hexosamines. It regulates redox balance by generating glutathione as well [31,38,39].

Due to the important roles played by increased glutamine utilization in cancer metabolism, significant efforts have been made to develop compounds that inhibit specific steps of glutaminolysis. As the rate-limited enzyme of glutaminolysis, glutaminase is probably to date the most extensively studied drug target. Although a variety of small molecule inhibitors have been developed such as DON, BPTES, CB968 [40,41], none of them has been tested in PCa. Zacharias and colleagues [23] found that the androgen-independent PC3 cells and even more aggressive, metastatic subline PC3M cells demonstrate increased glutamine utilization. More importantly, these advanced PCa cells are sensitive to a novel glutaminase inhibitor CB839 which is a modified selective glutaminase inhibitor with strong efficacy in inhibiting triple-negative breast cancer [23,42].

Inhibition of the cell surface transporters to block glutamine uptake is another possible therapeutic approach. ASCT2 has been identified as the primary glutamine transporter in certain types of cancer cells. Indeed, ASCT2 expression increases in primary PCa, as well as in recurrent PCa tissues, despite an obvious decline after hormone therapy [26]. Moreover, targeting ASCT2 by a competitive inhibitor benzylserine (BenSer) significantly reduces glutamine uptake and thus inhibits PC3 cell proliferation *in vitro* and xenograft tumor growth *in vivo* which indicates another new approach for therapeutic intervention in recurrent PCa [26]. Recently, Schulte and coworkers [43] revealed a competitive small molecule antagonist (V-9302) of transmembrane glutamine flux that selectively targets ASCT2 [43]. Pharmacological blockade of ASCT2 with V-9302 resulted in antitumor responses *in vitro* and *in vivo* in cancer cells.

Epigallocatechin gallate, a flavonoid from green tea, and purpurin can both bind and inhibit glutamate dehydrogenase (GDH), another important enzyme in

glutaminolysis [44–46]. In addition to GDH, glutamate-dependent transaminases have also been considered as drug targets for modulating glutaminolysis [47,48]. However, there is no evidence showing whether targeting glutamine metabolism is effective for the treatment of PCa.

2.5. Lipid metabolism

The link between PCa development and lipid metabolism is well established, with AR intimately involved in a number of lipogenic processes [49]. It has been observed that advanced prostate tumors accumulate lipid droplets and increase lipid metabolism which in turn aids androgen synthesis [50]. PCa cells tend to use lipid more than glucose as an energetic substrate through increased β-oxidation which is different from benign cells [22]. With increased evidence linking PCa to lipid metabolism, a number of treatment strategies that target various steps of the lipid pathways have been investigated.

SREBP1 is a master regulator of lipogenesis. Although it is a transcription factor, not an enzyme, it can bind sterol regulatory element-1 (SRE1) sites governing lipid homeostasis and metabolism as well as sterol biosynthesis [51,52]. Recently, silibinin, which is isolated from the seeds of the milk thistle plant, has been shown to inhibit SREBP via both protein expression and nuclear translocation and subsequently down-regulate the downstream gene expression [53]. Thus, silibinin acts to reduce lipid and cholesterol accumulation specifically in androgen-independent PCa cells through the inhibition of SREBP1 [53]. It inhibits cell proliferation, causes cell cycle arrest and consequently, prevents the development of androgen-resistance in PCa [53].

Fatty acid synthase (FASN) is a key enzyme of the lipid synthesis pathway. It functions as an oncogene to help synthesize long-chain fatty acids. A common phenotype within many cancers including PCa is the upregulation of FASN activity and expression [54]. Moreover, in cancer cells, FASN appears to no longer require other external stimuli to be activated except the transcription factor SREBP1 [54]. Inhibition of FASN has been found to result in apoptosis of cancer cells [55]. A naturally derived inhibitor, cerulenin, and its synthetic analog C75 could suppress FASN function by binding the β-ketoacyl synthase domain [56]. Furthermore, a novel combination strategy with co-inhibition of FASN and AMPK pathway has been explored, combining the use of AMPK inhibitor compound C with C75. This combination results in an accumulation of toxic reactive oxygen species (ROS) which breaks homeostasis balance including apoptosis and arrest of tumor cell proliferation [21]. Currently the treatment methods mentioned above have not been widely used due to the compound's instability and toxicity.

2.6. DNA damage repair-related therapeutic strategies

DNA damage, induced by exogenous stimuli including toxins, ultraviolet (UV) radiation, mutagenic chemicals and ionizing radiation [57], or endogenous insults such as ROS

and reactive nitrogen species (RNS) released from immune cells [58], is a major contributor to carcinogenesis [59]. In order to maintain genomic integrity, cells have evolved an elaborate repair system to fix these DNA damages [59,60]. Clinical observations have revealed a comparatively high incidence of alterations in DNA repair genes (DRG) in advanced PCa [61]. Additionally, distinct DNA repair pathways implicate that germline mutations in homologous recombination (HR), which is a typical repair pathway for double-strand breaks, are associated with disease progression, therapeutic response and overall survival of PCa [62,63]. One study that compared the aberrational burden in CRPC to primary PCa revealed many more genetic abnormalities in DNA repair factors in CRPC (23/50) than in untreated localized tumors (3/11) [64].

Among those DNA damage repair genes, *BRCA2* mutation is the most frequent in CPRC patients [10]. Because these mutations likely result in loss-of-function which would predict for defective HR, the *BRCA2* mutations have been reported to be associated with poor outcomes after prostatectomy or radiation in localized diseases. Furthermore, *BRCA2* mutation, coupled with other genetic defects, facilitate primary PCa development and metastasis [65]. *ATM*, another DNA repair gene, is the second most commonly altered gene in the International Stand Up to Cancer Dream Team study of 150 metastatic CRPC (mCRPC) patients [66]. Similarly, a genomic sequencing study conducted by Beltran and collaborators [67] reported that the mutation rate of *ATM* is 8%.

Olaparib, a PARP inhibitor, received a United States Food and Drug Administration (FDA) breakthrough therapy designation for the treatment of patients with *BRCA1/2* or *ATM* gene-mutated mCRPC. A phase II clinical trial found that men with mCRPC and genetic mutations in *DRG* had a nearly 90% overall response rate to olaparib treatment [68]. Other recent findings suggest that loss of *CHD1*, which is often detected in advanced PCa, increases the response to PARP inhibitors [69]. Taken together, olaparib may represent the beginning of a new class of promising drugs to prolong the survival of a subset of CRPC patients with an acceptable adverse effect profile.

2.7. Immunotherapy

Immune cells have been proposed to be important mediators of PCa development, which leads to a multitude of clinical trials by targeting these cells with a variety of approaches. As a therapeutic vaccine, sipuleucel-T was the first immunotherapy approved by FDA for PCa. The pharmacological rationale is to fight cancerous cells that express high levels of prostatic acid phosphatase which is usually overexpressed in PCa cells [70]. Sipuleucel-T has shown an overall survival advantage in mCRPC patients with only mild, manageable side-effects. Nevertheless, due to the high cost and a complicated processing procedure, its clinical use is quite limited [71,72].

Pharmacological inhibition of immune checkpoint receptors or their ligands represents a transformative breakthrough in the management of many cancer types. Programmed death-1 (PD-1)/ligand-1 (PD-L1) have become important therapeutic targets. However, the role of

checkpoint inhibitors for the treatment of mCRPC is less clear [73], as it has been shown that PD-L1 is hardly expressed in primary PCa or CRPC [74]. However, another study showed that in primary PCa specimens obtained from radical prostatectomy, 52.2% and 61.7% of cases expressed moderate to high PD-L1 levels in a training cohort ($n = 209$) and a test cohort ($n = 611$), respectively, and their positivity correlated with proliferation, Gleason score and AR expression [75]. More importantly, PD-L1 was shown to be an independent prognostic factor for biochemical recurrence [75]. Another preclinical study showed that enzalutamide resistant tumors expressed significantly increased levels of PD-L1. Importantly, this overexpression was independent of AR [76].

These findings provide evidence that PD-1/PD-L1 may be potential treatment targets independent of the classical AR pathway. Several clinical trials have been undertaken to study the efficacy of PD-1/PD-L1 inhibitors in advanced PCa. Pembrolizumab is a highly selective humanized monoclonal antibody which can block the interaction between PD-1 receptor and its ligands. For the pembrolizumab monotherapy trial, 23 patients with PD-L1 positive metastatic PCa were enrolled, all of whom had received prior treatment with either hormonal therapy or docetaxel. The results showed a 13% overall response rate and a 59-week median duration of response [77]. In addition to pembrolizumab, other PD-L1 inhibitors, such as atezolizumab and durvalumab, have also been evaluated in metastatic PCa [78]. Although most of the studies are only in phase I/II stages, there is a potential to establish companion diagnoses and establish predictive biomarkers for patient selection, and for understanding the role of immunotherapy in therapy-resistant PCa.

2.8. Chemotherapy

Chemotherapy is another non-AR axis-targeted approach to mCRPC. Docetaxel and cabazitaxel are two taxane drugs that have been approved by the FDA for mCRPC patients. A phase III TAC 327 trial reported that with prednisone, docetaxel achieved a better outcome than mitoxantrone showing a 25% lower risk of death and 3-month longer median overall survival in mCRPC patients [79]. Cabazitaxel is considered a chemotherapeutic treatment option post-docetaxel because in the randomized multicenter TROPIC study, cabazitaxel resulted in a 30% reduction in the risk of death and an improved median overall survival compared with mitoxantrone even in patients with disease progression during docetaxel treatment and in those who received high cumulative doses of docetaxel [80].

Although PCa-related treatment typically consists of monotherapies prescribed in sequence, the efficacy of taxanes in both hormone-sensitive and hormone-resistant settings warrants consideration of effective combination therapies [81]. For instance, taxane-induced shifts in AR nuclear localization may serve as a biomarker of clinical benefit in patients treated with taxane-based therapy [82]. Furthermore, the combination of docetaxel and carboplatin was associated with >50% prostate-specific antigen (PSA) decline in about 20% of mCRPC patients who were refractory to docetaxel chemotherapy [83]. However,

developing such new treatment regimens requires more studies in various clinical settings.

2.9. Radium-223

For mCRPC patients with bone metastasis, radium-223, an α-particle-emitting bone-targeted agent, provides consistent improvement in bone pain and overall survival [84]. Following phase I and phase II clinical trials showing promising efficacy and safety of radium-223, a multi-center phase III trial, ALSYMPCA, was conducted [85]. This study enrolled 921 CRPC patients with symptomatic bone metastases. The patients were randomized to receive best standard of care (SOC) including all types of hormonal therapy (abiraterone or enzalutamide were not available at that time), bisphosphonates, or external beam radiation, combined with up to six injections of radium-223. Control patients received placebo injections every 4 weeks. The results demonstrated a longer overall survival benefit in the patients treated with SOC + radium-223 compared to those who received placebo (14.9 months *vs.* 11.3 months). Analysis of all the secondary efficacy points also showed advantages of radium-223 in terms of longer time to symptomatic skeletal events (SSEs), longer time to an increase in the total alkaline phosphatase (ALP) level, longer time to an increase in the PSA level and lower incidence of the SSEs [85].

Another single-arm phase IIIb trial was conducted to include AR inhibition along with radium-223. Similar to ALSYMPCA, adding radium-223 with abiraterone or enzalutamide showed greater benefits in overall survival and biological response (lower ALP and PSA levels) [86].

Because radium-223 treatment causes unrepairable double-strand DNA breaks, it is theoretically possible that tumors with DNA damage repair defects are more vulnerable to this agent [87]. A recent published study addressed this hypothesis. Of a total of 28 mCRPC patients who received radium-223, 10 of which had DRG mutations whereas the other 18 patients did not. The DRG mutated patients showed greater ALP responses, longer time to ALP progression, and a trend toward longer overall survival [88]. This finding suggests another novel therapeutic option to patients harboring DRG abnormalities.

3. Conclusions and perspectives

Although second generation antiandrogen drugs (*i.e.*, abiraterone and enzalutamide) have been widely used in the clinic, nearly all the CRPC patients will experience therapy resistance and disease progression including developing the extremely aggressive SCNC. Multiple molecular mechanisms contribute to disease progression. Better understanding of the molecular mechanisms including AR-dependent and AR-independent mechanisms will open new avenues for the design and development of novel pharmacological treatment. The advent of novel technologies such as high-throughput next-generation sequencing and liquid biopsies will allow us to more quickly uncover more therapy-resistant mechanisms and identify novel therapeutic targets. Moreover, characterizing specific

contributors of resistance in each patient will give us an opportunity to provide personalized treatments utilizing rational therapy combinations.

Author contributions

LX, HH and JH contributed to the writing of this manuscript. JC, WL and CL contributed to the clinic perspectives. All authors had read and approved the manuscript and agreed with the order of presentation of the authors.

Conflicts of interest

The authors declared no conflict of interest.

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