RESPONSE AND RESISTANCE IN CASTRATION-RESISTANT PROSTATE CANCER

EDITED BY: Hung-Ming Lam, Qi Cao and Renee de Leeuw
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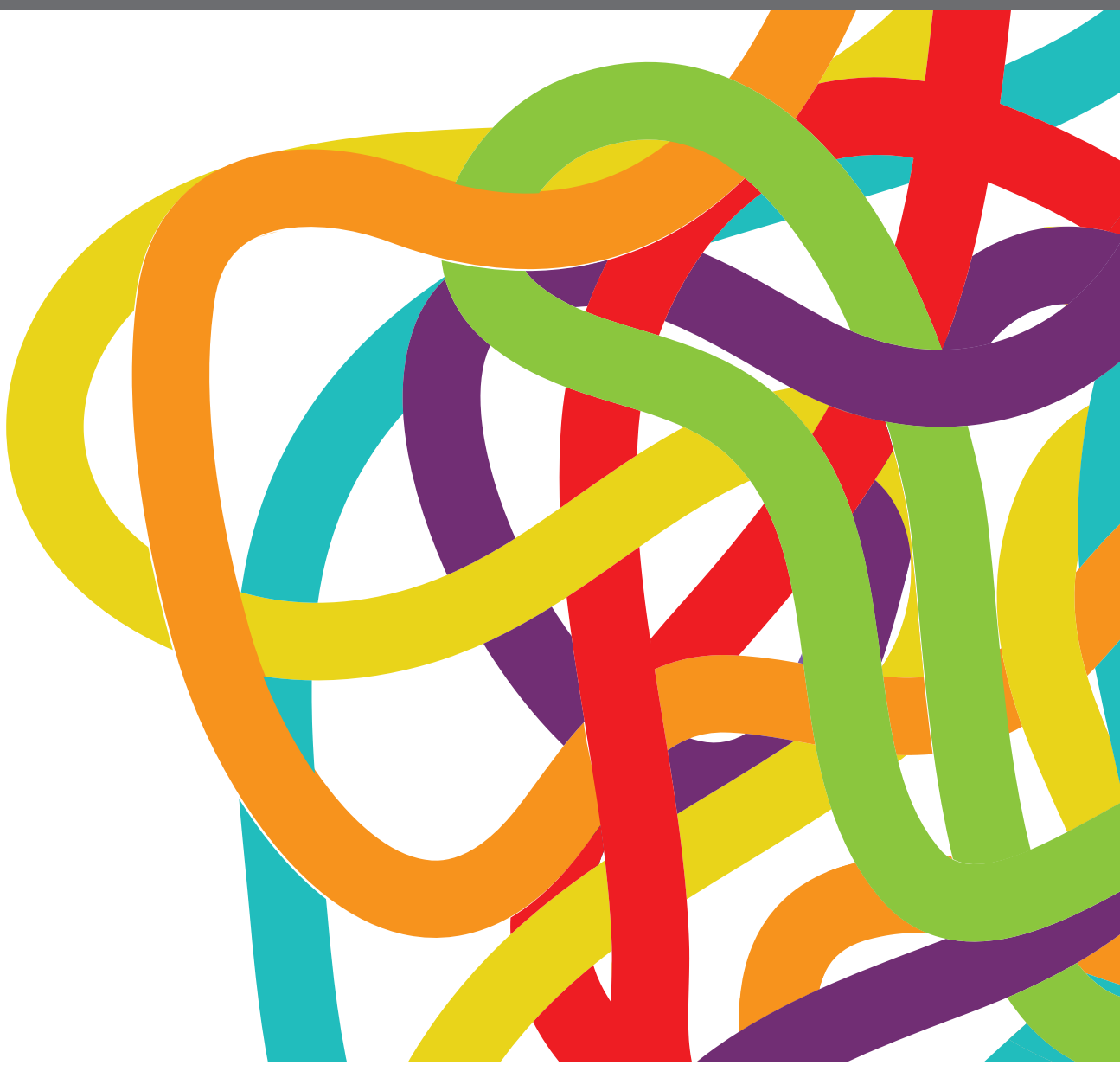

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RESPONSE AND RESISTANCE IN CASTRATION-RESISTANT PROSTATE CANCER

Topic Editors:
Hung-Ming Lam, University of Washington, United States
Qi Cao, Northwestern Medicine, United States
Renee de Leeuw, University of Illinois at Chicago, United States

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EDITORIAL
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Editorial: Response and Resistance in Castration-Resistant Prostate Cancer

Renée de Leeuw[1], *Qi Cao*[2] *and Hung-Ming Lam*[3]*

[1] Department of Pathology, University of Illinois at Chicago, Chicago, IL, United States, [2] Department of Urology and Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL, United States, [3] Department of Urology, University of Washington School of Medicine, Seattle, WA, United States

Keywords: neuroendocrine prostate cancer, castration-resistant prostate cancer, androgen deprivation therapy (ADT), new therapy, treatment resistance

Editorial on the Research Topic

Response and Resistance in Castration-Resistant Prostate Cancer

Androgen-deprivation therapy (ADT), often coupled with androgen receptor (AR)-targeted therapies, has been the mainstay therapy for patients with advanced prostate cancer (PC) for over seven decades (1). Inevitably, the disease will progress to castration-resistant prostate cancer (CRPC) in a median of 3–4 years. Second-generation AR-targeted therapies (abiraterone, enzalutamide) and chemotherapy (docetaxel, cabazitaxel) are effective in some patients. However, responsive tumors eventually develop resistance. In this Research Topic, we have organized a collection of opinion, review, and original research articles that discuss the history, biology, and therapeutic opportunities in CRPC.

PC can develop ADT resistance in multiple ways, such as CRPC or development of neuroendocrine PC (NEPC) (2, 3). Wang, Gao et al. demonstrated that Lysine specific demethylase 1 (LSD1) activated PI3K/AKT pathways in the absence of androgen and triggered AR transcriptional activity that drives PC initiation and progression to CRPC. They report that LSD1 transcriptionally regulates the expression of PI3K regulatory subunit, p85, and propose that this may occur through epigenetic reprogramming of the enhancer landscape in PC. This study suggests that LSD1 has dual functions in promoting PC development by enhancing AR signaling through its coactivator function and activating PI3K/AKT signaling.

Elevated expression of neuroendocrine markers and increased angiogenesis are the two hallmarks of NEPC. To date, the direct molecular links between these phenotypes of NEPC and their mechanisms remain largely unclear. Wang, Zhao et al. summarize the literature on proteins reported to regulate both phenotypes of NEPC, which include AURKA/B, CHGA, CREB1, EZH2, FOXA2, GRK3, HIF1, IL-6, MYCN, ONECUT2, p53, RET, and RB1. This review highlights the current efforts to target these proteins and potential therapeutic options to treat NEPC. Lee et al. discuss the potential role of a neuronal-specific RNA splicing factor, Ser/Arg repetitive matrix-4 (SRRM4), in reprogramming the transcriptome to facilitate the differentiation, proliferation, and survival of cells to establish a NEPC phenotype. This review explores the roles of SRRM4 with other pathways in driving a NEPC program as a coping mechanism for therapy resistance and defines potential therapeutic approaches targeting SRRM4 for treating NEPC.

To better understand cancer progression and therapy resistance, it is critical to investigate not only cancer-specific molecular alterations, but also global burden of genetic aberrations, genetic and non-genetic heterogeneity and dynamicity, and the cancer "ecosystem." Increased mutational burden does not appear to drive treatment-emergent NEPC. Ryan and Bose assessed published

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Edited and reviewed by:
Ronald M. Bukowski, Cleveland Clinic, United States

***Correspondence:**
Hung-Ming Lam
minglam@uw.edu

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PC cohorts for global burden of mutations and chromosomal structural variants across tumor stages, rather than individual aberrations. As anticipated, overall mutational burden, structural variants, copy number alterations all independently increase as disease progresses. However, this relationship does not appear to be linear. This review stresses that there is complexity beyond genomic alteration type, quantity, and clonality in the ability to predict cancer progression.

Jolly et al. propose alternate mechanisms that can layer on to genomic events to promote therapeutic resistance, including phenotypic plasticity and variability in genetically identical cells. This concept is better known from bacterial biology, where persisters survive antibiotic treatment and give rise to genetically similar populations. The authors build a compelling case for viewing heterogeneity beyond clonality, regarding tumors as an ecosystem that facilitates cellular phenotypic switching, allowing the tumor to withstand therapeutic assaults. Clinically, this idea of non-genetic, phenotypic plasticity in cancer is supported by positive responses after a "drug holiday." To further illustrate the relevance of non-genomic heterogeneity and plasticity, this review provides examples of therapeutic resistance that cannot be solely explained by clonal evolution.

CRPC generally shows sustained AR signaling which can be therapeutically exploited. Lam and Corey discuss the potential for paradoxically introducing androgen (i.e., testosterone) as a promising treatment for CRPC, even beyond second-generation AR-targeted therapies. Preclinical and clinical evidence have supported the use of supraphysiological testosterone to inhibit the growth of CRPC, and more recently abiraterone- and enzalutamide-resistant PC (4, 5). Despite encouraging efficacy in a subset of patients, treatment resistance develops. One pressing need on clinically managing CRPC is to identify response vs. resistance phenotypes to inform patients who will benefit from a treatment, and determine next line of therapy. While targeting AR is still under investigation in CRPC, Feng and He discuss the different AR dependent and independent paths to CRPC, and current pre-clinical and clinical developments aimed at mitigating disease progression. This review highlights advances and potential new opportunities for therapeutic intervention, including targeting different nodes of the AR signaling pathway, PARP inhibitors, and immunotherapy.

Although immunotherapy has not yet reached standard-of-care in CRPC, some patients respond exceptionally well. Ipilimumab is a human monoclonal antibody that binds to cytotoxic T-lymphocyte antigen 4 (CTLA-4). It blocks inhibitory signals expressed on activated T-cells and promotes anti-tumor activity (6). Phase III studies in PC have shown improved progression-free survival in some patients, albeit without an overall survival benefit (7, 8). Graff et al. identified 10 patients with metastatic PC with an incomplete response to ADT, and showed that three patients receiving ipilimumab achieved >50% PSA reduction with one patient achieving >90% reduction in PSA. Responders had an increase in effector memory T-cell subsets in blood and an increase in T-cell expression of T-bet, suggesting induction of a Th1 response. This study provides further rationale for future studies to identify a subset of CRPC patients who may respond to ipilimumab.

To identify new leads for CRPC treatment, Kim et al. used computational drug repositioning methods to repurpose existing drugs. The authors computationally integrated publicly available gene expression data of clinical CRPC, drug-induced gene expression data, and drug response data to determine key transcriptional perturbations in CRPC, then derived a computational reversal gene expression model to nominate drugs. Hence, they identified CRPC-associated genes MYL9, E2F2, APOE, and ZFP36 to be potentially reversed by existing drugs including sorafenib, olaparib, elesclomol, tanespimycin, and ponatinib. Importantly, lenalidomide combined with pazopanib was predicted to be the most potent therapy for CRPC.

CRPC continues to biologically evolve on treatment. With the appreciation of diverse genomic and molecular events driving CRPC progression, a multi-omics approach will be critical to define emerging CRPC phenotypes to predict therapeutic response and devise novel therapies.

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REVIEW
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Phenotypic Plasticity, Bet-Hedging, and Androgen Independence in Prostate Cancer: Role of Non-Genetic Heterogeneity

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Edited by:
Hung-Ming Lam, University of Washington, United States

Reviewed by:
Mark Labrecque, University of Washington, United States
Kouji Izumi, Kanazawa University, Japan
Daniele Baiz, Plymouth University, United Kingdom

***Correspondence:**
Prakash Kulkarni
pkulkar4@ibbr.umd.edu;
John Orban
jorban@umd.edu;
Herbert Levine
hl34@rice.edu

†Present address:
Prakash Kulkarni, Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, Duarte, CA, United States

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Mohit Kumar Jolly[1], Prakash Kulkarni[2*†], Keith Weninger[3], John Orban[2,4*] and Herbert Levine[1,5,6*]

[1] Center for Theoretical Biological Physics, Rice University, Houston, TX, United States, [2] Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD, United States, [3] Department of Physics, North Carolina State University, Raleigh, NC, United States, [4] Department of Chemistry and Biochemistry, University of Maryland, College Park, College Park, United States, [5] Department of Bioengineering, Rice University, Houston, TX, United States, [6] Department of Physics and Astronomy, Rice University, Houston, TX, United States

It is well known that genetic mutations can drive drug resistance and lead to tumor relapse. Here, we focus on alternate mechanisms—those without mutations, such as phenotypic plasticity and stochastic cell-to-cell variability that can also evade drug attacks by giving rise to drug-tolerant persisters. The phenomenon of persistence has been well-studied in bacteria and has also recently garnered attention in cancer. We draw a parallel between bacterial persistence and resistance against androgen deprivation therapy in prostate cancer (PCa), the primary standard care for metastatic disease. We illustrate how phenotypic plasticity and consequent mutation-independent or non-genetic heterogeneity possibly driven by protein conformational dynamics can stochastically give rise to androgen independence in PCa, and suggest that dynamic phenotypic plasticity should be considered in devising therapeutic dosing strategies designed to treat and manage PCa.

Keywords: bet-hedging, stochasticity, androgen independence, non-genetic heterogeneity, phenotypic plasticity, intermittent androgen therapy

INTRODUCTION

Phenotypic plasticity, the ability of cells/organisms in a population to switch states (phenotypes) in response to environmental conditions despite identical genetic contents, can have far-reaching consequences (1). In particular, it is widely acknowledged that the stochastic differentiation of a population of genetically identical cells (in other words, a clonal population) into distinct phenotypes can offer survival advantage in unpredictable fluctuating environments (2, 3). The phenomenon of bacterial persistence—the ability of a subpopulation of a clonal bacterial population to survive exposure to high concentrations of an antibiotic—is a striking example of the advantages of phenotypic plasticity (4). The existence of persisters protects the population from extinction under sudden harsh conditions and accounts for prolonged and recurrent infections (5). Recently, the concept of phenotypic plasticity has gathered much attention in cancer biology as well. Genetically identical cancer cells can manifest diverse phenotypes during tumor progression *via* mechanisms, such as epithelial–mesenchymal transition (EMT) (6), mesenchymal-amoeboid transition (6, 7), and neuroendocrine differentiation (8, 9). Such phenotypic plasticity can facilitate metastasis and therapeutic resistance

in cancer cells (10, 11). These examples have illustrated the dire unmet need to investigate the underlying mechanisms regulating phenotypic plasticity and consequent non-genetic heterogeneity.

BACTERIAL PERSISTENCE: A HALLMARK OF PHENOTYPIC PLASTICITY

Many clonal bacterial populations respond to antibiotic drug treatment in a biphasic manner; the initial steep decrease in survival (fast killing rate) of a "normal" (drug-naïve) bacterial population is followed by a much slower decrease (slow killing rate), revealing the existence of persisters (4) (**Figure 1A**). These persisters, when isolated and regrown in the absence of drug, give rise to a population that is strikingly similar to the original population. When this population is exposed to the same antibiotic treatment, a similar time-kill curve is reproduced which was observed in the initial population, thereby indicating that the slower rate of killing of the persistent population is not permanent (**Figure 1B**). Thus, the phenomenon of persistence is different than that of resistance (defined as inherited ability of microorganisms, often due to genetic mutations, to grow at high concentrations of antibiotic irrespective of the duration of treatment) (4) (**Figure 1A**). Instead, bacterial persistence has been reported to act as a "phenotypic switch" where individual *E. coli* persisters stochastically transit into an actively growing state with their growth rate indistinguishable from the non-persisters and *vice-versa* (12) (**Figures 1B,C**). A lack of change in the persisters' DNA sequence lends further credence to the idea that persistence is a non-genetic trait (13), i.e., the emergence of persisters need not depend on mutational or heritable changes in DNA sequence, but can result from diversity in cellular response to a repertoire of signals.

Direct single-cell and flow cytometry observations have suggested that persisters may arise as a subset of pre-existing dormant cells in an *E. coli* population (5). Specifically, some persister cells may have formed *a priori* even before the lethal antibiotic treatment. This pre-existing heterogeneity can be viewed as an example of "bet-hedging"—an evolutionary strategy that aims to maximize the fitness of an isogenic or a clonal population in dynamic environments through phenotypic heterogeneity, i.e., giving rise to two or more distinct subpopulations (14). Concomitant with this concept, bistability (existence of two distinct subpopulations that may reversibly transition to one another) in biochemical networks driving persistence has been proposed to give rise to persisters (15–17); this continued switching between different cell states can help to maintain a subpopulation of persisters (**Figure 1C**).

Another way of generating persisters is responsive diversification, where the application of sub-lethal levels of stress, including antibiotic treatment, can stimulate their formation (3, 5). Here, an initially homogeneous population can, while actively responding

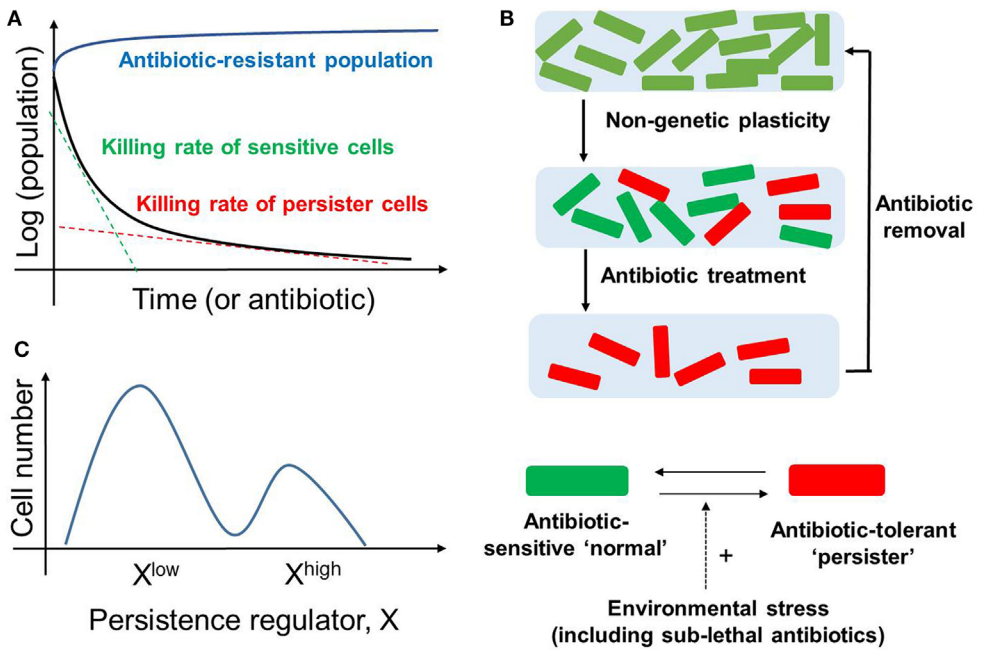

FIGURE 1 | Bacterial Persistence. **(A)** Biphasic time-kill curve in bacterial populations exposed to antibiotics: faster killing rate of sensitive cell (green dotted line) followed by a slower killing rate (red dotted line) of persisters. In contrast, the antibiotic-resistant population continues to grow in presence of antibiotic (blue curve). **(B)** (top) An isogenic population of antibiotic sensitive cells can give rise to persisters *via* non-genetic/phenotypic plasticity. These slow-cycling persisters survive in the antibiotic treatment and tend to resume growth and generate a new population identical to the original population upon antibiotic removal (bottom). Persisters and non-persisters can switch among one another; the switching rate can be influenced by external stress factors. **(C)** Non-genetic heterogeneity of a key regulator of persistence (say X) in an isogenic population may give rise to two (or more) subpopulations that may continue switching stochastically among themselves to maintain persisters.

to the environmental change, generate stochastically different subpopulations of cells, *via* induced bistability in the underlying networks (18). The above-mentioned bacterial responses highlight how bacteria can deal efficiently with multiple antibiotics. Besides generating persisters, bacteria have been observed to display advanced social community skills, such as quorum sensing and developing biofilms to enhance their survival (19).

DRUG-TOLERANT PERSISTERS (DTPs) AND MUTATION-INDEPENDENT PHENOTYPIC SWITCHING IN CANCER

More complicated and complex counterparts of the social features discussed earlier often drive adaptive tumor dynamics (20–23), for instance, cooperation among cancer cells in evading chemotherapy (24) and in successfully colonizing distant organs (25–28). "Complicatedness" refers to the number and diversity of components in a tumor microenvironment (TME) (29)—besides widespread intratumor clonal heterogeneity (30), TME contains diverse cell types, such as endothelial cells, macrophages, fibroblasts, and other immune cells (31). On the other hand, "complexity" refers to the gamut of regulatory connections among those components (29)—tumor cells communicate among themselves and with these stromal cells *via* multiple mechanical and/or chemical cues, and can thus alter cellular phenotypes reversibly (32–40). For example, M1 and M2 macrophages can affect epithelial–mesenchymal plasticity oppositely (34), whereas mesenchymal breast cancer cells can polarize macrophages toward M2 polarization (35). Nonlinear dynamics emerging from this multi-scale crosstalk defines the adaptive evolution of tumors and can dictate therapeutic response (41, 42). Thus, with this combination of clonal diversity and non-mutational mechanisms, such as dynamic phenotypic plasticity, the tumor, as an ecosystem, can withstand many therapeutic assaults and present clinically insuperable challenges of tumor relapse, metastasis, and therapy resistance (19).

While the implications of clonal diversity leading to therapy resistance and devising effective therapeutic strategies have been well-appreciated (43, 44), contributions of cellular plasticity driven by intrinsic (for example, the hypoxic or metabolic state of a cell) and/or extrinsic (for example, the chemokines or matrix stiffness a cell is exposed to) signals—without any essential complicity of genetic mutations (45–49)—have only recently begun to be elucidated. Here, we focus on the striking parallels between bacterial persistence and resistance of prostate cancer (PCa) cells against androgen deprivation therapy (ADT). These parallels aim to better understand how cancer, a community of heterogeneous subpopulations (19), may benefit from bet-hedging and thus evade multiple, potent-targeted therapies, and appreciate how cancer can exhibit traits of a robust, diverse, and adaptive social ecosystem.

Cancer has largely been considered a genetic disease driven by mutations (50). These primary and secondary mutations owing to clonal heterogeneity have been regarded as keystones of therapy resistance (51) (**Figure 2A**). However, the role of mutation-independent heterogeneity and phenotypic switching in cancer biology, such as cell-fate switching between a more dedifferentiated drug-resistant state and a well-differentiated drug-sensitive state in clonal or isogenic populations (32, 45), is gaining acceptance (46, 52). This dynamic cell-fate switching enables the emergence of multiple phenotypes from a single genotype, thus defying a precise linear genotype–phenotype mapping relationship and obfuscating the identification and targeting of mutations believed to be causal (53).

Striking recent observations in non-small cell lung cancer (NSCLC), melanoma, pancreatic ductal adenocarcinoma (PDAC), and breast cancer have illustrated the role of mutation-independent dynamic and adaptive phenotypic switching with implications in therapeutic design. For instance, treatment of multiple NSCLC cell lines sensitive to the epidermal growth factor receptor (EGFR) tyrosine kinase inhibition with a drug concentration 100-fold higher than the IC50 value led to the isolation of DTPs (45, 54). When propagated in drug-free media, DTPs resume growth and regain sensitivity to EGFR inhibition (45). This reversible phenomenon of persistence and the clonality of the population in which both persisters and non-persisters co-exist indicate that this phenotypic switching is mutation-independent (46). Similarly, some melanoma tumors that do not respond to B-raf proto-oncogene (BRAF) or mitogen-activated protein kinase inhibition may upregulate EGFR; this process can be reversed by discontinuing drug treatment, thereby re-sensitizing the apparently resistant cell population (54). Recent single-cell phenotyping and genome-wide transcriptomics reveal that in response to BRAF inhibition, many patient-derived BRAFV600-mutant cell lines undergo reversible cell-state transitions from a drug-naïve melanocytic state to a drug-resistant mesenchymal-like state (55). These transitions are driven not by selection of *de novo* genetically resistant clones, but instead result from the dynamics of underlying signaling networks (56) that can drive this adaptive transition (55). These instances of reversible and adaptive resistance against therapies are fundamentally different from *de novo* resistance (resistance due to "hard-wired" mechanisms, such as genetic mutations) and can help to explain clinical observations showing that some patients tend to regain sensitivity to BRAF inhibitor vemurafenib after a "drug holiday" (57). Furthermore, circulating tumor cells cultured from ER-positive/HER2-negative breast cancer patients revealed discrete HER2+ (proliferative) and HER2− (less proliferative, more drug-resistant) subpopulations that can interconvert spontaneously (58). Finally, a majority of PDAC cells were able to tolerate KRAS inhibition in both acute and sustained manner by adaptive switching through rewiring of signaling pathways (48). This switching did not invoke any significant mutational changes, underlining its non-genetic mechanism (48). These illustrative examples have motivated extensive investigations into phenotypic switching and DTPs in melanoma (47, 59, 60) and NSCLC (61), implying that drug resistance may be a reversible trait instead of a fixed modification, or that cells may dynamically enter and exit a window of drug resistance.

Similar mechanisms of phenotypic switching have been reported to regulate a dynamic equilibrium between cancer stem cells (CSCs)—a subpopulation with tumor-enhanced initiation potential and often enriched therapy resistance—and non-CSCs in breast cancer (62–64). These subpopulations have very similar, if not identical, genomic landscapes (62) and switching can be

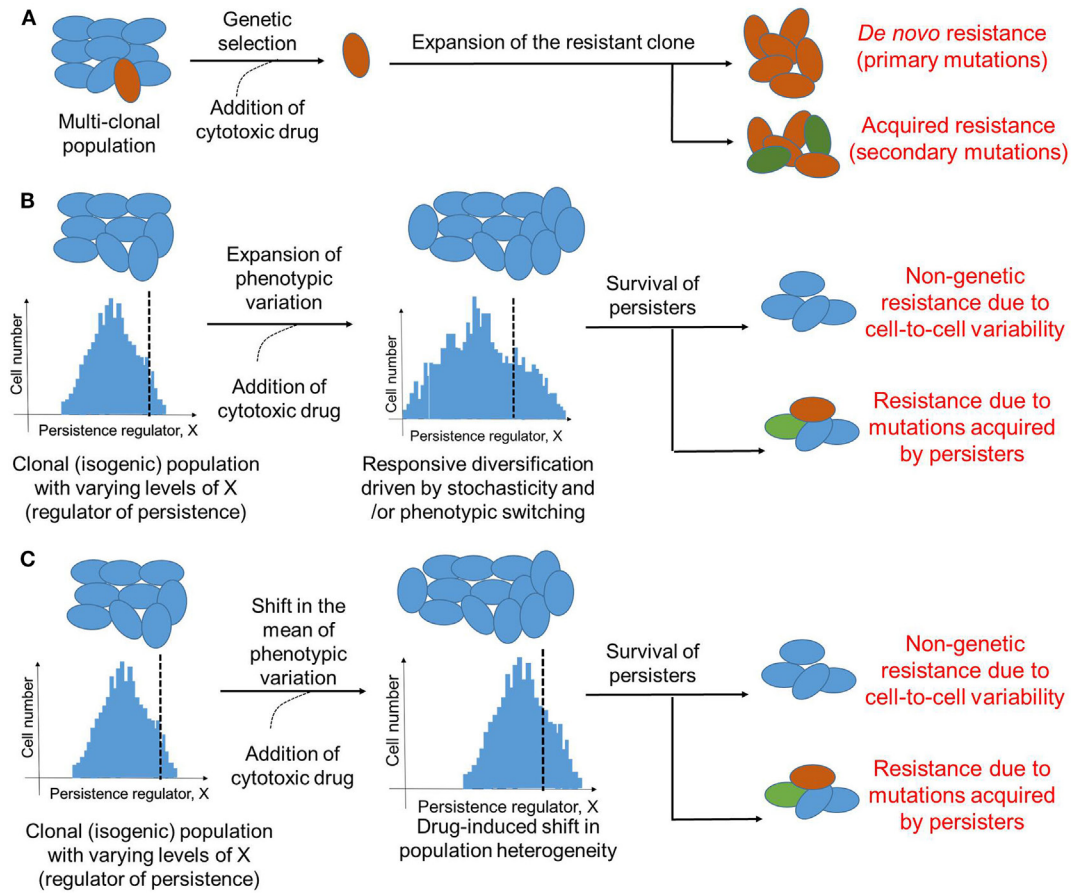

FIGURE 2 | Modes of therapeutic resistance in cancer cells. **(A)** Cancer cells may resist cytotoxic drugs due to genetic mutations either pre-existing (*de novo*) in different clones that together constitute the cancer cell population, or those acquired during expansion of the drug-resistant clone. **(B)** An isogenic or a clonal population may be able to survive therapeutic assaults due to stochastic cell-to-cell variability of a key player regulating the formation of persisters. Drug treatment may enhance this non-genetic heterogeneity due to responsive diversification and/or drug-induced cellular reprogramming. This non-genetic heterogeneity can survive addition of cytotoxic drug, however, it can also lead to different acquired mutations by drug-tolerant persisters (DTPs) that can then be inherited by DTP clones. Dotted black line indicates which cells are persisters (to the right of it) versus which are not (to the left of it). **(C)** Same as **(B)**, but without any change in variation of the levels of X; instead, the mean levels of X change. Cells shown in one color represent identical genetic makeup.

regulated by chromatin-mediated mechanisms (63), reminiscent of NSCLC studies (45), or cell–cell communication (32). Thus, similar to drug resistance, stemness need not be a static mutation-driven trait, but may be a functional reversible state that cancer cells can transiently adopt (46, 65, 66). Although the precise relation between DTPs and CSCs remains to be fully elucidated, mechanisms of drug resistance exhibited by CSCs and those by DTPs are remarkably similar (67).

ROLE OF STOCHASTICITY AND CELL-CELL COMMUNICATION IN GENERATING DTPs

Given that persistence tends to optimize the fitness of a clonal population by distributing the limited community resources into phenotypically distinct subpopulations (5), it is not surprising that cell–cell communication may be instrumental in generating DTPs and/or CSCs *via* bet-hedging and/or responsive diversification mechanisms. Similar to quorum sensing in bacterial persisters (5), cell–cell communication *via* soluble cytokines can maintain a dynamic equilibrium of CSCs and non-CSCs (32). Similarly, DTPs isolated from multiple breast cancer cell lines (68) display enhanced Notch-Jagged signaling (69), an evolutionarily conserved cell–cell communication pathway that can contribute to multiple hallmarks of cancer (70, 71), and potentially stabilize a persister cell state (72).

Further, similar to stress-induced dynamic responsiveness in bacteria, phenotypic transitions in cancer cells can be induced by therapy (68, 73, 74). One way these transitions could happen is by enhancing the pre-existing stochastic non-genetic heterogeneity (75–77) (**Figure 2B**); an alternative mechanism could be by altering the mean levels of a key regulator of cell survival (**Figure 2C**). Stochasticity is a fundamental feature of biological systems because all biochemical reactions may contain random fluctuations given that no two cells have the exact same number of key components, such as RNA polymerase, transcription factors, etc., that can

affect gene expression or activity (78). Such cell-to-cell variability has been implicated not only in modulating the probability of differentiation of embryonic stem cells into varied developmental lineages (79, 80), but also in improving population survival by diversifying cells to be able to survive stressful conditions (81, 82), i.e., by "bet-hedging". Stochastic single-cell behavior can also play a crucial role in recreating the population heterogeneity of breast cancer cells; apparently homogeneous subpopulations of breast cancer cells exhibiting distinct phenotypes, when cultured *in vitro* separately, often return to equilibrium populations over time (83). This inherent cell-to-cell variability can be enhanced by drug treatment by pushing a cell population to different cell states (55). Taken together, these observations argue for taking into account the inherent noise or stochasticity while assessing and optimizing anti-cancer therapies (84).

It should be noted that although DTPs are exemplars of non-genetic heterogeneity, genetic and non-genetic aspects of surviving therapeutic assaults may be intertwined. For instance, DNA damage—a key driver of genomic instability and genetic heterogeneity—can trigger persistence in *S. cerevisiae* by activating stress response (85). Similarly, induction of SOS response (response to DNA damage in which cell cycle gets arrested) increases the fraction of persisters in *E. coli* (86). On the other hand, EGFR T790M mutations were observed in NSCLC DTPs that were T790M-negative *a priori* (61, 87), indicating that DTPs provide a pool of cells from which various genetic modes of resistance can evolve (87) (**Figure 2B**). In the EGFR-addicted NSCLC cell line PC9 that initially revealed the existence of DTPs upon EGFR tyrosine kinase inhibition (45), 17 different persister-derived erlotinib-resistant colonies (PERCs) were established from a single persister (87). These PERCs displayed different genetic mechanisms of resistance, such as T90M mutation in EGFR and MET amplification (87). These two acquired resistance mechanisms account for over half of clinically reported cases that develop resistance against EGFR inhibitors (88). Furthermore, recent studies in melanoma, where vemurafenib treatment converted a transient transcriptional state in a clonal population into stable clones exhibiting resistance against vemurafenib (47) argue that genetic and non-genetic causes of resistance are not mutually exclusive. These observations are reminiscent of bacterial persisters acquiring stable resistance against antibiotics (89), and suggest that transient effects due to drug-induced cellular reprogramming and/or cell-to-cell heterogeneity may prevent cancer cells from extinction by giving them time to acquire inheritable secondary mutations that can stably drive the progression to relapse. Furthermore, given the growth-arrested state of persisters, the mechanism by which they gain mutation(s) may be independent of cell division, for instance, genome instability driven through DNA damage and consequent repair. Thus, preventing the formation of these persisters may contribute to reduced resistance.

NON-MUTATIONAL MECHANISMS OF ANDROGEN INDEPENDENCE IN PCA

Prostate cancer is a leading cause of cancer incidence and cancer-related deaths in men. The 5-year survival rate of patients with local and regional PCa is almost 100%, but this rate drops to 28% in patients with metastasis to a distant organ (90). The primary standard of care therapy for locally advanced and metastatic PCa is ADT—surgical or chemical castration that lowers testosterone levels by stably suppressing androgen secretion (91, 92). This treatment has been in place for over 75 years, since Charles Huggins and colleagues described its efficacy in 1941 (92). While PCa patients typically respond well to ADT, most patients experience recurrence of the disease—termed as castration-resistant prostate cancer (CRPCa)—within 2–3 years of ADT (91). New treatments for CRPC, such as enzalutamide and abiretarone have been approved, but they extend median survival by merely 2–8 months (91), thus illustrating CRPC as an unmet urgent need.

Multiple mechanisms have been reported to contribute to resistance against ADT, such as increased expression of androgen receptor (AR), mutations in the ligand-binding domain of AR, and production of splice variants of AR (91) that can be upregulated in CRPC (93). Most frequently observed genetic aberrations in metastatic CRPC occur in AR, TP53, ETS family, RB1, and PTEN (94). Loss of PTEN function—often achieved by somatic mutations—has been correlated with worse survival (95) and can suppress the levels of androgen responsive genes by modulating AR activity (96). Loss of RB1 function enhances AR mRNA levels significantly and can induce resistance against ADT (97). Fusion of ETS family members, such as ERG to androgen-regulated gene TMPRSS2 can attenuate AR transcriptional activity, and thus drive selective pressure for development of PCa resistant to ADT (98). Also, inhibition of TP53 may diminish AR-mediated signaling (99). Thus, while no universal mechanism has been identified to drive evolution to CRPC, the AR pathway usually plays a key role (100, 101).

However, other non-mutational-based mechanisms similar to bacterial persistence may also contribute to this aggressive behavior. Metastatic CRPC has been reported to contain a mixture of cells displaying a range of AR expression levels (92). It is thus possible that this heterogeneity may exist *a priori* before the onset of ADT and/or is a product of responsive diversification, i.e., ADT induces the formation of these subpopulations from a clonal population. Recent evidence supports at least the former possibility, i.e., an isogenic population of PCa cells harbors a continuum of phenotypes with varying sensitivity to ADT, or, in other words, varying androgen-dependence. Different subclones established from a parental LNCaP cell line that is generally thought to be androgen-dependent had varying androgen sensitivity and AR activity levels that correlated with their different invasive and proliferative potential (102). Given that most of the differentially expressed genes among these clones were located on regions where no copy number variation was observed (102), the existence of these subclones possibly indicates a role of stochasticity or cell-to-cell variability in the control of AR activity levels.

Stochasticity or noise in a cell can arise due to multiple reasons. Besides the well-characterized transcriptional noise (103), there may be random fluctuations in the interaction networks themselves, especially those that comprise intrinsically disordered proteins (IDPs)—proteins that lack rigid 3D structures either along their entire length or in localized regions (104). Such promiscuity in interactions may give rise to "conformational

noise" (104). IDPs have been found to be present as hub proteins in protein interaction networks from yeast to humans (105, 106), thus significantly impacting biological information transfer and propagating noise in signaling pathways. In contrast to well-defined energy landscapes of ordered proteins that determine their structure, IDPs may dynamically populate an ensemble of interconvertible structural conformations due to many local energy minima separated with low-energy barriers (107), especially when overexpressed (108). Several well-known oncogenes and tumor suppressor proteins, such as p53 (109), BRCA1 (110), PTEN (111), c-MYC (112, 113), and KRAS (114), and other key players regulating the formation of CSCs, such as LIN28, OCT4, NANOG, and SOX2 (115) have been reported to contain intrinsically disordered regions (IDRs). Further, many core modulators of EMT—a mechanism of phenotypic plasticity that shares molecular and functional overlaps with CSCs (116)—was predicted to contain IDRs (117). In the context of PCa, a striking example of an IDP is the key target of ADT itself, AR (118). Similarly, a majority of cancer/testis antigens (CTA)—a heterogeneous group of proteins that are typically expressed in testis with little or no expression in most somatic tissues, but aberrantly expressed in PCa—have been reported as IDPs (119).

Intrinsically disordered proteins may undergo a disorder-to-order transition to varying extents upon interacting with a cognate ligand, or upon specific post-translational modifications prior to ligand interaction (113, 120–122). Moreover, IDPs tend to have faster kinetics of interaction with their partners (faster binding/unbinding rates) (123), potentially amplifying promiscuity in interactions, and increasing stochasticity by allowing more flexibility in conformational switching. Considered together, these observations underscore the role of IDPs/IDPRs in phenotypic switching and thus the adaptability of biological systems in hostile environments (124, 125).

Our recent work employing multiple biophysical approaches illustrated how intrinsic disorder in a CTA named prostate-associated gene 4 (PAGE4) (126) can lead to its different conformations with implications for response to ADT (127). PAGE4 is a stress-response protein that is upregulated in response to many stress factors, such as inflammation; it is undetectable in normal adult glands, but aberrantly expressed in diseased gland and in prostatic lesions infiltrated with inflammatory cells (128). Epithelial PAGE4 correlates with and is an independent predictor of survival for patients with hormone-naïve PCa (129). PAGE4 is associated with attenuated AR signaling (129); one of the underlying mechanisms appears to involve the ability of PAGE4 to potentiate the transcription factor activator protein-1 (AP-1) (130) that can negatively regulate AR activity (131, 132). PAGE4 is phosphorylated by another component of the stress-response pathway homeodomain-interacting protein kinase 1 (HIPK1) predominantly at T51 which is critical for its ability to potentiate the transactivation of c-Jun (133), the most potent transcriptional activator of the AP-1 complex (134). PAGE4 is hyper-phosphorylated by CDC-like kinase 2 (CLK2) at many S/T residues, including T51. The interaction of PAGE4 with these two kinases leads to opposite functions. HIPK1-phosphorylated PAGE4 (HIPK1-PAGE4) potentiates c-Jun, while CLK2-phosphorylated PAGE4 (CLK2-PAGE4) attenuates c-Jun activity. This functional difference most likely arises from the different conformations of the PAGE4 ensemble, as elucidated using small-angle X-ray scattering, single-molecule fluorescence resonance energy transfer, and multidimensional NMR. HIPK1-PAGE4 exhibits a relatively compact conformational ensemble that binds AP-1, but CLK2-PAGE4 is more expanded and attains a random-coil conformation with less affinity for AP-1 (127).

As mentioned above, AP-1 can inhibit AR activity; moreover, AR can transcriptionally inhibit CLK2 (127), thereby forming a negative feedback loop in PAGE4/AR/AP-1 interactions. A recent mathematical model has predicted that this feedback loop can give rise to sustained or damped oscillations in the levels of AR activity, HIPK1-PAGE4 and CLK2-PAGE4 (**Figure 3A**), suggesting that androgen dependence of a cell can be a dynamic trait. Therefore, as the intracellular levels of HIPK1-PAGE4 and CLK2-PAGE4 vary dynamically, cells can go on phenotypic excursions with varying insensitivities to ADT [cells "resistant" to ADT have typically increased AR activity as an adaptive auto-regulatory mechanism (135)]. Additional interactions of these components could convert these oscillations into a multistable system. As already emphasized above, this heterogeneous population can thus potentially better evade the effects of ADT as compared to a homogeneous PCa population. This non-genetic mechanism is in contrast with the Darwinian clonal evolution model (136) which assumes the existence of mutually exclusive androgen-dependent

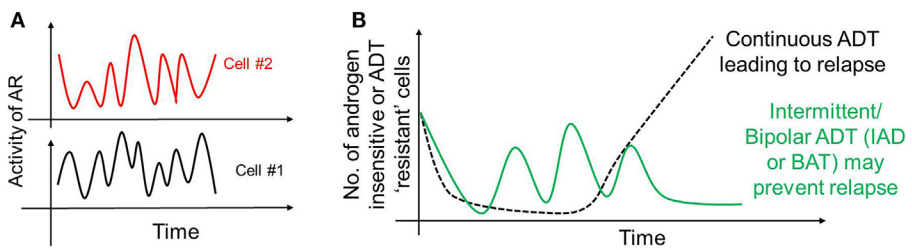

FIGURE 3 | Non-genetic heterogeneity in prostate cancer. **(A)** Androgen receptor (AR)/prostate-associated gene 4 (PAGE4)/activator protein-1 (AP-1) circuit can give rise to oscillations of AR activity in a cell that can dynamically vary its dependence on androgen. These oscillations need not be synchronized across the population. **(B)** These oscillations, together with any other mechanisms of persistence, may survive a continuous androgen deprivation therapy and eventually regrow the entire population leading to tumor relapse (dotted black curve). However, "drug holidays," such as intermittent androgen deprivation or bipolar androgen therapy may convert persisters to drug-sensitive cells, thus always keeping the number of androgen-independent (resistant) cells in check (solid green curve).

and androgen-independent clones. Thus, in addition to genetic changes, phenotypic plasticity in PCa may be driven by underlying dynamics of the PAGE4/AP-1/AR circuit.

Another plasticity mechanism that has been recently reported to be associated with PCa relapsing from antiandrogen therapies is where PCa cells acquire morphologic features of neuroendocrine carcinoma, a cell lineage whose survival no longer depends on AR (137, 138). Similar to the observed plasticity between epithelial and mesenchymal phenotypes in breast cancer (6, 52), between proneural and mesenchymal phenotypes in glioma (139), and between neuroendocrine and mesenchymal phenotypes in small cell lung cancer (9), this more macroscopic plasticity in PCa may mediate cellular response to multiple therapies (8, 140) and serve as a hallmark for aggressive disease progression (141).

IMPLICATIONS OF DYNAMIC PHENOTYPIC PLASTICITY AND STOCHASTIC STATE SWITCHING IN THERAPEUTIC DESIGN

Resistance against various therapies can unquestionably result from secondary mutations (142–145) and/or pre-existing clones with specific genetic changes (43, 146–149). But, non-mutational stochastic cell-to-cell variability that can affect drug response and therapy-induced cellular reprogramming can also drive acquired resistance (45, 46, 52, 60, 61, 68, 73, 87, 150–152). Thus, similar to precision medicine attempts focusing on genomic landscape differences (153), effective therapeutic dosing strategies, and target identification calls for considering the effects of non-genetic heterogeneity and therapy-induced phenotypic plasticity that may give rise to persisters.

The existence of these persisters may offer a plausible explanation for the success of interval dosing therapeutic strategies in stalling tumor growth in many cancer types (57, 154–156). Such discontinuous treatment regimens may exploit the fitness disadvantage typically exhibited by the DTPs in the absence of drug (157), thereby leading to a regression of persister subpopulation. Particularly, in the context of PCa, treatment paradigms that involve cycles of ADT followed by no ADT [referred to as intermittent androgen deprivation (IAD)] (158) or ADT followed by supra-physiological dose of androgen [referred to as Bipolar ADT or bipolar androgen therapy (BAT)] (159) may be as good in terms of disease-free survival rates. Continuous ADT can result in a sustained pool of PCa persisters that may provide a latent reservoir of cells that can eventually acquire diverse genetic mutations accounting for stable drug-resistance, while intermittent ADT may discourage the maintenance of persisters, thus restricting phenotypic heterogeneity and resulting in higher disease-free survival rates (**Figure 3B**). Thus, an intermittent approach is likely to be more potent in targeting the vulnerabilities of different subpopulations at once, as compared to a continuous therapy treatment that can not only spare a set of recalcitrant population, but also stabilize a transient mechanism of drug resistance (160).

An alternative approach to intermittent or discontinuous dosing strategy is combinatorial therapy. A recent study that analyzed both human clinical trial data and the drug responses of various patient-derived xenografts (PDXs) highlighted how combinatorial therapy can be beneficial even without any synergy in drug actions, due to patient-to-patient variability (161). Combinatorial therapy has also shown initial promise in PTEN-deficient PCa, where PI3K and AR signaling inhibit each other, potentially generating multiple subpopulations (162). Inhibiting either pathway singly activates the other, enabling adaptive response. However, pharmacological inhibition of both these pathways causes almost complete regression of the disease both in PTEN-deficient PCa mouse models and in human prostate PDXs (162).

Combinatorial therapy can also help to target the vulnerabilities of DTPs. Goldman et al. (68) observed that the treatment of breast cancer cells with high concentrations of taxanes generates persisters that drive aggressive tumor formation *in vivo*. These persisters display activated Src family kinase/hemopoietic cell kinase pathways whose pharmacological inhibition in a temporally constrained manner led to enhanced apoptosis (68). Similarly, Deb et al. (163) identified two mutually exclusive clonal subpopulations in altered signaling states—one with upregulated pSTAT3 and the other with downregulated SMAD2/3—and targeted STAT3 and BCL6 (a transcription factor downstream of SMAD2/3) in a combinatorial manner to overcome non-genetic heterogeneity. Furthermore, dual inhibition of Wnt and Yes-associated protein (YAP) signaling can restrict the population of both epithelial-like and mesenchymal-like CSCs (164). These combinatorial therapies are reminiscent of combinations of drug pyrazinamide (that specifically targets *M. tuberculosis* persisters) with other canonical treatments (165). However, persisters in both bacterial and cancer cell populations can often be heterogeneous in their mechanisms and extent of drug-tolerance (4, 47, 150, 166). In fact, the very idea of defining IC_{50} (50% inhibitory concentration—the drug concentration where the viability of the population is half as that of the control case) implies that individual cells in a given population exhibit heterogeneous response to treat the cells with drug concentrations considered to be lethal (165). Valuable insights into the extent of heterogeneity can be gauged by other pharmacological parameters, such as the variability in maximum susceptibility of all cells in a given population to cell death, and the range of doses over which different subpopulations get killed (165).

Given that ADT has remained the primary standard of treatment for advanced/metastatic PCa for more than 75 years, we envisage that the conceptual framework outlined above can help to guide alternative treatment options. For example, clinicians may consider prescribing IAD or BAT, thus sparing the patient of the huge costs and undesirable side effects of chronic androgen deprivation. Indeed, in a recent report, albeit on just three cases, non-metastatic PCa patients were treated effectively with long-term primary IAD (167). Although IAD is not a standard therapy for patients with non-metastatic PCa, this exploratory clinical study underscores the benefits of challenging the cancer cell's adaptive robustness due to its innate phenotypic plasticity. While the debate over the merits and controversies of administering either ADT regimen continues, and convincing data are needed to favor one over the other (157, 168), we trust the arguments presented here may inspire clinicians to reconsider treatment options and management of PCa that is currently estimated to strike one in every six men in the USA.

Phenotypic plasticity need not be a mechanism specific to PCa—it may also help normal prostate cells to cope with the significant diurnal variation (20–25%) in circulating testosterone levels in men (169). Thus, it is possible that as an adaptive evolutionary mechanism, PCa cells may be highly adept in phenotypic plasticity and persisting as a response to chronic and high fluctuations in hormonal levels and aggressive ADT—both of which represent the frequency of stressful conditions that can tune the rates of switching, and hence the frequency of persisters (170).

CONCLUSION

Phenotypic plasticity allows for a clone to sample many phenotypes—each with varying sensitivities—thus generating mutation-independent heterogeneity and enhancing clone survival. Therefore, phenotypic plasticity may serve as an effective bet-hedging strategy that may help overcome the varying selection pressures faced by a tumor (171). Here, we argue that besides genetically encoded resistance to ADT, PCa recurrence may also emerge from a phenomenon that bears a close resemblance to bacterial persistence—a bet-hedging strategy to face unpredictable harsh environmental fluctuations by generating non-genetic or mutation-independent phenotypic heterogeneity. Two crucial mechanisms underlying this heterogeneity—stochastic cell-to-cell variability and drug-induced cellular reprogramming—have already been implicated in forming DTPs. Here, we present one potential implementation strategy for generating cell-to-cell variability—the protein conformational dynamics of an intrinsically disordered protein PAGE4—that can generate dynamically varying AR levels in a cell, and thus give rise to different subpopulations, each with a varied sensitivity toward ADT. This "bet-hedging" may facilitate the presence of persisters—drug-tolerant reservoirs of cells from which multiple mechanisms of drug-resistance may evolve. Modulating inherent dynamic phenotypic plasticity and consequent heterogeneity may increase therapeutic efficacy.

AUTHOR CONTRIBUTIONS

MKJ, PK, and HL conceptualized the idea. MKJ and PK wrote the first draft. JO and KW contributed to revising the manuscript.

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Development of Neuroendocrine Prostate Cancers by the Ser/Arg Repetitive Matrix 4-Mediated RNA Splicing Network

Ahn R. Lee†, Nicole Che†, Jessica M. Lovnicki and Xuesen Dong*

Vancouver Prostate Centre, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada

While the use of next-generation androgen receptor pathway inhibition (ARPI) therapy has significantly increased the survival of patients with metastatic prostate adenocarcinoma (AdPC), several groups have reported a treatment-resistant mechanism, whereby cancer cells can become androgen receptor (AR) indifferent and gain a neuroendocrine (NE)-like phenotype. This subtype of castration-resistant prostate cancer has been termed "treatment-induced castration-resistant neuroendocrine prostate cancer" (CRPC-NE). Recent reports indicate that the overall genomic landscapes of castration-resistant tumors with AdPC phenotypes and CRPC-NE are not significantly altered. However, CRPC-NE tumors have been found to contain a NE-specific pattern throughout their epigenome and splicing transcriptome, which are significantly modified. The molecular mechanisms by which CRPC-NE develops remain unclear, but several factors have been implicated in the progression of the disease. Recently, Ser/Arg repetitive matrix 4 (SRRM4), a neuronal-specific RNA splicing factor that is upregulated in CRPC-NE tumors, has been shown to establish a CRPC-NE-unique splicing transcriptome, to induce a NE-like morphology in AdPC cells, and, most importantly, to transform AdPC cells into CRPC-NE xenografts under ARPI. Moreover, the SRRM4-targeted splicing genes are highly enriched in various neuronal processes, suggesting their roles in facilitating a CRPC-NE program. This article will address the importance of SRRM4-mediated alternative RNA splicing in reprogramming translated proteins to facilitate NE differentiation, survival, and proliferation of cells to establish CRPC-NE tumors. In addition, we will discuss the potential roles of SRRM4 in conjunction with other known pathways and factors important for CRPC-NE development, such as the AR pathway, *TP53* and *RB1* genes, the FOXA family of proteins, and environmental factors. This study aims to explore the multifaceted functions of SRRM4 and SRRM4-mediated splicing in driving a CRPC-NE program as a coping mechanism for therapy resistance, as well as define future SRRM4-targeted therapeutic approaches for treating CRPC-NE or mitigating its development.

Keywords: alternative RNA splicing, androgen receptor pathway inhibition, castration-resistant prostate cancer, lineage plasticity, neuroendocrine prostate cancer, resistant mechanisms, Ser/Arg repetitive matrix 4

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****Correspondence:***
Ahn R. Lee
alee@prostatecentre.com

†Co-first authors.

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INTRODUCTION

Prostate cancer (PCa) is not just a singular disease; it is many diseases that are interconnected through molecular, phenotypic, and functional heterogeneity not only between patients but also within the individual. This heterogeneity is one of the greatest challenges in developing therapeutic programs for PCa. Heterogeneity arises during the development of the cancer through genetic,

epigenetic, post-transcriptional, and post-translational alteration events in the tumor. In various malignancies, the fusion of both genetic and epigenetic adaptations promotes the cell to undergo processes of cellular plasticity, such as dedifferentiation or transdifferentiation, which in turn increases the rate of tumor growth, promotes resistance to therapeutics, and facilitates invasion and metastasis (1–5).

Clonal evolution theories suggest that random mutations and clonal selection generate the cellular heterogeneity seen in cancers (6, 7). This model is supported by the genetic diversity of subclones seen in primary and metastatic tumors of various cancers, including PCa (4). However, the mechanism by which this diversity in malignant cells emerges to form different subtypes of cancer remains unknown. Several mechanisms of heterogeneity establishment have been proposed including the capability of cancer cells to exhibit a remarkable degree of plasticity and the existence of cancer stem cells (1–5), although it is still controversial whether true cancer stem cells exists in PCa (8). In this section, we highlight the most recognized and supported mechanisms of lineage plasticity to promote tumor growth, metastasis, invasion, survival, and treatment resistance in PCa, with specific emphasis on the neuroendocrine (NE) prostate cancer (NEPC) subtype.

Heterogeneity of Castration-Resistant Prostate Cancer (CRPC)

The primary treatment for locally advanced or metastatic PCa is androgen receptor pathway inhibition (ARPI). This treatment is normally effective for many patients, but the benefits are short-lived as the cancer inevitably progresses to a more lethal CRPC status (9–12). Although more potent, new generation ARPI therapies, such as enzalutamide and abiraterone acetate, have been shown improved patient survival, resistance to these therapies inevitably occurs (13, 14). Overall, there are three main classifications of resistance mechanisms to ARPI that have been demonstrated to date: androgen-dependent AR signaling, receptor-dependent AR signaling, and bypass of AR signaling. In androgen-dependent AR signaling, tumor cells can restore the AR signaling pathway by increasing the synthesis of circulating androgens (15, 16, 17) or by acquiring AR gene overexpression, amplification, and mutations that allow AR activation by attenuated levels of androgens following castration or ARPI (18–22). By contrast, tumor cells can re-gain active AR signaling that is independent of androgen ligand-mediated activation of the AR by means of generating constitutively active splice variants of the AR (23–26), altering the mode of actions of the AR in a receptor-dependent manner (27), or by relying on the downstream signaling of other hormone receptor pathways, such as the glucocorticoid receptor (28). CRPC tumors that restore their AR signaling retain their luminal epithelial or adenocarcinoma (AdPC) phenotypes and are referred to as castration-resistant adenocarcinoma prostate cancer (CRPC-Ad) tumors. However, a subset of tumor cells will develop mechanisms that help them to bypass their dependency on the AR signaling altogether and progress into AR "indifferent" tumors. One subtype of AR indifferent CRPC that exhibits NE phenotypes is called treatment-induced castration-resistant neuroendocrine prostate cancer (CRPC-NE) (29–31). Generally, NEPC is defined by the expression of NE markers, such as synaptophysin (*SYP*), chromogranin A (*CHGA*), and neuronal-specific enolase (*NSE*), and the loss or low expression levels of epithelial makers, such as E-cadherin (*CHD1*), PSA (*KLK3*), and *AR* (32). Another subtype of AR indifferent CRPC that was recently reported by Bluemn et al. (33) is a double-negative AR-null and NE marker-null CRPC. Moreover, FGF and MAPK pathways have been reported to drive tumor progression, whereby activating the FGF pathway can bypass AR signaling and promote ARPI resistance in tumor cells.

Castration-resistant prostate cancer tumors do not exclusively use one of the three different mechanisms of ARPI resistance. It has been well established that CRPC tumors exhibit a varied range of AR expression levels, resulting in a significant degree of phenotypic, functional, and molecular heterogeneity seen within a tumor (8, 32). Furthermore, histopathological heterogeneity in the expression levels of various markers and genes has also been reported. For example, in AdPC tumors, NE foci (that are positive for NE markers) are observed in anywhere from 10 to 100% of the tumors examined (34–37). However, most of the tumors with NE differentiation were not confirmed to progress to CRPC-NE. In fact, it was recently demonstrated that a new genetically engineered CRPC mouse model with co-inactivation of *TP53* and *PTEN*, named *NPp53*, progresses to either CRPC-Ad tumors with NE foci that are non-proliferative or CRPC tumors explicit with NE differentiation that are highly proliferative, suggesting the importance of active proliferative genes during CRPC progression for the CRPC-NE phenotype to emerge (38).

Based on these observations in PCa, we hope that one can appreciate the vast degree of histopathological, molecular, phenotypic, and functional heterogeneity seen within not only CRPC subtypes but also individual NEPC cases. In fact, heterogeneity extends to the different subtypes of NEPC, such as AdPC with NE differentiation, AdPC with Paneth cell NE differentiation, carcinoid tumors, and small or large cell NEPC, which are classified by their histopathological characteristics (39). Furthermore, within individual CRPC-NE tumors, the expression levels of AR, as well as the expression of different NE markers, vary (40). This observation suggests that there are many complex mechanisms involved in the development of CRPC-NE; however, due to the limited understanding of the molecular underpinnings of NEPC development, the NEPC markers of detection and its various subtypes have not been well defined in the clinic or in the lab. Unfortunately, there is no gold standard to diagnose NEPC. Currently, in the clinic, SYP, CHGA, and NSE are the three main

Abbreviations: PCa, prostate cancer; NE, neuroendocrine; NEPC, neuroendocrine prostate cancer; AR, androgen receptor; ARPI, androgen receptor pathway inhibition; CRPC, castration-resistant prostate cancer; AdPC, prostate adenocarcinoma; CRPC-Ad, castration-resistant adenocarcinoma prostate cancer; CRPC-NE, treatment-induced castration-resistant neuroendocrine prostate cancer; SR, serine/arginine; RRM, RNA recognition motifs; RS, arginine/serine-rich; SCLC, small cell lung cancer; ASD, autistic spectrum disorder; HAT, histone acetyltransferase; SMI, small molecule inhibitors; ASO, antisense oligonucleotides; SSO, spice-switching oligonucleotides.

NE cells markers used to histologically detect NEPC tumors; although it has been shown that 10–40% of AdPC tumors are positive for these same markers, which demonstrates the relatively poor specificity of these diagnostic markers (41).

Overall, these findings confirm that CRPC heterogeneity develops as a result of survival mechanisms as a means to escape treatments, such as ARPI, either by progressing into AR-driven AdPC, AR "indifferent" CRPC-NE, or double-negative AR-null and NE marker-null PCa tumors.

MECHANISMS OF LINEAGE PLASTICITY

Lineage plasticity of PCa cells represents one of the greatest challenges in PCa therapeutics. It is described as a mechanism of ARPI resistance, whereby PCa cells with luminal epithelial phenotypes gain the ability to transform into other lineages or phenotypes, such as NE cell lineages. To date, several articles have reported that lineage plasticity can be used as a survival mechanism for cells to develop ARPI resistance by progressing into either AR-driven CRPC-Ad or AR "indifferent" CRPC-NE (32, 40, 42–44). How PCa cells gain this lineage plasticity and choose which lineage fate remains unclear; however, recent studies have demonstrated that genetic, epigenetic, and RNA splicing regulations, as well as tumor microenvironment factors, may influence the plasticity of PCa cells.

Genetic and Epigenetic Modifications Confer Plasticity of PCa Cells

Although the overall global genomic landscape (i.e., somatic copy number, point mutations, and polyploidy) between CRPC-Ad and CRPC-NE shows a significant overlap, some genetic alterations contribute to the lineage plasticity of PCa cells to produce the heterogeneity seen within PCa (40, 45). For example, loss-of-function alterations in *TP53*, *RB1*, and *PTEN* tumor suppressor genes are a common and frequent occurrence in CRPC-NE compared with changes in CRPC-Ad (46). Recent studies have also shown that a double knockdown of both *RB1* and *TP53* genes in the human LNCaP AdPC cell line facilitates resistance to ARPI (44). Furthermore, these cells display a degree of plasticity with increased expressions of basal epithelial and NE cell markers, as well as a decrease in expression of luminal epithelial cell markers (44). This article proposed a model of lineage plasticity in luminal epithelial cells, whereby cells undergo reprogramming and dedifferentiation from a luminal to a NE-like basal or mesenchymal lineage as a result of SOX2 deregulation, which is a putative developmental factor essential for self-renewal and pluripotency (44). Moreover, recent articles have reported the role of BRN2, a POU-domain transcription factor known to promote neuronal differentiation during neurogenesis, in driving NE differentiation of ARPI-resistant AdPC cells *via* SOX2 regulation (44, 47). This study demonstrated that BRN2 could promote augmented NE marker expression to drive a neural program together with SOX2 in ARPI-resistant AdPC cells to promote CRPC-NE development. In addition, a recent study by Zou et al. (38) revealed that the genetically engineered *PTEN*- and *TP53*-loss mouse model called NPp53 recapitulated human CRPC progression, whereby the tumors progressed quicker to this phenotype following ARPI treatment rather than show a positive response to the treatment. Following their analyses, these tumors had highly aggressive and proliferative phenotypes and displayed histopathological phenotypes similar to treatment-induced CRPC-NE. This group suggested that SOX11, a known target of p53 and found to be conserved in CRPC-NE tumors, could be responsible for promoting neuronal differentiation downstream of SOX2, which may be required earlier on to promote epithelial plasticity during CRPC-NE progression. In fact, predicted targets of SOX11 are BRN2 and N-Myc, which have also been shown to be drivers of CRPC-NE. This suggests that an initial establishment of epithelial plasticity and a degree of potency by early factors such as SOX2 and subsequent, downstream factors such as BRN2 and N-Myc are important in coordinating the NE cell lineage fate to promote the formation of CRPC-NE. These key findings suggest a role of the tumor suppressor genes *TP53*, *RB1*, and *PTEN*, as well as the essential temporal regulation of the SOX family of transcription factors, such as SOX2 and SOX11, in driving lineage plasticity of PCa cells.

Although there is large overlap in genomic landscapes between CRPC-Ad and CRPC-NE, there are significant differences in the epigenomic profiles of these two types of cancers. It is suggested that this marked difference in the genome-wide DNA methylation status between CRPC-Ad and CRPC-NE tumors is primarily driven by a histone methyltransferase called EZH2, where both its protein and mRNA levels are upregulated in CRPC-NE tumors (40, 48, 49). Recent findings by Dardenne et al. (50) have shown that N-Myc and EZH2 signaling activity is tightly coupled to drive a CRPC-NE molecular program (50). This study reported that the overexpression of N-Myc, a neuronal-specific transcription factor highly enriched in ~40% of CRPC-NE tumors and is associated with a range of neural cancers, increases EZH2 activity, which in turn, represses AR signaling and promotes an enhanced activation of the PI3K/AKT pathway to drive CRPC-NE development. Although further studies are required, these results suggest a potential mechanism by which N-Myc can promote an EZH2-mediated reprogramming of the epigenome to drive CRPC-NE development. In addition, Ku et al. (43) demonstrated that the lineage transition and ARPI resistance seen in the *RB1* and *TP53* double knockdown cell line is induced by EZH2, and treatment with EZH2 inhibitors could reverse this phenomenon (43). To date, these findings have shown the significance and interplay of genetic alterations and epigenetic modifications in driving the lineage plasticity, and thus, the emergence of CRPC-NE.

Alternative RNA Splicing Confers Plasticity of PCa Cells

Alternative mRNA splicing events in cancer cells have been found to facilitate the aggressive behaviors of cancers, which have been previously reviewed (51). In the context of PCa, it has been shown that tumor cells exploit splicing processes to promote tumor plasticity, treatment resistance, tumor growth, proliferation, and differentiation (44). For example, AR splicing products, such as

ARv7 (the most frequent variant of AR observed), have been shown to promote the resistance to ARPI and the proliferation of various cell line models in a ligand-independent, constitutively active manner (52–56). Various RNA splicing regulators have also been shown to change expression patterns in PCa. One of these regulators, Sam68, has been shown to be upregulated in PCa and promote cell survival and metastasis (57). For the purpose of this article, we will focus on a splicing regulator and driver of cellular plasticity in PCa cells and CRPC-NE development called Ser/Arg repetitive matrix 4 (SRRM4).

Lapuk et al. (58) have previously shown that DNA and RNA sequencing of CRPC-Ad and CRPC-NE tumors have an increased expression of SRRM4, a neural-specific mRNA splicing factor, which was unique to CRPC-NE (58). A follow-up study performed with deeper whole-transcriptome analyses on two CRPC-Ad/NE patient cohorts identified a CRPC-NE-specific RNA splicing signature that is predominately driven by SRRM4 (59). In their article, SRRM4 is responsible for the aberrant splicing of at least 16 key target genes involved in the transformation of AdPC to CRPC-NE under ARPI (59). Furthermore, SRRM4 is shown to drive the transformation of LNCaP AdPC to CRPC-NE xenografts when inoculated under the renal capsule of castrated mice. In addition, long-term studies of serial passaging subcutaneously inoculated LNCaP cells overexpressing SRRM4 in castrated mice generated a series of five treatment-induced CRPC-NE xenograft models called LnNE (60). As these tumors were passaged, they showed an increased expression of NE markers, grew more aggressively, and exhibited a decreased or no PSA expression, thus, mimicking characteristics of AdPC progression to treatment-induced CRPC-NE. Furthermore, recent findings have elucidated the role of SRRM4 in inducing NE-like phenotypes in an array of various cell types, such as PCa stromal cells, benign prostate hyperplasia cells, and normal prostate cells (Lee et al., unpublished manuscript). In this study, all of the cell lines overexpressing SRRM4 show an overall increase in NE markers and a decrease in AdPC markers, although heterogeneity in the expression of some markers were seen within the different cell line models overexpressing SRRM4. In addition, all cell lines express the CRPC-NE-specific RNA splicing signature previously reported in CRPC-NE tumors (59). These findings strongly support that SRRM4 drives NE differentiation of PCa cells and CRPC-NE tumorigenesis in a cell context-dependent manner.

Ser/Arg (SR) FAMILY PROTEINS: SRRM4

Ser/Arg repetitive matrix 4 belongs to a family of proteins involved in mRNA splicing called serine/arginine (SR)-related proteins. These proteins have a diverse range of functions in facilitating alternative splicing of genes, which, in turn, can have dramatic effects on the function, localization, stability, and/or expression of differentially spliced mRNA or its resulting translated protein (61). To fully elucidate the vast roles of SRRM4 in the pathological development of CRPC-NE, we must first identify and understand the SR family and SR-related family of proteins, as well as the normal biological and molecular functions of SRRM4 during neuronal development.

The SR Family and SR-Related Family of Proteins

It has been determined that 90–98% of the genes in the human body have alternative splice variants, emphasizing the importance that alternative splicing plays a critical role in normal development (62, 63). In fact, it has been proposed that alternative RNA splicing is the source of biological diversity and complexity within the human neural system (64, 65). The interactions between *cis*- and *trans*-acting factors are essential in regulating alternative splicing through either the repression or activation of splice-site selection. The factors essential for orchestrating splicing programs include RNA-binding domain-containing small nuclear ribonucleoproteins (also known as snRNP), such as U1, U2, U4, U5, and U6 [reviewed by Kramer (66), Will and Luhrmann (67)], and SR family and SR-related family of proteins (66, 67). These components make up a macromolecular complex called the spliceosome. The SR family of proteins contains one or two conserved RNA recognition motifs (RRM) at the N-terminus that are essential for RNA-binding specificity, as well as an arginine/serine-rich (RS) domain of varying sizes at the C-terminal end, which is important for promoting the protein–protein interactions and recruitment of the spliceosome complex (68, 69). The SR family of proteins has diverse functions in regulating not only pre-mRNA splicing but also post-splicing events, such as the exportation of mRNA, nonsense-mediated mRNA decay, and the translation of mRNA, which has been previously reviewed (70, 71). On the other hand, SR-related proteins contain an RS domain, but may or may not contain a defined RRM (72). Similar to SR family of proteins, SR-related proteins are found to play a role in not only splicing, but in other fundamental cellular processes, such as chromatin remodeling, cell cycle progression, and transcription (73). The regulation of SR protein splicing activity and its subcellular localizations, in part, depend on the dynamic cycle of phosphorylation and dephosphorylation of the serine residues in its RS domain (74). The phosphorylation statuses of RS proteins have a diverse effect in mediating the regulation of spliceosome complex assembly, recruitment, splicing activation, and splice-site selection *via* alterations to the protein–protein and protein–RNA interactions (75–77).

While SR proteins have many similar characteristics, SRRM4 (also known as nSR100) is particularly interesting as it has been suggested to be the source of proteomic diversity and functional complexity of the vertebrate nervous system, although its evolutionary origin is unclear (78). SRRM4 was identified as a neural tissue- and vertebrate-restricted SR protein involved in complex alternative splicing of neural-specific exons, which are essential for vertebrate nervous system development and neural cell fate differentiation (78). In addition, SRRM4 is uncharacteristically heavy, weighing 100 kDa, which is likely a result of a large RS region in the protein. The presence of large RS domains makes SRRM4 more highly phosphorylated than its family members, an important characteristic of splice-site selection. Furthermore, the RS-rich domains of SRRM4 have also been predicted to be responsible for protein–protein and/or protein–RNA interactions required for spliceosomal complex assembly to promote splicing that allows SRRM4 to regulate brain-specific exon inclusion of

genes associated with neuronal development (68, 79–81). In conclusion, SR proteins play an essential role in regulating development and key cellular processes, suggesting that the dysfunction of these proteins will result in unsuccessful development and abnormal cellular processes, which can lead to human diseases and cancers.

SRRM4 in Normal Development

Raj et al. (80) recently demonstrated that by knocking down or overexpressing SRRM4 *in vivo,* SRRM4 mediates the inclusion of 30–50% of conserved mouse and human brain-specific exons, manifesting their unique neural-splicing profiles (80). *In utero* SRRM4 knockdown studies in mice demonstrated a diminished differentiation of neural progenitors (82). Furthermore, knockdown of SRRM4 in neural cells and in zebrafish demonstrated impaired neurite morphogenesis and branching (78). In Calarco et al.'s (78) work, it was also revealed that SRRM4-mediated inclusion of neural-specific exons of genes encoding neuronal GTPase activity, which have putative roles in cytoskeletal remodeling and dendritic growth and branching, can alter protein-coding sequences, suggesting that these target exons may stimulate protein–protein interaction networks. Another study showed that an inactivating mutation in the *SRRM4* gene caused deafness and balance impairments in Bronx Waltzer mice (83). Importantly, transcriptomic analysis of the sensory neurons in these mice exhibited a distorted splicing signature when compared to normal mice (83). These findings suggest an essential role of SRRM4 function during neural development to promote neural cell fate differentiation, in particularly, its function in the splicing of neural-specific exons to regulate the overall resulting protein function from its processed pre-mRNA.

A specific, widely studied mechanism that heavily relies on SRRM4 function during neurogenesis to support that SRRM4 is implicated in the splicing of neural-specific exons is that it facilitates neuronal differentiation *via* a cross-regulatory mechanism with a transcriptional repressor called REST (78, 80, 82, 84). REST is a transcriptional repressor that binds to the RE-1 site in the regulatory region, upstream of a target gene, and inhibits the transcriptional activity of neuronal genes by facilitating repressive histone modifications *via* the recruitment of co-repressors and factors, such as HDAC1/2, coREST, and Sin3A, to the gene's promoter region. During neurogenesis, REST and SRRM4 antagonistically regulate the developmental process. Specifically, REST represses the transcription of genes that are important for driving the neuronal phenotype and has also been shown to directly inhibit SRRM4 expression as a means to prevent neurogenesis (82). Conversely, SRRM4 functions to facilitate the alternative splicing of *REST* into a splice variant called REST4. REST4 is a truncated isoform of the REST protein with reduced DNA-binding function. It has also been shown that REST4 isoforms can directly bind to REST to inhibit its function (84), thus, resulting in the transcriptional activity of REST-repressed target genes. In summary, high expression of REST suppresses the expression of neuronal-specific genes and inhibits SRRM4 activity in non-neuronal cells. On the other hand, to achieve neuronal cell differentiation, increased splicing activity of SRRM4 is required to increase the expression of the dominant-negative isoform REST4, which inhibits REST's function to activate the transcription of genes important for neuronal development. The critical roles of these genes in fostering and regulating normal neural development has also been demonstrated in studies that investigate the consequences of mutations within or dysregulations of these genes, which will be described within this article. In conclusion, these findings strongly support a key role of SRRM4 as a critical regulator of neuronal development and normal neuronal cell function.

SRRM4 in Pathogenesis

Based on the fundamental role of SRRM4 and its functions during normal neural development, it is clear that aberrant SRRM4 expression or function will promote pathogenesis. Aberrant RNA splicing has been demonstrated as a mechanism exploited by cells to promote the progression of many diseases, such as neurological diseases and cancers (44, 62, 81, 83, 85–87). In fact, SRRM4 has been associated with various diseases where the altered expression and splicing function of SRRM4 promotes the progression of these diseases, such as small cell lung cancer (SCLC), a NE cancer of the lung (59, 83, 88, 89). These neurological-related diseases with aberrant SRRM4 expression and function exhibited marked variations in their splicing programs. Therefore, studying the altered splicing profiles of diseases by SRRM4 will provide a new avenue to investigate the molecular mechanisms and outcomes of splicing during disease progression.

As mentioned earlier, our recent studies demonstrate that CRPC-NE tumors have a very conserved and unique splicing signature, where SRRM4 promotes the inclusion of microexons of genes that are highly enriched in neuronal functions (59). These results are supported by a previous study done on SRRM4 function and alternative splicing in autistic spectrum disorder (ASD) (85). In their study, Irimia et al. (85) observed a reduced expression of SRRM4 in ASD, which resulted in the dysregulation of the highly conserved SRRM4-mediated splicing program. Collectively, both studies revealed that the positions of the microexons in the SRRM4-targeted splice variants were near or overlapped regions of conserved domains and motifs that are important for protein–protein interactions during neural development (59, 85). This suggests that microexons are important in remodeling protein complexes and interaction networks, resulting in altered pathways to promote the neuronal lineage cell fate. To summarize, the consequences of the downstream spliced genes of SRRM4 were found to be important for several hallmarks in instigating CRPC-NE tumor establishment, such as driving NE lineage differentiation, stimulating neurite growth, evading apoptosis, promoting proliferation, and potentially regulating the epigenome. Further studies are essential in fully understanding the molecular mechanisms involved in CRPC-NE progression *via* SRRM4-mediated alternative splicing events.

ROLES OF SRRM4 IN CRPC-NE DEVELOPMENT

Recently, it has been shown that CRPC-NE patient tumors develop a unique splicing profile when compared with the

profiles of CRPC-Ad, whereby CRPC-NE tumors share 16 distinctly spliced genes that are primarily driven by SRRM4 (59). These spliced genes encode kinases that can activate major signaling cascades/pathways (such as the MAPK pathway), GTPases that can promote NE-specific morphogenesis, facilitators of proliferation and invasion, post-translational modifiers (such as acetylation and methylation), cell survival regulators, and neural differentiation fate factors. These CRPC-NE-specific spliced genes have recognized functions that are important for facilitating various aspects of neural programming in early development (80, 81). SRRM4-mediated RNA splicing of these genes includes, but is not limited to, *REST*, *Bif-1*, MYST/Esa1-associated factor 6 (*MEAF6*), and PHD finger protein 21A (*PHF21A*), all of which will be elaborated on how they promote CRPC-NE development downstream of SRRM4 in the following sections. However, it is also important to note that SRRM4 may facilitate CRPC-NE progression in conjunction with other known pathways and factors important for CRPC-NE development, such as the AR pathway, *TP53* and *RB1* genes, the FOXA family of proteins, and environmental factors. In this section, we will discuss how SRRM4 may be connected to multiple pathways of different known drivers of CRPC-NE progression and how SRRM4-mediated spliced variants facilitate the development of CRPC-NE tumors downstream of SRRM4.

SRRM4, the Androgen Receptor (AR), p53, and RB1

While primary *de novo* NEPC makes up <1% of PCa incidences, the majority of PCa cases are AR-driven PCa. However, after therapeutic treatments such as ARPI, tumors will inevitably gain resistance to these AR-targeted therapies. As previously described, some tumors will restore their AR signaling and some, such as CRPC-NE, will become AR "indifferent." The fact that CRPC-NE arises mainly after therapeutics shows that AR pathway regulation plays a fundamental role in the progression to CRPC-NE. In fact, sequencing studies revealed that nearly all CRPC-NE incidences arise as a result of the selection pressures of therapeutics (40, 44). Patient-derived xenograft CRPC-NE models reveal that AdPC tumors under ARPI conditions can transform to NEPC tumors (44, 45). Furthermore, studies have demonstrated that the AR pathway can suppress PCa lineage plasticity into NE or other cell lineages, where ARPI can reduce AR-mediated repression of SOX2 and induce BRN2, which in turn positively regulates the expression of SOX2, a fundamental regulator of stemness during embryonic development (47, 90, 91). These findings suggest a clear role of AR in conferring AdPC lineage plasticity and that the selection pressure of ARPI is imperative for the development of CRPC-NE.

Our lab has recently observed that SRRM4 mRNA is present in small populations of AdPC tumors, where the prevalence and mRNA expression of SRRM4 increases after therapeutic interventions such as ARPI in CRPC-Ad tumors, suggesting that active AR signaling can repress SRRM4 expression, and consequently function (Li et al., manuscript under review). In this study, we propose that one of the earliest initial molecular events in the emergence of SRRM4-driven CRPC-NE occurs in two possible ways. One way is that the existing SRRM4-positive population of PCa cells is selected for survival under the selection pressures of ARPI. Alternatively, prolonged ARPI can increase SRRM4 expression *via* epigenetic alterations regulated, in part, by AR. Although the specific molecular cross-talk mechanisms between AR and SRRM4 remain to be discovered, we hypothesize that, based on previous and current findings, the conjunction of ARPI and SRRM4 in the clinic promotes CRPC-Ad progression to CRPC-NE. However, it is noteworthy to add that PCa patients are treated with multi-therapeutics, where ARPI is commonly combined with chemo- or radiation therapies. Due to this, we cannot rule out other therapeutics that may contribute to the emergence of CRPC-NE.

The role of AR in conferring AdPC lineage plasticity has been shown to be augmented by functional inactivation of p53 and RB1 to promote the emergence of CRPC-NE. Recent whole-genome sequencing of CRPC-Ad and CRPC-NE tumors has revealed that ~55–75% of cases have concurrent functional mutations or deletions of the *RB1* and *TP53* genes, as opposed to the ~15–40% of cases seen in CRPC-Ad tumors (40, 44). This suggests that these genomic aberrations are highly correlated with CRPC-NE tumors and may play a role in the development of CRPC-NE. In fact, one of the earliest PCa transgenic mouse models, called TRAMP, demonstrated the implications of these tumor suppressors in the emergence of CRPC-NE tumors (92). This TRAMP mouse model expresses the transforming region of SV40 large T antigen, which acts to sequester and inactivate both p53 and RB1. These TRAMP mice spontaneously develop PCa that closely resembles the molecular and phenotypic characteristics and progression of hormone-naïve PCa to metastatic CRPC-Ad to CRPC-NE. However, the progression to CRPC-NE observed in the TRAMP mice models relied upon ARPI conditions. Indeed, recent research has demonstrated that ARPI treatment in conjunction with the loss of function of p53 and RB1 facilitates lineage plasticity of AdPC cells to basal, mesenchymal, or NE-like cells in various mice models (43, 93). A study by Mu et al. (94) demonstrated that the loss of function of p53 and RB1 in AdPC cells under ARPI conditions induced lineage plasticity, favoring the NE cell lineage *via* increased expression of SOX2 (44). However, these findings show that the acquired lineage plasticity by p53 and RB1 functional inactivation under ARPI is not limited to CRPC-NE specifically, suggesting other lineage directions. A prime example is brain metastasis-derived DU 145 AdPC cells that have inactivated mutations in *TP53* and *RB1* as well as an AR-null genomic profile. Furthermore, it is important to note the well-established function of p53 and RB1 in the emergence of CRPC-NE as a putative tumor suppressor of proliferation and survival. As their genomic and functional alterations are prevalent in CRPC-NE tumors, these alterations are important to the uncontrolled hyperproliferation observed in clinical NEPC tumors (95).

These findings suggest that, together with ARPI, inhibiting p53 and RB1 function increases lineage plasticity of PCa cells allowing for the differentiation of other cell types. In our recently generated CRPC-NE LnNE xenograft model, SRRM4-mediated transformation and tumor progression of AdPC tumors into CRPC-NE tumors under ARPI was augmented with the addition

of *TP53* knockdown (59). Throughout serial passaging of the LnNE xenografts in castrated hosts, these tumors recapitulated the progression of CRPC-NE as the expression levels of AR and PSA decreased over time. Moreover, in our recent work, SRRM4-overexpressing DU 145 cell lines transform into a tumor that closely reproduces clinical CRPC-NE in histopathology and exhibits an increased nucleus to cytoplasm ratio, decreased cell size, and dendritic outgrowths in culture (Lee et al., unpublished manuscript). Interestingly, this striking morphological change from a luminal epithelial lineage to a NE cell lineage was only observed in DU 145 cells, which have a unique genomic profile of *AR*-null and *TP53* and *RB1* functional mutations. However, as mentioned earlier, p53 and RB1 functional mutations are characteristic to DU 145 cells where no NE lineage differentiation is observed, and any known drivers of CRPC-NE are not expressed, supporting that p53 and RB1 function in NE lineage cell fate is cell-context dependent. As the AR-mediated repression of SRRM4 function is irrelevant in DU 145 cells, we suggest that the aberrant functions of p53 and RB1 in DU 145 cells primes the cells to be more susceptible to SRRM4-driven NE lineage fate determination. We hypothesize that SRRM4 may induce the expression of key stemness regulator genes such as *SOX2* in DU 145 to promote lineage plasticity and NE lineage differentiation to drive CRPC-NE progression. Furthermore, SRRM4 may promote lineage plasticity *via* a different mechanism. SRRM4-mediated splicing of the *MEAF6* gene creates a CRPC-NE-unique MEAF6-1 splice variant that can increase the expression of the inhibitor of differentiation-1, or *ID1*, in PCa cells (96). It has been previously demonstrated that RB1 function can be indirectly inhibited by ID1 through inhibition of ETS-mediated p16 activation, which the signaling cascade results in the activation phosphorylation of RB1 (97, 98). These findings suggest that MEAF6-1-induced expression of the *ID1* gene may indirectly inhibit RB1 function to further promote the development of CRPC-NE tumors *via* their putative functions in uncontrolled cell proliferation. This will be further described in the latter sections. Overall, these findings suggest that SRRM4 plays a role in the lineage plasticity of AdPC cells to confer NE cell fate through a potential RB1-SOX2 pathway and splicing of the *MEAF6* gene, which may in turn inhibit RB1 function *via* ID1. Although the details in the underlying molecular mechanisms must still be elucidated, these findings suggest that the AR signaling pathway as well as *TP53* and *RB1* genomic aberrations are implicated in facilitating lineage plasticity in non-neuronal cells where the conjunction of SRRM4 function can drive the NE cell lineage fate to promote the emergence of CRPC-NE.

SRRM4 and the FOXA Family of Proteins

The FOXA families of proteins are a family of transcription factors that have recently been implicated in the development of CRPC-NE. FOXA proteins are pioneer factors that modify and open chromatin to orchestrate the recruitment of transcription factors, such as the AR, to their intended target sites to regulate gene expressions (99). Two members of the FOXA family, such as FOXA1 and FOXA2, are important regulators of differentiation and development of the prostate during embryogenesis (100). Although very similar in structure, it was initially shown by Mirosevich et al. (100) that the functions carried out by these two proteins are unalike, as their temporal and spatial patterns are dissimilar during development (100). In this study, they report that FOXA2 is only expressed in the early stages of prostate development and only in a subset of cell within the basal layers, and colocalizes to cells positive for NE markers, whereas FOXA1 is expressed robustly throughout the development, growth, and adult differentiation of the prostate. Remarkably, the spatial and temporal patterns of FOXA1/2 expression were also observed in the TRAMP mouse model (101). This study detected FOXA1 expression in the normal prostate and throughout the progression of PCa, whereas FOXA2 was only expressed in the normal prostate. However, in the CRPC-NE progressed tumors of the TRAMP mouse model, FOXA2 was seen to be re-expressed (101). Supporting the findings of this study, a recent study done by Park et al. (102) revealed a unique expression of FOXA2 in PCa tumor microarrays of primary small cell NEPC as well as treatment-related CRPC-NE tumors (102). These findings are also consistent with previous findings of a positive correlation of FOXA2 expressions in NE cancers of the lung (103). Another recent study by Kim et al. (104) demonstrated that the inhibition of FOXA1 promoted NE differentiation in both PCa cell lines and mouse models (104). In this study, they also show that FOXA1 expression is downregulated in CRPC-NE tumor models such as the recently generated LnNE CRPC-NE xenograft model (58, 59). As mentioned earlier, this xenograft model was derived from LNCaP cells overexpressing SRRM4, suggesting a potential mechanism whereby SRRM4 may inhibit FOXA1 expression. Although further investigation is needed to fully elucidate the mechanism of FOXA1/2 in the NE differentiation of PCa cells, these findings suggest a contrasting role for FOXA1 and FOXA2 in regulating lineage plasticity where FOXA1 is involved in inhibiting NE-differentiation and promoting the differentiated state of AdPC cells. Furthermore, SRRM4 may regulate the expression of FOXA1 by inhibition to promote the emergence of CRPC-NE.

SRRM4-Mediated Alternative RNA Splicing of Master Neural Differentiation Regulator *REST*

REST can achieve NE differentiation suppression in AdPC cells. REST, as mentioned earlier, is a suppressor of neurogenesis where it acts as a transcriptional repressor of neuronal genes, such as SYP, through the recruitment of corepressors and histone methylation modifiers. During neurogenesis, SRRM4 creates a splice variant called REST4, which has been shown to antagonize REST protein and reprogram REST functions, resulting in the removal of neuronal transcriptional suppression in non-neuronal cells (105). This developmental mechanism is exploited in AdPC cells under ARPI to promote the NE lineage cell fate (58, 59). In these studies, decreased expression of REST and increased expression of the splice variant REST4 were identified as a CRPC-NE-unique transcriptomic signature. To support these findings, a study done by Zhang et al. (89) completed a microarray analysis on CRPC patient tumor samples and patient-derived xenografts and showed a positive

correlation with increased SRRM4 expression, loss of REST, and increased REST splicing and CRPC tumors with NE phenotypes (89). Similar to the mechanisms of neural developmental, SRRM4 was shown to promote NE differentiation of AdPC cells *via* direct binding and splicing of the *REST* gene and, in turn, inhibit the transcriptional repression of REST on neuronal genes *via* the dominant-negative function of the REST4 splice variant (59). These observations were also seen in SCLC, an NE cancer of the lung (88). Shimojo et al. (88) found that SRRM4 and SRRM4-target REST4 splice variant expression was high and REST expression was low in SCLC. The mechanisms by which SRRM4 regulated the alternative splicing in SCLC was similar to that in normal development (88). Apart from the SRRM4-mediated mechanism of RNA splicing to reprogram the function of REST to promote the emergence of CRPC-NE development, a recent study performed by Chen et al. (106) revealed that REST degradation or protein instability induced by PI3K/AKT pathway inhibition can facilitate a NE phenotype in AdPC cells which was observed by an overall increase in NE markers (106). Furthermore, the function of REST has been recognized to be an important facilitator of hypoxia-induced CRPC-NE progression as it is a master regulator of hypoxia genes, which will be discussed in the later section. In conclusion, research to date supports the critical role of REST in the emergence of CRPC-NE, where loss of REST-mediated repression on neuronal genes is important in facilitating the NE phenotype. The mechanisms in which REST is regulated has been shown at both the RNA and protein level, where SRRM4-mediated RNA splicing is key to the functional inactivation of REST and inhibition of the PI3K/AKT pathway promotes the degradation of the REST protein. Based on these current findings, it is clear that REST plays a critical role to the development and differentiation of CRPC-NE.

SRRM4-Mediated Alternative RNA Splicing of Apoptosis Regulator *Bif-1*

Neuroendocrine prostate cancer, like any other cancer, requires more than just differentiation to develop—resistance to cell death caused by ARPI, radiation, or chemotherapies is the prerequisite condition for CRPC-NE to develop. In fact, the Bax-interacting factor 1 (*Bif-1* or *SH3GLB1*) gene is spliced in CRPC-NE tumors (59). Bif-1 is an endophilin protein involved in apoptosis, autophagy, and mitochondrial functions. The function of Bif-1 is mediated *via* interactions with different co-factors and proteins such as UVRAG and Beclin-1, which form autophagosomes, regulate mitochondrial dynamics, and facilitate tumorigenesis (107). In addition, Bif-1 can interact with Bax to cause a conformational change to functional Bax activation *via* its N-BAR domain in response to apoptotic stress to promote mitochondrial membrane permeability for cytochrome *c* release (107, 108). The N-BAR domain of the Bif-1 protein is required for both the activation of Bax and promotion of mitochondrial lipid membrane remodeling (109). The *Bif-1* gene has three main splice variants, where the Bif-1a splice variant is expressed in all tissues and the Bif-1b and Bif-1c splice variants are brain tissue specific (110). In non-neuronal cells, Bif-1 promotes apoptosis under stress conditions; however, Bif-1 in neuronal cells promotes neuronal viability by increasing mitochondria membrane stability and inhibiting the release of cytochrome *c* (111). Interestingly, the inclusion of microexons 6 and 7 in the *Bif-1b* and *Bif-1c* genes, respectively, are within the N-BAR domain of the *Bif-1* gene (111). This suggests that the insertion of the microexons may potentially perturb protein–protein interactions or other related functions of the N-BAR domain of the Bif-1b and Bif-1c spliced variants, which may alter the function of Bif-1 into an anti-apoptotic function. Based on these findings and known functions of Bif-1 and its splice variants, we hypothesize that SRRM4 mediates the splicing of Bif-1 into CRPC-NE-unique splice variants Bif-1b and Bif-1c, and that these variants are important in helping PCa cells, particularly CRPC-NE cells, to escape apoptosis during CRPC-NE tumor progression. By contrast, we posit that the constitutive isoform, Bif-1a, which is highly represented in CRPC-Ad tumors, is a pro-apoptotic facilitator. Understanding the functions of Bif-1 splice variants will be important in elucidating the multifaceted molecular mechanisms in which SRRM4 and its RNA splicing activity drives the progression of CRPC-NE.

SRRM4-Mediated Alternative RNA Splicing of Epigenomic Regulators *MEAF6* and *PHF21A*

In addition to the role of SRRM4 in evading apoptosis *via* alternative splicing of the *Bif-1* gene, it has recently been demonstrated that SRRM4 can promote the proliferation of cells *via* splicing of the *MEAF6* gene (96). Among the CRPC-NE-unique gene signatures found by Li et al. (59), *MEAF6* gene was differentially spliced, and the splice variant MEAF6-1 was uniquely manifested in CRPC-NE tumors (59). MEAF6 is a component of four of five MYST families of histone acetyltransferase (HAT) complexes. HAT complexes have putative roles in key fundamental nuclear processes (i.e., transcription, DNA repair, and replication) *via* post-translational modifications of histones and transcriptional regulators, such as p53. These essential functions and components of HAT complexes are evolutionarily conserved from yeast to humans (112–114). This suggests and has been previously demonstrated that the deregulation or altered functions of the components that comprise the HAT complexes play an important role in cancer progression by altering the cancer epigenome. In fact, it was recently demonstrated by Lee et al. (96) that the neural-specific variant of the *MEAF6* gene, MEAF6-1, but not non-neural splice variant MEAF6-2 promoted cell proliferation (96). This was confirmed by BrdU incorporation assay's under 2D and 3D matrigel conditions. In addition, MEAF6-1 promoted colony formation, in colony number and size, and invasion and migration of PCa cells. Furthermore, MEAF6-1 promoted tumor growth in mice. Interestingly, microarray analyses showed that the functions of MEAF6-1 were mediated by ID1 and ID3, which was validated *in vivo* by silencing these genes in AdPC cells stably expressing MEAF6-1. Collectively, this study demonstrated that MEAF6-1, but not MEAF6-2, promotes cell proliferation, invasion, and migration of PCa cells as well as tumor growth of xenograft models. As MEAF6 is a component of HAT complexes, it would be interesting for future research to

study any potential variations in the protein–protein interactions between MEAF6 splice variants and the components of the HAT complexes and the function of the MEAF6-1 splice variant at the epigenomic level.

Another regulator of histone modifications that is a target of SRRM4 unique to CRPC-NE tumors is the *PHF21A* gene (58). PHF21A is a component of the histone modification complexes associated with LSD1, coREST, and HDAC1/2, which suppress the transcription of neuronal genes. As the DNA-binding activity of the complexes relies on the concurrent functions of both PHF21A and LSD1, knockdown of PHF21A results in the de-repression of LSD1 target genes (115–117). It was also previously shown that PHF21A functions as a negative modulator of REST-mediated repression of neuronal genes *via* inhibition binding to the REST protein (117). The human *PHF21A* gene encodes a neural tissue-specific splice variant in which the exon 12a is included, which has been shown to be a CRPC-NE-unique splice variant, and a ubiquitously expressed or constitutive variant in which the exon 12, not exon 12a, is included (58, 118). Previously, Iwase et al. (119) demonstrated that the neural-specific PHF21A splice variant had an increased binding to HDAC1/2 when compared with the PHF21A constitutive isoform (119). In this study, it was proposed that RNA alternative splicing of the PHF21A gene can reprogram the function of the PHF21A protein by altering the protein–protein interactions of PHF21A, HDAC1/2, and potentially other proteins. It is noteworthy to add that the constitutive PHF21A variant may facilitate altered histone deacetylation or demethylase activity as the inclusion of the alternatively spliced exon also disrupts an AT hook responsible for DNA binding, as well as one of the two predicted nuclear localization signals in the *PHF21A* mRNA (120). This supports the idea that the inclusion of the alternatively spliced exon facilitated by SRRM4 can reprogram protein function by altering protein–protein interactions or potentially altering the localization of the protein (85). Furthermore, the resulting reprogrammed function of the SRRM4-mediated PHF21A splice variant may also attenuate the transcriptional repression of REST and its associated cofactors, LSD1, coREST, and HDAC1/2 on neuronal-specific genes, whereby lifting the REST-dependent histone methylation repression of neuronal differentiation and increasing the likelihood of NE lineage cell differentiation in PCa cells.

Based on these findings, it is clear that SRRM4 mediates the splicing of genes that are CRPC-NE-specific and known to be highly enriched during neurogenesis. These newly spliced genes are important for promoting CRPC-NE tumor establishment, where the reprogrammed functions of CRPC-NE-unique MEAF6-1 splice variants can drive PCa cell proliferation, invasion, tumor growth, and, along with the PHF21A constitutive splice variant, may drive a neural program at the epigenomic level. Although further studies are needed to investigate the role of these splice variants in epigenomic modifications to facilitate CRPC-NE development, the fact that CRPC-NE-specific genes targeted by SRRM4 splicing are important epigenetic modifiers suggests that SRRM4 may function at the epigenetic level.

SRRM4 and the Microenvironment

Although further studies are required to elucidate the complexity of the mechanisms associated with environmental factor-mediated emergence of CRPC-NE, studies to date have suggested that the microenvironment is an important inducer of lineage plasticity and differentiation to the NE cell lineage. As previously discussed, stress factors from therapeutics such as ARPI have been demonstrated to induce CRPC-NE. Studies have revealed other various environmental factors important in NEPC development such as cAMP (121, 122), cytokines (i.e., IL6 and IL8) (123, 124), AKT inhibition (106), and hypoxia (125, 126). However, the induction of the NE phenotype by environmental factors such as cAMP (121, 122) is reversible, suggesting that other factors and molecular mechanisms are essential to the development of CRPC-NE tumors.

Alternative factors in the tumor stromal microenvironment that promote the emergence of CRPC-NE are mitogenic cytokines, such as IL8 and IL6 (104, 123, 124, 127). Early studies by Huang et al. (127) observed that benign NE cells in the prostate express high levels of IL8 (127). They also demonstrated that the expression of IL8 receptor, CXCR1, increased from low-to-none in benign epithelial cells to high in high-grade PCa to higher in metastatic PCa. This suggests a paracrine mechanism, whereby IL8 secretion from NE cells may stimulate the growth and proliferation of adjacent non-NE tumor cells. By contrast, a follow-up study demonstrated that NE cells express a different IL8 receptor called CXCR2, whereby NE cell quiescence is induced *via* the IL8–CXCR2–p53 pathway in an autocrine fashion in which mutant p53 can promote hyperproliferation of cells through inactivation of this signaling pathway (95). Parallel to this finding, a recent study by Kim et al. (104) reported that the inhibition of FOXA1, which is a transcriptional repressor of the *IL8* gene, promotes NE differentiation in both AdPC cell lines and mouse models by IL8-mediated activation of MAPK/ERK pathway and thus transcriptional activation of NE markers (104). Collectively, these findings demonstrate the implications of autocrine or paracrine IL8-mediated signaling pathways in promoting the emergence of NEPC. Furthermore, a mechanistic connection between mutant p53 and IL8, both of which are prevalent in CRPC-NE tumors, may drive hyperproliferation to promote the emergence of CRPC-NE tumors. Another important cytokine that has been shown to promote NE differentiation is IL6 (123). In fact, it has recently been demonstrated that IL6 can induce NE differentiation in LNCaP cells by suppressing REST function, whereas exogenous REST abolished the IL6-induced NE program (124). In this study, they also demonstrated that IL6-induced NE differentiation promoted REST protein degradation *via* the ubiquitin–proteasome pathway. Another study reported that REST function is essential for IL6-induced NE differentiation (128). Both IL6 and REST have both been implicated in hypoxia-induced NE differentiation (126). Hypoxia is another important regulator of NE differentiation in PCa cells. Interestingly, hypoxia-induced NE differentiation of PCa cells relies on the inhibition of REST, as REST is a master repressor of neuronal genes and thus a regulator of hypoxia-induced genes (126, 129). These studies suggest a possible mechanism of tumor microenvironment factors such

as hypoxia, cytokines, and REST functions in promoting NE differentiation. Furthermore, research by Qi et al. (130) demonstrated that ubiquitin ligase Siah2-expressing TRAMP mice was required for hypoxia-mediated CRPC-NE development (130). In this study, it was revealed that Siah2 regulation of HIF1a activity, a master regulator of hypoxia, which has been shown to be highly expressed in CRPC-NE tumors, is essential for the transcriptional activation of HIF1a/FOXA2 target genes such as SOX9 to promote the NE phenotype of CRPC-NE tumors. These findings indicate possible broader mechanistic pathway of NE differentiation that may involve the FOXA2–HIF1a hypoxia pathway and IL6-induced REST inhibition in concurrence with SRRM4-induced REST splicing and inhibition *via* REST4 function under stress conditions. Connecting these pathways together, it is possible that SRRM4 may contribute to the FOXA2–Saih2/HIF-1a pathway, where inhibition of REST function can be augmented by both SRRM4 function and FOXA to drive the NE lineage cell fate in AdPC cells. Consequently, it is possible that SRRM4 may act in conjunction or synergy with tumor microenvironment factors or regulators to further drive the development and progression of CRPC-NE.

SRRM4 AS A THERAPEUTIC TARGET FOR NEPC

Based on research findings, many factors are essential during CRPC-NE development. Research strongly suggest that ARPI treatment, genomic alterations, alterations in expression and function of histone modification enzymes, RNA splicing factors, and transcriptional factors are necessary for reprogramming the cell to gain lineage plasticity. The plethora of factors involved in CRPC-NE development emphasizes the heterogeneity of NEPC. Therefore, further investigation is needed to understand how these signaling pathways interplay with each other and understand the molecular mechanisms by which these factors promote CRPC-NE. Ultimately, this knowledge will provide insight for personalized medicine-based strategies for PCa patients. These efforts will rationalize SRRM4 and its CRPC-NE-unique splice variants as potential diagnostic or prognostic biomarkers, and SRRM4 as a therapeutic target in CRPC-NE patients.

In fact, SRRM4 fulfills several criteria to be a possible therapeutic target. Although knockdown of the *SRRM4* gene has been shown to be critical during neural development (78, 80, 82),

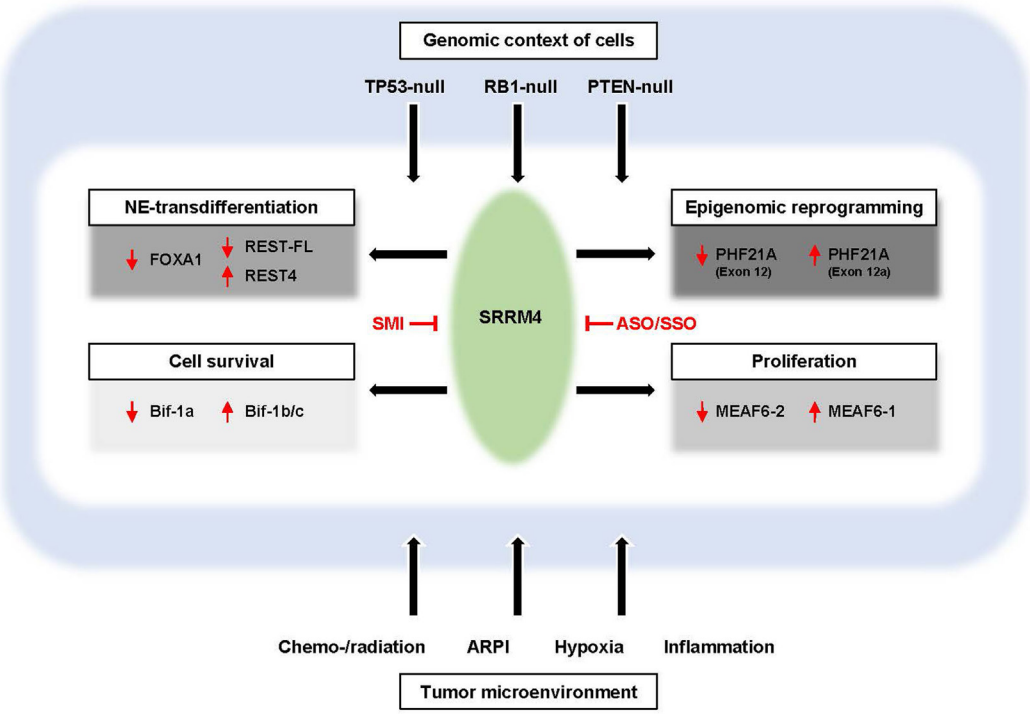

FIGURE 1 | Development of NEPC by the SRRM4-mediated RNA splicing network. The multifaceted roles of SRRM4 and SRRM4-mediated alternative RNA splicing of genes highly enriched in neuronal functions drives a CRPC-NE program *via* various aspects important for CRPC-NE progression. SRRM4-mediated spliced variants not only facilitate the NE-transdifferentiation of CRPC-Ad cells possibly through epigenetic alterations but also promotes cancer cell survival, proliferation, and tumorigenesis of CRPC-NE cells to establish CRPC-NE tumors. We propose that SRRM4 may function beyond its putative role in RNA splicing *via* mechanisms involving transcriptional factors (e.g., FOXA, REST, and AR), tumor suppressors (e.g., RB1 and p53), and microenvironment factors (e.g., therapeutics, hypoxia). Potential SRRM4-targeted therapeutic approaches for treating CRPC-NE or mitigating its development may be to inhibit the splicing or RNA-binding activity of SRRM4 *via* SMI or target the alternative splicing events *via* ASO. This multi-functional property of SRRM4 ultimately provides cancer cells the ability to develop therapy resistance and develop into CRPC-NE tumors. SMI, small molecule inhibitors; ASO, antisense oligonucleotides; SSO, splice-switching oligonucleotides; ARPI, androgen receptor pathway inhibition; CRPC-Ad, castration-resistant adenocarcinoma prostate cancer; NEPC, neuroendocrine prostate cancer; SRRM4, Ser/Arg repetitive matrix 4.

a partial deletion of 710 bp in the C-terminus of the *SRRM4* gene did not result in embryonic lethality in Bronx Waltzer mice (83). In these mice, the deletion impaired the splicing activity of SRRM4, but resulted in limited abnormal neural behaviors, such as deafness and impaired balance. This suggests that specific inactivation of the SRRM4 splicing activity while maintaining the majority of the protein may have limited side effects. Furthermore, SRRM4 is prominently expressed in neuronal cells and CRPC-NE tumors, but is rarely expressed in non-neuronal cells. SRRM4-specific inhibitors for CRPC-NE must be carefully designed to prevent entering the blood–brain barrier to limit off-target effects of SRRM4.

There are several ways to block SRRM4 function such as antisense oligonucleotides (ASO) or splice-switching oligonucleotides (SSO), which can redirect SRRM4-mediated alternative splicing events. Alternatively, small molecule inhibitors (SMI) can either target the RNA-binding domain of SRRM4 to prevent it from recognizing its RNA substrates or can directly inhibit the C-terminus domains important for the splicing activity of SRRM4. In fact, the first Food and Drug Administration-approved ASO/SSO called SPINRAZA™ has shown to be effective in patients with spinal muscular atrophy, a genetic disorder whereby altered RNA splicing patterns of the survival motor neuron (SMN) genes results in an unstable and dysfunctional SMN protein (131, 132). SPINRAZA™ targets the splicing events of the SMN genes by silencing the splicing silencer element in the intron region upstream of the alternatively spliced exon. Although further mechanistic studies are warranted, a similar method may be applied to mitigating CRPC-NE progression, whereby carefully designed ASO/SSO could inhibit SRRM4-recognized consensus RNA-binding UGC motifs in Ref. (80). Furthermore, although the exact mechanism of splicing inhibition is currently unknown, SMI compound called LMI070 is currently under clinical trials to treat spinal muscular atrophy, by binding to the RNA itself or RNA-binding domains of splicing factors (131). Before SRRM4-targeting SMI drug design, the SRRM4 protein must be crystalized *via* microfluidics reaction technology or cryo-electron microscopy methods. However, these techniques face many challenges, such as large amount of protein purification and low resolution, respectively. Following SRRM4 crystallization, SMI that bind to either the RNA-binding domain or the C-terminus domain may be designed. These methods highlight the potential for SRRM4 to be used as a target for future CRPC-NE therapeutic programs.

CONCLUSION

SRRM4 is an important driver gene for CRPC-NE. By regulating alternative RNA splicing, SRRM4 not only stimulates AdPC to undergo NE differentiation but also promotes cancer cell survival, proliferation, and tumorigenesis, illustrated in **Figure 1**. SRRM4 also interacts with other signal pathways including AR, p53, and RB1 to regulate phenotypical reprogramming PCa cells. This multi-functional property of SRRM4 ultimately provides cancer cells the ability to develop therapy resistance and develop into CRPC-NE. The information gathered for this article on SRRM4 in driving CRPC-NE progression will strengthen the rationale to design SRRM4 inhibitors that are expected to be effective to treat SRRM4-driven CRPC-NE.

AUTHOR CONTRIBUTIONS

AL contributed to the conception and design of the article and performed the necessary and required literature research to the drafting of the work. NC contributed to the conception by writing the first draft of the work. All authors revised the work critically for important intellectual content and contributed to manuscript revision, read, and approved the submitted version.

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OPINION
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Supraphysiological Testosterone Therapy as Treatment for Castration-Resistant Prostate Cancer

*Hung-Ming Lam and Eva Corey**

Department of Urology, University of Washington School of Medicine, Seattle, WA, United States

Keywords: supraphysiological testosterone, bipolar androgen therapy, castration-resistant prostate cancer, enzalutamide, abiraterone

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Fabio Grizzi,
Humanitas Research Hospital, Italy

Reviewed by:
Momoe Itsumi,
Tokyo Medical and Dental University, Japan
Benyi Li,
University of Kansas Medical Center, United States

***Correspondence:**
Eva Corey
ecorey@uw.edu

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Blocking androgen signaling has been the focus of treatment for advanced and metastatic prostate cancer (PC) for the past 70 years (1). First-line androgen deprivation therapy (ADT), either through surgical or medical castration (luteinizing hormone-releasing hormone agonists and antiandrogens), holds promise for PC patients; however, the disease inevitably progresses to castration resistance (2). Second-generation ADT, abiraterone acetate (AA), and enzalutamide (ENZ), have been effective for a subset of patients with castration-resistant PC (CRPC) with relatively short median survival benefits (~3–5 months) (3–5). Concerted effort in the field, including evidence from our group, clearly demonstrates a sustained AR activity in the CRPC tumors including (1) amplification of AR, (2) AR mutations, (3) expression of AR splice variants that are constitutively active, (4) altered milieu of AR coactivators and corepressors, and (5) intracrine synthesis of androgens to support CRPC progression (6, 7).

The addiction of PC to the AR signaling paradoxically creates a therapeutic vulnerability that has recently attracted increasing attention. While ADT causes regression of PC, high level of androgen can also inhibit PC progression. The concept of cancer suppression using excessive hormone therapy was introduced by earlier work from Huggins in 1940: "malignant cells can regress from too little or too much hormone" (8). In relation to PC, AR regulates proliferation as well as differentiation of prostate epithelial and cancer cells but it has not been established what conditions support one over the other. Interestingly activation of AR with excessive hormone (i.e., supraphysiological levels of testosterone; SPT) was shown to inhibit growth of CRPC *in vitro* by negative effects on proliferation and increased expression of some of the AR-regulated genes that are expressed in differentiated luminal epithelium, e.g., prostate-specific antigen. Multiple preclinical studies demonstrated that SPT inhibits growth of PC cells that express AR (9–21), with evidence suggesting that higher levels of AR might lead to more pronounced SPT effects in certain phenotypes of CRPC [reviewed in Ref. (22)]. However, AR by itself is not necessarily sufficient for the SPT-induced growth inhibition; cellular context (23) and AR-regulated transcriptome in its entirety will need to be assessed to delineate the molecular effect of SPT (24). Mechanistically, SPT-induced cell growth inhibition involves (1) cell-cycle arrest, (2) disruption of AR-mediated DNA licensing, (3) DNA damage, (4) transcriptional repression of AR and its variants, (5) transcriptional reprogramming, (6) cellular quiescence or senescence, and (7) induction of apoptosis [reviewed in Ref. (22)]. However, these effects were demonstrated exclusively in cell line models, and whether they play a significant biological role in SPT-induced tumor inhibition in patients remains to be determined.

Clinical use of testosterone (T) supplementation in PC has been limited and provided controversial results. Two older National Prostatic Cancer Project trials that used T-supplementation to normal levels with a goal to enhance the effectiveness of chemotherapy reported disappointing results (25, 26). Additional two phase I trials, which did not achieve consistent supraphysiological T levels, showed minimally reduced disease progression (27, 28). In contrast, several other studies showed that T-supplementation to normal-supraphysiological range (303−2637 ng/dl), specifically

in symptomatic hypogonadal PC patients, provided prolonged disease control (as measured by sustainably low-PSA level) (29–31). In our opinion, the lack of favorable response in some of the clinical trials is, at least in part, due to the absence of a supraphysiological level of T as well as the unselected patient population.

With advanced understanding of the biology and AR involvement in CRPC progression, leveraging the active AR signaling to explore therapeutic opportunity has recently received renewed attention in clinical settings. Dr. Denmeade's group at John Hopkins University pioneered a therapy termed "bipolar androgen therapy" (BAT) as a treatment for PC patients. With BAT treatment, PC patients receive intermittent T injections at doses shown to produce a spike in serum T to supraphysiological levels, followed by a decline to below normal at the end of a 28-day treatment cycle (32). This cycling strategy was developed based on the most common molecular hallmark of CRPC–overexpression of AR (33) and the potential growth inhibitory effect of SPT in AR-overexpressing PC. Rapidly cycling of T from SPT (~1,500 ng/dl) to below normal T levels (~150 ng/dl) was expected to blunt the adaptive changes in AR expression, thereby delaying the emergence of resistance. In these proof-of-principle BAT trials, one in CRPC showed radiographic response rates of ~50% in men (32), and one in hormone-sensitive PC showed favorable PSA responses (34). Promising results of these trials led to a new clinical trial, in which asymptomatic CRPC patients that progressed on AA or ENZ receive BAT, and after progression on BAT the patients are re-challenged with AA or ENZ. This trial aims to evaluate the efficacy of BAT in patients who progressed on secondary ADT and assess whether BAT re-sensitizes CRPC to secondary ADT. Recent data from this trial showed a PSA50 response in 9/30 ENZ-resistant patients on BAT, and, importantly, 15/21 patients who progressed on BAT showed a PSA50 response upon ENZ re-challenge (35). These results are encouraging. However, additional analyses and larger number of patients are needed to correlate tumor/radiographic vs. PSA responses in individual patients. One of the reasons is that PSA changes do not necessarily associate with tumor regression in advanced CRPC. PSA, an AR-regulated gene, is highly sensitive to AR activation/inhibition and can rise upon SPT and decline upon ADT. PSA response might not faithfully reflect radiographic responses in advanced CRPC which growth often does not rely solely on AR signaling [e.g., FGF signaling (36)].

Bipolar androgen therapy shows great clinical promise in a subset of patients. However, universal to all cancer treatment modalities, not all patients respond to this treatment and resistance to BAT develops. Therefore, there is an opportunity to improve this therapy. It is notable that a critical step in drug development, determining the optimal dosing schedule, was bypassed in the clinical development of BAT. Despite the clinical efficacy of BAT, there were by far no clinical data to support the hypotheses that cycling SPT (i.e., BAT) mitigates the development of resistance or that BAT represents the optimal mode for administering SPT. Notably, several preclinical studies have consistently demonstrated that SPT delivered on a continuous basis inhibits the growth of PC cells (13, 20, 21). While several small clinical trials of continuous T administration in men with CRPC have been carried out, they did not achieve SPT levels.

Cycling or not cycling—that is the question. While we currently do not have sufficient evidence whether BAT results in better clinical outcome than continuous SPT, it is possible that long-term continuous SPT and BAT could alter AR signaling differently. One would anticipate that continuous SPT might trigger more pronounced differentiation, potentially causing a change from a "low-T" oncogenic AR transcriptome to that of a more differentiating SPT transcriptome (24). Meanwhile, BAT might provide better efficacy if cell-cycle relicensing effects and DNA damage are the critical mechanism of action (37, 38). While BAT was associated with improved quality of life (34, 35), this effect diminished over the course of a cycle of BAT, presumably due to T levels falling below normal range. It is possible that quality of life metrics will be better with continuous SPT but there also might be increase in negative side effects. While T therapy has been reported to be generally safe, with a small subset of patients experiencing severe cardiovascular-related complications (27–32, 39–42), continuous SPT has not been tested and monitoring will be essential. Careful evaluation of effects of BAT vs. continuous SPT on tumor progression, as well as any potential health benefits or side effects will be required to make final decision.

Interestingly, cycling of ADT, intermittent ADT, has been evaluated in PC extensively since its introduction in mid-1980s. However, intermittent ADT was not found to be inferior to continuous ADT with respect to the overall survival but it was shown to improve patients' quality of life, and therefore it is thought to be a viable option for patients who experience significant adverse effects of continuous ADT [for review see Ref. (43, 44)]. In addition, intermittent AA therapy has been recently shown to delay the development of resistance from 16.5 (continuous treatment) to 27 months (45). Whether the intermittent therapy diversifies the residual tumor clones or re-sensitize the residual clones to a therapy that formerly failed remains scientifically and clinically important.

In summary, we will need to seek answers to multiple important questions before unleashing the full potential of SPT therapy in CRPC: (1) which mode of SPT, BAT or continuous SPT, represents the optimal administration regimen for tumor growth inhibition; (2) what population of patients will benefit from SPT therapy; (3) is there a way to prolong the treatment response; and (4) what are the mechanisms of resistance, as these will be diverse in different tumor phenotypes. To address these questions, systematic preclinical trials will need to be performed, and pre-treatment and on-treatment clinical specimens will be essential to identify mechanisms of SPT action and biomarkers that predict SPT response.

AUTHOR CONTRIBUTIONS

H-ML and EC jointly conceptualized and wrote the manuscript.

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Screening of Drug Repositioning Candidates for Castration Resistant Prostate Cancer

In-Wha Kim, Jae Hyun Kim and Jung Mi Oh**

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul, South Korea

Purpose: Most prostate cancers (PCs) initially respond to androgen deprivation therapy (ADT), but eventually many PC patients develop castration resistant PC (CRPC). Currently, available drugs that have been approved for the treatment of CRPC patients are limited. Computational drug repositioning methods using public databases represent a promising and efficient tool for discovering new uses for existing drugs. The purpose of the present study is to predict drug candidates that can treat CRPC using a computational method that integrates publicly available gene expression data of tumors from CRPC patients, drug-induced gene expression data and drug response activity data.

Methods: Gene expression data from tumoral and normal or benign prostate tissue samples in CRPC patients were downloaded from the Gene Expression Omnibus (GEO) and differentially expressed genes (DEGs) in CRPC were determined with a meta-signature analysis by a metaDE R package. Additionally, drug activity data were downloaded from the ChEMBL database. Furthermore, the drug-induced gene expression data were downloaded from the LINCS database. The reversal relationship between the CRPC and drug gene expression signatures as the Reverse Gene Expression Scores (RGES) were computed. Drug candidates to treat CRPC were predicted using summarized scores (sRGES). Additionally, synergic effects of drug combinations were predicted with a Target Inhibition interaction using the Minimization and Maximization Averaging (TIMMA) algorithm.

Results: The drug candidates of sorafenib, olaparib, elesclomol, tanespimycin, and ponatinib were predicted to be active for the treatment of CRPC. Meanwhile, CRPC-related genes, in this case *MYL9*, *E2F2*, *APOE*, and *ZFP36,* were identified as having gene expression data that can be reversed by these drugs. Additionally, lenalidomide in combination with pazopanib was predicted to be most potent for CRPC.

Conclusion: These findings support the use of a computational reversal gene expression approach to identify new drug and drug combination candidates that can be used to treat CRPC.

Keywords: drug repositioning, castration resistant prostate cancer, gene expression, drug activity, synergic effect

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Edited by:
Scott T. Tagawa, Department of Medicine, Weill Cornell Medicine, Cornell University, United States

Reviewed by:
Young E. Whang, University of North Carolina at Chapel Hill, United States
Masaki Shiota, Kyushu University, Japan

****Correspondence:***
In-Wha Kim
iwkim2@hanmail.net
Jung Mi Oh
jmoh@snu.ac.kr

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INTRODUCTION

Drug repurposing or repositioning is a strategy for identifying new indications for approved or investigational drugs that are outside the scope of the original medical indication (1). This strategy offers an advantage in that the cost of bringing a repurposed drug to market has been estimated to be US$300 million on average, compared to estimates of approximately $2–3 billion for a new drug (2).

Drug–disease similarity approaches aim to identify shared therapeutic applications for drugs (3) while drug–drug similarity approaches aim to identify shared mechanisms of action for drugs (4). Recently, interest in the use of genomics-based drug repositioning to aid and accelerate the drug discovery process has increased (5). Drug development strategies based on gene expression levels are advantageous in that they do not require a large amount of a priori knowledge pertaining to particular diseases or drugs (6, 7). Large public datasets such as the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) (8), the Cancer Cell Line Encyclopedia (CCLE) (9), and the Library of Integrated Network-Based Cellular Signatures (LINCS) (10, 11), describing chemical and biological disease entities or gene expression data and the relationships between them, provide an efficient approach by which to reposition existing drugs for new indications (5, 12). Recently, the reverse gene expression scores (RGES) computation method was developed as a one of the powerful drug repositioning tools to predict drug candidates (13). The RGES computation method was applied to find drug candidates for CRPC in this study.

Prostate cancer (PC) was the cancer with the highest incidence worldwide and the leading cause of cancer deaths for men in 2015 (14). Although PC mortality in Western countries has declined due to early diagnosis and treatment, incidence rates of PC continue to increase in the developing countries (15). Androgens and androgen receptors (ARs) may play key roles in the initiation and progression of PC (16). As Huggins and Hodges discovered that androgen-deprivation therapy (ADT) with surgical castration to reduce testicular testosterone could suppress PC progression (17), ADT has been the standard therapy to treat PC (16, 18).

Different therapeutic approaches to target androgen and AR signals after surgical or chemical castration were developed by combining ADT with various anti-androgens, including the steroidal anti-androgens cyproterone acetate (19) and megestrol acetate (19), and non-steroidal anti-androgens, including nilutamide (20), flutamide (21), and bicalutamide (22). Most PCs initially shrink in response to ADT, but eventually most types of ADT with anti-androgens fails and PC patients develop castration resistant PC (CRPC) (16). When this occurs, chemotherapeutic approaches such as docetaxel may be considered (23). Although recently enzalutamide, apalutamide, and abiraterone acetate were approved for the treatment of CRPC (24, 25), available drugs that have been approved for the treatment of CRPC patients are limited (26). Therefore, repositioning for CRPC is challenging and numerous preclinical or clinical trial studies deriving from repositioning approaches, including those focusing on itraconazole (27), phentolamine (28), and niclosamide (29), have been continually conducted.

Primary PC has relatively few genomic aberrations compared to other cancers (30). However, detailed spatial sampling and sequencing of prostate tumors has identified significant heterogeneity within multifocal tumors in the same patient (31, 32). In the case of CRPC, it has been shown to remain dependent on the AR signaling pathway by various mechanisms even during the systemic depletion of androgens (33).

The purpose of this study is to predict drug candidates that can treat CRPC using a computational method that integrates publicly available gene expression data of tumors in CRPC patients, drug-induced gene expression data and drug response activity data.

MATERIALS AND METHODS

Collection of Gene Expression Data

Publicly available gene expression data for CRPC related studies were identified in the GEO database hosted by the NCBI (http://www.ncbi.nlm.nih.gov/geo). A search of the GEO database was conducted in July of 2018 using "prostate cancer," "castration," "resistance," and "refractory" as a key search phrases. The results were filtered using the search terms *Homo sapiens*, expression profiling by array, and expression profiling by high throughput sequencing. Only original experimental datasets that compared the expression levels of mRNAs between CRPC tumor and normal or benign tissues were selected in CRPC patients. Additionally, gene expression data of human prostate adenocarcinoma cell lines were downloaded from CCLE (version 2.7. updated 2015 https://portals.broadinstitute.org/ccle) (9).

Preprocessing of Gene Expression Data

The GEO accession number, Gene Expression Omnibus platforms (GPL) access number, number of cases and controls, sample type, and gene expression data were extracted from each of the identified datasets. Gene expression data were individually log2-based transformed and normalized. If there were multiple probes for the same gene, the probe with the highest interquartile range was selected for that gene expression level. All probe sets on different platforms were re-annotated to use the most recent NCBI Entrez Gene Identifiers (Gene IDs), and the Gene IDs were used to cross-map genes among the different platforms. Only genes present in all selected platforms were considered. The R packages MetaQC (34) was used for quality control (QC) of gene expression data. The mean rank of all QC measures in each dataset was also determined as a quantitative summary score by calculating the ranks of each QC measure among all included datasets.

Identification of Disease Gene Expression Signatures

The R package MetaDE (35–38) was used to identify the DEGs in meta-analysis of CRPC. A moderated *t*-statistic was used to calculate *P*-values for each dataset, and a meta-analysis was conducted with a fixed effect model (39). Additionally, the degree of similarities among the gene expression data between CRPC

tumor samples from the GEO and PC cell lines from the CCLE were assessed.

Identification of Compound Gene Expression Signatures

The L1000 landmark transcript values (Level 4) of 978 landmark genes from LINCS as of May of 2018 were downloaded from LINCS cloud storage (http://lincscloud.org/) hosted by the Broad Institute (40). Cell lines described in LINCS, CCLE, and ChEMBL (version 24 1st Sep 2017, https://www.ebi.ac.uk/chembl/) (41) were mapped using PC cell line names followed by manual inspection. Meta-information for compound-induced gene expression values, in this case cell line types as well as treatment durations and drug concentrations, was retrieved. Only L1000 signatures with the annotation "is gold," indicating the highest quality of aggregate data were used for further analyses.

Collection of Compound Activity Data

Compound activity data, described as the half maximal inhibitory concentrations (IC_{50}) in PC cell lines were retrieved from ChEMBL. As the IC_{50} values for a given compound could vary for the same cell line across different studies, the median IC_{50} value was used. Compounds included in the ChEMBL and LINCS were manually mapped using International Union of Pure and Applied Chemistry International Chemical Identifier keys. Additionally, the area-under-the-curve (AUC) values for compound activity data in the PC cell lines were retrieved from the Cancer Therapeutic Response Portal (CTRP ver 2, https://portals.broadinstitute.org/ctrp.v2.1/) (42). Sensitivity levels were measured in the form of cellular ATP levels as a surrogate for cell number and growth using CellTiter-Glo assays (43). A compound-performance score was computed at each concentration of compound. Median AUC values across various cell lines were used. Compounds were categorized into active (IC_{50} $<10\,\mu M$) and inactive groups ($IC_{50} \geq 10\,\mu M$) based on their activities in cell lines. An IC_{50} value of $10\,\mu M$ was chosen as an activity threshold given that compounds with $IC_{50} \geq 10\,\mu M$ in primary screenings are often not pursued (44).

Computation and Summarization of RGES

The method used to calculate the reverse gene expression score (RGES) was adapted from the previously described Connectivity Map method (45) and RGES computational method (13). Briefly, genes were initially ranked according to their expression levels for each compound. An enrichment score for each set of up- and down-regulated genes in CRPC was computed based on their positions in the ranked list. RGES values emphasize the reversal correlation by capturing the reversal relationship between the DEGs and compound-induced changes in the gene expression levels. Therefore, a lower negative RGES indicates a higher likelihood of reversing changes in the gene expression of CRPC, and *vice versa*. In addition, the Spearman's correlation coefficient, the Pearson correlation coefficient, and the cosine similarity were computed between DEGs in CRPC and compound activities, as an alternate method for computing the reversal relationship between DEGs and the active compounds (46). The databases used can list multiple gene expression levels associated with one compound due to testing involving different cell lines, treatment concentrations and durations of compounds. This resulted in multiple RGESs for one compound that could reverse disease gene expression. Given these variations, summarized RGESs (sRGES) were weighted and calculated. Results obtained for a $10\,\mu M$ drug concentration and a 24 h treatment time were used to define the reference conditions.

Identification of Reversed Genes

In cases for which multiple gene expression values yielded multiple RGES values for one compound, a median RGES value was calculated from the PC cell lines. In cases for which multiple compound activity IC_{50} data were available for one compound, median IC_{50} values were calculated. Each gene expression datum was sorted by its expression value. Upregulated genes were ranked highly (i.e., on the top), whereas downregulated genes were assigned a low rank (i.e., on the bottom). Among the upregulated genes, reversal genes were defined as those that were ranked lower in the active group ($IC_{50} < 10\,\mu M$) than in the inactive group ($IC_{50} \geq 10\,\mu M$). In contrast, among the downregulated genes, the reversal genes were defined as those that were ranked higher in the active group than the inactive group. A leave-one-compound-out cross-validation approach was used to find genes having reversed expression (47). For each trial, one compound was removed and the reversed genes were then identified using the approach described above. Only those genes that were significantly reversed in all trials were retained. The genes with adjusted $P < 0.25$ in all trials were considered as reversal genes.

Prediction of Synergic Effects

To predict the synergic effects of drug combinations based on the interactions between drugs and the identified targets, RGES values was used for Target Inhibition Inference using the Maximization and Minimization Averaging (TIMMA) algorithm (48). The synergic scores were calculated with the TIMMA-R package (49). The synergic addictive score was defined as $S_a(i,j) = y(i,j) - (y(i) + y(j))$ and the synergic multiplicative score was defined as $S_m(i,j) = y(i,j) - (y(i) \times y(j))$. The synergic highest agent score was defined as $S_l(i,j) = y(i,j) - \max(y(i), y(j))$. An average synergy score was defined as $S(d1, d2) = \frac{1}{n} \sum_{i \in d1,\, j \in d2} S(i,j)$. The predicted sensitivity was defined as $Sensitivity = expectation + synergy$.

Statistical Analysis

The degree of similarity in gene expressions between CRPC tumor samples from the GEO and PC cell lines from the CCLE were assessed by a Spearman's rank correlation test, as were these similarity degrees between RGES and IC_{50} values from ChEMBL or AUC values from CCLE. A Wilcoxon signed-rank test was used to assess differences between RGES across active ($IC_{50} < 10\,\mu M$) and inactive compounds ($IC_{50} \geq 10\,\mu M$), the same and different cell lines, higher ($\geq 10\,\mu M$) and lower ($<10\,\mu M$) drug concentrations, and longer (≥ 24 h) and shorter (<24 h) treatment durations. *P*-values were adjusted with the Benjamini and Hochberg's false discovery rate method to correct for multiple testing.

RESULTS

Inclusion of CRPC Gene Expression Datasets

The study selection process for finding CRPC disease signatures is outlined in **Figure 1**. A total of 224 GEO Series Experiments (GSEs) were searched. A number of GSEs were excluded due to duplicated data ($n = 21$), disease ($n = 179$), non-human ($n = 14$), non-control (neither normal nor benign tissues) ($n = 4$), and number of genes <5,000 ($n = 2$). Finally, the four datasets of GSE3325, GSE35988, GSE70768, and GSE80609 were selected for further analysis after a MetaQC analysis (**Supplementary Table S1**). Detailed information about the downloaded CRPC gene expression datasets is summarized in **Supplementary Table S2**. GSE35988 contained gene expression data from the GPL6480 and GPL6848 platforms. Tumor gene expression signatures in CRPC were analyzed for 178 samples by comparing RNA expression data for 64 tumors and 114 normal or benign tissues from those four datasets.

Gene Expression Signatures of CRPC

The workflow for the exploration of the compounds using the calculated RGES values is presented in **Supplementary Figure S1**. Corresponding probes on each platform were re-annotated with the most recent NCBI Entrez Gene IDs and then mapped to yield 7,825 unique common genes across the five different platforms. A fixed-effect model method was used by combining the *P*-values using the MetaDE package. Among the gene expression signatures, 53 genes showed increased expression levels in tumors compared to normal or benign tissues (adjusted $P < 0.001$, $\log_2$foldchange > 1.5), whereas 42 genes showed decreased expression levels in tumors compared to normal or benign tissues (adjusted $P < 0.001$, $\log_2$foldchange < -1.5; **Supplementary Table S3**).

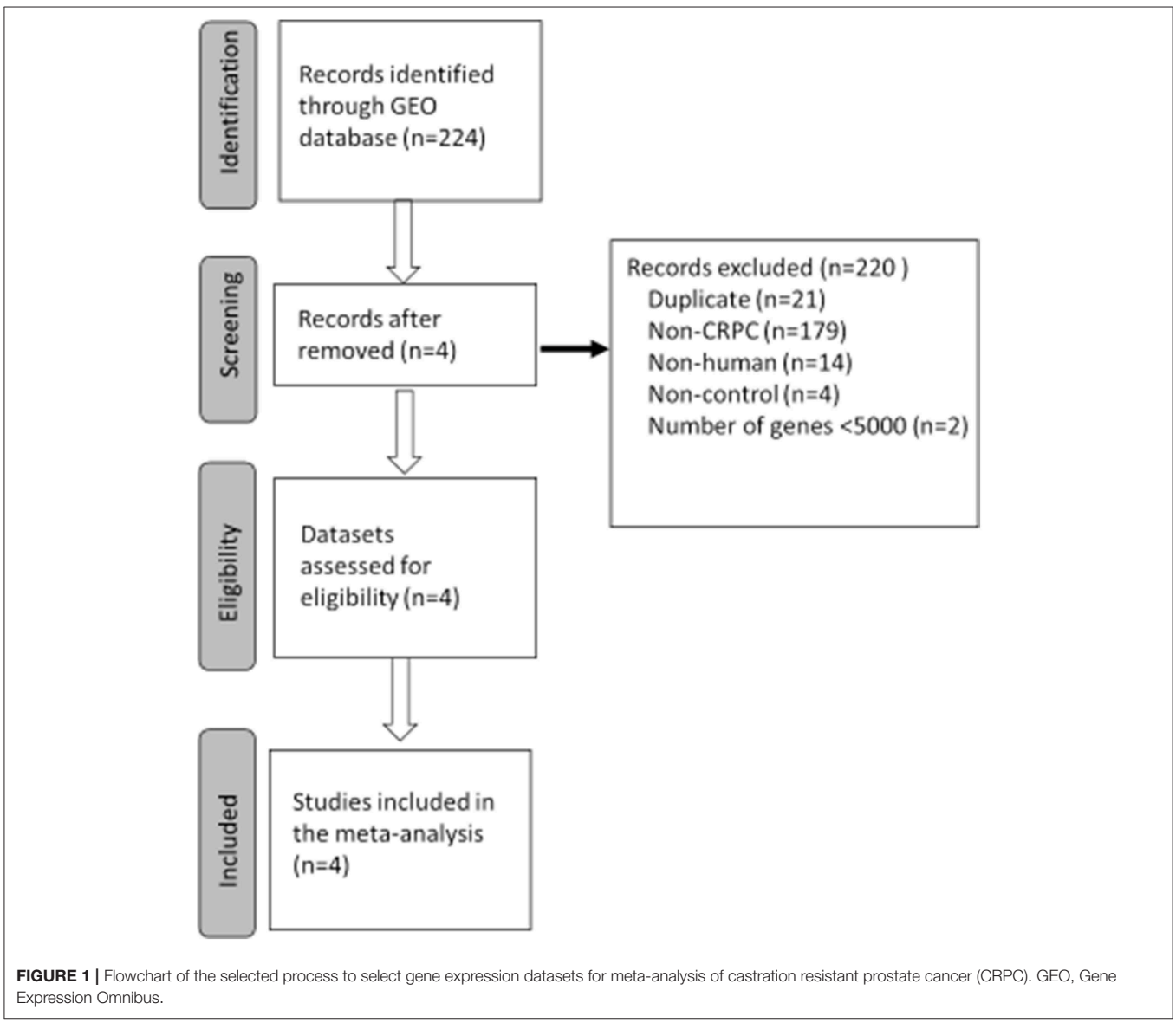

FIGURE 1 | Flowchart of the selected process to select gene expression datasets for meta-analysis of castration resistant prostate cancer (CRPC). GEO, Gene Expression Omnibus.

Similarity in Gene Expressions Between Tumor Samples and PC Cell Lines

The degree of similarity in the gene expression levels between CRPC tumor samples from the GEO and PC cell lines from the CCLE was assessed by a ranked Spearman's correlation test. Gene expression data for eight PC cell lines were included in the CCLE (**Supplementary Table S4**). The top 5,000 genes in these cell lines were ranked according to their interquartile range across all PC cell lines used. Among them, <0.1% of genes had expression levels in tumor samples from the GEO that did not correlate with those in these cell lines.

Computation of RGES Values

Changed expression values of 978 landmark genes after a compound treatment of human prostate adenocarcinoma PC3 cell lines with 172 compounds in the LINCS data as drug signatures were used for the computation of the RGES values. The median IC_{50s} values for 12,895 compounds used to treat PC cancer cell lines listed in the ChEMBL were used for the RGES computation. The changed expression values of 95 DEGs after extraction from the set of LINCS landmark genes as disease signatures were also used for computation. Variations in the RGES values were evaluated under various biological conditions. The RGES values showed larger variations across different cell lines relative to those within different replicates of the same cell line when the same concentration and treatment duration for a compound were used ($P < 2.2 \times 10^{-16}$; **Figure 2A**). In addition, higher compound concentrations (>10 μM) had lower RGES values than lower concentrations (<10 μM, $P = 1.46 \times 10^{-4}$; **Figure 2B**) when a compound was tested on the same cell line at the same concentration. Likewise, longer treatment durations (≥24 h) were associated with lower RGES values compared to shorter durations (<24 h) ($P < 2.2 \times 10^{-16}$; **Figure 2C**). The RGES values for the compounds were evaluated by examining the correlations with their activities in the same cell line. Finally, the RGES values were correlated with the IC_{50} values for the compounds (Spearman correlation rho = 0.19, $P = 1.43 \times 10^{-2}$; **Figure 3A**).

Summarization and Evaluation of RGES Outcomes

sRGES values were computed by weighting various cell lines, compound concentrations, and treatment durations. A number of known methods were used to summarize the RGES outcomes and obtain sRGES values (**Supplementary Table S5**). The calculated sRGES values for each compound were significantly correlated with drug activity levels (Spearman correlation rho = 0.21 and $P = 8.06 \times 10^{-3}$; **Figure 3B**). Additionally, CTRP was used as an external data set to confirm the correlation between the reversal potency and the compound activity. Compound activity data expressed as AUC values for 558 compounds tested in PC cell lines were collected from CTRP. After the sRGES computations, the median AUC values across multiple cell lines were used to evaluate the sRGES outcomes. The

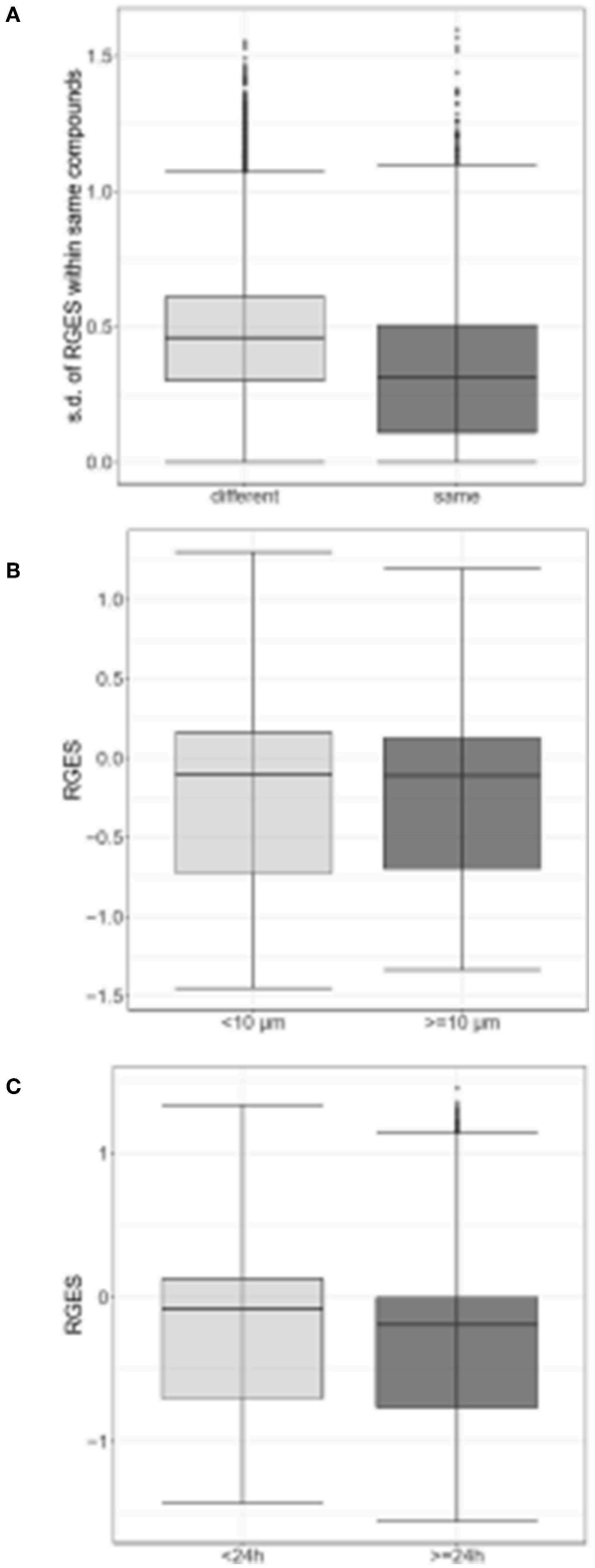

FIGURE 2 | Differences in reverse gene expression scores (RGES) under the various biological conditions. **(A)** Standard deviation (s.d.) of reverse gene expression scores (RGES) of individual compounds across different cell

(Continued)

FIGURE 2 | lines (gray) vs. across replicates within the same cell line (black gray). **(B)** RGES distribution between drug concentrations $<10\,\mu M$ (gray) and $\geq 10\,\mu M$ (black gray). **(C)** RGES distribution between treatment durations <24 h (gray) and ≥ 24 h (black gray). Treatment duration and compound concentration were categorized based on compound data in LINCS. *P*-value was calculated using a Wilcoxon signed-rank test.

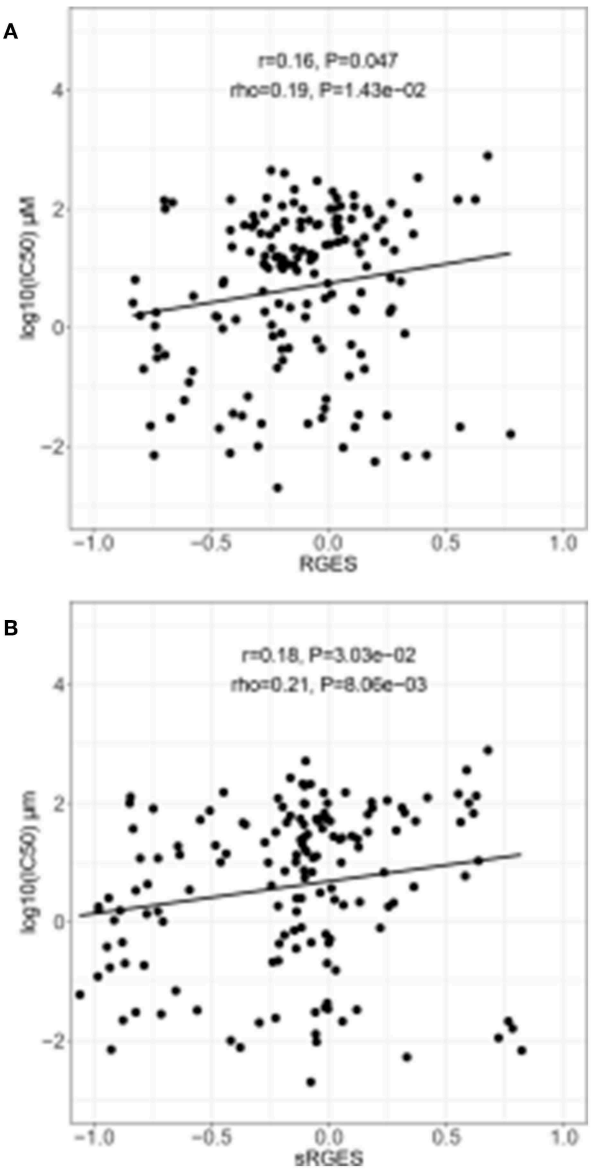

FIGURE 3 | Correlation between drug efficacy and reverse gene expression score (RGES) in PC3 cancer cell lines. **(A)** Correlation between RGES and drug efficacy (IC_{50}) by linear regression and Spearman's correlation tests. **(B)** Correlation between drug efficacy (IC_{50}) and summarized reverse gene expression score (sRGES) for all cancer cell lines using a linear regression and a Spearman's correlation tests. IC_{50}, half maximal inhibitory concentration.

sRGES values were significantly correlated with the AUC values (rho = 0.29, $P = 2.91 \times 10^{-5}$; **Figure 4**).

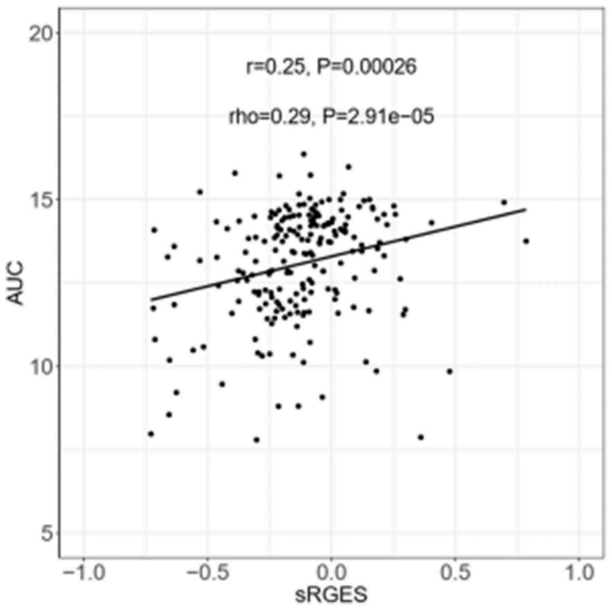

FIGURE 4 | Correlation between AUC and sRGES of compounds. AUC data were retrieved from CTRP. Sensitivity levels were measured in the form of cellular ATP levels as a surrogate for cell number and growth using CellTiter-Glo assays. A compound-performance score was computed at each concentration of compound. Median values were used to summarize AUC across all prostate cancer cell lines examined. A Spearman's correlation test was used to analyze the correlation between sRGES and AUC. AUC, areas under the concentration-response curve. CTRP, the cancer therapeutic response portal.

Identification of Reversed Genes and Predictions of Compounds

Using the correlation between the sRGES values and the compound activity, compounds having high reversal potency for PC were identified. Next, genes having expression levels that were reversed by the active compounds were predicted by a leave-one-compound-out approach. The four genes that showed significant reversal of expression following treatment with PC cell lines with the active compounds included the following: (i) myosin light chain 9 (*MYL9*), (ii) DNA topoisomerase 2-binding protein 1 (*TOPBP1*), (iii) Apolipoprotein E (*APOE*), and (iv) zinc finger protein 36 (*ZFP36*) (**Figures 5A,B**). Fifty compounds were determined to be active compounds against CRPC (**Figure 5A**), while 48 compounds were determined to be inactive compounds (**Figure 5B**, **Supplementary Table S6**). The active drugs against CRPC identified by our analysis contained the tyrosine kinase inhibitors dasatinib, lapatinib, lestaurtinib, and saracatinib; the histone acetylation inhibitors belinostat, entinostat, mocetinostat, panobinostat, trichostatin-A, and vorinostat and the heat shock inhibitors elesclomol and geldanamycin.

Prediction of the Synergic Effect

Combination of 98 drug candidates were employed the TIMMA. Of all these drug combinations, the highest synergy sensitivity

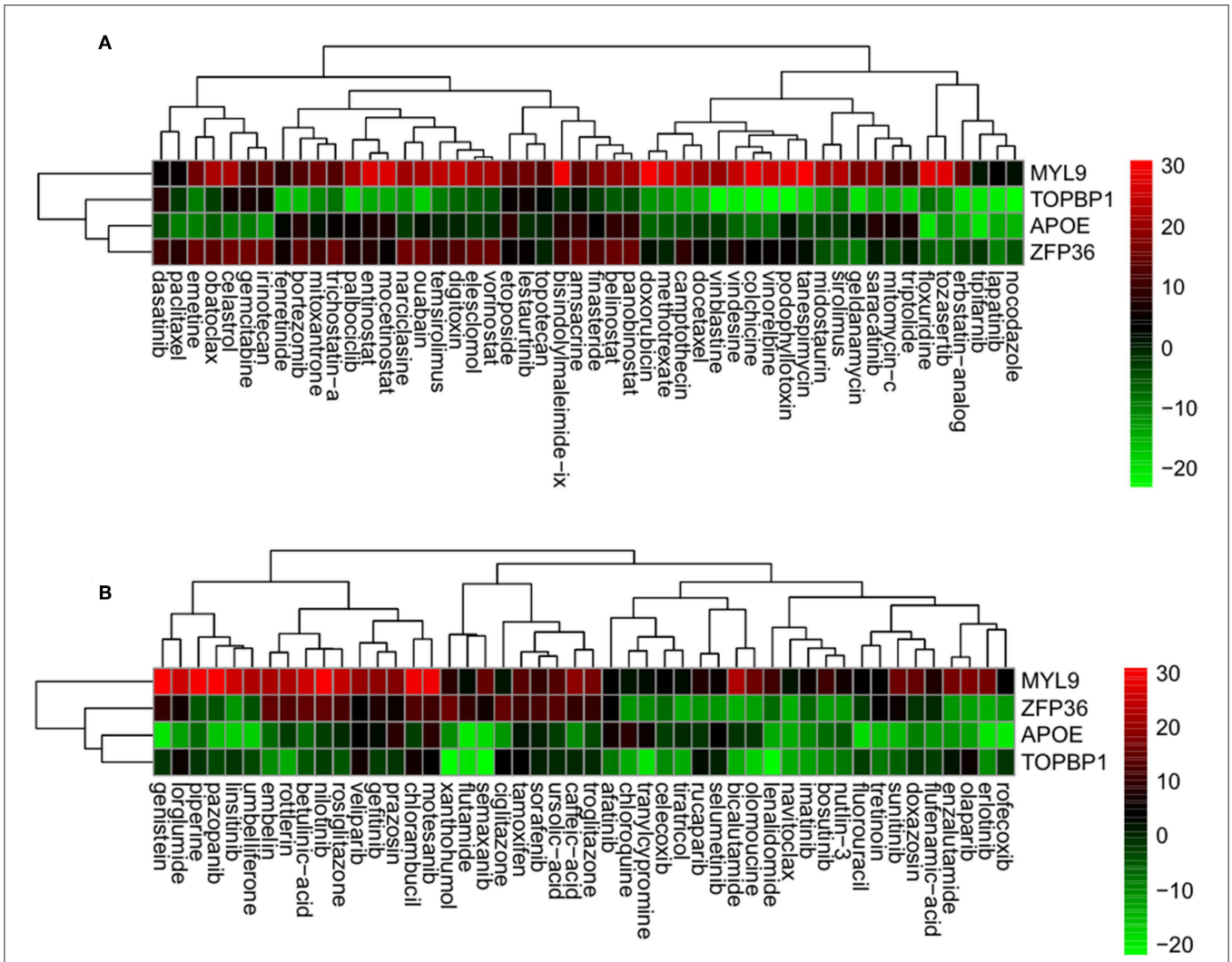

FIGURE 5 | Genes having reversed expression in response to treatment with active **(A)** and inactive **(B)** compounds. Low rank and high rank suggests that the gene expression is down- and upregulated, respectively, by the corresponding compound. The heatmap indicates the relative position of a gene in ranked drug expression data. Position are normalized and compound columns are ordered according to IC_{50}. Red and green colors indicate up- and down-regulation, respectively, after compound treatment.

score was predicted for the combination of lenalidomide and pazopanib (**Figure 6**, **Supplementary Table S7**). Next, the synergic sensitivity score orders were lenalidomide combined with olapanib, nocodazole, tipifarnib, and imatinib and their corresponding targets were *APOE*, *ZFP36*, *E2F2*, and *MYL9*.

DISCUSSION

The computational approaches used in systemic analyses of large amounts of data such as gene expression values, genotypes, and chemical structure similarities for predictive repositioning offer a relatively quick and mechanistic method of identifying new application of existing drugs that may be translated into clinical applications (50). In this study, computational methods using public database were used for the purpose of identifying drug repositioning candidates for treatment of CRPC. Several drug candidates were identified, as well as DEGs in CRPC whose expression can be reversed by these agents. We used public cancer genomic and pharmacologic databases to demonstrate the reversal potency relationship between DEGs and drug activities, and to predict potential new drug candidates for CRPC.

Our results showed that the ability of drugs to reverse DEGs was correlated with drug activity in CRPC, although this correlation was highly dependent on the cell lines as well as the treatment concentration and duration of the drugs. The positive correlation between sRGES and IC_{50} values indicated that combining disease gene expression data derived from clinical samples with drug gene expression data obtained from results with *in vitro* cell lines could be used to predict drug activities.

In our study, four genes, *MYL9, E2F2, APOE,* and *ZFP36*, showed reversed expression in response to 50 active compounds in CRPC. To the best of our knowledge, this is the first study focusing on drug repositioning using a computational reversal gene expression approach in relation to CRPC. *MYL9* (myosin

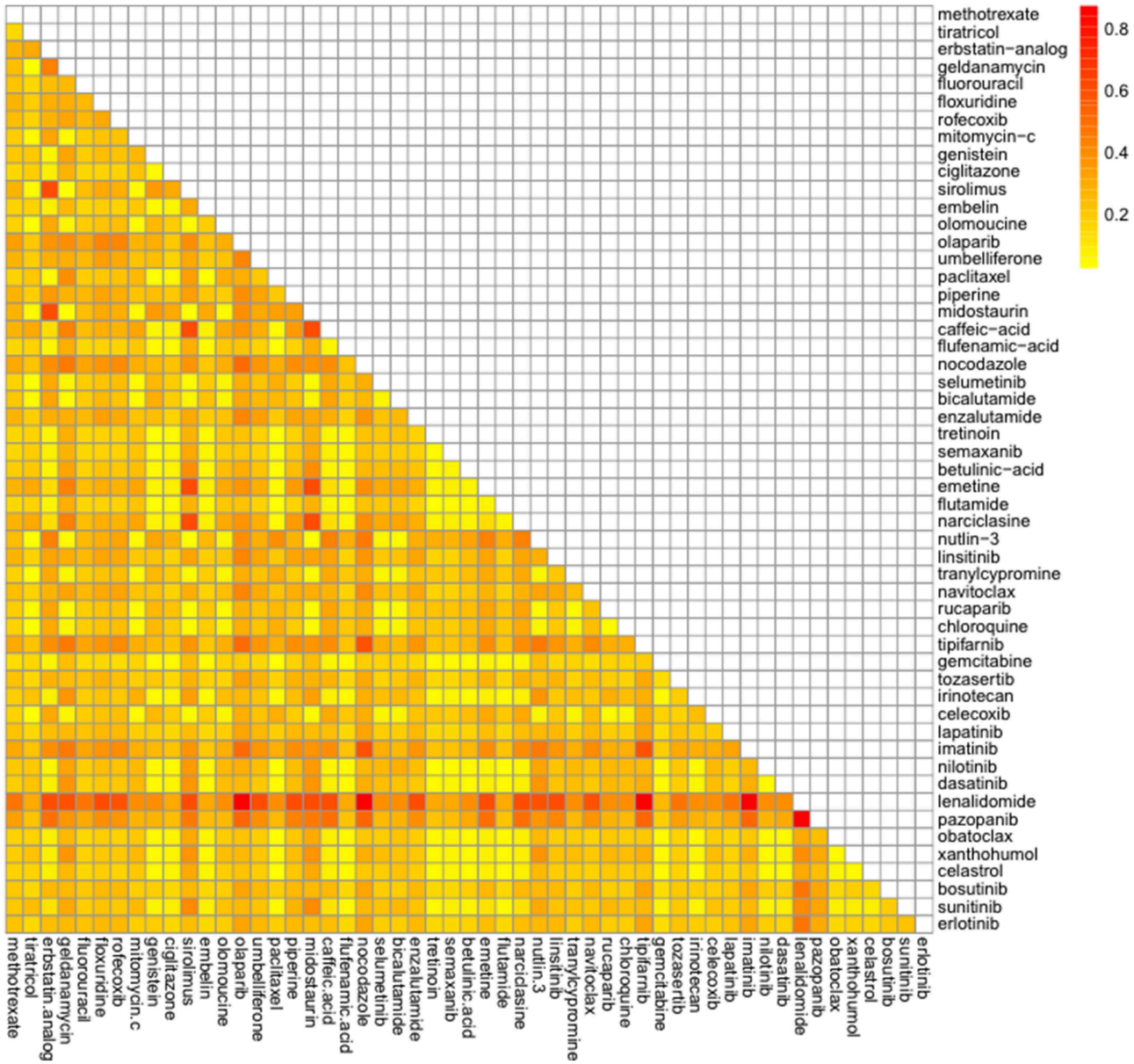

FIGURE 6 | Predicted synergic drug combinations against castration resistant prostate cancer. Predicted sensitivity scores were calculated by the Target Inhibition Interaction using Maximization and Minimization Averaging algorithm. The bar on the right shows predicted synergic scores.

light chain 9) has been reported to play an important role in tumor progression in PC (51). Loss of the RB function facilitates the development of CRPC via E2F-mediated upregulation of the AR (52). *ZFP36* is reportedly involved in the progression and prognosis of PC (53). As cholesterol is known to be a potential target for CRPC, *ApoE* has been suggested to play a potential role in prostate cancer progression (54). These genes showed reversed expression levels and thus may be feasible as therapeutic targets for CRPC.

Among the active drugs against CRPC identified by our analysis, the histone deacetylase inhibitors, vorinostat (55), and panobinostat (56), the tyrosine kinase inhibitors, dasatinib (57) and lapatinib (58), and a poly (ADP-ribose) polymerase (PARP) inhibitor, olaparib (59), have gone through phase I and phase II clinical trials for CRPC patients. Additionally, the docetaxel FDA-approved for metastatic CRPC (60) was identified as an active drug in our results. Bicalutamide and flutamide used as a hormonal therapy against CRPC have low cytotoxic activities, resulting that they have been identified as inactive drugs in our study. The cytotoxic chemotherapy is most effective when given in combination to achieve an additive or synergistic effect and a targeted therapy involving more than one drug

increases powerful antitumor effects and overcomes resistance mechanisms (61). A combination of high-throughput screening of all licensed drugs has been carried out in an attempt to discover synergistic interactions (62). Therefore, the synergic effects of the candidate drugs identified were examined in our study. The immunomodulatory, drug lenalidomide combined with a tyrosine kinase inhibitor, pazopanib was most potent against CRPC in our result. Several clinical trials of lenalidomide- or pazopanib-based regimens for CRPC were conducted (63–65). The phase I/II study of lenalidomide in combination with sunitinib was conducted in patients with advanced or metastatic renal cell carcinoma (66). Despite the fact that the combination of pazopanib and lenalidomide has been reported to induce synergistic cytotoxicity in multiple myeloma (67), this drug combination has not yet been tested yet in CRPC cases.

After CRPC development, PC switches from an endocrine-driven disease to a paracrine- or autocrine-driven disease. Therefore, CRPC represents an increased opportunity to accumulate genomic aberrations and is riddled with aggressive and heterogeneous clones. Strengths of this work is that it leverages publically available datasets to identify candidates for drug repositioning targets and theoretically, this could be a much cheaper and faster way to identify promising new leads. A limitation of this study is that the CRPC disease gene expression datasets from the GEO are not uniformly associated with clinical outcomes or CRPC etiologies. CRPC is a heterogeneous disease that might be driven by different pathways, depending on prior treatments and this approach essentially treats CRPC as a single entity. The drug efficacy of the predicted compounds may also vary because the CRPC tissue states varied for individual patients. Therapeutic efficacy is more complex than a simple correlation of gene expression levels with drugs and diseases. Therefore, our findings for drug candidates will require further preclinical testing and demonstration in clinical trials. As performing randomized trials in relation to a rare cancer disease is challenging, a computational drug repositioning approach with public gene expression databases may become a quite useful strategy for treating rare types of cancer.

In summary, our computational approach combined disease gene expression with drug-induced expression data in CRPC to identify new drugs and target genes as CRPC therapies. This approach, can also be used to predict the efficacy of new drug candidates to treat CRPC. This computational approach could be broadly applied to other rare forms of cancer for which reliable gene expression data are available.

AUTHOR CONTRIBUTIONS

I-WK and JO contributed conception and design of the study. I-WK organized the database and wrote the first draft of the manuscript. I-WK and JK contributed to data collection and performed the statistical analysis. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00661/full#supplementary-material

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LSD1 Activates PI3K/AKT Signaling Through Regulating p85 Expression in Prostate Cancer Cells

*Zifeng Wang, Shuai Gao, Dong Han, Wanting Han, Muqing Li and Changmeng Cai**

Center for Personalized Cancer Therapy, University of Massachusetts Boston, Boston, MA, United States

Lysine specific demethylase 1 (LSD1) functions as a transcriptional repressor through demethylating active histone marks such as mono- or di-methylated histone 3 lysine 4 (H3K4) and interacting with histone deacetylases. However, LSD1 can also act as an activator through demethylating repressive histone marks and possibly non-histone proteins. In prostate cancer (PCa) cells, LSD1 mediates the transcriptional activity of androgen receptor (AR), a ligand dependent nuclear transcription factor that drives PCa initiation and progression to the castration-resistant prostate cancer (CRPC). However, it is unclear whether LSD1 also regulates other growth promoting pathways independent of AR signaling in PCa cells. In this study, we show that LSD1 can activate PI3K/AKT pathways in absence of androgen stimulation, and we further demonstrate that LSD1 transcriptionally regulates the expression of PI3K regulatory subunit, p85, possibly through epigenetic reprogramming of enhancer landscape in PCa cells. Our study suggests that LSD1 has dual functions in promoting PCa development, that it enhances AR signaling through its coactivator function, and that it activates PI3K/AKT signaling through increasing p85 gene expression.

Keywords: KDM1A, LSD1, PI3K, AKT, p85, PI3K regulatory subunits, prostate cancer, androgen-deprivation therapy

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Hung-Ming Lam,
University of Washington,
United States

Reviewed by:
Shashwat Sharad,
Center for Prostate Disease Research (CPDR), United States
Zoran Culig,
Innsbruck Medical University, Austria

****Correspondence:***
Changmeng Cai
changmeng.cai@umb.edu

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INTRODUCTION

Lysine specific demethylase 1 (LSD1/KDM1A), a specific demethylase of mono- or di-methylated histone lysine 4 (H3K4me1,2, enhancer-associated histone marks), was first identified as a component of REST repressor complex through interaction with CoREST and histone deacetylases 1, 2 (HDAC1,2) (1, 2). LSD1 is also found in Mi-2/nucleosome remodeling and deacetylase (NuRD) repressor complex with interaction with MTA proteins (3, 4). While LSD1 is well-known for its transcription repressor activity, it also activates gene transcription through demethylating repressive histone marks, such as methylated histone 3 lysine 9 (H3K9me1,2), and other non-histone proteins (5–8). In prostate cancer (PCa) cells, LSD1 functions as a major androgen receptor (AR) coactivator (5, 9). This activity was thought to be attributed to androgen-induced phosphorylation of histone 3 threonine 6 and 11 (H3T6/T11ph), which lead to the switch of LSD1 substrate from H3K4me1,2 to H3K9me1,2 (5, 10–12). However, our recent study indicates that the H3K4 demethylase activity of LSD1 persists at AR-mediated enhancers marked with H3T6ph, suggesting additional mechanism(s) mediating its coactivator activity of AR (9). Indeed, we reported that LSD1 interacts and colocalizes with FOXA1, a pioneer factor of AR, at AR-mediated enhancers, and that this interaction may facilitate AR transcription activity (9). Nonetheless, since

AR signaling is critical to PCa development and progression to the lethal stage of castration-resistant PCa (CRPC) (13), studies from us and others highly suggest that targeting LSD1 may be a potential treatment strategy for PCa and particularly CRPC, where AR signaling is commonly restored. However, whether LSD1 regulates other major tumor-promoting pathways in PCa cells remains to be determined.

From the RNA-seq analyses of LSD1 inhibitor treated PCa cells, we found that LSD1-activated genes were enriched for PI3K/AKT pathway (14) in absence of androgen stimulation and we further confirmed that LSD1 inhibition significantly decreased AKT phosphorylation independent of DHT treatment. Through functional annotation analyses and subsequent validations, we identified the regulatory subunit of PI3K, p85α (and possibly its isoform p85β), as a critical transcriptional target of LSD1 that mediates its effect on PI3K/AKT pathway activation. Based on these findings, it is plausible that the effectiveness of LSD1 inhibitor treatment in CRPC may be due to inhibition of both AR signaling and PI3K/AKT signaling pathways. As LSD1 inhibitors are currently being tested in clinical trials of leukemia and small cell lung cancer, our studies can be rapidly translated into clinical trials of CRPC.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The LNCaP and CWR22-RV1 cell lines were recently authenticated using short tandem repeat (STR) profiling by DDC Medical (Fairfield). Both cell lines were cultured in RPMI with 10% FBS (fetal bovine serum). For androgen stimulation assays, cells were grown to 50–60% confluence in medium containing 5% charcoal stripped serum (CSS) for 3 days (d) and then treated with DHT or inhibitors for 24 h.

Chromatin Immunoprecipitation (ChIP)

For preparation of ChIP, dispensed cells were formalin fixed, lysed, and sonicated to break the chromatin into 500–800 bp fragments, followed by immunoprecipitation. Anti-H3K4me2 (Milipore) and anti-V5 (Sigma) are used for immunoprecipitation. The qPCR analysis was carried out using the SYBR Green method. The primers are listed as following: *PIK3R1*-enh: forward, 5′-GTGGAAGAACAGCTTTGGGG-3′, reverse, 5′-TCAAGGCAACTTACTTTGCAGG-3′. *PIK3R1*-LBS: forward, 5′- TTGTTGATTTCCCCACCCCTC-3′, reverse, 5′- TCCCAAGCTGGGCTCTATTTG -3′.

RT-PCR and Immunoblotting

RNA was extracted with TRIzol Reagent (Invitrogen) following manufacturer's protocol. The expression of genes was measured using real-time RT-PCR analyses with Taqman one-step RT-PCR reagents (Thermo Fisher Scientific) and results were normalized to co-amplified GAPDH. The primer and probe set for the following genes: *FKBP5 (Hs01561006_m1), PIK3R1 (Hs00933163_m1), PIK3R2 (Hs00178181_m1),* and *GAPDH (4310884E)* were purchased as inventoried mix (Applied Biosystems at Thermo Fisher). For immunoblotting, anti-AKT (Cell Signaling), anti-phosohoylated-473-AKT(Cell Signaling), anti-p85α (R&D), anti-p85β (R&D), anti-H3K4me2 (Milipore), anti-LSD1 (Abcam), anti-V5 (Sigma), anti-HDAC1 (Abcam), anti-GAPDH (Abcam), or anti-β-Tubulin (Abcam) antibodies were used. The inhibitors used are GSK2879552 (Selleck), ORY-1001 (Selleck), S2101 (Calbiochem), tranylcypromine (Calbiochem), and BKM120 (Selleck). Immunoblotting results shown are representative of at least 3 independent experiments.

Generation of LSD1 Knockout Cell Lines

The effective guided RNA to target LSD1: forward, 5′-CACC GGGGGCCTGGCGGAACCGCCG-3′, reverse, 5′-AAACCGGC GGTTCCGCCAGGCCCCC-3′. The sgRNAs sequences were inserted into lentiGuide-Puro system (Addgene) following the manufacture protocol. Lenti-Cas9-2A-Blast and lentiGuide-Puro vectors were transfected as 1:1 ratio into 22Rv1 cells by Lipofectamine 2000 (Thermo fisher) for 24 h. Cells were then co-treated by blasticidin (1 ug/ml) and puromycin (5 ug/ml) to select single clones. LSD1 knockout in the selected clones was determined by immunoblotting of LSD1.

Cell Viability Assay

PCa cells were maintained in RPMI-1640 supplemented with 5% CSS. Cell were plated into 96-well plates at ∼5,000–10,000 cells/well. After 24 h, cells were treated with DMSO, ORY-1001, and/or BKM-120 for 3 days. The cell viability was then examined by using CellTiter-Glo luminescent cell viability assay (Promega, USA).

Xenografts

CWR22-RV1 derived xenograft was established in the flanks of castrated male SCID mice by injecting ∼2 million cells mixed with 50% Matrigel. LuCaP35CR xenograft tumors were established in the flanks of castrated male SCID mice by transplantation. Tumor volume was measured by manual caliper. Frozen sections were examined to confirm that the samples used for RNA and protein extraction contain predominantly non-necrotic tumor.

Statistical Analysis

Data in bar graphs represent mean ± SD of at least 3 biological repeats. Statistical analysis was performed using Student's *t*-test by comparing treatment vs. vehicle control or otherwise as indicated. p-value < 0.05 (*) was considered to be statistically significant. For animal studies, one-way ANOVA was performed for the tumor volume data measured at the final day of the treatments.

RESULTS

LSD1 Inhibitor Treatments Decreased AKT Phosphorylation in PCa Cells

To identify the additional target pathways of LSD1 independent of androgen treatment, we treated LNCaP cells (an androgen-responsive PCa cells line with *PTEN* loss) under hormone-depleted condition with a clinical tested LSD1 inhibitor, GSK2879552 (Phase I for small cell lung cancer) (15), at lower doses (1 or 5 μM) but for extended period of

time (∼2 weeks), and then carried out RNA-seq analyses. This treatment resulted in a significant decrease of cell growth regardless of DHT stimulation (**Figure 1A**). KEGG pathway analysis (provided by DAVID) was performed to identify the enriched functions/pathways in LSD1-activated (LSD1 inhibition-downregulated) and LSD1-repressed (LSD1 inhibition-upregulated) gene subsets. While LSD1-repressed genes were enriched for neuronal and immune responses (data not shown), a consistent finding with its classic function and a recent study (16), LSD1-activated genes were significantly enriched for PI3K/AKT signaling pathway (**Figure 1B**), which plays a critical role in driving PCa development (14). To determine whether PI3K/AKT pathway is activated by LSD1, we examined Ser473 phosphorylation of AKT, a marker for its full activation (17, 18), in LNCaP cells treated with GSK2879552 (same strategy as in **Figure 1A**) and our data indicated that LSD1 inhibition markedly decreased AKT phosphorylation (**Figure 1C**). This result is in sharp contrast to the effects of treating LNCaP cells with an AR antagonist, enzalutamide, or androgen deprivation, which led to increased AKT phosphorylation (**Figure 1D**) (19).

As we have previously observed that short-term treatment of LSD1 inhibitors required much higher doses (∼50–100 μM) to reach the similar effects on cell growth and AR activity in comparison with the prolonged treatment with lower doses (not shown), we next examined whether the high dose treatment can similarly affect AKT phosphorylation in LNCaP cells. As seen in **Figure 1E**, treating cells with 50 μM GSK2879552 caused rapid inhibition of AKT phosphorylation (4 and 48 h). We then determined whether other LSD1 inhibitors can result in the similar effect on PI3K/AKT signaling. As seen in **Figures 1F,G**, treatments of two structurally related LSD1 inhibitors, TCP (tranylcypromine) and S2101 (20), resulted in the similar inhibitory effect on AKT phosphorylation at ∼100 μM, which also suppressed DHT-induced PSA expression (a classic target of AR). Moreover, we have also examined the effect of another clinical tested LSD1 inhibitor, ORY-1001 (Phase II for AML) (21), on AKT activation. As seen in **Figure 1H**, ORY-1001 decreased Ser473 phosphorylated AKT at ∼25 μM. Furthermore, since LSD1 inhibitor treatment was recently reported to inhibit the growth of AR-negative PC-3 cell-derived xenograft tumors (22), we next examined the effect of LSD1 inhibition on AKT activation in PC-3 cells. As seen in **Figure 1I**, LSD1 inhibition repressed AKT phosphorylation in PC-3 cells, indicating that this oncogenic activity of LSD1 is distinct from its activity on mediating AR signaling. Overall, these results demonstrate that LSD1 activates PI3K/AKT pathways independent of AR signaling in PCa cells.

LSD1 Transcriptionally Regulated p85α Expression

Since AKT can be methylated by SETDB1 (although this methylation promotes AKT activity) (23, 24), we first examined whether LSD1 can directly interact with AKT to remove its methylation. However, AKT was not coimmunoprecipitated with LSD1, or vice versa, in LNCaP cells (**Figure 2A**), indicating that LSD1 is unlikely to demethylate AKT. Therefore, we next hypothesized that LSD1 may transcriptionally regulate an upstream component of PI3K/AKT pathway. Through KEGG analyses, we have identified a subset of LSD1-activated genes that were involved in PI3K/AKT pathways (see **Figure 1B**). Amongst these genes, *PIK3R1* encodes for regulatory subunit alpha of PI3-Kinase, p85α. In cells, p85 regulatory subunit and p110 catalytic subunit form heterodimer of PI3K, which functions to phosphorylate $PI(3,4)P_2$ to $PI(3,4,5)P_3$ (25). Although several isoforms of p85, including p85β, are found in PCa cells, p85α is generally the most highly expressed PI3K regulatory subunit (14). Significantly, the expression of *PIK3R1* strongly associated with the expression of *KDMA1* (encoding for LSD1) in MSKCC PCa dataset (using cBioPortal) (26–28) (**Figure 2B**). We next examined whether p85α expression is regulated by LSD1. As seen in **Figure 2C**, LSD1 inhibition significantly decreased the mRNA expression of *PIK3R1* but not *PIK3R2* (encoding for p85β). As a result, the protein expression of p85α was markedly reduced by LSD1 inhibitor treatment (**Figure 2D**). Examining ChIP-seq of H3K4me2 in LNCaP cells, we identified an enhancer site (named *PIK3R1*-enh) located at the gene body of *PIK3R1*, where the level of H3K4me2 was decreased by LSD1 inhibitor treatment (**Figure 2E**). Surprisingly, using a published ChIP-seq dataset of LSD1, we did not find any LSD1 binding peak at this enhancer. There was only one nearby LSD1 binding site (named *PIK3R1*-LBS) located at the downstream of *PIK3R1* locus, but the level of H3K4me2 is barely detected, indicating that this site is unlikely an active enhancer. Nonetheless, we performed ChIP-qPCR in LNCaP cells to examine the H3K4me2 level at these two sites. As seen in **Figure 2F**, H3K4me2 was decreased at *PIK3R1*-enh by the LSD1 inhibitor treatment, consistent with the finding from H3K4me2 ChIP-seq. Interestingly, while H3K4me2 was low at *PIK3R1*-LBS, it was increased by LSD1 inhibition, supporting that this site is occupied by active LSD1, which can demethylate H3K4me2. To further determine if LSD1 binds to *PIK3R1*-enh, we generated a stable cell line expressing doxycycline-regulated V5-tagged LSD1 and then performed V5 ChIP. As seen in **Figure 2G**, LSD1 binding at *PIK3R1*-LBS but not *PIK3R1*-enh was induced by doxycycline treatment, confirming that LSD1 does not directly bind to this enhancer of *PIK3R1*. Overall, these results indicate that LSD1-mediated transcription of *PIK3R1* is possibly due to an indirect activation of a *PIK3R1* enhancer, which may be a consequence of previously reported LSD1-mediated epigenetic reprogramming (29).

The Combination Treatment of a PI3K Inhibitor With a LSD1 Inhibitor More Effectively Suppressed PCa Cell Proliferation

We next selected an AR-positive CRPC cell line, CWR22-RV1 cells, to further study the LSD1 function on PI3K/AKT pathway. Interestingly, this cell line appeared to be more sensitive to the LSD1 inhibitors as both p85α expression and AKT phosphorylation were decreased by ∼5–10 μM of GSK2879552 or ORY-1001 (**Figures 3A,B**). The heregulin-induced AKT activation (through activating EGFR and ErbB2 receptors) (30, 31) was also decreased by LSD1 inhibition (**Figure 3C**). Interestingly, unlike in LNCaP cells where LSD1 inhibition

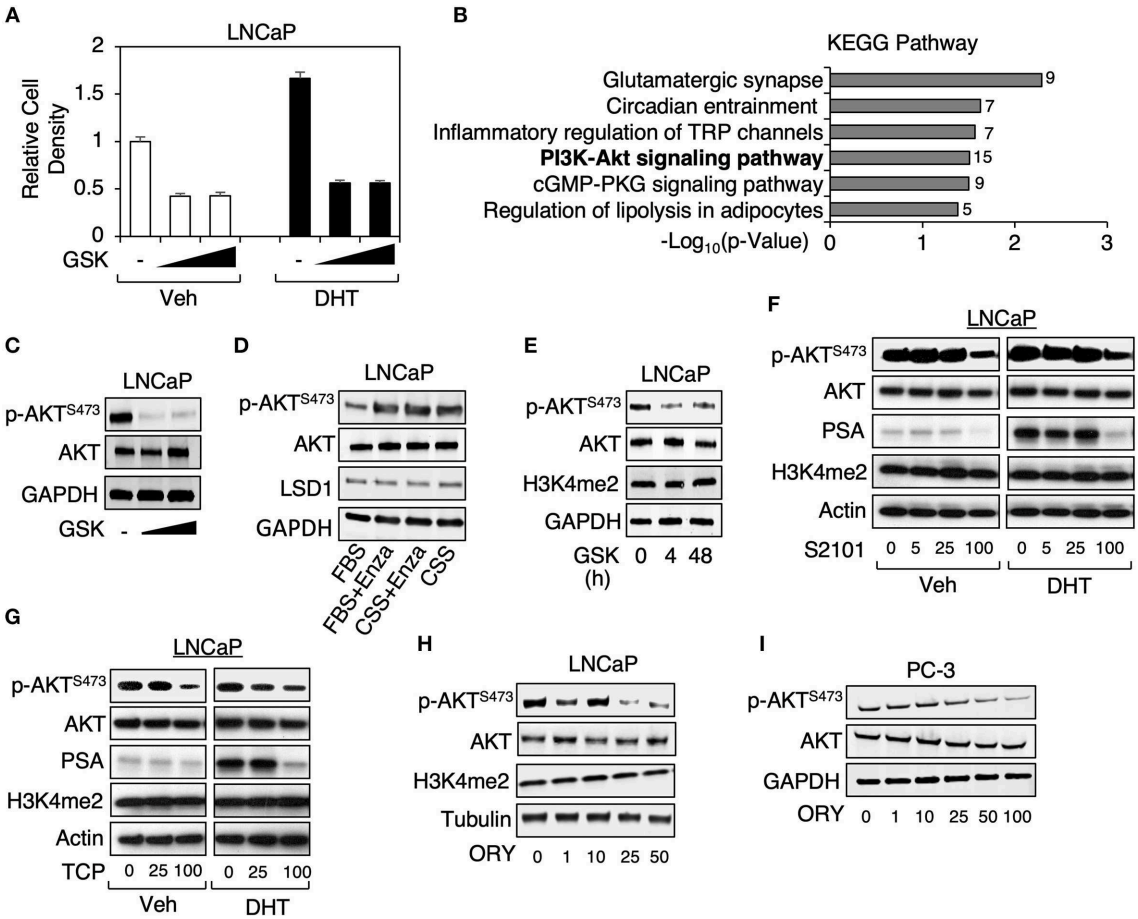

FIGURE 1 | LSD1 inhibitor treatments decreased AKT phosphorylation in PCa cells **(A)** LNCaP cells were maintained in medium containing 0–5 μM GSK2879552 for ~2 weeks (w) and then treated with/out 10 nM DHT for 4 days (d). Cell density was examined under the indicated conditions. **(B)** GSK2879552 (1 μM) downregulated (LSD1-activated) genes (identified from RNA-seq analyses, GSE114268) were analyzed by KEGG pathway annotation. **(C)** Immunoblotting for Ser473-phosphorylated AKT (p-AKT^{S473}) and total AKT in LNCaP cells with prolonged treatment of GSK2879552 (0–5 μM). **(D)** Immunoblotting for p-AKT^{S473}, total AKT, and LSD1 in LNCaP cells treated with/out 10 μM enzalutamide for 10 days in medium containing full serum (FBS) or hormone-depleted serum (CSS). **(E)** Immunoblotting for indicated proteins in LNCaP cells treated with/out 50 μM GSK2879552 for 0–48 hours (h). **(F–H)** Immunoblotting for indicated proteins in LNCaP cell treated with **(F)** 0–100 μM S2101, **(G)** 0–100 μM TCP, or **(H)** 0–50 μM ORY-1001 in absence or presence of 10 nM DHT for 48 h. **(I)** Immunoblotting for indicated proteins in PC-3 cell treated with 0–100 μM ORY1001 for 48 h.

only repressed p85α expression, in CWR22-RV1 cells LSD1 inhibition decreased the mRNA expression of both α and β subunits (**Figure 3D**).

PI3-kinase inhibitors, such as BKM120, have been tested in clinical trials of metastatic PCa, but failed to demonstrate significant activity in men with CRPC (32). However, our finding that LSD1 regulates p85 expression in PCa cells suggests that the combined treatment of PI3-kinase inhibitor and LSD1 inhibitor may be more effective in inhibiting downstream AKT activation while still maintain suppression effect on AR signaling. To test this hypothesis, we performed cell viability assays in CWR22-RV1 cell line as well as LNCaP cell line treated with BKM120 alone, ORY-1001 alone, or the combination. As seen in **Figures 3E,F**, the combination treatment was more effective in suppressing cell growth than the single agent treatment, indicating that this treatment strategy may be potentially used in men with CRPC.

LSD1 Gene Knockout Suppressed PI3K/AKT Signaling in CRPC Cells

Since the above used LSD1 inhibitors may target additional proteins, such as monoamine oxidases, we decided to use CRISPR/CAS9 approach to genetically silence LSD1 expression and then to examine the effect on PI3K/AKT signaling. Two stable clones (LSD1-KO-1 and LSD1-KO-2) were established in CWR22-RV1 cells and selected for the subsequent study (**Figure 4A**). The AR activity (AR regulation on FKBP5) was markedly impaired in LSD1-KO cells and the cell growth (in absence of androgen stimulation) was significantly reduced (**Figures 4B,C**). Importantly, AKT phosphorylation and the expression of p85α and p85β were all suppressed by LSD1 knockout (**Figures 4D,E**), consistent with the inhibitor effects. Furthermore, we also generated the xenograft tumors by injecting the control or LSD1-KO cells in castrated male SCID mice. The tumor growth of LSD1-KO line was much slower and

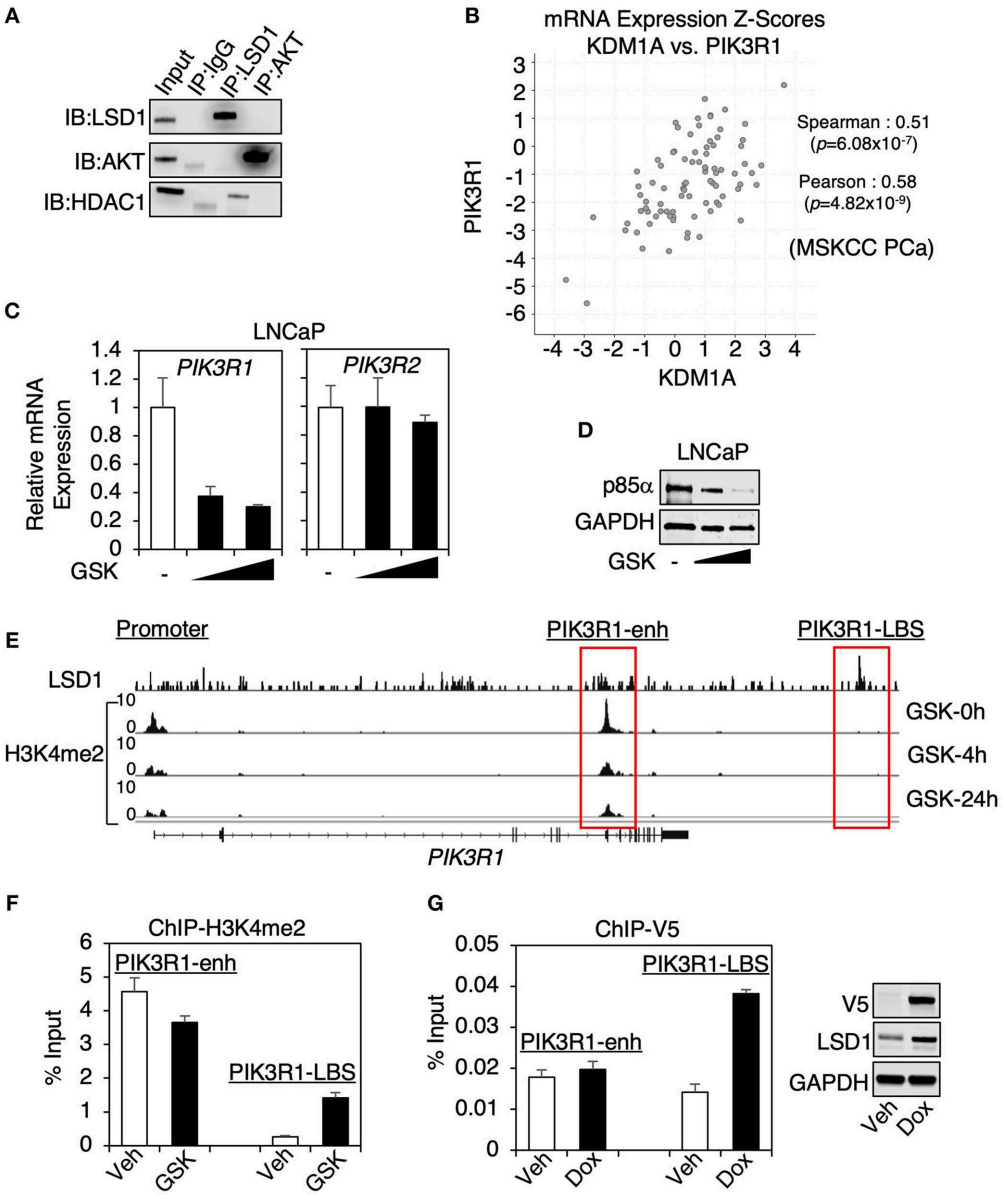

FIGURE 2 | LSD1 transcriptionally regulated p85α expression **(A)** LSD1 or AKT immunoprecipitation was performed in LNCaP cells, followed by immunoblotting for LSD1 and AKT. **(B)** The mRNA expression correlation analyses on KDM1A (LSD1) vs. PIK3R1 (p85α) using MSKCC PCa dataset ($N = 150$) from cBioPortal. The Pearson and Spearman rank correlation coefficient and p-value were shown. **(C)** Real-time RT-PCR (qRT-PCR) for PIK3R1 or PIK3R2 in LNCaP cells with prolonged treatment of GSK2879552 (0–5 μM). **(D)** Immunoblotting for p85α in LNCaP cells with prolonged treatment of GSK2879552 (0–5 μM). **(E)** ChIP-seq of H3K4me2 in LNCaP cells treated with 50 μM GSK2879552 for 0–24 h (GSE114268), aligned with public dataset of ChIP-LSD1 (GSE52201) at PIK3R1 gene locus. **(F)** ChIP-qPCR for H3K4me2 binding in LNCaP cells treated with/out 50 μM GSK2879552 for 24 h. **(G)** ChIP-qPCR for V5 (V5-LSD1) in LNCaP-C4-2 cells stably overexpressing tetracycline-regulated V5-tagged LSD1. Doxycycline-induced expression of V5-LSD1 was confirmed using immunoblotting.

when the tumors in control group exceeded the size limit, we sacrificed the mice and measured the tumor weight. As shown in **Figure 4F**, the average tumor weight for LSD1-KO line was significantly reduced. This tumor regression effect can be seen by Ki67 immunohistochemistry (IHC) staining (**Figure 4G**, upper panel). Importantly, the levels of phosphorylated-AKT were significantly reduced in LSD1-KO line (although it was not completely eliminated) (**Figure 4G**, lower panel), indicating that LSD1 activates AKT pathway *in vivo*. Overall, these results clearly demonstrate that the expression of p85 isoforms and subsequent AKT phosphorylation were specifically regulated by LSD1.

PI3K/AKT Signaling Was Repressed by LSD1 Inhibition in a Castration-Resistant PDX Model

We next determined whether LSD1 inhibitor treatment can suppress PI3K/AKT signaling in CRPC xenograft models.

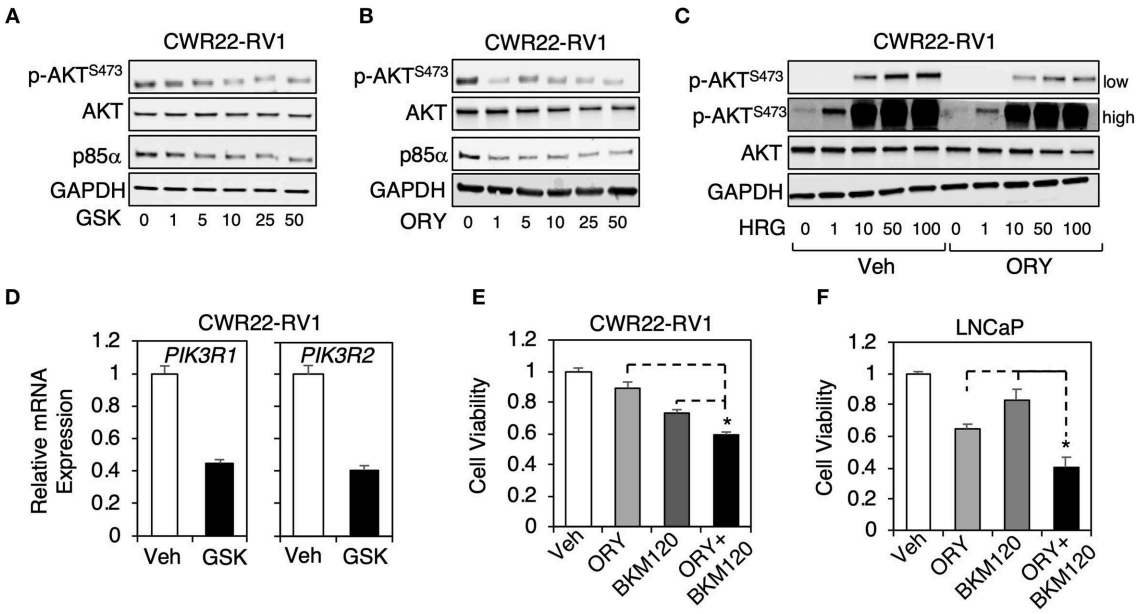

FIGURE 3 | The combination treatment of a PI3-kinase inhibitor with a LSD1 inhibitor more effectively suppressed PCa cell proliferation **(A,B)** Immunoblotting for indicated proteins in CWR22-RV1 cells treated with 0–50 μM **(A)** GSK2879552 or **(B)** ORY1001 for 48 h. **(C)** Immunoblotting for indicated proteins in CWR22-RV1 cells are pre-treated with 10 μM ORY1001 for 48 h and then stimulated by 0–100 ng/ml heregulin-β1 for 10 min. **(D)** qRT-PCR for PIK3R1 or PIK3R2 in CWR22-RV1 cells treated with/out 25 μM GSK2879552. **(E,F)** Luminescence based cell viability assay was performed in **(E)** CWR22-RV1 cells (hormone-depleted serum) or **(F)** LNCaP cells (full serum) treated with vehicle, ORY-1001 (50 μM) alone, BKM120 (1 μM) alone, or the combination of ORY-1001 and BKM120 for 3 days.

LuCaP35CR is a previously described patient-derived xenograft (PDX) model that is *TMPRSS2-ERG* positive and *PTEN*-negative, and resistant to androgen deprivation treatment (33, 34). Our study using this model indicates that the tumor growth was inhibited by 3-week GSK2879552 treatment (**Figure 5A**). Therefore, we examined how this treatment affects PI3K/AKT signaling. As seen in **Figure 5B**, GSK2879552 treatment significantly decreased AKT phosphorylation, suggesting that the impairment of PI3K/AKT signaling may be one mechanism contributing to the tumor regression effect by LSD1 inhibition. Consistently, *PIK3R1* expression was decreased by the LSD1 inhibitor treatment although it was not statistically significant due to the variations in samples (**Figure 5C**). Overall, these *in vitro* and *in vivo* studies demonstrated that LSD1 inhibition represses AKT signaling in CRPC cells with different genetic background.

DISCUSSION

Multiple studies have demonstrated LSD1 as a critical AR coregulator through its activator function, which is independent to its H3K4 demethylase activity (5, 9). This provides a strong molecular basis to treat PCa tumor with LSD1 inhibitors and we are currently testing LSD1 inhibitor treatments in preclinical models of CRPC. However, whether the tumor response to the inhibitors is solely dependent on suppressing AR signaling remains unclear. In this study, we show that LSD1 inhibitors markedly suppressed another major cancer-promoting pathway, PI3K/AKT signaling, in androgen-dependent PCa and CRPC cells, a consistent finding with a study using LSD1 inhibitor S2101 in an ovarian cancer cell line (35). We further demonstrated that this function of LSD1 is, at least in part, due to the transcriptional activation of the regulatory subunit of PI3-kinase, p85α. Interestingly, this activation function of LSD1 appears to be indirect as we observed decreased H3K4me2 by the LSD1 inhibitor treatment at an enhancer of *PIK3R1* (*PIK3R1*-enh), which was not occupied by LSD1 (see **Figure 2**). One hypothesis is that the distal LSD1 binding site (*PIK3R1*-LBS) can communicate with and subsequently activate *PIK3R1*-enh through chromatin looping and H3K4me-independent activity of LSD1, such as demethylating H3K9 or non-histone proteins or acting as a critical scaffold protein (5, 6, 9, 16, 22, 36). Another hypothesis is that LSD1 can induce an epigenetic reprogramming (29, 37, 38) that would reshape the enhancer landscape in PCa cells. Therefore, this decreased level of H3K4me2 at *PIK3R1*-enh by LSD1 inhibition might be a result of such epigenetic reprogramming. Nonetheless, it remains unclear how LSD1 performs such activation function on the *PIK3R1* enhancer and this unidentified mechanism clearly needs to be determined in the future studies.

Recent studies have indicated that AR signaling and PI3K/AKT signaling are reciprocally regulated by each other in PCa cells (19). Therefore, castration or the standard AR antagonist treatments, such as bicalutamide and enzalutamide (39), commonly result in the unwanted activation of PI3K/AKT signaling (also see **Figure 1D**), which may lead to anti-apoptotic activity in PCa cells and thus render tumor cells resistant to

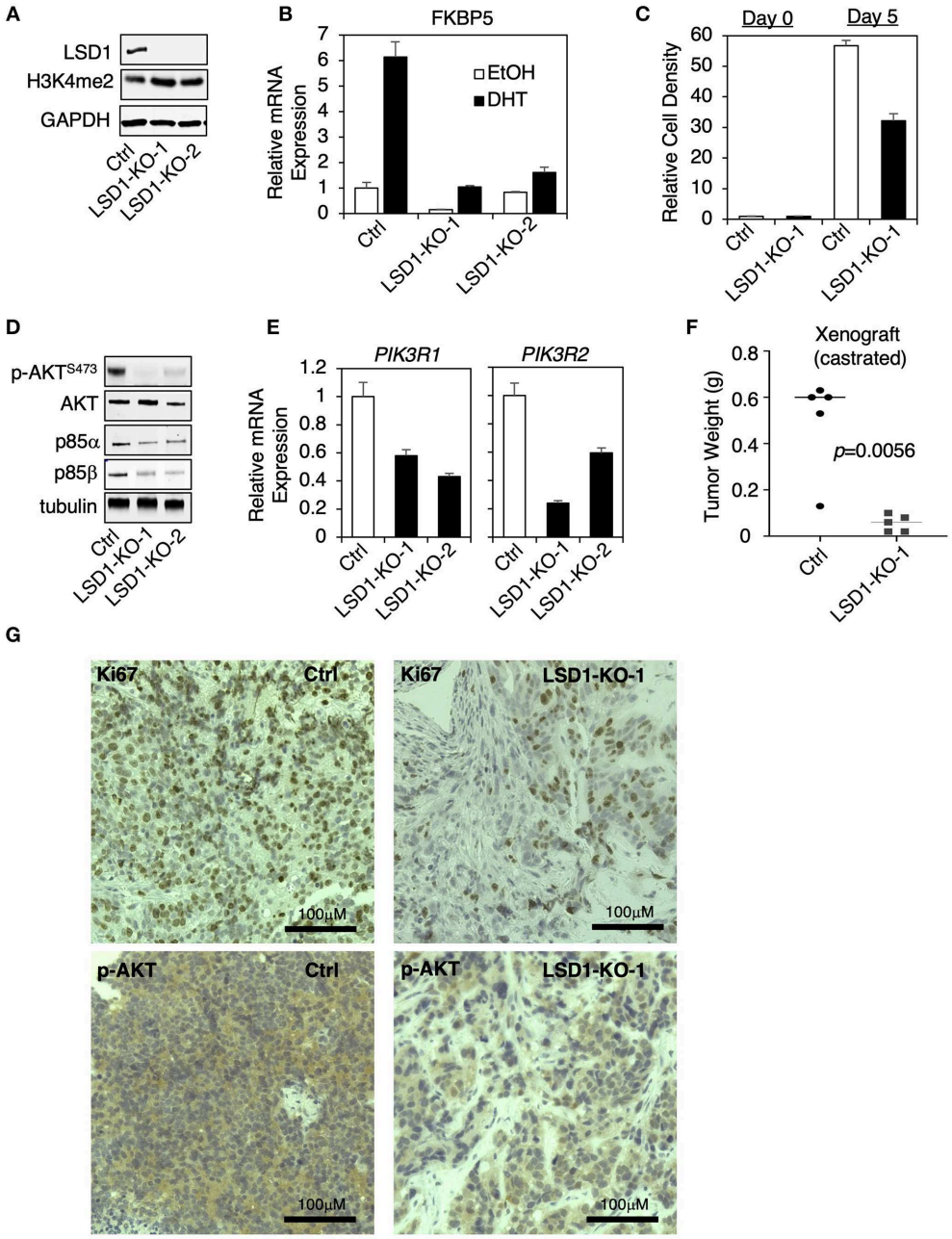

FIGURE 4 | LSD1 knockout suppressed PI3K/AKT signaling in CRPC cells **(A–E)** LSD1 knockout lines (using CRISPR/Cas9 approach) were established in CWR22-RV1 cell. **(A)** LSD1 silencing was confirmed in two knockout clones (LSD1-KO-1 and LSD1-KO-2) compared with parental control line (Ctrl). The following experiments were then performed: **(B)** qRT-PCR for FKBP5 in cells treated with/out 10 nM DHT for 12 h; **(C)** cell viability was measured under the indicated conditions; **(D)** immunoblotting for indicated proteins; and **(E)** qRT-PCR for PIK3R1 and PIK3R2. **(F)** Male SCID mice ($n = 5$) were subcutaneously injected with control line (left flank) and LSD1-KO-1 line (right flank). The development of xenograft tumors was monitored for over 6 weeks and the mice were sacrificed when the tumors at any side reached the size limit. Tumor weight was measured, and tumor biopsies were collected. **(G)** IHC staining of Ki67 and p-AKT^{S473} in tumor samples from control line vs. LSD1-KO-1 line was shown.

the therapies. In contrast, the LSD1 inhibitor treatments have dual functions on inhibiting both AR signaling and PI3K/AKT pathways, which provides an advantage to androgen deprivation treatments. In particular, PTEN deficient or PI3K activating mutations are commonly found in primary PCa and CRPC (40, 41) and this subset of PCa have been adapted to the overactivation of PI3K/AKT signaling. Therefore, the LSD1 inhibitor treatments might be more effective to treat this subset of tumors as they target both AR and PI3K/AKT signaling. In addition, since LSD1 regulates p85 gene expression, the combined treatment of PI3K inhibitors which target PI3K enzymatic activity and LSD1 inhibitors may achieve synergistic

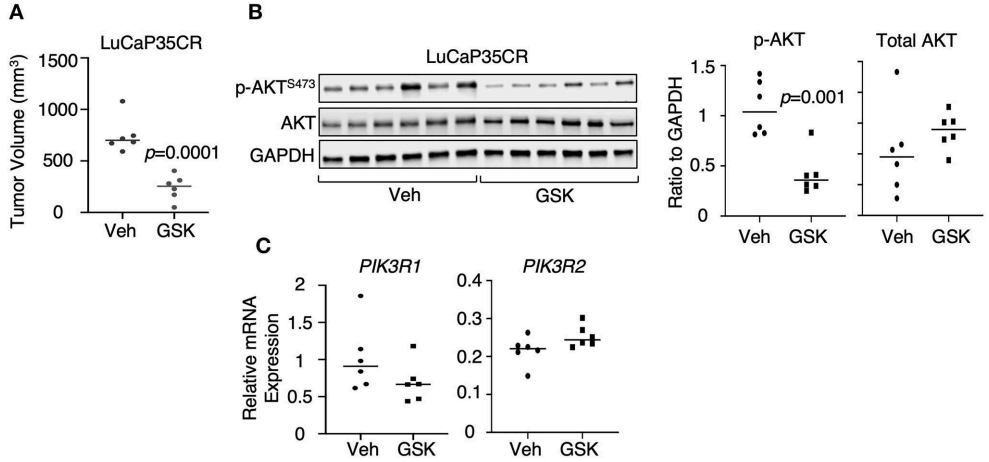

FIGURE 5 | PI3K/AKT signaling was repressed by LSD1 inhibition in a CRPC PDX model **(A)** Castrated SCID male mice bearing LuCaP35CR xenograft tumors (at ~5–7 mm) received daily DMSO ($n = 6$) or GSK2879552 (33 mg/kg) ($n = 6$) via i.p injection for 19 days. The mice were then scarified, and tumor biopsies were collected. The tumor volume at 19 day of treatment was shown. **(B)** p-AKT^{S473} and total AKT were examined by immunoblotting and quantified by ImageJ in tumor tissue samples. **(C)** qRT-PCR for PIK3R1 and PIK3R2 in extracted RNA samples from tumor biopsies.

effect in treating PCa patients and such treatments need to be further tested in the pre-clinical animal models of CRPC. Overall, this study provides novel insights on identifying the downstream effectors of LSD1 in PCa cells and the study will have a strong translational impact as it indicates that the LSD1 inhibitor treatment may be effective in delaying the progression of CRPC as it targets both AR signaling and PI3K/AKT signaling pathways.

DATA AVAILABILITY

The RNA-seq data used for this study can be found in GSE114268.

AUTHOR CONTRIBUTIONS

CC, SG, and ZW designed the study and wrote the manuscript. ZW, SG, DH, ML, and WH performed experiments and analyzed the results. All authors discussed the results and commented on the manuscript.

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

Qin Feng[1]* *and Bin He*[2,3]*

[1] Department of Biology and Biochemistry, Center for Nuclear Receptors and Cell Signaling, University of Houston, Houston, TX, United States, [2] Departments of Surgery and Urology, Immunobiology & Transplant Science Center, Houston Methodist Cancer Center, Houston Methodist Research Institute, Houston Methodist Hospital, Houston, TX, United States, [3] Department of Medicine-Cancer Biology, Weill Cornell Medicine, Cornell University, New York, NY, United States

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

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Joaquin Mateo, Vall d'Hebron Institute of Oncology (VHIO), Spain
Devin B. Lowe, Texas Tech University Health Sciences Center, United States

***Correspondence:**
Qin Feng
qfeng4@central.uh.edu
Bin He
bhe@houstonmethodist.org

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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 5α-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 NH_2-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 NH_2-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 NH_2-terminus and LBD (9). Moreover, the androgen receptor can also recruit an AR-specific MAGE-A11 coactivator through its extended NH_2-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).

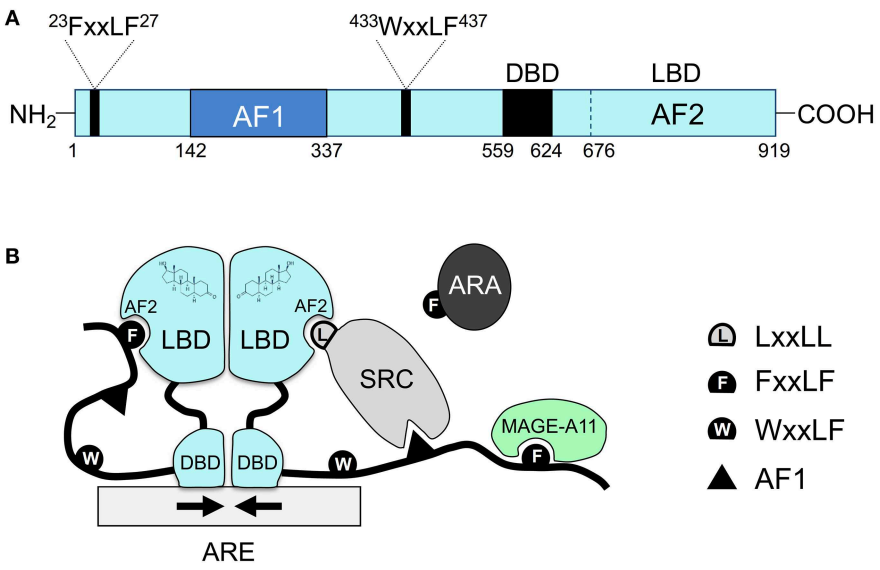

FIGURE 1 | The unique molecular features of the androgen receptor and its coregulator recruitment. **(A)** The primary sequence of the androgen receptor contains several functional domains: NH_2-terminal Activation Function 1 (AF1), the central DBD, the carboxyl-terminal LBD, and two AR-specific FxxLF and WxxLF motifs. **(B)** Schematic diagram of homodimeric androgen receptor bound to a palindromic androgen response element (ARE). Dimerization of the androgen receptor is mediated by both DBD and LBD. Shown in the diagram are FxxLF motif-mediated N/C interaction, recruitment of the SRC/p160 by AF1 and AF2, recruitment of FxxLF motif-containing ARA proteins by AF2, and recruitment of MAGE-A11 through the AR NH_2-terminal extended FxxLF motif. Competition likely exists among different FxxLF, WxxLF, and LxxLF motifs for binding to the same AF2 site on AR LBD (1). SRC, steroid receptor coactivator; ARA, AR-associated protein; AF1, activation function 1; AF2, activation function 2, a hydrophobic cleft in the LBD; ARE, androgen response element; DBD, DNA binding domain; LBD, ligand binding domain.

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 Ki of ∼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 NH_2-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,

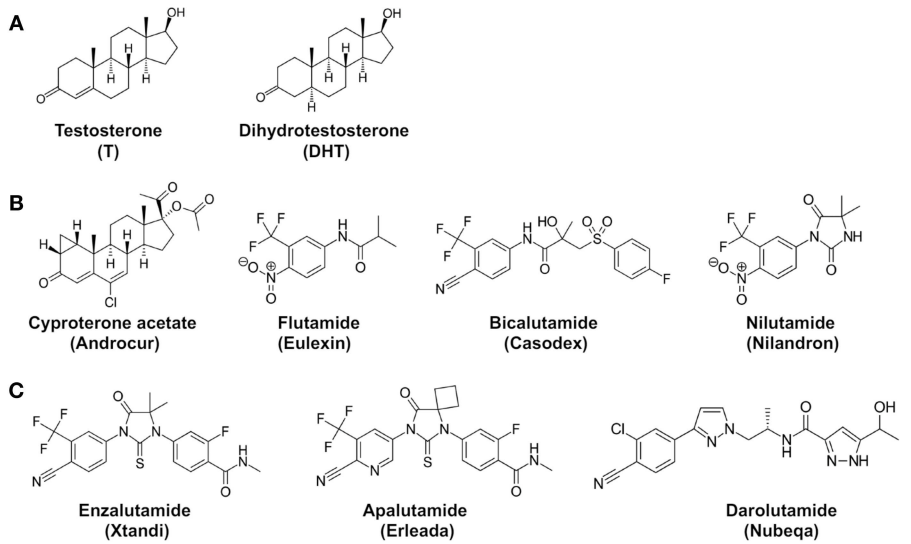

FIGURE 2 | Chemical structures of androgens and antiandrogens. **(A)** Two main androgens, testosterone (T), and dihydrotestosterone (DHT). **(B)** First-generation antiandrogens, cyproterone acetate (Androcur), Flutamide (Eulexin), Bicalutamide (Casodex), Nilutamide (Nilandron). 2-hydroxyflutamide is 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 RORγ in metastatic castration-resistant prostate cancer (101). RORγ 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 RORγ 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

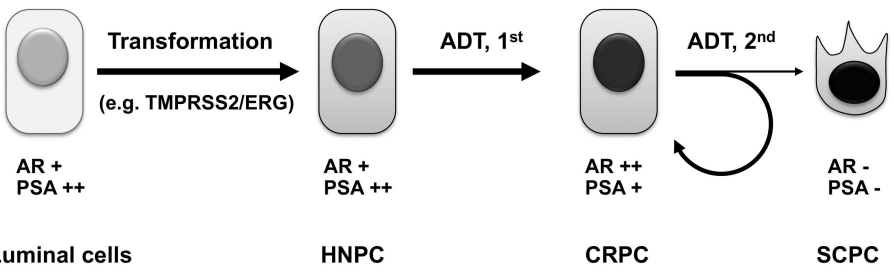

FIGURE 3 | Evolution of prostate cancer under androgen deprivation therapy. Androgen sensitive primary prostate cancers arise from prostate luminal epithelial cells, which have undergone genetic alterations, such as mutation of PTEN tumor suppressor (107) or chromosomal rearrangement resulting in the TMPRSS2/ERG chimeric gene (108). Upon androgen deprivation including castration and the first-generation antiandrogen treatment, most HNPC will develop into CRPC, whose survival and growth still depends on androgen receptor signaling. After treatment with more potent androgen deprivation therapies such as second-generation antiandrogens, the majority of CRPC manages to develop novel mechanisms to maintain active androgen signaling axis to confer resistance, whereas a subset of CRPC will irreversibly lose androgen receptor expression, undergo divergent clonal evolution or de-differentiation, and become truly androgen-independent small-cell prostate cancer. ADT, androgen deprivation therapy; HNPC, hormone-naïve prostate cancer; CRPC, castration-resistant prostate cancer; SCPC, small-cell prostate cancer.

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.

AUTHOR CONTRIBUTIONS

QF and BH conceived and wrote the manuscript.

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Genomic Alteration Burden in Advanced Prostate Cancer and Therapeutic Implications

Matthew J. Ryan[1,2,3] *and Rohit Bose*[2,3,4,5,6]*

[1] Medical Scientist Training Program, University of California, San Francisco, San Francisco, CA, United States, [2] Biomedical Sciences Graduate Program, University of California, San Francisco, San Francisco, CA, United States, [3] Department of Anatomy, University of California, San Francisco, San Francisco, CA, United States, [4] Departments of Medicine and Urology, University of California, San Francisco, San Francisco, CA, United States, [5] UCSF Helen Diller Family Comprehensive Cancer Center, San Francisco, CA, United States, [6] UCSF Benioff Initiative for Prostate Cancer Research, San Francisco, CA, United States

The increasing number of patients with sequenced prostate cancer genomes enables us to study not only individual oncogenic mutations, but also capture the global burden of genomic alterations. Here we review the extent of tumor genome mutations and chromosomal structural variants in various clinical states of prostate cancer, and the related prognostic information. Next, we discuss the underlying mutational processes that give rise to these various alterations, and their relationship to the various molecular subtypes of prostate cancer. Finally, we examine the relationships between the tumor mutation burden of castration-resistant prostate cancer, DNA repair defects, and response to immune checkpoint inhibitor therapy.

Keywords: prostate cancer, tumor mutation burden, copy number alteration, structural variants, aneuploidy, mismatch repair deficiency, immune checkpoint inhibitor

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***Correspondence:**
Rohit Bose
rohit.bose@ucsf.edu

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INTRODUCTION

Prostate cancer is the second-most common cancer in men worldwide (1), and advanced forms of the disease cause debilitating bone pain, pathologic fractures, and severe anemia. The era of profiling patients' tumors using next generation sequencing (NGS) has yielded both scientific and clinical advances including: the comprehensive detection of *BRCA* mutations for PARP inhibitor therapy; the identification of poorer prognosis *RB1* mutations; and the full extent of *AR* genomic alterations associated with androgen receptor signaling inhibitor (ARSI) resistance (2–6). Equally important, NGS profiling has expanded our insight beyond a handful of known loci to capture our first snapshots of the prostate tumor genome in its entirety. This raises the question: does the global burden of mutations and chromosomal structural variants reveal information beyond individual driver mutation analysis?

Here we examine the genomic alteration burden in various states of prostate cancer, a disease with a heterogenous clinical course. Next, we delve into the various mutational processes underlying those alterations and highlight associations with molecular subtypes. Finally, we evaluate how a tumor's mutation burden may help predict response to certain therapies. There are several caveats: factors beyond the tumor genome, such as the transcriptome, epigenome, and the microenvironment are undoubtedly relevant, but beyond the scope of this mini review. Secondly, the analyzed cohorts are predominantly comprised of patients of European ancestry. Finally, this review of global genomic alterations is simply designed to augment, not supersede, the relevance of individual mutations and traditional clinical parameters.

BURDEN OF GENOMIC ALTERATIONS IN DIFFERENT CLINICAL STATES

Tumor mutation burden (TMB) (7) is measured differently among various prostate cancer cohorts. Sometimes, it is reported as the load of non-synonymous mutations (NS) with a minimum allele frequency of 0.5–10%. Other times, it is reported as the load of any single nucleotide variants (SNVs). Some studies additionally report the rate of indels (8, 9). The TMB of unselected and usually treatment-naïve locoregional prostate adenocarcinoma cohorts typically falls between 0.94 and 1.74 NS per megabase (Mb) (**Table 1**). Average TMB appears to correlate with the patient's age at diagnosis (∼0.5 NS/Mb for those diagnosed in their 40s vs. ∼0.9 NS/Mb in their 60s) (12). Primary tumor grade is a major clinical feature and described by the Gleason score (currently being updated to the Grade Group system) (33). The SNV burden has been reported as 1.5× higher in intermediate pattern Gleason 7 tumors vs. well-differentiated pattern Gleason 6 tumors ($p = 1.05 \times 10^{-3}$) (16), consistent with other reports (12). Interestingly, a small cohort of South African patients of African ancestry with high-risk locoregional disease were found to have a roughly 4-fold increase of TMB (3.0–4.7 SNVs plus indels/Mb) (**Table 1**) compared with a control cohort of European ancestry (23). On the other hand, a study of African-American men with primary prostate cancer had a rate of 0.83 SNVs/Mb, in line with cohorts of predominantly European-Americans (17).

Prostate cancer that presents as *de novo* metastases, or reappears as macro-metastases following definitive prostatectomy/radiotherapy, is termed metastatic castration-sensitive prostate cancer (mCSPC) (34–36). Just as the pattern of individual mutations is similar between locoregional disease and mCSPC, so is the mean TMB (1.74 vs. 2.08 NS/Mb) (13). Likewise, a separate study showed that patients presenting with markedly elevated PSAs (≥15) and a biopsied MRI-positive primary lesion had no significant TMB difference compared to those found to have mCSPC disease (20). However, as the disease advances beyond mCSPC, so too does the TMB. Metastatic castration-resistant prostate cancer (mCRPC) can no longer be controlled with androgen ablation and is the most morbid and lethal clinical state. Several groups have noted that the TMB of mCRPC is accordingly increased (4.02 vs. 2.08 NS/Mb in mCSPC in one study) (**Table 1**) (13, 18, 27, 29, 31).

However, analyzing prostate tumor genomes solely via TMB misses many alterations, since the disease has a higher burden than many other cancers of chromosomal structural variants including insertions, deletions, inversions, translocations, gene-fusions, and tandem duplications (14, 37, 38). Locoregional prostate cancer cohorts have a highly variable structural variant burden, with a median of 19 structural variants per genome (range between 0 and 499, **Table 2**) (16). Like TMB, the structural variant burden correlates with Gleason score (17 in Gleason 6 disease compared to 22 in Gleason 7, $p < 0.001$) (**Table 2**) (16). The mCRPC cohorts have a much higher structural variant burden than in locoregional disease; median lies between 230 and 337 per study (**Table 2**), keeping in mind structural variant measurement is not standardized (9, 28, 29).

At the chromosomal level, mCRPC genomes frequently demonstrate polyploidy and/or aneuploidy. There are several NGS studies confirming that roughly ≥40% of mCRPC samples are triploid or more (9, 27, 43), a status itself associated with more translocations and SNVs (9). Regarding aneuploidy, about 75% of locoregional prostate cancer genomes have chromosomal arm-level alterations, and 23% possessed ≥5 arm-level alterations (44). As with TMB and structural variants, the degree of arm-level alterations correlates with Gleason score: only ∼3% of Gleason 6 tumors have ≥5 arm-level alterations compared to ∼40% of the very poorly differentiated Gleason 9–10 tumors. Even after adjusting for Gleason score, the degree of tumor aneuploidy predicted future lethal prostate cancer risk with a median follow-up of 15 years: patients with ≥5 arm-level alterations had a odds ratio for lethality of 5.34 (95% CI 2.18–13.1) compared with those with no aneuploidy (44).

The majority of NGS-based clinical testing involves targeted panels, rather than whole genome sequencing (WGS), making direct detection of some structural variants challenging. However, copy number alterations (CNA) of individual genes and genomic regions can be robustly detected, and they are an indirect measure of unbalanced structural variants and aneuploidy. The tumor CNA burden (TCB) is reported as the fraction of the measured genome with broad CNA. The median TCB of locoregional disease is ∼7% of the genome altered (12, 13, 45). TCB differs statistically with age at diagnosis and Gleason score in a similar way to TMB: those diagnosed in their 40s have a median TCB of ∼2% genome altered whereas those diagnosed in their 60s have ∼9% altered (12). Gleason 6 tumors have median TCBs of ∼1% genome altered, whereas Gleason ≥8 tumors have ∼13% altered (12), consistent with other reports (46). The TCB of tumors confers considerable prognostic information (43, 45, 47, 48): it is significantly associated with biochemical recurrence (each 1% increase in TCB was associated with a 5–8% decrease in 5-years relapse-free survival) and future metastasis (45). This was independently verified (43), even after adjustment for Gleason score and TMB (47). TCB was also found to be associated with prostate cancer-specific death after adjustment for clinical parameters, such as CAPRA (CAncer of the Prostate Risk Assessment) score (49) or Gleason score (per 5% TCB, HR 1.49; 95% 1.30–1.70) (47). Unlike TMB, median TCB of mCSPC tumors is higher than locoregional disease (20–30% genome altered) and even higher in mCRPC tumors (cohort medians between 23 and ∼38%) (9, 12, 13, 20, 24). TCB is negatively associated with overall survival in metastatic tumors in multivariate analysis even after adjustment for TMB (per 5% TCB, HR = 1.08; 95% CI 1.02–1.15) (47).

There is a subset of prostate cancer that emerges clinically in the treated mCRPC state, whereby the dominant metastatic histology is now either small-cell carcinoma or possesses neuroendocrine features. This treatment-emergent small-cell/neuroendocrine prostate cancer (t-SCNC) (30, 50) has both characteristic molecular and aggressive clinical features: there is an enrichment for RB1/TP53 genomic alterations and rapidly progressive visceral metastases. In one analysis, there were no statistically significant differences between TMB, TCB, or ploidy between t-SCNC vs. mCRPC adenocarcinoma (30).

TABLE 1 | Tumor mutation burden (TMB) in locoregional, metastatic castration-sensitive (mCSPC), and metastatic castration-resistant (mCRPC) prostate cancer samples.

Clinical state	TMB (mutations per Mb or sample)	Method of sequencing	Algorithm for somatic mutation calling	Cohort (number of samples)[a]	References
Locoregional	0.94 NS/Mb[b]	WES	MuTect (10)	TCGA, *Cell* 2015 ($n = 333$)	(11)
	1.36 NS/Mb[c]	WES	MuTect (10)	MSKCC/DFCI, *Nature Genetics* 2018 ($n = 1013$)	(12)
	1.74 NS/Mb[c]	Gene panel (MSK-IMPACT)[d]	MuTect (10)	MSKCC, *JCO Precis Oncol* 2017 ($n = 504$)	(13)
	33 NS/sample[c,e]	WGS	MuTect (10)	Broad/Cornell, *Cell* 2013 ($n = 57$)	(14)
	0.53 SNVs/Mb[b]	WGS	SomaticSniper (15)	CPC-GENE, *Nature* 2017 ($n = 477$)	(16)
	0.83 SNVs/Mb[b]	WES	MuTect (10)	Cornell/Karmanos, *Cancer Discov* 2017 ($n = 102$)	(17)
	0.93 SNVs/Mb[c]	WES	Used own method	MCTP, *Nature* 2012 ($n = 61$)	(18)
	0.93 SNVs/Mb[b]	WES	VarScan (19)	PROGENY Study, *Ann Oncol* 2017 ($n = 49$)	(20)
	1.4 SNVs/Mb[b]	WES	MuTect (10)	Broad/Cornell, *Nat Genet* 2012 ($n = 112$)	(21)
	3.0–4.7 SNVs plus indels/Mb	WGS	MuTect, Strelka, VarScan (10, 19, 22)	SAPCS, *Cancer Res* 2018 ($n = 15$)	(23)
mCSPC	2.08 NS/Mb[c]	Gene panel (MSK-IMPACT)[e]	MuTect (10)	MSKCC, *JCO Precis Oncol* 2017 ($n = 504$)	(13)
mCRPC	4.02 NS/Mb[c]	Gene panel (MSK-IMPACT)[e]	MuTect (10)	MSKCC, *JCO Precis Oncol* 2017 ($n = 504$)	(13)
	4.1 NS/Mb[b]	WGS	MuTect, Strelka (10, 22)	SU2C/PCF Dream Team, *Cell* 2018 ($n = 101$)	(9)
	44 NS/sample[c,e]	WES	Used Own Method	Fred Hutchinson CRC, *Nat Med* 2016 ($n = 176$)	(24)
	2.00 SNVs/Mb[c]	WES	Used Own Method	MCTP, *Nature* 2012 ($n = 61$)	(18)
	2.3 SNVs/Mb[b,d]	WGS	Freebayes, Pindel (25, 26)	UMichigan, *Cell* 2018 ($n = 360$)	(27)
	3.6 SNVs/Mb[c]	WGS	MuTect (10)	MSKCC/DFCI, SU2C/PCF Dream Team, *Cell* 2018 ($n = 23$)	(28)
	4.4 SNVs/Mb[c]	WES	MuTect (10)	SU2C/PCF Dream Team, *Cell* 2015 ($n = 150$)	(29)
	41 SNVs/sample[b,e,f]	WES	MuTect (10)	Multi-Institute, *Nat Med* 2016 ($n = 114$)	(30)
	98 SNVs/sample[c,e]	WGS	CaVEMan (31)	PELICAN Study, *Nature* 2015 ($n = 10$)	(31)

[a] *TCGA, The Cancer Genome Atlas; MSKCC, Memorial Sloan Kettering Cancer Center; DFCI, Dana-Farber Cancer Institute; PROGENY, PROstate cancer GENomic heterogeneitY; CPC-GENE, Canadian Prostate Cancer Genome Network; SAPCS, Southern African Prostate Cancer Study; MCTP, Medicaid Cancer Treatment Program; SU2C, Stand Up to Cancer; PCF, Prostate Cancer Foundation; CRC, Cancer Research Center; PELICAN, Project to ELIminate lethal CANcer.*

[b] *These are median values as reported.*

[c] *These are mean values as reported.*

[d] *Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) is a targeted panel (32).*

[e] *The human genome is ∼3 Gb. The exome is about 1% of the genome, or ∼30 Mb.*

[f] *Many samples in this cohort are neuroendocrine prostate cancer, rather than prostate adenocarcinoma.*

This is consistent with the postulation that neuroendocrine transdifferentiation may be driven substantially by epigenetic mechanisms (51).

Longitudinal analysis of prostate tumor genomes reveals further complexity in interpreting TMB and TCB, since mutational processes are dynamic, interrelated, can arise in a multi-focal setting, and evolve with different degrees of clonality (13, 31, 46, 52). One study reports that 40% of primary prostate tumors appear to be monoclonal i.e., one dominant clone is detected (46). The remaining 60% of primary tumors demonstrate subclonal populations (78% biclonal, 20% triclonal) originating from an ancestral clone. These polyclonal tumors have inferior clinical outcomes with respect to biochemical relapse following definitive prostatectomy/radiotherapy (HR 2.64; CI 1.36–5.15), and persisting after adjustment for standard clinical parameters and TCB (46). The polyclonality-related

TABLE 2 | Structural variant burden (SVB) in locoregional and mCRPC samples.

Clinical state	Structural variant burden (SVs per sample)	Method of determining SVs	Cohort[a]	References
Locoregional	19 SVs/sample[b,c]	Delly v0.5.5 (39)	CPC-GENE, *Nature* 2017 ($n = 477$)	(16)
mCRPC	230 SVs/sample[c,d]	SvABA (40); GROC-SVS (41); Long Ranger v2.1.2 (https://support.10xgenomics.com/genome-exome/software/pipelines/latest/using/wgs)	MSKCC/DFCI, SU2C/PCF Dream Team, *Cell* 2018 ($n = 23$)	(28)
	337 SVs/sample[b,c]	Manta v1.1.1 (42)	SU2C/PCF Dream Team, *Cell* 2018 ($n = 101$)	(9)

[a] *CPC-GENE, Canadian Prostate Cancer Genome Network; MSKCC, Memorial Sloan Kettering Cancer Center; DFCI, Dana-Farber Cancer Institute; SU2C, Stand Up to Cancer; PCF, Prostate Cancer Foundation.*
[b] *These are median values as reported.*
[c] *The human genome is ∼3 Gb, of which the exome is about 1%, or ∼30 Mb.*
[d] *These are mean values as reported.*

risk appears to be additive to those derived from the combination of TMB and TCB. Interestingly, triclonal tumors have a higher median PSA level at diagnosis (9.7 vs. ∼7 for monoclonal/biclonal; $p < 0.01$), and polyclonal primary tumors are also more likely to develop metastases later on (OR = 4.01; $p < 0.05$). In polyclonal primary tumors, most of the TMB is truncal (median 87% of total SNVs) whereas the TCB is more evenly distributed between being truncal (55%) vs. branch-specific (45%). Moreover, the individual truncal CNAs are larger (median 11.5 vs. 6.5 Mb for branch-specific CNAs) and biased toward deletions (84% of all deletions are truncal). CNAs are also observed at chromosome ends, and the median telomere length of polyclonal tumors is 500 bp shorter than monoclonal tumors (46).

Altogether, the burden of genomic alterations correlates with key clinical information for prostate cancer patients. The TMB, structural variants, and TCB all tend to increase with advancing clinical state, Gleason score, and age. However, clonality analysis hints that how these mutational processes combine during tumor evolution is quite complex.

MUTATIONAL PROCESSES UNDERLYING PROSTATE CANCER GENOMIC ALTERATIONS

Next, we examine the biologic processes that generate these genomic alterations, starting with SNVs. Comparison of tumor-derived patterns of SNVs within their trinucleotide context to pre-defined signatures (53) can suggest the underlying etiology; for example, cancers with known exogenous risk factors reveal robust signatures associated with tobacco or UV exposure. However, the majority of mCRPC tumors with intact DNA repair pathways reveal a robust signature that is endogenous, age-related and likely results from deamination of 5-methylcytosine to thymine at mCpG dinucleotides (COSMIC signature 1) (12, 54). If not repaired before DNA replication, this results in a permanent C > T transition. This signature is contributory in most cancer types and the frequency of associated SNVs correlates with the age at diagnosis in pan-cancer analysis, although not necessarily meeting statistical significance when analyzing each tissue individually (54). Nevertheless, the rate of prostate cancer SNVs attributed to this age-related signature loosely fits a slope of ∼6 SNVs/Gb/year and may contribute to the increased TMB of patients diagnosed at older ages. Clonality analysis reveals that this age-related signature is most dominant early in prostate tumor evolution (46).

In prostate tumors possessing DNA repair defects, SNVs are associated with different dominant signatures. A recent NGS analysis of one cohort revealed 3% of genomes possess somatic DNA mismatch repair defects (MMRD) (55) caused by loss-of-function mutations in the canonical genes *MLH1*, *MSH2*, *MSH6*, or *PMS2*, and consistent with other cohorts (56–58). If one of the allelic mutations is germline, the patient has Lynch syndrome and possesses increased lifetime risk for several cancer types including prostate cancer (59, 60). These tumor genomes are 10- to 100-fold less likely to repair base pair substitutions prior to DNA replication, and their TMB is elevated (20–80 SNVs/Mb in mCRPC) although not necessarily as high as other MMRD cancer types (29). Analysis as above reveals dominant SNV signatures associated with MMRD, as expected (9, 27, 55, 61). MMRD tumors also possess high rates of indels (9), leading to higher instability of DNA microsatellite lengths, a way in which such tumors can be detected (55). MMRD tumors have distinct genomes from those that are MMR proficient: they are usually diploid, and have the lowest TCB (27). We further discuss MMRD tumors in the next section.

A third class of SNVs is observed in tumors with homologous recombination deficiency (HRD) from 6 to 20% of patients with either somatic or germline alterations of *BRCA1* or *BRCA2*, frequently biallelic (9, 13, 27, 29, 62). Since DNA homologous recombination coordinates the repair of double stranded DNA breaks, HRD not only results in a high TCB, but also a reliance on alternative error-prone DNA repair pathways (63) and a distinct dominant group of SNV signatures (9, 27). Accordingly, *BRCA*-mutant tumors in mCRPC possess the highest SNV rate among MMR proficient tumors (7.0 muts/Mb), in addition to higher TCB (9).

There are other SNV signatures observed to varying degrees in prostate tumors, some of which have not yet been associated with an etiology (27, 53). Moreover, SNVs are not evenly distributed throughout a given tumor's genome, but rather dependent on many interrelated factors, including the underlying mutational process, the timing of the locus within DNA replication, as well as whether the locus affects transcription and/or translation. The phenomenon of localized regions of SNV-based hypermutation is called kataegis, and is found in 23% of primary tumors (16); it is coincident with genomic instability, likely altered DNA repair (64), and enriched for deletions of the chromatin remodeler *CHD1* (33% of kataegis-positive tumors compared with only 11% of kataegis-null tumors) (16). Kataegis is associated with increasing Gleason score, and present in 40% of Gleason 4 + 3 tumors.

Just as specific processes lead to increased TMB, others lead to increased TCB. For example, *BRCA*-mutant tumors have markedly higher frequencies of copy number deletions as well as classic genomic "scars" due to their HRD (9). On the other hand, specifically in HR proficient tumors, chromothripsis can occur: evidence of "shattering" of regions in one or a few chromosomes followed by intrachromosomal reassembly in a stochastic manner, resulting in large numbers of both deletions and inversions (9). It is found in 20% of non-indolent primary prostate cancer samples (16) and 23% of mCRPC samples (9). Although the exact mechanism is unknown, there are some clear correlations: chromothripsis positive genomes are enriched for biallelic *TP53* loss (83% of chromothripsis positive tumors vs. 35% of chromothripsis null tumors), although this event is not likely sufficient to cause chromothripsis (9, 16). Others have noted a correlation between genomic loss of *CHD1* and chromothripsis (14). From a clinical standpoint, chromothripsis is associated with the primary tumor T-stage, but was not found to differ by age or Gleason grade (16).

About 5% of mCRPC cases have a significantly higher number of genomic tandem duplications, and 90% of these genomes have biallelic *CDK12* alterations (27, 28). In such cases there is a median of 150 tandem duplications per sample with a median duplicated region size of 1.3 Mb (28). Accordingly, such tumors possess large numbers of focal CNAs, and also have the highest gene-fusion burden (100 per tumor vs. 25 in other tumors), due directly to the genomic duplication phenomenon (27). *CDK12*-mutant tumors are usually diploid and trend toward mutual exclusivity from HRD biallelic *BRCA*-mutant tumors (9). Clonality analysis reveals that *CDK12* alterations are usually truncal; in these samples, the accompanying SNVs are more likely to occur after tandem duplication than before, and in many cases in branch-specific subclones (28). It is unknown whether *CDK12* alterations directly cause the tandem duplications, or are merely associated with it, but there is evidence to support the former (28).

Finally, some mutational processes occur without directly affecting TMB or TCB. The most common gene fusions in prostate cancer occur between androgen-driven upstream elements of genes like *TMPRSS2*, and oncogenic ETS transcription factors like *ERG*, and are present in up to 50–60% of men of European descent (65). The underlying chromosomal rearrangements that cause such gene fusions are initially balanced, frequently complex and involve multiple chromosomes in a phenomenon termed chromoplexy (14). Some degree of chromoplexy is present in 50–90% prostate tumors (9, 14). Moreover, in tumors possessing ETS gene fusions, the chromoplexy has more than double the number of interchromosomal rearrangements compared to ETS fusion-null tumors (14). There is evidence of successive rounds of chromoplexy occurring, for example initially leading to ETS fusion formation, and then subsequently to inactivation of tumor suppressor genes. It is not known exactly how chromoplexy occurs; there is no enrichment for *TP53* mutations in such tumors, but the process may be related to androgen-related chromatin configuration (9, 14). Notably, the small cohort of South African men possessed lower frequencies of larger genomic rearrangements, such as chromothripsis and chromoplexy, and lower frequencies of ETS gene fusions, than the comparable cohort with European ancestry (23).

In summary, we have just begun to understand the processes that contribute to the burden of prostate cancer genomic alterations. SNVs possess distinct mutational signatures including those associated with aging and DNA repair defects; moreover, many tumor genomes have localized hypermutated regions. Complex chromosomal alterations, such as chromothripsis, tandem duplication, and chromoplexy tend to stratify by specific alterations in *TP53*, *CHD1*, *CDK12*, and *BRCA1/2* and underlie many CNAs and fusion events, such as the canonical *TMPRSS2-ERG* fusion.

PROSTATE TUMOR MUTATION BURDEN, DNA REPAIR DEFECTS, AND THERAPEUTIC RESPONSE

The initial trials of immune checkpoint inhibitors in unselected prostate cancer patients (66–68) demonstrated no global clinical benefit in prostate cancer. Nevertheless, interest in such therapies remained strong, given case reports of impressive and durable responses among individual prostate cancer patients (69, 70). Experiences from other tumor types illuminated patient subtypes that may derive clinical benefit from existing therapies. Efforts to identify a specific predictive biomarker, such as PD-L1 expression have been challenging (71, 72); however, the association with global genomic processes has been clear. Patients with non-small cell lung cancers (73), bladder cancers (74), and melanoma (75–77) that have a high TMB derive increased clinical benefit to immune checkpoint inhibitors compared to those with low TMB. There are markedly different numerical thresholds of what constitutes a high TMB, with the highest quintile within a given histology usually being associated with longer overall survival when treated with immune checkpoint inhibitors (78). Increased non-synonymous mutations and indel frameshifts lead to increased neoepitopes within MHC Class I-loaded peptides and it is hypothesized these serve as neoantigens in the context of immunotherapy (79–84). Ongoing prostate cancer immunotherapy trials are now beginning to incorporate TMB analyses (85).

As described above, the prostate tumors with the highest TMB are those with MMRD. A series of trials treating patients with MMRD tumors with pembrolizumab, regardless of histology, reported a 53% objective radiographic response rate, and a 21% complete response rate (86). This led to the FDA approval of pembrolizumab for any MMRD metastatic/unresectable solid tumors and reinforces the importance of testing such prostate cancer patients for MMRD. In a recent study of mCRPC patients with MMRD tumors and treated with immune checkpoint inhibitors, 55% achieved a PSA response >50%, and 45% of patients had durable clinical benefit (55). A smaller study revealed that three out of four patients with MMRD tumors achieved soft tissue tumor responses upon treatment with immune checkpoint inhibitors (56). In a separate large analysis of mCRPC samples, MMRD tumors were predicted to have median neoantigen burdens of ∼10,000 vs. 1,000 in MMR proficient tumors (27). Approximately 10% of these are further predicted to be "strong binders" of MHC Class I. MMRD prostate tumors were also found to have high degrees of immune infiltration (20, 27), the highest number of T-cell clonotypes, and the highest percent of expanded T-cell clones (27). Other studies have showed a complex relationship between predicted neoantigen load with immune infiltration (20), as well as considerable heterogeneity of tumor T-cell infiltration in MMRD cases (87). The exact mechanism of how predicted neoantigens stimulate a clinically-relevant immunologic response, and how this might inform the next generation of immunotherapies remains an active area of study (77).

Prostate tumors with MMRD may have other unique molecular and biological features, compared to MMR proficient disease. One case series reported an enrichment of MMRD among ductal adenocarcinoma of the prostate, a rare aggressive subtype of prostate adenocarcinoma (about 3% incidence, compared to the common acinar adenocarcinoma) with poor prognosis (56, 57, 88). Ultimately, it is important to understand the natural history of prostate cancer patients with MMRD tumors, particularly prior to any potential treatment with immune checkpoint inhibitors. One study of patients with recurrent disease reports a longer progression free survival following androgen-deprivation therapy when MMRD is detected (median 66 months compared to only 27 months in MMR proficient cases), as well as longer responses to first-line ARSI agents when used (56). On the other hand, among patients with clinically aggressive tumors (56% having metastatic disease at diagnosis), a different retrospective study of clinically aggressive CRPC noted the median overall survival for the MMRD cases was significantly shorter (3.8 years from androgen ablation) than MMR proficient groups (7.0 years), in both univariate and multivariate analysis (87). The studies above cannot be directly compared, but perhaps the biologic context is key to interpreting the clinical relevance of MMRD.

Beyond MMRD, prostate cancers with other DNA damage repair defects are being explored for their responses to immunotherapies. Because *CDK12*-mutant tumors have increased rates of gene fusions, they possess higher predicted neoantigen burdens (median ∼2,000) than other MMR proficient tumors (27). They may also possess high degrees of T cell infiltration and expanded T cell clones. These findings have led to a Phase II trial evaluating the efficiency of combination nivolumab plus ipilimumab in mCRPC patients with *CDK12*-mutant tumors (89). There are also several immune checkpoint inhibitor therapy combinations being explored, such as one in which the second agent, a PARP inhibitor, alters how the genome repairs itself (90). Interestingly, a recent phase Ib/2 study showed some interesting clinical responses to the combination of pembrolizumab and olaparib, despite no BRCA mutations being detected in the biopsies tested (91). Whether this clinical response is due undetected HRD, or whether the PARP inhibitor synergizes with the immune checkpoint inhibitor by altering the presented neoepitopes or an unknown mechanism remains to be determined.

While high TMB, particularly in the context of altered DNA repair, is important regarding successful immune checkpoint therapy in prostate cancer, it is certainly not the whole story. Roughly half of MMRD prostate tumors do not exhibit substantial clinical responses to such therapy despite relatively high TMB (55). Moreover, when clinical responses are observed, the TMB is often lower compared to that observed in other MMRD cancer types e.g., in a preplanned interim analysis of a small phase II mCRPC study of combination nivolumab plus ipilimumab, responses were observed in tumors above a modest TMB threshold (85). Identifying other genomic factors that modify response to immune checkpoint inhibitors and determining whether they map to specific genes and/or global processes, remains an active area of investigation. Due to NGS-based analysis of patients' tumors, we are just beginning to obtain a comprehensive snapshot of the prostate tumor genome in differing clinical states. A deeper understanding whether and how global genomic measures, such as TMB, TCB, gene-fusion burden and clonality affect responses to targeted and immuno-therapies will help us shape future prostate cancer investigations.

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MR and RB drafted, revised, and approved the submitted version.

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MINI REVIEW
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Molecular Links Between Angiogenesis and Neuroendocrine Phenotypes in Prostate Cancer Progression

Zheng Wang[1], *Yicheng Zhao*[1], *Zhiqiang An*[1,2] *and Wenliang Li*[1,2]*

[1] Texas Therapeutics Institute, Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center at Houston (UTHealth), Houston, TX, United States, [2] MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences (GSBS), Houston, TX, United States

As a common therapy for prostate cancer, androgen deprivation therapy (ADT) is effective for the majority of patients. However, prolonged ADT promotes drug resistance and progression to an aggressive variant with reduced androgen receptor signaling, so called neuroendocrine prostate cancer (NEPC). Until present, NEPC is still poorly understood, and lethal with no effective treatments. Elevated expression of neuroendocrine related markers and increased angiogenesis are two prominent phenotypes of NEPC, and both of them are positively associated with cancers progression. However, direct molecular links between the two phenotypes in NEPC and their mechanisms remain largely unclear. Their elucidation should substantially expand our knowledge in NEPC. This knowledge, in turn, would facilitate the development of effective NEPC treatments. We recently showed that a single critical pathway regulates both ADT-enhanced angiogenesis and elevated expression of neuroendocrine markers. This pathway consists of CREB1, EZH2, and TSP1. Here, we seek new insights to identify molecules common to pathways promoting angiogenesis and neuroendocrine phenotypes in prostate cancer. To this end, our focus is to summarize the literature on proteins reported to regulate both neuroendocrine marker expression and angiogenesis as potential molecular links. These proteins, often described in separate biological contexts or diseases, include AURKA and AURKB, CHGA, CREB1, EZH2, FOXA2, GRK3, HIF1, IL-6, MYCN, ONECUT2, p53, RET, and RB1. We also present the current efforts in prostate cancer or other diseases to target some of these proteins, which warrants testing for NEPC, given the urgent unmet need in treating this aggressive variant of prostate cancer.

Keywords: new therapeutics, molecular mechanisms, angiogenesis, neuroendocrine prostate cancer, neuroendocrine phenotype

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Qi Cao,
Northwestern Medicine, United States

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Yajia Zhang,
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Medical University of Gdansk, Poland

***Correspondence:**
Wenliang Li
wenliang.li@uth.tmc.edu

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INTRODUCTION

In the United States, prostate cancer is responsible for the second most cancer death in men, behind lung cancer. It is estimated that about 31,620 deaths in 2019 in USA are caused by prostate cancer (www.cancer.org). Androgen deprivation therapies (ADT) that target the androgen receptor (AR) is the main treatment for prostate cancer (1–4). ADT is effective initially. However, the majority of

tumors invariably relapse and progress, becoming castration resistant prostate cancer (CRPC) (1–4). Frequently associated with ADT resistance is the emergence of neuroendocrine prostate cancers (NEPC) that have a poor prognosis with no effective treatment (5–8). With the common use of new generation potent ADT into clinic, the incidence of NEPC is rising (6, 9–12).

NEPC are highly vascularized (13, 14). Increased angiogenesis and expression of NE markers are two prominent phenotypes of NEPC (13–16) and are expected to be molecularly linked. However, direct molecular connections between these two phenotypes in NEPC remain largely unclear. The main purpose of this review is to summarize the reported and potential connections between the regulation of increased angiogenesis and expression of NE markers. Further, we analyze the implications of these connections for prostate cancer. Our goal is to identify key regulators of both characteristics as potential targets for NEPC, with the hope of hitting two birds with one stone to achieve better therapeutic efficacy and fewer side effects.

NEUROENDOCRINE PHENOTYPE IN PROSTATE CANCER

Approximately 20% of lethal CRPCs have a neuroendocrine (NE) phenotype, and thus are called NEPC or CRPC-NE (5, 17–19). NEPCs often lose AR signaling, become resistant to ADT, and express NE markers, such as neuron-specific enolase 2 (ENO2), synaptophysin (SYP), chromogranin A and B (CHGA and CHGB) (5–8). Features of NEPC include elevated angiogenesis, high proliferative rates, and metastatic propensity (20). Unfortunately, there is no effective therapy against NEPCs. They respond only transiently to chemotherapy (17, 20–24).

Clinical data, including genomic analyses of metastatic tumors, and preclinical studies suggest an evolution of CRPC-NE from a prostate adenocarcinoma precursor (25–27). Researchers are beginning to unfold the signaling events involved in NEPC development (6, 17, 24). General knowledge of NEPC has been elegantly reviewed (19, 20, 24, 28–32). A number of proteins have been reported to contribute to NEPC progression. These proteins include Aurora kinase A and B, BRN2, CREB1, DEK, EZH2, FOXA2, GRK3, HIF1, IL-6, MYCN, ONECUT2, PEG10, p53, REST, RB1, SRRM4, SOX2 et al. (33–44).

ANGIOGENESIS IN PROSTATE CANCER

As a basic physiological process, angiogenesis usually occurs in embryonic development, tissue repair and fertility to form new blood vessels resulted from the extension of pre-existing vasculature. In addition, angiogenesis is also accompanied by chronic inflammation, tumor growth and metastasis (19). Actually, angiogenesis is a dynamic process involves interaction between endothelial cells and their extracellular environment. There are two main types of angiogenesis *in vivo*, including sprouting angiogenesis (sprouting of vascular endothelia from pre-existing capillary endothelia into the surrounding tissues) and non-sprouting angiogenesis (division of pre-existing capillaries by tissue pillars into new daughter vessels) (19, 45–47). The formation of new blood vessels depends on a balanced process that are regulated by many factors (48). Angiogenic activators include angiopoietins, CCL2, EGFL6, endothelins, FGF, HIF1, IGF1, MMPs, PDGF, TGF, VEGF, and et al. (48–56). On the other hand, angiostatin, endostatin, TSP1, and PAI2 are among the endogenous inhibitors of angiogenesis (57–60).

Angiogenesis is involved in prostate cancer survival, progression, and metastasis (61). Its importance in prostate cancer has been established (62, 63). Higher microvessel density is associated with worse prognosis in prostate cancer (64, 65). VEGF as well as some neurosecretory peptides, e.g., serotonin, bombesin, and gastrin, have been shown to boost angiogenesis in NEPC (15). We recently reported that ADT repression of thrombospondin 1 (TSP1 or THBS1), a potent endogenous angiogenesis inhibitor, contributes to angiogenic phenotype in NEPC (66). Several reviews have already described the current knowledge and therapeutic development targeting angiogenesis in prostate cancer (61, 67, 68).

CLINICAL CORRELATION OF NEUROENDOCRINE PHENOTYPE, ANGIOGENESIS AND PROGNOSIS IN PROSTATE CANCER

Several research groups have shown positive correlations between NE marker expression and angiogenesis in prostate tumors. Higher neovascularization and VEGF staining are observed in prostate tumors with more NE tumor cells (16, 69, 70). Grobholz et al. detected NE marker CHGA and angiogenic marker CD34 in 102 prostatectomy prostate tumor specimens. They found that poorer pathological staging correlates with increased neovascularization and stronger NE marker expression (16). Harper et al. found a positive correlation between VEGF and CHGA levels in 45 prostatic carcinoma specimens (67, 70–72). Borre et al. analyzed VEGF and CHGA expression in 221 prostate tumors (62). They found only tumors with strong expression of both VEGF and NE showed significantly poor clinical characteristics such as higher microvessel density, T stage, dedifferentiation, and shorter disease-specific survival.

PROTEINS AND PATHWAYS REGULATING BOTH NE PHENOTYPE AND ANGIOGENESIS

It remains largely unclear whether neuroendocrine differentiation and angiogenesis regulate each other in NEPC. It is also unclear what proteins directly link these two prominent characteristics of NEPC. Our literature search did not yield reports showing direct involvement of pro-angiogenic factors VEGF and neurosecretory peptides (serotonin, bombesin, and gastrin) in promoting NE marker expression. On the other hand, among the NE marker proteins, only CHGA (73, 74) has been shown to directly contribute to angiogenesis. As summarized below and depicted in **Figure 1**, several signaling proteins have

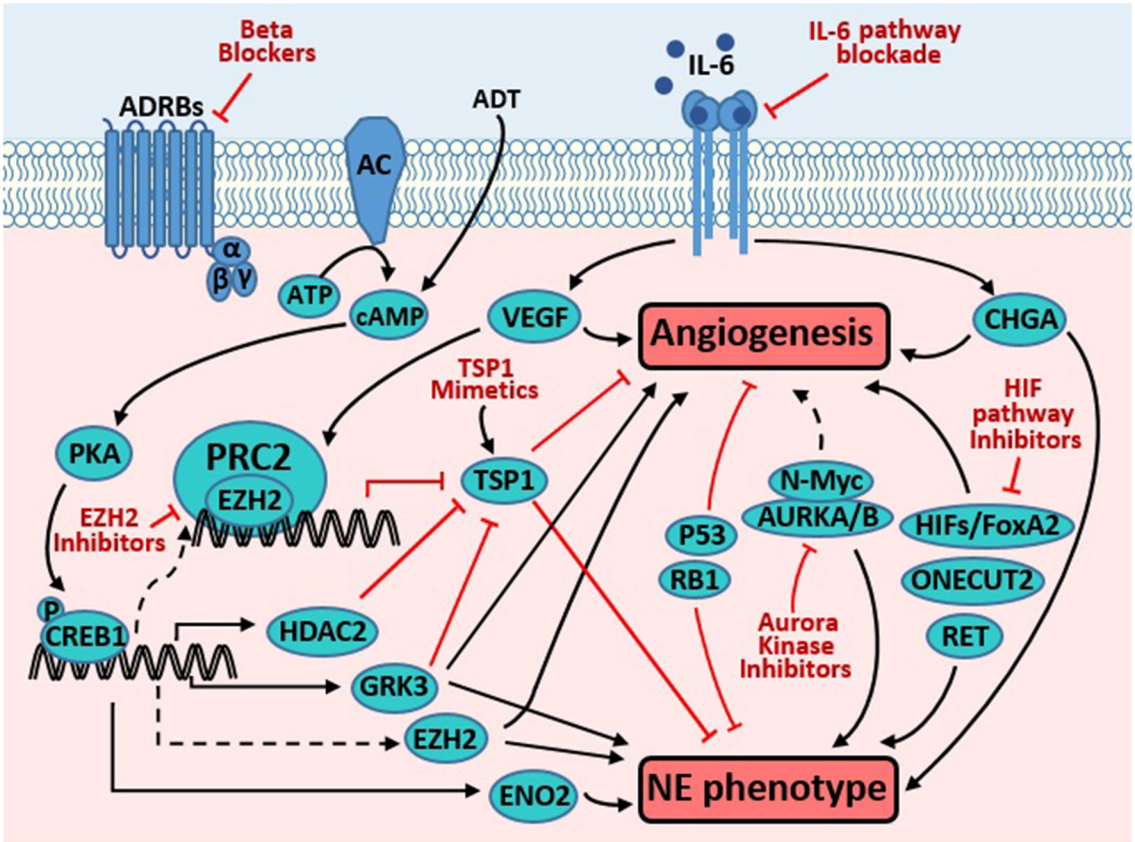

FIGURE 1 | Targeting molecules common to pathways promoting angiogenesis and neuroendocrine phenotype in prostate cancer. Androgen derivation therapy (ADT) elevates cAMP level, which activates PKA, resulting in phosphorylation and activation of CREB1. Activated CREB1 directly induces transcription of several genes involved in neuroendocrine differentiation (NED) and angiogenesis, such as VEGF, ENO2, GRK3, and HDAC2. VEGF is a potent pro-angiogenic factor, while ENO2 is a neuroendocrine marker. GRK3 promotes angiogenesis, NE marker expression, and prostate cancer progression. HDAC2 is critical for prostate cancer progression that is induced by chronical bio-behavioral stress and signals from beta adrenergic receptors (ADRBs). GRK3 and HDAC2 promotes angiogenesis, at least in part through downregulating TSP1. TSP1 is well-established as an anti-angiogenesis factor. Through unclear mechanisms, CREB1 activation enhances the PRC2 function of EZH2, which is critical for NED and angiogenesis induced by ADT. In endothelial cells, VEGF induces EZH2 expression and activity, which contributes to VEGF's action in promoting angiogenesis. Loss of p53 and RB1, alone or in cooperation, promote angiogenesis and NE phenotype through multiple mechanisms (detailed in text). IL-6 pathway activation enhances angiogenesis (through inducing VEGF) and NE phenotype (through inducing CHGA). AURKA interacts with N-Myc and regulates the stability of the latter, which promotes NED. AURKA and AURKB regulate angiogenesis in endothelial and neuroblastoma cells. HIF1A promotes angiogenesis through inducing VEGF. Moreover, it also cooperates with FoxA2 to promote NED and tumorigenesis. ONECUT2 has recently emerged as a master regulator of NED. Recent studies have also implicated receptor tyrosine kinase RET in regulating NED and angiogenesis. Novel strategies targeting the proteins and pathways that regulate both prominent phenotypes may be effective to treat NEPC (detailed in text).

been reported to regulate both angiogenesis and NE marker expression, often in separate diseases or biological contexts. These proteins are potential molecular links between the two important characteristics of NEPC.

CHGA

CHGA is one of the classic markers for NEPC. It is a secreted glycoprotein that shows paradoxical properties in angiogenesis (71, 73–75). Recent studies showed CHGA can be proteolytically cleaved into active peptides by thrombin. This cleavage shifts its function from anti- to pro-angiogenesis under pathophysiologic conditions, which could be observed in prothrombin activation or multiple myeloma (73, 74). Its function in angiogenesis in NEPC is still unclear.

p53 and RB1

p53 and RB1, two most prominent tumor suppressors, have been implicated in both angiogenesis and NE marker expression in separate studies. Mutations and loss of p53 or RB1 are common alterations in prostate cancer patients (76). Tumors containing p53 mutations are usually more vascularized than tumors harboring wild type p53. This pattern has been observed in several independent clinical studies on prostate, colon, and breast cancers (77–80). Some basic mechanisms underlying p53's inhibition of angiogenesis have been detailed. Ravi et al. found that, under hypoxic conditions, p53 inhibits the HIF1A activity

that is required for VEGF transcription (81). Besides VEGF, p53 also inhibits other pro-angiogenetic factors, such as bFGF-BP (bFGF-binding protein) and COX-2 (cyclooxygenase-2). In addition, p53 also induces anti-angiogenetic factors, such as TSP-1 and EPHA2 (ephrin receptor A2) (82). However, it is not clear whether or how p53 itself plays a role in regulating NE phenotype.

RB1 has also been reported to regulate tumor angiogenesis (83–85). For example, Lasorella et al. reported Id2 (inhibitor of differentiation 2), a target of RB1, mediates angiogenesis of pituitary tumors from $Rb1^{+/-}$ mice (86). RB1 loss is one of the most critical events in neuroendocrine carcinoma (12, 87, 88), but the mechanism by which RB1 contributes to NE phenotype is largely unclear. A recent study reported RB1 takes part in regulating both angiogenesis and NE phenotypes. Labrecque et al. found, under hypoxic conditions, RB1-loss deregulates the expression of genes that govern angiogenesis, metastasis and NE differentiation. These effects led to a more invasive phenotype as well as NE protein markers expression in human prostate cancer cells (40).

Growing evidence implies a cooperative function of p53 and RB1 in tumor angiogenesis. Martinez-Cruz et al. found that combinatorial deletion of p53 and RB1 augmented tumor angiogenesis in a spontaneous squamous cell carcinoma mouse model, comparing with loss of p53 alone (89). Similarly, inactivation of p53 and RB1 leads to a pro-angiogenic transcriptional response in keratinocytes (90). In a xenograft model of retinoblastoma, p53 was shown to increase VEGF expression and promote angiogenesis in cells deficient for p21/RB1 pathway (91). All these observations underline the possibility of p53 and RB1 cooperation in promoting prostate cancer angiogenesis.

Interestingly, p53 and RB1 are also both connected to NE marker expression in prostate cancer. In a NEPC xenograft model LTL-331R that relapsed upon castration resistance of prostate adenocarcinoma patient-derived xenograft LTL-331, genomic alterations of both p53 and RB1 were observed (39). Of note, Beltran et al. showed (25) that "concurrent loss of RB1 and p53 was present in 53.3% of NEPC patient tumors vs. 13.7% of CRPC-adenocarcinoma samples ($P < 0.0004$, proportion test)." In a classic NEPC genetically engineered mouse (GEM) model called TRAMP, p53 and RB1 are both inactivated in the prostate by SV40 large T antigen oncoprotein, which induces the development of prostate cancers that subsequently progress to NEPC (92). Using GEM model and human cell models, loss of p53 and RB1 has been shown to promote linear plasticity and a phenotypic shift from AR-dependent luminal epithelial cells to AR-independent NEPC with resistance to enzalutamide (an antiandrogen drug) (26, 36).

PKA-CREB1 Axis

Both angiogenesis and NE marker expression can be induced by increased cellular cAMP level (93–95). Androgen deprivation therapy (ADT) increases cAMP level in prostate cancer cells, which activates the PKA-CREB1 pathway that in turn regulates both phenotypes. VEGF and ENO2 have been identified as targets of CREB1 and regulate angiogenesis and NE marker expression, respectively (96–98). However, targets of CREB1 that regulate both phenotypes were largely unknown. We recently reported two direct targets of CREB1, GRK3 (G protein coupled receptor kinase 3) and HDAC2 (histone deacetylase 2). GRK3 was shown to promote both angiogenesis and NE marker expression in separate studies (detailed below). Induction of HDAC2 by CREB1 is critical for prostate cancer progression promoted by chronical bio-behavioral stress that activates PKA-CREB1 pathway though beta adrenergic signaling (99). It is still unknown whether HDAC2 is involved in NE phenotype regulation in prostate cancer. In another study, we found that PKA-CREB1 signaling enhances the epigenetic repressive activity of EZH2 (enhanced zeste homolog 2) that in turn induces NE phenotype and angiogenesis (detailed below). In short, the PKA-CREB1 axis seems to be a master upstream regulator for both NE phenotype and angiogenesis in prostate cancer.

GRK3

We initially uncovered GRK3 as a key regulator of the progression of prostate cancer through unbiased shRNA and focused cDNA screening of human kinases (100). GRK3 is essential for metastatic prostate cancer cells in culture and in mouse xenografts. Further, its overexpression promotes orthotropic prostate tumor growth in mouse xenografts. Mechanistically, GRK3 promotes prostate cancer progression in part through repressing two anti-angiogenic factors TSP1 and PAI2, thus inducing angiogenesis in prostate cancer cells (100). Genomic profiling and immunohistochemical staining of human prostate cancers showed that GRK3 is upregulated in advanced prostate cancers (100, 101). Of note, we found a strong trend between GRK3 protein level and glomeruloid microvascular proliferation, a marker of VEGFA–driven angiogenesis, in prostate cancer patient samples. This result further supports a role of GRK3 in stimulating angiogenesis.

We recently reported that GRK3 promotes ADT resistance and NE marker expression of prostate adenocarcinoma cells (101). The kinase dead form of GRK3 abolished these phenotypes, indicating a requirement of GRK3's kinase activity (100, 101). Moreover, GRK3 is positively associated with NE marker expression in human cancer cell lines and patient tumors. Upon GRK3 silencing, expression of NE markers induced by ADT was reduced. These results suggest that GRK3 is a key regulator of both NE phenotype and angiogenesis in prostate cancer. It is worth further investigating the molecular mechanisms of GRK3 and the potential of inhibiting GRK3 as a novel strategy to block NEPC.

EZH2

Polycomb repressive complex 2 (PRC2) is another important regulator for both angiogenesis and NEPC. PRC2 usually renders transcriptional repression by tri-methylating lysine 27 of histone H3 (H3K27me3) on target genes (102, 103). As the key catalytic subunit of PRC2, EZH2 is widely overexpressed in many tumors, including prostate cancer (102). Overexpression of EZH2 and elevated PRC2 activity promote prostate cancer cell proliferation and migration (103). Clermont et al. found that EZH2 is one of the most upregulated epigenetic regulators in NEPC across multiple datasets from clinical to xenograft

tissues (104). Dardenne et al. reported that high catalytic activity of EZH2 promotes N-Myc-AR-PRC2 complex formation and promotes NE phenotype (37). Ku et al. emphasized that overexpressed EZH2 in prostate-specific *Pten-Rb1-p53* triple knockout mice plays a pivotal role in promoting prostate cancer lineage plasticity, antiandrogen resistance, and neuroendocrine phenotype (26). We recently demonstrated that EZH2 presents a critical molecular link for NE phenotype and angiogenesis, downstream of ADT-activated PKA-CREB1 signaling (66). EZH2 is activated by ADT and PKA-CREB1 signaling, which in turn induces NE markers and reduces TSP1 in prostate cancer cells. Our study also fills in a gap of knowledge how EZH2 overexpression in cancer cells directly contributes to tumor angiogenesis. Lu et al. have showed that EZH2 is induced by VEGF in endothelial cells, which contributes to angiogenesis (105).

TSP1

TSP1 is found to have various specific biological activities in different specific tumor environments. The role, regulation and expression patterns of TSP1 in human malignancies are highly context dependent and complicated. On general knowledge of TSP1 in urological cancers, please refer to this outstanding review (106). TSP1 is the first identified endogenous inhibitor of angiogenesis. It suppresses endothelial cell proliferation, migration, and tube formation, as well as induces endothelial apoptosis (107–109). While TSP1's role in angiogenesis is well-known, we recently established its role and regulation in NEPC (66). As expected, TSP1 inhibits angiogenesis induced by NEPC cells. Furthermore, the expression of TSP1 in NEPC is significantly lower than that in CRPC-adenocarcinoma, and NE markers negatively correlate with TSP1 in several prostate cancer datasets (66). Interestingly, TSP1 silence increase NE marker expression in PC3 prostate cancer cells, which suggests that TSP1 may directly regulate NE phenotype. This intriguing observation supports an intimate relation between NE phenotype and angiogenesis in prostate cancer cells (66). The molecular mechanisms underlying TSP1's role of NE phenotype warrants further investigation.

IL-6

As a pro-inflammatory cytokine, interleukin-6 (IL-6) is expressed in both of prostate tumors and the stromal tumor microenvironment. IL-6 is well-known to participate in cellular angiogenesis. Recently, Culig and Puhr have elegantly reviewed the role and regulation of IL-6 in prostate cancer (110). Several signaling pathways downstream of IL-6 orchestrate angiogenesis and NE phenotype in prostate cancer. For example, Ishii et al. showed that IL-6 promotes angiogenesis by up-regulating VEGF through PI3K/AKT pathway (111). On the other hand, IL-6 boosts NE phenotype by inducing CHGA and ENO2 expression through JAK/STAT3 and MAPK pathways (112, 113), as well as AMPK activation and autophagy induction (114). Detailed molecular mechanisms that connect IL-6 induced angiogenesis and NE phenotype need to be further elucidated.

MYCN

As a key oncogene driver in neuroblastoma, MYCN (N-Myc) is also a critical regulator of NEPC and SCLC (small cell lung cancer, a poorly differentiated neuroendocrine lung cancer) (21, 37, 71, 115). While convincing evidence supporting a direct role of N-Myc in regulating angiogenesis is scarce, NDRG1 (N-Myc downstream-regulated gene 1) has demonstrated pleiotropic roles in angiogenesis and cancer progression, depending on cancer types (71, 116).

Aurora Kinases A and B

Aurora kinase A and B (AURKA/B) phosphorylate and stabilize N-Myc protein, which sustains N-Myc function in promoting NE phenotypes in neuroblastoma (117). AURKA and AURKB have been shown to regulate VEGF production and angiogenesis in endothelial cells directly and in neuroblastoma cells (118, 119). It is postulated that AURKA and/or AURKB may regulate angiogenesis of NEPC, although direct evidences are needed.

HIF1A-FOXA2 Axis

HIF1 and HIF2 are well-known key regulators of angiogenesis (48, 50, 120). Recent studies have also implicated them, especially HIF1A, in regulating neuroendocrine phenotype in prostate cancer. HIF1A cooperates with FOXA2, a transcription factor expressed in NE tissue, to induce several HIF1A target genes that are required for hypoxia-mediated NE phenotype and metastasis in prostate cancer (41, 43).

ONECUT2 (OC2)

According to recent reports by Rotinen et al. and Guo et al., ONECUT2 plays a critical role in poorly differentiated neuroendocrine prostate tumors as a master transcriptional regulator (41, 121). As a survival factor in mCRPC models, ONECUT2 depresses AR transcription-related program and activates NE differentiation genes and progression to lethal disease (121). Besides, overexpression of ONECUT2 in prostate adenocarcinoma under hypoxia condition is able to inhibit AR signaling and induce NE phenotype (41). Given the crucial role of hypoxia in angiogenesis, we postulate that ONECUT2 may also contribute to the angiogenic phenotype of NEPC, which warrants further study. One study in ovarian cancer demonstrated that silencing ONECUT2 reduces VEGF expression and vascularization in xenograft tumors (122).

RET

RET mutations are found to enrich in lung adenocarcinoma with NE differentiation (123, 124). Knockdown of RET inhibits prostate tumor growth *in vivo* (125). A recent study from Justin Drake's lab has showed that RET phosphopeptides and mRNA levels are higher in NEPC than in prostate adenocarcinoma, while RET inhibitor AD80 blocks NEPC cell growth in culture and in mouse xenografts (126). Further experiments on gain and loss of function of RET protein will need to be carried out in NEPC cell models. While a role of RET in angiogenesis is well-established in medullary thyroid cancers (127), it is still unclear whether it is critical for the angiogenic phenotype in poorly differentiated neuroendocrine tumors, such as NEPC.

TARGETING THE MOLECULAR LINKS BETWEEN ANGIOGENESIS AND NE PHENOTYPE FOR DEVELOPING NEW THERAPIES

As summarized above, elevated angiogenesis and NE marker expression are two important interconnected phenotypes. Targeting key molecules linking these two phenotypes may be effective therapeutic strategies for neuroendocrine prostate cancers. Potential therapeutic agents targeting some of these molecules include beta blockers inhibiting PKA-CREB1 signaling, TSP1 mimetic peptides, inhibitors of EZH2 and HIF1 pathway, and IL-6 pathway blockade. It is paramount to evaluate these and other related agents, alone and in combinations, for NEPC, given the reported contributions of their targets in this lethal variant of prostate cancer that has no effective treatment.

Beta Blockers

Beta blockers which inhibit beta adrenergic signaling and PKA-CREB1 activation, have been used to treat patients with cardiovascular diseases for decades. According to epidemiology studies, cancer patients who have used beta blockers for cardiovascular diseases have better clinical outcomes than the matched patients who do not use, in multiple cancer types, including melanoma, prostate, lung, and breast cancers (128–130). Results from these retrospective investigations are consistent with emerging evidences supporting anti-tumor effects of beta blockers in cancer cells *in vitro* and in mouse xenografts (99, 131–133). Because that beta blockers have been already applied in hypertension and heart diseases for years, they may also become efficient and safe therapies for NEPC. Beta blockers propranolol and carvedilol are tested in several cancer clinical trials (clinicaltrials.gov). However, major obstacles of beta blockers in clinical studies include incomplete understandings of their mechanisms of action in cancers, as well as a shortage of biomarkers for patient selection and efficacy monitoring (129, 134). We recently reported that propranolol downregulates NE marker expression and inhibits angiogenesis and growth of NEPC cell-derived xenografts by blocking a critical pathway CREB1-EZH2-TSP1 (66). This finding suggests that this pathway's activity level may serve as a biomarker for future cancer clinical trials of beta blockers. The therapeutic value of propranolol and other PKA-CREB1 signaling inhibitors in prostate cancer treatment should be further tested.

EZH2 Inhibitors

Based on the driving role and significant overexpression of EZH2 in many tumors, several inhibitors targeting EZH2 have been developed, such as GSK126, GSK343, GSK503, EPZ6438, CPI-1205, PF-06821497, and DZNeP. Some of these EZH2 inhibitors have demonstrated anti-tumor activity against NEPC *in vitro* and *in vivo*. Beltran et al. found that GSK343 preferentially inhibited cell viability of NEPC cells, while minimally affecting non-NEPC cells (25). Ku et al. reported GSK503 restored enzalutamide sensitivity of prostate tumors from castrated *Pten-Rb1* double knockout mouse (26). DZNeP has also shown some anti-tumor activity in preclinical studies of several cancer types, including prostate cancer (135, 136). We recently demonstrated that conditioned media from prostate cancer cells expressing EZH2 shRNA or treated with GSK126 or EPZ6438 inhibit *in vitro* angiogenesis of endothelial cells (66). In addition, GSK126 and DZNeP were shown to decrease NE marker expression (66). Several EZH2 inhibitors are currently in clinical trials for multiple types of lymphoma, synovial sarcoma, and solid epithelial tumors: NCT03010982 and NCT01897571 (EPZ6438), NCT03480646 (CPI-1205), and NCT03460977 (PF-06821497). It is conceivable that the existing EZH2 inhibitors or other new drugs under development may have positive efficacy targeting NEPC.

HIF Pathway Inhibitors

Pathways of hypoxia-inducible factors (HIF) play key roles in development of resistance to different treatment modalities. Thereby, HIF pathway inhibitors targeting advanced cancers warrant further clinical studies either as a single agent or in combination with other therapeutic agents (137). Specifically for prostate cancer, two mCRPC clinical trials of HIF pathway inhibitors, including 2ME2 nanocrystal dispersion (panzem) and 17-AAG (tanespimycin), have been reported, which unfortunately showed little efficacy (138, 139). However, given the critical roles of HIF in control both angiogenesis and neuroendocrine phenotypes in NEPC, future testing of other inhibitors of HIF pathway, alone or in combinations, is still justifiable for NEPC. Interestingly, Guo et al. recently showed that TH-302 (evofosfamide), a prodrug activated by hypoxia, significantly inhibits NEPC tumor growth (41). An ongoing immunotherapy study combines ipilimumab (targeting CTLA-4) and evofosfamide for the treatment several solid tumor types, including confirmed metastatic or locally advanced prostate cancers (NCT03098160).

Aurora Kinase Inhibitor

Phase II trial of Alisertib (MLN8237), an Aurora Kinase A inhibitor, for castration resistant and neuroendocrine prostate cancers was recently completed (140). Although the report did not meet its primary endpoint of significantly extending 6-month radiographic progression-free survival (rPFS), a subset of advanced prostate cancer patients with AURKA and N-Myc activation achieved significant clinical benefits.

TSP-1 Mimetic Peptides

ABT-510, a TSP-1 mimetic peptide, has been tested in phase I and II clinical trials for many cancer types, including soft tissue sarcoma, metastatic melanoma, renal cell carcinoma, and advanced solid tumors (141–144). ABT-510 failed to show significant clinical benefits as a single agent, suggesting a combinatory strategy is needed. Combination of ABT-510 and cytoxan leads to a delay in progression of PC-3 tumor xenografts (145). Notably, in a phase I study of glioblastoma, combination of ABT-510 with temozolomide and radiotherapy moderately extended overall survival time (146). These findings suggest

that combination of ABT-510 with other standard anti-tumor therapies may be an effective strategy to yield better clinical efficacy. Recently, a new TSP-1 mimetic peptide, ABT-898, with greatly increased potency over ABT-510, has been generated. Its efficacy has been tested in rodents and dogs (147–149), and have showed more notable antiangiogenic efficacy than ABT-510 (147). Investigation of the therapeutic potential of ABT-510 and ABT-898 in prostate cancers, especially in NEPC, warrants additional studies.

IL-6 Pathway Blockade

Given its critical contributions to cancer progression, IL-6 signaling pathway (IL6-/IL6R/JAK/STAT3) is being actively pursued for novel cancer therapies. Recent progress and obstacles in targeting IL-6 to treat cancers have been well-summarized (150–152). Agents blocking IL-6/IL-6R or inhibiting JAK/STAT3 to block tumor progression have been or are being tested in clinical trials, such as siltuximab (an anti-IL-6 mAb), tocilizumab (an anti-IL-6R mAb), Ruxolitinib (a JAK signaling inhibitor). Although many evidences confirmed a key role of IL-6 cascades in regulating the growth of malignant cells in preclinical studies, anti-IL6 or anti-IL6R mAbs have not demonstrated clinical efficacy in several cancer types. The lack of efficacy of IL-6 pathway blockade in cancer is likely due to tumor cells plasticity, displaying different tumor clones in tumor samples *in vivo* (153). Testing IL-6 pathway inhibitors, in combination with standard or other targeted therapies, is still favored for NEPC.

FUTURE DIRECTIONS

Besides the knowledge gaps and future directions abovementioned for individual regulators or therapeutic developments, we believe that the following three directions warrant further investigation to fully understand and target the molecules common to pathways promoting angiogenesis and neuroendocrine differentiation of prostate cancer.

Do Neuroendocrine Differentiation and Angiogenesis Promote Each Other?

We have described several genes reported to regulate both neuroendocrine and angiogenic phenotypes. Much of the knowledge for both phenotypes was in different biological contexts or cancer types. It is largely unclear whether induction of one phenotype leads to increase in another phenotype in the same biological system, such as in NEPC. It is conceivable that induction of neuroendocrine phenotype may promote angiogenesis, in part due to secretion of pro-angiogenic factors by neuroendocrine cells, such as VEGF and neuropeptides bombesin and gastrin, although the roles of these factors in neuroendocrine phenotype are still unclear (15).

Do Critical Regulators Established in One Phenotype Contribute to the Other Phenotype?

This review mainly focuses on genes that have been implicated in regulating both angiogenesis and neuroendocrine differentiation, although in separate contexts for many genes. To better understand these two phenotypes and to facilitate the development of effective treatments for NEPC, a systematic investigation is necessary to define the roles of these regulators in a shared context. Moreover, studies have characterized the function of several other proteins in regulating either neuroendocrine differentiation (such as BRN2, PEG10, SRRM4, REST, and DEK) or angiogenesis (such as FGF, TGF, EGFL6, PDGF, MMPs, and CCL2). Given the intimate links between the two characteristics as we summarize, it is worthwhile to investigate the roles of critical regulators of neuroendocrine differentiation in regulating angiogenesis, and vice versa.

Anti-angiogenesis Therapy and Combination Treatments for NEPC?

Positive results in anti-angiogenic therapy were observed in pancreatic neuroendocrine tumors (PNET), another type of neuroendocrine tumors that are well-differentiated with better prognosis than SCLC and NEPC. Sunitinib is a multi-targeted tyrosine kinase receptor inhibitor of VEGFR1-3, PDGFR, c-kit, RET, CSF-1R, and FLT3. It has demonstrated direct antitumor and antiangiogenic effects, and has received FDA approval for the treatment of locally advanced or metastatic PNETs (154, 155).

In SCLC, it was demonstrated that higher VEGF is associated with poor prognosis, which makes it a reasonable strategy to block VEGF pathway for inhibiting angiogenesis and tumor progression. However, only limited clinical benefits in this attempt was observed (156). As far as we know, no result has been reported on clinical trials of anti-angiogenic therapy for NEPC. Due to the striking pathological similarity between SCLC and NEPC, it is likely that, for NEPC, finding the right combinations of anti-angiogenesis and other therapies will be key to achieve significant efficacy for NEPC. Several strategies of combining anti-angiogenic regimens with targeted/chemo/immune therapies have been or are being tested clinically in several cancer types (59). These strategies include combining different anti-angiogenic regimens, simultaneously inhibiting angiogenesis and driving oncogenes, or combining anti-angiogenic regimens with immunotherapy. It is conceivable that similar combinatorial strategies are applicable to NEPC.

Another strategy for NEPC is to target key regulators for both NEPC phenotypes that we have discussed, i.e., neuroendocrine differentiation and angiogenesis, hitting two birds with one stone. In section Targeting the Molecular Links Between Angiogenesis and NE Phenotype for Developing New Therapies, we have summarized some opportunities for developing therapeutics to target pathways involved in both angiogenesis and neuroendocrine phenotypes. It may be necessary to co-target multiple key regulators of both phenotypes to simultaneously block alternative pathways that NEPC cells may use to escape.

CONCLUSION

NEPC is lethal without effective treatment. It is still poorly understood. They often have both elevated neuroendocrine

marker expression and increased angiogenesis, the mechanisms of which remain largely elusive. Here, we summarize the literature on several proteins and pathways that regulate both angiogenesis and neuroendocrine phenotype in prostate cancer and other contexts. Bridging the mechanistic gaps between regulation of angiogenesis and neuroendocrine phenotype will facilitate better understanding of NEPC progression. We also discuss the opportunities of targeting some of these key regulators to inhibit both angiogenesis and neuroendocrine phenotype for treatments of patients with NEPC. Furthermore, many of the molecular mechanisms that we discuss here for NEPC are also dysregulated in small cell lung cancer (SCLC), a poorly differentiated aggressive neuroendocrine lung carcinoma. Therefore, we expect that much of the current knowledge and new therapeutic potentials summarized here for NEPC are relevant to SCLC.

AUTHOR CONTRIBUTIONS

ZW, YZ, ZA, and WL wrote the paper.

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Phase II Study of Ipilimumab in Men With Metastatic Prostate Cancer With an Incomplete Response to Androgen Deprivation Therapy

Julie N. Graff[1,2], Mark N. Stein[3], Rishi Surana[4], Luai Al Rabadi[1,2], Eric Liu[5], Lawrence Fong[5], Shawna Bailey[2], Emile Latour[2], Timothy A. Newby[2], Amy E. Moran[2] and Tomasz M. Beer[2]*

[1] VA Portland Health Care System, Portland, OR, United States, [2] Knight Cancer Institute, Oregon Health & Science University, Portland, OR, United States, [3] Columbia University Medical Center, New York, NY, United States, [4] MD Anderson, Houston, TX, United States, [5] Division of Hematology/Oncology, Department of Medicine, University of California, San Francisco, San Francisco, CA, United States

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Edited by:
Hung-Ming Lam,
University of Washington,
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Cancer Center, United States

***Correspondence:**
Julie N. Graff
graffj@ohsu.edu

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Background: Phase 3 studies of immune checkpoint inhibitors have not shown a survival benefit in prostate cancer, but some patients have a profound anticancer response.

Patients and Methods: We evaluated the efficacy of the CTLA-4 targeted agent, ipilimumab, in metastatic prostate cancer patients who had an incomplete biochemical response to initial androgen deprivation therapy (ADT) alone. Ten patients were enrolled, each treated with ipilimumab 10 mg/kg (every 3 weeks for up to 4 doses) with maintenance ipilimumab every 12 weeks for non-progressing patients. The primary endpoint was proportion of patients with an undetectable PSA. The total sample size was 30 patients, but there was an interim analysis planned at 10 for futility. If none of the 10 patients achieved an undetectable PSA, the study would be halted.

Results: The study was halted at the interim analysis as none of the 10 patients achieved the primary endpoint, but 30% of patients demonstrated a >50% reduction in PSA, with one patient achieving a >90% reduction in PSA. Peripheral blood mononuclear cells (PBMC) examined by mass cytometry showed that patients with clinical responses had an increase in effector memory T-cell subsets as well as an increase in T-cell expression of T-bet, suggesting induction of a Th1 response.

Conclusions: This study provides further evidence that ipilimumab has activity in some patients with prostate cancer and provides further rationale for the development of future studies aimed at identifying a subset of patients with CPRC that are more likely to derive a benefit from treatment with ipilimumab.

Implications for Practice: There is insufficient evidence to use ipilimumab in prostate cancer in routine practice.

Trial Registration: ClinicalTrials.gov, NCT01498978. Registered 26 December 2011. https://www.clinicaltrials.gov/ct2/show/NCT01498978?term=julie+graff&rank=3.

Keywords: prostate cancer, immunotherapy, CTLA-4, metastatic, checkpoint inhibitor

BACKGROUND

Prostate cancer is currently the second leading cause of cancer deaths in men with more than 33,000 deaths in the United States from prostate cancer expected in 2020 (1). The backbone of therapy for incurable prostate cancer remains androgen deprivation therapy (ADT) with the degree of initial response to ADT strongly correlating with survival. Specifically, those patients who achieve a prostate specific antigen (PSA) level $\leq$0.2 ng/ml after 6–7 months of ADT have a significantly longer survival compared to those who had PSA nadir >0.2 ng/ml (2). Treatment with ADT, however, is not curative, and most patients will eventually progress to develop metastatic castration-resistant prostate cancer (mCRPC). One approach to address this has been intensification of primary therapy through the addition of chemotherapy or more potent androgen signaling inhibitors (3–8). Such approaches delay, but do not prevent, progression to metastatic CRPC. Thus, there is a need to identify patients at highest risk for developing metastatic disease and to develop treatment modalities that delay progression to mCRPC.

While immunotherapy with checkpoint inhibitors has heralded a new era of cancer treatment and revolutionized the treatment of multiple malignancies including metastatic melanoma and renal cell carcinoma, results in prostate cancer have been equivocal (9–12). Ipilimumab, is a first-in-class fully human monoclonal antibody that binds to cytotoxic T-lymphocyte antigen 4 (CTLA-4) and blocks inhibitory signals expressed on activated T-cells and depletes intratumoral T regulatory cells, promoting anti-tumor activity (13, 14). Results from two phase III trials, published after our study began, demonstrated antitumor activity, and increased progression free survival (PFS) in patients with mCRPC treated with ipilimumab compared to placebo without an improvement in overall survival (OS) (11, 12). Despite these results, we have reported on some exceptional responders to immunotherapy, including one patient with mCRPC treated with ipilimumab who had a profound and durable anti-cancer response, suggesting that there may be a subset of patients very well-suited for checkpoint inhibitors (15).

Our study tested the hypothesis that treatment with ipilimumab in patients with an incomplete response to ADT (PSA >0.2 ng/ml) could lead to complete PSA response and improved survival.

METHODS

Patient Selection

This single arm, dual center, phase II trial enrolled men $\geq$18 years of age, with an Eastern Cooperative Oncology Group (ECOG) performance status $\leq$1, who had histologically confirmed adenocarcinoma of the prostate, castrate levels of testosterone (<50 ng/mL), and a PSA level of >0.2 ng/mL after 6–18 months of ADT utilizing a luteinizing hormone-releasing hormone (LHRH) agonist/antagonist with or without the use of an antiandrogen. ADT was continued throughout the study. If an anti-androgen were stopped prior to enrollment, then it had to be stopped 4 weeks prior to enrollment for nilutamide and flutamide and 6 weeks prior for bicalutamide to ensure an appropriate washout period. All patients had radiographic evidence of distant or regional metastasis at the time of enrollment as detected by computed tomography and/or Technetium-99 bone scan.

Exclusion criteria included prior treatment with ipilimumab or any other CTLA-4 targeting agent (e.g., CD137 agonist) or concomitant therapy with any non-study immunomodulatory agent, radiation therapy to any area of the body within 28 days of enrollment, other active malignancies or autoimmune disorders, leucopenia, neutropenia, platelets $<50 \times 10^3$/uL, hemoglobin <8 g/dL, creatinine >3.0 × ULN, AST/ALT >2.5 × ULN.

Study sites participating in this study included Oregon Health and Science University (OHSU) and Rutgers Cancer Institute of New Jersey (CINJ). The trial was IRB approved and registered on clinicaltrials.gov (NCT01498978) prior to subject recruitment.

Study Design

The study design consisted of 4 phases. In the induction phase, all patients received up to four doses of ipilimumab 10 mg/kg IV (administered over 90 min) every 3 weeks. Patients then entered a follow up phase where they were monitored for PSA or radiographic progression. If progression did not occur, patients entered the maintenance phase of the study with the possibility four additional doses of ipilimumab occurring every 12 weeks. The final phase consisted of active follow up of patients until the time of radiographic and/or PSA progression.

The primary endpoint of the study was the proportion of patients who achieve an undetectable PSA (<0.2 ng/mL) after initiation of ipilimumab. Secondary endpoints included time to PSA progression, time to radiographic progression, time to progression by any clinical assessment, time to death from any cause, and maximum percent reduction in PSA.

PSA response was measured using Prostate Cancer Clinical Trials Working Group 2 (PCWG2) 2007 definitions with progression defined as a PSA increase of $\geq$25% and at least 2 ng/mL from baseline or nadir PSA (confirmed by a second measurement at least 3 weeks later) (16). PSA assessment occurred every 3 weeks during the first 4 cycles and every 6 weeks during weeks 12–24, then every 3 months during the follow-up phase of the study. Radiographic assessments were conducted every 12 weeks via bone scan and a computed tomography (CT) scans of the chest, abdomen, and pelvis using a modified version

of RECIST (mRECIST) based on PCWG2 (16). To account for potential "tumor flares," patients who demonstrated tumor progression or lack of laboratory parameter response prior to week 12 but without rapid clinical deterioration were allowed to continue to be treated with ipilimumab.

Correlative Endpoints

Serum and PBMCs were collected on day 1 of each cycle and cryopreserved. Samples were then thawed, barcoded, and stained with mass-labeled antibodies. These samples were then washed and data acquired on a mass cytometer (Helios, Fluidigm). Statistical Scaffold was used to generate clustering maps based on marker similarities (to determine cell types) and maps them onto a grid based on manually gated landmark populations (17). Gating strategy was as follows: singlets, live, CD45+CD61-CD235ab–, CD3+CD19– (T cells), CD3+TCR+ (T cells), CD3+CD56 (NK T cells), CD3+CD4+ (CD4 T cells), CD3+CD56-CD8a+ (CD8 T cells), CD3+CD56–TCR–CD8+CD45RO+CD127+ (CD8 Central Memory), CD3+CD56–TCR–CD8+CD45RO+ (CD8 Effector Memory), CD3+CD56–TCR–CD8+CD45RA+CD127+ (CD8 Naïve), CD3+CD56–TCR–CD4+CD25^{hi}Foxp3+ (regulatory T cells), CD3+CD4+CD56–TCR–Foxp3–CD45RO+CD127+ (CD4 Central Memory), CD3+CD4+CD56–TCR–Foxp3–CD45RA+CD127+ (CD4 Naïve). Clusters were assigned vectors associated with the average median value of defined protein markers (to evaluate functional status of each cell type) and edges, which are defined as similarity between vectors to produce graphs which show the relationships between different clusters. Cluster frequencies and boolean expression for certain markers for each cluster were passed through the Significance Across Microarrays algorithm and results were formulated into the Scaffold maps for visualization (github.com/nolanlab/scaffold).

Statistics

Ten patients were initially accrued using the Simon two-stage design for phase II trials. At least two patients with an undetectable PSA were required within the initial 10 patient cohort to continue to stage II of the patient accrual with a total planned enrollment of 30 patients. The Kaplan-Meier method was used to estimate median time-to-event outcomes and to generate survival curves.

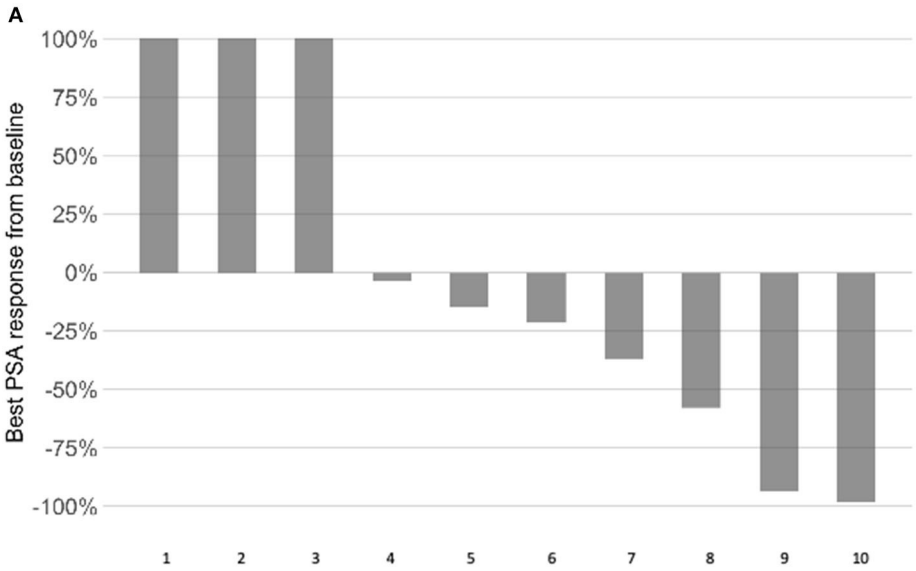
RESULTS

Ten patients were enrolled in this study from September 2012 to June 2015. The data cutoff used in this analysis is May 9, 2019. The median age of patients at enrollment was 65 years with a median PSA of 14 (**Table 1**). Prior to this study, 6 participants had nadired to a PSA 0.2–4 ng/ml, and 4 never got to 4 ng/ml. All patients had radiological evidence of metastases at enrollment. A median of 3 cycles of ipilimumab were administered with two patients completing 2 cycles of ipilimumab, four patients completing 3 cycles, two patients completing 4 cycles, and two patients receiving the full course of 8 cycles. None of the patients enrolled reached the primary endpoint of complete biochemical response evidence by undetectable PSA (<0.2 mg/mL). Thus, per protocol, this study did not move to stage II. Although the primary endpoint was not reached, there was evidence of clinical responses. Three of the ten (30%) experienced a >50% decline in PSA level, two of these demonstrating a decline of more than 90% and one of these a decline of >98% (**Figure 1**). The median time to PSA progression was 17.2 months (95% CI 2.53 to NR) (**Figure 2A**). Median time to radiographic progression was not reached. However, all patients eventually demonstrated biochemical progression (**Figure 2A**). Three out of 10 (30%) remain alive with follow-up ranging from 44.1 to 58.0 months and with a median overall survival of 53.6 (95% CI: 15.4 to NR) (**Figure 2B**). Median OS was 42.2 months in patients with a PSA nadir prior to study of 0.2–4.0 ng/ml and 64.5 months in patients with a PSA that did not nadir to 4 ng/ml or lower. None were related to immune-mediated complications or treatment-related side effects.

TABLE 1 | Baseline Characteristics (ADT, androgen deprivation therapy; mHNPC, metastatic hormone naïve prostate cancer).

Enrolled	**10**
Age	
Median	64.5 years (56–69)
Race	
Caucasian	9
Asian	1
ECOG	
0	7
1	3
Stage at initial presentation	
Localized, node negative	1
Localized, node positive	4
Distant metastatic disease	5
Gleason score	
6	1
7	3
8–10	6
Prior treatment to the primary	
Surgery	3
Radiation	1
None	6
Sites of metastatic disease	
Bone Only	3
Lymph only	1
Bone and lymph	6
Visceral	0
PSA nadir on ADT	
0.2–4 ng/ml	6
>4 ng/ml	4
PSA at cycle 1	
0.2–4 ng/ml	2
>4 ng/ml	8
Chemotherapy for mHNPC	1

A



B

	Diagnosis: Stage/Treatment of Primary/ Gleason grade (PSA at dx)	Response to ADT: PSA nadir (ng/ml)/time to castration resistance	Cancer Characteristics time of study entry: PSA (ng/ml)/sites of disease	Immune related adverse event
1	cTxN1M1/None/4+4	3.07/8 mos	3.28/bone, lymph, lung	None
2	cT4N1M1/None/5+4	1.09/6 mos	52.63/bone, lymph	None
3	pT2cN0M0/RP/4+3	2.9/12 mos	36.4/bone	G2 rash
4	pT2cN0Mx/RP/5+4	2.06/6 mos	17.37/bone, lymph	G2 diarrhea
5	cT3bN1M0/RT/4+5	3.09/24 mos	5.29/bone, lymph	None
6	cTXNXM1/none/4+5	6.45/12 mos	16.36/bone	G3 rash
7	cT2cN1M1/none/4+3	0.38/24 mos	0.72/bone, lymph	None
8	cT2bNxMx/RT/3+3	48/6 mos	77/bone, lymph	G2 colitis
9	cT4N0M1/None/4+4	5.74/10 mos	7.78/bone	G2 hepatitis
10	pT2N0M0/RP/4+3	8.8/14 mos	191/bone, lymph	G1 rash

RP: Radical prostatectomy; RT: radiation therapy; mos: months; G: grade

FIGURE 1 | (A) Best PSA response on study; **(B)** tumor and patient factors according to cancer response.

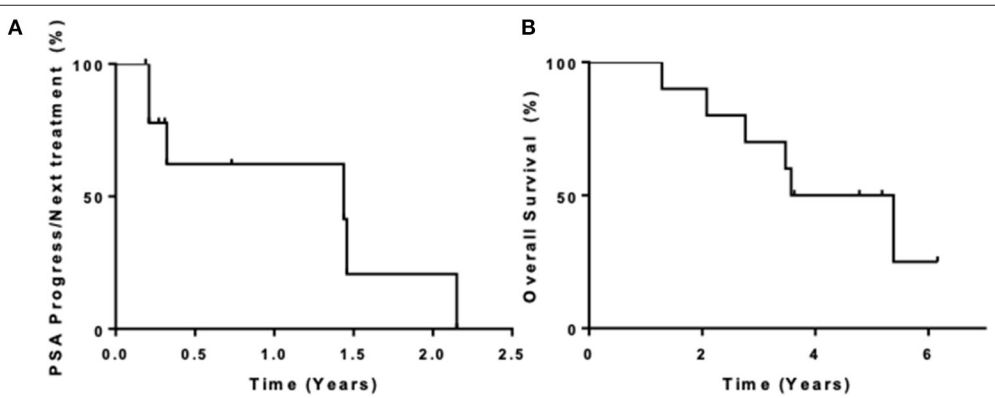


FIGURE 2 | (A) PSA progression and time to next therapy; **(B)** overall survival.

TABLE 2 | Adverse events (AE): (A) non-immune related; (B) immune related (AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase).

Adverse event	Grade 1	Grade 2	Grade 3
(A)			
Number of patients			
Pruritis	5		
Diarrhea	5	4	
Rash	1	4	1
Fatigue	2	2	
Fever	3		
Myalgias (leg cramps)	4		
Dry skin	1		
Hypotension	1		1
Vomiting		1	
Increased creatinine		1	
Flu like symptoms		1	
Elevated AST	1	1	
Elevated ALT	1	1	
Elevated alkaline phosphatase	1		
Colitis			1
Adrenal insufficiency		1	
Nausea	1	1	
Chills	1		
Anorexia		1	
Bloating		1	
Malaise	1		
Hyponatremia	1		
Hypokalemia	1		
Dupuytren's contracture	1		

(B)	
Immune-related adverse events	**Grade (number of patients if more than 1)**
Colitis	3
Maculopapular rash	1,2,3
Diarrhea	1(2), 2
Adrenal insufficiency	2
Pruritis	1
Fever	1
Elevated AST	2
Elevated ALT	2
Elevated alkaline phosphatase	1

Adverse events were common and consistent with previous ipilimumab experience (**Table 2**). Overall, six patients (60%) developed one or more adverse events. One patient developed multiple immune-related adverse events (irAEs) most prominently grade 2 hepatitis and rash that required treatment with steroids and eventually with infliximab. Notably, this patient also had the largest absolute decline in PSA mentioned above. Another patient developed grade 2 adrenal insufficiency requiring steroid replacement while another experienced an acute kidney injury for which cycle 3 was withheld. Four patients developed two or more irAEs. No patients in this study experienced a grade 4 or higher adverse event (**Table 2**). There was not a significant association between irAEs and response.

Serial PBMC were available for eight of the study subjects. We performed immune phenotyping by mass cytometry from the patients to assess for treatment induced effects. In response to treatment, there was an increase in Ki-67 across multiple PBMC subsets when comparing pre-treatment to post-treatment samples (**Figure 3A**). In exploratory analyses, we compared the immunologic profiles in patients with PSA responses (>50% declines) vs. non-responders. In the pre-treatment PBMCs, our analysis revealed that patients with PSA responses had significantly higher T-bet and PD-1 expression in CD4 T-cells (**Figures 3B,C**, respectively). Finally, we observed an overall increase in the percentage of T-bet positive T-cells suggesting expansion of Th1 cells was favored in patients who had PSA declines vs. those who did not (**Figure 3C**).

DISCUSSION

This study examined the effect of CTLA-4 blockade in men with metastatic prostate cancer who did not achieve an undetectable PSA, and it failed to meet its primary endpoint. Since this study was designed in 2009, management of metastatic prostate cancer has changed dramatically. First, we have five new therapies that prolong survival in mCRPC. Second, we now treat newly diagnosed metastatic prostate cancer more aggressively. Third, we have more information about how checkpoint inhibitors perform in mCRPC, and we know that single-agent therapy may be inferior to combination approaches. Fourth, we are more attuned to the mutational landscape of mCRPC and are pairing patients to treatments based on our findings (e.g., mismatch repair deficiency and DNA repair defects). Therefore, it is possible outcomes would be different if we used this information to enroll for this trial today.

Since 2009, there have been 5 trials in mCRPC with ipilimumab (**Table 3**). A phase I/II study of patients receiving ipilimumab (10 mg/kg) with external beam radiation therapy showed that chemotherapy naïve patients may have more PSA responses (6 responders out of 23 patients, 26%) compared to chemotherapy experienced patients (2 responders out of 27 patients, 7.4%) (19). Similarly, a phase III study randomized 799 patients with mCRPC previously treated with chemotherapy to either ipilimumab plus radiation vs. placebo plus radiation. Although the primary endpoint of improved OS in the ipilimumab treated patients was not met, the ipilimumab arm had a superior progression free survival (PFS) (4.0 vs. 3.1 months) (11). A *post-hoc* analysis suggested a benefit with ipilimumab in patients with more favorable prognostic factors, specifically alkaline phosphatase ≤1.5 times the upper limit of normal, hemoglobin 11 g/dL and the absence of visceral metastases (11). In a second phase III study, using chemotherapy naïve patients who were either asymptomatic or minimally symptomatic, those with visceral metastases were excluded. This study randomized

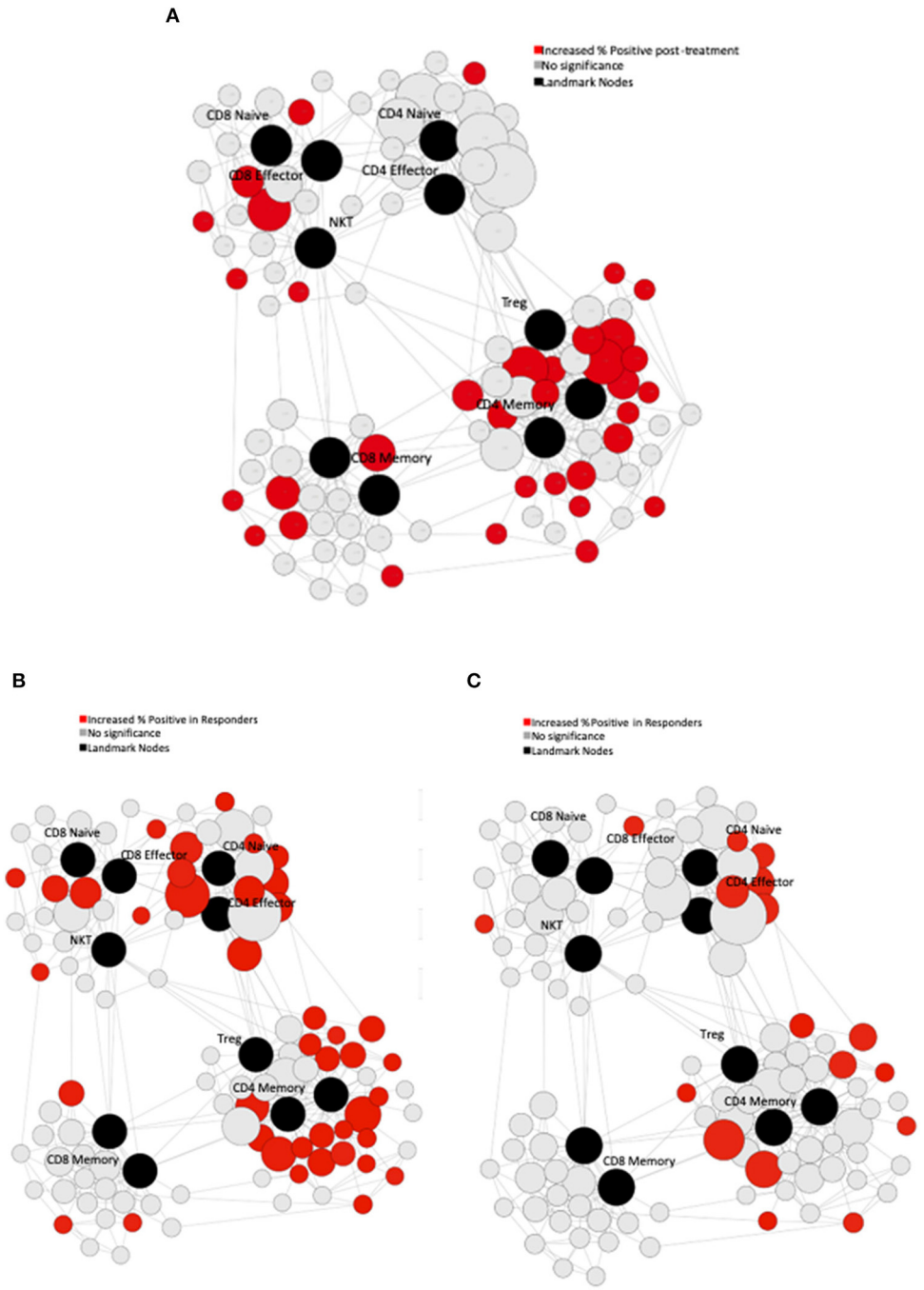


FIGURE 3 | Immune Characteristics. CyTOF assessment of peripheral blood mononuclear cells (PBMCs) from treated patients. **(A)** Pre-treatment PBMCs were compared to post-treatment PBMCs using Statistical Scaffold analysis. Landmark nodes are denoted in black and serve as reference points representing pre-determined cell subsets. Sample cluster sizes generated from distinct cell populations surround the landmark nodes and edges connect clusters to one another based on similarity to guide the development of Scaffold maps. Sample cluster sizes are scaled according to the population of each cluster. Clusters with statistically significant increase in the % of cells that are Ki-67 positive in post-treatment samples are denoted in red ($q < 0.05$). **(B)** Pre-treatment PBMCs in patients with PSA responses were compared to the PSA non-responders with Statistical Scaffold. Clusters with statistically significant increase in t-bet pre-treatment are denoted in red ($q < 0.05$). **(C)** Clusters with statistically significant increase in PD-1 pre-treatment are denoted in red ($q < 0.05$).

602 patients to receive ipilimumab 10 mg/kg every 3 weeks for four doses or placebo. No difference in OS was observed, but patients treated with ipilimumab had a longer PFS (5.6 vs. 3.8 months, 95% CI, 0.55–0.81, HR 0.67) and were more likely to have a PSA response (23 vs. 8%) (12). Additionally, there is a study with finite ADT with ipilimumab in men with

TABLE 3 | Studies of Ipilimumab in mCRPC and PSA response rates (OS, Overall survival; PFS, Progression-free survival; PSA, Prostate specific antigen).

Study/Intervention	Patients	Outcomes
Phase I: Ipilimumab + Poxviral vaccine Escalating Ipi: 1, 3, 5, 10 mg/kg; 6 or more doses Recombinant fowlpox PSA-Tricom: monthly boosts (18)	Asymptomatic 6 post docetaxel 24 docetaxel naïve	Post docetaxel: 1 (17%) of 6 had a PSA response; median PFS = 2.4 mos (1.5–3.7) Chemotherapy naïve: 14 (58%) of 24 had PSA response; 6 (25%) of 24 had PSA declines >50%; median PFS 5.9 (3.4–8.8)
Phase I/II: Dose escalation study with Ipilimumab +/– radiation (8Gy/lesion) (19)	Asymptomatic, +/– prior treatments	In the 10 mg/kg ipilimumab +/– radiation cohort (n = 50), 8 had confirmed PSA decline (6 prior chemo, 2 chemo naïve)
Phase I: Dose escalation study with Ipilimumab (up to 10 mg/kg) + Sargramostim (20)	42 patients, chemotherapy naïve	5 patients experienced > 50% decline in PSA (2 of them in 10 mg/kg ipilimumab cohort)
Phase II: Single-Arm Ipilimumab (3 mg/kg IV every 3 weeks, up to 4 doses) (21)	30 patients with mCRPC	Median PSA PFS: 1.7 mo; median radiographic PFS: 3.0 mo; median OS 24.3 mo
Phase III: Ipilimumab (10 mg/kg) vs. placebo (12)	602 patients, chemotherapy-naïve, asymptomatic (or minimally symptomatic)	Median PFS: 5.6 mo in ipilimumab cohort vs. 3.8 mo in placebo OS: No statistically significant difference between two cohorts
Phase III: Ipilimumab (10 mg/kg) vs. placebo (11)	799 patients, all had prior radiation treatment	Median PFS: 4.0 mo in ipilimumab cohort vs. 3.1 in placebo OS: No statistically significant difference between two cohorts

metastatic castration sensitive disease; 27 patients received 8 months of ADT with ipilimumab before the early termination of the study due to grade 3 irAEs in more than 40% of subjects (22). The 18 patients who did not progress during ADT had their ADT discontinued. The median time to PSA progression was 10.0 months following day 1 of ADT, and there were complete responses in 2 patients. The investigators found that clonal expansion of CD8+ T cells preceded the development of severe irAEs.

Although no patient met the primary endpoint of PSA <0.2 ng/dl, 30% of patients achieved a >50% decline in PSA while on study demonstrating antitumor activity in this patient population. The primary endpoint chosen for this trial is unusual, although there is rationale to using it. If this study had been powered with a different primary endpoint in mind, it might have yielded more clinically informative data. In this small study, those who had a PSA nadir of 0.2–4 ng/ml did not do better than those who never reached 4 ng/ml. A key determinant in identifying potential responders to checkpoint-inhibition, and what likely drove the lack of complete PSA response observed in this study, is both intra- and inter-tumor heterogeneity that likely drives systemic anti-tumor immune responses. Treatment with anti-CTLA-4 resulted in alteration of the phenotype of effector T cells. Specifically, higher percentage PD-1+ CD4 T cells correlated with clinical response, which is consistent with prior studies demonstrating that pre-existing CD4 T cells expressing PD-1 correlated with overall survival (23). Furthermore, the data suggest that a higher abundance of T-bet positive Th1 T cells correlated with PSA response both before and after treatment, while there seemed to be an inverse relationship between the effector and central memory T-cells; multi-dimension analysis tools demonstrated that a higher frequency of CD4 effector memory cells correlated with a PSA response, whereas a high proportion of CD4 central memory cells correlated with no PSA response. These data expand on existing literature that suggests that patients with a specified pre-treatment immunologic signature may be more likely to respond to treatment with checkpoint inhibition (24). Clearly there needs to be prospective study with tumor biopsies to determine if these changes in the peripheral blood are reflected in the tumor microenvironment.

Relative to other genitourinary malignancies, trials investigating single-agent checkpoint inhibitor immunotherapy in prostate cancer have been disappointing. However, ongoing work combining checkpoint inhibitor therapy with other known active prostate cancer agents is tantalizing. In one phase II study, the PD-1 inhibitor pembrolizumab was combined with enzalutamide in 28 patients with metastatic castrate resistant prostate cancer (mCRPC) who were progressing on enzalutamide (25). Five of 28 patients (18%) reached a PSA of 0.2 ng/dl. Similarly, in another phase I/II study, 17 patients with mCRPC who had progressed on enzalutamide and/or abiraterone were treated with durvalumab plus the poly (ADP-ribose) polymerase inhibitor, olaparib, until disease progression, or unacceptable toxicity (26). Notably in the second study, seven patients had deficiencies in homologous recombination (BRCA2), for which olaparib is a treatment, and this makes determination of relative contribution of the checkpoint inhibitor difficult. In this study, 9 of 17 (53%) patients had a radiographic and/or PSA response (PSA decline of ≥50%). In addition, the efficacy of combination immunotherapy with nivolumab and ipilimumab has been evaluated in two clinical trials. The preliminary results of one trial of 78 patients with a minimum of 6 months follow up demonstrated an ORR of 26% (6 of 23) and 10% (3 of 30) among patients that were chemotherapy naïve and those previously treated with taxane-based therapy, respectively (27). The second trial focused on patients with AR-V7 mutations and showed a PSA response in 2/15 (13%) participants (28).

There are trials accruing for men with mCRPC that include ipilimumab. The CheckMate 650 trial is a phase II trial planning to enroll 618 participants, who will be randomized to receive nivolumab with ipilimumab, ipilimumab monotherapy, or cabazitaxel. The primary endpoints are objective response rate and overall progression free survival (NCT02985957). Another randomized phase II study will examine the effects of abiraterone, prednisone, apalutamide with or without ipilimumab (NCT02703623).

CONCLUSION

In conclusion, this trial evaluated the utility of early initiation of ipilimumab in patients with an incomplete response to ADT. The primary endpoint of complete biochemical response was not met, but there was evidence of clinical activity of ipilimumab in a subset of trial participants. This is consistent with finding of studies in CRPC and extends those findings into patients at a somewhat earlier point in their disease course. More work is needed to identify a subset of patients with CRPC that will likely benefit from checkpoint inhibition. These studies will likely include assessments of immune response before and after treatment, understanding resistance mechanisms (e.g., upregulation of other checkpoint molecules), understanding the fundamental tumor biology that specifically predisposes some tumors to be susceptible to checkpoint inhibition (e.g., microsatellite instability), and evaluating combination therapy with immunotherapy and other known active agents in prostate cancer.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by OHSU Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. Cancer Institute of New Jersey (CINJ) also had IRB approval. MS was there when he enrolled patients.

AUTHOR CONTRIBUTIONS

Trial conceived of and designed by JG and TB. Data collected by JG, MS, SB, TN, and TB. Analysis was performed by JG, MS, RS, LA, ELi, LF, SB, ELa, TN, AM, and TB. Paper written and approved by JG, MS, RS, LA, ELi, LF, SB, ELa, TN, AM, and TB. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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