Review

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Review

CRISPR/Cas9 Technology Providing a Therapeutic Landscape of Metastatic Prostate Cancer

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Abstract: Prostate cancer (PCa) is the most prevalent malignancy and the second leading cause of cancer-related death in men. Although current therapies can effectively manage the primary tumor, most patients with late-stage disease manifest with metastasis in different organs. From surgery to treatment intensification (TI), several combinations of therapies are administered to improve prognosis of patients with metastatic PCa. Due to the high frequency of mutation during the metastatic phase, the Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genetic engineering tool can accelerate the effects of TI by enhancing targeted gene therapy or immunotherapy. This review describes the genetic backgrounds of metastatic PCa and how CRISPR/Cas9 technology can contribute to the field of PCa treatment development. It also discusses current limitations of conventional PCa therapy and the potential of CRISPR-based-PCa therapy.

Keywords: metastatic prostate cancer (mPCa); gene therapy; synthetic lethality; CRISPR-Cas9; DNA damage repair (DDR)

1. Introduction

Due to its high prevalence, prostate cancer (PCa) is the second most diagnosed solid-organ cancer in men after lung cancer [1,2]. Based on the GLOBOCAN 2020 report, there were 1,414,259 new cases of PCa and 375,304 deaths [3]. Although the incidence of PCa has been remained stable from 2014 to 2018, its prevalence accounts for 29% of all malignancies, and the incidence of advanced PCa in the USA has been increasing by 4%–6% annually since 2011. Additionally, the prevalence of PCa in men aged >65 years is approximately 60%. Furthermore, the mortality rate of PCa from 2017–2021 and the expected number of deaths in 2024 in the USA are 18.8% and 35,250, respectively [4].

More than 95% of PCa cases are adenocarcinomas, with an acinar origin being more common than a ductal origin. Additionally, almost 80% of PCa cases develop from the luminal or basal (with a lesser prevalence) epithelial cells in peripheral regions that occupy >70% of the prostate gland.

Approximately 80% of patients with PCa have prostate-limited PCa [5]. If PCa is diagnosed at an early stage, life expectancy may be as high as 99% for >10 years [6]. Data from the Cancer of the Prostate Strategic Urological Research Endeavor registry showed that despite conducting PSA screening, approximately 40% of new cases manifest with intermediate-risk localized disease [7].

Furthermore, 8% of men with PCa have distant metastases (often in multiple sites), while 13% present with locoregional metastases. If PCa is diagnosed when distant metastases has occurred, the overall survival rate is only 34% for 5 years [8]. Metastatic PCa (mPCa) accounts for >400,000 deaths annually and is expected to increase by two-fold or more by 2040 [9].

Although PCa is usually diagnosed at an early stage, the risk–benefit ratio of treatment remains uncertain. Treatment of PCa is one of the most challenging due to the significant morbidity that results from therapy [10,11]. As approximately 20%–30% of patients develop metastases, and that development of metastatic castration-resistant prostate cancer (mCRPC) results in drug resistance, it is important to study the mechanisms of PCa metastasis to overcome drug resistance as well as to personalize therapy. Due to the high mutation burden of mPCa, identifying and targeting genes that induce metastasis is important for advancing personalized medicine. CRISPR/Cas9 technology offers

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a platform to detect metastasis drivers and provides tools for clinical treatment through gene therapy. This review focused on the comprehensive analysis of the cause of mPCa and the latest developments in its treatment, including experimental trials in PCa research. Additionally, this review also included a brief introduction to CRISPR technology and how it can be employed in PCa research. All data were prepared by searching the literature from PubMed, clinicaltrials.gov, and Web of Science.

2. Biology of Metastatic PCa

mPCa is a serious health issue due to the increasing prevalence of advanced disease as well as its effects on quality of life and as a cause of mortality. PCa metastasis is mostly associated with spread to the locoregional lymph nodes and/or hematogenously to the stroma of the bone marrow [10]. Uncommonly, PCa metastasis is associated with spread to distant visceral sites. Overall, >80% of distant metastatic lesions occur in bone tissues [10], with osteoblastic bone lesions to the axial skeleton being the most common metastatic sites in advanced PCa [12]. Bone metastases commonly present as osteoblastic lesions with mixed osteolytic features that cause severe pain, hypercalcemia, and frequent fractures (Figure 1).

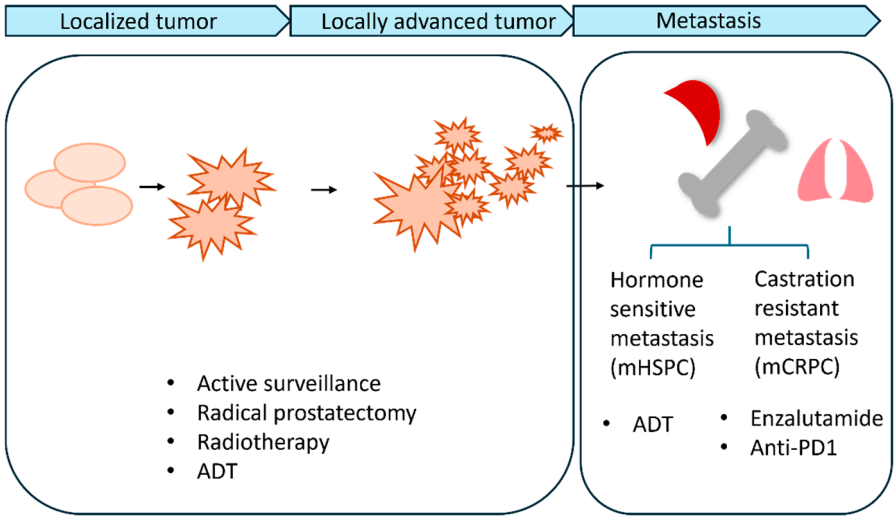

Figure 1. Progression of prostate cancer and the development of mCRPC. Localized adenocarcinoma can progress to invasive carcinoma and spread to distant organs such as lymph nodes, bones and lungs. Standardized treatments are effective in early-stage of cancer, but many metastatic patients develop drug resistance and experience significant increase in mutational burden. Therefore, combination of standard treatment with gene therapy could enhance the overall prognosis of mPCa patients.

Tumor cells undergo epithelial–mesenchymal transition (EMT), leave the primary site, and enter the circulation as circulating tumor cells (CTCs). However, only a small proportion extravasates at a distant site and persists as disseminated tumor cells (DTCs). Of these DTCs, an even smaller proportion can metastasize. Once PCa cells colonize the bone marrow, the interaction between cancer cells and the bone microenvironment leads to a "vicious cycle" of bone formation and destruction, contributing to cancer cell survival and tumor growth. PCa cells also compete with hematopoietic stem cells (HSCs) for the occupancy of limited niches in the bone marrow [13,14], and the reduction of the niche size hampers dissemination [15]. Once DTCs occupy the vascular niche, they acquire a stem cell-like phenotype [16]; together with the protective microenvironment, this results in DTCs that are highly resistant to therapy [17,18].

Almost all patients with mPCa experience castration-resistant PCa (CRPC) that is refractory to androgen deprivation therapy (ADT), which is the primary causes of morbidity and mortality [10]. mCRPC eventually becomes therapy- and castration-resistant PCa (t-CRPC), which is considered as end-stage disease due to the unavailability of effective treatment options [11,12].

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3. Genetics of Metastatic PCa

Many genetic factors are involved in PCa metastasis. Unlike advanced mPCa that has several point mutations, early-stage PCa has a relatively lower frequency of point mutations that include large-scale chromosomal rearrangements such as ETS family gene fusion [19]. *The most common genetic alteration is gene fusion between the androgen receptor (AR)-regulated transmembrane serine protease (TMPRSS2) and transcription factor erythroblast transformation-specific (ERG) genes (>50% of primary tumors). TMPRSS2-ERG fusion belongs to ETS family rearrangement and accounts for 90% of the total ETS family fusions [20]. Although TMPRS-ERG fusion is strongly correlated with the stage and prognosis of PCa [21] [22], the significance of TMPRSS2-ERG fusion in tumorigenesis of PCa remains unknown [23] [24]. Nevertheless, this gene fusion upregulates ERG expression and reactivates AR signaling in tumor cells, with amplification and/or mutations of AR strongly correlating with the onset of mPCa [5].*

The AR gene in chromosome X (Xq11-12) is the most researched molecular factor in PCa research and reportedly promotes CRPC. AR is a ligand-dependent nuclear transcription factor; binding to its ligands, namely testosterone or dihydrotestosterone (DHT), results in the transcription of AR-responsive genes that induce proliferation and promote survival of prostate epithelial cells. Approximately 20% of patients with CRPC have X chromosome rearrangement and subsequent AR amplification, resulting in increased levels of AR proteins in tumor cells [25]. Alterations in AR signaling is the driver of resistance to ADT in patients with mCRPC [9].

Recurrent hot spot mutations in Speckle Type BTB/POZ protein (SPOP) (~10%), Forkhead Box A1 (FOXA1) (~5%), Phosphatase and Tensin Homolog (PTEN) (40%), and Tumor Protein 53 (TP53) (~50%) are also enriched in patients with mCRPC (Table 1) [26] [27].

Table 1. Frequency of somatic and germline mutations in prostate cancer stage. Reprinted from the Lancet, 398, Sandhu et al., Prostate cancer, 1075-90 [9], Copyright 2021, with permission from Elsevier. *Castration sensitivity was not defined in this study.

Somatic mutations	Localized (n=333) [28]	Metastatic castration sensitive (n=140) [29]	Metastatic castration resistant (n=444) [30] and (n=101) [31]
TMPRSS2-ERG fusion	46.0%	Not reported	41.0% and 43.0%
Other ETS family gene fusions	14.0%	Not reported	10.0% and 15.0%
SPOP mutation	11.0%	11.0%	5.0% and 6.0%
CHD1 deletion	7.0%	6.0%	23.0% and 33.0%
FOXA1 mutation	4.0%	10.0%	9.0% and 19.0%
PTEN deletion	17.0%	17.0%	32.0% and 45.0%
TP53 mutation or deletion	8.0%	30.0%	40.0% and 57.0%
RB1 deletion	1.0%	7.0%	12.0% and 13.0%
PI3K mutation	3.0%	5.0%	5.0% and 5.0%
AKT mutation	1.0%	2.0%	1.0% and 2.0%
BRCA1 mutation or deletion	1.0%	1.0%	1.0% and 2.0%
BRCA2 mutation or deletion	3.0%	7.0%	10.0% and 11.0%
ATM mutation	1.0%	2.0%	1.0% and 2.0%
CDK12 mutation	2.0%	6.0%	3.0% and 7.0%
Mismatch repair mutation	5.0%	5.0%	4.0% and 5.0%
APC deletion	5.0%	13.0%	8.0% and 9.0%
CTNNB1 mutation	2.0%	6.0%	4.0% and 6.0%

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MYC gain-of-function	7.0%	6.0%	23.0% and 33.0%
AR amplification or mutation	1.0%	4.0%	59.0% and 70.0%

Germline mutations	Localized (n=499) [32]	Metastatic* (n=692) [32]
BRCA1	**0.6%**	**0.9%**
BRCA2	**0.2%**	**5.3%**
ATM	**1.0%**	**1.6%**
CHEK2	**0.4%**	**1.9%**
PALB2	**0.4%**	**0.4%**
RAD51D	**0.4%**	**0.4%**
Mismatch repair (Lynch syndrome)	**0.6%**	**0.6%**

SPOP encodes an E3 ubiquitin ligase, and its mutation prevents the degradation of the ERG and AR proteins [33]. SPOP also acts as a negative regulator of PCa cell proliferation through the activation of both phosphytidylinositol 3-kinase (PI3K)-AKT serine/threonine kinase (AKT)-mammalian target of rapamycin (mTOR) and AR signaling [34], with SPOP mutated PCa cells being resistant to bromodomain and extra-terminal motif (BET) inhibitors [35]. Studies showed that the SPOP mutation sensitizes cancer cells not only to AR inhibitors but also to poly (ADP-ribose) polymerase inhibitors (PARPi) by repressing homology recombination (HR) and promoting non-homologous end joining (NHEJ) DNA repair [36].

PTEN mutation is another hallmark of human malignancies and is a key determinant of metastasis. PTEN suppresses the PI3K-AKT-mTOR signaling pathway, which regulates cell proliferation and energy metabolism [37].

Loss-of-function mutations in the cyclin-dependent kinase 12 (CDK12) gene represent a specific subtype of mCRPC [38]. Compared with primary PCa, mCRPC is enriched with CDK12 mutations (6.9% vs 1.2% of 360 vs. 498 patients) that mostly harbor truncated kinase domain (amino acids 728–1020) [39] [38]. CDK12 is involved in i) regulation of RNA polymerase II transcription by phosphorylating serin residues of the hepta-peptide repeats (YSPTSPS) in the C-terminal domain of RNAPII that allows entry into the elongation phase of transcription [40] and ii) regulation of expression of DNA damage repair (DDR) genes (BRCA1, FANCD2, FANCJ, ATR) [41]. CDK12 loss is mutually exclusive from other primary genetic drivers (PGD) such as ETS fusion, SPOP mutations, and mismatch-repair (MMR) deficiency, and it is associated with high genome-wide frequency of focal tandem duplications [38]. A study also described the distinct pattern of CDK12-mutated mCRPC, showing the high chromosomal breakage numbers by exome sequencing and worse prognosis compared to controls [42].

Aside from PGDs, DDR pathway-related genes are highly mutated in 655 patients with mCRPC as revealed by multi-institutional clinical sequencing projects [39]. A report from the International Stand Up to Cancer/American Association for Cancer Research Prostate Cancer/Prostate Cancer Foundation Team (SU2C-PCF) showed genetic alterations of DDR genes in 23% of 150 metastatic biopsy samples [43]. In 2018, the prevalence of MMR defects in PCa was established in a large series involving 1,033 patients. Inherited mutations in genes involved in MMR, namely MLH1, MSH2, and PMS2, also increase the risk of PCa [44].

HR pathway alterations are early events during the evolution of aggressive PCa. The Cancer Genome Atlas (TCGA) reported the molecular analysis of 333 primary prostate tumors, with 19% of

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them, including BRCA2, BRCA1, ATM, CDK12, and FANCD2, or Rad51C, harboring alterations in DDR genes [28]. BRCA2, which is a critical regulator of the HR repair pathway, is the most frequently mutated DDR gene in PCa. In total, 13.3% of patients with advanced PCa harbor BRCA2 alterations, with the BRCA2 mutations resulting in sensitivity to PARPi treatments [45] [46]. A report on 1,211 men with PCa undergoing active surveillance, including 11, 11, and 5 with BRCA1, BRCA2, and ATM germline carriers, respectively, revealed that BRCA2 carriers are more likely to undergo a tumor grade re-classification in subsequent biopsies [47]. Another retrospective study that evaluated the outcomes of 1,302 patients reported that after radical treatment, BRCA1/2 carriers developed metastasis significantly earlier than non-carriers (13.2 vs. 28 months, P = 0.05) [48]. A prospective study also reported that among 53 patients with de novo metastatic hormone sensitive prostate cancer (mHSPC), the time-to-castration resistance (TTCR) was significantly shorter in 11 cases with somatic and/or germline DDR alterations. Men with germline BRCA1 or BRCA2 mutations have a three- to eight-fold higher lifetime risk of PCa that can behave aggressively because of additional MYC activation in combination with inactivation of TP53 and PTEN [49,50].

4. Current Standard Treatments

An important characteristic of PCa is its hormone responsiveness. Similar to normal prostate cells, PCa cells need androgens for growth [51]. Hence, the primary treatment for advanced or metastatic PCa is ADT through surgical or pharmacological castration [52]. Decreasing testosterone levels is achieved by surgical removal of the testicles or treating with luteinizing hormone-releasing hormone agonists, anti-androgens, and estrogens. Although ADT reduces the severity of symptoms and attenuates tumor growth, ADT resistance can develop, leading to mCRPC recurrence. Therefore, single-drug treatments should not be considered for mPCa [31].

Enzalutamide, which is a potent second-generation AR antagonist, is used for mCRPC therapy, resulting in significant improvement in patient survival rates [53]. A large-scale randomized trial reported that enzalutamide extends the time to metastasis and increases overall survival rates of patients with non-mCRPC [54]. However, most patients eventually developed resistance to enzalutamide, warranting alternative therapies. AR-independent enzalutamide-resistant mechanisms are characterized by the bypassing of AR signaling via other hormone nuclear receptors, such as the glucocorticoid receptor [55], or by developing lineage plasticity traits through the expression of neuroendocrine and stem cell-related genes [56] [57].

Palliative treatment is essential for patients with bone metastasis and should aim to relieve pain, enhance mobility, and prevent complications such as pathologic fractures or epidural cord compression [58]. As the histopathology of end-stage bone metastases samples acquired at autopsy or from surgical resections of the spinal cord shows heterogeneity of bone metastasis [59] [60], it should be noted that nuclear AR-negative tumor cells are present in both CRPC and treatment-naïve mPCa [61]. Heterogeneity of metastatic disease indicates that second-generation AR-directed therapies, such as abiraterone and enzalutamide, will most likely require additional therapies, such as bone-targeting therapies, and those directed against non-AR pathways.

5. DNA Repair Inhibition/Targeted Therapy

Due to the significant mutational burden of mPCa, targeted gene therapy offers a complementary approach to ADT for patients with mCRPC wherein the concept of synthetic lethality can be employed (Figure 2).

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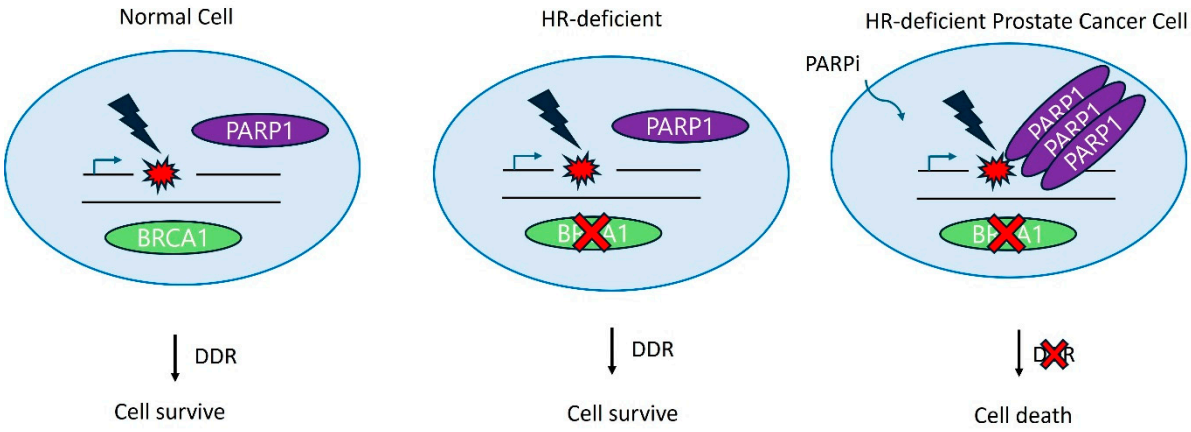

Figure 2. Schematic diagram of synthetic lethality in cancer cell treatment and the mode of action of PARP inhibitors. In HR-deficient cells, single alteration of DDR gene (BRCA1) can allow survival, whereas simultaneous alterations in both partner genes (BRCA1 and PARP1) by application of PARP inhibitors can lead to the death of HR deficient prostate cancer cells. PARP inhibitor work by trapping PARP proteins at the site of DNA damage, which prevents proper DDR and ultimate.

5.1. Ly Results in Cancer Cell Death

Cancer cells harbor mutated genes; if its partner genes are suppressed by inhibitors, specific cancer cell death may occur while sparing normal cells [62]. The first synthetic lethality targeting drugs were PARP inhibitors for BRCAness patients [63]. PARP1 senses DNA lesions, such as single-strand DNA break (SSB) and double-strand DNA break (DSB), inducing self-activation through poly (ADP-ribosy)lation (PARylation). PARylated PARP1 then recruits other DDR factors and promotes downstream signaling for repair pathways [64].

Various genomic studies have reported that 15%–35% of mCRPC cases harbor DNA repair defects, including in BRCA1/2, ATM, ATR, and RAD51 (TCGA Research Network 2015) [39]. Germline mutations in BRCA genes are correlated with an increased risk for PCa development or a more aggressive phenotype as well as worse outcomes [65] [66]. Currently, olaparib, rucaparib, niraparib, and talazoparib are the only FDA-approved PARPi in the USA. These inhibitors trap PARP1 and PARP2 at SSB that result in stalled and collapsed replication forks. Consequently, SSBs are converted to DSBs, resulting in inefficient repair by HR-deficient cells and causing catastrophic DNA damage, cell cycle arrest, and cell death of tumors [67]. Olaparib and rucaparib are approved by FDA for mCRPC with deleterious germline and/or somatic mutations in BRCA1/2 [45,46]. In phase II and III trials, olaparib for mCRPC resulted in high response rates as evidenced by prolonged progression-free or increased overall survival rates [68,69].

Beyond PARPis, extensive research has been conducted to develop synthetic lethality that targets other metastasis drivers in cancer cells. PTEN loss is another hallmark of mPCa that hyperactivates PI3K/AKT signaling and stimulates tumor cell survival and metastasis in vitro. In a genetically-engineered murine model, PTEN loss cooperated with RAS/MAPK signaling to promote EMT and macro metastasis [70]. Various reports suggest the synthetic relationship between PTEN and other genes. Zhao et al. conducted a large-scale genomic analysis of the TCGA database and reported that CHD1 is in a synthetic lethality relationship with PTEN deficiency. Functional PTEN promotes degradation of CHD1, whereas PTEN-deficient PCa shows stabilization of CHD1 and activation of the pro-tumorigenic TNF-NFκB signaling pathway [71]. These findings indicate trackable synthetic lethal targets in PTEN-deficient PCa.

Wu et al. conducted an integrative genomic analysis of data from 360 patients with mCRPC and revealed that CDK12 loss defines another subtype of mPCa that enables the application of the checkpoint inhibitor anti-PD1 as treatment in these patients [38].

Large-scale genomic analyses have been performed to reveal PGDs for targeted therapy. As genome-wide CRISPR/Cas9 screening can be employed, identification of targetable gene alterations

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required for cancer cell survival and the development of a synergistic treatment with existing therapies have become feasible [72].

6. CRISPR Technology for mPCa Therapeutics

A remarkable number of patients with mCRPC harbor defects in genes involved in the DDR pathway. Additionally, a significant proportion of alterations are present in the germline. Through genome editing tools, gene therapy has been developed and improved over the past few decades. CRISPR/Cas9 technology is one of the tools utilized in precision medicine that has the potential to be employed in cancer therapy due to its high accuracy and efficiency in gene alteration.

CRISPR-Cas9 is comprised of the Cas9 enzyme and guide RNA (gRNA). The Doudna group first synthesized single gRNA (sgRNA) that can target a specific DNA sequence and purified the Cas9 enzyme that cleaves DNA at a desired location [73]. Target binding is driven by an sgRNA, and the gRNA/Cas9 RNP complex hybridizes to an intended DNA region containing the sequence (protospacer) complementary to the gRNA and protospacer-adjacent motif (PAM). Through this method, it is possible to perform DNA editing, including insertion, deletion and modification at the level of single base pairs [74] [75]. This programmable gene-editing technology revolutionized various fields, including medicine and agriculture (Figure 3A).

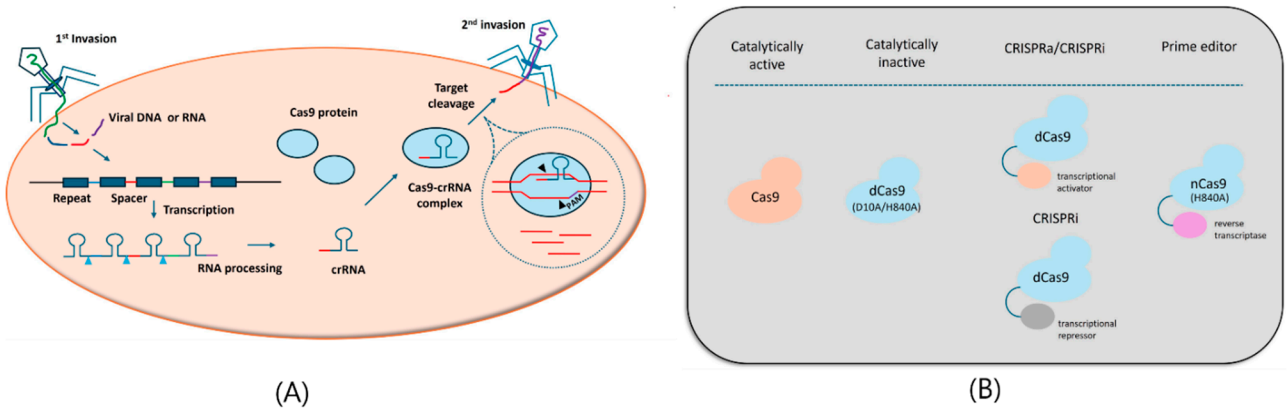

Figure 3. CRISPR-Cas9 system: (A) Mechanism of bacterial immune defense system CRISPR/Cas9. Bacteria develops adaptive immune system to defend themselves from virus invasions. i) Adaptation: the first vial genetic materials (spacer) are integrated into the host genome within the CRIPSR array, separated by palindromic repeats. Ii) Expression: the CRISPR array is transcribed into RNA, followed by RNA processing generating CRISPR RNAs (crRNA). iii) Interference: when the second vial invasion occurs, crRNA guides a bacterial CRISPR-associated protein 9 (Cas9) protein to the viral DNA/RNA and cleaves it to deactivate. This mechanism is applied to the generation of revolutionary gene editing tool, CRISPR-Cas9 technology to modify DNA/RNA in various organisms. (B) Representative engineering of CRISPR/Cas9 technology. Catalytically active SpCas9 is the most widely used Cas9 as an editing tool. It is mutated to make catalytically dead Cas9 (D10A/H840A) which lacks endonuclease activity but still can bind to DNA, acting as a locator for specific genomic loci. dCas9 can be fused to different effector proteins such as transcriptional activator (CRISPRa) or repressor (CRISPRi) to multiply regulate the target gene expressions. The most recently developed editing tool is prime editor that is composed of nickase Cas9 (H840A) fused to reverse transcriptase and edit the DNA at single base level without double strand breaks (DSBs).

A representative Cas9 engineering model is catalytically deactivated Cas9 (dCas9), which is mutated on two amino acids (D10A/H840A) of SpCas9 [74]. dCas9 acts as a locator that searches for specific genomic loci under sgRNA guidance, and it can be conjugated to other effector proteins that perform enzymatic functions on the genome differently. For example, the CRISPRa and CRISPRi systems are composed of dCas9 fused to a transcription activator and repressor, respectively [74] (Figure 3B). Along with the CRISPR KO library, these screening tools can be employed to not only

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multiply, activate, or repress target genes under certain conditions, such as during therapy, but also identify genes required for cancer cell survival as candidate targets.

The recent application of the CRISPR system can be categorized into three groups: mechanism of drug resistance, metastasis, and treatment (Table 2). Various CRISPR-based strategies have been proposed, including i) suppression of oncogenes or repair of genetic mutations such as BRCA1 and BRCA2 mutations [76], and ii) enhancement of immune response to cancer cells by engineering T cells using CRISPR technology.

Table 2. The recent application of CRISPR technology in the field of prostate cancer research.

Subject	Organism	Target	Methods	Genetic factors		Ref.
Drug resistance	*in-vitro*, *in-vivo*	PARP inhibitor sensitivity and resistance	CRISPR KO library	MMS22L KO	CHEK2 KO	Tsujino et al [77] (2023)
				Increasement of sensitivity to PARPi	Increasement of resistance to PARPi	
	in-vitro	AR inhibitor resistance	CRISPR KO library	CDK12 KO		Lei et al [78] (2021)
				Synergistic effect with ARi		
	in-vitro, *in-vivo*	AR inhibitor resistance	CRISPR KO library	CK1α KO		Liu et al [79] (2023)
				Increasement of sensitivity to ARi		
Metastasis	*in-vivo*	Lung metastasis	CRISPR KO library	KMT2C		Cai et al [80] (2024)
				Driver of lung metastasis		
	in-vivo	Bone metastasis	CRISPRa/CRISPRi library	CTIED2		Arriaga et a [81]l (2024)
				Driver of bone metastasis		
	in-vitro	Cancer cell proliferation and migration	CRISPR KO library	MMP9, miR-21		Camargo et al [82] (2023)
				Driver of metastasis		
Treatment	*in-vivo*	Synthetic lethal target dentification	CRISPR KO library	BRG1 KO		Ding et al [83] (2019)
				Inhibition of PTEN-deficient Pca progression		
				TP53 KI		

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	in-vitro	Nanotherapeutics Correction of oncogene TP53	PEI-GQD/CRISPR RNP	Induction of apoptotic cell death of prostate cancer cell	Lee et al [84] (2023)

6.1. Drug Resistance

Although AR signaling and PARP inhibitors prolong the progression-free and overall survival of patients with mPCa, drug resistance frequently develops and has become a serious concern. To overcome drug resistance, CRISPR/Cas9 technology can be employed to identify novel targets that can synergize the treatment effects using conventional mono-treatment.

Some germline alterations, specifically those involved in HR, can act as predictors of response to PARP inhibition. Tsujino et al. revealed that loss of checkpoint kinase 2 (CHEK2) confers resistance to PARPi through the upregulation of BRCA2 expression [77]. They also conducted CRISPR KO screening in four BRCA1/2 deficient PCa cell lines (LNCaP, C4-2B, 22Rv1, and DU145) with and without olaparib. The CRISPR KO library targets over 18,000 protein-coding genes, and negatively and positively selected gene KOs confer sensitivity and resistance, respectively, to olaparib. Gene Ontology (GO) analysis revealed that 67 negatively selected common heats shared by at least two cell lines are related to DNA repair and replication. Meanwhile, 103 positively selected genes that shared at least two cell lines were enriched in cell cycle phase transition and positive regulation of gene expression. Among them, a loss of MMS22L, which is a component of TONSL that recognizes and repairs DSB at stalled or collapsed replication forks [85], increases the PARPi response due to impaired HR function, and its effect is dependent on TP53.

Furthermore, they also revealed that the loss of CHEK2 enhances HR function through E2F7-controlled BRCA2 expression, resulting in olaparib resistance. CHEK2 is a BRCAness gene due to its phosphorylation of BRCA1 gene that promotes HR, and it has been utilized as a biomarker for olaparib treatment in clinical trials [86] [46]. Hence, olaparib resistance conferred by CHEK2 loss in mCRPC cell lines was a surprising result. This finding represents the value of the proper use of CRISPR/Cas9 screening as it revealed novel information that can be contradictory to traditional perspectives.

Regarding AR signaling inhibitors, Lei et al. utilized CRSIRP/Cas9 screening under AR suppression with enzalutamide treatment, revealing that CDK12 is required for PCa cell survival while its inhibition suppresses proliferation and induces apoptosis of PCa cells [78]. Although this finding is inconsistent to previous reports regarding CDK12 loss-of-function mutations, the synergistic anti-PCa effect is obvious when a CDK12 inhibitor and AR antagonists are combined. The effects may be due to attenuated H3K27ac signaling on AR targets and intensive super-enhancer-associated apoptosis pathways. Notably, that was the first report that showed CDK12 as a conservative target of PCa using the CRISPR/Cas9 screening system, and that CDK12 may be a potential therapeutic target for PCa treatment.

Liu et al. also utilized CRISPR KO screening to identify casein kinase 1α (CK1α) as a therapeutic target to overcome enzalutamide resistance in mPCa [79]. Depletion or inhibition of CK1α stabilized the ATM protein through phosphorylation and activated downstream DDR signaling, resulting in sensitization to enzalutamide.

6.2. Metastasis

Several studies have employed CRISPR technology to identify key drivers of metastasis in PCa. Cai et al. developed a mouse model that allows the development of simultaneous and multiple gene mutations in the epithelia of the prostate [80]. To observe the effects of conditional loss of specific genes in mouse prostate, intercrossing of multiple mouse strains, which is extremely laborious and

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time consuming, was previously necessary. However, CRISPR with an adeno-associated virus (AAV) delivery system allows for the simultaneous mutation of five tumor suppressors (TP53, PTEN, Rb1, Stk11, and RnaseL), resulting in the creation of a rapid, invasive, and androgen-independent tumor mouse model. Three additional gene knockouts (Zbtb16, KMT2C, and Kmt2d) showed that loss of KMT2C was essential to induce lung metastasis but not tumor progression. KMT2C is a histone methyltransferase found mutated in many types of cancers [39] [87], but its role in PCa remained elusive. This study not only provides a novel PCa mouse model for CRPC but also suggests new factors required for tumor progression and metastasis.

Another study revealed a molecular driver of bone metastasis in PCa using CRISPR [81]. In that study, genome-wide CRISPRa (activation) and CRISPRi (inhibition) libraries each containing 5 sgRNAs targeting 18,915 genes with 1,895 non-targeting control sgRNAs in 22Rv1 cells, a human PCa cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, and an androgen-dependent CWR22 xenograft were created. The nuclease-dead dCas9 was coupled to a transcriptional activator (sunCas9) or repressor (dCas9-KRAB) and integrated into 22Rv1 labeled with GFP-Luciferase [88]. Genome-wide sgRNA libraries were then packaged into lentiviruses that infected the target cell followed by implantation into mice. Subsequent development of metastatic tumors was visualized by GFP signals, and tumor samples were sequenced to identify enriched sgRNAs. Using this system, it was revealed that the CITED2 gene is a driver of bone metastasis in PCa. CITED2 is a transcriptional co-activator that promotes metastasis in other cancers [89] [90]. The results successfully confirmed the possible role of CITED2 in PCa metastasis as suggested in previous research using the CRISPR system.

Exploring the role of non-coding RNA in mPCa using CRISPR has been attempted. Camargo et al. revised metalloproteinase 9 (MMP9) and microRNA (miR) miR-21, revealing that they attenuate PCa metastasis [82]. The ECM-degrading enzyme MMP9 contributes to the infiltration of tumor cells into other organs. Thus, alteration of MMP9 expression may contribute to PCa evolution and affect its metastatic potential [91]. miR-21, which upregulates MMP9, is highly expressed in PCa, and its inhibition reduced metastasis in a PCa xenograft model, leading to downregulation of reversion inducing cysteine rich protein with kazal motifs (RECK) signaling [92]. miR-21 also regulates B-cell translocation gene 2 (BTG2), which is linked to PCa progression [93], and myristoylated alanine-rich protein kinase c substrate (MARCKS), which controls cellular invasion [94]. sgRNAs targeting MMP9 and miR-21 sequences were inserted into a PX-330 plasmid and transfected into DU145 and PC-3, resulting in attenuation of cell proliferation and invasion and induced apoptosis through the upregulation of RECK expression

6.3. Treatment

For targeted therapy, CRISPR/Cas9 screening can be used in mPCa to identify novel targets to induce synthetic lethality. Ding et al. performed CRIPSR KO screening with or without PTEN knockdown to determine epigenetic regulators that induce synthetic lethality with PTEN deficiency in mPCa [83]. Their results revealed that SWI/SNF subunit Brahma-related gene 1 (BRG1) (SMARCA4) knockdown results in decreased cell proliferation of PTEN-negative cells (LNCaP, C4-2, and PC3) but not PTEN-competent cells (22Rv1, BPH-1, and LAPC4). In a PTEN-null pre-clinical model, treatment with a BRG1 antagonist inhibited progression of PTEN-deficient PCa. Mechanistically, upregulated BRG1 expression in PTEN-deficient cell line causes chromatin remodeling, thereby stimulating pro-tumorigenic transcription.

Delivery of the CRIPSR system into the human body is another concern in terms of treatment efficiency. Although an AAV viral delivery system may be the most promising candidate due to its reduced risk of genomic integration, inherent tissue tropism, and clinically manageable immunogenicity, it may cause carcinogenesis and has a limited loading capacity and restricted scalability for use in the human body. Therefore, the CRISPR non-viral delivery system has been extensively developed. Lee et al. developed a nanomaterial polyethylenimine (PEI)-derived graphene quantum dots (PEI-GQD)-CRISPR RNP system to overcome physiological barriers and to enable the visual tracking of genes of interest [84]. GQD is highly scalable due to the ease of synthesis and widely

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available precursor materials. With this delivery system, TP53 gene mutations in the PC-3 cell line were successfully converted to WT, cancer cell viability was dramatically reduced to 60%, and the increase in apoptosis signal was similar to that following staurosporine treatment (apoptosis inducer) without significantly affecting HEK293T cells. These results suggest a promising avenue for GQD delivery of CRISPR-Cas9 therapeutics in vivo.

7. Conclusions and Future Directions

Although there are no on-going clinical trials using CRISPR technology in PCa, CRISPR may potentially be used to treat PCa based on its successful use in other cancers, with immunotherapy being the most recent development using CRISPR technology.

The remarkable effects of chimeric-antigen receptor (CAR)-T cell therapies in hematological malignancies have inspired its development and use for the treatment of mCRPC. As solid tumors have an immunosuppressive tumor microenvironment (TME) [95] [96], strategies to improve the function of T cells should be developed for successful immunotherapy. Clinical trials of CAR-T cells against solid tumors, including PCa, are being performed, and several studies have already shown the possibility of CAR-T cell therapy in mCRPC by engineering T cells [97] [98].

CRISPR technology can improve the persistence of CAR-T cells to target and kill cancer cells [99]. A breakthrough discovery in cancer research is the blockade of interaction between programmed cell death protein (PD-1) on T cells and programmed cell death ligand (PD-L1) on tumor cells [100] [101]. In a syngeneic immunocompetent mouse model, Dötsch et al. revealed that CRISPR/Cas9-mediated PD-1 KO CD19-CAR-T cells are continuously exposed to antigens and survive over 390 days [102]. Another study revealed that CRISPR-mediated LAG-3 knockout CAR-T cells displayed robust antigen-specific antitumor activity in a cell culture in vitro and in a mouse xenograft model [103].

CRISPR/Cas9 technology can potentially enhance targeted gene therapy as well as immunotherapy. It can also reveal novel genetic alterations in mPCa that can be targeted to improve the effectiveness of CAR-T therapy through the genetic engineering. Due to its high accuracy and efficiency, CRISPR/Cas9 technology has advantages over other genetic engineering tools for personalized medicine. CRIPSR/Cas9 is expected to offer new hope to PCa patients by providing them with effective and affordable personalized treatment.

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