CRISPR-Cas System and Urological Malignancies

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Urological cancers account for a significant portion of cancer diagnoses and mortality rates worldwide. The traditional treatment options of surgery and chemoradiation can have significant morbidity and become ineffective in refractory disease. The discovery of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system has opened up new avenues for cancer research by targeting specific genes or mutations that play a role in cancer development and progression.

prostate cancer bladder cancer renal cell cancer screening gene editing CAR T cell treatment CRISPR

1. Prostate Cancer

Prostate cancer (PCa) is the second-most-frequent cancer and the fifth-most-common cause of cancer death in men [1]. Androgen deprivation therapy (ADT) is the mainstay of treatment in advanced and metastatic hormone-sensitive PCa (mHSPC). However, over time, these men will inevitably progress to metastatic castration-resistant PCa (CRPC). This has led to the development of novel treatments including androgen receptor (AR)-targeted inhibitors and combination chemotherapy. However, there remains an ongoing challenge of identifying new therapeutic targets with a genetic emphasis in mCRPC. Given the move towards personalised medicine, this approach aims to avoid the ‘one size fits all’ treatment.

The early research utilising Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 included targeting the AR, which has been long-known to play a role in PCa carcinogenesis. Wei et al. (2017) designed three sgRNAs to target the AR gene in the LNCaP human cell line. This AR-sgRNA-guided CRISPR-Cas9 system was able to disrupt the AR at specific sites and inhibit the growth of androgen-sensitive PCa cells, demonstrating decreased cell proliferation due to apoptosis [2]. Similarly, Kawamura et al. (2015) used CRISPR-Cas9 to target the proteins NANOG and NANOGP8, which have been shown to be over-expressed in tissues with a higher Gleason score [3]. NANOG and NANOGP8 knockout in DU145 PCa cell lines reduced the malignant potential, including sphere formation, anchorage-independent growth, migration capability, and drug resistance [4]. This anti-tumourgenic effect was subsequently replicated in an in vivo murine model, thus demonstrating its potential as a therapeutic target in PCa.

In a recent study by Warner et al. (2020), CRISPR-Cas9 gene editing was used to demonstrate that estrogen receptor β (ERβ) may be a tumour suppressor gene for PCa [5]. Previous disagreements about the phenotype ERβ knockout mouse prompted researchers to use CRISPR-Cas9 technology to delete the entire ERβ gene from the mouse genome. They subsequently confirmed the role for ERβ in controlling growth of ventral prostate epithelium, where it opposes AR signalling. From a clinical perspective, this suggests ERβ can be targeted as a novel approach to treatment of PCa, which is an AR-driven disease. There are already ERβ agonists that have been synthesised and found to have a favourable safety profile [6][7].

Batir et al. (2019) evaluated the efficacy of CRISPR-Cas9 in repairing the dysfunctional mutant tumour protein p53 (TP53), a highly prevalent cancer-related mutation found in at least 50% of all human cancer cell lines [8]. These mutations of the TP53 gene result in impairment to, or loss of, p53 function, which is responsible for the transcriptional activation of apoptosis, cell cycle arrest, DNA repair, and senescence-related genes. By using the lentiviral delivery of sgRNA accompanied by single-stranded oligodeoxynucleotide (ssODN), the researchers were able to effectively repair the TP53 414delC gene region with an efficacy of 26%, resulting in increased apoptosis and reduced cell proliferation in the PC-3 cell line [8]. Although with a modest efficacy, this study represents a novel approach for the CRISPR-directed restoration of a mutant gene in a human PCa cell line and reveals the in vitro potential of targeting TP53.

PTEN (phosphatase and tensin homolog) is another tumour suppressor gene that has recently been inactivated using CRISPR-Cas9 by Takao et al. (2018) [9]. The knockout of PTEN from a murine prostate cell line leads to the activation of cyclin D1 expression and RAC-alpha serine/threonine-protein kinase phosphorylation; these are critical genes for cancer cell survival [9]. While knocking out a tumour suppressor gene could seem counterproductive, PTEN-CRISPR knockout has subsequently facilitated the discovery of genes such as TUBB3 and TCEAL1, which have been shown to overcome docetaxel resistance in laboratory settings [10][11]. TUBB3 knockdown enhanced PTEN expression, which, in turn, reversed docetaxel-resistance in cell lines. Interestingly, knockdown even re-sensitised docetaxel-resistant cells to cabazitaxel, indicating that TUBB3 mediates cross-resistance between both chemotherapeutic agents. The reverse was true, as PTEN knockout enhanced TUBB3 expression.

Whilst the above CRISPR knockout models have proven their value in oncological research by completely removing a target gene from the genome, this loss-of-function approach may not always reflect the true physiological effect of altering its expression levels in vivo. In contrast, a gain-of-function screen such as CRISPR activation (CRISPRa) allows for the targeted upregulation of specific genes without eliminating their function, enabling the identification of potential drug targets by determining genes that, when overexpressed, lead to therapeutic effects. Unlike traditional overexpression methods, CRISPRa can activate the expression of genes without the need for exogenous expression constructs or the potential for nonspecific effects [12]. CRISPRa provides immediate functional validation, which traditional whole-genome sequencing cannot do. More recently, Rodriguez et al. (2022) applied an in vitro genome-wide CRISPRa screen in the androgen-sensitive LNCaP cell line to identify genes that confer enzalutamide resistance. They identified the Paired Related Homeobox-2 (PRRX2) transcription factor as one of the top hits. Subsequently, they showed that PRRX2 is an oncogene in PCa

and that PRRX2 overexpression mediates enzalutamide resistance, which can be overcome via BCL2 and CDK4/6 inhibition [13].

Chimeric antigen receptor (CAR) T cells, based on the genetic engineering of the patient's own T cells for targeted tumour cell lysis, have great potential in immunotherapy for PCa [14]. A number of PCa-relevant antigens have been targeted by CAR T cell approaches, including prostate stem cell antigen (PSCA) and prostate-specific membrane antigen (PSMA). Although considerable clinical success has been seen in lymphoid malignancies, its use in solid tumours such as PCa has been limited by the hostile immunosuppressive tumour microenvironment. To overcome this, Ren et al. (2017) used CRISPR-Cas9 technology to eliminate PD-1 expression in PSCA-targeted CAR-T cells, which were subsequently infused into mice that had previously been injected with PC-3 tumour cells [15]. Whilst PSCA-CAR-T cell therapy alone reduced the tumour volume by 67%, the addition of CRISPR-mediated PD-1 knockout reduced the tumour volume by 88% compared to the control after 52 days. This CRISPR-enhanced anti-tumour activity of CAR-T cells during co-culture demonstrates the potential of combining both technologies.

2. Bladder Cancer

Given that close to a third of bladder cancer (BCa) presents as muscle-invasive with poor prognosis, there is an ongoing need for the development of therapeutic agents [16]. In the setting of metastatic disease, platinum-based therapy is the mainstay of treatment. Unfortunately, the overall response in clinical settings is less than 50% [17]. With the drive for personalised medicine, genetically emphasised treatments have become increasingly important.

Serving as key transcriptional co-activators, chromatin remodelling binding protein (CBP) and p300 are important in tumorigenesis, with recent genome-wide sequencing indicating that somatic mutations of these genes lead to multiple cancers, including BCa [18]. Using a CRISPR interference system, Li et al. (2019) selectively suppressed CBP and p300 expression, leading to BCa cell death in vitro; hence, this may be an attractive and novel strategy for prevention of BCa progression [19]. Similarly, engineered CRISPR-Cas13d sensing human telomerase reverse transcriptase (hTERT) selectively suppressed BCa progression in human BCa cell lines T24 and 5637 due to hTERT effects on maintaining cancer cell immortalisation, cancer growth, and metastases [20]. Unlike the more well-known Cas9 enzyme, which is commonly used in CRISPR gene editing, Cas13d is primarily known for its RNA-targeting capabilities rather than DNA editing.

Another popular target for the prevention of BCa progression is long non-coding RNA small nucleolar RNA host gene 3 (LncRNA SNGH3), which has been found to affect gene transcription, the post-transcriptional process, and, similarly, chromatin modification, with high expression in BCa cells. Hence, dysregulation and aberrant expression have been noted to promote tumorigenesis [21]. Unsurprisingly, the repression of the SNGH3 gene demonstrated reduced progression of BCa, similar to previous studies. However, the translation of such treatment targets remains to be seen.

Che et al. (2020) went one step further and utilised 38 BCa patients' cells to determine in vivo the effects of SMAD enhancer RNA (SMAD7) knockdown in a mouse xenograft model [22]. SMAD7e is known to antagonise

transforming growth factor β1 and facilitate cancer cell growth in colorectal, pancreatic, prostate, and lung cancer [23]. Hence, the authors examined SMAD7 enhancer's significance in BCa and the effects of knockdown on the proliferation, apoptosis, migration, and invasion of BCa cells. The authors concluded that SMAD7e knockdown mediated by CRISPR-Cas13a reduced oestrogen's cancer-promoting ability in vitro and in vivo in BCa cells and thus may represent an attractive target for treatment.

The cisplatin-based chemotherapy response in BCa is unsatisfactory due to genomic differences, pathological subtypes, and eventual drug resistance. Hence, Shi et al. (2022) attempted to elucidate the associated cisplatin resistance genes in BCa using high-throughput genome-wide CRISPR screening in human BCa cells and tumour xenograft mice models [24]. This method of CRISPR screening utilises large libraries of guide RNAs to target thousands of genes simultaneously and comprehensively explore gene functions and interactions on a genome-wide scale. Using this unbiased and systematic screening method, authors identified the Heterogenous Nuclear Ribonucleoprotein U (HNRNPU) gene and used in vitro and in vivo experiments to demonstrate HNRNPU function and depletion in cisplatin sensitivity. HNRNPU was highly expressed in tumour cells, and the subsequent knockout correlated with the inhibition of cell proliferation, invasion, and migration with apoptosis promotion in cisplatin-treated cells. Furthermore, HNRNPU knockout enhanced cisplatin sensitivity through the regulation of DNA damage repair genes. Hence, authors suggest that HNRNPU inhibition may a useful target in cisplatin-resistant BCa [24].

More recently, Neyret-Kahn et al. (2023) used CRISPR-Cas9 to establish a novel integrated epigenetic map for BCa and demonstrated a link between tumour subtypes. The group found that the long-term inactivation of FOXA1 alone through CRISPR mutation was sufficient to induce a shift from the luminal to basal subtype in luminal cells [25]. This finding is oncologically significant, as basal bladder tumours are typically of a high grade and stage, with a reduced response to chemotherapy and an overall poorer prognosis than luminal tumours. Not only did the study highlight the role of FOXA1 as a key transcription factor in subtype determination, it also induced ZBED2 overexpression, which plays a role in dampening the inflammatory response in cancer cells [25]. CRISPR has therefore demonstrated its ability to further our understanding of transcriptional regulation by identifying super-enhancer pathways providing potential targets for the treatment of aggressive disease.

3. Renal Cell Cancer

Patients presenting with metastatic renal cell cancer (mRCC) face a poor prognosis, with a five-year survival of less than 15%. Whilst mRCC tyrosine kinase inhibitors (TKIs) and checkpoint inhibitors (CPIs) have shown promising efficacy, treatment failure following use leads to a poor prognosis [26]. The lack of enduring interventions to combat mRCC underscores the need for better models to characterise this immunogenic malignancy and new insights into the mechanisms driving this condition.

The majority of RCCs are of the clear cell (ccRCC) subtype, much of our understanding of which is derived from studies investigating the von Hippel–Lindau (VHL) tumour suppressor gene. Schokrpur et al. (2016) used CRISPR-Cas9 to knockout VHL from RENCA mice. The RENCA murine model is a widely used xenograft model in which

tumour cells from the RENCA cell line are implanted under the kidney capsule of immunocompetent mice and subsequently metastasise to sites seen in human ccRCC, including the lungs, liver, and lymph nodes, despite expressing wild-type VHL [27]. This research found that the loss of VHL led to morphologic and molecular changes indicative of the epithelial mesenchymal transition (EMT) phenotype, which, in turn, drives increased metastasis through stabilisation and therefore the oncogenic action of hypoxia-inducible factors-1α (HIF-1α) in mRCC [28]. A better understanding of this mechanism could lead to treatments to reduce the risk of progression to metastases in RCC in the first instance.

In a study by Yoshino et al. (2017), CRISPR-Cas9 was used to edit endogenous small non-coding RNAs, also known as microRNAs (miRNAs), that have been previously identified as highly upregulated in the RCC cell lines 786-O, A498, and Caki2 [29]. Upon deleting miR-210-3p from multiple RCC cell lines, the authors surprisingly found that its downregulation resulted in significantly increased cell invasiveness in vitro and promoted tumorigenesis in vivo in a mouse xenograft model. Although initially contradictory, these findings can be explained by earlier research showing *miR-210-3p* to be downregulated in high-grade late-stage ccRCC compared to low-grade early stage ccRCC [30]. Therefore, authors postulate that *miR-210-3p* expression has dual consequences in tumorigenesis and metastasis. Upregulation may be necessary to establish tumorigenesis in ccRCC. However, to then achieve EMT and metastasize, *miR-210-3p* needs to be downregulated in order to release the suppression of Twist-related protein 1 (*TWIST1*). These findings were supported by real-world patient data in the Cancer Genome Atlas database, where high *TWIST1* and low *miR-210-3p* expression were associated with poorer overall and disease-free survival, suggesting that RCC progression is promoted by *TWIST1* suppression mediated by *miR-210-3p* [31]. The success of these studies demonstrates that CRISPR-Cas9 gene-editing techniques can be applied to not only detect genes that cause RCC but also understand the complex mechanism by which they may progress to mRCC.

As with PCa, CRISPR-Cas9 has also been used to knockout the tumour suppressor gene PTEN from RCC cell lines. PTEN knockout was found to promote spheroid formation and decreased sensitivity to the commonly used TKIs sunitinib and sorafenib, suggesting that PTEN may be a biomarker and therapeutic target in patients with mRCC [32]. More recently, Makhov et al. (2020) used CRISPR-Cas9-based high-throughput loss-of-function screening to identify the cellular factors involved in the resistance to sunitinib [33]. In this type of screen, individual genes are targeted using CRISPR to disrupt their function, usually by creating small insertions or deletions (indels) that result in frameshift mutations and non-functional proteins. Cells with knocked-out genes are then subjected to a particular assay, and the changes in phenotype or behaviour are analysed. Farnesyltransferase was identified among the top hits contributing to the sunitinib-resistant phenotype in ccRCC. This was subsequently validated in cell and animal models of ccRCC by combining the farnesyltransferase inhibitor lonafarnib with sunitinib, and a significantly augmented anti-tumour efficacy was found both in vitro and in vivo mouse models [33]. This highlights the ability of CRISPR-Cas9 to identify and validate the druggable factors involved in resistance to targeted therapeutics.

4. Testicular Cancer

In men aged 20–34, testicular cancer is the most common malignancy. It represents one of the most curable cancers when identified and treated promptly. Although rarer than the above cancers, the incidence has doubled over the past 40 years for unknown reasons, with increasing significance due to the long impact of both the disease and treatment on a younger age group [34]. In the setting of CRISPR, testicular cancer has been investigated less compared to the other common urologic cancers mentioned above, with the majority of the literature currently available focusing on the targeting of genes leading to chemotherapy resistance. However, given the younger age group, increasing life expectancy, and unexplained rising incidence, the development of screening tools and treatment efficacy is at the forefront of early diagnosis, the investigation into the rising incidence, disease monitoring, and personalised medicine.

Interestingly, filamin A (FLNA) has been found to be crucial in balancing stem cell characteristics and invasive properties in seminoma cells and possibly testicular germ cells [35]. Welter et al. (2020) investigated FLNA due to its abundance in seminoma TCam-2 cells using FLNA knockout via the CRIPSR-Cas9 system [35]. Given its importance in the mechanosensitive properties of cells, FLNA loss subjected the cell to actin cytoskeletal irregularity, leading to mechanical instability and impaired adhesive properties and ultimately disrupting migratory ability. FLNA knockout was able to reduce the invasive capacity of testicular tumorigenesis, thus demonstrating potential as a target in future therapeutics.

In an attempt to identify biomarkers to predict the efficacy of DNA-damaging drugs (genotoxins), Constantin et al. (2020) utilised the whole-genome CRISPR-Cas9 gene knockout screen to identify ASH2L [36]. As a core component of the H3K4 methyl transferase complex, which is required for bleomycin sensitivity, ASH2L knockdown rendered testicular cancer cells resistant to bleomycin, etoposide, and cisplatin. The authors also note that testicular cancer patients with ASH2L gene alterations are more likely to relapse than those without, based on the Tumour Cancer Genome Atlas. Hence, this research concluded that ASH2L levels may serve as a screening tool to predict response to genotoxins. Interestingly, the sensitivity toward ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia- and Rad3-related (ATR) inhibitors was not affected in ASH2L knockdown cells, suggesting that its use in genotoxin-resistant patients may be more efficacious.

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