

Progress of CRISPR Technology in Cancer Treatment

Junxin Zhang*

Shenzhen College of International Education, China

*Corresponding author: s21507.zhang@stu.scie.com.cn

Abstract. The CRISPR (Cluster of regularly interspersed short palindromic repeats) technology is the nowadays trend of genetic engineering. The simplicity of the system has made genetic engineering more applicable and convenient for bioengineers, in comparison to ZFNs and TALENs. It is now used in a wide range of aspect, by inserting, deleting, silencing, mutating the target gene. However, this technology still has drawbacks. Off-target affect, one of the most common shortcoming has made this technology often considered to be too risky to be put in actual treatment. CRISPR technology holds a great promising prospect in cancer treatment and immunotherapy through enhancing the direct or indirect inhibition of cancer growth. This article will provide an overview of the CRISPR system, its application in studying and screening of cancer cells, improving the efficacy and specificity of cancer immunotherapy, and possible future direction of cancer treatment, for example editing or modifying oncogene and drug resistant genes.

Keywords: CRISPR technology, Cancer, CAR-T therapy, TCR-T therapy.

1. Introduction

Before CRISPR technology was used in genetic engineering, Zinc Finger Proteins (ZNFs) and Transcription Factor Like Nucleases (TALENs) were the two popular tools used. ZNFs were first found in immature *Xenopus* oocytes, with repeating motifs in the protein. They contain finger-like modules, and zinc ions [1]. It has the ability to grasp the DNA through specific hydrogen-bond interaction between the amino acids at the helical positions on the ZNF and the triplet bases on the DNA strand [2]. The application of ZNFs includes a wide range of aspects, including inhibition of HIV expression, disruption of the effective cycle of infection of HSV, activating the expression of vascular endothelial growth factor-A in a human cell line, and so on [3].

Another genome editing tool, TALENs, also has central repeats that are nearly identical. The variation between the repeats is the amino acids called repeat variable di-residues (RVDs) that recognize the bases on the DNA strand [4]. It serves a similar function in gene editing, usually with a higher efficiency than ZNFs.

Both ZNFs and TALENs were made by the combination of either Zinc Fingers or TAL effectors with restriction endonuclease Fok1, which cleave the DNA. Due to that catalytic activity requires the Fok1 catalytic domain to dimerize, ZFNs and TALENs must bring Fok1 to the domains on both strands of DNA. This makes the editing process relatively complicated and slow.

The discovery of the gene CRISPR was from an experiment analyzing the 'iap gene' responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*. In the 3' end flanking region of iap, five highly similar sequences totaling 29 nucleotides were organized as direct repetitions with a gap of 32 nucleotides. This is the first case of Cluster of regularly interspersed short palindromic repeats being observed and recorded [5]. However, the biological significance of this unusual DNA sequence remained unclear until the breakthrough in 2005, Francisco Mojica implied a relationship between CRISPR and immunity followed by several similar discoveries. He investigated the identities of the CRISPR spacers from various strains and found a high rate of similarities of spacers between the strains. By further looking at the sequence and source of the spacer DNAs, most of them were found to be corresponded to bacteriophages or conjugative plasmids. Based on the results, it is hypothesized that the CRISPR system plays an essential role in immune response against phage infection and extrachromosomal elements in bacteria [6,7,8].

In 2008, Marraffini and Sontheimer stated that the crRNA transcribed from the CRISPR spacer targets DNA molecules in the CRISPR interference on conjugation and plasmid transformation in *S.*

epidermidis [9], instead of RNA as assumed by most of the researcher before then. This discovery brought up the possibility of using CRISPR as a genetic editing tool in the field. As the researchers went further down to the mechanism of the CRISPR-Cas system, they found a way to let it function heterologous in other species in 2011, providing a path for the development of CRISPR technology in the following years [10].

CRISPR with a quite different mechanism from the other genetic tools is usually easier to engineer. Instead of using amino acids to target the DNA bases, it uses single strand guide RNA (sgRNA) as the recognition site. In the CRISPR/Cas9 nuclease system, Cas9 is activated, targets and, cleaves the DNA under the guidance of sgRNA. The convenience and simplicity of engineering compared to the other editing tools have made the CRISPR/Cas protein system popular in the field of genetic engineering.

CRISPR/Cas system has a promising therapeutic potential in treating genetic diseases. It could be used in pulmonary and gastrointestinal diseases, hematologic diseases, viral diseases, vector diseases, Autoimmune and inflammatory diseases, primary immunodeficiency, cancer, and so on. It functions by knocking out the gene responsible for the disease, adding a protective mutation to the genome, or introducing a therapeutic transgene [11].

In terms of cancer, tumor gene editing and T cell-based immunotherapy are both prospective directions for cancer treatment. The targeted gene in the tumor gene editing therapy can vary according to the type of cancer. Common examples include restoring abnormal TP53, the gene that codes for tumor suppressor protein, down-regulating the HER2 gene in breast cancer, knocking out genes responsible for the tumor's drug resistance, and so on.

T cell-based immunotherapy uses TCR-engineered T lymphocytes (TCR-T Cells) and Chimeric Antigen Receptors T cells (CAR T Cells) to eliminate tumors. In the usual immunotherapy, using autologous T cells causes a great inconvenience due to time, quality, and quantity requirements. Allogenic T cells could bring up a few problems, including alloreactivity and graft-versus-host disease (GVHD) caused by the endogenous MHC class I and TCR on the lymphocytes. TCR-T immunotherapy expresses the transgenic TCR, which is specific against a tumor-associated antigen (TAA) and derived from other patients or animal models, by the patient's T cells. Meanwhile, CAR-T immunotherapy engineering the lymphocytes to produce CAR that binds to antigens on the cancer cells [12].

2. CRISPR/Cas System

2.1. Classification of CRISPR/Cas System

2.1.1. Class 1

Type I: Type I CRISPR system is the most common CRISPR/Cas system found in bacterial and archaeal adaptive immune system. Cas3, the signature gene of type I, codes for a protein with a helicase that is activated by single-stranded DNA (SS DNA)-stimulated ATPase activity and is linked to the unwinding and degrading of RNA-DNA and DNA-DNA duplexes [13]. Cascade effector complex working with cas3 helicase-nuclease can identify and targets the DNA to preform DNA interference [14]. The helicase domain is usually linked to an HD family domain that cleaves the targeted DNA. The HD domain is either encoded by a different gene within the same locus as the cas3 helicase, or it is found at the N-terminus of cas3 proteins [13].

It is less frequently used in genetic editing due to the complexity in the cascade effector complex. However, by modifying the complex it is shown to be possible to improve the efficiency of genetic editing using type I CRISPR/Cas system. For example, by fusing the cascade complex with FokI nuclease domain, the efficiency could be high up to ~50% in editing human cell lines [14].

Type III: Cas10 is the signature gene in type III systems. Its codes for a multidomain protein with 4 domains, including the palm domain, which is predicted to be active and retains the catalytic residues; the N-terminal cyclase-like domain, which is now believed to have no enzymatic activity;

the C-terminal alpha helical domain matching the thumb domain of A-family DNA polymerase; and Cmr5, an alpha helical protein. Type III CRISPR-Cas systems frequently use crRNAs produced from CRISPR arrays connected to type I or type II systems instead of encoding their own cas1 and cas2 genes. However, the cas1, cas2, and cas6 genes are associated with a type III system that is projected to be completely functional in many genomes lacking type I and type II systems [13].

Type IV: The csf1 gene is considered to be the signature gene in type IV system. The whole system's effector complex includes: a reduced large subunit (csf1), two genes encoding RAMP proteins from the Cas5 (csf3) and Cas7 (csf2) groups, and, in certain situations, a gene encoding a small subunit make up the effector complex of type IV systems. This system often appears independent from CRISPR arrays and lacks the cas1 and cas2 genes like most of the type III CRISPR Cas system [13].

2.1.2 Class 2

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Type II: Cas 9 is the signature gene for type II CRISPR systems. It produces a multidomain protein that integrates every property of effector complexes and target DNA cleavage. Research indicates that Cas9 assembles into a two lobed structure, with the target DNA and sgRNA situated in the interface between the two lobes. The recognition of the PAM is facilitated by the loops in both lobes [13].

The HNH (McrA-like) nuclease domain, which is positioned in the middle of the protein, and the RUVF-like nuclease (RNase H fold) nuclease domain are both present in the Cas9 protein. Target DNA cleavage requires both of the nucleases. Still, Cas9 cannot be utilized as the only marker for the identification of type II systems as it tends to have homologs with very similar sequences but are unrelated to CRISPR-Cas [13].

It is favored due to its simple design and better foundation of understanding about its structure and activities. The CRISPR/Cas9 system is the most commonly used CRISPR system in genetic engineering, screening and other areas of genetic studies.

Type V: Type V CRISPR system is relatively simple. The majority of CRISPR systems classified as type V have exhibited focused RNA-guided dsDNA cleavage activity. The structural and functional properties of Cas12a (type V-A), Cas12b (type V B), and Cas12e (type V-E) have been thoroughly investigated. Following the formation of a binary complex by the effector protein binding to the gRNA, the effector protein selectively detects the 5'T-rich PAM. Notably, the RuvC domain cleaves the NTS and target strand (TS) at PAM remote sites in a sequential manner to create a staggered incision with 5, 7, or 10 nt 5' overhangs. This is distinct from Cas9, which generates a blunt end by acting on the PAM-proximal link [15].

Type VI: Type VI CRISPR system exclusively target single-stranded RNA (ssRNA). Up until now, four subtypes (AD) have been identified; subtypes VI-A, VI-B, and VI-D have all been functionally characterized in conjunction with their corresponding effectors, Cas13a, Cas13b, and Cas13d. The activated Cas13-rRNA interference complex cleaves nonspecifically both the crRNA-bound complementary ssRNA sequence and the crRNA spacer-complementary

dsDNA sequences (i.e., target DNA), in contrast to Cas9, which cleaves these sequences specifically. The application of type VI CRISPR system is still in an early stage. Current application includes transcriptome editing by Cas13 effectors, ssRNA cleavage that could be used in disease diagnosis, nucleic acid imaging and antiviral applications [17].

2.2. Mechanism

The CRISPR immunoresponse involves three stages called in many ways: Spacer acquisition or adaptation, CRISPR/Cas expression and DNA interference.

The homologous DNA from bacteriophage or plasmid DNA is inserted into the loci in CRISPR, usually using two proteins, Cas1 and Cas2. The complex contains two DNA binding regions, one for protospacer, the other is for CRISPR array binding [17]. The process is followed by the replication of a repeat, forming a complete CRISPR unit. In type I CRISPR system, the presence of a protein called integration host factor (IHF) binds to the IHF-binding site on the leader sequence on a CRISPR array, which induce a distortion in CRISPR array DNA. The distortion enables Cas1-Cas2 integrase to target specifically [17]. For type II, an alpha-helix called leader anchoring sequence (LAS) functions similarly to assist the specific binding of Cas1-Cas2 with leader DNA [17]. Some type III CRISPR system, however, node genes that is the fusion of Cas1 and a reverse transcriptase, which indicate the possibility of obtaining the spacer from RNA [18].

The spacer selected, called protospacer, is determined by the recognition of short DNA sequence, protospacer adjacent motifs (PAMs) in the invading plasmid or genome. The possibility of CRISPR-Cas systems self-targeting and self-cleaving is eliminated by the absence of PAM in the CRISPR loci [19]. Type I's Cas1-Cas2 complex sequence-specific interacts with the PAM sequence selectively, which bias the PAM adjacent-protospacer to be integrated. In contrast, the selective of PAM in type II is enforced by Cas9 [17].

In the process of DNA interference, there are different application processes according to different type.

Type I: The crRNA guides the cascade to locate the target DNA site. The binding of DNA strand resulting an R-loop would be formed if the valid PAM sequence is present. This R-loop serves as a loading site for the Cas3 protein. When Cas3 attaches to the single-stranded DNA, it cuts both strands of DNA in the protospacer region. This action induces ATPase/helicase activity, which evidently helps in modifying the Cascade structure. After the DNA strands within the protospacer are split, Cas3 moves along the non-target strand in the presence of ATP, cutting the strand with the assistance of its HD-nuclease domain [22]. Conversely, in the absence of ATP, the Cas3 nuclease domain (HD) recognizes PAM and attaches to DNA, then cleaves a displaced non-target strand inside a protospacer to produce nicked DNA [22].

Type II: Type II systems consist of the combination of crRNA and tracrRNA, as previously stated. The Cas9 protein aids in the formation of the specific crRNA: tracrRNA duplex during this stage. The tracrRNA, which is bound to Cas9, stabilized the crRNA and functions as a framework for the binding. Similar to type I, the ternary Cas9-crRNA-tracrRNA complex in this case identifies the PAM sequence and then targets and binds to a protospacer sequence in the DNA. The process of the attachment to the target sequence in the DNA leads to the formation of an R-loop structure, in which one strand of the DNA is displaced and the complementary strand is banded to the crRNA. Subsequently, in the presence of Mg²⁺ ions, both strands of DNA are cleaved within a protospacer, specifically 3 nucleotides upstream of the PAM region, resulting in blunt DNA ends [22].

3. CRISPR in Cancer Treatment

3.1. Immunotherapy

T-cell receptors (TCRs), different from CARs, binds to the MHC on cells, instead of some particular antigens. The interaction between TCRs and peptide-major histocompatibility complexes (pMHC), which are complexes made up of peptides bound to MHC, determines the efficacy of TCRs [23]. The pMHCs are MHC molecules bounding to peptide. Intracellular antigens are cut into peptide chains and displayed by MHC molecules. Class I MHC proteins, often defective ribosomal translation products, are bound to class I MHC proteins on nucleated cells' cell surface. Meanwhile, antigen-presenting cells (APCs) express class II MHC proteins, which are internalized by endocytosis and

cleaved into peptide chains for binding with these proteins [24]. Chimeric antigen receptors, CARs are engineered receptors that work to redirect lymphocytes, usually T cells, so they can identify and destroy cells with a particular target antigen. CARs utilize an antibody-antigen recognition mechanism, involving a scFV derived from an antibody, to bind to antigens on the target cell's surface, activating immune responses [24]. CAR binding induced anti-tumor responses and T cell activation occurs independently of the MHC receptor, to target antigens produced on the cancer cell surface.

Despite the fact that treatment with CAR-T cells has shown significant clinical outcomes with some cases of B cell leukemia or lymphoma, the therapeutic efficiency of CAR-T cells in solid tumors and hematological malignancies is still limited by numerous obstacles. Limitations of CAR-T and TCR-T therapies includes but not limited to low affinity of receptor with targeted antigen or pMHC, inability to cross barriers of solid tumor, T-cell activities inhibited by immunosuppressive environment, and T-Cell related toxicity. To enhance the efficiency of targeting and binding, and to address the issues of current CAR-T therapies, possible modifications done with the help of CRISPR technology were purposed [24,25].

The immunosuppressive environment created in the microenvironment of solid tumor is stated to be responsible for the T cell exhaustion [26]. The prolonged expression of inhibitory receptors like PD-1, CTLA-4, LAG3, and TIM3 is a feature of T-cell exhaustion. Upon attaching to their ligands, inhibitory signals are conveyed into T cells and promote cell exhaustion. Immune checkpoint inhibitors have been shown in clinical trials to considerably lessen T-cell depletion and increase tumor patients' survival.

Numerous research endeavors have examined how CRISPR/Cas9 disrupts immunological checkpoints in T cells. Studies show that PD-1 disruption in EGFRvIII-CART cells enhances cytotoxicity and reduces exhaustion in glioblastoma, breast carcinoma, and fluid tumors. PD-1-deleted GPC3-CART cells presents anti-tumor activity without impairing cell activation and proliferation. In general, studies prove that CRISPR/Cas9-mediated PD-1 disruption is effective in suppressing CAR-T cell exhaustion and prolonging persistence [27].

To provide immunostimulatory effects, T cell engineering is an additional intriguing strategy. Instead of concentrating on inhibitory signals, studies have highlighted the expression of pro-inflammatory cytokines that boost T cell survival, proliferation, antitumor activity, and rebalance the tumor microenvironment [25]. "On-target, off-tumor" is a major cause of normal cell death in T-cell therapy. In some cases, the targeted antigen or pMHC is present on both cancer cells and normal cell, causing T-cells to bind and damage the normal cell. Through replacing the endogenous TCR with heterogenous TCR via genetic knockout, lower frequency of mispairing and competitive binding is observed in the binding of CD3 complex [28].

Additionally, cytokines toxicity and neurotoxicity are major challenges in CART therapy clinical practice. A feasible solution should reduce downstream signaling of key cytokines, which do not significantly impact the therapeutic efficacy. Cytokines, such as IL6, are secreted by tumor cells and CART-secreted cytokines, activating immune cells. Study using CRISPR to engineered CAR-T with cytokine knockout revealed low level of IL6 and other toxic cytokines, and re-expansion of CAR-T cells is also observed [29].

In addition to repression of cytokine production, suicide gene is also purposed to have the potential to reduce T-cell cytotoxic effect. Secondary inducing agent is also considered to have the potential to decrease engineered cells at adverse events. Examples include independent expression or CAR constructs with full-length CD20 or CD20 mimotopes [30]. However, the slow onset of antibody-mediated depletion may limit its efficacy in patients requiring immediate reversal during severe cytokine-mediated toxicities [25]. An alternative method involves introducing a gene encoding the modified human CYP4B1 enzyme into engineered T cells. This results in the bioactivation of the protoxin 4-ipomeanol, hence inducing T-cell death [31].

Epigenetic control has the power to improve effector function and change the course of T-cell development. The enzyme TET2, which facilitates DNA demethylation, is essential for the alteration of chromatin in cells. The epigenetic profile of TET2-disrupted CD19-CART cells promotes long-

term cancer remission [32]. Furthermore, compared to the wildtype, DNMT3A, the DNA methyltransferase (DNMTs) deletion exhibits a substantial anti-tumor activity [33]. Various studies focusing on different effector enzymes show that CAR-T cells can be made more resistant to exhaustion and more effective in treating patients via epigenetic engineering [27].

3.2. CRISPR Based Tool in Cancer Treatment

The inactivation mutation on the nuclease domains, RuvC and HNH on Cas9, disable the protein from DNA cleavage activity and make it become a tool that targets DNA specifically [34]. In the process called CRISPR interference (CRISPRi), dCas9 inactivate or down regulate a gene, by binding to the DNA strand in its promotor region, which prevent the binding of transcription factors or RNA polymerase [35]. Furthermore, by tethering transcriptional (trans) effectors to the dCas9, it is also plausible to target specifically and regulate gene transcription [36,37].

Similar to the former mechanism, dCas9 could also be tethered with an epigenetic modifier to regulate gene expression. By fusing dCas9 with an epigenetic modifier, activation and silencing of gene could be performed [38]. As mentioned before, the application of epigenetic modification could be used in enhancing T-cell proliferation, which further improves the treatment efficacy.

It is the interference of RNA achieved by the CRISPR system. Cas13a is found to be efficient in cleaving targeted RNA under the guidance of sgRNA. It could be used to do specific knockdown of mRNA [39]. It has the potential to perform human genotyping from cell-free DNA and the diagnosis of cancer [40].

It is another crispr that is independent from breaking double strand of DNA. There are two classes of base editing tool derived from crispr: cytosine base-editors and adenine base-editors. It is achieved by fusing base deaminase to the terminus of a mutated Cas9, usually Cas9 nickase (nCas9).

Importantly, about half of all pathogenic point mutations can theoretically be reversed by employing adenine base-editors (ABE) to change an A-T base pair back into a G-C base pair. This is because methylation cytosines are susceptible to high rates of spontaneous cytosine deamination. As a result, the discovery of a novel class of adenine base-editors that could cause A to G conversions greatly enhanced base-editing capabilities and the research of genetic disorders. However, the base mutation is limited with the change T to C, C to T, A to G, and G to A [41].

While some technologies, such as saturation mutagenesis and saturation genome editing (SAGE), are capable of screening gene variations on a wide scale, they either rely on ineffective HDR technologies or exogenous overexpression of a variant. Thus, CRISPR-mediated base editor screening presents a very promising solution to those problems [42].

The CRISPR system, specifically CRISPR/Cas9 is widely used in screening of cells in cancer. Cancer cells that have a dependency to oncogenes are more susceptible to treatments that target certain driver genes. CRISPR-cas9 screening has been applied to diverse tumor types and biological environments to find novel targets and genes that are responsible for drug resistance is also curtail in cancer treatment development, including identifying the key factors in T cell activation and drug dependence in specific, essential drivers in group 3 medulloblastoma tumorigenesis and vemurafenib resistant genes in A375 melanoma cells and so on [43--45]. Although there's still limitation of careening using CRISPR, for instance, the off target and specificity of the system, CRIPR holds a great prospect for enriching the knowledge and data of essential genes in a diverse type of cancer [46].

Prime editing compensated the limitations of base editing. Prime-editors employ a prime-editing guide RNA (peRNA) and an engineered reverse transcriptase joined to Cas9 nickase. The complementary sequence to the target sites that guides nCas9 to its target sequence and an extra sequence that spells the desired sequence alterations are both present in the peRNA. Prime-editors broaden the scope of DNA editing beyond minor insertion and deletion mutations and some transition and transversion alterations [41].

Prime editing is progressively developing into a useful method for genome editing, and much research is being done on its potential as a therapy to treat cancer and other deadly diseases. This

method can be used to detect if cancer has the capacity to revert mutations. A study showed how to use nickase Cas9 fused to reverse transcriptase to edit the target site in human organoids to introduce cancer-related mutations and then repair CFTR gene alterations brought on by cystic fibrosis. Their study demonstrated PE's therapeutic promise in the treatment of disorders in organoid-based models; however, there were several issues with off-target effects [47].

Targeted chromosomal structural modification is a major problem since chromosomal abnormalities are frequently associated with various cancer types and other illnesses. Originally, radiation or chemical mutagens were used to induce chromosomal rearrangements in mammalian cells in an aimless manner [48]. However, this method's use is limited because it is impossible to foresee where these rearrangements will occur when radiation or mutagens are applied. Inversions could be efficiently produced by simultaneously inducing two double stranded breaks utilizing the CRISPR/Cas system. Translocations, implicated in the formation of many leukemia species and sarcomas, also demonstrated to be achievable using the CRISPR system [48].

4. Conclusion

Overall, the crisper technology shows a great prospect in cancer treatment including improving the immunotherapy strategies, and also providing alternative methods of cancer treatments by using engineered CRISPR/Cas that has specific functions targeting either the immune cell or the cancer cell.

In addition to improving the specificity of crisper Cas system, there's still some fields of this technology that is not yet throughout studied. For example, by investigating the exact function of reverse transcriptase presence and the type III CRISPR system, it is possible to make use of the mechanism in genetic engineering, cancer diagnosis, and so on. Furthermore, discovering some new and more efficient CRISPR systems that can be used in different conditions could bring more possibilities to the genetic engineering field, as there are organisms that are not compatible to Cas9.

In addition, more detailed modifications on the immunotherapy could be made by investigating the relationship between the details on the CARs or TCRs such as hinge areas and spacer length and the treatment efficacy and efficiency.

Despite all the benefits the system has brought, there are still some drawbacks that are not yet eliminated in CRISPR technology development. One of the most essential and common drawbacks is the off-target affect. Any therapy or experimental treatment that involves the introduction of foreign materials into human cells carries an expected risk of off-target effects. Not only have off-target effects been noted and well documented for genome editing technologies (CRISPR, meganucleases, ZFNs/TALENs, etc.), but they have also been reported for gene knockdown technologies like antisense and RNA interference (RNAi). Because both in vivo and in vitro clinical trials will probably involve the genetic change of very large cell populations, off-target effects in genome editing pose a severe risk. This is because there is a greater chance that even an uncommon off-target risk could result in some sort of damaging outcome [49].

Common factors affecting the off-target effect includes the sgRNA, PAM sequence, the Cas protein, methylation on target DNA, and so on [50]. According to studies, chemical modification of sgRNA, controlling duration or does of cell exposure to the system, using Cas9 mutants that has a greater specificity, for example, fusing catalytically inactive Cas9 with FokI nuclease domain [50], and changing delivery method of the system, could reduce off-target effect [51]. In conclusion, the CRISPR system plays a significant role in the development of therapeutic applications and will continue to influence its development in the future, potentially creating new treatment methods that have a curtail impact on cancer treatment.

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