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Optimizing CAR-T Therapy through CRISPR technology

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Abstract. Cancer continues to be the leading cause of death worldwide, driving an urgent need for effective and precise therapeutic strategies. The combination of CRISPR-Cas9 gene-editing technology with chimeric antigen receptor T-cell (CAR-T) therapy has represented a breakthrough in cancer treatment. This paper offers an in-depth overview of the application of CRISPR technology in enhancing CAR-T therapy. Through targeting and disrupting genes such as TRAC and B2M in T cells, universal CAR-T cells are developed, mitigating immune rejection issues in allogeneic treatments. Epigenetic editing is utilized to modulate proto-oncogene enhancers, inhibiting tumor growth. Moreover, controllable Cas9 systems, including the Rimiducid safety switch, are employed to finely tune CAR-T activity, reducing side effects like cytokine release syndrome. Studies demonstrate that CRISPR technology has significantly improved the precision, safety, and adaptability of CAR-T therapy. However, challenges remain, such as off-target effects, insufficient delivery efficiency, and limited efficacy in treating solid tumors. Looking ahead, it will be crucial to develop high-fidelity CRISPR tools like base editors, optimize targeted delivery methods, and expand clinical trials for solid tumors. As technology advances, CRISPR-enhanced CAR-T therapy is expected to become a key component of personalized cancer treatment, providing patients with better chances of recovery.

Keywords: CRISPR/Cas9; CAR-T; tumor therapy.

1. Introduction

Cancer ranks as one of the primary causes of death globally. As reported by the WHO, it accounted for roughly one in six deaths worldwide in 2020. With the rising incidence of cancer cases and fatalities, there is a pressing demand for effective, precise, and personalized cancer therapies. Since the introduction of Chimeric Antigen Receptor T-Cell (CAR-T) immunotherapy in 1989, gene-editing technology has been recognized for its significant potential in cancer treatment.

The CRISPR-Cas system represents a groundbreaking biotechnology that enables scientists to conduct accurate gene-editing research. This system comprises two key components: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas). CRISPR sequences function as an innate immune mechanism in bacteria and archaea, capable of recognizing and cleaving invading viral DNA. Cas proteins serve as enzymes that precisely cut DNA, thereby inducing modifications in the genome. CRISPR sequences are short, highly repetitive DNA segments arranged systematically within bacterial genomes. The regions between these sequences, known as spacers, originate from fragments of DNA derived from past viral or plasmid invasions of bacteria. When the same virus reinvades, bacteria employ CRISPR sequences as a defense strategy by producing RNA molecules that identify and degrade viral DNA. Cas proteins are a class of enzymes that bind to CRISPR sequences and are responsible for cutting DNA. Currently, Cas9 (SpCas9 or SpyCas9) from Streptococcus pyogenes (S. pyogenes) is the most widely used and well-studied multidomain CRISPR/Cas protein for genome editing applications.

CRISPR technology has shown great potential in cancer treatment research. It is mainly applied to immunotherapy optimization, such as enhancing CAR-T cell activity by knocking out PD-1. Or knocking out PD-L1 to enhance tumor antigen presentation, gene repair therapy such as repairing cancer gene mutations such as RB1 and TP53 to inhibit tumor growth, drug development support such as screening targets and studying drug sensitivity, and clinical trial verification. Despite its

preliminary safety and efficacy, off-target effects and delivery difficulties remain challenges to be solved.

This paper, using the application of the CRISPR system in CAR-T as a foundation, examines common optimization strategies for CAR-T therapy, aiming to offer insights for the advancement and clinical implementation of next-generation CAR-T therapies.

2. CRISPR Gene Editing Technology

SpCas9 is an RNA-guided DNA endonuclease characterized by two distinct nuclease domains: RuvC and HNH. These domains work together to introduce double-strand breaks (DSBs) in specific DNA sequences. The targeting specificity of SpCas9 is determined by various forms of guide RNAs (gRNAs). For example, a guide RNA may consist of a CRISPR RNA (crRNA) complex that plays a crucial role in specifically recognizing the target sequence. Additionally, trans-activating crRNA (tracrRNA) assists in binding to the enzyme and in processing precursor crRNAs. Alternatively, a single-guide RNA (sgRNA) can also serve as a guide, combining the functions of both crRNA and tracrRNA into a single molecule [1].

The application of SpCas9 in gene editing carries a certain degree of off-target activity. Notably, even with discrepancies of up to five mismatches, the Cas9 protein can still effectively modify the DNA target guided by RNA [2-4]. This off-target effect has been the focus of considerable research through various in vitro and in vivo experiments. The specificity of the SpCas9 system can generally be summarized as these four parts: (1) In most instances, the system fails to target DNA sites with more than three mismatches in a part; (2) The CRISPR system does not recognize or edit mismatched DNA sites situated near the PAM region, which is within 10-12 bps; (3) Elevated concentrations of the CRISPR/Cas9 complex increase the likelihood of non-specific activity; (4) In studies conducted in bacteria and in vitro, the CRISPR/Cas9 system is capable of targeting NAG-PAM sites, although with much reduced affinity compared to NGG-PAM sites.

SpCas9 is an RNA-guided DNA endonuclease that features two distinct nuclease domains: RuvC and HNH. These domains collaborate to generate double-strand breaks (DSBs) in specific DNA sequences. The targeting precision of SpCas9 is dictated by various forms of guide RNAs (gRNAs). For instance, a guide RNA may comprise a CRISPR RNA (crRNA) complex, which is significant in recognizing the target sequence. Additionally, trans-activating crRNA (tracrRNA) aids in binding to the enzyme and processing precursor crRNAs. Alternatively, a single-guide RNA (sgRNA) can also function as a guide by integrating the roles of both crRNA and tracrRNA into a single molecule [1].

There is a certain level of off-target activity in the use of SpCas9 in gene editing. Notably, even with up to five mismatches, the Cas9 protein can still effectively modify the RNA-guided DNA target [5,6]. This off-target effect has been extensively studied through numerous experiments, both in vitro and in vivo. Four aspects can generally be outlined to describe the specificity of the SpCas9 system: (1) In most cases, it does not tend to target DNA sites with too much mismatches in a given region, and the number is considered as three; (2) It fails to recognize or edit mismatched DNA sites located near the PAM region, specifically within 10-12 base pairs; (3)As the concentration of the CRISPR/Cas9 complex increases, more non-specific activities will be observed; (4) In bacterial and in vitro studies, it can target NAG-PAM sites, albeit with significantly lower affinity compared to NGG-PAM sites.

NGS methods, including GUIDE-seq and ChIP-seq [7-9], have been employed to detect the off-target effects of it. These high-throughput analyses confirmed that Cas9 demonstrates off-target activity. In response to these challenges, various Cas9 variants have been identified, developed, and engineered from diverse organisms. These novel CRISPR systems exhibit significant promise for applications in clinical medicine.

3. Research Progress and Clinical Translation of CAR-T Therapy

In the field of tumor immunotherapy in recent years, CAR-T can be regarded as one of the most breakthrough technologies. In this therapy, autologous or allogeneic T cells are genetically engineered to specifically recognize CAR, so as to achieve precise targeted therapy for malignant tumors. Since 2017, when the Food and Drug Administration (FDA) approved the first two CAR-T products (tisagenlecleucel and axicabtagene ciloleucel) for the treatment of relapsed/refractory B-cell malignancies, CAR-T therapy has shown significant clinical efficacy in hematological tumors, with objective response rates (ORR) of up to 60%-90% [10-12].

Structural development has roughly divided chimeric antigen receptors (CARs) into four generations. The first-generation CAR only contains the antigen-binding domain, transmembrane region, and CD3ζ signaling domain. The introduction of co-stimulatory molecules such as CD28, 4-1BB, or OX40 respectively constitutes the second and third-generation CARs. The third-generation CAR has two co-stimulatory molecules, while the second-generation CAR has only one. Such designs allow T cells to proliferate more persistently and survive longer [13, 14]. The fourth-generation CAR is named "armored CAR-T", which significantly enhances anti-tumor activity by integrating cytokines or immune checkpoint inhibitors such as IL-12 and PD-1 antibodies [15]. In addition, the advancement of universal CAR-T (UCAR-T) technology provides new solutions to the high cost and long preparation time of personalized treatment [16].

Like all other therapies, CAR-T also has its limitations. For instance, it faces many challenges in the treatment of solid tumors. Most of these challenges stem from immune suppression in the tumor microenvironment (TME), antigen heterogeneity, and the on-target/off-target toxicity of CRISPR technology itself [17]. In recent years, preliminary clinical potential has been observed in addressing these issues through strategies such as combination with radiotherapy, use of immune checkpoint inhibitors (ICIs), or design of bispecific CARs [18, 19]. Additionally, the development of emerging gene editing technologies has significantly enhanced the safety and efficacy of CAR-T [20].

4. CRISPR/Cas9 Modification for CAR-T Therapy

4.1. Development of Generic CAR-T Cells

CAR-T cell therapy has achieved substantial advancements in cancer treatment; however, traditional autologous CAR-T therapy faces challenges such as high manufacturing costs, lengthy production times, and inconsistent T-cell quality. As a result, the development of universal CAR-T cells has become a critical priority. Advances in CRISPR gene-editing technology offer a promising solution to address the issue of immune rejection between the host and transplanted cells.

During the process of T cells recognizing antigens, the T cell receptor (TCR) plays a crucial role, with the key lying in the α chain encoded by the TRAC gene. In general, CAR-T therapy, due to the difference between the donor and the recipient, the donor's TCR recognizes the host tissue antigens. We call this graft-versus-host disease (GVHD). Another key step in CAR-T is the presentation of endogenous antigens on the surface of T cells, which is dependent on MHC class I molecules. The components of these molecules include β2-microglobulin, which is encoded by the B2M gene. The host immune system may also attack donor T cells because MHC class I molecules may be regarded as foreign antigens, leading to the rejection of donor T cells. Therefore, knocking out the TRAC and B2M genes is of great significance for constructing universal CAR-T cells. In 2016, Liu et al. researchers used the CRISPR-Cas9 system to simultaneously disrupt the TRAC and B2M genes and successfully generated universal CAR-T cells. Mouse experiments showed that these universal CAR-T cells neither attacked host tissues nor were easily cleared by the host immune system. Ren et al. researchers went further and simultaneously knocked out the PD-1 gene, TRAC and B2M genes. Compared with universal CAR-T cells that only knocked out TRAC and B2M genes, these triple-knockout universal CAR-T cells showed stronger anti-tumor effects in lymphoma mouse models and Nalm6-PDL1 leukemia models [21, 22].

4.2. Epigenetic Editors

The dCas9 system developed from the CRISPR/Cas9 system has made great progress in the study of endogenous gene expression regulation. The first-generation dCas9-VP64 and dCas9-KRAB can only maintain high efficiency in the promoter region adjacent to the target gene, and rapidly decrease with the increase of the distance between the sgRNA target and the promoter, such as the distal cis-regulatory element (enhancer). The newly developed second generation of dCas9-activated system integrates multiple activating effectors to greatly improve the efficiency of transcription activation. However, these studies all regulate gene expression by targeting promoters. Enhancers are also important non-coding sequences that regulate gene expression. They have remote regulation functions without direction dependence, and their mutations may lead to decreased expression of tumor suppressor genes or overexpression of proto-oncogenes, which in turn cause cancer. Through epigenetic editing to change the histone marks of enhancers, it is possible to activate or inhibit the expression of target genes, which provides a new strategy for disease treatment. In 2020, Li et al. fused the CRISPR-dCas9 system with two effectors (VP64/p300 or LSD1/KRAB) to construct the activation (enCRISPRa) and inhibition (enCRISPRi) systems to achieve epigenetic editing of enhancers [23]. In T-cell acute lymphoblastic leukemia (T-ALL), there are nucleotide insertions of different lengths at the 8 kb upstream enhancer of TAL1 proto-oncogene. In Jurkat T-ALL cell model, a 12-bp heterozygous mutation at the enhancer of TAL1 gene introduced a binding motif of the MYB proto-oncogene, leading to the formation of a super-enhancer (SE). In this experiment, sgRNAs targeting the super enhancer of TAL1 proto-oncogene in T-cell acute lymphoblastic leukemia (T-ALL) were used to demonstrate that this program could regulate TAL1 expression and control tumor progression in allogeneic transplantation. Furthermore, enCRISPRi and enCRISPRa can be used to epigenetically edit enhancers in vitro, xenografts and in vivo, and further study the role of non-coding regulatory elements in disease and development.

4.3. Controllable Cas9 System

From an immunological standpoint, CAR-T represents a novel approach to cancer treatment and has shown significant therapeutic potential, particularly in addressing hematological malignancies. However, severe side effects such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) continue to pose the greatest challenge to its advancement in clinical medicine. In response, scientists have devised several small-molecule-based safety mechanisms to enable precise control over CAR-T cell functionality. Among them, Rimiducid, also known as AP1903, effectively regulates treatment-related toxicity by inducing dimerization of caspase-9, thereby promoting apoptosis of over-activated CAR-T cells [24]. Rimiducid is a chemically induced dimerization (CID) small molecule whose mechanism of action is based on binding to the FKBP12-F36V variant in modified caspase-9, such as inducible caspase-9 (iC9). When present, Rimiducid can induce the dimerization of caspase-9 and activate the downstream caspase cascade (including caspase-3, 6 and 7), which ultimately leads to apoptosis. This mechanism makes Rimiducid an effective "off" type safety switch capable of rapidly eliminating CAR-T cells in the event of severe toxic reactions.

The safety-switch mechanism of Rimiducid has been confirmed through various clinical trials. For instance, in managing relapsed acute leukemia and the severe CRS and GvHD linked to allogeneic hematopoietic stem cell transplantation (haploHSCT), Rimiducid successfully regulated the toxicity of CAR-T cells. The findings indicated that Rimiducid could trigger apoptosis in approximately 90% of CAR-T cells within a brief timeframe of around two hours, thus swiftly halting the toxic effects.

5. Conclusion

The combination of CRISPR-Cas9 gene editing technology and CAR-T cell therapy has opened up new possibilities in the field of cancer treatment. This article systematically discusses the application of CRISPR technology in optimizing CAR-T therapy, and reveals its significant advantages in

improving the accuracy, safety and versatility of treatment. By knocking out the immune rejection-related genes (such as TRAC and B2M) in T cells by gene editing, researchers have successfully constructed universal CAR-T cells, which effectively solves the problems of graft-versus-host disease and host immune rejection in allogeneic therapy. In addition, CRICRIS-mediated epigenetic editing (such as targeting super enhancers to regulate the expression of oncogenes) and the introduction of controllable Cas9 systems (such as Rimiducid safety switches) further enhance the anti-tumor activity and clinical controllability of CAR-T cells, providing a solution to deal with serious side effects such as CRS.

However, the clinical application of CRISPR-Cas9 technology still faces multiple challenges. First, off-target effects may lead to unexpected gene mutations, and potential safety risks need to be mitigated by optimizing gRNA design, developing high-fidelity Cas9 variants (e.g., SpCas9-HiFi), or using genome-wide off-target detection technologies (e.g., Guided -seq). Secondly, the efficiency and specificity of the delivery system still need to be improved, especially for the targeted delivery of solid TME. In addition, the efficacy of CAR-T therapy in solid tumors is limited. Therefore, it is necessary to combine multi-target design, immune microenvironment regulation, and combination therapy (such as ICIs) to break through the existing bottlenecks.

Future research should focus on the following directions: first, the development of more accurate and less toxic CRISPR tools, such as base editors or epi-editing systems, to reduce irreversible modification of the genome; The second is to explore modular delivery platforms such as lipid nanoparticles or viral vector optimization, polymer vectors, etc., to improve the efficiency and tissue specificity of gene editing. The third aspect involves advancing the validation of clinical trials, with a particular focus on long-term safety and efficacy assessments in the treatment of solid tumors. As technology continues to evolve and interdisciplinary collaboration deepens, CRISPR-enhanced CAR-T therapy is anticipated to become a central approach for personalized cancer treatment, offering a curative potential to a greater number of patients.

References

[1] Nishimasu H, Ran FA, Hsu PD, et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell, 2014, 156(5): 935-949.

[2] Ding Y, Li H, Chen LL, Xie K. Recent advances in genome editing using CRISPR/Cas9. Frontiers in Plant Science, 2016, 7: 703.

[3] Cradick TJ, Fine EJ, Antico CJ, Bao G. CRISPR/Cas9 systems targeting β-globin and CCR5 genes have substantial off-target activity. Nucleic Acids Research, 2013, 41(20): 9584-9592.

[4] Fu Y, Foden JA, Khayter C, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature Biotechnology, 2013, 31(9): 822-826.

[5] Hsu PD, Scott DA, Weinstein JA, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nature Biotechnology, 2013, 31(9): 827-832.

[6] Mali P, Aach J, Stranges PB, et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nature Biotechnology, 2013, 31(9): 833-838.

[7] Pattanayak V, Lin S, Guilinger JP, et al. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nature Biotechnology, 2013, 31(9): 839-843.

[8] Tsai S, Zheng Z, Nguyen N, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nature Biotechnology, 2015, 33: 187-197.

[9] Kuscu C, Arslan S, Singh R, Thorpe J, Adli M. Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. Nature Biotechnology, 2014, 32(7): 677-683.

[10] Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. The New England Journal of Medicine, 2018, 378(5): 439-448.

[11] Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. The New England Journal of Medicine, 2017, 377(26): 2531-2544.

[12] Locke FL, Ghobadi A, Jacobson CA, et al. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. The Lancet Oncology, 2019, 20(1): 31-42.

[13] Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor design. Cancer Discovery, 2013, 3(4): 388-398.

[14] June CH, Sadelain M. Chimeric antigen receptor therapy. The New England Journal of Medicine, 2018, 379(1): 64-73.

[15] Pegram HJ, Lee JC, Hayman EG, et al. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. Blood, 2012, 119(18): 4133-4141.

[16] Qasim W, Zhan H, Samarasinghe S, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Science Translational Medicine, 2017, 9(374): eaaj2013.

[17] Newick K, O'Brien S, Moon E, Albelda SM. CAR T cell therapy for solid tumors. Annual Review of Medicine, 2017, 68: 139-152.

[18] Adusumilli PS, Cherkassky L, Villena-Vargas J, et al. regional delivery of mesothelin-targeted CAR T cell therapy generates potent and long-lasting CD4-dependent tumor immunity. Science Translational Medicine, 2014, 6(261): 261ra151.

[19] Rafiq S, Yeku OO, Jackson HJ, et al. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. Nature Biotechnology, 2018, 36(9): 847-856.

[20] Stadtmauer EA, Fraietta JA, Davis MM, et al. CRISPR-engineered T cells in patients with refractory cancer. Science, 2020, 367(6481): eaba7365.

[21] Ren J, Liu X, Fang C, et al. Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. Clinical Cancer Research, 2017, 23(9): 2255-2266.

[22] Liu X, Zhang Y, Cheng C, et al. CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. Cell Research, 2017, 27(1): 154-157.

[23] Li K, Liu Y, Cao H, et al. Interrogation of enhancer function by enhancer-targeting CRISPR epigenetic editing. Nature Communications, 2020, 11(1): 485.

[24] Zheng Y, Nandakumar KS, Cheng K. Optimization of CAR-T cell-based therapies using small-molecule-based safety switches. Journal of Medicinal Chemistry, 2021, 64(14): 9577-9591.