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CRISPR Technology in Tumor Immunotherapy: Application, limitations, and Improvement Methods

Shenghan Gao [1, *], Xuru Miao [2] and Jiaqi Liu [3]

[1] School of Harbin Medical University, Harbin, 150081, China

[2] School of Jinling Institute of Technology, Nanjing, 730050, China

[3] School of Nanjing No.13 High School, Nanjing, 210008, China

* Corresponding author: 2022152521@hrbmu.edu.cn

Abstract. The CRISPR/Cas system is a natural immune mechanism present in prokaryotes, which protects itself by specifically cutting off viral DNA that invades the bacterial body. Under the research of scientists, CRISPR technology has developed into a powerful tool in tumor immunotherapy. It can reduce the inhibitory effect of tumor microenvironment on T cells and enhance tumor immunity by specifically knocking out genes at immune checkpoints (such as PD-1/CTLA-4) on T cells cultured in vitro. However, CRISPR technology also has significant limitations, with the main limitation being its off-target effect - CRISPR is prone to mistakenly identifying and cutting off genes similar to the target gene sequence, including the normal human genome. Once the off-target effect occurs, it will destroy normal genes other than the target gene and cause irreversible damage. Through research, it has been found that CRISPR combined with other technologies can effectively avoid off target effects and reduce treatment risks. This article elaborates on the specific mechanism of CRISPR technology combined with other technologies to avoid off target effects, and explores potential improvement methods.

Keywords: CRISPR screen; tumor immunotherapy; off-target effect; improved method.

1. Introduction

As the biggest threat to human health in the world, the treatment of tumors is still facing a severe situation. The birth of CRISPR technology has opened up a new path for tumor immunotherapy. The main predisposing factors for cancer are mutations and abnormalities in a range of genes [1]. There are many ways to treat tumors, including inhibiting the growth and development of tumors and correcting mutated tumor-associated genes. Currently CRISPR screening technology has been involved in a variety of basic cancer research in order to identify genes that are important for cancer initiation, progression and drug escape [2]. It mainly focuses on the improvement of T cell function, including the generation of CAR-T cells in vitro after the collection of patients' autologous T cells, or the knockout of PD-1 gene before the infusion of T cells to activate T cells [3,4]. For ethical and social reasons, such as considering the patient's own condition, although crisprcas9 technology is not suitable for direct application in human body, it can be reused for perfusion therapy after in vitro editing has been completed . Examples include in vitro editing of Til and CAR-T [5,6]. Although CRISPR technology has shown exciting effect and efficacy in cancer treatment in preclinical research, there are still many issues that need to be addressed before it can be applied to clinical treatments [6]. The biggest impact on the therapeutic effect is the off-target effect, that is, editing the target while editing other parts of the genome, which will reduce the accuracy of gene editing and bring risks. The key to determining the upper limit of CRISPR technology application is to effectively avoid the occurrence of off target effect. Once all these problems are finally solved, CRISPR-Cas9 technology will show exciting potential for clinical applications beyond being limited to a single disease treatment. In this review, we describe the original mechanism of CRISPR, and introduce its application in tumor immunotherapy and the causes of off target effect. Finally, we summarize the most effective methods to reduce the off-target and its mechanism.

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2. CRISPR System and Working Principle

The CRISPR-CAS system is mainly divided into two categories, based on the different roles of CAS proteins in the process of bacterial immune defense.

In the first category: the effectors of their cleavage of foreign nucleic acids are complexes formed by multiple CAS proteins, including type I, type III and type IV.

The second category: uses relatively single CAS proteins as acting factors, such as type V CPF protein and type II cas9 protein. As shown in figure1, the mechanism of CRISPR-cas9 consists of three stages [7]. The first stage is the acquisition of highly variable spacers of CRISPR (capture of foreign DNA, registration of "blacklist") CRISPR works by integrating a small portion of the DNA sequence of exogenous DNA into the host's genome to obtain a high variable spacer, then integrate it between two repeats located at the 5' end of CRISPR. Therefore, the invasion order of the exogenous genetic material determines the order in which their DNA fragments are arranged from 5' to 3' on the CRISPR locus. The acquisition of new interval sequences may be divided into three steps. First, CRISPR sends cas1 and cas2 proteins to scan the invading DNA and recognize the protospacer-adjacent motif (PAM), and then uses the DNA sequence adjacent to the PAM as a candidate protospacer. PAM is similar to ID card, and the protospacer sequence is similar to ID card number. PAM is usually composed of three bases of NGG (n is any base). Cas1 and cas2 proteins are the core proteins that help CRISPR obtain new spacer sequences. In addition, the study found that CSN2 protein in the CRISPR/CAS system is also necessary for the acquisition of new spacer sequences. Then, the cas1/cas2 protein complex shears the protospacer sequence from the foreign DNA and inserts it downstream of the leading region of the adjacent CRISPR sequence with the participation of some enzymes.

Finally, in order to close the open double-strand gap, the DNA will be repaired. This is where the new spacer sequence in the CRISPR sequence comes from. The second stage is the expression of CRISPR sites. Through the regulation of the lead region, the CRISPR sequence is transcribed and produces pre-crRNA while producing tracrRNA. Pre-crRNA pairs with tracrRNA to form double-stranded RNA and assembles into complexes with the cas9-encoded protein. The complex selects the appropriate spacer RNA for different invaders and, with the assistance of RNase III, cleaves this spacer into a short crRNA. Eventually, tracrRNA, crRNA, and cas9 are assembled together to form a shearing complex in preparation for the next shearing.

The third stage is when the CRISPR/CAS system performs targeted interference. The complex of tracrRNA, crRNA, and cas9 precisely attacks exogenous DNA. The complex scans the entire exogenous DNA sequence to identify protospacer sequences that are complementary to the crRNA. At the same time, the complex will replace the PAM/protospacer sequence region, allowing the DNA duplex to unravel and remain in a free state. Then the crRNA will pair with the complementary strand in the double strand, while the other strand will remain free.

Subsequently, the cleavage site of the cas9 protein, located upstream of the PAM, forms a blunt end product. And the HNH domain of the cas9 protein cleaves the DNA strand that is complementary to the crRNA, while the ruvc domain cleaves another non-complementary DNA strand. Finally, the action of cas9 and DNA double-strand breaks (DSBs) silences the expression of exogenous DNA and annihilates invaders in one fell swoop.

Figure 1. Working principle of CRISPR [7].

3. The Principle of CRISPR in Tumor Immunotherapy

Figure 2 illustrates the various immune cells in the tumor microenvironment. The tumor microenvironment contains a variety of immune cell subsets, among which Th1 cells, macrophages, CD8 T cells, and NK cells promote tumor killing, while MDSC, immature DC cells, Treg cells, and TAMs promote tumor escape. So far, CRISPR screening based on CD8 T cells, Tregs, and macrophages has been carried out in tumor treatment, which has provided some help for tumor treatment.

Figure 2. Various immune cells in the tumor microenvironment [1].

As shown in figure 3, it is a pattern diagram of CRISPR screening of tumor cells in vitro and in vivo. Based on the gene pool of interest, the sgRNAs of these genes are designed and transduced into tumor cells by lentivirus, and then the tumor cells are selected by different resistances. In the selected tumor cells, the target genes are processed by CRISPR, and screened and obtained by different tumor cells according to the needs of different cell subsets of interest, such as improving ICB therapeutic ability, NK cell or T cell killing in vitro, tumor sensitive drugs, and reduce tumor metastasis and enhance the sensitivity of tumors to ICB therapy and act therapy in vivo [1]. Finally, analysis is performed to identify latent functional genes.

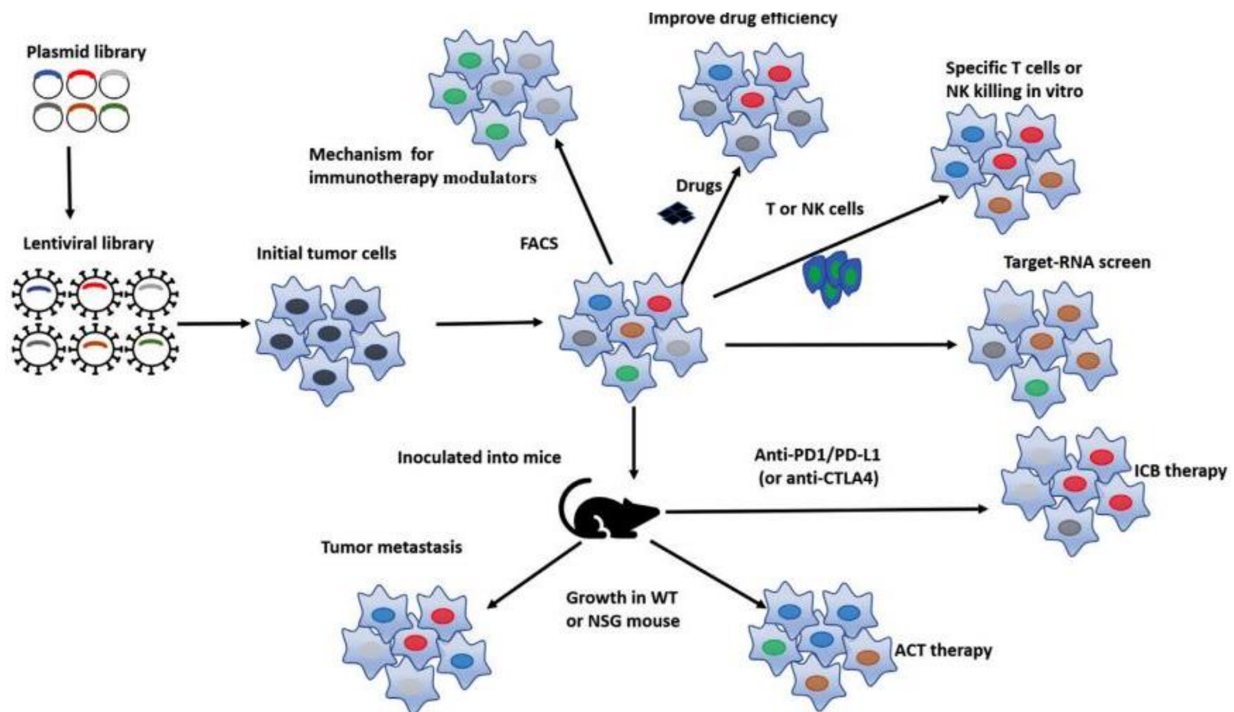

Figure 3. CRISPR designs and prepares chronic disease sgRNAs targeting these genes for the gene pool of interest [1].

4. Off-target Effects of CRISPR

Although CRISPR-cas9 gene editing technology has shown immeasurable prospects, it still has obvious limitations. The most important of these limitations is off target effect [2, 6]. sgRNAs sometimes recognize and bind to gene sequences outside of the target sequence and direct the cas9 protein to edit them known as off-target editing [8]. Improving targeting is a decisive factor for basic research and clinical application.

So why does off target effect occur? CRISPR/CAS system is an innate immune mechanism existing in prokaryotes. It protects itself by specifically cutting off viral DNA that invades bacteria. Viruses are constantly mutating and evolving, and their DNA will also change. In order to track the evolution of viruses and better protect bacteria, CRISPR/CAS system can cut DNA sequences slightly different from target genes, which is beneficial to bacteria, but harmful to human body [9]. Theoretically, after CRISPR-cas9 gene editing cleaves the DNA duplex, the host will attempt to repair, but the repair is highly uncertain. Deletions or insertions of large or small gene segments can lead to unknown changes in the function of the entire genome. And it may lead to serious consequences, such as activation/inactivation of off-target genes, resulting in a lethal or undesirable phenotype, or activation of oncogenes that cause cancer in animals [8].

5. Improved Method of CRISPR Off Target Effect

5.1. CRISPR Hybrid RNA-DNA Guides Technology

The current study shows that CRISPR hybrid RNA-DNA guides technology can significantly reduce the off target effect [10]. By comparing the editing activity and specificity of multiple independent genomic loci cas9 with chRDNA or whole RNA guided in human primary T cells. Among the multiple genomic targets of primary human T cells, researchers have found that 2'- deoxynucleotide (DNT) localization affects guidance activity and specificity in a target dependent manner, which can be used to design chRDNA guidance and reduce off target effects. chRDNA guidance enables highly specific cas9 mediated editing with little interference with targeting efficiency; However, whole RNA guidance will trigger multiple unexpected off target events in the whole genome. This study also showed that in the design of chRDNA, the number and position of DNA nucleotides can be adjusted for each target site to maximize the discrimination between on target and off target sites. Structural analysis of the target binding cas9 chRDNA complex revealed that chRDNA led to changes in the geometry of the interface between chRDNA and target DNA, which was accompanied by structural rearrangement of cas9 protein. These changes slow down cas9 cleavage activity and promote the dissociation of off target substrates.

5.2. Newly Designed SuperFi Cas9

CRISPR-Cas9 targets Cas9 protein to bind to the target DNA sequence through gRNA to cleave the double strand of the target DNA, thereby achieving gene editing. In this process, gRNA containing 20 base pairs identifies the sites that need to be edited by complementary pairing with the target DNA base. Research has found that when gRNA mismatches at the 18th to 20th bases, the pairing structure becomes looser. But Cas9 protein did not stop, but instead firmly bound to this mismatch region through a "finger like structure" (Figure4), stabilizing the RNA DNA double stranded structure at the molecular level, making it appear to be the correct pairing, and then continuing to cleave the DNA, ultimately leading to the occurrence of off target effects [11].The research team redesigned the Cas9 protein by modifying its finger like structure to be away from DNA. In this way, when a mismatch occurs, the Cas9 protein cannot "reinforce" the mismatched structure, thereby avoiding the cleavage and editing of DNA sequences at the mismatched sites, solving the problem of off-target at the molecular level.We refer to the newly designed Cas9 protein as' SuperFi Cas9 [12],which is a high-fidelity protein variant with a probability of being off target about 4000 times lower than natural Cas9 protein, and the editing speed is as fast as natural Cas9 protein, greatly improving the safety of gene editing.

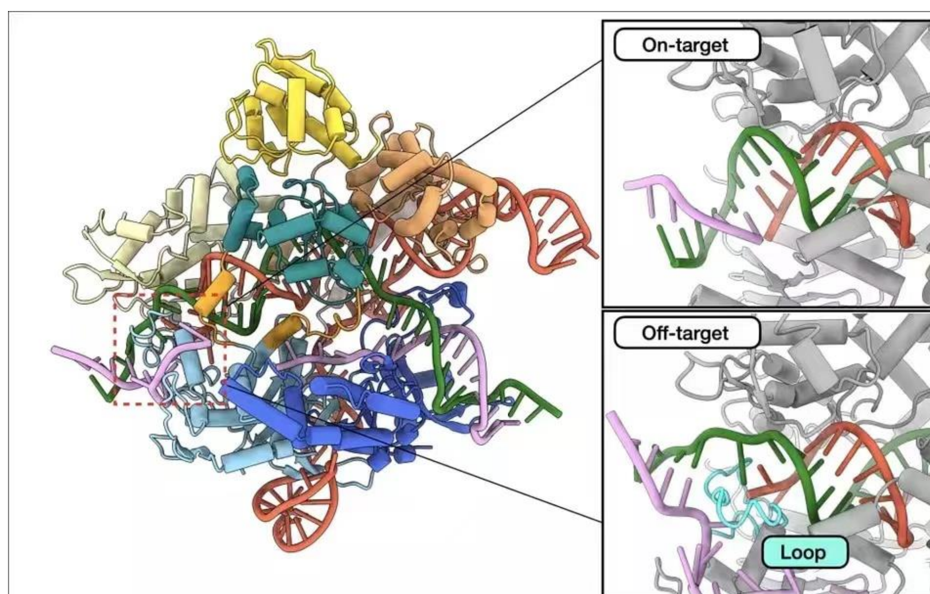

Figure 4. Finger like structure [12]

5.3. CRISPR Combined with Anti CRISPR Protein

Anti CRISPR proteins (also known as Acr family proteins) are a class of proteins evolved by bacteriophages to overcome the bacterial CRISPR system. It can block the function of CRISPR in various ways [13]. For example, AcrIIA4 can block the PAM recognition site by blocking the interaction of Cas9 with the PAM site by inhibiting DNA binding [14]; The interaction of AcrIIC2 with Cas9 inhibited crRNA loading; AcrIIC1 inhibits DNA cleavage by binding to the HNH endonuclease domain of Cas9 [13].

Research has shown that at least 50% of off target effects occur after 6 hours [15]. And as Cas remains active in cells for a longer time, the likelihood of off target editing increases. Adding Acr family proteins after editing CRISPR for 6 hours can block the effect of CRISPR, thereby reducing the probability of off target occurrence. Through experiments, it has been proven that this method can reduce the probability of off target effects by one fourth of the original.

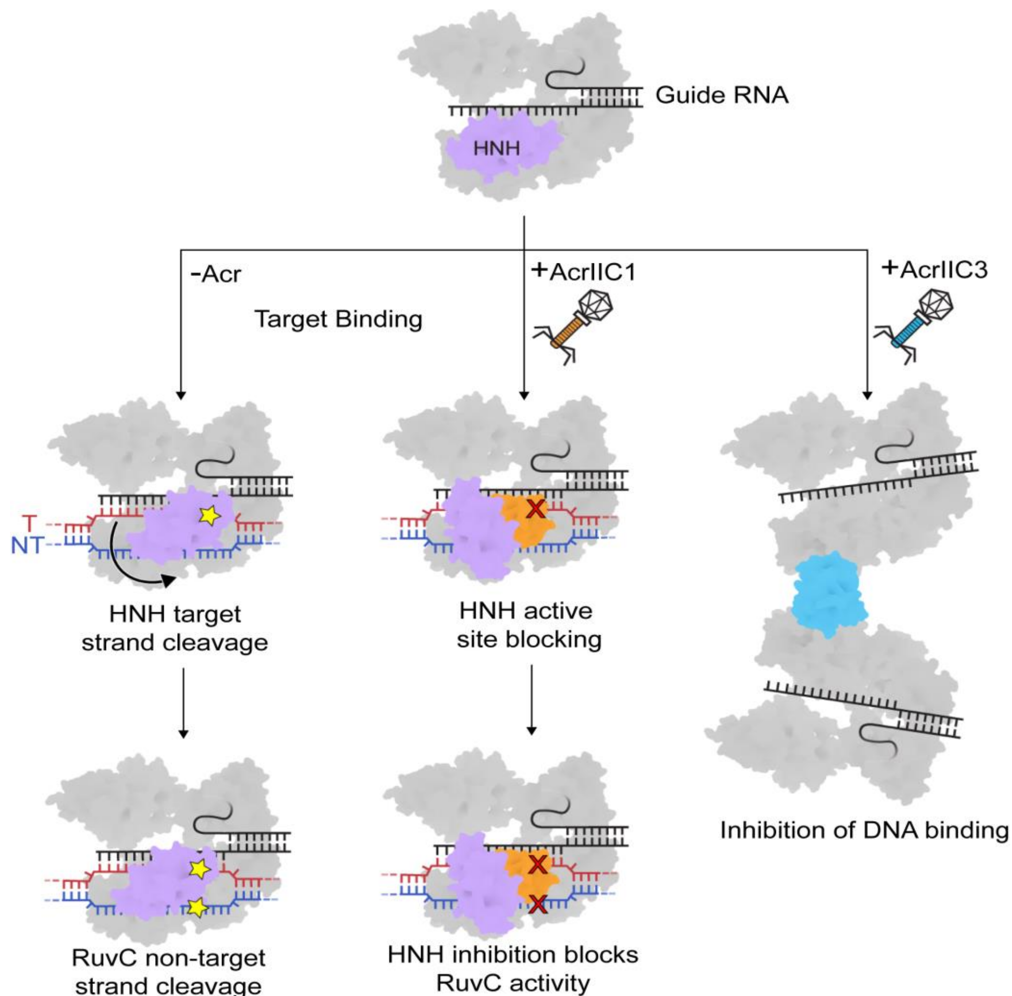

Figure 5. Mechanism of action of AcrIIC1 and AcrIIC3 on inhibition of Cas9 [15].

6. Conclusion

CRISPR technology has taken a big step forward in cancer research, providing significant help in understanding the pathogenesis of tumors and the treatment methods. In particular, it plays a key role in individualized treatment, diagnosis and tumor prediction. But the limitations of CRISPR, especially off-target effects, limit its application to a large extent. To address off-target, scientists are maximizing its specificity through a variety of studies, which are important in research and clinical treatment, and play a key role in ultimately transforming a patient's tumor into a manageable chronic disease. It is hoped that in the near future, scientists will be able to completely eliminate these

limitations, so that CRISPR can help in the treatment of various human diseases without worries and benefit all mankind.

Authors Contribution

All the authors contributed equally and their names were listed in alphabetical order.

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