The application and therapeutic potential of CRISPR/Cas9 technology in diseases

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Abstract. The application of CRISPR technology is one of the focuses of current research orientation. At present, CRISPR technology has reached a new level, but there are still some challenges in the clinical application of human diseases. This paper introduces the background and principle of CRISPR technology and cites two specific diseases for case analysis. It discusses the feasibility, limitations and possible solutions of CRISPR technology in the clinical treatment of actual diseases. Studies have found that CRISPR technology has the ability to cut HIV virus genes that have been integrated into the host genome, which is expected to eliminate the potential viral reservoir to achieve the goal of complete cure. In the treatment of DMD, CRISPR technology can restore the expression of dystrophin, so that the patient can be completely cured. However, safety evaluation is still needed before practical clinical application. Finding alternative vector materials, editing the components of CRISPR system, and maintaining long-term efficacy have become the main problems to be solved in practical applications.

Keywords: CRISPR/Cas9 technology; HIV/AIDS; Duchenne Muscular Dystrophy (DMD); Off-target effect.

1. Introduction

With the development of science and technology, traditional methods of disease treatment have gradually exposed their disadvantages: long treatment cycle and poor treatment effect. In contrast, gene therapy is beginning to be applied to rescue major diseases and human defects, in which the discovery and continuous innovation of CRISPR system is a focus. CRISPR system, also known as Clustered regularly interspaced short palindromic repeats. It was identified in E. coli, and research has shown that CRISPR is a kind of adaptive immune system that exists in bacteria and archaea to protect against foreign mobile genetic elements [1]. The CRISPR/Cas9 technique has made rapid progress because of its simplicity and efficiency for decade. Then it was used in gene editing and treatment of diseases. However, with the continuous development of research, it has also exposed some limitations and defects, such as serious off-target effects and promoter inefficiency. These problems still need to be studied and solved. CRISPR-Cas immunity consists of three main sequence steps: adaptation, expression/maturation, and interference (Fig. 1), each of which requires a specific Cas protein and additional accessory proteins [2-4]. In adaptation phase, it consists of identifying and extracting a protospacer from the invading gene and subsequently incorporating it into a CRISPR array. And the Cas1-Cas2 adaptation complex performs these functions [1]. Protospacer identification begins when PAM is identified by an adaptive complex [3], then a spacer is incorporated and a conserved repeat sequence will be duplicated. The PAM sequence is defined as recognition motifs that can identify target nucleic acids for degradation [3, 5, 6]. In expression/maturation phase, CRISPR array initiates transcription and generates long strand RNA precursor transcript, which is then processed into shorter RNA molecules and cleaved by related enzymes and proteins in bacteria to form mature crRNA. The mature crRNA is composed of a spacer and a portion of repetitive sequences. In the final steps, interference will occur, following the formation of a complex of crRNA with Cas protein, functioning as endonuclease that is guided by RNA. The endonuclease is directed by the crRNA, and once it recognizes the PAM, it binds to the target DNA using its spacer sequence, leading to cleavage of the target DNA sequence [1]. Versatile gene editing technology has great potential in clinical treatment. Committed to technological innovation and upgrading can offer a new

direction in diseases treatment and solve the potential risks of diseases. CRISPR technology is developing rapidly, but it is not without challenges in the direction of disease treatment. In this study, we studied some cases of diseases treated with CRISPR technology. To analyze its advantages and limitations, and seek the feasible development direction of CRISPR technology in the future. This paper reviews the background and principle of CRISPR technique and discusses the therapeutic efficacy and poor prognosis of CRISPR in treating the related diseases. At the same time, we tried to explore the feasibility research direction to solve the adverse effects. In terms of research methods, case analysis was used to find the advantages and limitations of CRISPR technology through case analysis of specific diseases. The study aims at finding the limitations of CRISPR gene therapy and explore new research directions through the practical analysis of CRISPR technology. Furthermore, it aims at discovering the clinical potential of gene editing technology by solving the problems such as CRISPR targeting defects and promoter inefficiency.

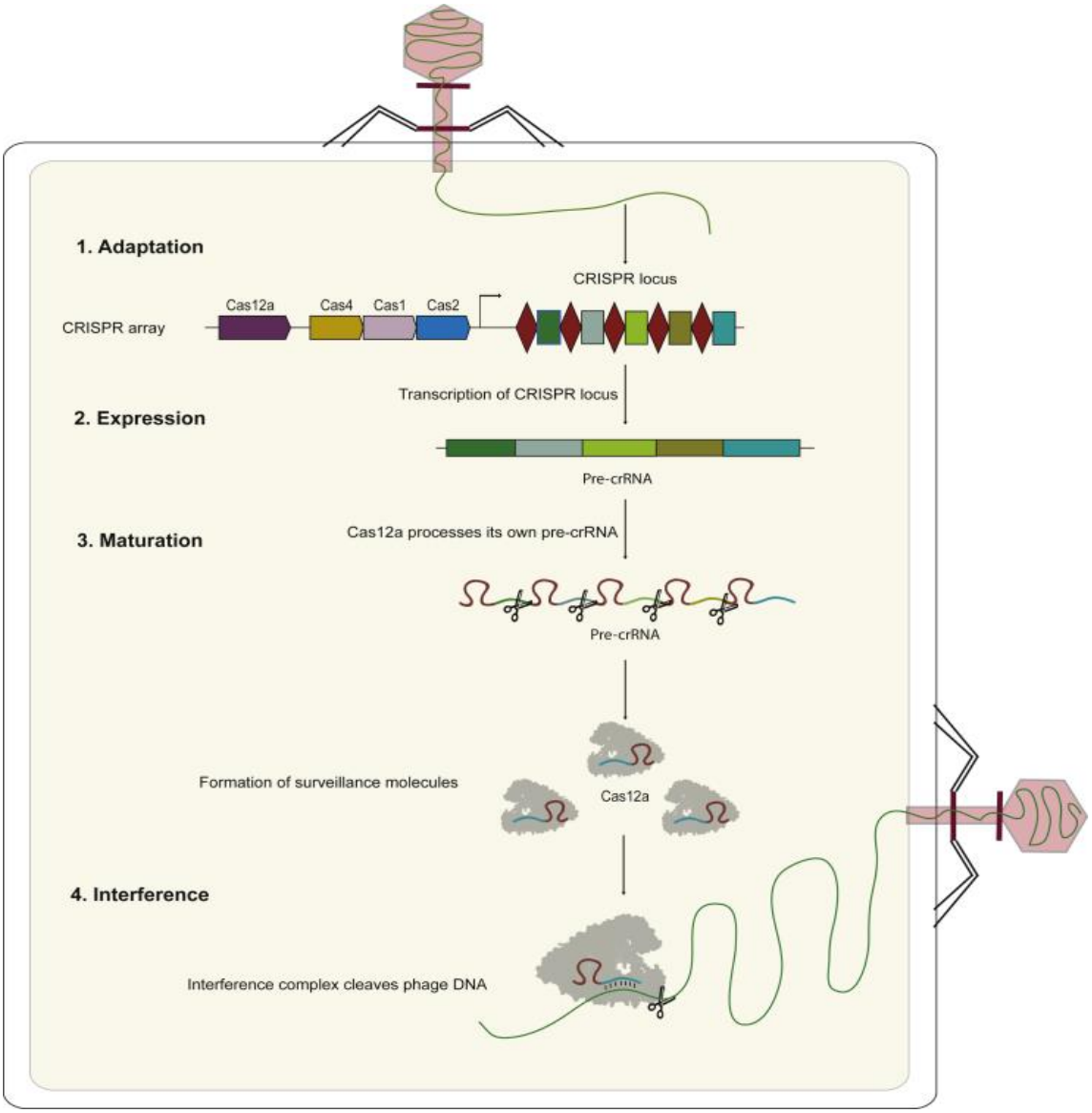


Fig 1. The CRISPR-Cas immune stages: adaptation, expression/maturation, interference [1].

2. Application in diseases

2.1. Applying in HIV-1/AIDS Therapy

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) is a type of human diseases that cannot been completely cured [7]. Statistically, in 2022, 1.3 million new cases

of HIV and 630,000 AIDS-related deaths. By July 2023, Geneva: The Joint United Nations Program on HIV/AIDS (UNAIDS) reports that there are still 39 million people living with HIV globally, 29.8 million of whom are on highly active antiretroviral therapy (HAART). At present, HAART remains a major therapeutic strategy for HIV-1, which can decrease the incidence and death rate of HIV-related diseases. In fact, HAART is unable to effectively remove latent virus reservoirs, rendering AIDS requires long-term and even lifelong treatment [8-10]. In addition, there are many challenges in HIV-1/AIDS treatment, including the high costs, drug toxicity, and tolerance [11]. In recent years, research in the area of genetic engineering has made significant advancements at a fast pace. A variety of nuclease-mediated gene editing tools, led by CRISPR/Cas9, are used extensively to treat HIV. The CRISPR/Cas9, has the merits of accurately inserting, deleting and replacing the target DNA sequences comparing with TALENs and Zinc Finger Nucleases (ZFNs) [12]. In 2013, CRISPR/Cas9 was formally applied to cure HIV-1 infection by interfering the latent HIV-1 virus [13].Following this, CRISPR/Cas9 technology has been applied to study the complete viral genome clearance of HIV-1/AIDS and has shown its great potential.

Research on using CRISPR technology to treat AIDS has been going on for a decade. CRISPR/Cas9 has successfully suppressed HIV-1 gene expression in Jurkat cell lines. Two potential targets for CRISPR technology in treating HIV are the NF-κB binding site and TAR sequences. The NF-κB binding site T6 gRNA also significantly inhibited the activity of the LTR promoter. This effectively inhibits the transcription and replication of HIV-1 provirus. More importantly, the CRISPR/Cas9 technique is able to remove the integrated virus gene from the host cell chromosome. These specially designed nucleases are able to identify and cut particular nucleotide sequences within target genomes, leading to the generation of different types of mutations, including substitutions, deletions, and insertions caused by the host's repair mechanisms [13]. The Cas9/gRNA system was utilized by researchers to target the HIV-1 LTR U3 region. This will lead to the restricted viral gene expression in infected host cells, such as T cells while minimizing genotoxicity. Furthermore, no off-target editing was detected [14]. Research found that improving the efficiency of excision and destruction of provirus genomes can be achieved by increasing the targeting sites within the HIV-1 genome [15]. Additionally, when two sgRNAs were used as cleavage tools, viral replication was effectively inhibited, and at the same time, the possibility of virus escaping cleavage was reduced [16]. CRISPR/Cas9 editing induced by a single sgRNA can also result in inactivation of HIV-1 proviral mutations, but the virus is able to evade cleavage mediated through a single gRNA. The cleavage efficiency of double sgRNAs was higher than that of single sgRNAs on the HIV-1 reporter plasmid that is not integrated. Combination therapy has greater potential and prospects in the clinical treatment of HIV-1/AIDS in the future.

The primary challenge in utilizing CRISPR for HIV/AIDS treatment lies in the potential off-target impact, which has the potential to induce genetic mutations or chromosomal translocations within the host cells. In the clinical application of CRISPR, experiments find that the off-target phenomenon cannot be avoided even if six or more sgrnas are used. At present, there are three main methods to solve the off-target effect. The first one is to design gRNA appropriately. Secondly, the efficiency and accuracy of cleavage can be influenced by the type of nuclease chosen. Opting for high-fidelity Cas9 nuclease variants, such as HypaCas9, can effectively minimize off-target cleavage occurrences without compromising on-target editing capabilities. Different types of CRISPR nucleases are shown. Finally, the timing of CRISPR nuclease exposure can also influence off-target effects. The investigators manipulated the CRISPR/Cas9 system by inserting the Cas9 gene into a minimal HIV-1 promoter. This strategy enabled infected cells to express appropriate levels of Cas9 protein and reduced the problem of Cas9 excess [17]. After editing the target DNA, Cas9 rnp is degraded, which can minimize the off-target effect.

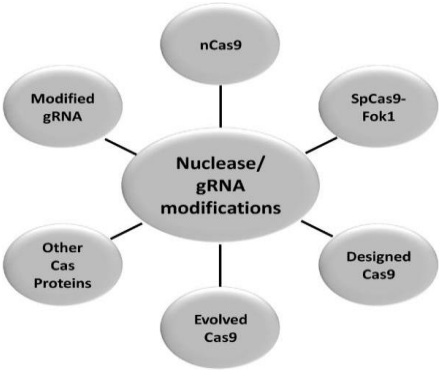


Fig 2. Summary of gRNA and Cas9 proteins that were designed and edited [18].

Another significant obstacle in the use of CRISPR/Cas9 is finding an effective method to deliver this complex into cells infected with HIV-1. At present, the primary delivery vehicles consist of adenovirus, lentivirus, and adeno-associated viral vectors. Adenoviruses have the ability to incorporate large DNA fragments, but the generation of recombinant adenoviral vectors faces many problems. Lentiviral vectors can effectively transport the CRISPR/Cas9 system into cells with long-lasting expression, although they may potentially elevate off-target effects. Adeno-associated viral vectors are known for their safe, minimal toxicity, and effective delivery capabilities [19]. However, it cannot accommodate large foreign genes and may induce immune responses.

So far, the CRISPR/Cas9 technique has been widely used in the inhibition of HIV-1 infection. Restriction factors can inhibit HIV infection, for example, the recently discovered restriction factor, serine binding factor 5 (SERINC5), which inhibits virus infection by blocking viral and cell fusion [20]. By using CRISPR/Cas9 technique, the expression of various restriction factors in infected cells can be activated to function during different viral infection stages, and it may even produce functional coupling to enhance the therapeutic effect. This idea may offer a novel strategy for treating HIV-1.

2.2. Applying in DMD Therapy

Duchenne muscular dystrophy (DMD) is a debilitating disease that worsens over time and ultimately leads to death. It is characterized by the loss of skeletal muscle, difficulty breathing, and heart muscle problems [21]. The genetic basis for this is a mutation in the dystrophin gene, leading to the abnormal expression of dystrophin protein, which locates at the X chromosome exclusively [22]. As there is only a single X chromosome in the male body, it has a high incidence in the global male population. DMD occurs in childhood and mainly generates from gene out-of-frame mutations that lead to muscle degeneration. Subsequently, because the body does not have a functioning dystrophin, the patient is unable to walk upright, and is prone to cardiomyopathy, with a high mortality rate in early adulthood. The structure of the Dystrophin protein structure is shown in Fig .3. Although the use of corticosteroids can reduce the symptoms of DMD, it cannot be cured completely [23]. Treatment of DMD with CRISPR is expected to become the mainstream method of clinical treatment in the future. CRISPR technology can completely cure DMD by permanently correcting DMD mutations and restoring the open reading frame (ORF) that can produce functional dystrophin.

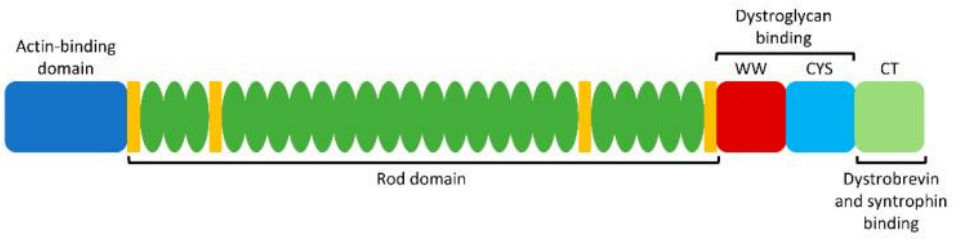


Fig 3. Dystrophin is composed of four separate functional sections. The repetitive regions within the central rod domain can be removed through clipping. It expressed dystrophin with unchanged function but shortened length. In the redundant domain, exons 45-55 are more prone to mutation [24].

Recombinant adenovirus is often used as a vector for the delivery of CRISPR/Cas9 gene editing components. Successfully transformed Cas9 proteins can cause genes to generate DSBs, and two major endogenous repair pathways, non-homologous end joining (NHEJ) or homologous directed repair (HDR), can repair the cleaved fragment. HDR has the advantage of precise editing, but this repair modality cannot repair large deletions on multiple exons. Therefore, the practical application of NHEJ is more extensive, leading to the creation of small insertions or deletions. And there are various mechanisms which can facilitate the restoration of dystrophin expression [23, 25, 26].

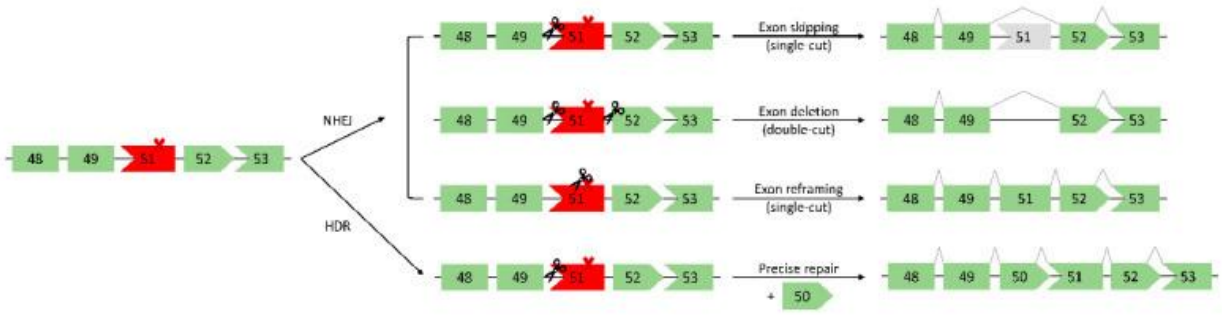


Fig 4. Two major endogenous repair pathways for repairing DSBs: NHEJ and HDR [24].

2.2.1. exon skipping (single cut)

In exon skipping, the acceptor and donor sites of the exon are the cleavage site and the sgRNA is the tool [24]. The sgRNA/Cas9 complex cleaves at the exon junction, resulting in skipping the current exon and splicing to the next available exon. This method reduces the vector requirements that package and deliver compared with double-cut approaches. In addition, the single induction of DSB production reduces the possibility of total DNA aberrations or deleterious mutations.

2.2.2. exon deletion (double cut)

Because most DMD patients have multiple exon deletions, the multiple exon deletion strategy is mainly applied to the exon 45 to 55 mutation region. The double-cut exon strategy, which cuts both sides of the exon simultaneously, removes multiple exons, but the resulting dystrophin protein may be shorter. But it has a high off-target rate because of the use of two sgRNAs. At the same time, the two DSBs generated after cleavage carry the risk of mutation or rearrangement. In addition, efficiency and delivery difficulty also need to be considered.

2.2.3. exon reframing (single cut)

Single exon rearrangement attempts to recover an ORF by producing small indices. This approach is able to preserve the large dystrophin protein but produces insertions or deletions with only a one-third chance of successful restoration of the dystrophin gene. Exon rearrangement also needs one sgRNA [24]. Because exon rearrangement generates small indels, it has a high possibility to maintain the maximum genomic sequence of dystrophin and avoid the DMD mutation [23].

CRISPR/Cas9 therapy has the potential to completely cure DMD, but there are also some challenges. More research and experimentation are still needed before clinical translation. A major concern with CRISPR therapy is delivery vector capacity. The intrinsic size of SpCas9 is already close to the inherent packaging limits of AAVs. Delivering the components into host cells requires individual delivery with multiple AAV vectors. This requirement adds difficulty to delivery. Apart from size constraints, the virus vector and the CRISPR system components can trigger an immune response. Furthermore, the accumulation of AAV vectors in the body can lead to potential side effects, and it is difficult to maintain the long-term DMD normal. Satellite cells do not usually undergo CRISPR/Cas9 modification, so the modified nucleus can be diluted during the formation of new muscle cells [27]. The immune response generated by the AAV vector would preclude secondary treatment with CRISPR. Therefore, CRISPR/Cas9 therapy can only be used once, and how to increase the long-term efficacy is the challenge.

In terms of vectors, lipid or gold nanoparticles are expected to be alternatives to viral vectors. Nanoparticles can be taken up by endocytosis, which has the advantages of reducing the risk of off-target mutations, being cost-effective, and being less immunogenic [24]. Modifying the Cas9 protein can reduce the immune response elicited by the internal components. Epitope modification of Cas9 enzyme can reduce the likelihood of recognition by the immune system of DMD patients. In addition, studies have shown that some patients with DMD are treated with corticosteroids as an anti-inflammatory therapy, which may inhibit a mild immune response.

3. Summary

Through research, this paper finds that CRISPR technology has the potential to completely cure HIV and DMD. At present, the difficulty of HIV cure lies in the inability to eliminate the latent virus reservoir. As a "gene scissor", CRISPR can be incorporated into a host cell's genome, targeting the integrated viral genes, cutting and degrading the viral sequence, so as to achieve the purpose of a complete cure. However, the off-target problem of CRISPR has been criticized for a long time and is still difficult to solve. Off-target phenomena may cause adverse reactions and even genomic mutations. Therefore, safety in clinical use needs to be fully evaluated. It can be promising for using the CRISPR technique in DMD rescue. The mutated dystrophin gene can be expressed normally after being cleaved by CRISPR/Cas9 complex. Experiments have proved that only restoring part of the dystrophin gene can make the level of dystrophin in the body meet the demand, and maintain the body walking and normal life. The recovery of cardiomyocytes has prolonged the life span of patients. But the renewal of cells in other parts of the body, such as skeletal muscle, makes long-term maintenance a new problem. Limited-dose CRISPR/Cas9 therapies need to be considered to increase long-term efficacy. This article presents two case studies of using the CRISPR technique to treat human diseases, showing the possibility of CRISPR to cure incurable diseases. In addition, it also puts forward possible directions for how to improve and promote CRISPR technology in the future: Alternative materials for vectors and design of internal components of the CRISPR system, respectively. In the future, continuous innovation and improvement in CRISPR technology will make it possible to completely cure various diseases.

4. Discussion

Since the CRISPR system was discovered in the E. coli, it has subsequently become a major tool for gene editing. Recently, it is believed that the CRISPR technique will be able to completely cure some incurable diseases. However, the off-target effects and delivery difficulty of CRISPR system are the major problems that need to be solved. For off-target effects, gRNA was designed to reduce the number of mismatched bases. Combination therapy with other drugs or gene editing methods can also be used to reduce the occurrence of off-target phenomena. To solve the delivery problem, the appropriate vector needs to be selected, and lipid vectors have the potential to replace viral vectors.

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