Application of CRISPR-Cas System in the Treatment of Human Viral Disease

Weijia Lin*

Xiamen No.1 High School of Fujian, Xiamen, 361001, China

Abstract.. CRISPR-Cas systems, consisting of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas), are the latest generation of gene editing technology and have been widely used in molecular biology research. CRISPR-Cas systems also have unlimited potential in the field of medicine, especially in the treatment of human viral diseases, such as blocking virus invasion, interfering with virus replication, and eliminating viral genome and sequelae of virus infection. In this article, the latest research progress of CRISPR-Cas9 system and other CRISPR systems in treatments of several viral diseases are reviewed. In addition, the advantages and potential problems of CRISPR systems as treatment options are analyzed to provide ideas for subsequent related research.

1. Introduction

Researchers have further demonstrated their close association with the adaptive immune system in prokaryotes since clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) were discovered in 1987 by scientists in Osaka [1]. On that basis, scientists developed a new gene-editing technology, CRISPR-Cas systems, which is now widely applied. The CRISPR-Cas system, except for gene editing, shows multiple functions, for instance, targeted silencing in the transcription of prokaryotic and eukaryotic cells, namely CRISPR interference (CRISPRi), which has enriched the research technology of molecular biology and been applied to various types of research, providing a complementary technology to RNA interference (RNAi) [2].

CRISPR-Cas systems are composed of single guide RNA (sgRNA) and Cas protein in molecular technology. The most classic and commonly used system is the CRISPR-Cas9 system, and the gene editing functions of CRISPR-Cas system are used through the Cas9 protein. Cas9 protein has endonuclease activity. When guided by sgRNA, Cas9 protein recognizes and binds to the target site, thus cleaving the target site and creating double-strand DNA breaks (DSBs). Editing of any specific sequence can be achieved through homology-directed repair (HDR) or nonhomologous end-joining (NHEJ) of cells If the catalytically inactive Cas protein is used, the elongation of RNA polymerase transcripts is prevented by steric hindrance because of the binding of Cas-SgRNA complex to DNA elements, thus the transcriptional repression of specific genes can be achieved. In addition, other Cas proteins can cleave RNA, such as Cas13, constituting other CRISPR-Cas systems. This system is simple in design and widely applied in biomedicine.

The viral disease is one of the major diseases threatening human health and an important research area in biomedicine. Up to now, scientists have utilized CRISPR-Cas system to provide novel treatments for diseases such as viral infections. In this article, the application of CRISPR-Cas system genome targeted editing and transcriptional inhibition in viral diseases, as well as its research progress are reviewed to provide a reference for better exploring the potential of this technology in disease treatment.

2. Application of CRISPR-Cas9 in Virus Infectious Diseases

2.1 Human Papilloma Virus (HPV)

The incidence of cervical cancer is high around the world. According to the World Health Organization, there were about 600,000 new cases and 340,000 deaths in 2020 [3]. Cervical cancer shows the highest incidence rate in women in 23 countries, as well as the leading cause of death in women in 36 countries [4]. In recent years, the incidence and mortality rates of cervical cancer have shown an increasing trend among young women. The

* Corresponding author's e-mail: 451627649@qq.com

occurrence of cervical cancer is closely related to the infection of HPV. HPV is a kind of non-enveloped and double-strand DNA virus with epithelioid properties and has a circular genome of about 8,000 bp. Some subtypes of HPV can be randomly integrated into host cells genome when immunity function lessening. Among them, the E2 region is the region has the highest frequency of integration between the virus and the host cell genome occurs. It is also the region where deletion or breakage is most likely to occur during the integration. The disruption of the E2 gene induces overexpression of E6 and E7 genes, which in turn induces cell canceration.

To disturbing deterioration of HPV, the CRISPR-Cas9 system can be used to block the expression of E6 and E7 proteins by targeting the gRNA of E6 and E7 genes, thus restoring the normal activity of cancer suppressor gene p53 and pRb, and causing apoptosis of cancer cells. At the same time, CRISPR-Cas9 cuts the viral E6/E7 genes and blocks the replication and proliferation of the virus, which is conducive to the effective elimination of HPV by bodies.

Mcmillan et al. [5] used polyethylene glycol (PEG)-based liposomes to deliver non-viral CRISPR-Cas9 against tumors and found a significant decrease in tumor volume compared to negative controls after 46 days of detecting tumor volume. The immunohistochemical staining of tumor tissue showed a significant decrease in cancer cell activity, which demonstrates that CRISPR/Cas9 treatment can eliminate tumors in the body. However, due to the small size of such liposome vectors, it is not possible to deliver all CRISPR/Cas9 system components in one vector. Therefore, Eric et al. [6] transformed non-viral CRISPR into an integrated delivery system, namely high-capacity adenovirus vector (HCAdV), and constructed a CRISPR-Cas9 system with complete expression, which contains Cas9 protein and gRNA against HPV16-E6 or HPV18-E6. The HPV-specific HCAdV-CRISPR was also used to transfect the HPV-positive cervical cancer cell lines HeLa, SiHa, and CaSki and the HPV-negative lung cancer cell line A594. The results showed that CRISPR successfully induced the predicted target site mutations in HeLa and SiHa cell lines compared with HPV-negative cell lines. Further research showed that HPV16-E6 and HPV18-E6-specific CRISPR-HCADV significantly inhibited the proliferation of HPV-positive cell lines. In addition, Western Blot analysis showed that p53 content in HPV-positive cell lines obviously increased, while the A594 cell line showed no change. All these suggest that this system can be used as a specific treatment for cervical cancer caused by HPV infection.

Zhen et al. reported that HPV16 E6/E7 CRISPR-Cas9 was used as the sensitizer in combination with cisplatin [7], and in vivo experiments showed that the survival rate of cells with E6/E7 CRISPR-Cas9 alone was 100% and the relative survival rate of cells with cisplatin plus E6/E7 CRISPR-Cas9 was 20%, which significantly inhibited the cell growth. In another research by Zhen et al., researchers found that HPV16 E6/E7 CRISPR-Cas9 in combination with radiotherapy [8] also provided better tumor inhibition than that in CRISPR-Cas9 or radiotherapy alone. Thus, CRISPR-Cas9 has the potential to be an effective mean of treating HPV-infected disease, either as a single treatment or in combination with conventional treatment options.

2.2 Human Immunodeficiency Virus (HIV)

HIV is a kind of retrovirus which could attacks T cells in the immune system. After T cells are destroyed in large numbers, cellular and humoral immunity cannot be carried out properly, thus leaving bodies without protection from immune system and leading to acquired immune deficiency syndrome (AIDS). HIV has the common characteristic of retrovirus and can integrate into the host DNA to become a provirus. It also has an incubation period, during which DNA copy of the provirus is dormant. However, when T cells are activated, they can reproduce replication-competent viruses. Hence, current mainstream treatment, namely active anti-retroviral therapy (ART), is ineffective in eradicating HIV and prones to appear recurrence after treatment interruption. CRISPR-Cas9 system can remove the DNA copy of the provirus integrated into the host cell, providing a new idea to address the relapse of AIDS and prevent AIDS.

Rafal et al. [9] tested the ability to eliminate HIV-1 gene copies in the human T-lymphocyte cell line 2D10 with the CRISPR-Cas9 system that could recognize a specific DNA sequence within the HIV-1 promoter that spans the 5' long terminal sequence (LTR). This cell line contains a complete copy of a single HIV-1 and a gene encoding the green fluorescent protein (GFP) tagged to replace the latent Nef protein. 2D10 cells treated with pro-inflammatory agents and the untreated group represented the activation and latency states of the virus respectively. The results showed that the percentage of GFP-positive cells in the latent virus group not expressing gRNA was 1.28%, while it decreased to 0.4% in cells expressing gRNA. The contrast was even more obvious in the activated virus group, where the percentage of GFP-positive cells decreased from 94.1% to 0.9% in gRNA-expressing cells compared to non-expressing cells. Subsequent PCR results also confirmed the effective elimination of HIV-1 genomic DNA in cells containing the intact CRISPR-Cas9 system.

The research of Hsin-Kai et al. [10] showed two possible mechanisms for Cas9-mediated inactivation of viral gene expression. First, Cas9 can directly target and destroy viral genomic DNA after the viral reverse transcription forms dsDNA but before it is integrated into the host genome. Second, excision of the integrated proviral DNA. In subsequent experiments, exploration of the time course also showed that despite transient expression, CRISPR/Cas9 can mediate permanent inhibition of viral expression through provirus disruption on the DNA level, unlike other methods of transient inhibition for viral expression (e.g. RNAi).

In addition, the neutralizing antibody that can produce HIV is one of the effective means of preventing ADIS. However, how to induce specific and neutralizing antibodies to highly variable viruses is a long-standing

challenge in medicine. And B-cell engineering based on CRISPR-Cas9 technology provides an effective means for this treatment. Alessio D. Nahmad [11] et al. demonstrated the feasibility of this approach through in vivo experiments in mice. Two kinds of recombinant adeno-associated viruses (AAVs) were designed in the experiment: First, CRISPR-Cas9 gene editing systems, including CMV/SFFV promoter-driven SaCas9 and U6 promoter-driven sgRNA. Second, the HIV-bNAb-3BNC117 homologous directional repair template (HDRT) for targeting the immunoglobulin gene. The experimental data showed that both B cells and non-B cells can express the relevant antibodies on the membrane after the CRISPR-Cas9-mediated targeted integration, for example, the system can accurately introduce antibody genes to the desired sites in B cells genome. However, only B cells are able to proliferate after antigen binding. Follow-up tests showed that all treated model animals responded with a significant amount of the required antibodies in their blood. By extracting the antibodies from the blood, the scientists found that they were effective in neutralizing the HIV virus in culture dishes. This demonstrates the feasibility of in vivo B-cell modification engineering for the prevention of HIV infection and the elimination of HIV viral particles from the bodies.

2.3 Hepatitis B Virus (HBV)

HBV is the predominant pathogen of liver disease around the world. In recent years, there have been about 500,000 new cases and 820,000 deaths per year [12]. The HBV genome is a circular DNA molecule with a gap, and when HBV enters the cell, the viral genomic DNA is transported into the nucleus and repaired with the help of viral proteins and host cell factors into covalently closed circular DNA (cccDNA). cccDNA is the template for the replication of viral pregenomic RNA and is highly stable.cccDNA will continue to replicate and produce new HBV viral particles as long as it is in the nucleus of hepatocytes. This mechanism is the main reason for the rebound of HBV levels after general antiviral treatment. Therefore, the basic idea for the eradication of HBV is to remove cccDNA.

Ramanan et al. [13] from the University of Cambridge designed 24 sgRNAs targeting the HBV genome and screened the three sgRNAs (sg6, sg17, and sg21) that inhibited viral proliferation most significantly. Subsequently, the functional HBV integration form of Cas9's Cas9-2A-Puro lentivirus transduction and cccDNA's HepG2.2.15 cells were used for further experiments. The results showed that Cas9/sgRNAs strongly inhibited HBV DNA release (77-95% reduction in different sgRNAs), HBeAg secretion, and viral mRNA production (> 50%). Later, the experiment was conducted immediately after HBV infection to verify whether Cas9 acts directly on free cccDNA. Meanwhile, the levels of both HBV surface antigen and cccDNA were significantly reduced. These results suggest that the CRISPR-Cas9 system can target the free virus and exert anti-HBV effects through direct cleavage of cccDNA.

To better deliver CRISPR-Cas9 components into hepatocytes, Jiang et al. [14] developed an optimized formulation of lipid-like nanoparticles (LLNs) and injected LLNs with Cas9 mRNA and sg21 by caudal vein. The results showed that the efficacy of Cas9+sg21 LLNs in reducing HBV viral load was comparable to previous research with the hydrodynamic injection of plasmids of Cas9 and sg21. By sequencing the first three predicted off-target sites, the scientists evaluated the potential off-target effect of Cas9+sg21 TT3 LLNS treatment and found no significant insertional deletions, which suggests a low off-target rate. It demonstrates that the optimized TT3 LLNS can effectively deliver Cas9 mRNA and sgRNA to mouse livers. LLNS-based delivery was highly specific for the liver [15], which shows that LLNS-mediated CRISPR/Cas9 delivery is expected to be a safe and effective option for the treatment of HBV.

3. Application of Other CRISPR Systems

In addition to the CRISPR-Cas9 system, other CRISPR-Cas systems composed of other Cas proteins also hold great promise in the treatment of viral diseases. For example, Cas13, an RNA-guided RNA endonuclease that specifically cleaves single-strand target RNA under the guidance of crRNA and can remain active and cleave other non-target RNAs after completion of cleavage, which can be used to fight against RNA virus. Cas12, which can recognize target dsDNA under the guidance of crRNA only, targeting the protospacer adjacent motif （PAM） site rich in T cells, it can specifically cleave target sequences and incidental non-specific ssDNA activity and can be used as a novel molecular diagnostic technology that gives rapid results for virus detection and typing.

3.1 Prevention and Treatment of CRISPR-Cas13 for COVID-19

At present, COVID-19 is one of the most worrisome epidemics around the world. SARS-COV-2, as an RNA virus, has increased high variability and potentially enhanced antibody dependence, and poses great challenges for vaccine development. Therefore, it is critical to discover new antiviral drugs across multiple strains.

The CRISPR-Cas13 system has great potential in the field of anti-SARS-CoV-2. Cas13 is a kind of CRISPR-associated proteins with RNA enzyme activity in targeting RNA fragments that cleave specific sequences. Blanchard et al. [16] from the Georgia Institute of Technology and Emory University in the USA developed a new treatment with the CRISPR-Cas13 system, which can stop the replication of SARS-CoV-2. More importantly, it can deliver the drugs to lungs through the nebulizer, which makes it possible for patients to be treated easily at home. In the research, the team introduced Cas13a mRNA into host cells for expression, as well as designed crRNA. The experimental results showed a significant reduction at 57% in the copy number

of SARS-COV-2 in animals treated with targeting N2.3 (nucleocapsid) compared to the non-targeted crRNA group and the virus-only group, and all crRNAs targeting other sequences also had a treatment effect of inhibiting viral replication. It shows that the Cas13a protein can be guided by crRNA to destroy the RNA replicase of the virus and mRNA of other important proteins, thus effectively blocking the proliferation of the virus in the host cell.

The anti-SARS-CoV-2 strategy of the CRISPR-Cas13 system is able to target not only the viral genomic RNA but also the virus-associated receptors of the host cell. The entry of SARS-CoV-2 into the host cell requires the help of a host cell-specific protease, namely cathepsin L (CTSL). Therefore, the inhibitors of this enzyme can be used for SARS-CoV-2 treatment. However, scientists have not found good inhibitors that can be used for such treatment. CRISPR-Cas13 system provides a new idea from the perspective of CTSL expression. Cui et al. [17] from Duke University used an encapsulated CRISPR-Cas13d chemical engineering nanometer system which contains the mRNA of lung tissue CTSL that could target to reduce CTSL expression in the lungs of normal mice effectively, safely, and specifically, thus creating an environment in the lungs unsuitable for SARS-Cov-2 infection, blocking SARS-CoV-2 infection in mice, reducing viral infection in virally infected mice and prolonging the survival of mice infected with lethal SARS-CoV-2.

3.2 Rapid Detection of CRISPR-Cas12 for COVID-19

Molecular detection technology has always played an important role in disease prevention and public health. In 2003, the SARS epidemic prompted RT-qPCR technology to become the most important standard for molecular detection. The recent outbreak of COVID-19 is more potent and insidious, which has posed new challenges to traditional molecular detection technology. Traditional detection technology including RT-qPCR, isothermal PCR, and sequencing all have their own limitations. RT-qPCR and sequencing techniques have high requirements in equipment and operator, and isothermal PCR is prone to non-specific amplification, resulting in false positives. Therefore, there is an urgent need to develop a novel molecular detection technology that is easy to operate, rapid, sensitive, and inexpensive to address the strong demand for molecular detection technology under COVID-19. The potential of the CRISPR-Cas12 system in providing a novel molecular detection technology has attracted the attention of scientists. In 2016, researchers discovered that the Cas12 and Cas13 families possess the ability of collateral cleavage (or cleavage in trans). This means that after the Cas protein-crRNA binary complex recognizes and binds substrates, they could cleave not only substrates but also arbitrary substrates that are free within the environment [18]. The principle of CRISPR-Cas12-based molecular detection is as follows. The substrate DNA or RNA is first amplified by recombinase polymerase amplification (RPA) or reverse transcription RPA (RT-RPA) to increase the substrate concentration. Mix with Cas12 and crRNA reaction solution. When the Cas12-crRNA complex recognizes the substrate DNA, cis and trans cleavage occurs and collateral cleavage breaks the ssDNA cleavage reporter within the system. The reporter 5′ end is attached to a chromophore (e.g., FAM) and the 3′ end is attached to a fluorescence quenching group, (e.g., BHQ) thus the reporter molecule does not fluoresce when it is not broken. After breaking, the fluorescence and quenching groups are separated and are able to emit a fluorescent signal [19].

Numerous researchers validated the true effectiveness of this molecular detection method in the diagnosis of SARS-CoV-2 in the recent COVID-19 test. Lucía Ana Curti et al. [20] used the Lyo-CRISPR SARS-CoV-2 kit to detect nose swabs. The overall Kappa index was estimated to be 0.991 compared to the results of RT-qPCR, reflecting an almost perfect agreement between the two detections. Cross-reaction with other respiratory pathogens was also not detected in subsequent experiments. Positive results were still detected in the dilution test, demonstrating the sensitivity of the CRISPR-Cas12 system in molecular diagnosis. Compared with conventional RT-qPCR, the CRISPR-Cas12 system kit is more time-saving, easier to operate, and less prone to show false positives. It is expected to replace traditional nucleic acid testing in the COVID-19 epidemic, achieving in time reduction and cost savings in nucleic acid testing.

3.3 Replication and Inhibition of CRISPR-Cas13b for Zika Virus

Zika virus (ZIKV) is a single-strand RNA virus that is transmitted through mosquito bites. From 2015, ZIKV crossed the Pacific Ocean and caused more than 1 million cases of infection in Brazil. As of May 2019, the virus spread rapidly to 84 countries and regions and has become a worldwide public health problem. During the ZIKV pandemic in 2015 in Brazil, many newborns with microcephaly were found, which is thought to be caused by ZIKV infection. ZIKV may also cause Guillain-Barré syndrome, leading to neurological and immune system complications. However, there is no effective drug or vaccine, and the placental barrier poses a great challenge to the drug research process. Fortunately, the CRISPR-Cas13 system provides a solution for the inhibition of ZIKV.

Chen et al. [21] demonstrated the ability of CRISPR-Cas13b against ZIKV in 293T cells through a series of experiments. First, an efficient ZIKV infection system, namely the 293T cell line, was designed in the experiment. Second, a CRISPR-Cas13b expression system with Cas13b fused to GFP was constructed and crRNA was designed based on 1138 highly conserved regions of the ZIKV genome sequence. The researchers used the ZIKV-mCherry fluorescent reporter gene to characterize the antiviral activity of CRISPR-Cas13. Next, the researchers also evaluated the efficiency of crRNAs to interfere with ZIKV in 293T-DC-SIGN cells and found that the crRNAs were significantly effective in the replication and

inhibition of ZIKV. It illustrates the great potential of the CRISPR/Cas13b system for treatments of ZIKV infection.

In addition to the Cas13 and Cas12 proteins mentioned above, Cas14 with a smaller molecular weight, it has greater specificity for ssDNA recognition and is not restricted by PAM sequences, exhibiting great potential in molecular diagnosis. Due to its small size and ease of delivery, it also leaves room for the delivery system to install other regulatory elements, making gene editing more efficient [22]. There is even a Cas3 system that can perform large segmental elimination in embryonic stem cells, which holds great promise for the removal of ectopically integrated recalcitrant viruses such as HSV, EBV, and HBV.

In recent years, several research teams have regulated the transcription of target genes by fusing the mutationally inactivated Cas9 (dead Cas9, dCas9) with other functional proteins. When dCas is fused to a structural domain belonging to a variety of transcriptional repressor proteins, namely the Kruppel association box (KRAB), it is possible to inhibit the transcription of specific genes on the genome based on sgRNA, a system called CRISRPi. When dCas9 is fused to Suntag, the fusion sequence contains multiple copies of yeast transcriptional activator proteins - general control protein 4 (GCN4) activator recruitment sequence, this system activates specific gene transcription and is therefore referred to as CRISPR activation (CRISPRa) [23]. CRISRPi and CRISPRa can bring gene transcription under control by several orders of magnitude and have shown a less off-target effect, with great potential for improving clinical diagnosis and treatment options.

Other CRISPR systems in viral disease treatment research are still at the cellular research level, and most of them have not yet entered the in vivo experimental stage. However, as scientists continue to deepen their experiments and more and more Cas are discovered, it is believed that the potential of the CRISPR system will be fully shown to the public in the future.

4. Conclusions and Prospects

The CRISPR system allows rapid and precise gene editing, which has already led to transformative breakthroughs in molecular biology and a whole new vision in medicine - treatment at the molecular level. There is tremendous potential in the treatment of cancer, genetic diseases, and viral diseases. In terms of viral disease treatment, the CRISPR-Cas system has excellent results in virus typing and diagnosis, blocking virus entry into cells, inhibiting virus replication, and treating the sequelae of virus infection. Because the CRISPR system is directly manipulated at the molecular level, modulation of all stages and aspects of life is possible, which is the biggest difference from conventional treatments. The components of the CRISPR system can be flexibly modified, allowing a wide variety of subsystems of the system with unlimited application possibilities to achieve more functions, such as introducing the CRISPR system by virus into specific tissues or cells, or the use of tissue-specific promoters [24, 25], which can be of great help in the research of disease mechanisms and treatments.

However, there are still many problems that need to be solved before the CRISPR system can officially become a mainstream treatment option. First, the off-target effect of the CRISPR system cannot be ignored. Due to the limited bases in the pair-binding region of sgRNA, there may be multiple segments of DNA sequences paired with it in the genome, leading to incorrect cleavage. However, if the localization region of sgRNA is designed to be too long, a part of the sequence will lose its function. And in the case of local unpaired, the localization region of sgRNA may also bind to similar DNA sequences, resulting in cutting the wrong gene. sgRNA (crRNA) has a segment of seed sequence near the PAM, and if any mismatch occurs in the seed sequence, it can lead to a big drop in target-cutting efficiency or even disappear. The larger the genome, means more dangerous to the organism, especially in humans with 3.16 billion bases. The off-target effect may lead to the generation of false phenotypes, and the more worrisome consequence is the emergence of pseudophenotypes, with the resulting toxicity to the organism being unpredictable. Therefore, the design optimization of sgRNA remains a long-term work. Second, the complete and efficient delivery of CRISPR systems also remains to be solved. There is still no ideal delivery system, i.e., the lack of vectors with both high specificity and high efficiency. This is one of the problems that must be solved before CRISPR treatment can enter clinical trials.

In my view, there are still many ethical problems and safety risks associated with human gene-level editing treatments. At present, gene editing therapy is not mature, most of the experiments are carried out on animals, lack of a lot of clinical trial evidence and treatment experience, whether there are side effects when using this effect remains to be verified. The potential adverse effects of gene editing therapy are also unknown. Adverse consequences are difficult to detect in a short period of time, and it is difficult to hold accountable for related medical errors. It is extremely irresponsible and unethical to test gene editing therapy in humans before the complex role of all human genes is fully understood. In addition to the technical risks, there are also ethical ones. As human gene-editing technology matures, it could also be used to create so-call “designer human”, which could be a huge social problem. How to avoid the application of unethical technology in real life and how to correctly use science and technology are not only related to researchers in the field of biology, but also the common responsibility of human beings. The government should also take certain compulsory measures and legislate to avoid the application of technology that violates ethics. And it is only after social awareness and laws are well established that CRISPR system treatment options can truly enter the public domain.

This article mainly focuses on the application prospects of CRISPR-Cas9 in the treatment of several viral diseases that seriously endanger human health and possibilities of other CRISPR systems in the treatment of viral diseases. It is believed that in the near future, CRISPR systems will contribute more and more to the

research of viral diseases treatment and have more breakthroughs in disease mechanism exploration and other diseases treatment.

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