The Current Applications and Feasibility of the Crispr-Cas9 Complex (1)

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Abstract: Clustered regularly interspaced palindromic repeats (CRISPR) is a gene-editing technology that has revolutionized the biotechnology industry. With a guide RNA and an endonuclease protein termed CRISPR associated (Cas) protein, the CRISPR-Cas technology is the most programmable and flexible editing technology in comparison to previous alternatives like zinc finger proteins (ZFN) and TALENs. Specifically, CRISPR has made strides in the battle against cancer, HIV, and single-point mutation diseases. However, due to the technology's recency and relative lack of research, any given treatment is projected to be extremely expensive. This paper explores not only the recent research on treatable diseases but also the affordability and ethics of gene-editing with CRISPR as a whole.

1. Introduction

Clustered regularly interspaced palindromic repeats (CRISPPR) technology has revolutionized the biotech industry, being awarded the Nobel Prize in Chemistry in 2020. First found in Escheria coli[1], the CRISPR system originally serves as bacteria's immune response to viral phages. As researchers identified its role and increased its targeting specificity, the current CRISPR-Cas9 system has been developed to consist of two parts: a single guide RNA (sgRNA) that is complimentary to the particular targeted DNA strand and a CRISPR-associated protein 9 (Cas9) which can bind to sgRNA and DNA, with restriction domains that cut double-stranded DNA at a specific site[2] . By working together, these two components introduce a double-strand break (DBS) at the targeted locus on the genome, initiating innate DNA repair mechanisms such as base excision repair, non-homologous end joining (NHEJ), and homology directed repair (HDR) 3,4. NHEJ and HDR can generate random nucleotide insertions and deletions (indels) that alter the gene sequence downstream of the break site, or insert a gene from doner DNA.[5,6] As a result, the transcription of the entire gene sequence is altered and the gene is transcribed incorrectly, knocking out the gene[5,6] CRISPR is also flexible in application due to the low cost and relative ease to design. The sgRNA can be edited to target any nucleotide sequence, allowing researchers to program different protein complexes for research7 theoretically; most importantly, the CRISPR-Cas9 system can be used to treat genetic diseases such as cystic fibrosis and HIV as well combat various types of cancer. All of the afore-mentioned diseases involve with DNA mutations as the main pathway for these diseases to take root. As a result, CRISPR has significant potential in clinics as a genome editing tool.

Unfortunately, much research is required before CRISPR is implemented into everyday clinics. Issues arising from experimental design and specificity of the sgRNA, potential off-target effects, and the delivery methods to introduce the CRISPR-Cas9 technology into eukaryotic cells are still under research. Most significantly, the price of CRISPR treatment remains a barrier for administrations to most patients. An emphasis will be placed upon diseases that can be treated by CRISPR in the near future due to the significant amount of research conducted in their fields, mainly single-point mutation diseases, HIV, and various forms of cancers.

2. Main

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According to a plethora of research[2,5,7–10], CRISPR is a repeating sequence of noncoding DNA within the genome of bacteria and serves as an immune response to viral phages.1 Bacteria can insert viral infecting DNA into their own genome at specific loci called protospacers. Each protospacer is separated from the next by nonrepeating sequences called spacer sequences.[7,8] The bacteria also includes trans-activating CRISPR RNA (tracrRNA) that binds to the non-repeating sequence as a key mechanism that allows the entire system to base pair with the target DNA.7,8 First, when an invasive DNA sequence is detected, each individual protospacer-spacer sequence serves as the blueprint for a target gene. Once transcribed, the sequence becomes pre-CRISPR RNA that binds with tracrRNA; afterwards, the combined sequence becomes mature CRISPR-RNA (crRNA) that can be recognized and bind by a CRISPR-associated (Cas) protein[7,8] Notably, each invasive DNA sequence must also include a 3 to 5 nucleotide sequence on the 3' end called the protospacer adjacent motif (PAM) sequence that plays a crucial rule in recognizing the binding site of the complex.[8]

3. Current Clinical Trials

3.1 Hiv – What is It?

HIV is a retrovirus that infects around 38 million individuals around the world. 5,11 A retrovirus is a class of virus that uses RNA as its genetic material and can conduct retro-transcription, changing RNA into DNA in host cells.[12] It targets a range of white blood cells, from lymphocyte T-cells to macrophages that are crucial in our immune response, making it a difficult infection for our body to fight on its own.[5] Notably, the virus injects its RNA into the host cells and releases the reverse transcriptase enzyme that converts the injected RNA into DNA that is then integrated into the host's genome.[5] HIV then utilizes the host cell's replication and protein synthesis machinery to replicate itself.[5] Because the virus essentially becomes part of the host's genome and infects the pathogen-fighting cells of the host, HIV is an insidious disease that cannot be fought with the native cell defense machinery. Thus, patients must rely on drugs and therapies, like anti-retroviral therapy (ART). Given that the host genome is permanently altered, HIV infection is irreversible.

Because of the versatility and genome editing properties of CRISPR-Cas9 system, it can be used as a novel tool to help treat HIV. The HIV sequences integrated into the host DNA can be permanently knocked out. Current researchers have developed methods to treat HIV infection in all of its reproductive stages with CRISPR-Cas9: binding, integration, replication, and post-integration.

3.2 Hiv – Crispr in Disrupting Binding

The infection of HIV to lymphatic T-cells and macrophages requires co-receptors that exist on the host membrane, the CCR5 coreceptor and the CXCR4 coreceptor respectively. Each coreceptor involves a different strain of HIV, the R5 tropic HIV and R4 tropic HIV (R5 tropic viruses bind to CCR5 coreceptors on T-cells and R5 tropic viruses bind to CXCR4 on macrophages).[5] Lusvarghi and Wang et. al found that the expression of the coreceptor proteins can be decreased by using the CRISPR-Cas9 system to cut the sequences that would be translated into CCR5 proteins, causing up to 74.1% of mutations in the CCR5 genes.[13,14] Furthermore, CCR5 inhibition has been tested in rats injected with cells with ablated/damaged CCR5 coreceptors. After being introduced to the R5 tropic virus, Xu et. al revealed that the presence of viral RNA within the rats was significantly reduced; in fact, these edited cells showed no traces of HIV even after 12 weeks.[15] Given that clinical trials were given to human patients, CCR5 treatment will be an invaluable addition to the world of HIV treatment[15] Experiments designed to treat CXCR4 tropic viruses also worked in a similar fashion by reducing expression of the coreceptor proteins. Additionally, scientists have tested the simultaneous use of both coreceptor inhibitions, which showed a decrease in reduction of both gene expressions.

3.3 Hiv – Crispr in Disrupting LTR's in Hiv Pre-Integration

The long-terminal repeats (LTR) in retroviruses are a series of long, repetitive nucleotide bases on the retrovirus genome. They act as promoters, DNA sequences that the hijacked host-transcription machinery bind to in order to begin reverse transcription of the RNA into DNA. Along the sequence are also locations which enhancers and other transcriptional factors can attach to.[16,17] Notably, the CRISPR-Cas9 system can be used to cut and induce indels in locations along the LTR's, inhibiting reverse transcription of the viral RNA as well as the replication of the virus. For example, because the LTR in HIV acts as a promoter for human RNA polymerase II,[17] if the LTR were altered by the Cas9 protein, it could potentially prevent the polymerases from being attached to the HIV genome and halt viral reverse transcription. In fact, researches have shown that by using the CRISPR-Cas9 complex on LTR's, gene expression crucial for the replication of HIV along the LTR has decreased tremendously[18–20].

3.4 Hiv – Crispr and LTR's, Post-Integration

Even if HIV progress beyond the integration stage, its spread can be stunted with the use of the CRISPR-Cas9 system to generate DSB's in the integrated LTR, causing indels to knockout targeted genes through NHEJ and random mutations.[5]For example, Liao et. al conducted an experiment using Cas9, LTR, and green fluorescent protein (GFP), a reporter protein, to quantitively measure the gene expression. [18] After infecting HEK293T cells, a strain of human embryotic kidney cells, the researchers introduced gRNA-Cas9 complex via plasmids into the stem cells. [18] (The reporter protein lies along the integrated viral RNA as to measure the gene expression.). They reported that there was significantly less GFP expression, indicating that transcription of the viral RNA was hindered and the production of the virus after integration was inhibited by 48-92%.18 Most significantly, they found that the number of targeted genes and general efficacy of the treatment was directly proportional to the number of times the Cas9-treatment was injected into the patient, suggesting that regular doses of the CRISPR treatment over time could eventually remove HIV from the body with minimal side effects. [5,18] Additionally, experiment results have indicated that the entire LTR sequence could be knocked out with the CRISPR-Cas9 system. This finding is significant because complete eradication of the infection/integration sequence, the LTR, from the host ensures that there is no future reactivation of the virus. A once-permanent disease is now permanently removed from the system. [21]

More information about specific trials conducted on the LTR can be found in previous review and research articles. [18–20,22–24, 5,25]

4. Cancer/Oncology

4.1 Car-t Therapy

The most significant application of CRISPR-Cas9 technology in oncology is the application in lieu with chimeric antigen receptor (CAR) T-cell therapy. A T-cell is a white blood cell that can directly kill pathogens (including carcinogenic cells), and the chimeric antigen receptor are genetically engineered receptors with an extracellular domain that can bind specifically to cancer antigens. Most significantly, they also have an internal receptor domain that can activate the T-cell and kill the host once the cancer antigen is recognized. Structurally, this domain possesses a transgene encoding antigen receptor (CARs), a genetically engineered T-cell, that expresses modified T-cell receptors (T-cell) that utilize single chain variable antibodies (scFv's), various ligands, and an extracellular antigen-recognition moiety to increase the bonding efficiency of the cell to the cancer cell. Given its highly specific targeting and patient-specific design, CAR T-cell therapy has shown immense success in treating lymphoma, leukemia, and various forms of cancer.

4.2 Car-t Therapy in the Current Medical Field

Currently, the T-cells are extracted from the patient's body. Given the cost of the surgery as well as the time that it takes to conduct the treatment, CAR T-cell therapy is extremely expensive. [26,27] As a result, researchers are looking to develop universal T-cells that come from healthy host

donors. Afterwards, these T-cells can be mass produced in the laboratory and become a mainstream off the shelf substance for researchers and doctors. [26,27] However, there are a few factors that hinder the development of universal T-cells. The most prominent is graft-versus-host disease (GVHD), where innate human T-cells trigger an immune response with the donor CAR-T cells because of specific antigens on patient immune cells called human leukocyte antigens (HLA's) that treat the donor T-cells as foreign. [26,28] In order to address this problem, multiple studies have shown that the CRISPR-Cas9 system can be used to knockout gene expression of vital subunits of HLA's26; significantly, they found that the experimental T-cells showed no signs of GVHD. [29–31]

To boost the efficacy of CAR-T cell therapy, CRISPR-Cas9 technology has also been applied to genetically edit T-cell inhibitory receptors that tumor cells can manipulate to shut down and exhaust T-cells.

Single-Point Mutation Diseases

Finally, CRISPR-Cas9 system can serve as an extremely effective tool for single-point mutation diseases like sickle cell anemia, muscle atrophy, and down syndrome. A single-point mutation disease is a swapping of a nucleotide within a sequence. Because an amino acid is coded by a codon, a specific sequence of three nucleotides, the amino acid that forms as a result of the swapping is different from the original, which, in turn, causes a mutation in the translated product. This altered and malfunctioned protein can have many detrimental impacts on the body.

One example is muscular dystrophy, which appears in the form of Becker and Duchenne dystrophy. The two diseases differ in the location of mutation within the dystrophin gene, which codes for a crucial cytoskeletal protein that promotes muscle integrity; without the dystrophin protein, all muscle control in the body fails. [32] Becker and Duchenne's muscular dystrophy (BMD and DMD) defers in the location within the genome sequence that the mutation occurs. The genome is like a puzzle piece, with different lengths of different nucleotide sequences separated into introns (non-expressed sequences) and exons (expressed sequences). During RNA splicing, the introns are removed and the exons are linked together. However, mutations can either prevent exons from binding with the next by disturbing the nucleotide bonds, while other mutations may simply delete an exon and the protein remains relatively unchanged. [32,33] In BMD, a mutation occurs so that one exon within the entire sequence is removed but the adjacent exons can still bond to each other and form a functional dystrophin protein. However, in DMD, a mutation occurs on an exon that prevents the adjacent exons from binding. [32]

One possible solution of muscular dystrophy is exon skipping. Exon skipping involves placing a molecular plaster, or a "dummy" sequence of nucleotides as a placeholder for the mutation in DMD, so that adjacent nucleotides can bind to the plaster and form a functional protein. [32] Three articles published in 2016 showed that introduction of the CRISPR-Cas9 system to guide the plaster has succeeded in mice, providing a significant milestone in introducing the technology in humans. [33]

Other single point mutation diseases can be potentially cured similarly. With sickle cell anemia and down syndrome, one can combat the disease by utilizing gene therapy on fetal stem cells to make sure the fetus can grow without the mutation, or to change fetal expression of the red blood cells to circumvent sickling. [34,35] Overall, there are many potential methods through which CRISPR-Cas9 can be applied, including cell transduction, expression, and DNA methylation.

4.3 Crispr Setbacks/Areas of Improvement

Most pivotal factors to the appearances of CRISPR in day-to-day clinics is the process is the delivery mechanisms that must be employed to deliver the CRISPR system into the host cell. Delivery methods can be classified into 2 categories: viral and non-viral delivery.

4.4 Delivery – Viral Delivery

Viral delivery involves using "nature's" packaging, including virus capsids and vectors that have evolved to deliver exogenous genetic material through the host membrane, then cytoplasm, and finally nuclear membranes. Most notably, adenoviruses (AV) and adeno-associated viruses (AAV)

show tremendous potential in in vivo delivery of exogenous material. [36,37] AV and AAV are viruses that inject their double-stranded DNA into host cells. Notably, their genetic material is not integrated into the host genome; still, it is transcribed and translated like host genes. [38] However, the host's immune system reacts violently with the introduction of AV: thus, researchers mostly use adenoviruses in lab experiments with petri dishes. On the contrary, AAV's have a low immunogenicity, making them the most likely candidate if used in clinical trials. However, the size of their capsid (20 nucleotides), or protein shell, is smaller than that of the adenovirus (100 nucleotides), limiting the total size of gRNAs and the Cas9 protein that can be delivered in the same virus. [38]

Delivery – Nonviral Delivery

Nonviral delivery involves the usage of synthetic elements that are hand-created by the researchers in laboratories along with implementation of different methods of injection of the CRISPR-Cas9 system such as hydrodynamic injection and electroporation. [37,39] The main forms of delivery appear in the forms of plasmid-based DNA delivery, mRNA delivery, and protein-based DNA delivery. In conjunction with the two above-mentioned injection techniques, plasmid-based DNA delivery involves the introduction of Cas9 DNA encapsulated by a plasmid into the nucleus of the host cell.40 As the name suggests, mRNA delivery involves the delivery of the transcribed CRISPR-Cas mRNA by the same means. [40]

Beyond delivery mechanisms, researchers are still fining tune the CRISPR-Cas9 technology. Specifically, they are determining models that can accurately determine the most efficient method of designing the complex itself. From the length of the gRNA, the multiple PAM's, the nucleotides within both sequences, and the interaction of all of these factors, researchers have to condense an almost infinite list of possibilities when creating a CRISPR-Cas9 treatment for clinics, given the length of our genome and the combinations of nucleotides that result from it.

Given CRISPR's current status, how will it compare to current gene therapies?

5. Discussion

Indeed, the CRISPR/Cas9 system is a robust and ever-improving tool in the field of genome editing. Given its flexibility and unlimited potential for applications, CRISPR is the future. Before then, however, potential issues must be addressed. sgRNA design, potential off-target effects, and delivery of Cas9 all act as barriers for CRISPR's entrance into the medical field [37,41].

5.1 Current Gene Therapies

Unfortunately, all forms of gene therapy are extremely expensive. Given that the majority of genetic disorders occur in populations of low socioeconomic standing, those that need the treatment the most have no access to it. Let's take a look at current existing gene therapy treatments.

For HIV, the most efficient and prominent treatments anti-retroviral therapy (ART). ART involves the intake of specific drugs that can inhibit the enzymes such as reverse transcriptase and integrase that HIV utilizes in its replication cycle in order to propagate. [42 The CDC finds that current treatments for ART cost on average about $41,667 for each diagnosis of HIV, with lifetime treatment with ART to cost around $379,668 in 2010.43] Furthermore, HIV lifetime treatment varies with different programs that a hospital may offer. CDC's uses an example of 2 programs: one with an annual cost of 500,000 with 12 diagnoses per year, and another program with annual cost of 37 million with 5000 diagnoses per year. [43] Although unrealistic, this CDC case study points out the disparity in affordability between potential programs of HIV treatment. Since multiple screenings allows for better combatment of HIV, this would mean that those who can afford the treatment have better quality treatment, while those that cannot afford the expensive treatment must settle for less.

Current cancer treatment also faces a similar problem of lack of access. Current treatments of chemotherapy, immunotherapy, and radiology therapy can cost up to 12,000, 9000, and 12,500 dollars respectively. [44] Even more, research indicates that preventable cancers such as lung cancers can cost up to a total of 280,000 dollars over a lifetime, and even those with health care

coverage, whether Medicaid (7.5% of patients), Medicare (16.8%) or private health insurance (48%), struggle with payment costs because of "coverage gaps, deductible requirements, copayments, and out-of-pocket expendentures." [45,46] Individuals over 65 years old make up 56% of all cancer cases in the US and 69 percent of cancer mortalities, highlighting the helpless situation of many Americans. [47] Given that targeted drug therapies are increasing in quality and are less invasive, treatment for cancers may just gradually increase over time, such as the highly successful cancer immunotherapy treatment CAR T-cell therapy.

Other forms of treatments to genetic diseases are even more outrageous. Nusineren, a gene therapy used to cure a patient of spinal muscular atrophy, has allowed previously bed-ridden, paralyzed patients a second chance to stand up in life. [48] Other leukemia treatments, hereditary skin diseases, etc. have demonstrated the power that gene therapy has over previously life-debilitating diseases. [48] Yet, the cost of the treatments around $500,000 to $1,500,000, with Nusineren being priced at $750,000 the first year and $375,000 every year after until death. [49] Luxturna, a gene therapy that helps with patients with inherited blindness, costs $800,000 to $1,000,000 for both eyes. [50] Glybera, a treatment for lipoprotein lipase deficiency, was charged at $1,000,000. [49,50] A study from MIT provides light onto the situation, claiming that the fewer the number of patients, the higher the price. [50] This fact suggests that those suffering from etic diseases are unable to afford the treatment to most simply because current gene therapies are too expensive for companies to produce; given that the genetic diseases are rare in number, the tiny target market disincentivizes pharmaceutical companies to continue research and production of the disease.

5.2 Future Models (Ethical Dilemmas + Policy)

So is there hope for gene therapy and CRISPR? Will the current pharmaceutical industry continue to function only for profit, overpricing a cure after monopolizing a novel treatment for a disease? Or will researchers forfeit the project altogether, unwilling to outmaneuver the rarity and consequent low customer base? Only the interactions of governmental regulations of CRISPR-gene therapy and the pharmaceutical industry will determine the future of medicine.

Harvard Business Review summarizes 3 economic models in making these overpriced treatments more affordable. The entire article can be found here. [51]

Yet it's also important to consider the ethics of genome editing. Both the public and private sector need to consider the ethical concerns that may arise from authorizing CRISPR for clinical trials. Pharmaceutical companies need to judge the cost and profit of the CRISPR-Cas9 based therapies, and governments must consider the moral concerns of gene editing. Critics and skeptics are reluctant to accept the technology for its potential impact on future eugenics. As the system's application strays from medicine and into cosmetic and arbitrary usage for the wealthy, social inequality will only be exacerbated. "Superior genes" would only stretch the gap between the rich that could afford the treatments and the poor that are virtually isolated from these opportunities. Can individuals select to almost design themselves and their offspring? What if someone desired blue eyes? Red hair? A narrow nose? Taller height? Increased muscle density? What would the future of our world look like if we could artificially alter our bodies to suit our different needs? Is it possible?

The answer is yes… and no. Certainly, with enough research and dedication, researchers can decode the entire human genome, track all of the different interactions and combinations of genes and produce these results. However, this would only happen in the far, distant future in the next few centuries. CRISPR was originally discovered in 2011, and by 2020 we are still struggling with the above-mentioned problems of packaging, delivery, design, off-target effects, etc. In the meantime, the CRISPR-Cas9 technology and gene editing in general can be developed to aid individuals afflicted by once-before uncurable patients. Those suffering from debilitating single-point mutation diseases now can live a normal life. HIV patients can finally get rid of the terminal illness, and the CRISPR-Cas9 technology can be the future cure to cancer. The technology's potential to save lives and benefit our society far outweighs the far-off possibility of gene editing's application in cosmetics. All in all, it is more ethically sound to approve the treatment and save the countless lives

in the near future than to reject for the possibility that gene editing to other industries in the next one hundred years.

6. Conclusion

Thus, CRISPR has an enormous potential in the medical field. Among its many potential future applications, it can be manipulated to treat previously untreatable diseases such as HIV and genetic diseases that are permanent and act as a diverse toolkit in treating various forms of cancers. After several talks with the WHO and a few more years of research, CRISPR is certain to make its grand entrance into the clinical world and revolutionize medicine.

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