How CRISPR/Cas9 Can Be Used to Treat Cystic Fibrosis

Nandita Rajagopal

Abstract:

From its discovery in the immune systems of prokaryotes, the clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas9) system has revolutionized gene editing. With these advances in gene editing, there come many potential medical applications of the CRISPR/Cas9 technology to treat genetic diseases. This paper describes methods of using CRISPR/Cas9 to treat the genetic disorder Cystic Fibrosis (CF). CF is caused by a mutated cystic fibrosis transmembrane conductance regulator (*CFTR)* gene, affecting many of the body's cells. People carrying this mutation have thick and sticky mucus which can build up in the digestive tract and lungs, leading to severe blockages and damage. These blockages can lead to trouble breathing and a higher possibility of infection. This paper will examine the current cystic fibrosis treatment, including the available drugs and their limitations. CRISPR/Cas9 has the potential to completely cure the disease in one treatment through the modification of the *CFTR* gene in the patients' cells. This paper describes delivery mechanisms and mechanistic details of the gene editing process and how it can be applied to cystic fibrosis patients.

Introduction:

CF occurs in about 1/3500 births (De Boeck, 2020). While this number may not seem like much, CF is actually the most common autosomal recessive genetic disorder in the world. In addition to the high number of people that have the disease, 90% of patients die due to complications associated with the disease (Rafeed & Aly Sayed Murad, 2017).

CF is a genetic disease, caused by a mutation in the *CFTR* gene; two copies of the mutated gene must be inherited for someone to have CF. The *CFTR* gene encodes the CFTR protein which is an anion channel that conducts chloride and bicarbonate/regulates water and ion transport. The action of this protein is necessary for unfolding mucins and defending against bacteria in the airways. Additionally, the CFTR channel is required to enable pancreatic enzymes for digestion (De Boeck, 2020). When the channel is affected by a mutation in the *CFTR* gene, respiratory health and the digestive tract are negatively affected. There have been thousands of different *CFTR* mutations discovered, each affecting the CFTR protein in different ways (De Boeck, 2020; Graham & Hart, 2021). The many types of mutations can make CF a difficult disease to treat.

Symptoms of CF include a multitude of conditions. The most frequent symptoms seen soon after birth are numerous respiratory infections and issues with weight gain. Pancreatic insufficiency, a high sweat chloride concentration and poor respiratory function are other common symptoms of CFn(De Boeck, 2020; Graham & Hart, 2021). There are many fatal complications that can occur with CF, and they can affect nearly every organ in the body. These complications can be anything from dangerous lung infections of unusual bacteria to liver disease and diabetes (De Boeck, 2020). Due to the life-threatening nature of CF, newborn babies are screened for the disease (De Boeck, 2020). The multifaceted nature of this disease can make the quality of life for those who have it extremely difficult. Currently, the average lifespan for a person with CF is about 40 years, highlighting the need for a new approach to curing this disease (Graham & Hart, 2021).

Section 1: An Overview of Cystic Fibrosis

CF was first identified in 1935 (De Boeck, 2020). At the time, children diagnosed with CF would die early due to pancreatic insufficiency. Treatments have greatly developed since this time. At first, treatments included pancreatic enzyme supplements to prevent patients from dying of malnutrition along with antibiotics to fight against lung infections (De Boeck, 2020; Graham & Hart, 2021). As drugs to treat CF have developed, more patients have been able to survive to adulthood. Treatments in the modern world have been geared toward specific types of CF mutations (Rafeed & Aly Sayed Murad, 2017).

There are seven basic classes of *CFTR* mutations. Class I mutations lead to an absence of the CFTR protein; these mutations are often caused by frameshift mutations. Class II mutations involve incorrect processing of the CFTR protein. Class III mutations deal with improper gating of the CFTR channel. Class IV mutations lead to fewer ions passing through the channel. Class V mutations lead to less of the CFTR protein being present. Class VI mutations have a less stable CFTR protein; Class VII mutations have no mRNA present (De Boeck, 2020; Graham & Hart, 2021; Rafeed & Aly Sayed Murad, 2017).

The different classes can be caused by different kinds of mutations. Frameshift mutations are when a nucleotide is inserted or deleted from the DNA sequence, causing the rest of the nucleotides in the sequence to shift a base pair away from its normal position. These mutations can be very harmful, as can be seen through the adverse effects of Class I mutations. While there are many *CFTR* mutations, the most common is the F580del mutation; it falls under Class II *CFTR* mutations (Graham & Hart, 2021).

To treat the multiple classes of *CFTR* mutations, there are some drugs that have been approved as a treatment for CF. Ivacaftor, lumacaftor, and tezacaftor are some of these drugs. They are called modulators; some (ivacaftor) improve channel opening while others (lumacaftor and tezacaftor) improve the folding of the CFTR protein (Graham & Hart, 2021). There are other drugs that have also been approved to treat CF that are mutation specific.

While great advancements have been made, modern drug therapies for CF have many limitations for patients (De Boeck, 2020). One of the most prevalent limitations is that none of the treatments are permanent. CF patients are required to take these drugs daily to keep their disease in remission. In essence, none of the aforementioned treatments can fully cure the disease.

Additionally, not all classes of CF mutation can be treated by drug therapies. For modulator drugs to be effective, there must be CFTR present in the cells; without CFTR, there is no protein on which the drug can act. Class I CF mutations result in a lack of the CFTR protein in cells, meaning that modulator drugs would not be very effective. Treating Class VII mutations is more complicated than Classes I-VI. The lack of mRNA makes this mutation more unique and difficult to treat than others (De Boeck, 2020). Using modulator drugs is not a viable treatment for this mutation.

In addition to the restrictions of drug therapies, CF can be a difficult disease to treat due to the nature of how it is inherited and the sheer number of CF mutations. CF is an autosomal recessive disease; therefore, a person will have the disease only if they have two mutated *CFTR* genes inherited from their parents. With the numerous different kinds of mutations, there is a high likelihood that the two mutations that the patient receives will not be the same. There is a possibility that they may not even be within the same class.

To account for the restrictions of drugs, researchers have begun looking at other treatments for CF. A viable option for future treatments is gene therapy (Graham & Hart, 2021).

Gene therapy is any treatment of a disease on the genetic level, and it has the potential to completely cure a patient of CF. In addition to this, gene therapy would be able to treat any CF mutation, including rare and undruggable ones.

Section 2: An Overview of CRISPR/Cas9

The discovery of CRISPR/Cas9 has revolutionized many fields of science. CRISPR and Cas systems have the ability to edit sequences of DNA, including mutations in the genetic code. This has huge implications for diseases caused by genetic mutations, such as CF; correcting the mutations that cause CF could completely cure a patient of the disease, and allow them to live a healthy life.

Found in the immune system of prokaryotes, the CRISPR/Cas9 system has helped make major advancements in the field of biology and medicine (Jinek et al., 2012; Martin Newman & Frederick M. Ausubel, 2016). CRISPR/Cas systems are a defense mechanism in prokaryotes to prevent viral attacks by cleaving DNA. CRISPR is the arrays in the bacterial genome that encode for RNA molecules that can guide Cas9 to the right location, and Cas9 is an enzyme that can cut DNA (a nuclease (Jinek et al., 2012)). CRISPR/Cas9 is a type of RNA-guided site-specific nuclease (RGN)— or a type of nuclease that uses an RNA complementary to the target strand of DNA to guide it to the correct place. Once the RGN is in the correct place, it will introduce a double-stranded break (DSB) in the DNA, allowing for the cleavage of DNA. In its natural setting, the cut made by CRISPR/Cas9 in the viral DNA will result in the degradation and inactivation of the virus (Martin Newman & Frederick M. Ausubel, 2016).

This system has 2 RNA molecules that bind to Cas9; the CRISPR RNA (crRNA) —a twenty nucleotide sequence that corresponds to viral DNA— and trans-activating CRISPR RNA (tracrRNA)**.** Viral sequences of DNA that are complementary to the crRNA are targeted. The crRNAs are seen in Figure 1A associating with Cas8, which allows for the Cas9 to cut at the complementary site. These sequences are targeted only if they are 5’ to an “NGG” sequence. This “NGG” sequence is known as the protospacer adjacent motif (PAM) sequence. Without the presence of the PAM sequence, a DSB will not be introduced (Jinek et al., 2012).

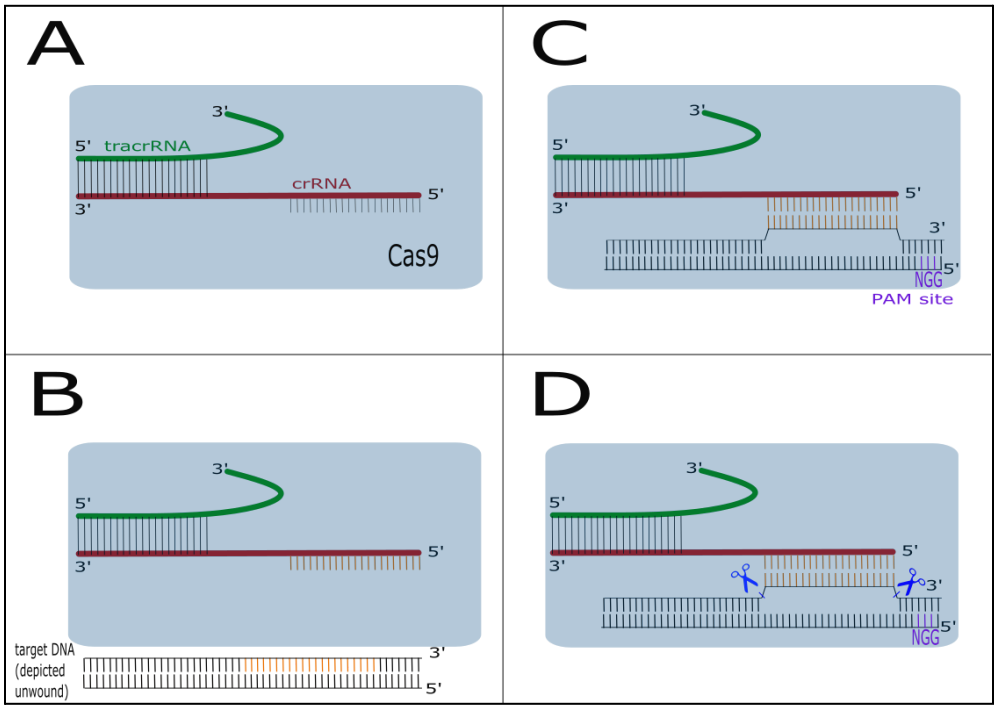

Figure 1: (A) Association between the crRNA (red) and tracrRNA (green). (B) The crRNA is complementary to the target strand of DNA. (C) The crRNA binds to the target sequence upstream from the PAM sequence (NGG). (D) The DSB is made 3-8 base pairs upstream from the PAM site.

Further advancements have been made in a laboratory setting to this system. One single "chimeric RNA" can be created (as seen in Figure 2). This chimeric RNA contains components of both crRNA and tracrRNA to guide the system rather than using two separate molecules of RNA. The crRNA and tracrRNA would be fused at the 3' end of the crRNA and the 5' end of the tracrRNA (Jinek et al., 2012). Using only one RNA molecule instead of two makes using CRISPR/Cas9 for gene editing much easier.

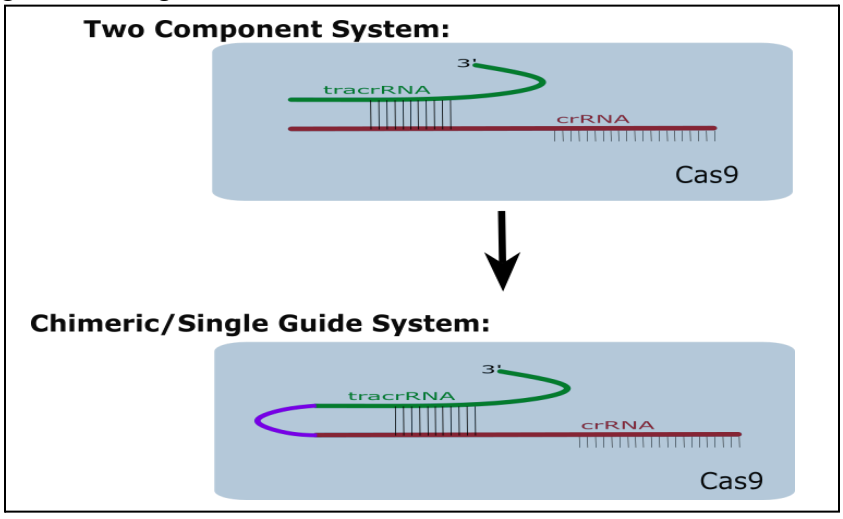

Figure 2: Depiction of how tracrRNA and crRNA are fused to make a single gRNA.

Once Cas9 has been directed to the target site, the cell is confronted with a double-stranded break in the DNA. There are two main pathways that cells can use to repair DNA damage, and these pathways have been used to achieve edits in the DNA sequence in laboratory settings. Homology directed repair (HDR) is a natural repair technique where a homologous chromosome is used as a template for rebuilding another. This can be used by researchers to repair DNA after cuts with CRISPR/Cas9, and should researchers additionally introduce a repair template that mimics a homologous chromosome, they can introduce whatever sequence they desire into the target organism (Graham & Hart, 2021). Non homologous end joining (NHEJ) techniques can also be used. NHEJ occurs when two ends of DNA are rejoined (Sanchez et al., n.d.). This is also used to repair DNA after a DSB. However, NHEJ mechanisms can often introduce random insertions or deletions into the gene sequence. Such alterations can result in a frameshift mutation or can even knockout the function of the gene.

CRISPR/Cas9 offers the prospect of a single, long-term treatment for some genetic diseases as an alternative to numerous short-term treatments. These treatments come with many risks, such as malignant off-target edits by CRISPR/Cas9. An off-target edit may accidentally inactivate a tumor suppressor gene, for example, which could result in the patient developing cancer. Others have ethical concerns regarding the use of gene editing in medicine, requiring careful consideration when it comes to human trials. Yet despite the dangers of using CRISPR/Cas9, there are also boundless possibilities.

One such disease that could potentially be treated through gene modification is Cystic fibrosis. CF is an excellent example of a disease that could be cured through CRISPR/Cas9 (Sanchez et al., n.d.). A genetic disease, CF would require the correction of the mutation in the *CFTR* gene.CRISPR/Cas9 has thus far been used to edit the genes of numerous living organisms and tissue cultures from humans. Numerous genetically inherited diseases could be treated, and possibly cured. Illnesses from sickle cell disease to cancer have the possibility of being treated, and clinical trials have already started for some. Because cystic fibrosis is genetically inherited and has no permanent cure, only partially effective treatments, CRISPR/Cas9 gene editing should be considered to treat CF in human patients

Section 3: Using CRISPR/Cas9 to Treat CF

CRISPR/Cas9 has great potential for curing CF, but one of the main obstacles of this treatment is the delivery of CRISPR/Cas9 to the cells. The mutation that causes CF is encoded in the DNA within each cell which is packaged in the nucleus, meaning that the machinery needed to edit the DNA would need to be imported into the cell and get into the nucleus to make an actual edit.

Section 3.1: Delivery of CRISPR/Cas9 into Cells

One method of delivering CRISPR/Cas9 into the cells is through the use of plasmids. Plasmids are circular rings of DNA often found in bacteria. The CRISPR/Cas9 gRNA and the Cas9 gene can be encoded onto a plasmid, and then the plasmid can be transfected into the cells (Graham & Hart, 2021). Then, the cell can use its own transcription and translation machinery to make the gRNA and Cas9, allowing the edit to proceed. Treatment involving the use of plasmids as a vector has many disadvantages, including low rate of edits (Firth et al., 2015; Sanchez et al., n.d.). In addition to this, there is a delay in the expression of Cas9, a long-term expression of Cas9. This is often due to

the accidental integration of the plasmid into genomic DNA, making the transformed cells continue to express Cas9 and increasing the risk of an off-target edit. Furthermore, the plasmids often have genes for antibiotic resistance, which can have harmful effects in a clinical setting. However, plasmids have highly specific edits; in one case, there were no off-target edits within 300 base pairs of the target site (Firth et al., 2015).

In addition to plasmids, mRNA and ribonucleoprotein complexes (RNPs) can be used as a delivery mechanism for CRISPR/Cas9. Messenger RNA and RNPs can be put into the cells. In human upper and lower airways, RNPs cut 80% of the time, giving it rather high efficiency. Homologous recombination occurs over 43% of the time (Sanchez et al., n.d.).

The mRNA has also been used for *in vivo* and *in vitro* delivery. An in vivo treatment will occur inside the patient. Using lipids that can enter the cells, the mRNA encoding Cas9 can be introduced to the cell and then translated into Cas9 to cut in the proper place (Sanchez et al., n.d.). This treatment has been observed in mice and other animal models (Graham & Hart, 2021).

While in vivo delivery methods have advantages, they can be dangerous in certain cells. Additionally, it may be difficult to reach the cells in a patient's lungs in vivo. As an alternative, there are ex vivo treatments, in which the cells are edited outside the body and then implanted into the patient's organs.

Section 3.2: How CRISPR/Cas9 is Used with Point Mutations

For some mutations, base editing is a viable option as well. Some versions of Cas9 contain a mutation in the catalytic domain that allow them to change one type of nucleotide into another—usually Cytosine to Thymine or Adenine to Guanine. This tool can be guided to a specific region of the DNA to change a single nucleotide, which can potentially correct certain kinds of CF mutations, such as those involving a premature stop codon or other point mutations. This does not create a DSB, so off-target edits are less likely to occur (Graham & Hart, 2021).

Section 3.3: Where the Cells to Edit with CRISPR/Cas9 are from

There are many ways to get the cells to edit with CRISPR/Cas9. Sometimes, epithelial cells can be scraped directly from the airway or researchers can take a biopsy to isolate stem cells from the patient, including basal epithelial cells. The cells would be treated with different chemicals and edited before being re-implanted (Firth et al., 2015). One challenge with this treatment is that its efficiency can be low. Repopulation of the edited cells would also need to occur for the treatment to be successful. First and foremost, for this treatment, success is dependent on the safe implantation of the cells. Additionally, these procedures can often be traumatic, often including potentially harmful chemicals (Sanchez et al., n.d.).

Intestinal stem cell organoids are a potential way to use CRISPR/Cas9 to treat CF. Intestinal stem cell organoids are organ-like tissues used to model diseases. In this approach, the CFTR channel was studied in small intestinal (SI) and large intestinal (LI) stem cells (Schwank et al., 2013). SI and LI stem cells were obtained from two pediatric CF patients, both homozygous for the F508del mutation (Schwank et al., 2013). After confirming that the patients both lacked functioning of the CFTR channel, *CFTR* exon or intron 11 was targeted with a donor plasmid containing the wild-type *CFTR* sequence

(Schwank et al., 2013). In addition to the corrected gene, the silent mutation was introduced to confirm that the sequence was edited. The organoid cells were confirmed to have been edited, with the correction of the F508del allele (Schwank et al., 2013). In the future, the goal would be to transplant these edited cells into human patients. However, like the previously mentioned approach involving plasmids, this too has low efficiency.

Another option would be to use induced pluripotent stem cells (iPSCs) from a CF patient. Researchers can take cells from a patient (such as skin fibroblasts) and reprogram them into iPSCs that are capable of differentiating into any cell (Sanchez et al., n.d.). The patients in this study had a homozygous F508del mutation. Researchers used CRISPR/Cas9 to correct this mutation in the extracted iPSCs; the gRNA that guides the system was encoded into a plasmid to be delivered to the cell. After the correction, the resulting clones were screened via Polymerase Chain Reaction (PCR) to see if the gene was edited properly, and 6 of the 36 clones that were analyzed were correctly edited, with an efficiency of 16.7% (Firth et al., 2015; Sanchez et al., n.d.). The iPSCs differentiated into lung epithelial cells, and they were capable of proper function (Firth et al., 2015).

Another approach is using embryonic stem cells that would be used in ex vivo editing, differentiation, and eventual implantation into the pateint.

Section 3.4: Scheme of CRISPR/Cas9 Edits for CF

While all these methods can change a patient's DNA, the edit would need to cure CF. There would be five steps involved in the process of curing CF in a patient, outlined in Figure 3.

1. You would need to find out what specific mutation the patient has. This is done by sequencing the *CFTR* gene and comparing it against known mutations. There are many *CFTR* mutations, and sometimes the different mutations can require different edits. Additionally, patients often have two different *CFTR* mutations. However, oftentimes only one mutation would need to be edited (CF is a recessive trait, meaning people with one *CFTR* mutation are still healthy). This can affect how the treatment proceeds, as it may be easier to edit one mutation over the other.
2. Once the mutation has been identified, a specific gRNA and repair template must be designed for the mutation.
3. Cells from the patient must be isolated using methods mentioned in Section 3.3.
4. The edit must be performed using one of the approaches described in Section 3.1, and validated in vitro using techniques such as PCR to validate that the edit has occurred.
5. Once this step has occurred, the cells can be transplanted back into the patient. As the gene that causes CF would be corrected, the patient would potentially be cured of CF.

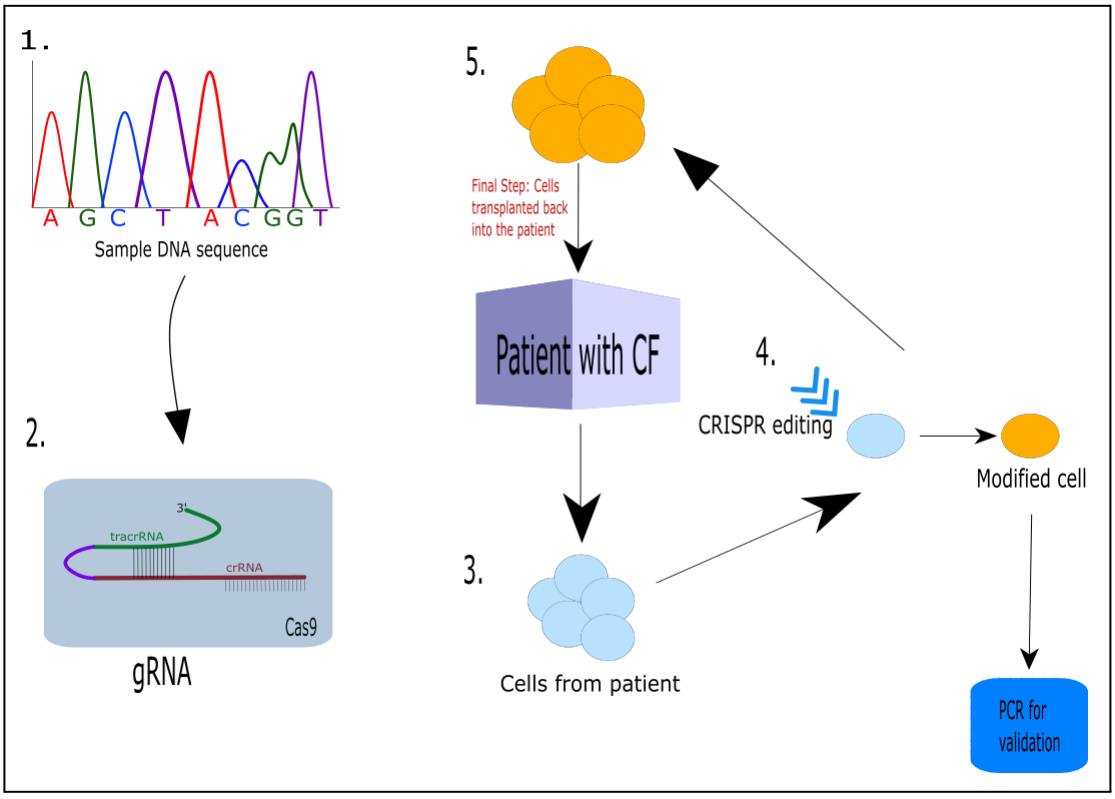

Figure 3: Visual Diagram of the steps involved in making a CRISPR/Cas9 edit to correct *CFTR* mutations. (1) A sample of Sanger sequencing to determine which *CFTR* mutation the patient has. (2) A gRNA that must be designed. (3) Cells from the patient. (4) CRISPR edits the patient's cells with the mutation (blue); the cells with the corrected mutation (orange) are validated through PCR. (5) The modified cells are transplanted back into the patient.

This technology has not been successfully used in people/animals yet, but it still remains a potential cure for CF patients in the future.

This paper has thus far considered the use of CRISPR/Cas9 to cure CF in somatic cells, but there is also the possibility of edits in the germline. These edits would follow similar strategies as what is listed here, but edits in the germline come with their own ethical concerns, discussed in detail below

Section 4: Ethical Debates About Using CRISPR/Cas9

Gene editing is a relatively new technology, and though there is a vast array of beneficial possibilities that come with this discovery, there is also the possibility that it will be misused. The strategies mentioned in the last section for curing CF are edits that occur in somatic cells. However, there has been some speculation on curing CF in future generations through editing germline (reproductive) cells or embryos (Ledford, 2019).

Germline edits are a very controversial topic. While this could offer parents the chance to give their children a life without disease or disability, any changes made would be permanent. For better or worse, the child will have the modified genes, and they will pass these genes on to *their* offspring (Shinwari et al., 2017). For instance, if parents decide to edit their child's genes to

cure them of CF, but there is a harmful off-target edit, the child will have to live with the new issue. Additionally, it is unclear if the parents should have the right to modify their child's genetic code. The parents would be changing their child's genetic makeup without the child's consent, but the child would be unable to give consent as it would not have been born yet. Some say that the parents should not be able to permit such treatment without the child's permission, yet others believe that the parents/guardians can act on behalf of the child (Shinwari et al., 2017). This can be compared to how parents can approve medical procedures for their children.

Additionally, there have been discussions about the idea of eugenics, or the purification of a gene pool (Carolyn Brokowski & Adli, 2018). This technology makes genetic purification a possibility. Someone can select a trait, and then edit the genome of those without the trait to not allow them to reproduce (Carolyn Brokowski & Adli, 2018). In the context of CF, if there was a law passed that parents with CF cannot reproduce, this could be enforced on the genetic level. Although frightening, the same technology that can be used to save lives can have such applications. Despite the debate, in all countries abiding by the World Health Organization guidelines, germline editing is forbidden (Cyranoski, 2019).

Discussion:

The strategies to treat CF using CRISPR/Cas9 are not currently able to be used in a clinical setting. However, there is a large possibility that these treatments may be seen in the near future.

Before CRISPR/Cas9 can be used on humans as a treatment, certain conditions must be met for the safety of the patient. As this paper has discussed, one of the risks of using CRISPR/Cas9 to edit genes is the risk of off-target edits. Before gene therapy techniques can be used as treatments with CRISPR/Cas9, they should prove that they have few off-target edits. Any potential treatments must also show how they can be used to treat different mutations, as one of the main benefits of using CRISPR/Cas9 is that it can be used to treat any mutation of the *CFTR* gene. Finally, this cure must be shown to be permanent. CRISPR/Cas9 is a good option for treatment of genetic diseases because it has the potential to permanent cure patients; if the cure does not last long, then it is not much better than current CF treatments.

Many of the studies on using CRISPR/Cas9 to cure CF show promising results. Many of these studies show how you can use CRISPR/Cas9 in multiple contexts. As CF is a disease that does not just affect one area of the body, the versatility in these techniques could prove to be beneficial for patients. For example, scientists might be able to use CRISPR/Cas9 to edit both lung cells and intestinal cells, treating both these aspects of the disease. While there is still much we do not know about gene therapy, its potential to cure CF is high.

Conclusion:

CF is a deadly disease and is the most common autosomal recessive disease. While treatments have progressed since CF was first identified, more can still be done to treat this disease. Current treatments are limited in that they cannot completely cure the disease and cannot treat some classes of CF mutations. The future may bring a potential cure to CF through the use of CRISPR/Cas9 genome editing. There are many methods that could possibly utilize CRISPR/Cas9 as a cure for this disease. Using plasmids, RNAs, and RNPs are all potential solutions with CRISPR/Cas9. As technology develops, the potential to make edits in the germline may become possible in order to cure future generations of CF. At this time, such edits are generally not permitted, but they have sparked an ethical debate over whether they should

be allowed. As more research is being done with CRISPR/Cas9, curing genetic diseases such as CF becomes a more feasible possibility every day.

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