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RESEARCH ARTICLE

CRISPR: POTENTIAL USES AND CHALLENGES

Abishay Santram

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Abstract

CRISPR Cas is a genome editing tool, that has the potential to revolutionize the medical world. As a cheap and simple alternative to other gene editing technologies, it has grabbed scientists' attention and is being actively studied. Its uses range from the removal of disorders caused by genetic mutations to developing rapid diagnostic tests. As well as a potential cure for cancer.This paper aims to understand CRISPR's potential along with the challenges it may face while providing a brief explanation of its mechanism.

Introduction:-

Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR is a genome editing system that acts as an immune system against invading viruses and plasmids in prokaryotic organisms such as bacteria and archaea.The CRISPR system involves two components: a single guide RNA (sgRNA) with complementarity to any sequence in the genome, and the Cas9(**C**RISPR-**as**sociated) endonuclease, which associates with the sgRNA at the genomic target sequence(Min et al., 2019).Francisco Mojica first identified it in Escherichia coli bacteria (present in the human body) in 1993. While sequencing the DNA of a halophilic microorganism of the archaea group, he observed regular spaced repeats which would later be called CRISPR. Precision targeting and the creation of double-strandbreaks for gene manipulation are the two most vital requirements in genome editing, and CRISPR fulfils both, making it a revolutionary discovery.

In 2020, Jennifer Doudna and Emmanuelle Charpentier won a Nobel Prize in Chemistry for their landmark paper published in 2012, which popularised CRISPR. They had identified a key component in the CRISPR system other than theCas protein, an RNA molecule involved in recognizing phage sequences. The paper proved that although CRISPR is not the first gene editing tool, it could revolutionize the field with its ease of functionality, quick results, and minimal expenses(Jinek et al., 2012).The CRISPR gene-editing tool has numerous medical applications,such as the treatment of HIV infection, resistance against malaria, a possible cure for cancer, the potential eradication of genetic disorders,and many more.

In 2013, the CRISPR tool was further developed and applied to modify genes in several model organisms such as **Drosophila**, **Caenorhabditis elegans**, and zebrafish. Simply injecting Cas9 mRNA and gRNA into early embryos resulted in efficient genome editing(Singh et al., 2015)..β-thalassemia, one of the most common genetic diseases worldwide, is caused by mutations in the human haemoglobin beta (**HBB**) gene. In 2014 researchers successfully edited the first non-viable human embryo using CRISPR to correct the mutation that leads to beta-thalassemia(Xie et al., 2014). Also, in 2019 researchers figured out CRISPR could also correct mutations in the X-linked dystrophin gene that cause Duchenne muscular dystrophy (DMD). DMD is the most common lethal monogenic disorder, primarily affecting boys owing to their single X chromosome. The incidence of the disease is

Corresponding Author:- Abishay Santram

estimated at 1:5,000 boys worldwide. Approximately two-thirds of DMD mutations are inherited by the sons of mothers who are unknowing carriers of dystrophin mutations. (Min et al., 2019)

CRISPR-Cas modules are adaptive antivirus immunity systems based on the self-nonself discrimination principle. This system is divided into two classes. Class I and Class II. Class I is divided into Types I, III, and IV. These are further divided into subtypes. Class II is divided into Type II, Type V and Type VI. Even with such variety, due to the simplicity, high efficiency, and multiplexing capability of the type II CRISPR/Cas system, it has been adopted as the genome editing technology of choice. The type II system utilizes a single Cas9 protein nuclease sufficient to cleave the target DNA specified by crRNA. (Singh et al., 2015)

The purpose of this paper is to elaborate upon the understanding of the various Classes and types of the CRISPR-Cas system as well as the implementation of CRISPR in the field of medicine, particularly finding cures to genetic disorders that were previously considered untreatable. It will also talk about CRISPR's challenges and the questions it faces.

How does CRISPR work?

The CRISPR-Cas system is like a pair of biological scissors that can cut a part of the genome and edit it. The CRISPR/Cas system consists of two main components the guide RNA (gRNA) and the Cas protein.The small RNAs are responsible for sequence-specific detection and silencing of foreign nucleic acids(Jinek et al., 2012).The systems are comprised of a CRISPR array and one or multiple **cas** genes transcribed independently or as an operon. An operon is a cluster of genes that are transcribed (transcription is the process of making an RNA copy of a gene's DNA sequence) together to give a single messenger RNA (mRNA) molecule that encodes multiple proteins. The CRISPR array is composed of identical repeats interspaced with short unique sequences called spacers. The spacers originate from mobile genetic elements and function as memory devices that allow recognition of the invaders upon reinfection.CRISPR-Cas systems act in three stages: (1) adaptation, (2) CRISPR RNA (crRNA) biogenesis and (3) interference. To infect an organism, viruses allow their genetic material to pass through the cell. This hijacks the cellular processes to produce a virally encoded protein replicating the virus's genetic material (Cohen, 2016). The adaptation stage involves the insertion of a new spacer, derived from the invading genetic material, into the CRISPR array. In the second stage, the CRISPR array is transcribed as a precursor CRISPR RNA (pre-crRNA), which is then processed into mature crRNA containing a part of the repeat and the spacer. In the final stage, interference, a complex formed by the mature crRNA with single or multiple Cas proteins, recognizes spacer-complementary sequences (protospacers) on the invading nucleic acids and mediates their cleavage. Immunity requires a sequence match between the invasive DNA and the spacers that lie between CRISPR repeats. This subsequently leads to the destruction of foreign genetic material. In some cases, a short protospacer adjacent motif (PAM) sequence located next to the targeted protospacer is necessary for both adaptation and interference stages. In PAM-dependent CRISPR-Cas systems (namely types I, II and V), the PAM sequence, present on the foreign DNA but absent from the CRISPR array, enables self- vs non-self-discrimination. All nucleated cells of the body have unique surface molecules that it identifies as self. If the cell does not recognize a foreign molecule, it triggers an immune response. The foreign molecules are called non-self or antigens. PAM-independent systems have evolved strategies to avoid self-targeting, such as a protospacer flanking site in some type VI systems. (le Rhun et al., 2019b)

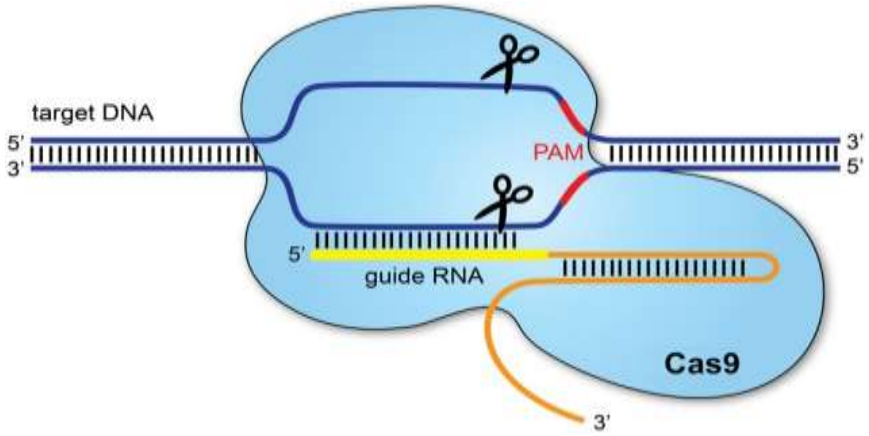

Figure 1:- Working of CRISPR (Redman et al., 2016).

The different types of CRISPR

There are many different variants of the CRISPR-Cas system, and it is therefore divided into many different types based on the effector molecules, mainly type I, type II and type III. (Makarova et al., 2011a)

Type I

Typically, the type 1 loci contain the cas3, which encodes a large protein with separate helicase and DNA activity. All cas3 loci can unwind double-strand DNA and RNA-DNA complexes to facilitate target cutting. In **E. coli**, the Cascade complex alone is capable of generating crRNA, but it requires Cas3 to achieve immunity against foreign DNA. Hence, Cas3 appears to be an essential protein of the CRISPR system, which is necessary for crRNA-guided interference of virus proliferation (Sinkunas et al., 2011). Although Type 1 is prevalent in bacteria and archaea, it is not as popular as Type II CRISPR.

Type II

Type II is the most popular type of CRISPR-Cas due to its potential in genome editing. This is because of cas9 a single, substantial protein sufficient for generating crRNA and cleaving the target DNA. Cas9 contains at least two nuclease domains, a RuvC-like nuclease domain, aRuvC nuclease initiates cleavage of the DNA strand not complementary to the guide RNA,near the amino terminus and the HNH nuclease domain in the middle of the protein. Still, the function of these domains remains to be elucidated. However, as the HNH nuclease domain is abundant in restriction enzymes and possesses endonuclease activity, it is likely to be responsible for target cleavage.(Makarova et al., 2011a).In the past few years, crystal structures of several Cas9s have been solved. These include SpyCas9, another type II-A ortholog from Staphylococcus aureus (SauCas9), and type II-B Cas9. These crystal structures have provided crucial information about the structural basis for sgRNA recognition, DNA recognition and cleavage.(Mir et al., 2018) Researchers investigate the role of a specific gene by analyzing the effect that its inactivation or overexpression has on an organism. Therefore, advancements in biological sciences have been tightly linked to developing tools that allow facile and accurate genetic manipulation. Genome-engineering techniques enable the modulation of a particular gene (or a set of genes) while maintaining the context relatively unchanged. Furthermore, precise modification of the genetic content and/or its expression is an attractive strategy for treating various genetic diseases, viral infections, and cancers.(le Rhun et al., 2019a)The CRISPR-Cas system has been realized to be the perfect tool to understand the role of genes because of its ability to cut a part of the DNA or particular genes. The synthesis of cas9 is much simpler and cheaper than any other genome editing tool or cas protein, making it the best choice for scientists.

Type III

Type III CRISPR-Cas system can recognize both RNA and DNA.It contains polymerase and RAMP modules in which at least some of the RAMPs seem to be involved in processing the spacer–repeat transcripts, analogous to the Cascade complex. RAMPs are defined as highly evolutionarily conserved, multi-functional, constitutively expressed proteins whose immunoregulatory activity is dependent upon their rapid decompartmentalization from the intracellular environment either actively, following cell stress or passively via necrotic cell death(Shields et al., 2011). Type III is further divided into subtype III-A and subtypes III-B. Subtype III-A systems can target plasmids, and it seems plausible that the HD domain of the polymerase-like protein encoded in this subtype might be involved in the cleavage of target DNA. The subtype III-B system is the one that has shown strong evidence for targeting RNA. The study of the Type III system is still in the early stages, but it is interesting to see that both subtypes target different nucleic acids.There is still room to know more about Type III.(Makarova et al., 2011b)

Application of CRISPR in medical sciences

The CRISPR system, a genome editing tool, could have many benefits in the medical sciences, particularly in removing genetic disorders and/or mutations.We are creating cures and preventions for various diseases. Some of the applications are listed below.

CRISPR in drug implementation

The discovery and development of drugs is a long and complex process to bring them to the market. The process typically begins with the hypothesis that perturbing a particular biological target will produce a beneficial effect that changes the course of a disease. Genome engineering is particularly useful in drug discovery as it can identify the genes that are responsible for a particular disease. However, this is commonly a laborious and time-consuming process. The identification of unknown genes and determination of their function is commonly carried out with high-throughput genetic screening platforms. Mutagenesis screenings have been successfully used to discover many

basic biological mechanisms and signalling pathways, and through this approach, one can determine which genes are responsible for a given phenotype. However, the main limitation of mutagenesis screenings for target drug discovery is the generation of heterozygous mutants with unknown random mutations. Implementing the CRISPR/Cas9 system can accelerate the identification and validation of high-value targets as one way to overcome the limitations is the use of targeted RNA interference (RNAi).(Martinez-Lage et al., 2018)

CRISPR in cancer treatment

CRISPR can potentially solve one of medicine's biggest problems: a cure for cancer. Cancer is a complicated and highly dynamic disease. Tumorigenesis comprises a multi-step process involving a complex interplay between cancer cells and the host immune system. Over the past few decades, targeted therapies brought hope for treating many types of cancer. However, a common complication is that the drugs eventually stop working in many patients, partly due to the complex mutational patterns in tumours and heterogeneity within the microenvironment.(Huang et al., 2018). Cancer is generated by oncogenes or tumour suppressor genes for various genetic and epigenetic reasons. Therefore, the CRISPR system could potentially treat the disease by generating accurate mutations by turning off the oncogenes or turning on the tumour suppressor genes.

There is an increasing number of clinical trials utilizing CRISPR/Cas9 technology to treat cancers of different origins. Most of these trials are based on genetically engineered T cells for cancer immunotherapy rather than targeting a specific gene in the tumour cells. (Liu et al., 2019). According to various genetic and epigenetic factors in cancer development, cancer modelling using the CRISPR system is expected to play an important role in detecting and identifying factors involved in cancer. (Mahmoudian-sani et al., 2018)

CRISPR in treating blood disorders

Transfusion-dependent Beta-thalassemia (TDT) and sickle disease (SCD) are some of the most common monogenic disorders in the world. Mutations cause both in the haemoglobin beta subunit gene (HBB). Two cases of TDT and SCD were observed by(Frangoul et al., 2021) and the use of CRISPR cas9 to treat the same after the administration of CTX001 showed both patients had early, substantial, and sustained increases in foetalhaemoglobin levels with more than 99% pan cellularity during 12 months. These findings, which indicate that CRISPR-Cas9–edited HSPCs underwent engraftment that was durably maintained, are consistent with an expected survival advantage of erythrocytes with a high level of foetalhaemoglobin.TDT, SCD and other disorders caused by mutations can be detected and/or cleaved with the CRISPR system.

CRISPR vs COVID-19 and other influenza strains

During the pandemic, researchers at Stanford University developed a technology called PAC-MAN (Prophylactic Antiviral CRISPR in human cells). The technology includes the Cas13 enzyme and a gRNA specific to nucleotide sequences in the SARS-CoV-2 genome. PAC-MAN eliminates the viral threat by preventing viral replication as it targets and destroys the viral genome. Such methods can be used for other viral infections as well. (Straiton, 2020) CRISPR's nucleotide-targeting ability makes it optimal for detecting the presence of viral RNA, giving researchers hope to develop a rapid at-home diagnostic test. This would help to keep up with the mass testing protocols that many experts have deemed necessary to control the spread of the virus**,** thus helping to prevent any future pandemics.

CRISPR as a diagnostic tool

Rapid nucleic acid detection is vital in identifying infectious diseases, agricultural pathogens, or circulating DNA or RNA associated with the disease. CRISPR-based technologies are rapidly advancing in molecular diagnostics and may soon take over PCR-based diagnostic tools. Enzymes from CRISPR–Cas systems have been adapted for the specific, rapid, sensitive, and portable detection of nucleic acids. Recently, a large set of different CRISPR-based methods used to detect nucleic acids has been described.Early technologies utilized the canonical Cas9 protein of type II CRISPR-Cas systems or its modified nucleolytically null, or dead, Cas9 (dCas9) protein. A massive leap toward developing CRISPR-based molecular diagnostics was the discovery of protein collateral activity of Cas12 and Cas13. Cas13 subtypes have different prefaces cleaving at specific dinucleotide motifs. In addition, Cas13 subtypes differ in size, direct repeat (DR) sequence, and CRISPR RNA (crRNA) structure. In 2017, researchers presented the CRISPR-Cas diagnostic tool DNA endonuclease-targeted CRISPR trans reporter (DETECTR). This method depends on the collateral activity by Cas12, which is activated after the recognition of target RNA by Cas12a. If the reaction with Cas12a protein and targeting crRNA is complemented by single-stranded DNA reporters (probes) and then mixed with the biological sample, crRNA-dependent recognition of pathogenic nucleic

acids by Cas12a turns on collateral activity that destroys DNA probes. Extraordinarily the DETECTR analysis takes 1 hour to complete.

In 2018, Specific High-sensitivity Enzymatic Reporter un-LOCKing (SHERLOCK)was presented. This diagnostic tool works on the CRISPR-Cas Type VI system. Its principle is the same as DETECTR it depends on activity from the Cas13 nuclease. Cas13 only recognizes and cleaves RNA, unlike Cas12 which also recognizes DNA. Isothermal amplification by RPA can be used to enrich target molecules and increase sensitivity. The amplified RNA fragments are mixed with Cas13 protein crRNA and fluorescent RNA probes.

CRISPR-Cas-based systems are superior to PCR-based methods due to the use of specific primers during isothermal amplification and the precision in spotting target templates via the Cas-sg/crRNA complex. Another significant fact regarding SARS-CoV-2 is that the viral load can vary during the day and at different stages of infection. Thus, a quantitative reverse transcriptase PCR (RT–PCR) diagnostic method could be negative at the time when the viral load is low and fails to identify infection, and thus a more accurate test is required.(I & M, 2021)

Challenges of CRISPR

CRISPR technology has been a ground-breaking discovery with great potential. However, it is a very new field in biology and, thus, faces challenges as researchers actively study it.

Off-target mutations

Off-target mutations are a major concern about CRISPR/Cas9 mediated genome editing. Large genomes often contain multiple DNA sequences that are identical or highly homologous to target DNA sequences. Besides target DNA sequences, CRISPR/Cas9 cleaves these identical or highly homologous DNA sequences, leading to mutations at undesired sites called off-target mutations. These off-target mutations can cause cell death or transformations. The dosage of CRISPR/Cas9 is also a factor for off-target mutations. Xiao **et al**. recently developed a flexible searching tool CasOT, which could identify potential off-target sites across whole genomes. (Zhang et al., 2014)

Delivery method

There are a lot of complications with the delivery of CRISPR components in the target cell. One of them is that the CRISPR components are too big, which complicates reaching the target cell. This problem, however, has been addressed by researchers at Stanford with the development of synthetic molecules called Liptoids, which hold potential as an effective delivery tool.(Straiton, 2020)

Questions also remain regarding the delivery methods of CRISPR/Cas9 into organisms. DNA and RNA injection-based techniques are used for CRISPR/Cas9 delivery, such as injection of plasmids expressing Cas9 and gRNA and injection of CRISPR components as RNA. The efficiency of delivery methods depends on the target cells and tissues. More attention should be paid to developing novel, robust delivery methods for CRISPR/Cas9.(Zhang et al., 2014)

Misuse and ethicality

The biggest question against any gene editing tool is its ethicality. Is it ethical to edit a person's genome and "go against nature"? Also, society's perception of CRISPR and gene editing differs greatly from reality. It is almost hyperbolic. People do not tend to see the positives of it, only what can be done wrong with it. This is discussed in (McCauley, 2019). Another major challenge is the possible misuse and/or purely commercial use. CRISPR being used for cosmetic purposes would greatly diminish its vast potential.

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