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# CRISPR/Cas9 Technology: Challenges and drawbacks

**Ayan Ghorui[1] and Sibashish Baksi[2*]**

[1]Department of Microbiology, Swami Vivekananda University, West Bengal-700121

[2] Department of Biotechnology, Swami Vivekananda University, West Bengal-700121

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**ABSTRACT**

Genome engineering has been transformed in recent years by the introduction of the CRISPR technology for a variety of cancer research projects spanning from fundamental science to translational medicine and precision cancer treatment. Although there have been tremendous advancements in this area, a number of technical issues still need to be resolved, including off- target activity, inadequate indel or poor homology-directed repair (HDR) efficiency, in vivo distribution of the Cas system components, and immunological reactions. Chromosome rearrangements brought on by off-target effects might unintentionally affect some poorly matched genomic locations and restrict the use of CRISPR-Cas editing technologies for therapeutic reasons.Studies have shown that CRISPR-Cas tools may be more susceptible to off-target effects than some of the other common gene-editing techniques because a Cas protein is a monomer that might accidentally enhance the identification of shorter target sequences, whereas the TALEN and ZFN assemblies are dimeric. Off-target effects often come from Cas enzymes that cleave bystanders (not intended targets) and guide RNA to recognize mismatches. CRISPR systems delivered in vivo cantrigger immune responses against foreign substances by significantly increasing people's innate immunity and/or adaptive immunity. Guide RNAs may be used to initiate innate immune responses. This article provides an overview of CRISPR-Cas applications from the lab to the clinic, identifies current barriers that may restrict the use of CRISPR-Cas systems as gene-editing toolkitsin precision medicine, and offers some perspectives on how to address these barriers and speed uptechnical advancement.

**Keywords:** CRISPR, RNA, Epigenetic, Cas

**Introduction:** Cancer is one of the primary causes of disease-associated death with a rising incidence throughout the world. Large-scale sequencing databases have shown that genetic changes, whether exclusive to one form of tumor or common to several, play important roles in carcinogenesis. Research into cancer is being advanced by identifying the structural and functional characteristics of mutant genes, especially long-tail molecular modifications, in cancer genome

variants. Directly targeting and altering the genomic sequence is now possible because of the advent of designed nucleases such as effector nucleases that resemble transcription activators (TALENs) and zinc finger nucleases. The recent advancement of clustered regularly interspaced short palindromic repeat (CRISPR) technology has expedited genome engineering. This toolkit has been greatly and continually developed since the initial use of CRISPR/CRISPR-related proteins (CRISPR/Cas) as a tool for genome modification in 2013 in mammalian cells. Currently, CRISPR/Cas systems may introduce and target site-specific epigenetic and transcriptional alterations in addition to changing the gene sequence in organisms and animals (Yang *et al*., 2021; Wang *et al*.,2022).

* Corresponding author; email: sibashishb@svu.ac.in, sibashish.baksi@gmail.com

CRISPR-Cas systems have been classified into six unique varieties (Type I-VI, each having trademark Cas nuclease components) and two broad classes (Class 1 or Class 2). The effector complexes in Class 1 systems (Types I, III, and IV) are composed of numerous Cas proteins (including one or more nuclease components) that are tightly bound to the crRNA. Class 2 systems (Types II, V, VI) have CRISPR RNA-Cas effectors that contain just a single multi-domain effector protein with nuclease activity (Asmamaw and Zawdie, 2021).

**Mechanism of CRISPR- CAS:** A schematic representation of CRISPR-Cas's fundamental process may be found in Figure 1. The two fundamental parts of the CRISPR/Cas-9 system are guide RNA (gRNA) and CRISPR-associated (Cas-9) proteins. Known as a genetic scissor, the Cas-9 protein is a large, multi-domain DNA endonuclease consisting of 1368 amino acids that cleaves target DNA to create a double-strand break. The nuclease (NUC) lobe and the recognition (REC) lobe are the two sections that make up Cas-9. RuvC, HNH, and domains that interact with protospacer adjacent motif (PAM) make up the NUC lobe, whereas the REC lobe is made up of the REC1 and REC2 domains, which bind guide RNA. CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) are the two components that make up guide RNA. While tracrRNA is a lengthy loop that acts as a binding scaffold for the Cas-9 nuclease, crRNA is a 16–20 base pair molecule that identifies the target DNA by partnering with the target sequence (Asmamaw and Zawdie, 2021; Liu *et al*., 2020; Mei *et al*., 2016). Three basic processes make up the CRISPR/Cas-9 genome editing mechanism: recognition, cleavage, and repair. The sgRNA that was created guides Cas-9 and identifies the target sequence in the desired gene by means of its complementary base pair component, 5′crRNA (Ming *et al*., 2016).

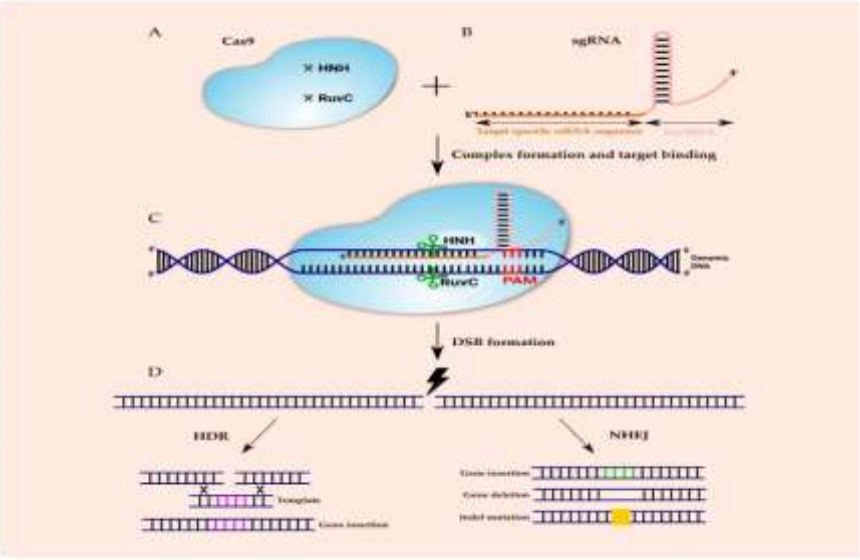

**Fig. 1** CRIPR-Cas Mechanism: A schematic diagram [Adopted from (Janik *et al*., 2020) as it is)

When sgRNA is absent, the Cas-9 protein is dormant. At a location three base pairs upstream of PAM, the Cas-9 nuclease creates double-stranded breaks (DSBs). The length of the PAM sequence, which is a short-conserved DNA sequence (two to five base pairs) that is located downstream of the cut location, changes according on the kind of bacterium. After locating a target site with the proper PAM, Cas-9 initiates local DNA melting, which is followed by the creation of an RNA-DNA hybrid. The Cas-9 protein is then made ready to cleave DNA.

The target DNA's complimentary strand is cut by the HNH domain, while the non-complementary strand is cut by the RuvC domain, resulting in DSBs that are primarily blunt-ended. Ultimately, the host cellular machinery fixes the DSB. Mechanisms of Double-Stranded Break Repair The two methods for repairing double-strand breaks (DSBs) produced by the Cas-9 protein in the CRISPR/Cas-9 mechanism are homology-directed repair (HDR) and non-homologous end joining (NHEJ) (Mei *et al*., 2016; Ceasar *et al*., 2016; Jiang *et al*., 2017).

**Challenges and limitation:** CRISPR/Cas-9 technology has shown considerable promise as a genome-editing system, but its use has been hindered by a number of issues that need to be resolved [Refer **Fig. 2**] (Rasul *et al*., 2022).

In complex eukaryotic organisms, off-target effects remain a significant problem, notably in vivo for therapeutic uses. The gRNA of the Cas9 and PAM sequences, as well as off-target cleavage in the genome, determine the targeting specificity. Several web-based editing tools have been created and effectively applied to detect and anticipate off-target cleavages in silico. These methods, however, are restricted to analyzing homologous genes and have limitations when it comes to anticipating changes such as epigenetic alterations. Dosage influences a number of factors, and in some applications, the cleavage target specificity might be crucial. As an alternative, Cas9 systems' target selectivity can be increased by directly controlling the activity of the genome-editing Cas9 proteins, which decrease activity in response to target locus alterations (Yang *et al*., 2021; Yang *et al*., 2018; Zischewski *et al*., 2017).

Cas9 and a single guide RNA (sgRNA), for example, must be effectively delivered to the target cell for CRISPR/Cas9 treatment in vivo. The delivery strategy should be very effective at editing, have a low immunogenicity, and target the target organ or cell type precisely. The plasmid-based production of Cas9 and sgRNA, which is effective for in vivo applications in model species like mice, has been used in the first-generation genome editing procedures in mammalian cells. However, editing effectiveness and targeted delivery are inefficient, and Cas9 activity is difficult to manage. However, the effectiveness of Cas9/sgRNA delivery in vivo has been improved by the development of several viral and non-viral delivery techniques (Yang *et al*., 2021; Cong *et al*., 2013; Mali *et al*., 2013).

The CRISPR/Cas9 components may be delivered by viruses like as lentivirus, adenovirus, and adeno-associated virus (AAV). AAVs are presently the most sophisticated approach among them for in vivo gene delivery. AAV is an appropriate gene therapy vector due to its non-pathogenic nature in humans, several serotypes for cell-type targeting, little immune response, and proven efficacy and safety in both animal models and approved clinical trials. Their tiny package size, which necessitates the use of many viruses to transport all of the CRISPR/Cas9 components (Cas9, sgRNAs, and donor DNA if needed), is one of the main disadvantages of their employment, though, and further reduces the editing efficiency. AAV technology, in contrast to certain other

techniques, allows CRISPR components to be expressed persistently in altered cells, thus increasing the risk of immunological reactions or unfavourable off-target genomic consequences (Yin *et al*., 2016; Yin *et al*., 2017; Follenzi *et al*., 2007; Ahi *et al*., 2011).

Lentivaunts and adenoviruses are able to infect cells that are dividing as well as those that are not; however, unlike lentiviruses (and AAV sometimes), adenoviruses do not integrate into the recipient cell's DNA. Furthermore, other viral proteins, such as the G-protein of the vesicular stomatitis virus (VSVG), can modify the tropism of lentiviruses. Nevertheless, because lentivirus and adenovirus both trigger robust immune responses, there are certain disadvantages to their usage (Follenzi *et al*., 2007; Ahi *et al*., 2011; Kotterman *et al*., 2014).

Another issue that needs to be taken into account when implementing CRISPR-Cas9 technology in clinical settings is the immunogenicity of the Cas9 nuclease. Certain donors have naturally occurred Cas9 antibodies in their blood; 79% of them have anti-saCas9 antibodies and 65% have anti-SpCas9 antibodies. T cell immunological memory against SpCas9 was present in 96% of the donors examined. Low editing efficiency and a potentially dangerous immunological storm are caused by the human anti-Cas9 immune response in patients undergoing CRISPR-Cas9 therapy (Charlesworth *et al*., 2019; Wagner *et al*., 2019).

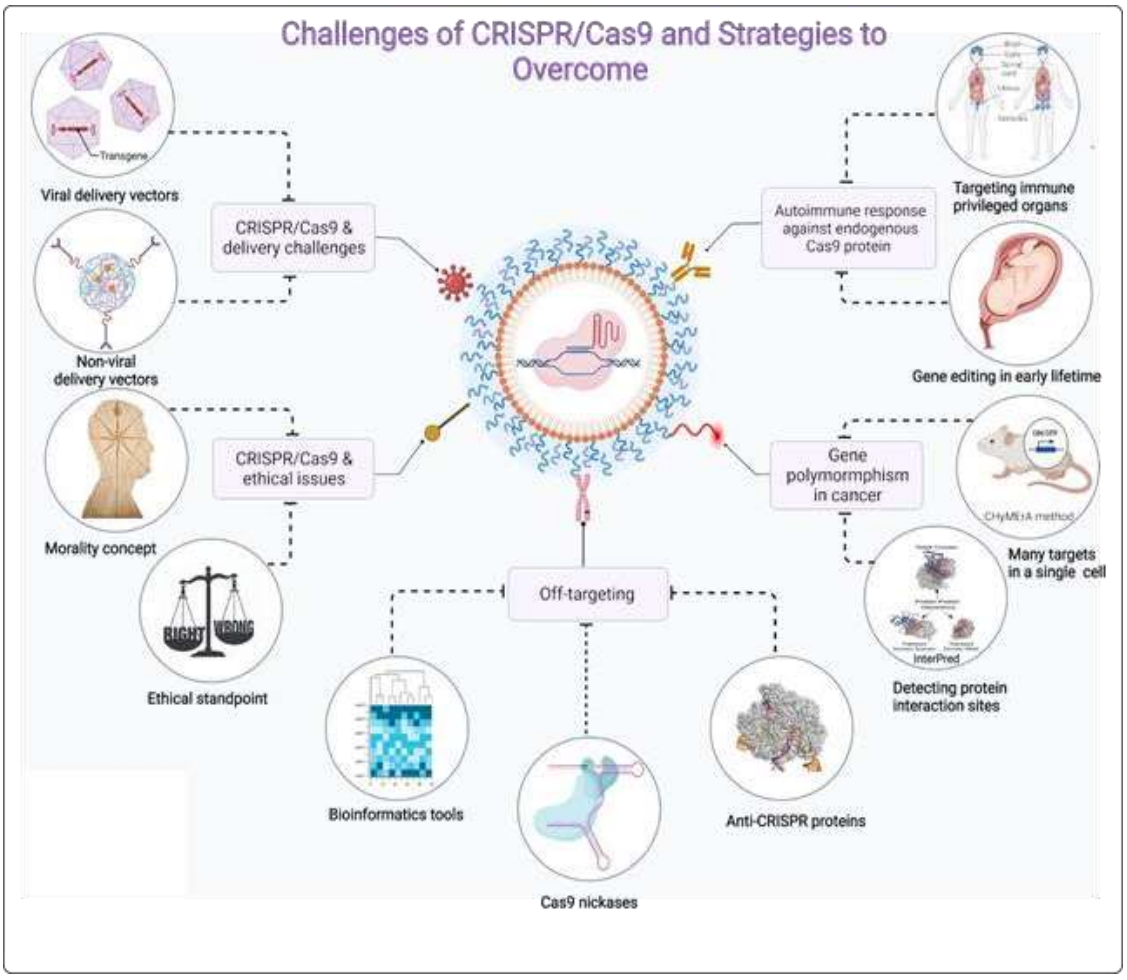


**Fig. 2 Challenges and limitations of CRISPR/Cas9** [Adopted from (Rasul *et* al, 2022) as it is]

The effectiveness and generalizability of CRISPR-Cas9 technology is a last major worry. For example, precise CRISPR-Cas9 genome editing requires protospacer adjacent motif (PAM; NGG) sequences at target locations. The widespread use of this technique has been restricted by the fact

that Cas9 has only recently been identified in a small number of PAM sequences. Thankfully, the range of PAM sequences that recently created xCas9 can recognise has increased (to NG, GAA, and GAT), quadrupling the potential applications of CRISPR-Cas9 technology. Moreover, the effectiveness of accurate CRISPR-Cas9-mediated genomic insertions via HDR is low; nonetheless, this efficiency can be raised by nucleofection delivery, NHEJ inhibition, and the use of single-stranded DNA (ssDNA) donors rather than double-stranded DNA (dsDNA) (You *et al*., 2019).

**Possible overcome strategies:**

***Autoimmune response against endogenous Cas9 protein:*** However, Cas9 may be destroyed by the immune system's response, which would restrict gene editing. Two strategies to get around this are to focus on immune-privileged organs and to start using the CRISPR/Cas system for gene editing as early as possible in infancy. Gene editing in the early stages of development can identify disorders in children and block molecular pathways in prevalent tumors. The likelihood of autoimmune illness can be decreased by concentrating on immune-privileged tissues such as the brain, placenta, fetus, and testicles. However, more abilities and methods are needed to lower the chance of off-targeting andDNA fragment loss (Rasul *et al*., 2022; Alanis-Lobato *et al*., 2021).

***Off-targeting*:** Due to the high rate of off-targeting, which can result in mutations developing in undesirable genomic sites, the use of CRISPR/Cas9 has raised concerns. Tools for bioinformatics have been created to assist in predicting and minimizing off-targ*et al*terations. Designing more precise gRNA and lowering the size of gRNA to fewer than 20 nucleotides are two methods to combat off-targeting. By changing one nuclease domain in only one strand of DNA, Cas9 nickases can also lessen off-targeting. Following site-specific targeting, Cas9 protein inactivation may also lessen off-targeting. The CRISPR system can be enhanced by utilizing Aca proteins to inhibit Acr proteins and phage-based anti-CRISPR proteins to overcome drug resistance (Hazafa *et al*., 2020; Chung *et al*., 2020).

***The delivery challenges*:** Although Adeno-Associated Viruses (AAV) are a potential method for CRISPR/Cas9 technologies, there are difficulties due to their tiny package size and restricted targeting. Strategies include dividing the Cas9 protein into two AAV vectors and using ribonucleoprotein complexes can be used to get around these difficulties. These techniques can increase delivery effectiveness while lowering the dangers of off-targeting and mutation. The overall delivery efficiency of cancer treatment is still low, though. The best delivery method should be chosen according to the unique requirements and possible dangers of AAV distribution (Rasul *et al*., 2022; Chew *et al*., 2016; Yin *et al*., 2014).

***Ethical issues and CRISPR/Cas9 technology:*** The potential of gene editing technologies, like CRISPR/Cas9, raises serious ethical and safety concerns. The technology's uses in molecular biology research, such as developing more powerful police dogs and resistant crops, create ethical, moral, and safety issues. Concerns regarding hazards to human safety and dignity as well as the possibility of genocide are brought up by the possibility of human germline alteration. Evaluating possible risk-benefit ratios and the intricacy of the relationship between genetic information and biological phenotypes are key moral concerns in biomedicine. The right use of CRISPR/Cas9 technology and its ability to enhance the quality of life, however, is key to its potential to improve health and wellness (Rasul *et al*., 2022).

**Conclusion and Future Perspectives:** Due in large part to the relatively effective delivery methodologies developed for these systems, the field of therapeutic genome editing has advanced rapidly in recent years, moving from basic research through preclinical development and human

trials, especially for ex vivo HSC and T cell editing and in vivo liver genome editing. However, before the biological potential of genome editing can be completely realized, a number of significant difficulties must be overcome.

First, one of the most difficult issues in the field of gene therapy, if not the most difficult, has always been delivery. Significantly, better delivery efficiencies are required to make up for the poor HDR and even knockout efficiencies that exist in many tissues at the moment.

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