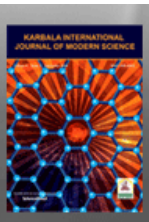
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CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) is an exponentially growing tool with wide-spread applications in therapeutics like gene modifications that focus on altering the hereditary material to repair or eliminate any defective gene-causing diseases like cancer, AIDS (Acquired immunodeficiency syndrome), etc. It also includes the identification of the target sequence with the help of sgRNA followed by the substitution of a malfunction-ing gene with a normal version. It offers high efficiency, specificity, and post-gene-editing efficacy, but have also some off-target impressions, and immunogenic effects. The contribution of CRISPR/Cas9 has already been proved primarily in in-vitro studies using animal germ cell lines but translation in in-vivo models is still not much supported due to ethi-cal considerations. The recent advances include studies and clinical trials focusing on the treatment of various diseases of genetic origin. For instance, CRISPR gene knock-in technique was applied for in-vivo Leber Congenital Amaurosis 10 treatment, where CRISPR components were delivered via sub-retinal injection to correct the mutation in CE9290. The current paper recapitulates the capability of CRISPR/Cas9 in in-vivo gene therapy for various disorders like cancer, AIDS, sickle cell disease and the most recent COVID-19. The insights presented herein are poised to contribute signifi-cantly to the advancement of the field, fostering a deeper understanding of CRISPR/Cas9 technology and accelerating its clinical transition. Ultimately, this review paper serves as a valuable resource for researchers, clinicians, and policy-makers invested in the continued evolution of gene therapy and responsible utilization of CRISPR/Cas9 for human welfare

Keywords

CRISPR/Cas9; gene editing; healthcare; disease treatment; gene therapy

REVIEW ARTICLE

Recent Advances in CRISPR/Cas9-assisted Gene Therapy

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Abstract

CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) is an exponentially growing tool with widespread applications in therapeutics like gene modifications that focus on altering the hereditary material to repair or eliminate any defective gene-causing diseases like cancer, AIDS (Acquired immunodeficiency syndrome), etc. It also includes the identification of the target sequence with the help of sgRNA followed by the substitution of a malfunctioning gene with a normal version. It offers high efficiency, specificity, and post-gene-editing efficacy, but have also some off-target impressions, and immunogenic effects. The contribution of CRISPR/Cas9 has already been proved primarily in in-vitro studies using animal germ cell lines but translation in in-vivo models is still not much supported due to ethical considerations. The recent advances include studies and clinical trials focusing on the treatment of various diseases of genetic origin. For instance, CRISPR gene knock-in technique was applied for in-vivo Leber Congenital Amaurosis 10 treatment, where CRISPR components were delivered via sub-retinal injection to correct the mutation in CE9290. The current paper recapitulates the capability of CRISPR/Cas9 in in-vivo gene therapy for various disorders like cancer, AIDS, sickle cell disease and the most recent COVID-19. The insights presented herein are poised to contribute significantly to the advancement of the field, fostering a deeper understanding of CRISPR/Cas9 technology and accelerating its clinical transition. Ultimately, this review paper serves as a valuable resource for researchers, clinicians, and policymakers invested in the continued evolution of gene therapy and responsible utilization of CRISPR/Cas9 for human welfare.

Keywords: CRISPR/Cas9, Gene editing, Healthcare, Disease treatment, Gene therapy

1. Introduction

Errors in genetic information transfer due to monogenic disorders, multifactorial inheritance disorders, and mutations lead to genetic illnesses. Certain disorders like sickle cell disease are present by birth while some are acquired during life span like cancer. For instance: AIDS, cystic fibrosis, cancer, hematological and neurological disorders have adverse effects on a large number of people globally [1]. Safer and more effective remedies have become the need of the hour. Nanotechnology has been proven effective in treating ailments with much lower doses of medicine and with the least side effects. Several nanomaterials and composites with biomaterials like chitosan, hydroxyapatite [2,3], and metal-based nanostructures [4–6] have proved effective against cancer. However, gene therapy eliminates the disease from the root level.

The basic idea behind gene therapy is to rectify the defective gene/s by transferring new gene/s to treat non-life-threatening as well as life-threatening ailments [1]. Considering the results from the trials, 27 cellular and gene therapies have already been approved by the FDA (Food and Drug Administration) [7]. CRISPR-associated (Cas) nuclease systems, meganucleases, zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs) are some of the most prominent methods for gene therapy [8].

CRISPR/Cas9, one of the advanced gene therapy variants has potential applications outside the realm

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of traditional gene therapy techniques [9]. CRISPR/Cas covers more than 50% of bacterial and archaeal genomes and is responsible for adaptive immunity in prokaryotes [10]. It uses RNA-guided nucleases to detect and chop foreign genetic material. The hypervariable locus, created by CRISPR absorbed by the host and utilized for the development of acquired immunity [11]. CRISPR was designated as the breakthrough of 2015 by Science and in 2020, Jennifer Doudna and Emmanuelle Charpentier shared the Nobel Prize in Chemistry [12].

CRISPR/Cas is widely considered to acquire disease-related models for ailments including Alzheimer's disease [13], brittle bone [14], X-linked adrenoleukodystrophy [15], and aniridia-related keratopathy [16]. In addition to this, CRISPR/Cas has also shown the contribution of CRISPR in monogenic human genetic illnesses treatment, which is one of the fascinating and most promising developments of this technology. For instance, as the paper highlights the ongoing advancement of CRISPR in cancer treatment, diagnostic and therapeutic applications in COVID-19 disease, and gene therapy approaches in viral diseases like AIDS and hereditary disorders like SCD, this technology not only overcomes the shortcomings of conventional therapies but also paves the way to new and advanced methodologies for research studies and clinical trials with successful results as is presented in this review paper. Drawing from a plethora of recent studies and breakthroughs, the paper systematically presents different challenges, emerging strategies, and solutions aimed at enhancing the precision, efficacy, and safety of in-vivo CRISPR/Cas9 applications. Scientists have evaluated several mouse and animal models against different diseases (Table 1). This paper acknowledges the previous reviews done on the subject [17–20] and continues the work by providing an improved, detailed, and recent review on different aspects of CRISPR/Cas9 which includes recent and ongoing clinical trials using this technology, detailed view of diseases and their treatment and ethical concerns surrounding CRISPR/Cas9 along with current international standing and regulatory norms of different countries and stakeholders on gene editing. It focuses on the convergence of scientific progress and ethical discourse essential for the continued success and acceptance of CRISPR-based therapies.

2. Gene editing using CRISPR/Cas9: mechanism

During a pathogen attack in a bacterium, the CRISPR acts as a genomic memory from the previous

Table 1. CRISPR/Cas9 application in healthcare and disease treatment.

Model	Disease	Target	Mode	Result	Reference
Human	Metastatic lung Cancer	PD-1 edited T cells	Ex-vivo	17 (of 22 patients) had higher modified T-cells for infusion.	[37]
	β-thalassemia	BCL11A Hematopoietic stem and progenitor cells	In-vitro	Indel frequency of 93.0% (SpCas9)	[39]
	HIV-1 and acute lymphoblastic leukemia	CCR5-ablated hematopoietic stem and progenitor cells	Ex-vivo	Complete remission of acute lymphoblastic leukemia	[40]
	Transthyretin amyloidosis	Transthyretin (TTR)	In-vivo	Dose-dependent decrease in serum TTR	[41]
	Renal cell carcinoma	CD70	Ex-vivo	Ongoing (NCT04438083)	[42]
	Multiple myeloma	BCMA	Ex-vivo	Ongoing (NCT04244656)	[43]
	Hematopoietic malignancies	HPK1	Ex-vivo	Ongoing (NCT04037566)	[44]
Mouse	Hereditary tyrosinemia type I	FAH hepatocytes	Ex-vivo	2.6% of alleles corrected.	[45]
	X-linked retinitis pigmentosa	Retinitis pigmentosa GTPase regulator	In-vivo	Significant preservation of photoreceptors observed	[46]
	Duchenne muscular dystrophy	Exon 23 from dystrophin gene (Mdx mouse)	In-vivo	Partial recovery of functional dystrophin protein	[47]
Mice	Huntington's disease	Huntingtin (HTT) gene	In-vivo	Lowered mutant HTT mRNA and related protein aggregates.	[48]
Rat	Amyotrophic lateral sclerosis	Superoxide dismutase 1	In-vivo	~50% improved motor neurons at the end stage.	[49]
	Hypertension	AGT gene	Ex-vivo	40% reduction of AGT expression.	[50]

exposure to the pathogen that is used by Cas proteins and guides RNA to recognize the foreign DNA followed by its inactivation via endonuclease activity [21]. The same mechanism for gene editing and altering the genomic target of Cas9 proteins can be used for human welfare by simply modifying the target sequence.

Genome editing with CRISPR/Cas9 is usually classified into three steps [22]: The first stage (recognition) engineered sgRNA directs the Cas9 protein toward the target sequence. In the second stage, RuvC and HNH domains of Cas9 protein form double-stranded break (DSBs) at target loci, upstream to a protospacer adjacent motif (PAM) site [23–25]. In the final stage (rejoining), created breaks are repaired either via homology-directed repair (HDR) or Non-homologous end joining (NHEJ) [26]. NHEJ while the repair of DSBs may introduce small insertions or deletions (indels) randomly that make the process more error-prone than HDR [24].

3. Applications of CRISPR/Cas9 in healthcare and treatment

CRISPR has shown a promising way to treat some of the serious and lethal diseases and aid in saving lives. However, the animal and human model trials are still not getting support due to ethical concerns. This section summarizes the advancements in CRISPR/Cas9 and its contribution to some health ailments:

3.1. Cancer

Abrupt genetic changes and certain environmental factors induce tumorigenesis [27]. Current treatment methods involve chemotherapy, radiation therapy, surgery, hormonal therapy, and molecular targeted therapy or their combinations which mainly suffer from poor efficiencies, side effects, and possible recurrence [28]. Antitumor immunotherapy relies on the identification and targeting of immunological checkpoints such as the cluster of differentiation *i.e.* CD152 (also called CTLA-4) and CD279 (also known as PD-1) [29,30] and the use of immunological checkpoint inhibitors. Gene therapy for the modification of immune cells like Chimeric antigen receptor T-cells (CAR-T cells) has shown great potential [31] but is ineffective against solid cancers [32] due to heterogeneous antigen expression [33], T cell fatigue [34], poor expression of MHC (major histocompatibility complex), high tumor mutational burden, and immunosuppressive elements in the tumor microenvironment [35] that ultimately results in immune escape.

CRISPR has offered multiplexing capability, especially against genetic changes related to cancers, and become a potent tool to restore the normal state of genes [36]. The first clinical trial with CRISPR against cancer was performed in China in 2016 by editing immune cells against lung cancer. The PD-1 gene's exon 2 was targeted using sgRNA1, and sgRNA2, followed by cotransfection of Cas9 and sgRNA plasmids into T-cells via electroporation. In comparison to unedited cells, modified T cells had a significantly higher percentage of CD8+IFN-γ+ cells [37]. This study has raised the hope for the clinical applications of CRISPR. Li et al. [38], have prepared CD133 knockout cells by targeting exon 1 gene using LentiCrispr V2 followed by transfection into HEK-293T cells. Modified CD133+ colon cancer cells have shown a significant decline in colony-forming ability, cell proliferation, and inhibition of cell migration and invasion.

Feasibility and safety assessment of multiplex CRISPR editing and T-cell designing was conducted with three patients having refractory cancer. The T-lymphocytes from the patient were edited ex-vivo to target TRAC, PDCD1 and TRBC genes and then infused back. The prolonged activity of designed T-cells has suggested the potential application of CRISPR gene editing in cancer immunotherapy [51]. Besides gene editing, CRISPR/Cas9 system is also capable of identifying the chemo-resistant genes that are the main obstacles in cancer treatment [19].

3.2. Corona-virus disease of 2019

The latest pandemic COVID-19 was brought on by the SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) virus. The World Health Organization (WHO) estimates that as of February 23, 2023, COVID-19 has infected 757, 264, 511 people and caused 6,850,594 deaths. The virus has rapid transmission via direct contact, air, bacteria, and droplets [52]. Long COVID has shown new-onset illnesses including cardiovascular and cerebrovascular diseases [53], type 2 diabetes [54], and dysautonomia [55]. Vaccines, antiviral medications, monoclonal antibodies (mAbs), and non-pharmaceutical measures like isolation, lockdowns, social distancing, contact tracing, and intensive care have been used to prevent transmission as well as lower the number of casualties [56,57].

The advancements in CRISPR-Cas9 technology allows the development of time-effective assays and diagnosis kits along with providing therapeutic tool. The detection assays are around 95% accurate and completed within 40 min of turnaround time [58]. Specific high-sensitivity enzymatic reporter

unlocking (SHERLOCK) is another detection approach that provides a framework for COVID testing with paper strips [59] that generates visually readable output by detecting specific nucleic acid sequences. In addition, CRISPR can also be employed as a gene therapy tool in Covid-19 treatment with RNA-guided RNA-targeting endonuclease Cas13. PAC-MAN (Prophylactic Antiviral CRISPR in huMAN cells), a VI-D CRISPR/Cas13d variant from *Ruminococcus flavefaciens* can disrupt RNA simultaneously at multiple regions. This approach has higher efficiency and can complete a relatively high rate of mutation and recombination of SARS-CoV-2 [60].

3.3. Acquired immunodeficiency syndrome (AIDS)

Despite several advancements in medical and healthcare, viral infection like COVID and HIV (human immunodeficiency virus) AIDS remains a serious health concern across the globe. The major issue with HIV infection is incomplete eradication and retention of the virus in HIV reservoirs, like astrocytes (human brain) [61], peripheral blood [62], and lymphoid tissues [63] that may show recurrent infection [15,63]. Lifelong antiviral medication may be suggested but it has shown serious side effects like the risk of fractures, central nervous system, cardiovascular, hepatic, and renal systems [64] associated ailments.

The emerging CRISPR system might help in eliminating HIV-integrated genomes as well as the complete elimination of HIV from reservoirs [65]. Previous studies revealed that multiplexed CRISPR can selectively target multiple conserved sequences and eliminate the elevated level of the pre-integrated proviral genome along with prolonged protection from HIV-1 infection [66]. In August 2013, the CRISPR system was used to engineer the mutated long terminal repeat sequence (LTR) of HIV-1 in an in-vitro model which resulted in the elimination of proviral DNA integrated into the infected host cell and blocked the virus expression. The gRNA expression vector was constructed with a human U6 polymerase III promoter to target HIV-1 LTR considering target 5 (located in the R region, at TAR sequence) and target 6 (located in the U3 region at NF−κB binding sequence). LTIG vectors, pseudotyped with VSV-G envelope protein were used to infect HeLa cells and 293 T to evaluate the impact of the CRISPR. It successfully disrupts the expression of active HIV-1 provirus but prevents latent infection [67]. In another study, TALEN+ and CRISPR were compared to edit the human CCR5 gene (transmembrane co-receptor critical for HIV-1 entry into target CD4+ cells). It turned out that CRISPR/Cas9 facilitated the sorting of cells with 4.8 times higher gene editing than TALEN + transfected cells [68]. Teque et al. [69], reprogrammed peripheral blood mononuclear cells of HIV-infected patients by introducing bi-allelic CCR5Δ32 mutational changes using Sendai virus vector (SeVdp) or EBNA1/OriP episomal vectors. All created iPSC lines and altered cell lines maintained their normal karyotype and showed no signs of HIV integration.

3.4. Sickle cell disease (SCD)

It is a hereditary monogenic autosomal recessive illness caused due to point mutation in the hemoglobin β subunit gene (HBB) [70]. Polymerization of deoxygenated sickle hemoglobin resulted in anemia, erythrocyte distortion, hemolysis, excruciating vaso-occlusive events, and irreparable end-organ damage [71]. The only treatment possibilities available are hydroxyurea, transfusions, and pain management. FDA has approved only four medications *i.e.* voxelotor (2020), crizanlizumab-tmca (2019), L-Glutamine (2018), and hydroxyurea (1998) to minimize acute complications.

One of the therapeutic approaches involved ex-vivo β-globin gene repair in hematopoietic stem and progenitor cells (HSPCs) using adeno-associated virus serotype 6-mediated HBB gene repair with chemically modified guide RNAs. The preclinical trial results confirmed the reproducibility, safety, and efficacy to start 1/2 phase clinical trial [72]. Wu et al. [73], amplified sgRNA with promoter T7 from pX458 plasmid followed by in-vitro transcription. The strategy didn't have any off-target impact even after the alteration of more than 80% of alleles. It enhanced fetal hemoglobin without any transfusion and vaso-occlusive abolition.

4. Challenges in gene editing with CRISPR/Cas9

CRISPR is one of the efficient methods used for gene editing but suffers from some bottlenecks like off-target effects, off-target binding, and editing [74] which may result in serious complications. In comparison to zebrafish and mice, human cells are more sensitive to off-target mutations [75,76]. CRISPR-assisted gene editing may have unintentional complex rearrangements, large deletions, and inefficient DSB repair which result in adverse impact and cell apoptosis [77,78].

In the past few years, multiple techniques *i.e.* IDLVs (integrase-defective lentiviral vectors) [79], DISCOVER-seq (discovery of in-situ Cas off-targets

and verification by sequencing) [80], CIRCLE-seq (Circularization for in vitro reporting of cleavage effects by sequencing) [81], digenome-seq [82], HTGTS (high-throughput, genome-wide, translocation sequencing) method [83], GUIDE-seq (genome-wide, unbiased identification of DSBs enabled by sequencing) [84], etc., have been immersed to detect off-target genome modifications. Some of the methods include the engineering of Cas9 nucleases [85], dimeric Cas9 nuclease [86], Cas9 coupled with artificial inhibitory domains [87], optimized sgRNA design [88] "hit-and-run" approach by transferring Cas9 protein in the place of Cas9 gene [89] and non-viral delivery [90] to minimize the off-target effects. The use of dCas9 (dead Cas9) was more efficient in its therapeutic applications as it can modify the expression of target genes without the introduction of DSBs [91] while Cas9n uses only single-stranded breaks (SSBs). The upgraded approach has contributed to the development of prime editors and base editors for the safer applicability of CRISPR in gene editing [17]. Besides, stability of alterations and efficiency in genes also is a major concern [74]. For example, in some cells, the edited genome gives out a growth advantage. In a mouse model of hereditary tyrosinemia [92], liver cells were modified with CRISPR. Initially, only 0.25% of liver cells were genetically corrected which survived and after 33 days, this proportion reached 33.5%, which was enough for the disease phenotype to be rescued. In some cases, the edited genome has also shown growth disadvantages like in the case of inactivating oncogenes. In comparison to edited cells, unedited cancer cells have malignant capacities and acquire growth advantage. To counter this, repeated treatment episodes and highly specific editing efficiency must be required [74].

Some immunogenic effects because components used in CRISPR-based in-vivo gene therapy like Cas nucleases [18], and gene delivery vehicle [90] are exogenous e.g. humans have frequent exposure to Cas9 nucleases from *Streptococcus pyogenes* and *Staphylococcus aureus* [93]. In such cases, subjects already have pre-existing immunity against these bacterial orthologs: SaCas9 and SpCas9 [94]. Studies have confirmed that mouse models can tolerate the presence of bacterial Cas9 orthologs: SaCas9, SpCas9 and CjCas9 (*Campylobacter jejuni* Cas9) [95] but humans already possess antibodies against SaCas9 and SpCas9, only CjCas9 left as an option. Removal of Cas9 epitope nuclease [18] and selection of nonimmunogenic delivery vehicles like nanoparticles or lipid-based mRNA or protein delivery vehicles can be employed to prevent immunogenic responses [96,97]. Besides, the engineering of Adeno-associated viruses (AAVs) with the insertion of human epidermal growth factor receptor 2 specific ligands into the AAVs capsid increased the tropism of the virus for tumor cells in-vivo by approximately 20-fold [98].

5. Ethical concerns

Application of CRISPR Cas9 as a gene editing and a potential gene therapy tool has posed plenty of ethical concerns regarding the transmittance of undesirable changes into future generations along with several side-effects and unintended mutations [99], non-therapeutic applications of CRISPR [100] and evolution of the "designer babies" [101]. One more problem associated with CRISPR is the cost of treatment which makes its availability fractional [102]. To avoid the misuse of CRISPR Cas9 technology, socially acceptable and ethically sound regulations and policies are necessary [101]. The National Guidelines for Gene Therapy Product Development and Clinical Trials, framed by the Indian Council of Medical Research and Department of Biotechnology, Ministry of Science and Technology, Government of India, clearly states that the ideal gene therapy product must not be teratogenic, excessively immuno-stimulating, mutagenic, or with undesirable host immune response. The Department of Health Research, Ministry of Health and Family Welfare, Government of India has also constructed a specialist national committee 'Gene Therapy Advisory and Evaluation Committee (GTAEC)' that evaluates various aspects of biomedical research, concerned government bodies and other stakeholders involved [103]. The international standpoint and regulatory approaches regarding gene editing in various countries can be accessed from the research project report presented at the Third International Summit on Human Genome Editing (Francis Crick Institute, London UK) [104].

6. Conclusion

The scope of CRISPR/Cas9 in the future is vast, as the current exploratory research and application-based studies are just a drop in the ocean and it has barely scratched the surface in terms of its future in medical research. The intricate interplay of gene editing, precision medicine, and ethical considerations have been examined indicating the need for a multidisciplinary approach to advance the field. It is quite possible that CRISPR-based genome editing will soon be widely used in clinical practice, even

though efficacy and safety concerns remain a major issue at present. However, the scientific breakthroughs and clinical successes over the past years demonstrate the capacity of CRISPR/Cas-mediated gene therapy to provide lasting benefits to human health, which justifies increasing efforts and continued optimism towards incorporating these therapies into our standard treatment option and forward-thinking strategies that maximize precision, efficacy and patient safety. The prospects of this technology have resulted in an explosion in its application across multiple fields but most importantly it could lead to becoming a one-stop solution for any disease that has a genetic origin. As we stand at the threshold of transformative advancements, we encourage the community to harness the insights presented here as catalysts for innovative research, actionable solutions, and a shared commitment to realizing the full potential of CRISPR/Cas9 in shaping the future of personalized medicine.

Conflicts of interest

The authors have no conflict of interest to declare.

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