Strategies to overcome the main challenges of the use of CRISPR/Cas9 as a replacement for cancer therapy

Mohammed Fatih Rasul1, Bashdar Mahmud Hussen2,3, Abbas Salihî3,4, Bnar Saleh Ismael5, Paywast Jamal Jalal6, Anna Zanichelli7, Elena Jamali8, Aria Baniahmad9, Soûdeh Ghaîouri-Fard10, Abbas Basiri11 and Mohammad Taheri9,12*

Abstract
CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9) shows the opportunity to treat a diverse array of untreated various genetic and complicated disorders. Therapeutic genome editing processes that target disease-causing genes or mutant genes have been greatly accelerated in recent years as a consequence of improvements in sequence-specific nuclease technology. However, the therapeutic promise of genome editing has yet to be explored entirely, many challenges persist that increase the risk of further mutations. Here, we highlighted the main challenges facing CRISPR/Cas9-based treatments and proposed strategies to overcome these limitations, for further enhancing this revolutionary novel therapeutics to improve long-term treatment outcome human health.

Keywords: CRISPR, Cas9, Cancer therapy, Gene editing, Gene modification challenges

Background
Cancer is one of the leading causes of disease-related death, increasing worldwide incidence [1]. At the same time, advancements have been achieved in the prevention and therapeutic approaches, resulting in longer lifetimes or even cures for certain patients with cancer. Unfortunately, chemotherapy and radiotherapy, the two gold stones in cancer treatment, are also painful for patients and cause severe side effects [2]. Therefore, developing innovative anti-cancer therapies with less side effects needs a comprehensive understanding of cancer biology. The most recent advancements in sequencing technology have made it possible to study the cancer genome more effectively and at a lower cost than ever before. The use of an integrated strategy that incorporates genomic and transcriptomic advancements can provide a comprehensive view of an individual's genome. Additionally, this method is used to make valuable decisions relating to patient therapeutic options [3].

Different genomic engineering tools have been performed in cancer therapy such as ZFNs and TALENs by targeting DNA domain-binding proteins. Still, their efficacy was limited due to the inability to target epigenetic modification that arises in tumorigenesis [4]. Recently, a more flexible genome editing technique, CRISPRs linked with HNH domain protein Cas9, promises efficient, long-term safety cancer treatment [5]. The CRISPR/Cas9 system, unlike previous genome editing methods that used protein-DNA interactions to mediate sequence recognition, uses an RNA molecule to mediate binding. CRISPR loci, which are made up of alternating repeat-spacer units, and CRISPR-associated (Cas) proteins, are derived from a prokaryotic
host defense system that protects against viral genomes and plasmids [6]. Based on the method of recognition and cleavage, CRISPR/Cas systems are divided into two classes, which are further divided into six types and various subtypes [7]. Class 1 systems cleave with protein complexes, whereas Class 2 systems only cleave with one protein, creating an opportunity for genome engineering [8]. However, certain targeting limitations apply to all Class 2 systems (types II, V, and VI). For example, a protospacer flanking sequence is recognized by Type VI systems, which use Cas13 to cleave RNA [9]. In addition, type II and Type V systems recognize the adjacent protospacer motif (PAM), a conserved 2–5 bp sequence [10]. For example, the Cas12a/Cpf1 protein uses a simple crRNA and recognizes a PAM directly before the protospacer, such as T-rich PAMs (TTTN) [11]. Conversely, type II Cas9 nuclease recognizes PAM sequences downstream of the protospacer [12]. The most well-characterized and broadly applied CRISPR system is the type II CRISPR/Cas9 system.

Cas9 is an RNA-guided endonuclease that recognizes and cleaves target DNAs that have template strand pairing to the guide RNA, and it requires RNA molecule known as the trans-activating crRNA (tracrRNA). TracrRNA promotes crRNA binding and processing. Moreover, a linker can join the tracrRNA and crRNA into a single molecule known as the single guide RNA used in genome editing (sgRNA) (Fig. 1).

Cas9 is an RNA-guided endonuclease that recognizes and cleaves target DNA that have template strand pairing to the guide RNA, which is composed of Crispr RNA (crRNA) and tracrRNA [13]. crRNA, which has a [17–20] nucleotide sequence that is complementary to the target DNA, and tracrRNA, which acts as a Cas nuclease binding scaffold [14].

The CRISPR/Cas9 system has been successfully applied to \textit{in vitro} cancer research by inhibiting one or more oncogenic molecular pathways (Table 1). However, the \textit{in vivo} use of the CRISPR/Cas9 system has faced many challenges such as the occurrence of off-targeting modifications, the possibility of causing autoimmune diseases, the identification of a proper delivery technique, and, lastly, ethical concerns. As a result, research scientists follow different procedures and investigate various bioinformatics tools to prevent, or at least reduce, these obstacles to make the CRISPR/Cas9 system more suitable for treating cancer in the human body. This review summarizes some of the main limitations of using CRISPR/Cas9 in clinical trials and some of the strategies applied in previous studies to overcome these limitations. Hopefully, this study provides a comprehensive overview of the main roadblocks to implementing this promising technique \textit{in vivo}, helping future researchers focus their efforts on tackling them and making CRISPR come alive as a powerful strategy to treat cancer.

**Innovative advances in CRISPR/Cas9 gene-editing technology**

When Japanese scientists found several previously undiscovered tandem repeats in the \textit{E. coli} genome in 1987, they didn’t report the biological relevance of those findings [15]. However, the role of these sequences remained unknown until they were termed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in 2002 [16]. Then, in 2005, the CRISPR loci were shown to play a significant role in adaptive immunity by three different study teams [17–19]. In 2007, Barrangou and his team revealed that viral gene sequences integrated by bacteria might modify the bacterium’s resistance to phages [20]. Brouns et al. in 2008 discovered that non-coding RNA produced from the CRISPR incorporating short fragments might direct the CRISPR-associated (Cas) proteins to the target-specific portion of DNA, allowing it to perform a protective function [21]. Deltcheva et al. discovered that trans-coding crRNA (tracrRNA) was related to the maturation and processing of pre-crRNA, and their research revealed new destinations for crRNA development [22]. \textit{In vivo} studies in 2012 showed that mature crRNA produced two unique RNA structures when base-paired with tracrRNA, guiding CRISPR-associated protein Cas9 to create double-stranded (ds) DNA cleavage [23]. Subsequently, Cong and Mali teams made genome editing with the CRISPR/Cas9 system possible, who used two different type II Cas systems to make DNA cuts in cell cultures [24, 25]. Once the CRISPR/Cas9 technology was developed, many CRISPR/Cas9-based tools for gene editing at the DNA and RNA levels were created by 2020, with fast advancements in the technology since [26, 27] (Fig. 2).

**Overview of CRISPR/Cas9-based genome editing**

CRISPR is a response of the bacterial and archaea immune system to protect themselves from virus infections [28]. Approximately half of the bacteria have a CRISPR/Cas system [29, 30] a defense mechanism that allows the bacterial cell to memorize, recognize and beat recurrently infecting agents [31]. In this system, short guide CRISPR RNAs (crRNA) interfere with invading nucleic acids in a sequence-specific manner. CRISPR/Cas is composed of a genomic locus termed CRISPR that contains harsh repeating elements separated by unique sequences (spacers), which derive from Mobile genetic factors like phages, plasmids, or transposons. An AT-rich region is typically found at the beginning of Cas genes, which encode Cas proteins [32]. Nowadays, according to the structure and function of the Cas protein, the
CRISPR/Cas systems can be divided into two classes (class I, class II), which are further categorized into six types (type I–VI) [33]. Class I consists of multiprotein complexes responsible for the cleavage of nucleic acid.

In contrast, in class II, only a single protein, Cas9, is used to read, identify and cleave the DNA target sequence [33]. In CRISPR technology, a single protein method is more effective than a multiprotein approach, hence the class II system is more often used, especially in research [10]. Figure 1 illustrates the details of the Type II CRISPR/Cas9 system. For instance, deactivated Cas9 can be utilized to target the epigenome by inhibiting the enzymatic activity of HNH domains without causing sequence disruption [34]. The guide RNA is composed of two core parts; the first is required to bind the RNA to the Cas protein, and the second part, called a spacer, consists of about 20 nucleotides and is responsible for identifying and binding to the targeted site [35]. Furthermore, the PAM sequence is a short DNA sequence usually between 2 and 6 nucleotides that is also required to identify the exact target site on the DNA, and it is located three base pairs from the site where the DNA will be cut, and the mutation will be introduced [10] (Fig. 1).
Anticancer application of CRISPR/Cas9 gene editing and clinical trials

Cancer initiation and spread are mediated by mutations and dysregulation of a variety of genes [36] such as oncogenes, tumor suppressor genes, and stem cell-associated genes, chemo-resistant genes and metabolic genes. Cancer treatment's primary goal is to halt cancer cell growth and development by repairing mutations and restoring dysregulated gene expression. Since its inception, the CRISPR/Cas9 gene-editing method has been widely used in cancer research, with promising results. Georgiadis et al. recently demonstrated that fratricide-resistant T cells can be generated by removing and replacing the TCR/CD3 and CD7 with lentiviral-mediated production of CARs specific for the CD3 or CD7 [37]. Table 1 lists some of the target genes, tumors, and studies that show the effectiveness of CRISPR/Cas9 in correcting these alterations. Based on promising pre-clinical results, the CRISPR/Cas9 system can be used in clinical settings to target cancer-causing genes (Fig. 3). The efficacy of CRISPR-based cancer therapeutics is now being investigated in a number of clinical trials (Table 2).

The programmed cell death-1 (PD-1) protein expression is being targeted by several of these clinical studies. For example, a monoclonal antibody against PD-1 called pembrolizumab exhibits anti-tumor activity in Non-Small Cell Lung Cancer (NSCLC), suppressing the immune system's ability to produce PD-1 and PD-L1 (programmed death-ligand 1), dramatically improves patients' survival rate [38]. Because the FDA has approved PD-1 inhibitors for cancer immunotherapy, PD-1 is an intriguing target for immunotherapy. In addition, CRISPR/Cas9has been used in patients to begin targeting PD-1 (NCT02793856). They used CRISPR/Cas9 to suppress PD-1 expression in metastatic cells from NSCLC patients. The cells were cultured and modified before being reintroduced into the patient [39].

PD-1 knockout-engineered immune cells to treat metastatic NSCLC will be tested for safety in a dosages trial. Additional trials targeting PD-1 expression in T-cells are currently done in other types of cancer such as renal, bladder, and prostate malignancies [40]. Similarly, PD-1 deletion has been used in T-cells in phase II clinical trials for esophagus cancer (NCT03081715). Furthermore, the ability of CRISPR gene editing for cancer immunotherapy to persist for up to 9 months, suggests that immunogenicity is low under these settings and demonstrates the practicality of CRISPR gene editing for cancer immunotherapy [41]. Clinical experiments are also using CRISPR/Cas9 to create chimeric antigen receptor (CAR) T cells.

The first-in-human trial was conducted by scientists from the university of Pennsylvania applying CRISPR/Cas9 genome-edited NY-ESO-1 TCR cells for cancer patients [42] including advanced multiple myeloma (MM) myxoid/round cell liposarcoma (MRCL), and synovial sarcoma (NCT03399448). They showed that T cells were proven to be safe, viable, and long-lasting [42]. Furthermore, using CRISPR to eliminate endogenous TCR and PD-1 might improve tumor rejection activity [40]. Additionally, the allogeneic CAR T-cells targeted to the CD19 antigen were produced by combining the lentivirus-delivered CAR receptors and electroporation-delivered CRISPR RNA to alter the natural TCR and B2M genes. For patients with leukemia, this strategy may help avoid the host's immune system and hence avoid graft-versus-host-disease complications.
Consequently, additional CRISPR clinical trials (phase III) used CRISPR-edited CAR T-cells with dual specificity for CD19 and CD20/CD22, which can identify and destroy CD19-negative malignant cells by identification of CD20/CD22 (NCT03398967). This may have been a helpful adjunctive treatment for an extensive range of the population. In another work, Chen et al. applied CRISPR/Cas13a to disrupt human papillomavirus16/18 E6/E7 mRNAs using an emerging programmed CRISPR technology. They revealed that HPV 16/18 E6/E7 mRNA was successfully and selectively knocked down using a modified CRISPR/Cas13a system, causing growth suppression and cell death in HPV 16 and 18 positive SiHa and HeLa cell lines, but not in the HPV-negative C33A cells [43]. An additional CRISPR clinical study has been planned to test new medications and determine their effectiveness (NCT03332030). In this study, patients with Neurofibromatosis type 1 (NF1) were used to create an induced pluripotent stem cell bank (iPSC) (NF1). NF1 is a common neurocutaneous disease that frequently develops tumors of both benign and malignant types [44]. The main method that used in vivo and in vitro CRISPR/Cas9 study to treat diseases showed in Figs. 3 and 4.

To identify a particular target drug for NF1, CRISPR/Cas9 was used to create NF1 homozygous (NF1-/-) and NF1 heterozygous (NF1+/-) cell lines, as well as NF1 wild type (NF1+/*). The discovery of NF1-targeted therapies may be aided by the opposite or alleviated characteristics. Despite promising clinical trial results, more research is needed to ensure that CRISPR/Cas9 is a safe and effective method of treating human cancers [45]. On the other hand, CRISPR Cas9 indirectly can be used in cancer therapy to find out the drug-resistance mutation in a short period of time. For example, through applying CRISPR Cas9, only in 40 min can determine the FLT3-F691L with a sensitivity of 0.1% [46].

Challenges of CRISPR/Cas9
Even though the previous explanation suggests that CRISPR/Cas9 is a promising approach, this editing system still has a number of limitations and risks that make it challenging to use in clinical trials due to its recent discovery and use in humans. Immunogenicity,
off-targeting, polymorphism, delivery method, and ethics are only several major concerns with the CRISPR/Cas9 system highlighted with the list of strategies that has been developed and can be used to overcome those limitations (Fig. 5).

Autoimmune response against endogenous Cas9 protein
The Cas9 protein is one of the three main components of the structure of the CRISPR system, and it has a fundamental role in binding double-stranded DNA, paired with the mRNA guide, and cutting it at a specific site, expressly 3 bases before the PAM sequence [104]. This protein derives from *Streptococcus pyogenes*, a bacterium that is the cause of many common infections in humans. It is recognized by the body as an antigen, developing an immune response against it [105]. Similarly, the existence of a pre-existing immune response to the homologous Cas9 protein in *Staphylococcus aureus* has been reported [106]. Indeed, both *Staphylococcus aureus* and *Streptococcus pyogenes*, from which the main Cas9 proteins are obtained, SaCas9 and SpCas9, have infected humans for a long time [106]. Thus, the human immune system recognizes these proteins as foreign and develops an immune response against them upon injection, which leads to fast degradation of the Cas9 protein, preventing it from performing the gene-editing function [107].

Strategies to overcome immunogenicity
Several strategies have been proposed to overcome limits posed by immunogenicity against Cas9. Here, we are giving an overview of the main ones offered; (i) implementing the CRISPR/Cas system for gene editing early in a lifetime; (ii) targeting immune-privileged organs (Fig. 6).

Gene editing in early lifetime
Even before birth, various types of disease can be detected in children, and preventing or treating those diseases will save thousands of lives worldwide. The CRISPR/Cas system has been successfully applied in treating various types of inherited diseases in children, such as cystic fibrosis, thalassemia, and sickle cell anemia, Mucopolysaccharidosis type IVA [108–113]. Furthermore, CRISPR/Cas9 can inhibit different molecular pathways of various common types of cancer in children, such as neuroblastoma and lymphoma [114, 115]. Moreover, treating these defects by CRISPR Cas system after diagnosed can be done before the infant is immunized with anti-Cas protein.

Targeting immune-privileged organs
Another practical approach to overcoming the risk of autoimmune disease is gene editing by CRISPR Cas9 techniques in those organs recognized as immune-privileged organs. An Immune privileged organ can be defined as a site in the body where a graft tissue can be implanted without being rejected by the organism due
Table 1 list of genes that were knocked out using CRISPR/Cas9 technique in different types of cancer and their effects

| Types of Cancer | Target Gene(s) | Cell line | Animal model | Mode of action | Delivery Method | Function |
|-----------------|----------------|-----------|--------------|----------------|----------------|----------|
| Breast Cancer   | P53, PTEN, RB1, NF1 | -         | Mice         | Knockout       | Lentiviruses   | For both endocrine and chemotherapy, mutated organoids had a greater response rate for mutated organoids. (47) |
| Breast Cancer   | miR-23b and miR-27b | MCF-7     | Mice         | Knockout       | Lentiviruses   | miR-23b and miR-27b have been shown to be oncogenic miRs, and miR-27b reduces tumor development after knockout. (48) |
| Breast Cancer   | PTEN, AKT1, PKCα | NIH3T3    | Mice         | Knockout       | Lentiviruses   | We show that somatic base editing is feasible and effective at installing defined missense and nonsense mutations at endogenous loci in a mouse model of TNBC. (49) |
| Breast Cancer   | CBEs            | HEK293-T, MDA-MB-231, MCF-7 | -           | Knockout       | Lentiviruses   | For ER-driven breast cancer cell growth, unique CTCF-mediated chromatin configurations are required. (50) |
| Breast Cancer   | AURKA           | HEK293-T, MDA-MB-231, 98BR3, MCF-7 | -           | Knockout       | Lentiviruses   | CHR-6494 might be used in conjunction with MLN8237 to enhance its anti-cancer benefits. (51) |
| Types of Cancer | Target Gene | Cell line | Animal model | Mode of action | Delivery Method | Function |
|----------------|-------------|-----------|--------------|---------------|----------------|----------|
| Breast cancer  | CXCR4 and CXCR7 | MDA-MB-231 | - | Knockout | Lentivirus | The knockout of CXCR4 and CXCR7 genes reduces the binding ability and activities of CXCL12, slows the growth of TNBC cells, and may be used to treat TNBC. [52] |
| Breast cancer  | PARP1 | MDA-MB-231, MDA-MB-468 | - | Knockout | | The effectiveness of PARP1 inhibition with chemotherapy for TNBC treatment varies. [53] |
| Breast cancer  | BRCA1 | MDA-MB-231, ASC | - | Knockdown | LentiCRISPRv2 vector | Breast cancer development is promoted by BRCA1 mutation in the tumor microenvironment. [54] |
| Breast cancer  | APOBEC3G | MCF-10A and HCC1806 | - | Knockout | Lipofection | Multiple clones evaluated for APOBEC3G gene knockout success. [55] |
| Breast cancer  | CDK4, SRPK1, DNMT1 | MCF-10A-HEK293T and GP2-293 | Mice | Knockout | Lentivirus | Transcriptional epistasis influences around 30% of differentially expressed genes in cancer cells. [56] |
| Breast cancer  | CDH1 | MCF-7 | Rats | Knockout | Plasmid Transfection | It is possible to target cancer-related genes using any genome editing technique. [57] |
| Breast Cancer  | OPN | MDA-MB-231 | - | Knockout | GRD2 transformation | Inactivating osteopontin with CRISPR/Cas9 may overcome radioresistance in breast cancer. [58] |
| Breast Cancer  | BRCA | MDA-MB-231 | - | Knockout | Lentivirus | Targeting a group of genes offers new possibilities for PARPi combination treatments. [59] |
| Breast Cancer  | TNEM106A | MDA-MB-231, MDA-MB-468 | - | Knockout | - | In breast cancer, TNEM106A inhibits WDR77 translation. [60] |
| Breast and Lung cancer | CDK4, p107, TGFβ1 | A549 and MCF7 | - | Knockout | - | After being challenged with CRISPR-Cas9 libraries, both cell lines showed a considerable decline in cell count. [61] |
| Lung cancer    | PKP2 | H1299, A549, H460 | - | Knockout | - | Methylation of PKP2 plays an essential factor in radioresistance by stabilizing catenin by CRISPR/Cas9 library screening. [62] |
| Lung Cancer    | Trp53, KRas | HT-29, SW480 and A549; and CFPAC-1 | Adult Mice | Knockout | Lentivirus | Using the CRISPRi toolset, researchers may rapidly build novel, therapeutically relevant alternative models for biomedical research. [63] |
| Colon Cancer   | KRA5 | HT29, WDR, HCT116, LS174T, and HBE293T; SW480 and A549, and CPI56-5 | - | Knockout | Two-vector lentivirus system | GPER-Rh1 has a critical axis for RTK tolerance. PAK1 and thus a suitable target for synergizing MEK inhibitors in CRC patients with KRA5 mutations. [64] |
| Colon Cancer   | Klotho | Caco-2 | - | Knockout | - | By causing apoptosis, Klotho gene expression in Caco-2 cells by CRISPR/Cas9 inhibits cell growth. [65] |
| Colon cancer   | uPAR | O RL1619, CCL247 | - | Knockout | Okayama-Berg vector | Knockout of the uPAR gene leads to tumor growth inhibition, EGFR downregulation, and an increase in stemness markers. [66] |
| Types of Cancer | Target Gene | Cell line | Animal model | Mode of action | Delivery Method | Function |
|----------------|-------------|-----------|--------------|---------------|----------------|----------|
| Prostate cancer | Tceal1 | Mouse: SP1, Human: PC3, LNCaP, DU145, CWR22, RWPE | - | Knockdown | Lentivirus | TCEAL1 deletion causes a different cell cycle profile than docetaxel alone, with more subG1 cell death and polyploidy. [67] |
| Prostate cancer | mRNA (mR205, mR221, mR222, mR30c, mR224, mR453, mR23b, mR505) | LNCaP | - | Knockout | Lentivirus | Functional classification of prostate cancer-associated mRNAs through CRISPR/Cas9-mediated gene knockdown. [68] |
| Prostate cancer | BRAF | CWR22 | - | Knockout | Lentiviral | MAPK/AR co-targeting may help patients with active MAPK pathways, especially those with oncogenic BRAF mutations. [69] |
| Prostate cancer | TP53 | PC-3 | - | Knockdown | Lentiviral | The impact of CRISPR/Cas9 guided mutant TP53 gene repair in PC-3 human prostate cancer cells. [70] |
| Prostate cancer | ECE1, ABCA12, BPH2, EEFDAT1, RAD9A, and NPSNAP1 | DU145 and PC3 | - | Knockdown | Lentiviral | Prostate cancer metformin Resistance related gene screening using CRISPR-Cas9. [71] |
| Ovarian cancer | EGFL6 | SHG17 | - | Knockout | Lentivirus | EGFL6 knocked out by CRISPR/Cas9 inhibited tumor angiogenesis. [72] |
| Ovarian cancer | ZNF587B and SULF1 | A2780, SKOV3, IOSE80 | - | Knockout | Lentivirus | Based on genome-scale CRISPR/Cas9 screening, loss of ZNF587B and SULF1 led to changes in resistance. [73] |
| Ovarian cancer | AR and Nanog expression | A2780, SKOV3 | - | Knockdown | Lentiviral | Nanog interaction with androgen receptor signaling axis regulates ovarian cancer stem cells using CRISPR/Cas9. [74] |
| Ovarian cancer | ITK | SHG17 | Human | Knockout | Lentivirus | For ovarian cancer metastasis, ITK has a possible role in lymph node metastasis. [75] |
| Thyroid cancer | AXIN1 | ACT-1 | - | Knockout | Viral vector | CRISPR-Cas9 has been used to effectively create an ACT-1 knockdown thyroid cancer cell line lacking the AXIN1 gene. [76] |
| Liver Cancer | PTPMT1 | HCC | - | Knockout and knockdown | Lentivirus-Blast vector | CRISPR-Cas9 knockdown of PTPMT1 in normal liver cells may improve survival in hypoxia. [77] |
| Liver cancer | Pten, Rb1, and Ctnnb1 | Pig | Knockout | CRISPR/Cas9 edited liver cancer mouse model: Longitudinal imaging of liver cancer using NanoCT and nanoparticle contrasting agents. [78] |
| Liver Cancer | Traf3 | HepG2 | - | Knockout | Lentiviral | The CRISPR/Cas9 method improved HepG2 cell proliferation, migration, and invasion and provided a helpful tool for researching Traf3 function and mechanism. [79] |
| Liver cancer | ARID1A | HCC | Pig | Knockout | - | CRISPR/Cas9 editing of pig liver cancer cells to create genetically customized cancer cells. [80] |
Table 2 The efficacy of CRISPR-based cancer therapeutics in several different clinical studies

| Clinical Trials Identification | Country | Developer | Disease | Number of participants | Target Gene/modification | Delivery | Study Phase | Estimated Study Completion Date | References |
|-------------------------------|---------|-----------|---------|------------------------|--------------------------|----------|-------------|----------------------------------|------------|
| NCT03655678                  | Canada, Europe | Vertex Pharmaceuticals Incorporated | Beta Thalassemia | 45 | Analogous CD34+ HSPCs that have been reprogrammed at the BCL11A gene enhancer. | Ribonucleoprotein electroporation | Phase 2, Phase 3 | August 2024 | [81, 82] |
| NCT03728322                  | Unknown | Allife Medical Science and Technology Co., Ltd. | Beta Thalassemia | 12 | HBB gene is corrected in iHSCs patient-specific | Unspecified | Early Phase 1 | January 31, 2021 | - |
| NCT04205435                  | China | Bioray Laboratories | Beta Thalassemia Major | 12 | Autologous hematopoietic stem cells gene-edited with β-globin restoration. | Unspecified | Phase 1, Phase 2 | December 1, 2023 | [81] |
| NCT04211480                  | China | Bioray Laboratories | Thalassemia Major | 12 | Gene-edited autologous hematopoietic stem cells with γ-globin expression | Unspecified | / | June 1, 2023 | - |
| NCT03745287                  | US, Europe | Vertex Pharmaceuticals Incorporated | Severe Sickle cell disease | 45 | Autologous CD34+ HSPCs modified at the enhancer of the BCL11A gene | Ribonucleoprotein electroporation | Phase 1, Phase 2 | October 2024 | [81–83] |
| NCT04774536                  | US | Mark Walters, MD | Sickle cell disease | 9 | Autologous CD34+ HSPCs modification | Ribonucleoprotein electroporation | Phase 1, Phase 2 | December 1, 2026 | - |
| NCT04037566                  | China | Xijing Hospital | Leukemia Lymphocytic Acute (All) Refractory | 1. Leukemia Lymphocytic Acute (All) Refractory 2. Lymphoma, B-Cell (1) B cell leukemia, (2) B cell lymphoma | CRISPR gene-edited to eliminate endogenous HPK1(XFY19 CAR-T cell) | Ribonucleoprotein electroporation | Phase 1 | August 2024 | - |
| NCT03398967                  | China | Chinese PLA General Hospital | 1. B cell leukemia, (2) B cell lymphoma | 80 | Allogenous CD11 and CD20/22 directed CAR T cells | Unspecified | Phase 1, Phase 2 | May 20, 2022 | - |
| NCT03166878                  | China | Chinese PLA General Hospital | (1) B cell leukemia, (2) B cell lymphoma | 80 | Allogenous CD19-directed CAR T cells; TCR and B2 M disruption | RNA electroporation | Phase 1, Phase 2 | May 21, 2022 | - |
| NCT03690011                  | US | Baylor College of Medicine | T-cell Acute Lymphoblastic Leukemia | 21 | Anti-CD7 CAR T cells, CD7 KO | Unspecified | Phase 1 | May 1, 2038 | - |
| Clinical Trials Identification | Country | Developer | Disease | Number of participants | Delivery | Target Gene/ modification | Study Phase | Estimated Study Completion Date | References |
|-------------------------------|---------|-----------|---------|------------------------|----------|--------------------------|-------------|---------------------------------|------------|
| NCT02793856                  | China   | Sichuan University | Metastatic Non-small Cell Lung Cancer | 12 PD-1 KO T Cells | Unspecified | GD112-CAR cells | Unspecified | - | [83–92] |
| NCT03081715                  | China   | Hangzhou Cancer Hospital | Advanced Esophageal Cancer | 16 PD-1 KO T Cells | Unspecified | CRISPR-edited targeting CD19 | - | Completed (March 17, 2020) | [85–92] |
| NCT03044743                  | China   | Yang Yang, Nanjing University Medical School | 1. Stage IV Gastric Carcinoma 2. Stage IV Naso-pharyngeal Carcinoma 3. T-Cell Lymphoma 4. Stage IV Adult Hodgkin Lymphoma 5. Stage IV Diffuse Large B-Cell Lymphoma | 20 PD Knockout EBV-CTL | Unspecified | CRISPR-edited targeting CD19 | - | Phase 2 March 2022 | [93–98] |
| NCT04426669                  | US, UK  | Intima Bioscience, Inc. | Gastrointestinal cancers | 20 Gene Encoding autologous CISH-inactivated TILs | Unspecified | CRISPR-edited targeting CD19 | Phase 1 | Phase 2 October 2022 | - |
| NCT03872479                  | US      | Editas Medicine, Inc. | 1. Blindness 2. Leber Congenital Amaurosis 3. Eye Diseases | 18 Eliminate the mutation on the CFTR gene | Unspecified | CRISPR-edited targeting CD19 | - | Phase 1 Phase 2 March 22, 2024 | AAV [99] |
| Clinical Trials Identification | Country | Developer | Disease | Number of participants | Target Gene/ modification | Delivery | Study Phase | Estimated Study Completion Date | References |
|-------------------------------|---------|-----------|---------|------------------------|--------------------------|----------|-------------|---------------------------------|------------|
| NCT04560790                  | China   | Shanghai BDgene Co., Ltd | 1. Viral Keratitis 2. Blindness Eye 3. Herpes Simplex Virus Infection 4. Cornea | 6 | BD11 CRISPR/Cas9 mRNA Instantaneous Gene Editing | Unspecified | Phase 1 Phase 2 | May 2022 | - |
| NCT03332030                  | US      | Roger Racker | 1. Neurofibromatosis Type 1 2. Tumors of the Central Nervous System | 20 | Fix NF1 mutation allele | Unspecified | - | July 1, 2025 | - |
| NCT03164135                  | China   | Affiliated Hospital to Academy of Military Medical Sciences | HIV-1-infection | 5 | CD34+ hematopoietic stem/progenitor cells From donor are treated with CRISPR/Cas9 targeting CCR5 gene | Unspecified | Not Applicable | May 20, 2021 | [100] |
| NCT04244656                  | Swiss/American | CRISPR Therapeutics AG | Multiple Myeloma | 80 | CTX120 B-cell maturation antigen (BCMA)-directed T-cell immunotherapy comprised of allogeneic T cells genetically modified ex vivo | Unspecified | Phase 1 | January 2027 | [101] |
| NCT04035434                  | Swiss/American | CRISPR Therapeutics AG | 1. B-cell Malignancy 2. Non-Hodgkin Lymphoma 3. B-cell Lymphoma 4. Adult B Cell ALL | 143 | CTX110 (CD19-directed T-cell immunotherapy comprised of allogeneic T cells genetically modified ex vivo | Unspecified | Phase 1 | August 2026 | - |
| NCT04767308                  | China   | Huazhong University of Science and Technology | 1. CD5+ Relapsed/ Refractory hematopoietic malignancies 2. Chronic lymphocytic leukemia (CLL) 3. Mantle Cell Lymphoma (MCL) 4. Diffuse large B-cell lymphoma (DLBCL) 5. Follicular lymphoma (FL) 6. Peripheral T-cell lymphomas (PTCL) | 18 | CT125A chimeric antigen receptor (CAR) T cells | Unspecified | Early phase 1 | December 2023 | - |
| Clinical Trials Identification | Country          | Developer                                      | Disease                       | Number of participants | Target Gene/ modification | Delivery       | Study Phase          | Estimated Study Completion Date | References |
|-------------------------------|------------------|------------------------------------------------|-------------------------------|-------------------------|---------------------------|----------------|---------------------|-------------------------------|------------|
| NCT04417764                  | China            | Central South University                      | Advanced Hepatocellular Carcinoma | 10                      | PD-1 KO T Cells          | Unspecified    | Phase 1             | December 31, 2021              |            |
| NCT03525652                  | The First Affiliated Hospital of Guangdong Pharmaceutical University | Prostate Cancer                |                              | 30                      | PD-1 KO T Cells          | Unspecified    | Phase 1 Phase 2     | August 30, 2021                |            |
| NCT04601051                  | Intellia Therapeutics | Hereditary Transthyretin Amyloidosis        | NTLA-2001                      | 38                      | lipid nanoparticles      | Phase 1        | March 2024          | [102, 103]                    |            |
to an immunological reaction formed against it [116].
Examples of immune privilege organs are eyes [117],
brain [118], placenta, fetus [119], and testicles [120].
Many congenital eye disorders lead to blindness and
other defects in the eyes, such as Leber congenital amaurosis type 10, retinal dystrophy caused by a mutation in the CEP290 [121]. Fortunately, many studies proved that the eyes are one of the immune-privileged organs that can successfully imply CRISPR Cas9 on it and edit a particular mutation there [122]. For example, Jain et al. employed CRISPR-Cas9 genome editing in human TM cells and in a POAG animal model to reduce the expression of mutant MYOC, resulting in a reduction in the stress on the ER [123].

CRISPR offers an excellent opportunity for scientists to reach high gene editing efficiency in fetuses and embryos, as the immune system has not yet reached maturity. Nevertheless, Because of the substantial danger of embryo off-targeting associated with its use in vivo, it is illegal in many countries. For example, CRISPR/Cas9’s off-targeting rate was 16% in a study aiming to target the POU5F1 gene in embryos [124]. Correspondingly, due to the cleavage of both alleles, off-target cleavage of Cas9 causes chromosomal loss and hemizygous indels [125]. These findings show that chromosomal content can be manipulated. Still, it requires other skills and strategies to reduce the high risk of off-targeting and loss of DNA fragments.

Additionally, testicles are another immuno-privileged organ that the gene editor can target to correct the mutated genes and deactivate oncogene in cancer patients [120]. These genes can be identified and reverted to their normal function through CRISPR Cas system. Sun et al. found that male fertility genes in mice can be dispensable for further fecundity by knocked out through CRISPR/Cas9 [126]. Furthermore, in mice, CRISPR/
Cas9-mediated gene editing uncovered 30 testis-enriched genes not required for male fertility [127]. Likewise, brain is another immune privileged organ, and several studies were performed in vivo without immune tolerance. Normalized FMR-1 gene expression was achieved by CRISPR/Cas9-mediated deletion of the CGG repeat in hiPSCs from fragile X syndrome patients, a change that was sustained even after differentiation into neural progenitor cells (NPCs) and mature neurons; in addition, hypermethylation of the CpG sites upstream of FMR-1 was reversed [128].

Off-targeting
Another main concern about using CRISPR/Cas9 in recent years is having a high number of off-targeting [129–131]. When implying the CRISPR Cas9 system in a complex genomic species such as mammalians, the gRNA might bring to a wrong target due to similarities within the genome, which may lead to further mutations being introduced in undesired genomic locations [132]. In recent years, many bioinformatics tools have been developed to help predict and reduce off-target modifications. These should be further improved to enable researchers to use them effectively in the development of new therapies.

Strategies to overcome off-targeting
The main strategies that have been successfully performed in previous studies can be classified into three main groups; (I) bioinformatics tools to design more accurate gRNA and predict off-targeting; (II) use of Cas9 nickases; (III) add anti-CRISPR proteins.

Bioinformatics tools
Bioinformatics tools play a crucial role in analyzing, predicting, and determining the CRISPR Cas system. Bioinformatics tools allowed Francisco Mojica to discover that the system previously found in bacteria also existed in archaea [133]. Further, bioinformatics tools help scientists design more efficient gRNAs, detect the accurate editing site within the whole genome, and evade off-targeting percentage probability (Table 3) [134]. Studies have shown that the gRNA is responsible for most of the off-targeting [135]. For example, many studies have shown a direct correlation between gRNA length and the number of off-targeting; thus, finding the perfect size of the gRNA is essential to reduce the off-targeting probability [136]. Such as reducing the length of gRNA to less than 20 nucleotides have a significant role in lowering off-targeting by about 5000 folds in the same efficiency of the longer gRNA [34, 122]. According to another study, most of the mismatches occur within the last three nucleotides placed
at the opposite side of the PAM sequence, thus removing these nucleotides and maintaining the length of gRNA about 17 nucleotides crucial role in the reduction of off-targeting [137]. On the other hand, gRNAs shorter than 15 base pairs are not safe as they would lose the specificity and could not bind the right target inside the nucleus [138].

**Cas9 nickases**

Another practical approach to reducing the number of off-targeting is mutating in one nuclease domain in just one strand of the DNA by CRISPR nickase, which crucial to create nick that quickly repaired in the cells nickase [155]. Cas9 nickase has a different breaking mechanism than the normal Cas9 protein; in particular, it breaks down just one strand of the DNA, and they use double adjacent gRNAs rather than sgRNAs (Fig. 7). Therefore, editing genes by using Cas9 nickase reduces further damage in the target DNA, and it has a significant role in reducing the number of off-targeting [156]. Furthermore, it was shown that paired nicking could reduce the risk of off-targeting by 50 to 1500 folds in cell lines, and in mouse zygotes, it allows the gene knockout without any effect on cleavage efficiency [155].

**Anti-CRISPR proteins**

Inactivation of Cas9 protein after targeting its site may also reduce the number of off-targeting [134]. It has been proven that the number of off-targeting is correlative increased as long as the Cas9 protein is expressed in the human tissue culture [137]. Deactivation of Cas9 protein can be obtained through using anti-CRISPR proteins (Acr) [157]. Acr proteins are produced in both bacterial and human cells and allow to disable CRISPR function [158]. Moreover, more than 50 anti-CRISPR proteins have been discovered so far, synthesized by viruses as a defense system against prokaryotic cells [159]. The first Acr protein discovered that deactivates the CRISPR type I system in *P. aeruginosa*, while the other Acr proteins can act on different types of CRISPR, such as types II, III, and V [31]. Acr proteins are about 52 to 333 amino acids, meaning they are tiny molecules and diverse with no sequence overlap with other proteins [159, 160]. Also, each Acr protein has a specific and unique sequence free of conserved sequences, which increases their diversity [161]. Having a small size and a unique genomic sequence make the recognition of Acr difficult by standard homology-based methods. Therefore, these proteins can target their aimed sequences before being recognized. Furthermore, using a different mechanism is one of the successful keys used by Acr to deactivate the CRISPR/Cas9 system [162]. For example, AcrIIA4 binds to both Cas9 and sgRNA rather than binding with just one of them [161]. The efficiency of Acr depends on three main mechanisms, which are the crRNA concentration, DNA binding obstruction, and DNA cleavage inhibition [163]. When the viral genome is injected into the phage, its Acr proteins in a small concentration make the host cells immunosuppression and prepare the bacteriophage for future infections by the phase [164]. Conversely, having a high concentration of Acr proteins and vulnerable bacteriophage disables the function of the CRISPR system from the infected bacteria [164]. Moreover, Acr proteins have a stronger binding affinity with CRISPR; thus it is required a small concentration disable the function of the CRISPR system. On the other hand, anti-CRISPR-associated (Aca) proteins work oppositely to Acr proteins by preventing the transcription of anti-CRISPR proteins [163]. Therefore, the CRISPR system can be improved by using Aca proteins to suppress Acr proteins. Also, the use of Acr proteins that imply phage instead of antibiotics may overcome the issue of drug resistance [165].

**Screening before the treatment**

Pre-existing mutations in genes like TP53 and KRAS may raise the risk of additional mutations during CRISPR Cas cancer therapy [166]. And the two primary ways for dealing with this problem are screening before using the CRISPR Cas system and monitoring the patient after injection.

**Polymorphism in cancer**

Unlike other genetic diseases such as Duchenne Muscle, Dystrophy, and cystic fibrosis, cancer relies on several mutations [167–170]. Moreover, dysregulation of the multiple genes leads to cancer most of the time. For example, mutations happen in approximately 190 codons in the human TP53 gene, and around 25% of the mutations occur in eight codons [171]. Hence, editing a single mutated nucleotide is not enough in most cases that are widely performed in gene therapy [170]. Correcting mutated nucleotide by knocking-in is much more challenging in CRISPR Cas9 since it is more precise than knocking out, which creates alterations, as knocking in, all of the cancer-causing genes takes longer and needs multi-guide RNA [172]. However, by CRISPR Cas9, knocking in is potentially helpful in many ways, such as studying particular gene variation to find out the gene regulation [172].

Correcting or editing the mutated nucleotides of tumor suppressor genes is one of the approaches that should be thought about to obtain the desired result in cancer therapy by knock-in in the mutated gene (Fig. 8). CRISPR/Cas9 technology targeted these
Table 3  Online bioinformatics tools detect the accurate editing site within the whole genome and evade off-targeting percentage probability

| Tool name         | Description                                                                 | Input                                                                 | Output                                                                                           | Maximum mismatches allowed | Supported nucleases                  | PAM sequence | References |
|-------------------|----------------------------------------------------------------------------|----------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|----------------------------|--------------------------------------|--------------|------------|
| CRISPResso2       | Genome editing and interpretation of amplicon sequencing                     | 1. Editing of tool specification  
2. Input sequences  
3. Amplicon sequence, sgRNA sequence | 1. indel sizes and positions  
2. HDR/NHEJ frequency  
3. sequence alignment with reference  
4. allele-specific quantification | Cas9, Cpf1                      | NGS                                   | [139]                     |
| Cas-Analyzer      | Genome editing and programmable nucleases                                   | 1. Fastq  
2. gzip-compressed | 1. indel sizes and positions  
2. HDR/NHEJ frequency  
3. sequence alignment with reference | up to a 1-nt                      | SpCas9, StCas9, NmCas9, SaCas9, CjCas9, AsCpf1, LbCpf1, paired nucleases: ZFNs, TALENs, Cas9 nickases, dCas9-FokI | NGS          | [140]     |
| CRISPR-GA         | Quantification of the edited site then analysis of the different alterations. | Paired-end reads | 1. indel sizes and positions  
2. HDR/NHEJ frequency | < 20                        | SpCas9, SaCas9, St1Cas9, NmCas9, AsCpf1, FnCpf1, LbCpf1 | Sanger sequencing         | [141]     |
| TIDE/TIDER        | Identification of major induced mutations in the editing site using specially developed decomposition algorithm | DNA from a pool of cells treated with RGEN Cas9  
and a character string representing the sgRNA sequence (20 nt) | 1. indel sizes and positions  
2. HDR/NHEJ frequency | ~1%                         | SpCas9, SaCas9, St1Cas9, NmCas9, AsCpf1, FnCpf1, LbCpf1 | Sanger sequencing         | [142, 143] |
| CRISPR-ERA        | Analyze gene editing and gene regulation                                      | Sequence starts with N20NGG | 1. gRNA design  
2. E score (efficacy score)  
3. S score (specificity score)  
4. E+S score: the sum of efficacy score and specificity score. | 3                          | CRISPR/Cas9                      | NGS          | [144]     |
| CRISPRseek        | Using various tools for the CRISPR editing including Base Editors and the Prime Editor for input target sequences, | The RNAs sequence is annotated with a total score of the top5 and topN off-targets and Cas9 | 1. gRNA design  
2. off-targeting count  
3. score on targeting  
4. find Spacer | 4                          | User customizable              | NGS          | [145]     |
| CHOPCHOP v3       | Web tool for selecting alternative transcription of RNA using CRISPER-CAS13 | gene name, genomic coordinates, or a pasted sequence (including ReSeq and ENSEMBL gene IDs) | 1. gRNA design, Off-targeting  
2. GC content (%)  
3. number of self-complementary  
4. efficiency | -                     | CRISPR effector (e.g., Cas9, CasX, or Cas13) | NGS          | [146]     |
| Tool name          | Description                                                                 | Input       | Output                                                                 | Maximum mismatches allowed | Supported nucleases      | PAM sequence | References |
|--------------------|------------------------------------------------------------------------------|-------------|------------------------------------------------------------------------|----------------------------|--------------------------|--------------|------------|
| E-CRISP           | Specific Algorithm used to target any nucleotide sequence ranging from single exons to entire genomes | FASTA       | 1. gRNA design (for various targeting purposes)                        | -                          | genomic context (e.g., exons, transcripts, CpG islands) | NGG, NAG     | [147]      |
| CRISPy-web        | Design sgRNAs                                                               | API         | gRNA design, Target Site selection                                     | 3                          | Cas9                     | NGG          | [148]      |
| CRISPR-P 2.0      | Genome editing in plant                                                      | Gene name, ID, position, and sequence | 1. on-target score 2. off-target score 3. GC content 4. restriction endonuclease site | -                          | Cas9                     | NGG, NAG     | [149]      |
| COSMID            | Validation and identification of off-target sequence                         | FetchGWI search program | Off-target score GC content (%)                                       | 3                          | CRISPR Off-target Sites with Mismatches, Insertions, and Deletions | NGG, NAG, NRG | [150]      |
| WU-CRISPR         | Gene editing and detection of CRISPR/Cas9 Knockout                          | Gene Sequence in FASTA format | 1. gRNA sequence 2. potency score 3. off-target status 4. BLAST alignment 5. coding sequence | -                          | NGG, NAG     |              | [151]      |
| Cas-Designer       | Selecting all RGEN targets via Microhomology-predictor                      | FASTA       | 1. RGENs 2. Cas-OFFinder 3. Cas-Designer 4. Cas-Database.              | 0-10                       | NGG, NAG, NNAGAAW, NNNNGMTT |              | [152] [153]|
| CRISPR MultiTargeter | Web program to detect the High identical site in multiple genes             | FASTA       | Multiple sequence Alignment                                            | 0-24                       | 1. SpCas9 (PAM ‘NGG’), 2. StCas9 (PAM ‘NNA-GAAW’), 3. NmCas9 (PAM ‘NNNNG-MTT’) | NGG          | [154]      |
tumor-suppressor genes to inhibit or reduce tumorigenesis by restoring the activities of tumor-suppressor genes [34]. However, as in cancer, there is plenty of mutations in tumor-suppressor genes, it requires a higher number of gRNA, and there is a higher risk of off-targeting. On the other hand, the CRISPR Cas system can disrupt the nucleotides located in the active site of the protein to suppress the activity of oncogenes, such as KRAS in pancreatic cancer and ATM in neuroendocrine cancer by deleting their inactivation sequences (Table 1) [34, 173]. On the other side, in TNBC cells, the deactivation of CXCR7 and the co-knockout of CXCR4 and CXCR7 have been shown to inhibit the expression of oncogenes and may have a potential target in TNBC treatment [52]. For the Cas system to be effective in knocking out oncogenes, the proper gRNA must be designed to target the binding site of oncogenes and prevent protein-protein interaction, which is an essential step in the molecular pathway of cancer progression [52].

Strategies
We described two primary solutions for managing polymorphism issues: CHyMErA and bioinformatics techniques to investigate the protein interaction site and forecast the results.

Performing CHyMErA
To edit many targets in a single mammalian cell, CRISPR may be utilized with various kinds and procedures, such as the CHyMErA (Cas hybrid for multiplexed editing and screening applications) method. CHyMErA depends upon two Cas proteins, Cas9 and Cas12a nucleases, rather than just the standard CRISPR/Cas9 gene editing (Fig. 9) [174]. Exons may be deleted using CHyMErA, which is helpful for the high deletion of gene sequences. As a result, employing CHyMErA to target multiple sites is one of the novel approaches to overcoming cancer polymorphism [175].

Detecting protein interaction site
Bioinformatics tools can have a crucial role in predicting and obtaining the desired results in knocking out. For example, different databases can be used for finding out the interaction site of the proteins, such as InterPred [176]. By using this platform, amino acids located in the active site of the proteins can be detected, and then gRNA is designed based on it. Moreover, different databases can be used for predicting the result of CRISPR knockout (Table 3).

In conclusion, knock-in and Knock-out for oncogenes and tumor suppressor genes are critical in gene editing using CRISPR Cas9. However, knocking in to edit a particular nucleotide should be performed more precisely. Indeed, having more than one mutation in cancer cells required performing other techniques such as ChyMErA, which can target multi targets by binding two cutting DNA enzymes Cas9 and Cas12a. On the other hand, creating one mutation in the protein active site of the oncogenes is enough to suppress its role, so performing Knocking out in cancer therapy to suppress oncogene is much more practical.
The delivery challenges
Choosing a proper safe, and precise delivery technique to carry the CRISPR system into the tumor site, especially in vivo, and targeting the right sequence inside the nucleus is another challenge that should be considered. CRISPR/Cas9 technologies are delivered through different approaches, such as viral, physical, and extracellular vesicle-base system delivery techniques [177]. Additionally, each method is used for a specific purpose and has its limitations. Thus, the main challenges while choosing the right vector are packaging, delivery, and targeting the right site [178, 179]. For example, viral vectors are used widely in both in vivo and in vitro, but it has many limitations, such as immune response and insertional limitation [177]. For instance, after implying viral vector in vivo, it exposes continuously for a long time and increases the risk of mutations and off-targeting [180].

Viral delivery vectors
Adeno-associated viruses (AAV) such as adenovirus and lentivirus have been utilized successfully in other research in vivo [181] and they do not cause any other diseases in humans, only a very few immune responses [178, 182]. On the other hand, the main disadvantage of AAV has a tiny packaging size, so more than one AAV is required to carry all the CRISPR systems such as gRNA and Cas protein [182]. Additionally, the maximum size that a single AAV vector can deliver is about 4.7 kbp, while the genomic size of SpCas9 alone is around 4.3 kbp [178]. Thus, more than one vector is necessary to hold all the systems.

Non-viral delivery vectors
Besides that, non-viral delivery vectors are another approach, such as lipid nanoparticles and inorganic nanoparticles [183]. In addition, a non-viral delivery approach, like nanoparticle-based delivery, allow for more frequent
administration of gene therapy with lower risk of immunogenicity, less exposure to nuclease and more accurate targeting [183, 184]. Furthermore, non-viral vectors have a greater capacity than viral vectors without integrating in the carried genome [185]. On the other hand, extracellular vesicle-based systems have been utilized in both *in vivo* and *in vitro* systems successfully and, compared to the other system, are safer and cheaper [186]. Another challenge is the delivery and efficiency percentage of the CRISPR system into the targeted size, especially in cancer therapy with total editing efficiency [178]. And according to Yin et al. (2014), the total delivery efficiency is 1 out of 250 in targeting liver cells using hydrodynamic injection [187] (Table 4).

**Strategies**

One of the effective ways to overcome the packaging challenge is splitting the Cas9 protein into two AAV (AAV-split-Cas9) vectors instead of one [188]. As previously explained, large size vectors increase the risk of off-targeting and mutation [189]. As a result, employing a smaller Cas9 protein and splicing it into two AAV vectors is critical for reducing off-targeting and increasing delivery efficiency [190]. Another option that can be used to reduce the risk of off-targeting associated with delivery techniques is the use of ribonucleoprotein (RNP) complexes, such as recombinant CRISPR-Cpf1 Ribonucleoprotein (CRISPR-Cpf1-RNP) suppressed off-target activity in mouse cells [191]. Furthermore, according to their results, Mout and his colleagues applied Cas9-RNP methods, which efficacy around 95% in cultured cells [192]. This approach also degrades after 24 to 48 h of injection [182] (Table 4). Thus, the risk of further mutations and off-targeting that occur due to the continuous expression of viral vectors is reduced significantly [177].

**Ethical issues and CRISPR/Cas9 technology**

Human genetic alterations have long been a source of ethical debate; CRISPR/Cas9-mediated genome editing has provided a new perspective. Considering the unpredictability and broad-reaching effects of this technology’s appealing applications, a thorough examination of its ethical and societal implications is required. The conceptions of various members of society, such as the public and religious academics, are fundamental.

**Current ethical standpoint**

The application scope of CRISPR/ Cas9 is expanding at an incredible rate. Switching genes on or off to investigate how they work or causing mutations in cells to
learn why and how they become malignant, are some of the opportunities it has opened up in molecular biology research. Gene editing can be used to create resistant crops and stronger police dogs, for example [210, 211]. Another highly contentious concept would edit the human genome permanently to eliminate disease-causing mutations or even improve or introduce desired features in offspring by inserting helpful genes and this is debatable [212]. Non-reproductive cell genome modifications are not heritable, whereas germ cell modifications can be passed down to the next generation. As a result, the attractive uses of this approach raise ethical, moral, and safety concerns [213]. Human germline modification using CRISPR/Cas9-based gene editing has raised concerns about threats to human safety and dignity, as well as the potential for genocide. There was an effort to halt human genome research until a national or global agreement on society’s acceptance of this new technology was reached [214].

Morality concepts
Morality concepts, particularly in biomedicine, are based on empirical research and entail evaluating potential risk-benefit ratios, to maximize the latter while decreasing the former. It is vital to assess the spectrum of conceivable outcomes, the likelihood of each occurring, and the various arguments for the outcomes of any one while making moral decisions. There are at least three major causes for ethical concerns concerning CRISPR genome engineering technology. Concerns have been raised about the power and technical limitations of CRISPR technology in the first concept. These drawbacks include a lack of on-target editing efficiency [215], incomplete editing (mosaicism) [216, 217], and inaccurate on-target or off-target editing [218, 219]. CRISPR experiments with animals and human cell lines have revealed these limitations. Technology, on the other hand, is evolving at a tremendous speed. The second concern is for the transformed species’ long-term survival: if they will be influenced indefinitely and whether the edited genes will be passed down through generations, perhaps influencing them in unanticipated ways.

Making precise predictions regarding the future of a modified creature and estimating potential hazards and advantages may be difficult, if not impossible, given the aforementioned technical constraints and the intricacies of biological systems. As a result, the uncertainty created by these circumstances makes precise risk/benefit assessments difficult, making moral decision-making more difficult [220]. Finally, even if the genome is altered as planned and the necessary functional output is achieved on time, the complicated link between genetic information and biological phenotypes is not fully understood, according to the skeptical viewpoint. As a result, depending on the circumstances, the biological impact of altering a gene in germline and/or somatic cells may be unknown. The intricate regulatory actions of many genes govern many biological features [221]. As a result, “designing” a biological phenotype at the organismal level is difficult, if not impossible.

Strategies
On the Brightside, it has the potential to make a significant difference in terms of health and wellbeing if used properly [222]. There are several reasons why this technology can be used correctly, although patient safety is one of the most important. One of the most compelling arguments in favor of allowing the use of this technology is the need to protect patients [212]. When germline editing research is applied in a clinical setting to avoid the inheritance of a specific genetic condition, it may alleviate the sorrow and anxiety that parents encounter in the life of the possibility that their child may be born with that genetic disease [214, 223]. Recently, Bioengineer Feng Zhang of MIT and Harvard has modified the Cas9 enzyme to limit mutations outside its target region [224]. Furthermore, the error rate of CRISPR/Cas9 might be further decreased to a safe range if further modifications are introduced [225]. Considering this, CRISPR/Cas9 mediated genome editing safety concerns might be overcome to some extent. Overall, CRISPR/Cas9 technology’s risk profile varies depending on the design. After overcoming some ethical and safety problems, some are approved or predicted to be used soon. On the Brightside, it may significantly improve health and quality of life, but still, it relies on how this technique is used.

Conclusion and future perspectives
The therapeutic genome editing field has made tremendous progress in recent years, progressing from essential investigation to preclinical development and into human trials, in particular for ex vivo HSC and T cell editing and also for in vivo liver genome editing, as a result of the relatively efficient delivery methodologies developed for these systems. However, several considerable challenges need to be addressed before the biomedical promise of genome editing can be fully realized. First, delivery has always been one of the most, if not the most, formidable problems in the gene therapy field. Importantly, HDR and even knockout efficiencies are currently low in many tissues, so higher delivery efficiency is needed to compensate. There should be an international law to ensure that gene editing does not harm humanity, and experiments should be restricted in a health care system.
| Therapeutic genome editing approaches | Delivery methods | Targets or disease | Genome editing accomplishments | Carrying capacity | Challenges | Strategies | References |
|--------------------------------------|------------------|--------------------|--------------------------------|-------------------|------------|-----------|------------|
| Viral vector                         | Adenovirus       | T cells            | CCR5 knockout is in clinical trials | 37 Kb             | In vivo, immunogenicity is a major restraint. | Targeting immune privilege organs such as eyes, brain, uterus | [193, 194] |
| AAV in vitro                         | T cells and HSCs | High genome editing rate as a donor; can be paired with non-viral nuclease delivery | 4.7 kb | HDR donor size is limited by vector carrying capability. | It is possible to generate donor templates for HDR-mediated methods by infecting AAV vectors with a ssDNA vector genome | [195–197] |
| AAV in vivo                          | brain, retina, Liver, heart, muscle | In animal models, knockouts and HDR have been produced, this can be used with non-viral nuclease delivery | 4.7 kb | 1. There are still issues with delivery efficiency and preexisting immunity to natural serotypes. 2. Exposes continuously for a long time after infusing in vivo and increase risk of off-targeting. 3. Having a small packaging size, 4.7 kbp, while the genomic size of SpCas9 alone is around 4.3 kbp 4. Hepatotoxicity | 1.a. To eliminate pre-existing immunity to AAV, it can be employed alone or in conjunction with other approaches. 1.b. Targeting immune privilege organs such as eyes, brain. 3.a. Splicing the Cas9 protein into two AAV vector (AAV-split-Cas9) can be performed. 3.b. Choosing a smaller size of Cas9 protein such as SpCas9 which is 1 kilo base shorter. | [183, 196, 198, 199, 178, 183, 188, 200] |
| Lentiviral vector                    | In retina and in vitro | Lentivirus with integrase defects utilized as a donor | 8 kb | The de novo expression of a protein lacking in the host may result in immune responses leading to the clearance of the transduced cells and the formation of antibodies that inhibit the activity of secreted factors. | Cyclosporine, tacrolimus, and cyclophosphamide can inhibit the synthesis and secretion of cytokines and prevent the activation and proliferation of T cells | [201–203] |
| Therapeutic genome editing approaches | Delivery methods      | Targets or disease                                                                 | Genome editing accomplishments                                                                                                                                                                                                                                                                                                                                                                                                   | Carrying capacity | Challenges                                                                                                                                                                                                                             | Strategies                                                                                                                                                                      | References |
|--------------------------------------|-----------------------|-------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Non-viral vector                     | Electroporation       | In vitro: T cells, HSCs; in vivo: muscle and kidney                                   | High genome editing efficiency in cells difficult to transfect                                                                                                                                                                                                                                                                                                                                                                                                                                         | -                 | 1. Only feasible in ex vivo applications; in vivo electroporation is limited to mice, unclear if possible in humans. 1.a. combining the CRISPR/Cas9 system and in utero electroporation is an effective and rapid approach to achieve brain-specific gene knockout in vivo. 1.b. electroporation does not require microinjection skills and can be used to treat 40–50 embryos simultaneously. | 1.a. combining the CRISPR/Cas9 system and in utero electroporation is an effective and rapid approach to achieve brain-specific gene knockout in vivo. 1.b. electroporation does not require microinjection skills and can be used to treat 40–50 embryos simultaneously. | [204, 205] |
| Lipid-based delivery vehicles        | PCSK9, TTR, TMC1       | High NHEJ efficiency for hepatocytes and hair cells in vivo. Minimize immunogenicity. | -                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | -                 | 1.a. Cas9 mRNA may activate TLRs. 1.b. Due to the constant positive charge, these formulations induce toxicity, adverse reactions, and immunogenic responses. | 1. Lipid nanoparticles (LNPs) based on ionizable cationic lipids were developed to circumvent these restrictions.                                                                                                   | [200, 206] |
| Microinjection                       | In vivo: zebrafish Caenorhabditis elegans | -                                                                                      | 1. Cell damage 2. Only a single cell can be targeted in each injection.                                                                                                                                                                                                                                                                                                                                                                                                                                         | -                 | 1. To reduce cell damage, a high level of sophistication and manual skills are required.                                                                                                                                            |                                                                                                                                                                                                                                          | [207, 208] |
| iTOP                                 | iTOP transduction is effective for intracellular delivery of the Cas9 protein and sgRNAs independently, or direct delivery of RNPs. | -                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | -                 | Lower efficiency in primary cells. Since it is only soluble at high salt concentrations, it is not adequate for in vivo.                                                                                                                                 |                                                                                                                                                                                                                                          | [209]      |
Abbreviations
CRISPR/Cas9: clustered regularly interspaced short palindromic repeats-associated protein9; ZFNs: Zinc-finger nucleases; TALEN: Transcription activator-like effector nucleases; TTN: T-rich PAMs; tracrRNA: trans-activating crRNA; crRNA: CRISPR RNA; PD-1: programmed cell death-1; PD-L1: programmed death-ligand 1; MRLC: myosin/roundcell liposarcoma, POAG: Primaryopen angle glaucoma; hiPSCs: Human induced pluripotent stem cells; NPs: neuronal progenitor cells; gRNA: Guide RNA; Acr: anti-CRISPR proteins; CXCR: Chemokine receptors; CrHyMEa: Cash hybrid for multiplexed editing and screening applications; AAV: Adeno-associated viruses; RNP: ribonucleoprotein; CRISPR-Cpf1-RNP; CRISPR-Cpf1 Ribonucleoprotein; HDR: homology-directed repair; NHEJ: non-homologous end joining; HSCs: hematopoietic stem cells.

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Authors’ contributions
MT and AB designed and supervised the study. SGE, AB, BMH, and MF wrote the draft and revised it. EJ, AS, BSI, PJJ, and AZ collected the data and designed the figures and tables. All the authors read the submitted version and approved it.

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Author details
1Department of Medical Analysis, Faculty of Applied Science, Tishk International University, Erbil, Kurdistan Region, Iraq. 2Department of Pharmacognosy, College of Pharmacy, Hawler Medical University, Kurdistan region, Erbil, Iraq. 3Center of Research and Strategic Studies, Lebanese French University, Erbil, Iraq. 4Department of Biology, College of Science, Salahaddin University-Erbil, Erbil, Iraq. 5Department of Pharmacology and Toxicology, College of Pharmacy, Hawler Medical University, Kurdistan region, Erbil, Iraq. 6Biotechnology Department, College of Science, University of Sulaimani, Sulaimani, Iraq. 7Department of Biomedical Sciences, University of Westminster, London, UK. 8Department of Pathology, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. 9Institute of Human Genetics, Jena University Hospital, Jena, Germany. 10Department of Medical Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. 11Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. 12Men’s Health and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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