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Gene Editing: a Powerful Tool for Cancer Immunotherapy

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Abstract: Recently, Gene editing has emerged as one of the recent promising tools for gene therapy. This technique is remarkable in biotechnology and medicine since it enables genomic editing *in vivo* with high accuracy. The most important gene-editing enzymes are zinc finger nucleases (ZFNs), homing meganucleases, transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9). The immune system plays an important role in the removal of abnormal and cancerous cells and their role in the defense against foreign pathogens. Immune cells routinely remove abnormal cells by screening cell function, gene mutations, and cancerous cell formation. The CRISPER technology was widely applied to introduce a therapeutic regimen in cancer treatment based on tumor genome editing. Additionally, it was used in cancer immunotherapy. For example, CRISPR technology has introduced an alternative to the conventional clinical drug, Herceptin, targeting HER2 in breast carcinoma. Moreover, it was used in CAR-T cell generation and immune cell checkpoint inhibition. Researchers are seeking to fight many hard diseases by using CRISPR technology. However, many challenges still exist. Some of these challenges include the requirement of PAM sequence, the possibility of on target deletion or addition, off-target effects, Cas9-DSB complex formation, the lack of perfect delivery methods, and low HDR output. In this review, we outline the application of CRISPR technology in cancer immunotherapy and the challenges that hinder the implementation of this technology.

Keywords: cancer immunotherapy; gene editing; CRISPR; immune checkpoints.

1. Introduction

Cancer is considered the major health problem worldwide, with more than 8 million deaths in 2015 (WHO). This great number of deaths makes cancer the second leading cause of mortality worldwide, where one out of six deaths is due to cancer (WHO). In 2010, 1.16 trillion USD was paid for cancer treatment which is a very high and significant cost [1,2]. The poor understanding of molecular aspects of the disease and the lack of effective treatment strategies cause a socioeconomic problem and increasing numbers of mortality globally. Variant mutations, therapeutic resistance, and tumor heterogeneity complicate the situation and limit the clinical outputs. All previously mentioned facts make new treatment an urgent demand to replace or be used in parallel with current regimens.

Gene therapy is classically defined as any drug which compromises an active molecule that contains any form of a recombinant nucleic acid (DNA or RNA) and is used to treat a

human being [3]. Gene editing is one of the recent promising tools from gene therapy tools. The controlled modification of a gene DNA sequence helps greatly understand its function within the cell. Gene editing enzymes include zinc finger nucleases (ZFNs), homing meganucleases, transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats(CRISPR)/CRISPR-associated nuclease 9(Cas9). All of them need reengineering of the enzyme for each individual targeted sequence except the Cas9 system [3-6]. Besides, genetic engineering techniques for the enzyme using homologous recombination have poor editing efficacy and consequently need a larger sample size [7]. Homologous recombinant (HR) has been used for targeting genes at specific sites of the organism's genome. HR is based on the creation of homologous DNA constructs that are used as templates and resemble the targeted genome sequence. However, this approach has limitations in mammalian cells and animal models.

CRISPR/Cas9 is a technology that is an easy, flexible engineering method [8, 9]. The CRISPR/Cas9 system is a naturally occurring immune defense against external invasions of viruses and genetic materials where it was first observed in microbes [10, 11]. This natural mechanism was extensively utilized for editing the mammalian genome with potent precision by designing specific breaks in the DNA double helix [12]. Similarly, like ZFNs and TALENs, Cas9 generates double-strand breaks at a specific genetic locus. However, Cas 9 has the advantage that editing is achieved by nuclease action guided by a single recombinant strand guided RNA (sgRNA) or a natural dual-RNA that binds specific sequences via Watson–Crick base pairing. CRISPR/ Cas9 gene-editing technology is a promising treatment option and helps better understand how certain genes participate in the initiation and progression of diseases in beings [3].

Oncolytic virotherapy is one of the most recent and highly efficient therapeutic strategies for treating different types of cancer. The oncolytic viruses are either native or genetically modified with the capacity to selectively replicate inside cancer cells but not in normal cells. Recently, the genetically-manipulated *Herpes simplex virus type 1*(HSV-1) is one of the most commonly investigated oncolytic viruses (OV) that has been efficiently utilized as a vector in cancer therapy [13].CRISPR/ Cas9 technique was reported to speed up the genetic manipulation of oncolytic viruses with higher efficiency than the traditional techniques. Furthermore, Vaccinia Virus (VACV) is an oncolytic virus with great therapeutic potential. CRISPR/Cas induces double knockout of both N1L and A46R genes (immune-regulatory genes), which is important in improving VACV immune response induction. Therefore, sgRNA-guided Cas9 is able to target multiple sites simultaneously on the VACV genome [14].

Abnormal cells are removed by immune cells routinely, which screen cell function, gene mutations, and cancerous cell formation. Normally, macrophages perform this routine check. The first line of defense is Neutrophils and natural killer (NK) cells, while the CD8+ cytotoxic T-cells represent the second line of defense.

The function of natural killer cells (NK) is greatly and negatively affected in the tumor microenvironment; consequently, their antitumor effect is inhibited to a great extent. Antigen-specific recognition criterion is deficient in NK cells, unlike T-cells [15]. Recently, many treatment modalities have been introduced to tackle the suppressed immune response in cancer and to upregulate NK cells target recognitions, such as the use of antibodies, cytokines, or gene-editing technologies [16, 17]. Recently developed flexible and potent CRISPR/Cas9 technology has been employed in this track [18,19]. The *ex vivo* NK cell gene editing and *in situ* editing of the tumor microenvironment to enhance NK potency and keep their survival

were successfully and promisingly achieved. However, *in situ* NK cells gene editing *in vivo*is still difficult to be achieved.

Natural killer cells mediate their antitumor effect either through their cytotoxic effects or by secreting cytokines. Upon target cell recognition, NK cells are activated through a balance between activation and inhibition signals. Using cell surface molecular patterns, NK cells recognize infected cells, transformed or even stressed [20]. After recognizing the target cell, NK cells exert their cytotoxic effects by secreting granzyme and perforin or by FasL, TNF-α,andTRAIL to erase the target cells. After that, target cells undergo DNA fragmentation, apoptosis, and death. Besides, NK cells produce cytokines such as IFN-γto enhance the cascade of the adaptive immunity events [21,22] and reconstitute the tumor microenvironment [23]. Recently, CRISPR/Cas9 editing technology was employed to reconfigure the NK immunity controlling pathways.

Macrophages and dendritic cells (DCs) are responsible for T-cell activation to achieve a potent immune response. Besides, DCs are considered tumor-associated antigens (TAAs) to T cells [24]. However, the tumor microenvironment evades the immune system by many strategies despite these strong immune defense mechanisms. For example, the tumor microenvironment may change the pattern of checkpoint markers to be unseen to the immune cells, polarize tumoricidal macrophages to be tumorigenic macrophages, or achieve cytotoxic CD8+T cell neutralized function. Finally, chemokines secretion, cytokines secretion, metabolic mediators, and signaling mechanisms are factors produced by a tumorigenic immune response and lead to an immunosuppressive tumor environment.

Gene editing technology was extensively used for genome-wide screening, disease models, and therapeutic options in many diseases. In the current review, gene-editing technology, especially CRISPR, will be discussed, and highlights will be focused on its application in cancer cell immunotherapy. Additionally, the limitations and challenges facing this promising technology and the future directions will be reviewed.

2. Cancer Immunotherapy

Recently, tumor-related immune cells' role and suppression of tumor enhancement have gained many interests. Many immune-suppressive treatments have been introduced and clinically applied to manage many types of solid and hematological cancers. Some of which target the evasion mechanisms of the tumor microenvironment, such as targeting the polarized microphage by the use of nanoparticles specific modifications or targeting CTLA-4, PD-1, and PD-L1 pathways. Besides, immune checkpoint inhibitor therapy has been extensively and clinically applied. Recently, a new hope was directed toward cancer vaccine, including cell-based, protein antigen, synthetic proteins, DNA vaccines, and antibodies. All the previously mentioned strategies for cancer cell immunotherapy will be discussed in detail in the next few points.

2.1. Nanoparticles specific modifications for targeting the polarized macrophage.

Cytokines and chemokines of the tumor environment are the main responsible factors for the development of polarized macrophages. Macrophages are considered a blade of double edges that can act both as pro-oncogenic and anti-oncogenic factors (Figure 1). Polarized M1-macrophages secretes pro-inflammatory cytokines like iNOS, IL-1, IL-23, IL-12, and IFNγ, hence enhancers of the anti-tumor-immune functions [25]. Consequently, activated M1

macrophages program the DC and CD4+ T cells to exert their tumoricidal immune response and introduce tumor antigens for cytotoxic CD8+ T initiated cell death. On the contrary, the tumor-associated macrophages and the pro-oncogenic M-2 secrete tumor stroma anti-inflammatory elements. Many studies confirmed that the formation of tumor-associated fibroblast, oncogenic addiction, and angiogenesis, which lead to the down regulation of adaptive immunity, are mediated by M1-macrophages and tumor-associated macrophage (TAM) [26]. An intelligent approach used a nanoparticle-specific modification of some subtypes of macrophages as a new cancer immune-therapy strategy. For example, the FDA-approved ferumoxytol nano-micelles iron supplement was able to enhance apoptotic protein upregulation by enhancing reactive oxygen species [27]. Moreover, the iron nanoparticle-induced pro-inflammatory macrophage polarization resulted in tumor growth suppression.

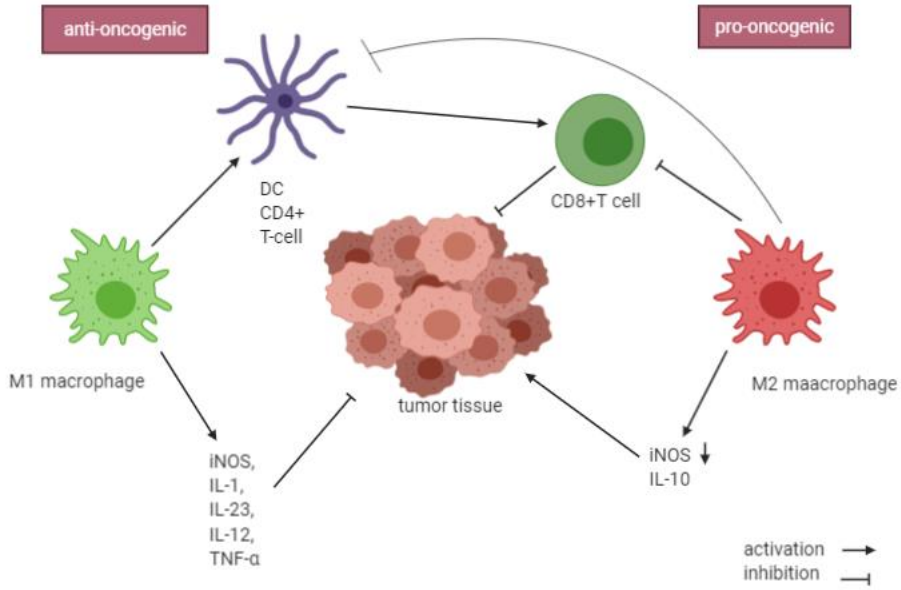

Figure 1. Dual functional role of macrophage as tumoricidal and tumorigenic factors, DC= dendritic cells.

2.2. CTLA-4, PD-1, PD-L1 pathway targeting.

CTLA4 mediates CD4+ T cells signaling, as it suppresses helper T cells and enhances regulatory T (Treg) cells. Consequently, CTLA4 mediates an immune-suppressive role in cancer cells. CTLA-4 binds to either the B7-1 or the B7-2 domains on the surface of the antigen-presenting cells. And hence blockers of CTLA4 aimed to enhance the immune-up-regulatory responses by enhancing CD4+ T cells and suppressing Treg cells [28]. The first FDA-approved drug for checkpoint inhibitor that targets CTLA4 was Ipilimumab, which was used in metastatic melanoma treatment.

Similarly, PD-1 is a checkpoint receptor having a hopeful tumor target. It is able to modulate antitumor immune responses. In normal cells, this checkpoint receptor hinders the cytotoxic effects of T cells against autoimmunity and inflammations. However, this regulation is transformed into immune resistance [29, 30]. When T-cell activates PD-1, it binds with one of its ligands, either PD-L1 or PD-L2. This conjugation is a crucial step in blocking many antitumor immune responses. It results in suppression of CD8+ T cells functions, upregulation of Treg cell infiltration, and reduction of the binding of antibody presenting cell with T-cell (APC) [31] (Fig 2).

Similarly, like PD-1, which is upregulated in most tumor-infiltrating lymphocytes (TILs), PD-L1 is overexpressed in myeloid cells, cancer cells, TILs, and tumor-associated

macrophages. It was reported that renal carcinoma with high expression levels of PD-L1 has a poor prognosis compared with the negative PD-L1 tumors [32]. PD-L1 is modulated by oncogenic kinases such as a signal transducer, activator of transcription 3, PI3K-AKT, and anaplastic lymphoma kinase in lung cancer, glioblastoma, and lymphoma [33].

2.3. Immune checkpoint inhibitor treatment.

2.3.1. PD-1/PD-L1 and CTLA-4 antagonist.

About 7 billion US dollars are directed annually toward immune checkpoint inhibitors markets and are expected to be 15 billion US dollars by 2024. Till now, five immune checkpoint inhibitors are commercially available. They are Nivolumab and Pembrolizumab, which target programmed cell death protein 1 (PD-1); Atezolimuab and Durvalumab targeting PD-L1; and Ipilimumab which targets cytotoxic T lymphocyte-associated protein 4 (CTLA4). More than ten immunotherapy-based clinical trials have been achieved, while more than fifty clinical trials are in progress [25]. Nivolumab (Opdivo; Bristol Myers Squibb, Princeton, NJ) was the first success story for targeting PD-1/PD-L1 interaction where it is a human monoclonal antibody against PD-1 and used in melanoma treatment. After that, it was approved for treating other cancers such as lung, colon, and renal cancers. This discovery represents a new hope of cancer treatment. Pembrolizumab was effective in treating many hematological cancers such as chronic lymphocyte leukemia, Hodgkin's and non-Hodgkin's lymphoma, and acute myeloid leukemia. Atezolizumab and Durvalumab,anti-PD-L1, was recently approved for treating several solid tumors [25].

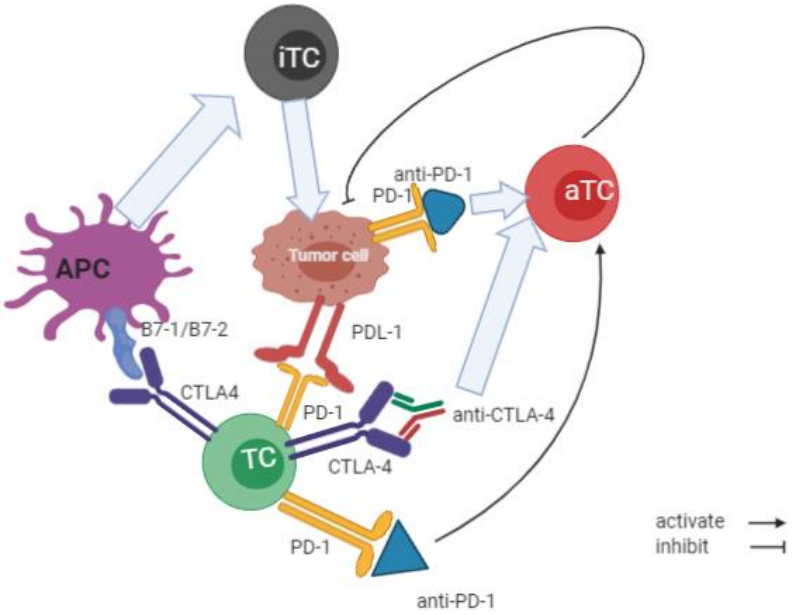

Figure 2. PD-1, PDL-1 and CTLA-4 function in the tumor microenvironment and interactions between cancer, T-cell, and antigen-presenting cell. APC= antigen-presenting cell, TC=T-cell, aTC= active T-cell, iTC= inactive T-cell.

2.3.2. siRNA for checkpoint knockdown.

RNA interference technology was used extensively to target many cancer-specific genes. However, many limitations hinder naked siRNA effectiveness, such as being liable to degradation by nucleases, short lifetime, and low stability. And hence, wrapping of siRNA with nanoparticles will greatly enhance the efficacy of siRNA [34]. siRNA could target either

immune suppression or immune-enhancing pathways. However, the most effective trials would induce an immune response and inhibit immune suppression [35].

2.4. Dendritic cell-based vaccine.

Dendritic cells (DC) play a crucial role in immunity by linking and communicating innate and adaptive immune responses. Besides, it is an important antigen-presenting cell; it also has the ability to induce or suppress the immune response depending on the type of antigens [36]. DC can enhance the main components of adaptive immunity by T-cell activation, in addition to B-cell differentiation [25].

Dendritic cells can be generated and differentiated *ex vivo* either from monocytes (CD14+) or stem cells (CD34+). Several studies referred that both populations generated DCs with similar morphology, phenotypic characteristics, phagocytic, and antigen presentation capacities. However, some studies reported that CD14+-derived DCs express a markedly higher costimulatory molecule B-7 than CD34+ -derived DCs. These cells can efficiently phagocyte both apoptotic and necrotic cellular debris. Internalization of exogenous antigens by DCs leads to the loading of tumor peptides to MHC Class II molecules; however, it can also lead to cross-presentation of peptides to MHC I molecules. Contrarily, endogenous antigens are loaded on both MHC Class I and II molecules [37]. DCs possess the capacity to prime both naïve T lymphocytes CD4+ and CD8+. Immature DCs are potent at engulfing dying cells and processing their antigens, while in their mature state DCs express high levels of both MHC class I and II antigens as well as a variety of costimulatory including B7-1/CD80 and B7-2/CD86 molecules needed to induce a primary T cell response [38, 39]. Moreover, the maturation of DCs is accompanied by their migration to lymphoid tissue to impulse naïve T-lymphocytes. In addition to stimulating adaptive T-cell responses, DC can trigger NK cells and induce B-cell proliferation through a B-cell activating factor (BAFF). Therefore, DCs have a dual role in innate and adaptive immunity with potential utility in tumor immunotherapy combined with alternative therapeutic modalities. Additionally, these treatments also can change the tumor microenvironment [40] via the release of pro-inflammatory cytokines such as tumor necrosis factor α, interleukin (IL-1), IL-12, and IL-6. Thus, the DC-based vaccine represents a useful tool to induce differential cell death via tumor destruction resulting from direct cytotoxic effects and the induction of a local inflammatory [41].

3. CRISPR/Cas 9 System for Gene Editing

CRISPR/Cas is used to refer to Clustered regularly interspaced short palindromic repeats-associated nuclease. It was discovered accidentally by Ishino and his team work in 1987. They were studying iap gene in the genome of E.Coli where they unintentionally cloned a certain part of the CRISPR. After that, they discovered that the bacterial genome consists of a successive array of repeats [42]. Besides, CRISPER sequences were also found in *Haloferax mediteranii*, which is an Archaea [43]. After that, another related species *Haloferax volcanii* was reported to have similar functions regulatory spaced repeats[44]. The average length of these repeats is about 32 bp. However, they may range from 21 to 47 base pairs. These repeated sequences were only reported in prokaryotes, while eukaryotes and viruses do not express these sequences. The sequence of these repeats is highly unique and conserved for one specific species. There are Four Cas genes in prokaryotes, Cas1–4 till the year 2002 [44]. After that, several CRISPR/Cas sequences and Cas proteins were discovered, in addition to many CRISPR

spacers that were identified in 2005 in plasmids and phages with the aid of computational and sequencing technologies [45]. The wonder of CRISPR was unveiled in the year 2007 by Barrangou *et al*.[46] who reported that CRISPER prevented the virus from attacking *Streptococcus thermophilus*. Moreover, the horizontal gene flow of Staphylococci was hindered by the CRISPER defense mechanism. Interestingly, CRISPR RNAs were found to regulate CRISPER interference. Bacteria utilize the CRISPR/Cas system in the cut specific sites in plasmid DNA and bacteriophages[45]

Thus, after the exact mechanism of CRISPR has been elucidated by the previously mentioned observations, the CRISPRCas9 system has been widely utilized in biomedical research. CRISPR-Cas9 system was widely utilized to modify and edit specific genomic regions and was also used to understand specific gene functions and diseases.

Gene editing could be achieved by using the CRISPR-Cas9 system, which can edit specific genome loci by making a double-strand beak in the DNA double helix. Gene editing studies have gained many benefits from the CRISPR-Cas9 system in many cell lines and organisms and clinical trials. Besides, a knockout library of the guide sequence was prepared with the aid of a genome-scale CRISPR-Cas9 to determine the cancer-related genes [47].

Activating cellular damage response, in response to DSB created by Cas 9 nuclease, is critical for preserving genome stability [48]. The host cell could heal this break, DNA double-strand break repair (DSBR), either by homology-directed repair (HDR) or non-homologs end joining (NHEJ). NHEJ pathway deals with the DSB through a template-independent error-prone mechanism, while HDR is considered an error-free mechanism and uses extensive homolog to heal the DSBR(Figure3). Decreasing Cas9 nuclease size, fidelity improving, and Cas targeting efficiency optimization are the main goals for CRISPR/Cas system reengineering [49]. Till now, there are three identified types of CRISPR systems, I-III. The CRISPR system contains the genes associated with CRISPER, non- coding RNA, and the direct repeats or the repetitive elements. The CRISPR RNA (crRNA) is formed of these direct repeats within which there are short variable sequences originating from the exogenous DNA targets and called protospacers [50]. In each CRISPR system and within the DNA sequence, there is an adjacent protospacer motif (PAM) [51].

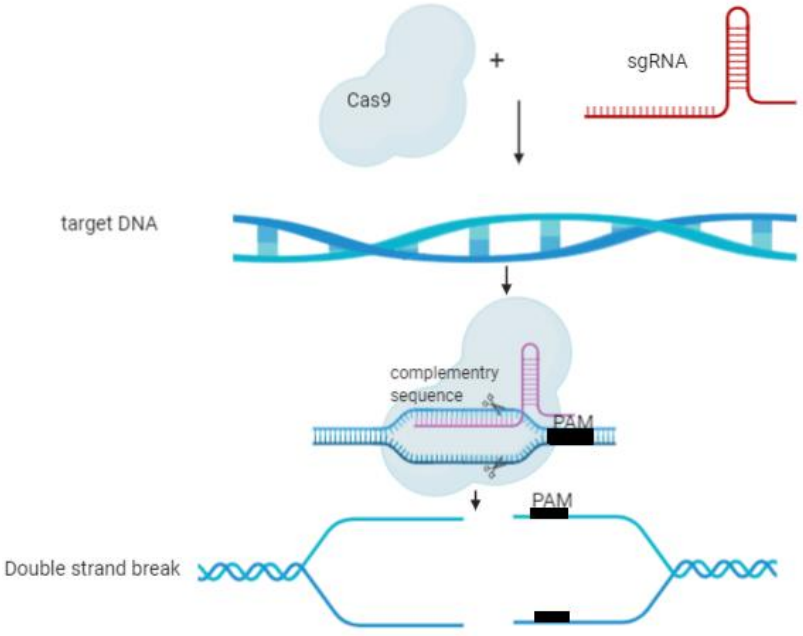

Figure 3. The mechanism of gene editing by CRISPR/Cas 9.

4. CRISPR/Cas System Classes

There are two distinct classes of the CRISPR/Cas system. The first class includes multiple Cas protein, class 1, while the second class contains a single Cas protein [2] (Table 1). These systems are further subdivided into types. Type I, III, IV belong to class 1 CRISPR/Cas system, while type II, V, and VI compose class 2 CRISPR/Cas system.

Type I, type II, and type III use Cas3 enzyme, Cas9, and Cas10, respectively. Additionally, type V uses Cpf1, C2c1, or C2c3, while type IV and VI use Csf1 and Cas13a, respectively. [52] The well-identified CRISPR system is type II, which contains Cas9 nuclease crRNA, in addition to trans-activating crRNA (tracrRNA). Both crRNA and tracrRNA are combined to form recombinant or chimeric sgRNA. This sgRNA is consisted of about 20 nucleotides guide sequences which could be tailored to target specific gene loci neighboring to a PAM sequence. This PAM sequence was found to be NGG for the Cas9 system [2].

This system is the best-characterized CRISPER system applied widely for gene editing studies in humans. Recently, another type of CRISPR system was well identified; type V uses Cpf1 nuclease, named Cas12a. This system, unlike CAs9, is guided by crRNA only and not by the fused crRNA and trancrRNA [53]. Besides, it recognizes a PAM sequence rich in T neighboring to the target DNA sequence and not a G-like Cas9 system. In contrast to Cas9, which produces a blunt end at PAM proximal area, Cas12a produces wavy ends with 4 or 5 nucleotides overhangs at the distal portion of its PAM sequence. Besides, Cas12a can deal with its specific crRNA array to form mature crRNAs [54]. Cas13a was recognized by a screening study using 15 orthologous from Leptotrichiawadei[55]. LwaCas13a is used in specific knockdown of internal targets like RNA interference technology.

Additionally, it showed better specificity with RNAase activity, which enables it from cleaving the target RNA site. Another Cas system was identified by mutating both domains of Cas9 protein and called dCas9, which has a catalytically dead protein. Consequently, it can bind target DNA sequence without cleaving it. dCas9 was used for gene activation, known as CRISPR activator (CRISPRa), in addition to its use for reversible interference (CRISPRi). Gene editing could be achieved by combining both CRISPR interference and CRISPR activation.

Table 1. Cas9 proteins and their functions in CRISPR editing.

Cas protein	CRISPR system	Guide RNA	Recognition sequence	Nuclease activity
Cas9	Type II	crRNA+transcrRNA	G-rich PAM	Yes
Cpf1	Type V	crRNA	G-rich PAM	Yes
Cas 13a	Type VI-A	crRNA+transcrRNA	Except G-rich	Yes
dCas9	TypeII	crRNA+transcrRNA	G-rich PAM	no
Cas3	TypeI	crRNA	T-rich PAM	Yes
Cas10/Csm1	TypeIII	crRNA	AT-rich PAM	Yes
dCas13a	TypeVI-A	crRNA+transcrRNA	Except G-rich	Yes

5. The use of CRISPR Technology in Genome Screening

Specific factors in certain pathways or interactions between their effectors are mediated by a gain of function (GOF) or loss of function (LOF) analysis or screening. RNA interference technology was used extensively for LOF screening; however, it showed many limitations. These limitations include high false positive and negative rates, off-target effects, high false-positive rates, high false-negative rates. Besides, it did not produce high throughput data because of its expensive costs and large libraries. Similarity, cDNA and open reading frame screening for GOF have many limitations. These include that they are time-consuming and are

not helpful in genome-wide screening. On the contrary, sgRNA genome screening is of high productivity due to the small length and uniformity of sgRNAs [2].

Regulators of PD-1, genes involved in drug resistance, lethal interactions, and other targets in cancer were elucidated by CRISPR screening. Besides, a huge number of sgRNA was screened by CRISPR knock out and CRISPRi. Pivotal genes involved in cell differentiation, survival, and signaling were identified by these techniques [56-58]. The most prominent application of using sgRNAs for LOF screening is the recognition of BCR and ABL genes as lethal hits in chronic myelogenous leukemia(CML); KBM7, KRAS, and PIK3CA cell lines and also as lethal hits in colorectal cancer; DLD-1 and HCT116 cell lines. [56, 57].

The gain of function screening was used to elucidate positive and negative effectors of cancer survival using CRISPRa for screening. For example, cell proliferation was suppressed in the CML K562 cell line by CRISPRa activation of tumor suppressor genes [59]. And hence a transient tumor suppressor inactivation during cancer initiation was postulated. Similarly, drug resistance activation for the BRAF, the inhibitor of vemurafenib in A375 cells, introduced a possibility of evading BRAF inhibition by two synergistic pathways, either by re-enhancing the MAPK pathway through BRAF or MAPK independently.

6. Transplant-based and Direct *in vivo* CRISPR Screening

An important question about cancer cells' behavior and possible therapeutic options could not be answered by the *in vitro*CRISPR-based screening, such as the possible crosstalk between the cancerous cell and its microenvironment. Consequently, the *in vivo* CRISPR screens were important to answer such essential questions. The first trial was achieved to mutagenize genes involved in tumor proliferation and metastasis to study their possible role [60]. *In vivo,* crisper screening could be either a transplant-based or direct screening. The transplant-based *in vivo* CRISPR is a two-step operation, including transfection of sgRNA library to cell line in culture. Then, transplanting these cells to mice to answer the specifically raised questions is done. Reduced or depleted sgRNAs are determined after cell isolation and sequencing. Several of these techniques have helped greatly elucidate oncogenes, anti-oncogenes, and synthetically lethal genes. However, this technique still has some pitfalls. First, the bulk of cancer cells does not resemble the *in vivo* environment. Second, orthotropic transplants to the specific organ could not be mimicked by subcutaneous transplantation. And lastly, transplantation is done in immune-deficient mice, and consequently, the immune interaction with cancer cells could not be clarified. Based on the previous limitations, direct *in vivo* CRISPR screening was developed to overcome such limitations.

In vivo, direct screening-based mutagenesis is done directly on the target organ [61]. For example, when sgRNA was embedded in a plasmid and injected in the tail vein of Cas9 mice, multiple mutageneses in hepatocytes were observed. Similarly, mutagenesis in Cas9 expressing lung epithelial cells was observed when a lentivirus containing sgRNA was delivered into the lung intratracheally. In addition, glioblastoma pathology in the human brain was mimicked by forming an AAV library consisting of 280 sgRNAs specific for 56 genes. Despite all the above-mentioned advantages of direct CRISPR screening, it still has limitations, including the veiled cell-cell crosstalk in the target organ, poor viral delivery efficacy, and immune intolerance [2].

7. Advantage of CRISPR CAS9over ZFNs and TALENs

CRISPR-Cas9 technique has several advantages over ZFNs and TALENs since it can be applied easily. ZFNs and TALENS need protein recoding by using long DNA sequences about (500-1500 bp) for every new target site. However, CRISPR-Cas9 can easily accommodate the target site of any genomic sequence by altering the 20-bp protospacer sequence of gRNA (the guided RNA)via subcloning of the new nucleotide sequence intosgRNA plasmid backbone without any change in the Cas9 component[62]. CRISPR-Cas9 is characterized by multiplexed genetic alteration by introducing specific mutations into multiple genes simultaneously by using multiple sgRNA in the same cell.CRISPR/Cas9 technique is super efficient and highly specific, although the target selection is limited by the need for PAM sequence and Off-target effect limited in ZFNs and TALENs systems [63].

8. CRISPR in Cancer Therapy

Parallel to the great productive screening applications of CRISPR/Cas9 technology, it was also used to generate disease models and introduce therapeutic options in cancer treatment. For example, CRISPR/Cas9 and TALEN were used to generate knock-in of degron tags (degron tags) to produce protein function inhibition through specific allele induction. This procedure helped in elucidated the function of PI3Kα and EZHH2 in cancerous cells. Besides, the link between possible SF3B1 oncogene hot spots mutations and splicing variations was clarified by the Degron-KI system[64]. . CRISPR/Cas9 was used to make some genomic rearrangements in lung cancer; such as KIF5B-RET and EML4-ALK inversion; in addition to CD74-ROS1 translocation [65]. In the same context, genome engineering to generate certain chromosomal translocations in cell lines was carried out using CRSIPR/Cas9 to elucidate events in the initiation and pathology of acute myeloid leukemia(AML) and Ewing's sarcoma [2]. CRISPR/Cas9 could also follow these *in vivo* screens to determine new therapeutic targets in AML. Interestingly, CRISPR technology introduced an alternative to the conventional clinical drug, Herceptin, targeting HER2 in breast carcinoma [66].

Several mutations were done in human intestinal epithelium using CRISPR/Cas9 editing in the organoid models. Besides, colon carcinoma was mimicked *in vitro* using CRISPR technology to make mutations in antitumor genes, APC, SMAD4, TP53, and pro-tumor genes KRAS and/or PIK3Ca. Additionally, the mismatch repair-deficient colorectal cancer was studied by using CRISPR/Cas9 to make multiple deletions of DNA repair genes in colon organoids. In pancreatic cancer, genome editing of PDAC driver genes using CRISPR–Cas9 clarified that its function is Wnt independent during the process of tumor formation [2].

Regarding the animal models, mutations in 5 genes (Uty-8 alleles, Tet1, 2, 3, and Sry) in mouse embryonic stem cells were done using CRISPR/Cas9 editing. Using CRISPR/Cas9 editing eliminates the need for multiple-genes cancer models. Another application of CRISPR/Cas9 editing was used in mice zygotes by direct fusion of Cas9 mRNA and sgRNA to introduce mutations in Tet1and Tet2 genes. In another approach, CRISPR/Cas9 was combined with Cre-dependent somatic enhancement of Kras oncogenes to produce a mouse lung adenocarcinoma model. Moreover, the liver cancer model was developed by direct liver hydrodynamic injection of a combination of a plasmid containing the Cas9 and sgRNAs specific for Pten and p53, individually and in combination.

Similarly, a direct liver hydrodynamic injection of Cas9 plasmid and sgRNA specific for mutated Ctnnb1was used to generate Ctnnb1 mutations in the liver. Moreover, mouse lung

cancer was developed by intratracheal injection of AAV with Cas9 and sgRNA for Em14 and ALK. Besides, invasive lobular breast cancer was developed by intraductal injection of lentivirus coding the Cas9 and sgRNA for Cdh1 specific alleles [2].

9. Applications of CRISPR Technology in Cancer Immunomodulation

9.1. CRISPR/ Cas9 for CAR-T cell generation.

Immunotherapy is becoming a recent fundamental trend in cancer treatment. As previously mentioned, immune checkpoints were extensively used in order to improve T-cell function, and hence, improve treatment outcomes in hematological and solid tumors[67]. Alternatively, recombinant T-cells such as chimeric antigen receptor T-cells (CAR-T) were used for cancer immunotherapy. Recently, CRISPR/Cas9 technology was used in CAR-T production. In this technique, patient-specific T-cells are subjected to genetic modification to produce CAR-T cells in vitro and then re-introduce to the patient to improve T-cell cancer recognition and killing.

T cell uses the CAR intracellular chimeric signaling domain to be activated as well as using the variable fragment of CAR for specific tumor antigen detection. The successful example of using CAR-T-based immunotherapy is recognizing the CD19 surface market in B cell tumors [68]. However, being a successful technology, CAR-T cell-based immunotherapy showed several limitations. For example, the patient-specific generation of CAR-T cells is very expensive. Besides, there are some technical limitations, and it is a very long procedure.

Consequently, recent trials were done using the CRISPR technology to overcome the CAR-T cell-based immunotherapy limitations. These trials aim to introduce universal T-cells obtained from healthy volunteers. These T-cells are genetically modified to lack the internal TCR (T cell receptor) and HLAI-I to prevent the host-graft interactions. In this technique, CRISPR/Cas9 was employed to interrupt many regions in T-cell receptors to generate a universal CAR-T cell. One of the examples that CRISPR/Cas9 targeted is the Fas receptor. This is due to when the FAS receptor is combined with its ligand (FASL), T-cell apoptosis is increased. And hence Fas targeted suppression by CRISPR/Cas-9 produced CAR-T cells having the potency to kill tumor cells and resulted in mice extended survival [69]. Similarly, CAR-T cells modified by CRISPR/Cas9 in the locus of T-cell receptor alpha constant resulted in a constant CAR presentation in T-cells. Additionally, it increased the potency of T-cells and their performance in the AML mouse model [70].

9.2. CRISPR/Cas9 for PD-1 and CTLA-4 knockdown.

Two important cell surface receptors were targeted by CRISPR/Cas9, namely PD-1 and CTLA-4, and resulted in a more efficient T-cell mediated immunotherapy. This trend was developed into the first clinical trial, which targeted PD-1 knockout in T-cells using CRISPR/Cas9 in lung cancer patients [71]. This was the first clinical trial using the CRISPR/Cas9 technology. After that, many clinical trials were conducted, such as targeting PD-1 in prostate cancer (NCT02867345), renal cell cancer (NCT02867332), and bladder cancer (NCT02863913). Additionally, four loci of PD-1 and CTLA-4 were knocked out using CRISPR/Cas-9 and produced universal chimeric T-donor cells used to immunize cancer cells.

The importance of PD-1, CTLA-4 surface receptors and, PD-L1 ligand arises from their interaction with many elements in different immunity-related pathways. The gene expression analysis tool from Reactome and IntAct databases were used to analyze the PD-1, PD-L1, and

CTLA4, which is a statistical hypergeometric distribution. This analysis test possibility of certain Reactome pathways being involved and interacting with these targets, according to Fabregat *et al.* [72]. As evident from Table 2, they have many interactions with many targets in different pathways. The listed pathways in Table 2 represent the most significant 25 pathways containing PD-1, PD-L1, and CTLA4 obtained by Reactome and IntACT analyses.They have significant interactions with elements of 24 pathways of the listed 25 pathways, which are: PD-1 signaling, CO-stimulation by the CD28 family, Nef Mediated CD8 Down-regulation, MET activates PTPN11, CTLA4 inhibitory signaling, Prolactin receptor signaling, Nef Mediated CD4 Down-regulation, Adaptive Immune System, Netrin mediated repulsion signals, Platelet sensitization by LDL, PECAM1 interactions, Activation of IRF3/IRF7 mediated by TBK1/IKK epsilon, Regulation of IFNA signaling, Nef-mediates down modulation of cell surface receptors by recruiting them to clathrin adapters, Interleukin-6 signaling, RET signaling, Transcriptional regulation by RUNX1, Signaling by Leptin, GP1b-IX-V activation signaling, Interleukin-6 family signaling, Tie2 Signaling, Regulation of RUNX1 Expression and Activity, Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA), and Amplification of signal from the kinetochores.

Table 2. Interactions of PD-1, PD-L1, and CTLA4 genes with the most significant 25 pathways, the data are corrected for false discovery rate using the Benjamani-Hochberg method. The analyses were done with the aid of reactome and IntACT databases(https://reactome.org/PathwayBrowser/#TOOL=AT).

Pathway	Targets found in this pathway	P-value	Interactions	Ratioof interactions
PD-1 signaling	PD-1 PD-L1	1.11e-04	1. PD-1- PDCD1	3.25e-04
RUNX1 and FOXP3 control the development of regulatory T lymphocytes (Tregs)	CTLA4	1.36e-04	----------	0.002
Costimulation by the CD28 family	PD-1 PD-L1 CTLA4	1.89e-04	1. CTLA4- CD8 2. PD-L1-PD-1 PD-1- PTPN11	0.003
Nef Mediated CD8 Down-regulation	PD-1	0.006	PD-1- ATP6V1H	2.44e-04
MET activates PTPN11	PD-1	0.013	1.PD-1- PTPN11	8.12e-05
CTLA4 inhibitory signaling	CTLA4	0.021	1.PD-1- PTPN11	4.06e-04
Prolactin receptor signaling	PD-1	0.021	1.PD-1- PTPN11	0.001
Nef Mediated CD4 Down-regulation	PD-1	0.022	1.PD-1- ATP6V1H	4.06e-04
Adaptive Immune System	PD-1 PD-L1 CTLA4	0.025	1. CTLA4- CD86 2. PD-L1-PD-1 3. PD-1- PTPN11	0.021
Netrin mediated repulsion signals	PD-1	0.034	PD1-PTPN11	3.25e-04
Platelet sensitization by LDL	PD-1	0.035	PD1-PTPN11	7.31e-04
PECAM1 interactions	PD-1	0.035	1. PD-1- PTPN11	5.69e-04
Activation of IRF3/IRF7 mediated by TBK1/IKK epsilon	PD-1	0.036	1. PD-1- PTPN1	7.31e-04
Regulation of IFNA signaling	PD-1	0.038	1. PD-1- PTPN11	4.06e-04
Nef-mediates down modulation of cell surface receptors by recruiting them to clathrin adapters	PD-1	0.039	PD-1-ATP6V1H	0.001
Interleukin-6 signaling	PD-1	0.04	PD-1-PTPN11	0.002
RET signaling	PD-1	0.045	PD-1-PTPN11	0.002
Transcriptional regulation by RUNX1	CTLA4	0.045	PD-1-PTPN11	0.011
Signaling by Leptin	PD-1	0.045	1. PD-1-PTPN11	0.002
GP1b-IX-V activation signaling	CTLA4	0.047	1.CTLA4-PIK3R1	5.69e-04
Interleukin-6 family signaling	PD-1	0.05	1. PD-1-PTPN11	0.003
Tie2 Signaling	PD-1	0.051	1.PD-1-PTPN11	0.001
Regulation of RUNX1 Expression and Activity	PD-1	0.053	1.PD-1-PTPN11	0.002

Pathway	Targets found in this pathway	P-value	Interactions	Ratioof interactions
Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA)	PD-1	0.056	1. PD-1-KAT5	8.12e-05
Amplification of signal from the kinetochores	PD-1	0.056	1.PD-1- AD1L1	3.25e-04

9.3. CRISPR/Cas9 based LAG3 and PTPN2 knockdown.

Immunotherapy targeting cell surface receptors did not produce efficient results in all patients. And consequently, searching for new targets is an essential need for successful immunotherapy against cancer. Zhang *et al*. [73] introduced CAR-T cells with a knockout of lymphocyte activating gene-3 (LAG-3) that showed better specificity and potency in killing cancerous cells in mouse xenograft models. Similarly, CRISPR–Cas9 mediated screens *in vivo* elucidated the protein tyrosine phosphatase non-receptor type 2 (PTPN2) to be a new target for cancer immunotherapy, which was accelerated by this deletion gene interferon-gamma modulated pathway [2].

10. Recent Trials in CRISPR Mediated Immunotherapy

Ye *et al*. [74] tested the hypothesis that membrane protein editing by CRISPR technology could increase T cell-based immunotherapy. They developed a mixed genetic screening system that is composed of adeno-associated virus (AAV), in which sleeping beauty transposon and sgRNA cassette are nested. These new hybrid gene-editing techniques increased the efficiency of membrane protein editing and screening readout,inCD8+ T cells of a glioblastoma mouse model. Screen hits were validated by illustrating that the new transfer of CD8+ T cells containing edited Lag3, Pdia3, Mgat5, or Emp1 improved the survival of glioblastoma mice. The authors declared that Pdia3 edited T-cells enhanced the effector functions by using cytokine assays, Transcriptome profiling, single-cell sequencing, and T cell signaling analysis. Engineered PDIA3 and mutant in EGFRvIII recombinant antigen T cells showed more antigen-specific killing potency of human glioblastoma cells.

Ju *et al.* [75] established enhanced CD8+ T cells in EG7 suspension cancerous cells through the application of multiplexed gene editing that is carrier-free. This multiplexed Cas9 ribonucleoproteins significantly knocked down the PD-1 and PD-L1 genes which are negative regulators of the immune response. This was achieved using engineered Cas9 protein with high penetration and complexation power. Besides, the electroporation technique was also used to enhance the efficacy of CRISPR editing. This method achieved gene editing at many sites of the genome of suspension cells. Using multiplexed Cas9 ribonucleoproteins against PD-1 and PD-L1enhanced the production of cytotoxic CD8+ T cells mediated by Th1-type cytokine production. This T-cell showed cytotoxic action against cancerous cells by enhancing the antitumor immunity.

Lu *et al*. [76] used a liposome-encapsulated CRISPR/Cas9 system to suppress the PD-1 gene in T cells to generate PD-1- T cells. When this cell is activated by dendritic cells fused withHepG2 tumor cells (by antigen presentation of DC/HepG2 FCs), they show a higher proliferation rate and increased pro-inflammatory cytokine IFN-γ. Moreover, it was cytotoxic against HepG2 cells *in vitro* and increased the apoptosis *in vivo* (in HepG2 xenograft). In addition to PD-1 and PD-L1 targeting to enhance the antitumor immunity, Bhate *et al*. [77] pointed to ADAR1 (RNA editing enzyme) as a promising target for cancer cell immunotherapy. They depended on the results of three recent studies by Ishizuka *et al*. [78],

Liu *et al*. [79], and Gannon *et al*. [80], who highlighted that deleting the ADAR1 gene could enhance higher cell toxicity and make cancerous cells more subjected to immunotherapy.

Dai *et al*. [81] established a system for the production of chimeric antigen receptor (CAR)-recombinant T cells by employing the CRISPR-Cpf1 system, which is a tracrRNA-independent system combined with adeno-associated virus (AAV). They successfully generated CAR-T cells with immune-checkpoint knockout and homology-directed-repair knock-in (KIKO cells). This AAV-Cpf1 KIKO system generates a flexible and efficient double knock-in of two CARs within a single T cell. This T-cell has many advantages compared to Cas9 CAR-T cells. It is much potent in cytokine production and cancerous cell cytotoxicity. Besides, it expresses lesser exhaustion markers.

11. Nanotechnology-based Delivery System of CRISPR/Cas9 for Cancer Treatment.

The safety and efficiency of delivery remain a major obstacle facing CRISPR/Cas9 applications in cancer treatment. The most promisingly used technique in cancer drug delivery is nanotechnology [82]. NTLA is a Very recent lipid nanoparticle-based CRISPR/Cas9 gene-editing system that was used for transthyretin amyloidosis (ATTR), clinical trial NCT04601051 [83]. Moreover, Lipofectamine and RNAiMAX are nanoparticles-based commercial components that are both used to transfect different cells with CRISPR/Cas9 system [82].

Recently, nanoparticles in the form of a gold/lipid-CRISPR system were developed to target PLK-1 in melanoma and the form of a Polymer-CRISPR system targeting MTH1 in ovarian cancer [84]. Indeed, the challenge for developing nanocarrier for CRISPR/Cas9 with safety and efficiency still exists because of many barriers in the *in vivo* applications. The first challenge is a large and charged surface system, making the efficient encapsulation of the CRISPR/Cas9 components very difficult [85]. At the same time, the second obstacle facing the CRISPR-loaded nanoparticle is the blood barrier where CRISPR/Cas9 system could be degraded by different enzymes in the plasma, besides its clearance by the mononuclear phagocytes or macrophages [86].

Due to its strong negative charge, CRISPR/Cas9 cargo, positively charged nanoparticles were used for encapsulation by electrostatic attraction. The positively charged components are polypeptides, cationic lipid-based nanoparticles, and polymers [87-91]. Additionally, polyethylene glycol PEG coating on nanoparticles was employed to protect CRISPR/Cas9 from denaturation and increase circulation time [92, 93]. Recently, nanocarriers with glutathione (GSH)-triggered release property were extensively used due to their sensitive reduction capabilities in tumor sites [94]. Consequently, it was applied extensively in the delivery of CRISPR/Cas9 [95].

12. The Beginnings of CRISPR Clinical Trials

As mentioned previously, the first clinical trial of CRISPR mediated therapy was against aggressive lung cancer (NCT02793856).CRISPR/Cas9 was utilized to disable the PD-1 protein in the T-cell surface in the *ex vivo* after its isolation from the blood of a donor patient. Another important clinical trial (NCT03057912) used a mix ofCRISPR/Cas9 and TALENs gene-editing tools to treat cervical carcinoma originating from HPV infections. Where in this approach, HPV16 and HPV18 E6/E7 DNA were targeted for the knockout. This is the first *in vivo* CRISPR/ Cas9 clinical trial where the CRISPR/Cas9 and the TALEN components are

proposed to be applied as a gel material directly to the infected cervix[2,96]. Recently, Stadtmauer *et al*. [97]introduced an in-human phase 1 clinical trial in three patients who have refractory cancer. The safety and applicability of engineer T cells were tested. T cells were edited using multiplex CRISPR-Cas9 targeting three genes: endogenous T cell receptor (TCR) chains, TCRα, TCRβ, and programmed cell death protein 1 (PD-1). The transfer of these engineered T cells into patients resulted in the sustainable expression of the three edited loci. However, chromosomal translocations were observed, its frequency was reduced by time.

Moreover, Edited T cells remained for 9 months in these patients. In October 2021, NTLA-2001(clinical trial NCT04601051) got FDA recognition as a potential promising single-dose, novel therapy for treating ATTR amyloidosis (Transthyretin amyloidosis). NTLA-2001 was used as an *in vivo* gene-editing therapy designed to treat ATTR amyloidosis by lowering the concentration of transthyretin (TTR) in serum[83]. Most recently, in November 2021, CTX110 (clinical trial NCT04035434) gained FDA regenerative medicine advanced therapy designation. It is applied for the Treatment of Refractory CD19+ B-cell malignancies. CTX110 is a donor-derived CAR-T therapy that targets CD19(a B cell-specific cell surface antigen) predominantly expressed in all B cell lineage malignancies [98].

13. Challenges Facing the Applications of CRISPR/Cas9 Technology

Although the success of CRISPR/Cas9 based genome editing in studying diseases' etiology and progression, in addition to its therapeutic applications, it still shows many pitfalls and limitations. Where it is an error-prone technique and showed a 15% failure, some of these limitations and challenges are listed here and summarized in Table 3.

13.1. The host immune response.

The immune response triggered against Cas protein is a major obstacle facing clinical applications of Crisper technology. This is because more than 50% of the human population is not responsive to nucleases due to resistance to Staphylococcus aureus and Staphylococcus pyrogens nuclease positive bacteria. Many studies are searching for new types of nucleases to transfer this technique from *in vitro* to *in vivo* applications [99].

13.2. PAM sequence requirement.

An important limitation of this technique is the need for a PAM [100], a criterion that limits the possible target loci. Many trials used a PAM containing nuclease to solve this limitation [99].

13.3. Formation of Cas9-DSB complex.

The unwanted permanent binding between the Cas9 protein and the DSB can hinder the action of the repair protein by preventing its access to the breaking site. And consequently, the repair efficiency is reduced, and this represents the main real limiting step in the *in vivo* applications. This could be solved by translocating the RNA polymerase at a certain action point in the polymerase on DSB [2].

13.4. On-target deletions or modifications.

The double-strand breaks made by the Cas9 enzyme could cause *in situ* deletions or genetic modifications. These were proved by using long-range PCR and long-read sequencing [101]. This finding highlights the possibility of tumor suppressor gene inactivation, activating dormant mutations, or resulting in any disease. These effects could be avoided by a few clones sequencing and excluding any undesired modification before expanding the application.

13.5. Off-target effects.

In spite of using a guided RNA sequence, sgRNA, many studies have reported the possibility of developing off-target effects, which may lead to chromosomal rearrangements and mutations. In the PAM distal part of the sgRNA, DNA mismatches could be added. Some protocols could be used to reduce these off-target effects, including the reduction of Cas9-sgRNA concentrations; the use of modified truncated 3'end of the sgRNA; replacing Cas 9 bydCas9; fusing dCas9 with FokI nuclease with a higher sequence specificity, and nonhomologous sequence selection[2]. Abudayyeh *et al*., [102]introduced RNA-guided RNA-targeting CRISPR–Cas effector (Cas13a8 the class 2 type VI6,7) as an engineered tool for mammalian cell RNA knockdown and binding. 15 orthologues were screened, and Cas13a from Leptotrichia wadei (LwaCas13a) was the most effective in an interference assay in Escherichia coli. Cas13a can be used for targeted knockdown with minimum off-target effects.

13.6. The delivery method.

One of the challenges facing the CRISPR technology is the choice of transfection method to deliver the sgRNA and the nuclease into the cell. Indeed, direct delivery of construct plasmid seems to be an easy method; it is not always suitable. Another suitable choice is using viruses, such as adeno-associated viruses. However, the large spCas9 (derived from streptococcus pyogenes bacteria) protein is difficult to integrate into the virus. This obstacle was avoided by generating other Cas proteins of smaller sizes, namely, nmCas9 and saCas9 (3.2 kb) (which are derived from Neisseriameningitides and Staphylococcus aureus bacteria, respectively). In addition to, direct delivery of the CRISPR/ Cas9 system by using microinjection, electroporation, or lipofection is still a valid option for delivery [99].

13.7. Low HDR output.

HDR repair mechanism has many advantages over NHEJ, which may cause undesired mutations in the cutting site. However, HDR repair has a low output. This output could be increased by optimizing the delivery method and manipulating cell cycle genes. Additionally, Cpf1 nuclease was recently used, which has a higher output than conventional nucleases [99].

Table 3. Challenges facing the applications of CRISPR/Cas9 technology.

Limitation	Causes	Solutions	References
1. Host immune response	Immune response stimulated against Cas protein. More than 50% of the human population are not responsive to nucleases due to resistance to *Staphylococcus ureus*and*Staphylococcus pyrogenes* nuclease positive bacteria.	Many studies are searching for new nucleases to transfer this technique from *in vitro* to *in vivo* applications.	[99]
2. PAM sequence requirement	The limitation of this technique is the need for a PAM sequence.	Many trials used a PAM containing nuclease to solve this limitation.	[99,100]

Limitation	Causes	Solutions	References
3. Formation of Cas9-DSB complex	The unwanted permanent binding between the Cas9 protein and the DSB can hinder the action of the repair protein by preventing its access to the breaking site.	Translocation of the RNA polymerase at a certain point of action in the polymerase on DSB.	[2]
4. On-target deletions or modifications	The double-strand breaks made by the Cas9 enzyme could cause *in situ* deletions or genetic modifications. These were proved by the use of long-range PCR and using long-read sequencing.	These effects could be avoided by a few clones sequencing and excluding any undesired modification before expanding the application.	[99]
5. Off-target effects	Many studies have reported developing off-target effects which may lead to chromosomal rearrangements and mutations. In the PAM distal part of the sgRNA, DNA mismatches could be added.	Reduction of Cas9-sgRNA concentrations; the use of modified truncated 3'end of the sgRNA; replacing Cas 9 bydCas9; fusing dCas9 with FokI nuclease with a higher sequence specificity; and non homologous sequence selection.	[2]
6. The delivery method	Direct delivery of construct plasmid seems to be an easy method; it is not always the suitable way. Another suitable choice is using viruses such as an adeno-associated virus. However, the large size of the spCas9 (derived from streptococcus pyogenes bacteria) protein is difficult to be integrated into the virus.	Generation of other Cas proteins of smaller sizes namely, nmCas9 and saCas9 (3.2 kb) (which are derived from Neisseria meningitides and Staphylococcus aureus bacteria, respectively). Moreover, direct delivery of the CRISPR/ Cas9 system by using microinjection, electroporation, or lipofection still valid options for delivery.	[100]
7.Low HDR out put	HDR repair mechanism has many advantages over NHEJ, which may cause undesired mutations in the cutting site. However, HDR repair has a low output.	Optimization of delivery method and manipulation of cell cycle genes. Additionally, Cpf1 nuclease was recently used, which has a higher output as compared to conventional nucleases.	[99]

14. Future Perspectives of CRISPR Technology

CRISPR-mediated gene editing has become a wonder in the biological sciences due to its enormous applications in screening and therapeutic models for different diseases, especially cancer. However, many studies are directed from now and ongoing to solve the previously mentioned limitations such as the delivery methods optimization and reducing the off-target effects. Additionally, some applications of CRISPR technology faced some ethical criticisms. Indeed, CRISPR was proved to cure mutations in hardly cured diseases such as Duchenne muscular dystrophy, thalassemia, and cystic fibrosis. Besides, it showed great promising results in curing lethal diseases such as cancer and HIV. Promisingly, scientists are motivated to completely eradicate hard diseases by the CRISPR applications in the near future.

15. Conclusions

Gene editing is one of the recent promising tools from gene therapy technologies. The crisper technology has introduced therapeutic options in cancer treatment, generally, and in cancer immunotherapy specifically. Researchers are highly motivated to introduce a cure for many hard diseases by the use of CRISPR technology. Many challenges still exist and need solutions for successful gene editing.

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Conflict of interest

The authors declare no conflict of interest.

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