

CRISPR-Cas9 in basic and translational aspects of cancer therapy

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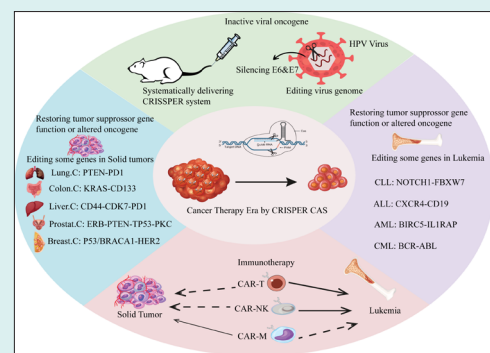
Abstract

Introduction: The discovery of gene editing techniques has opened a new era within the field of biology and enabled scientists to manipulate nucleic acid molecules. CRISPR-Cas9 genome engineering has revolutionized this achievement by successful targeting the DNA molecule and editing its sequence. Since genomic changes are the basis of the birth and growth of many tumors, CRISPR-Cas9 method has been successfully applied to identify and manipulate the genes which are involved in initiating and driving some neoplastic processes.

Methods: By review of the existing literature on application of CRISPR-Cas9 in cancer, different databases, such as PubMed and Google Scholar, we started data collection for "CRISPR-Cas9", "Genome Editing", "Cancer", "Solid tumors", "Hematologic malignancy", "Immunotherapy", "Diagnosis", "Drug resistance" phrases. Clinicaltrials.gov, a resource that provides access to information on clinical trials, was also searched in this review.

Results: We have defined the basics of this technology and then mentioned some clinical and preclinical studies using this technology in the treatment of a variety of solid tumors as well as hematologic neoplasms. Finally, we described the progress made by this technology in boosting immune-mediated cell therapy in oncology, such as CAR-T cells, CAR-NK cells, and CAR-M cells.

Conclusion: CRISPR-Cas9 system revolutionized the therapeutic strategies in some solid malignant tumors and leukemia through targeting the key genes involved in the pathogenesis of these cancers.



Introduction

DNA repair by deletion or replacement of the sequences involved in diseases and generation of nonpathogenic genes are the main areas of genetic manipulation in gene therapy. These therapeutic approaches are promising strategies for treating many non-curable human diseases, such as some hemoglobinopathies, acquired immunodeficiency syndrome (AIDS), diabetes, metabolic and neurodegenerative diseases, and cancer.¹⁻³ The precise manipulation of the target regions, including specific gene activation or inactivation, nucleotide sequence

replacement, gene deletion, and rearrangement, usually enables appropriate nucleases to be embedded in the editing platforms.³ At the first attempts, few products purported to be impressive in the treatment of patients with head and neck carcinomas and certain brain tumors. These products used a cut-and-paste technique to eliminate defects and insert the correct gene in the right place on the chromosome.³ However, these initial gene therapy tools integrated a copy of exogenous DNA into the genome with undesirable effects, including abnormal protein expression and insertion mutations. In recent



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years, to avoid these drawbacks, programmable nucleases have been widely used in gene editing platforms.³ This review briefly describes the basics of the most successful gene editing technologies, such as clustered regularly interspersed short palindromic repeats (CRISPR) –Cas9, their applications, and achievements in some prevalent cancer treatments. CRISPR-Cas9-based genome editing has generated massive interest in applied biology and medicine due to the easy targeting of Cas9, high effectiveness as a site-specific nuclease, and the potential for highly complex changes.

Different kinds of genome editing tools

Effective genome editing requires four main kinds of DNA-binding proteins: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), Meganucleases, and Cas9 nuclease (Table 1).⁴ The first programmable nucleases that alter the stem cell genome were ZFNs. 4 ZFNs contain two domains: a zinc finger domain that recognizes DNA targets and a nuclease domain, which is originated from the FokI restriction enzyme. The DNA-binding domain can bind to target sites which are about 18 bp in length or longer and allows each ZFN motif to recognize 3-6 bp. The second domain cleaves ZFN target sites, which are often rich in guanines and consist of 5'-GNN-3'.⁵ As ZFN heterodimers can be quickly packaged as 1 kb/monomer, they can be carried by adenoviral vectors. The limited targeting density and high off-target effects of ZFNs often hinder their usefulness due to resulting cytotoxicity.⁶

Another programmable nuclease is TALEN. TALENs consist of a FokI nuclease domain at the carboxyl terminus and a DNA-binding domain at the amino-terminal region. The first domain terminates almost 12–20 bps nearby the spacer sequence.⁵ The DNA-binding domains of TALEN contained nearly identical amino acid replicates of 34 residues in length, varying only at positions 12 and 13. This hypervariable di-residue area makes the specific nature of the TALEN editing tool. TALENs can modify any desired DNA sequence, and in spite of their enormous size, they can be delivered using various tools, namely codon-divergent repeat variable di-residues (RVDs), adenoviral vectors, and mRNA- or protein-based gene transfer procedures.⁵ However, the primary obstacles faced by TALEN approaches include the need for intricate molecular cloning for every new DNA target

and the limited effectiveness in screening genomes within targeted cells.⁷

Meganucleases are the other members of the endonuclease family that specifically recognize and cleave large segments of DNA (12-40 nucleotides). Although they have large recognition sites and are less cytotoxic than ZFNs, the number of naturally occurring meganucleases is limited and insufficient to support all the potential loci of interest.⁸ In addition, the expensive and time-consuming process of developing sequence-specific enzymes for all potential sequences makes meganuclease less favorable.⁹

By making double-strand breaks (DSBs) at particular genome sites, the mentioned gene editing tools tackled the major obstacle of genetic engineering. However, some severe limitations, such as the complex and time-consuming design of the constructs, remained significant challenges.

CRISPR-Cas9

The Cas9 nuclease combined with a short guide RNA, discerns the DNA target using the Watson-Crick base pairing model. The guide RNA sequence (corresponds to the phage sequences which is a natural mechanism of antiviral defense) can be simply replaced with the desired sequence and retarget Cas9. Multiplexed CRISPR-Cas9 gene targeting can now be controlled with a short guide RNA in place of several bulky proteins (Supplementary movie).¹¹

The history of CRISPR started with the study of the isozyme transformation of alkaline phosphatase by the intestinal alkaline phosphatase (IAP) enzyme in *Escherichia coli* in 1987.¹¹ Nakata et al detected 29 replicates of nucleotides downstream of the IAP gene, which, unlike most replication elements, were separated by interspaced sequences.¹² Afterwards, Mojica et al categorized repetitive interspaced sites as an unparalleled family of clustered repetitive elements that exist in more than 40% of sequenced bacteria and 90% of archaea. Jansen and Mojica used CRISPR to describe bacterial sequences containing an interspaced repeat array.¹² The importance of these sequences became apparent when the researchers realized the similarity of the phage genome sequence with the sequences between CRISPR loci. It was also discovered that the CRISPR locus is a part of adaptive immunity targeting foreign viruses.¹² Fig. 1 shows the timeline of the historical events surrounding the discovery

Table 1. Comparison of the four major classes of DNA-binding proteins

	ZFN	TALEN	Cas9	Meganuclease	Ref
Specificity	Low	Medium	High	High	9
Targeting constraints	Difficult to target non-G-rich sequences	5' targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM	Targeting novel sequences often results in low efficiency	9
Ease of engineering	Difficult	Moderate	Easy	Difficult	9,10
Cleavage efficiency	Low efficiency	Efficient	Efficient Highly	Low efficiency	9
Ease of multiplexing	Low	Low	High	Low	10

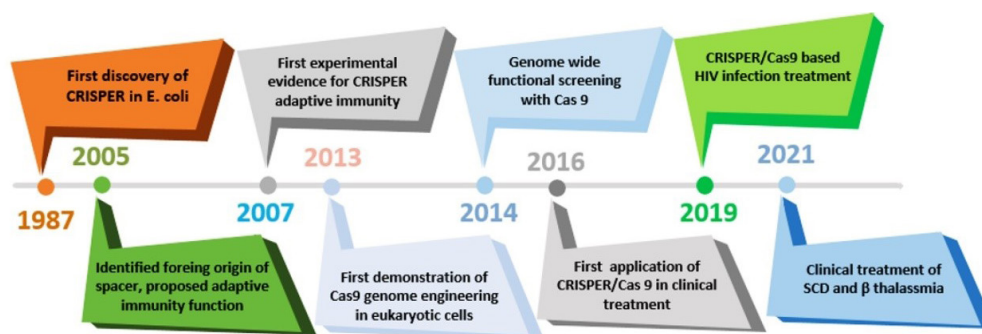


Fig. 1. A chronological overview of key milestones in the development of CRISPR-Cas9 genome editing technology.

and identification of the CRISPR–Cas9 system.

The CRISPR-Cas9 system functions as an acquired immune system against invading viruses and plasmids in bacteria and archaea.¹³ When a foreign DNA fragment enters the cells, it is cleaved by Cas nuclease enzymes, and a part called a spacer is placed in the locus of the crisper gene between two repetitive sequences (adaptation step).¹⁴ In the next step, known as the expression step, the spacer sequences are used as a template to generate short CRISPR RNA sequences (crRNA). In addition, after transcription of a part of the CRISPR gene, CrRNA-activating-trans (tracrRNA) is produced, then two sequences are joined. This complex is a guide sequence in the next step (interference) and guides the Cas protein to the foreign DNA. Once the Cas binds to the invading DNA, its HNH nuclease domain mediates the cleavage of the complementary crRNA strand, and the Like-RuvC1 nuclease domain cleaves the opposite strand. Since the discovery of the CRISPR system, 45 families of Cas proteins have been described with different roles, including crRNA synthesis, uptake and insertion of new spacer sequences, and invasive DNA cleavage.

The Cas9 protein is responsible for the cleavage of DNA, and the guide RNA (sgRNA) is comprised of crRNA and tracrRNA.¹⁵ The Cas9 protein is derived from *Streptococcus pyogenes* and is responsible for destroying two DNA strands and is delivered to the target site by sgRNA.¹⁵ This two-membered protein consists of target recognition and the nuclease lobe. The nuclease lobe also contains RuvC and HNH domains; both domains generate blunt-ended double-stranded DNA (DSB); RuvC (the retroviral integrase protein) cleaves non-target double-stranded DNA (DSB), whereas HNH cleaves targeted DNA strands. siRNA effectively identifies specific sites and directs Cas9 to the target region. The high specificity, efficiency, and accuracy of sgRNA are related to the 10-12 initial nucleotides at the end of '3, located upstream of the proto-spacer adjacent motif (PAM), and direct the Cas9 protein to the desired site.^{16,17} Eventually, the assembly of tracrRNA, crRNA precursor (pre-crRNA), and Cas9 forms a complex in which tracrRNA is responsible for activating RNase III to accelerate the maturation of pre-crRNA. The tracrRNA with a hairpin structure is transcribed from a

repetitive region.¹⁷

CRISPR-Cas9 delivery

CRISPR-Cas9 gene editing faces challenges in delivering its components. Viral vectors have been used for decades in genetic engineering, with recombinant retroviral vectors receiving much attention due to their low off-target effects, high accuracy, and specificity for targeting the cell genome. Lentiviruses have been selected as a suitable delivery option due to the large size of Cas9 (4.1 KB).¹⁸ However, several main problems, including the possibility of mutations and immunogenicity, have limited the use of adenoviral and lentiviral vectors. Plasmid DNA is another vehicle for delivery of CRISPR components, which has been used in *in vitro* and *in vivo* studies. However, problems such as the high persistence of CRISPR RNPs inside the cell, consequent rise in off-target effects, and probability of mutagenicity limit their optimal use.¹⁹ To overcome these challenges, researchers have introduced non-viral methods due to their advantages, including high packing ability, fewer side effects, high accuracy, and spatiotemporal specificity for targeting the genome. Several compounds, such as graphene oxide, liposomes, gold nanoparticles, zeolitic imidazole, DNA nanoclews, and cationic polymers, have been associated with promising results in delivering CRISPR-Cas9 components.²⁰ However, the large size of Cas9, the high negative charge of sgRNAs, the difficulty of protecting Cas9 and sgRNAs from degradation, and the Cas9-loaded non-viral particles process are also crucial concerns for non-viral carriers. Researchers have developed modified lipid nanoparticles that can deliver RNPs to modify gene expression and significantly reduce serum levels in mice. These results indicate the generalizable approach of these nanoparticles for editing different regions of the genome, biological modeling, and therapeutic interventions.²¹

Application of CRISPR-Cas9 in treatment of malignancies

Non-lethal genetic alterations are trigger points in cancers. This initial damage may be caused by environmental exposure (viral, chemical, toxic endogenous molecules, radiation, and unknown factors) or may be inherited in the germ lines of victims.²² At the beginning of cancer gene

editing, TALENs and ZFNs acted as a powerful class of tools for cancer therapy but their complicated design and time-consuming experimental processes hindered their clinical usage.¹⁸ RNA interference (RNAi) was another successful cancer treatment, but it was accompanied by unwanted gene insertion, low specificity, transfection, and a low immune response.²³ Genome editing by CRISPR-Cas9 has recently elucidated to be successfully applied in the correction of genes involved in initiation or driving in carcinogenesis. CRISPR-Cas9 has been a significant priority for cancer treatment due to its simplicity and high accuracy.²⁴

There are two main strategies for genetic editing in tumor cells: inactivation of active viral oncogenes and repairing altered oncogenes or silenced tumor suppressor genes.² As the CRISPR-Cas9 editing tool originates from the bacterial adaptive immune system, it has an inherent benefit in protecting against viral infections. This editing system can directly remove or inactivate viral infections by manipulating the virus genome and may offer new opportunities to prevent and treat virus-associated malignancies.² Several viruses have been associated with cancer development, including hepatitis B virus (HBV) and hepatitis C virus (HCV) in hepatocellular carcinoma (HCC), Epstein-Barr virus (EBV) in nasopharyngeal carcinoma, human papillomavirus (HPV) in uterine cervix squamous cell carcinoma, and human Herpesvirus-8 disease (HHV8) in Kaposi sarcoma. Eliminating or inactivating these carcinogenic viruses can intercept or reverse the process of tumor formation.² The studies targeting the genomic structure of viruses are limited but show promising results. Zhen et al transferred Cas9 and sgRNAs into HPV-16-positive cervical cancer cells (SiHa), targeting HPV16 E6 or E7 and observed inhibition of the proliferation of cancer cells in a mouse xenograft model.²⁵ Another study utilized the CRISPR-Cas9 system to inactivate E6 or E7 and induce senescence in HPV18 immortalized HeLa cells.²⁶ They reported that E6 or E7-knocked out HeLa cells showed senescence characteristics (such as large surface area and high expression of β -galactosidase) and increased expression of p53/p21 and pRb/p21 genes. In addition, Jubair et al systemically delivered the CRISPR-Cas9 system to an animal model of cervical cancer with high expression of the E6 and E7 proteins. They observed tumor omission and increased survival in the treated group compared to untreated animals.²⁷ Regarding EBV and cancer, a study investigated the role of latent membrane protein 1 (LMP1) in nasopharyngeal carcinoma (NPC) CNE-2 cell growth and the effects of LMP1-knockout on EBV infection and CNE-2 cell growth using the CRISPR-Cas9 system. The researchers found that LMP1 overexpression promoted CNE-2 cell growth compared to LMP2A overexpression. LMP1 knockout significantly hindered EBV proliferation in CNE-2 cells and markedly inhibited LMP1-mediated promotion of cell growth. The knockout of either

LMP1 or LMP2A blocked the activation of eukaryotic translation initiation factor 4E (eIF4E) induced by EBV infection or LMP1/LMP2A overexpression. The study concluded that LMP1-mediated promotion of NPC cell growth can be effectively blocked by CRISPR-Cas9-mediated LMP1 knockout, and precise LMP1 knockout might be a promising method for targeted inhibition of EBV infection and NPC cell growth.²⁸

These types of examinations aiming to abolish the viral oncogenes are limited and this era is open to be studied vastly in combatting virus-related malignancies.

Tumor cells may acquire several types of genetic alterations affecting proto-oncogenes, tumor-suppressing genes, DNA repair genes, or genes involved in apoptosis and cell survival. These alterations include point mutations, deletions, amplifications, and non-random chromosomal abnormalities such as translocations, deletions, and inversions. Different cancers usually exhibit distinct DNA fingerprints related to one (or more) of the mentioned genetic alterations. CRISPR-Cas9 technique can disrupt the activity of oncogenes and restore tumor suppressor gene activity (Fig. 2).²⁹ Some of these DNA alterations are the main genetic drivers of carcinogenesis in certain tumors, which can be regarded as hallmarks for molecular diagnosis and hotcakes in targeted tumor therapy.

In the following sections, we will notify some interesting reports on gene editing made by CRISPR-Cas9 on some of the major organs' cancers.

Cancers targeted by CRISPR-Cas9 in preclinical and clinical practice

Breast cancer

Today, breast cancer is the second leading cause of cancer-related deaths among females, with heterogeneous molecular characteristics, subtypes, and diverse clinical consequences.³⁰ According to the expression of estrogen receptors (ERs), the breast epithelium is classified into four subgroups: luminal A, luminal B, Her2-enriched, and triple-negative breast cancer.³¹ CRISPR-Cas9 has recently gained much attention for genetic manipulation targeting HER-2, MIEN1, and MASTL oncogenes as well as PTEN and BRCA1 tumor suppressor genes. Concerning the role of mir-23b and mir-27b, Hannafon et al knocked out these genes using CRISPR-Cas9 in the MCF-7 cell line, showing a potent oncogenic role for mir-23b and mir27b in breast cancer. These data were compatible with the *in vivo* results, showing a reduction in cancer progression in xenograft nude mice following miR-23b and miR-27b knockout.³² HER-2 was directly targeted via the CRISPR-Cas9 editing tool in Wang and colleagues' experiment. They demonstrated the efficient role of this technique in inhibiting the growth of BT-474, SKBR-3, and MCF-7 cell lines.³³ Mintz et al used 2D and 3D tumor-chip models to evaluate the synergistic effect of various chemotherapies and CRISPR-Cas9 in BRCA1

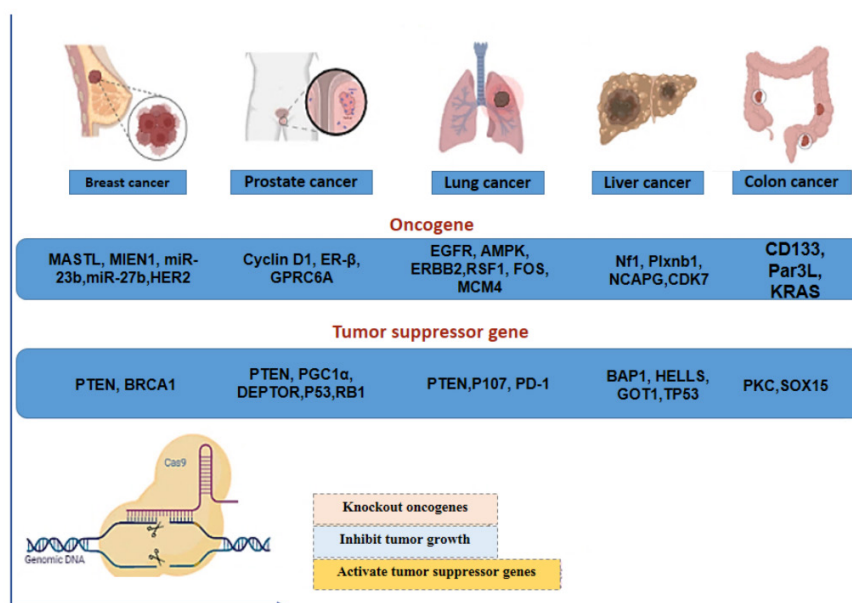


Fig. 2. The utilization of the CRISPR-Cas9 tool shows promise in treating various solid tumors by effectively targeting oncogenes and tumor-suppressor genes in cancer therapies, including those for breast, lung, liver, prostate, and colon cancers. Cas9 has extensively improved or suppressed the activity of different oncogenes and reduced tumor growth.

wild type and BRCA1 mutant (BRCA1m) cell lines, namely as MDA-MB-231 and MDA-MB-436, respectively (Fig. 3A).³⁴ BRCA1m displayed higher sensitivity to three indicative chemotherapy medicines, including docetaxel, gemcitabine, and doxorubicin in comparison to the wild-type form. Similarly, delivery of sgRNA to HCC1806 and MCF10A cells leads to considerable inactivation of both alleles of APOBEC3G by inhibiting cell proliferation and blocking the G1/S transition in breast cancer.³⁵ In another survey, the role of the Migration and Invasion Enhancer (MIEN1) gene in the development and spread of breast cancer was confirmed by gene deletion.³⁶ Likewise, Pulver et al reported that genome editing through lentiviral administration of the CRISPR-Cas9 targeting tumor suppressor genes in a mouse model significantly increased breast cancer resistance in triple-negative breast cancer.³⁷

Dai et al identified tumor regulatory functions for essential mTOR and Hippo pathways components in triple-negative breast cancer (TNBC). They showed the therapeutic relevance of their findings using *in vitro* drug-synergy matrix models and *in vivo* patient-derived xenografts. Moreover, they found that pharmacological inhibition of mTORC1/2 and oncoprotein YAP efficiently reduces tumorigenesis in TNBC, and torin1-mediated mTORC1/2 inhibition promotes macropinocytosis, further facilitating verteporfin uptake and enhancing its pro-apoptotic effects in cancer cells. Their study underscored the power and robustness of *in vivo* CRISPR genome-wide screening in identifying clinically relevant and innovative therapeutic modalities in cancer.³⁸ Inhibition of MASTL kinase effectively condensed human mammary tumor cell line growth, suggesting that MASTL interruption is a privileged choice for breast cancer treatment³⁹ and Guo et

al could interrupt the carcinogenesis of the proteasome-addicted human breast tumor cells *in vivo* by knocking out dual-specificity tyrosine-regulated kinase 2 (DYRK2) using the CRISPR-Cas9 technology (Fig. 3B).⁴⁰

Considering the role of tumor suppressor genes in breast cancer progression, Annunziata et al reported a novel approach to validate candidate tumor suppressors that may be involved in invasive lobular breast carcinoma (ILC) *in vivo*. ILC is known for the elimination of E-cadherin, the cell-cell adhesion molecule. To model ILC, lentiviral vectors encoding either Cre recombinase, the CRISPR/Cas9 system, or both were utilized to inject female mice with conditional alleles of the Cdh1 gene. By employing CRISPR-Cas9-mediated somatic gene editing, the PTEN gene was specifically disrupted in ILC-initiating cells.⁴¹

To assess the usefulness of targeted somatic manipulation of missense mutations in breast cancer, a knock-in mouse with the Cre-conditional expression of a cytidine base editor was created. To evaluate the impact of defined allelic variations on mammary carcinogenesis, a designed sgRNA-encoding vector was applied to introduce specific point mutations. This model was successfully applied in triple-negative breast cancer (TNBC) simulations.⁴² Table 2 delineates the studies with CRISPR-Cas9 system that had efficient results in antitumor therapy by targeting oncogenes and tumor suppressor genes in breast cancer.

Liver cancer

Hepatocellular carcinoma (HCC) is the one of the most common malignancies leading to death in both men and women.¹⁸ Some of the most significant mutations predisposing to HCC are activation of Plxnb1, NCAPG, CDK7, CD44, Nf1 oncogenes,^{18,43} and silencing of BAP1,

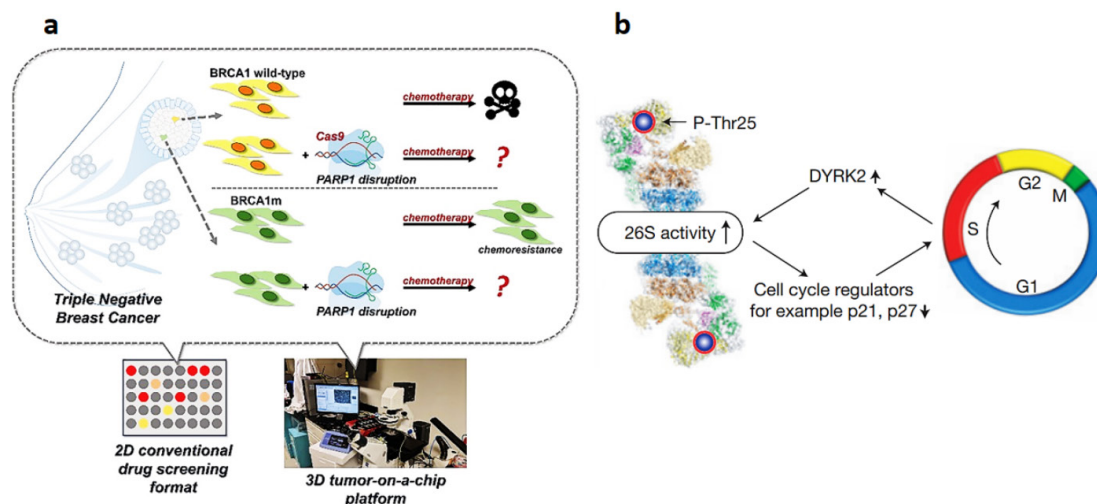


Fig. 3. (a) Tumor-on-chip models to examine the combined therapeutic effects of different types of chemotherapy treatments and CRISPR-Cas9 in two breast cancer cell lines. 34 (b) A model of the 26S proteasome's reversible phosphorylation. Cell cycle-dependent Rpt3-T25 phosphorylation controlled by DYRK2 facilitates the degradation of crucial proteins like p21 and p27, thereby promoting cell cycle progression 40 (Reprinted with permission under the Copyright Clearance Center).

HELLS, and P53 suppressor genes.⁴⁴⁻⁴⁶ Preliminary reports on the application of the CRISPR-Cas9 system to silence some of the genes involved in liver carcinogenesis are described in Table 2.

Feng et al conducted a genome-wide CRISPR-Cas9-based knockout screen and revealed a relationship between the anti-tumor effects of metformin and the levels of DOCK1. They established patient-derived HCC organoids to characterize the role of DOCK1 in determining metformin sensitivity. They also found that combining metformin with DOCK1 inhibition may provide a promising personalized therapeutic strategy for metformin-resistant HCC patients.⁴⁷ Wang et al gave an idea of lentiviral delivery of sgRNA into Huh7 and Hep38 liver cell lines and described CDK7, a cyclin-dependent kinase, as a hit target in hepatic cell growth.⁴⁸ Additionally, Huh7 and Hep38 growth rates were noticeably reduced by CRISPR-Cas9-mediated CDK7, indicating that this gene may be a good target for HCC treatment. Successful knockout of the CD44 gene in HCC, a transcriptional regulatory gene, was achieved by lentiviral delivery of sgRNA and Cas9 to C3A-iCSCs liver cancer stem cells (C3A-induced cancer stem cells). The results of this research indicate the function of CD44 in the poor differentiation of cancer stem cells, high malignant degree of tumor cells, and tumor growth.⁴⁹ Zhu et al knocked out the IncBRM gene in two liver cancer cell lines, Huh7 and Hep3B. They noted that knocking out the IncBRM gene affected the development of the serial sphere while not affecting the expression of nearby genes, suggesting that IncBRM has a trans role.⁵⁰ In addition, another experiment used the CRISPR-Cas9 system to reverse the limited benefits of sorafenib as the recommended treatment for people with advanced HCC. They suggested that inhibition of ERK2 (MAPK1) causes a variety of HCC cell lines to be susceptible to sorafenib. Thus, it

can be assumed that higher levels of ERK (more than 30% of all liver tumors) leading to a better response to this polytherapy, and inhibiting different kinases, such as ERK and MEK, represent a promising therapeutic tool in HCC treatment (Fig. 4A).⁵¹ Song et al used CRISPR-Cas9 and discovered that the genes *Plxnb1*, *B9d1*, *Flrt 2*, and *Nf1* inhibit liver tumors.⁵² When sgRNA and Cas9 were delivered to *p53*^{-/-}; *Myc*; Cas9 hepatocytes, the *NF1* gene was successfully knocked out, and subsequent liver cancer inhibition in a mouse model was achieved (Fig. 4B). An *in vivo* experiment used a lactose-derived branched cationic biopolymer (LBP) with the superior properties of gene transfection, biocompatibility, compacting, degradability, and HCC-targeting for the delivery of CRISPR-Cas9 to an orthotopic mouse model of HCC. Targeting survivin oncogenes exhibited excellent anti-cancer activities as well as effective gene editing performances.⁵³ Besides, systemic administration of E1E2-pseudotyped lentiviral vectors could deliver Cas9 and sgRNA-specific for kinesin spindle protein (KSP) to Huh7 tumors in orthotopic Huh7 mice. This particular delivery system effectively disrupted the KSP gene, thereby potentially suppressing the growth of HCC.⁵⁴ Due to the essential role of hypoxia-inducible factor 1- α (HIF-1 α) in the pathogenesis, invasiveness, and angiogenesis of HCC, it was knocked out in SMMC-7721 cells (liver cancer cells). The HIF-1 α knocked out tumor cells considerably and showed low invasiveness and migration. In addition, this strategy suppressed the engraft growth of SMMC-7721 tumors and significantly extended the survival of mice bearing HCC with a decreased CD31 expression level (a tumor angiogenesis marker) and enhanced apoptosis in the tumor cells.⁵⁵ A clinical trial [NCT04417764] started to knockout PD-1 receptors using the CRISPR-Cas9 system in autologous T lymphocytes taken from individuals with advanced HCC combined with trans-catheter arterial chemoembolization.⁵⁶

Table 2. Studies using CRISPR-Cas9 in the treatment of solid tumor cell line/animal model

	Cell line/animal model	Gene	Assay setting	Vector	CRISPR effect
Breast Cancer	MCF-7	miR-23b and miR-27b	<i>In vitro</i>	lentiviral vectors	Educed tumor growth in xenograft nude mice
	BT-474, SKBR-3, MCF-7	HER2	<i>In vitro</i>	Plasmid	Inhibits cell proliferation
	MDA-MB-231, MDA-MB-436	PARP1	<i>In vitro</i>	Plasmid	Knockout PARP1 inhibitors
	MCF10A, HCC1806	APOBEC3G	<i>In vitro</i>	pX459 Plasmid	Knockout of both alleles of APOBEC3
	MDA-MB-231	MIEN1	<i>In vitro</i>	PX458 and MLM3636 plasmids	Disruption of the MIEN1 gene
	K14Cre; p53F/F; Cas9 mouse model	BRCA1 or BRCA2	<i>In vivo</i>	lentiviral vectors	Somatic gene disruption of tumor suppressor genes
	Athymic nude mice	MASTL kinase	<i>In vivo</i>	pLKO.1 lentiviral plasmid	MASTL knockdown
	Athymic Nude-Foxn1nu mice	DYRK2	<i>In vivo</i>	Retroviruses	Knockout of DYRK2
	pSECC-sgPten in Cdh1F/F	Cdh1	<i>In vivo</i>	lentiviral vectors	ILC development
Liver Cancer	WB1P-Cas9 mice	BRCA1 and p53	<i>In vivo</i>	Lenti-sgRNA-Myc lentiviral vectors	Point mutations with high efficiency in one or multiple endogenous genes
	Huh7 and Hep38	CDK7	<i>In vitro</i>	lentiviral vectors	CDK7 knockout
	C3A	CD44	<i>In vitro</i>	Lentivirus plasmid	Knockout of CD44
	Huh7, Hep3B and PLC BALB/c mice	LncBRM	<i>In vitro/In vivo</i>	lenti Cas9-EGFPvector	Knockout of the LncBRM
	Hep3B, HepG2, SNU387, SNU398, SNU449, HuH7 and PLC/PRF/5 BALB/c nude mice	ERK2 (MAPK1)	<i>In vitro/In vivo</i>	lentiviral vectors	Sensitizes several liver cancer cell lines to sorafenib
	Hep3B, HepG2, and Huh7 p53 ^{flox/flox} mice	Nf1, Plxnb1, Flrt2, and B9d1	<i>In vitro/In vivo</i>	pX330 vector/ lentiV2	Inactivation of Plxnb1, Nf1, Flrt2, and B9d1
	HEK293 and BEL7402 nude mice	survivin	<i>In vitro/In vivo</i>	Lactose-derived branched cationic biopolymer (LBP)	Downregulate of survivin oncogene
	Huh7, HepG2, Hep3B, PCL/PLF5, and HEK293T orthotopic Huh7 mice	kinesin spindle protein (KSP)	<i>In vitro/In vivo</i>	E1E2-pseudotyped lentiviral vectors	Powerful KSP gene disruption
	SMMC-7721 BALB/c nu/nu mice	HIF-1 α	<i>In vitro/In vivo</i>	pLenti-Cas9-sgRNA719/720/721-egfp vectors	HIF-1 α knockout
Lung Cancer	T-cells	PD-1	Clinical trial	-	Recruiting
	A549, NCI-H460	PTEN	<i>In vitro</i>	Plasmid	Knockout of both alleles of PTEN
	NCI-H1975, NCI-H1650	EGFR	<i>In vitro</i>	lentiviral vectors	Knockout of EGFR
	634T, 821T4 and 807LN/ Genetically engineered mouse model	AMPK	<i>In vitro/In vivo</i>	lentiviral vectors	AMPK deletion in KrasG12D lung tumors
	Trp53 ^{flox/flox} , Rb1 ^{flox/flox} , Rosa26LSL Luciferase/LSL-Luciferase mice	p107	<i>In vivo</i>	Adenoviral vectors	Knockout of genes
Prostate Cancer	PC-3, LNCap, DU145, 22Rv1/ Athymic nude mice	GPRC6A	<i>In vitro/In vivo</i>	lentiviral vector	Blocking of GPRC6A
	PC-3 cells	TP53	<i>In vitro</i>	Plasmid	Repair the TP53 414delC gene region
	2924V cell line/	PTEN	<i>In vitro</i>	Plasmid	Inactivation of PTEN
	ER β ^{crispr-/-} mouse	ER β	<i>In vivo</i>	-	Delete the ER β gene
	Human PC cells, PC3 and DU145, & the murine PC cell line C57BL/6 male TRAMP mouse	IL30	<i>In vitro/In vivo</i>	lentiviral vector	IL30 deletion
LNCap, PC-3 nude mice	PLK1	<i>In vitro/In vivo</i>	A10-liposome-CRISPR/Cas9 chimeras	Silencing PLK1 gene	

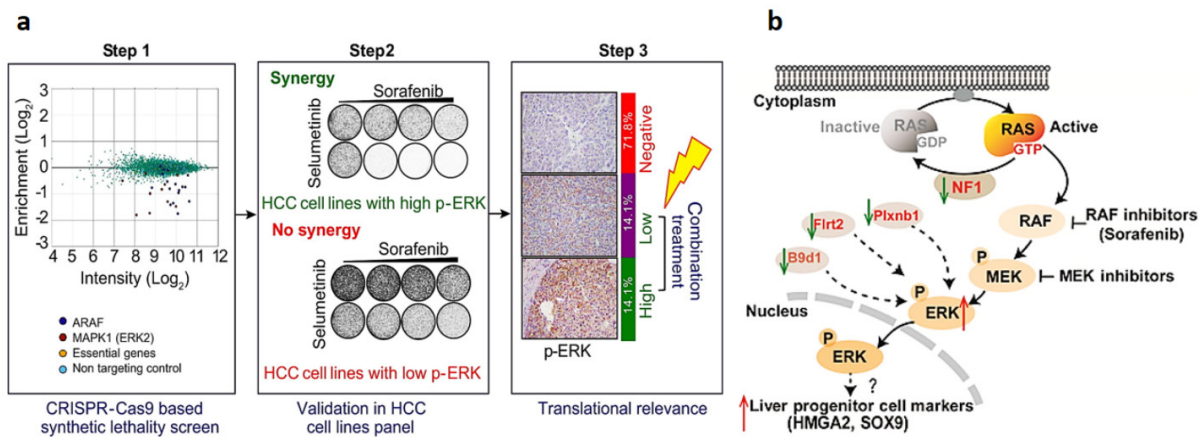


Fig. 4. (a) An *in vitro* and *in vivo* experiment employed CRISPR-Cas9 to assess the combined inhibitory effects of selumetinib and sorafenib in the treatment of liver tumors.⁵¹ In HCC cell lines with elevated p-ERK, Selumetinib augments the response to sorafenib, and inhibiting ERK2 enhances the sensitivity to sorafenib in HCC. Synthetic inhibition of ERK kinase results in a synthetic lethal effect. In cancers with high p-ERK levels, combination therapy is highly likely to yield benefits (Reprinted with permission under the Copyright Clearance Center). **(b)** Diagram showing how the loss of potential tumor suppressor genes causes the MAPK pathway to be activated and causes the liver progenitor cell markers HMGA2/SOX9 to be induced in liver cancer activity (Reprinted with permission under the Copyright Clearance Center).

Lung cancer

Many genes have been linked to the evolution and progression of different histological types of lung cancers, but there are limited studies using molecular manipulation of these genes by CRISPR-Cas9.^{57,58} Perumal et al utilized plasmid delivery (non-viral) of CRISPR-Cas9 to A549 and NCI-H460 (Slug/Snail non-small lung cancer cells) and successfully knocked out the PTEN gene. They disclosed that knocking out the PTEN gene was associated with high rates of cancer cell growth, metastasis, and invasion. Wild-type PTEN suppresses the phosphorylation of β -catenin while enhancing GSK-3 β and AKT pathways.⁵⁹ Another study showed that the presence of T > G, A > G, and C > G point mutations can be treated using CRISPR-Cas9 to target specific parts of the genome in L858R mutant lung cancer cells.⁶⁰ Eichner et al removed the murine AMPK genes ($\alpha 1$ and $\alpha 1$) and thereby the size of murine lung tumors (KrasG12D) was significantly reduced.⁶¹ In another survey, CRISPR-Cas9 helped to knockout p107 and related p130 genes (tumor suppressor genes) in a mouse model, resulting in the development and progression of small cell lung cancer (SCLC).⁶² They also showed the possibility of employing CRISPR-Cas9 technology to stimulate the deletion of tumor suppressor genes in animal models of SCLC. In 2020, an *in vivo* epigenetic CRISPR screen in a KRAS-mutant lung adenocarcinoma (LUAD) model identified Asf1a as an immunotherapeutic target. Li et al constructed an epigenetic-focused sgRNA library and performed an *in vivo* CRISPR screen in the KrasG12D/P53^{-/-} (KP) LUAD model. They found that loss of the histone chaperone Asf1a in tumor cells sensitizes tumors to anti-PD-1 treatment. Mechanistic studies revealed that tumor cell-intrinsic Asf1a deficiency induced immunogenic macrophage differentiation in the tumor microenvironment by upregulating GM-CSF expression

and potentiated T-cell activation in combination with anti-PD-1.⁶³ The outcomes of a phase I clinical trial in individuals with advanced non-small cell lung cancer [NCT02793856] revealed that infusion of PD-1-edited T cells modified by transfection of Cas9 and sgRNA to T cells by electroporation increased the median progression-free survival with no serious adverse event.⁶⁴ The effects of the CRISPR-Cas9 editing technology on different lung tumor cell lines are illustrated in Table 2.

Colon cancer

Colorectal cancer (CRC) cells exhibit increased fucosylation of the Lewis^x antigen, attributed to the abnormal expression of pertinent fucosyltransferases like fucosyltransferase 4 (FUT4) and fucosyltransferase 9 (FUT9).⁶⁵ In this regard, Blanas et al activated the expression of fucosyltransferases (Fut4 and Fut9) at the transcriptional level in a mouse colon cancer cell (MC38) via the CRISPR-d Cas9-VPR system, which resulted in the expression of functional Lewis^x antigen on the cell surface (Fig. 5A). In both MC38-FUT4 and MC38-FUT9 cells, Lewis^x was mostly transported by N-linked glycans and had a significant effect on MC38-glycovariants' core-fucosylation, sialylation, antennarity, and N-glycan subtypes. Data from this study demonstrated the CRISPR-dCas9-VPR system's use for amplifying the expression of glycosyltransferase as a propitious technique in glycobiology and oncology experiments.⁶⁵ Similarly, lentiviral delivery of the CRISPR-Cas9 system to colon cancer cells (LoVo) caused CD133 knockout and blocked tumorigenic features, cell migration, and metastasis.⁶⁶ Restoration of a loss-of-function mutation of Protein Kinase C (PKC) through CRISPR-Cas9 interrupted anchorage-independent proliferation in a patient-derived colon cancer cell line and decreased tumor proliferation

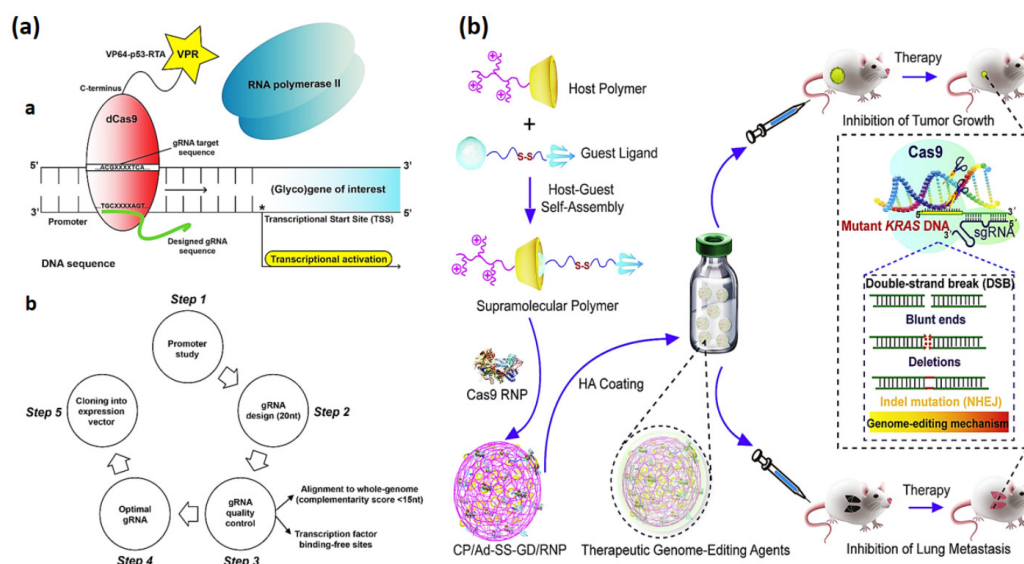


Fig. 5. (a) A model illustrating the CRISPR-dCas9-VPR system (a) and an overview (b) the CRISPR-dCas9-VPR technology's five-step experimental design, specifically applied to transcription.⁶⁵ (b) The administration of Hyaluronic acid-associated CP/Ad-SS-GD/RNP nano-complexes in a colorectal tumor mouse model (Reprinted with permission under the Copyright Clearance Center).

in a xenograft model.⁶⁷ In a different experiment, Cas9/sgRNA lentiviral delivery into the CaCO-2 cell line was able to remove the Par3L protein in colon cancer cells by suppressing cell growth, apoptosis induction, and activation of the cascade-3 pathway.⁶⁸ Due to the sensitivity of Par3L proteins to antitumor chemotherapies, the inactivation of Par3L by inhibiting AMPK signaling transduction can be a prospective target for treating CRC cell proliferation. Michels et al designed a platform for pooled CRISPR-Cas9 screening in human colon organoids to identify tumor suppressors. The researchers in this project developed optimal conditions and strict guide RNA requirements for screening in 3D organoids. They demonstrated that this technology permits unbiased detection of genes that confer positive selection *in vitro* and after xenotransplantation.⁶⁹ In an *in vitro* and *in vivo* experiment, delivery of hyaluronic acid (HA)-associated CP/Ad-SS-GD/RNP nanocomplexes to a CRC mouse model efficiently suppressed the growth and invasion of tumor cells by specifically focusing on the KRAS mutant gene using CRISPR-Cas9 (Fig. 5B).²¹ Additionally, the effective transport of Cas9 RNP through CP/Ad-SS-GD/RNP nanocomplexes to 293T cells and CRC cells leads to significant genome-editing capability *in vitro*. This particular delivery system is a supramolecular polymer to facilitate effective controlled delivery of Cas9 RNP. This system is formulated through the complexation of disulfide-bridged biguanidyl adamantine (Ad-SS-GD) with β -cyclodextrin-conjugated low-molecular-weight polyethyleneimine (CP) via supramolecular assembly, resulting in the formation of CP/Ad-SS-GD.

Prostate cancer

The second-leading cause of cancer-related mortality

in male is prostate cancer. Point mutations, structural rearrangements, single nucleotide polymorphisms (SNPs), and copy number variations in specific genes have all been implicated in the development of prostate cancer.¹⁸ GPRC6A, P53, PTEN, cyclin D1, and ER- β play significant roles in the pathogenesis of prostate cancer.^{18,70} The role of CRISPR-Cas9 as a therapeutic approach for prostate cancer has been proven in several studies due to its great precision and limited off-target effects. The GPRC6A receptor was noticeably inactivated after introduction of CRISPR-Cas9 through lentiviral delivery into the human xenograft mouse model (PC-3), and the mitigation of prostate cancer cells was diminished.⁷¹ Recently, Batir et al⁷² proved that lentiviral delivery of sgRNA to a human prostate tumor cell line (PC3) resulted in the successful repair of 26% of the 414delC mutation in TP53. Takao et al. knocked out the PTEN gene in mouse prostate tumor cells. This action activates cyclin D1 and phosphorylates the RAC-alpha serine/threonine kinase. According to their findings, prostate tumor cells lacking PTEN trigger some genes involved in cancer cell survival.⁷³ Fenner et al also determined the ER- β gene as a prostate tumor suppressor gene in mice with prostate cancer by CRISPR-Cas9.⁷⁴ An *in vivo* study in a murine prostate cancer model investigated the loss of functions of two FOX transcription factors: FOXA1 and FOXP1. The result showed that deficiency of Foxp1 increased proliferation in combination with loss of Pten, while loss of Foxa1 induced epithelial plasticity in prostate cancer. The researchers also found that Foxp1 is a repressor for the androgen-regulated target Tmprss2, and there is a negative correlation between FOXP1 and TMPRSS2 expression in a human prostate cancer dataset.⁷⁵ IL-30 stimulates the proliferation, invasion, and migration of prostate cancer cells. These effects are

associated with STAT1/STAT3 phosphorylation and upregulation of BCL2 and NFKB1 and some chemokines. Depletion of the IL-30 gene by the CRISPR-Cas led to upregulation of reduction of tumor suppressor genes like SOCS3 and downregulation of STAT3, NFKB1 BCL2, NFKB1, CXCL5, STAT3, IGF1, and CXCL5 in syngeneic and xenograft PC models.⁷⁶ Polo-like kinase 1 (PLK1), involved in prostate cancer pathogenesis, was remarkably inhibited by an aptamer-liposome-CRISPR/Cas9 chimera delivery system and caused tumor necrosis in treated mice compared to the control group.⁷⁷

Leukemia

Although chemotherapy and transplantation have improved cure rates of patients diagnosed with leukemia, there are still many patients showing relapse due to regrowth of minimal residual disease (MRD) or developing drug resistance.⁷⁸ Some specific and well-known genetic events are linked to most leukemia and lymphoma, rendering them appropriate targets for gene editing.⁷⁹ In the following paragraphs, we briefly notice the studies that directly address manipulating the main genes involved in the pathogenesis and aggressiveness of leukemia. Most of the achievements in treating acute (and less commonly chronic) leukemia have mainly been yielded by the discovery of CAR technology, which will be mentioned in the CAR-cell section. Table 3 summarizes some of the therapeutic benefits of the CRISPR-Cas9 system in several types of leukemia.

Acute lymphoblastic leukemia

Studies have shown that 9-O-acetylation of sialic acids (Sias) in ALL is crucial for lymphoblast medication resistance.^{79,80} Baumann et al showed the crucial role of

CASD1 (a sialate O-acetyltransferase) in the process of 9-O-acetylated sialoglycans synthesis by knocking out this gene.⁸¹ T315I mutation is resistant to all approved tyrosine kinase inhibitor. Tan et al⁸² reported that *in vivo* delivery of CRISPR-Cas9 efficiently postpones the rapid progression of Ph⁺ ALL (a subgroup of ALL with c-kit expression) with this mutation. McComb et al showed that the SMAC (second mitochondrial-derived caspase-activators) gene reactivates apoptosis of tumor cells mediated by RIP1. Knocking out this gene in refractory and relapsed ALL xenograft models represses RIP1 expression, impairing cell death.⁸³ To confirm the crucial role of CXCR4 in the pathogenesis of ALL, the deletion of CXCR4 in a B-ALL cell line was carried out, leading to a reduction in the *in vivo* aggressiveness of ALL.⁸⁴ During the patient recruitment phase of a clinical trial [NCT04557436], allogeneic-engineered human T-cells (also referred to as TT52CAR19+TCRαβ-) were employed in the treatment of CD19+ B cell leukemia. The lentiviral vector carrying CRISPR constructions was used for editing the CD52 and TRAC loci and transiently supplied Cas9. These constructions were applied to transduce the cells for the expression of an anti-CD19 chimeric antigen receptor (CAR19). Through T cell-mediated cytotoxicity, CD19+ leukemia and normal CD19+ B cells were eliminated in response to recognition by TT52CAR19 T-cells.⁸⁵ The other trial [NCT04154709] has been designed for the evaluation of the safety and feasibility of CTA101 (a CD19/CD22 dual-targeted CAR-T cell product developed using CRISPR-Cas9 technology) for treating relapsed or refractory CD19+ B-ALL.⁸⁶

Acute myelocytic leukemia (AML)

Targeting suppression of survivin gene expression in

Table 3. Therapeutic effects of CRISPR-Cas9 on various types of leukemia

	Cell line/animal model	Gene	Assay setting	Vector	CRISPR effect
	LM-TK-, TE671, HEK293T, CHO-K1, CHO lec1, and CHO 6B2	CASD1	<i>In vitro</i>	Plasmid	CASD1 knockout and reduction of 9-O-acetylation
	p210 BCR-ABL1 ^{T315I} mutation mouse model	BCR-ABL1 fusion gene	<i>In vitro/In vivo</i>	Lentiviral vector	Disrupts the BCR-ABL1 fusion gene
ALL	NSG mice	RIP1	<i>In vivo</i>	Plasmid	Disruption of RIP1
	9-15C/ non-irradiated NOD/SCID/IL-2rnull (NSG) mice	CXCR4	<i>In vitro/In vivo</i>	Plasmid	Deletion of CXCR4 gene
	T cells	CD52 and TRAC	Clinical trial	Lentiviral vector	Recruiting
	T cells	CD19	Clinical trial	-	Recruiting
AML	HL-60 and KG-1	BIRC5	<i>In vitro</i>	Lentiviral vector	Suppression of BIRC5
	BALB/cJ, NOD.Cg-Prkdcscid Il2rgtm1Wjl, NSG	IL1RAP	<i>In vivo</i>	lipidoid nanoparticle	IL1RAP knockout
CLL	MEC-1	Notch1	<i>In vitro</i>	Plasmid	Emulate NOTCH1 mutations
	HG3	FBXW7	<i>In vitro</i>	Plasmid	FBXW7 mutations increase in NOTCH1 target gene expression
CML	K562/ e NOD/SCID mice	BCR-ABL gene	<i>In vitro/In vivo</i>	PEG-PLGA nanoparticles	Knocked out the BCR-ABL fusion
	K562, K562/G01, U937, HL60 and CML stem/progenitor cells/ NOD/SCID mice	BCR-ABL gene	<i>In vitro/In vivo</i>	Plasmid	Disrupt BCR-ABL gene

AML, Narimani et al knocked out BIRC5 in AML cell lines, namely HL-60 and KG-1, through site-specific cleavage of its locus. Additionally, inhibition of BIRC5 caused cell viability to decrease and caused necrosis and cell death in the mentioned cell lines.⁸⁷ Moreover, HO et al introduced leukemia stem cells (LSCs) as a critical player in the pathogenesis and relapse of AML and removed them successfully as a therapy model. They utilized a reducible lipidoid-encapsulated Cas9/single guide RNA (sgRNA) ribonucleoprotein [lipidoid nanoparticle (LNP)-Cas9 RNP] to target the interleukin-1 receptor accessory protein (IL1RAP) and eliminate leukemic stem cells (LSCs). Loading of LNP-Cas9 RNP and the chemokine CXCL12 α onto mesenchymal stem cell membrane-coated nanofibril (MSCM-NF) was also done to mimic the environment of bone marrow and increase the capacity of LSC targeting. The *in vitro* data revealed that LSCs could migrate to MSCM-NF scaffolds, and LNP-Cas9 RNP-mediated gene editing reduced LSCs' capacity to form colonies.⁸⁸

Chronic lymphoblastic leukemia

Chronic lymphoblastic leukemia (CLL) is the most prevalent form of adult chronic leukemia characterized by a progressive increase in clonal mature-appearing CD5+ B cells in the bone marrow, lymph nodes, and peripheral blood cells. The most relevant mutated genes in CLL are Notch signaling genes, TP53, and ATM.^{89,90} Arruga et al used CRISPR-Cas9 technology to create several NOTCH1 variants. They demonstrated the role of NOTCH1 signaling in tumor growth and chemotaxis through the downregulation of the tumor suppressor DUSP22 and suggested the contribution of NOTCH1 mutations to the pathogenesis and prognosis in CLL.⁹¹ CRISPR-Cas9 technology was used to knockout FBXW7, another mutated gene in NOTCH1 signaling. Following truncation of this gene, the activity of the intracellular domain of NOTCH1, c-MYC protein, and HIF-1 α was increased in the HG3 cell line. It has also been reported that CLL patients with dysregulation of FBXW7 could be functionally related to NOTCH1 mutations.⁹²

Chronic myeloblastic leukemia

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder primarily identified by the reciprocal translocation between chromosomes 9 and 22 (BCR-ABL fusion).⁹³ CRISPR-Cas9 technology has received more attention as an exquisite therapeutic protocol for CML and as *in vitro* model development.^{94,95} For this purpose, Liu et al used an encapsulated CRISPR-Cas9 plasmid with poly (ethylene glycol)-b-poly (lactic acid-co-glycolic acid) (PEG-PLGA)-based cationic lipid-assisted polymeric nanoparticles (CLANs) to directly target the overhanging BCR-ABL gene fusion region in a mouse model. They effectively knocked out BCR-ABL fusion following the intravenous injection of CLANs carrying pCas9/gBCR-ABL in mice CML models. They

observed an improvement in the survival rate and proposed the CRISPR-Cas9 system utilization combined with nanocarriers as an efficient therapy for CML.⁹⁶ In another study, BCR-ABL fusion was stopped using FokI nuclease (RFN) delivery through the CRISPR-Cas9 editing system. This demonstrated that RFNs effectively impaired BCR-ABL and inhibited cell proliferation in CML lines (K562, K562/G01, U937, and HL60) and CML stem/progenitor cells. In an additional survey, RFNs remarkably suppressed the leukemogenesis capacity of CML cells in a xenograft model.⁹⁷ Table 3 details the therapeutic benefits of the CRISPR-Cas9 system in several types of leukemia.

CAR-T cells and CRISPR

Currently, novel and promising avenues for immunotherapy have been created by chimeric antigen receptors (CAR). This fusion protein comprises a selected single-chain variable fragment (ScFV) from a specific monoclonal antibody and one or more intracellular T-cell receptor-derived signaling domains.⁹⁸ Modification of autologous or allograft T-cells to express CAR (CAR-T cells) leads to an efficient and selective recognition of tumor-associated antigens (TAA).⁹⁹ By making a universal "off-the-shelf" cellular product or altering immune cells to overcome resistance in hematological or solid malignancies, CRISPR-Cas9-based genome editing provides the possibility of more effective immunotherapy.¹⁰⁰ The CRISPR-Cas9 system unquestionably helps address the unmet needs of CAR-T cell-based therapy for malignancies despite multiple roadblocks regarding the safety, effectiveness, and scalability.¹⁰¹ Template-free and non-DSB genome editing procedures, multiplex disruption of inhibitory molecules and signaling pathways, targeted gene knock-in, reducing the frequency of cytokine release syndrome (CRS) and GVHD, and manufacturing universal CAR T-cell products using normal donor leukocytes or iPSCs are some examples of such advancements of CRISPR technique.^{102,103}

Several preclinical studies have utilized the CRISPR technique, which has dramatically improved the function of CAR-T cells.^{104,105} Eyquem et al designed a dual strategy of CD19 CAR knock-in and TCR knockout, which reduced the likelihood of GVHD and simultaneously prevented CAR signaling by optimizing CAR internalization and re-expression kinetics.¹⁰⁶ Another study used CRISPR-Cas9 technology to simultaneously disrupt PD-1, endogenous T-cell receptor (TRAC), and beta-2 microglobulin (B2M) genes. The results indicated the long-term survival of glioma mouse models following intracerebral injection of this universal CAR-T cell product.¹⁰⁷

CRISPR-Cas9 application for generating feasible, effective, safe, and universal CAR-T cells has already entered the clinical phase (So far, 18 trials have been registered at clinicaltrials.gov). Most of these clinical studies are related to hematologic neoplasms with the aim of targeting CD19 markers (Table 4).

Table 4. Clinical trials based on the application of CRISPR-Cas9 strategy for improving the efficacy of CAR-T cell

Rank	Clinical Trial identifier	Status	Clinical trial phase	Disease	CAR Antigen	Target Locus of CRISPR	NK source	Number enrolled	Interventions	Dosage	Starting date	Location
1	NCT03166878	Unknown	Phase 1/2	CD19+ leukemia and lymphoma	CD19	Endogenous TCR and B2M genes	Allogeneic	80	Universal CRISPR-Cas9 Gene-Editing CAR-T Cells Targeting CD19	Unknown	June 2017	China
2	NCT03398967	Unknown	Phase 1/2	B-cell malignancies	CD19, and CD20 or CD22	Unknown	Allogenic	80	Universal Dual Specificity CD19 and CD20 or CD22 CAR-T Cells	Unknown	January 2, 2018	China
3	NCT03545815	Unknown	Phase 1	Solid Tumors	Mesothelin	PD-1 and TCR gene-knocked out	Unknown	10	anti-mesothelin CAR-T cells	Unknown	March 19, 2018	China
4	NCT03747965	Unknown	Phase 1	Solid Tumors	Mesothelin	PD-1 gene-knocked out	Unknown	10	Mesothelin-directed CAR-T cells	Unknown	November 2018	China
5	NCT05812326	Completed	Phase 1/2	MUC1- positive breast cancer	MUC1	PD-1	Autologous	15	AJMUC1- PD-1 gene knockout anti-MUC1 CAR-T cells	3x10 ⁵ /kg, 1x10 ⁶ /kg 3x10 ⁶ /kg	May 17, 2019	China
6	NCT04035434	Recruiting	Phase 1	B-cell malignancies	CD19	Unknown	Allogenic	143	CD19-directed CAR-T-cell (CTX110)	Unknown	July 22, 2019	Unknown
7	NCT04037566	Recruiting	Phase 1	CD19+ leukemia or lymphoma	CD19	Endogenous HPK1	Autologous	40	CD19-specific CAR-T cells with edited endogenous HPK1 (XYF19 CAR-T cells)	Unknown	August 2019	China
8	NCT04438083	Active, not recruiting	Phase 1	Renal Cell Carcinoma	CD70	Unknown	Allogenic	107	CRISPR-Cas9-Engineered T Cells (CTX130)	Unknown	June 16, 2020	USA
9	NCT04502446	Recruiting	Phase 1	T or B cell malignancies	CD70	Unknown	Allogenic	45	Anti-CD70 CRISPR-Cas9-Engineered T Cell	Unknown	July 31, 2020	USA
10	NCT04557436	Active, not recruiting	Phase 1	B-ALL	CD19	CD52 and TRAC loci	Allogenic	10	CRISPR-CAR Genome Edited T Cells (PBLT52CAR19)	Unknown	August 12, 2020	UK

Table 4. Continued

Rank	Clinical Trial identifier	Status	Clinical trial phase	Disease	CAR Antigen	Target Locus of CRISPR	NK source	Number enrolled	Interventions	Dosage	Starting date	Location
11	NCT04767308	Not yet recruiting	Early phase 1	CD5+ Hematopoietic Malignancies	CD5	Unknown	Autologous	18	Anti-CD5 CAR-T Cells	1×10 ⁶ /kg 2×10 ⁶ /kg 3×10 ⁶ /kg	March 2021	Unknown
12	NCT04637763	Recruiting	Phase 1	B cell non-Hodgkin lymphoma	CD19	Unknown	Allogeneic	72	CRISPR-edited allogeneic CAR-T cell therapy targeting CD19	Unknown	May 26, 2021	USA
13	NCT04976218	Recruiting	Phase 1	EGFR positive solid tumors	EGFR	TGF-β receptor II	Unknown	30	TGFβR-KO CAR-EGFR T Cells	1-2×10 ⁵ /kg 1×10 ⁶ /kg 1×10 ⁷ /kg	March 15, 2022	China
14	NCT05397184	Recruiting	Phase 1	T Cell Malignancies	CD7	Unknown	Allogeneic	10	Cryopreserved BE CAR7 T cells	Unknown	April 19, 2022	UK
15	NCT05037669	Withdrawn	Phase 1	CD19+ Leukemia and Lymphoma	CD19	Endogenous TCR, HLA-class I and HLA-class II	allogeneic	0	CRISPR-edited T Cells (PACE) Gene Edited to Eliminate Endogenous TCR, HLA-class I and HLA-class II and Engineered to Express Anti-CD19 CAR	Unknown	July 2022	Unknown
16	NCT05722418	Recruiting	Phase 1	Multiple myeloma	BCMA	Unknown	allogeneic	50	CRISPR-Edited Allogeneic Anti-BCMA CAR-T Cell	Unknown	February 6, 2023	USA
17	NCT05643742	Recruiting	Phase 1/2	B-cell malignancies	CD19	Unknown	allogeneic	120	Anti-CD19 Allogeneic CRISPR-Cas9-Engineered T Cells (CTX112)	Unknown	March 10, 2023	USA
18	NCT05795595	Recruiting	Phase 1/2	Solid tumors	CD70	Unknown	allogeneic	250	Anti-CD70 Allogeneic CRISPR-Cas9-Engineered T Cells (CTX131) i	Unknown	April 2023	USA

A part of the data from the phase 1 clinical trial [NCT04557436] was published in 2022. In this experiment, the CRISPR-Cas9 technology was applied to improve the efficacy of CAR19 T cells in such a way that T-cell receptor α -chain CD52 antigen was disrupted in CAR19 T cells (TT52CAR19 T cells) through lentiviral vector carrying Cas9 and sgRNA. Following the lymphodepletion regimen, six children with relapsed/refractory CD19-positive B-ALL received a single dose of CAR T-cells (spanning 0.8×10^6 to 2.0×10^6 /kg). Although no immediate toxicity was seen, grade II cytokine release syndrome, skin GVHD, and transient grade IV neurotoxicity were observed in some patients. Generally, safety, feasibility, and therapeutic application of CRISPR-engineered immunotherapy have been confirmed.¹⁰⁸ Additionally, during a clinical trial performed by Guo et al, TCR and B2M double-removed CAR-T cells were created by lentiviral and electroporation delivery of the CRISPR-Cas9 editing tool. In this study, the lymphodepletion regimen was followed by allogeneic CAR T-cell administration in two patients with R/R diffuse large B-cell lymphoma at a dose of 1.5×10^6 /kg and 1.21×10^6 /kg cells for each subject. Despite disease progression, both patients showed expansion and biological acting without GVHD.¹⁰⁹ It is charming to point to the results of phase I clinical trial [NCT04035434], which examined the effect of AAV-mediated anti-CD19 CAR-T cells and CRISPR-Cas9-disrupted TRAC and B2m genes. Although long-term outcomes are still pending, interim findings in NHL patients showed a 38% remission rate with no GVHD. Only one case of severe neurotoxicity related to viral reactivation has been reported.¹¹⁰ A similar approach was used to test the effect of CRISPR-Cas9 mediated-anti-CD70 CAR incorporation into the TRAC locus with a second edit TRAC, B2m, and CD70 in 15 T-cell lymphoma volunteers. The preliminary results of this trial showed complete remission (29%), no detectable GVHD, and a tolerable toxicity profile.¹¹¹

Finally, regarding improvements in accuracy and efficiency, as well as the possibility of multiplex targeting of genes, antigens, and checkpoint molecules,¹¹² it is hoped that positive data from these clinical studies will revolutionize the treatment of a larger variety of malignancies and ameliorate their outcomes.

CAR-NK cell and CRISPR

Natural killer (NK) cells are fundamental components of the innate immune system developing in the bone marrow (BM) and secondary lymphoid tissues.¹¹³ They play antiviral and antitumor roles in multiple ways, like releasing lysing granules (perforin and granzyme), cytokine secretion, and antibody-dependent cell cytotoxicity (ADCC).¹¹⁴ NK cells have the innate ability to identify and eliminate transformed cells without prior sensitization, independent of the MHC class antigen presentation. Furthermore, NK cells can distinguish abnormal cells from normal cells through KIR receptors

and ligand mismatch, resulting in targeted cytotoxicity and reduced off-target complications.^{115,116}

Given their specified biological characteristics, strong inherent ability to combat cancers, and favorable history of safety in medical practice, NK cells have attracted considerable interest as a promising alternative framework for CAR engineering in the last few years. It is possible to obtain pure populations of NK cells from both autologous and allogeneic sources, such as peripheral blood, umbilical cord blood, different kinds of stem cells (induced pluripotent stem cells and hematopoietic stem cells), and NK cell lines such as NK-92.¹¹⁷ Nevertheless, genetic engineering of NK cells faces some limitations, such as a high rate of cell apoptosis, low efficiency of transduction, and loss of transgene expression after expansion.¹¹⁸ Toward cancer immunotherapy research, CRISPR has proven to be effective in the construction of CAR and TCR structures and the identification of novel immune checkpoint genes.¹¹³ Regarding the application of gene editing of NK cells, CRISPR has been utilized for optimizing NK cell metabolism, targeting checkpoint molecules, enhancing antibody therapies, and producing of CAR-NK cells.¹¹⁹

Immunotherapy using CAR-NK cells has the advantage of being performed in HLA-unmatched individuals. However, the injected NK cells are recognized and destroyed by the immune system of the recipient limiting their lifespan in the body and reducing the chance of success in repeated injections. Hoerster et al. utilized CRISPR-Cas9 to suppress HLA class I molecule expression by deleting the B2M gene in NK cells, and then genetically modifying the NK cells to express a single-chain HLA-E molecule. Their study showed that it is possible to access non-HLA-matched primary human NK cells as an “off-the-shelf” product with a high level of safety.¹²⁰ In a one-step CRISPR delivery through a retroviral particle (RP) system, Jo et al successfully knocked out the TIGIT gene and simultaneously generated anti-epidermal growth factor receptor (EGFR)-CAR NK cells. They generated large amounts of gene-edited CAR-NK cells that have great potential to integrate site-specific CAR transgenes.¹²¹ Another targeted gene integration into NK cells was achieved through an innovative electroporation delivery system of Cas9/RNP and ssAAV6 or scAAV6 gene combinations.¹¹⁸ By leveraging the precise targeting capability of CRISPR-Cas9 for the selective introduction of genetic materials into primary NK cells, Kararoudi et al generated numerous high-purity CD33-targeting CAR NK cells with significant antileukemic activity. Cas9 ribonucleoprotein complexes in combination with single-stranded (ss) or self-complementary (sc) adeno-associated virus (AAV)-mediated gene delivery have been used for site-directed gene editing of NK cells.¹²² Their theory was confirmed in a subsequent study, and the RNP/scAAV6 combination was introduced as a promising approach for clinical application, owing to the stable transduction of

NK cells.¹²³ In this direction, the cytokine-inducible Src homology 2-containing (CIS) protein, a critical negative regulator of interleukin 15 (IL-15) signaling, was targeted using CRISPR. Coalescence of this strategy and CAR-NK cells indicated enhanced efficacy of natural killer (NK) cells against tumors in comparison to each modification in a lymphoma mouse model.¹²⁴ An *in vitro* study tested the effect of disrupting three distinct inhibitory checkpoints (CBLB, NKG2A, and TIGIT) on the functionality of NK cells using the CRISPR-Cas9 technique in combination with CD276-CAR or CD19-CAR NK-92 cells in U-937 or U-937 CD19/tag AML cell lines. It was concluded that the cytotoxicity of triple-knockout CD276-CAR-NK-92 cells markedly became greater when targeting different AML cell lines.¹²⁵

Thus, the CRISPR-Cas9 technique has received much attention because of its ability to increase the antitumor capabilities of NK cells. This can be achieved by inserting CARs into NK cells as well as by permanently disabling or integrating specific genes related to NK cell exhaustion, activation, and tolerance functions.¹²⁶

CAR-M cells and CRISPR

Macrophages (M) are long-lived phagocytic cells that reside in nearly all organs and are vital components of the innate immune system. Macrophages exhibit two functional phenotypes. Classically activated macrophages (M1 type) mainly produce NO and lysosomes, enhance their ability to kill microorganisms, and act as pro-inflammatory cells. The alternatively activated macrophages (M2 type) are principally involved in the repair process.¹²⁷ A significant portion of the macrophage population within the tumor microenvironment is composed of the M2 phenotype, probably to restore the destructive effects of tumor cells.¹²⁸ In addition, there is a unique subpopulation of macrophages in solid tumors known as tumor-associated macrophages (TAMs). These TAMs facilitate metastasis, promote invasion and angiogenesis, and augment immunosuppression.¹²⁹ Due to the infiltration of solid tissues, genetically manipulated macrophages (CAR-M) could be more effective than CAR-T or CAR-NK cells in eliminating solid tumors. However, the pivotal role of macrophages in combating tumor cells depends on their pro-inflammatory state.¹³⁰

Although the macrophages have been used to combat cancer for many years, early clinical trials showed that transplanting purified macrophages had little antitumor effects, suggesting that additional pro-inflammatory signals are required to battle against tumors.^{131,132} Opposing signals impede macrophages from destroying targeted tumor cells.¹³³ Cancer cells use some mechanisms (such as self-labeling) to avoid being destroyed by macrophages. One of these significant tools is the "don't eat me" signal between CD47 and the macrophage membrane receptor SIRPα. Exploring these positive and negative signals has attracted scientific attention in CAR-M therapy by

manipulating specific gene(s) to augment the ability of macrophages to bind to tumor cells (via antigen identification) and activate their activity in tumor lysis by activating certain enzymes.^{134,135}

One era of the CAR-M studies is to augment immune cell infiltration into tumors via the extracellular matrix (ECM) by producing CAR-147 macrophages. CD147 plays a crucial role in ECM remodeling via the expression of matrix metalloproteinases (MMPs).¹³⁶ CAR-iPSC-induced macrophages (CAR-iMACs) containing CD86 and Fcγ domains result in M2 macrophages. However, the addition of IFN-γ can polarize the macrophages to M1 to enhance the phagocytic capacity of CAR-iMACs against solid tumors.¹³⁷

Although the efficacy and safety profile of CAR-M cells have been studied in laboratory animals, their effects in humans are still unknown, and the use of viral transfection in CAR gene transfer may result in unpredictable effects on treatment.¹³⁸ Zhang et al applied CRISPR-Cas9 technology to insert a third-generation anti-GD2 CAR (14G2a-CD28-OX40-CD3ζ) into the AAVS1 locus of H9 human pluripotent stem cells (hPSCs) for the development of antigen-dependent antitumor macrophages. Their findings indicated that the antitumor activity of CAR-Ms was not affected by the M1/M2 phenotype induced by LPS, IFNγ, and IL-4. Additionally, when hPSC-derived macrophages were exposed to tumor cells, CAR-M expression led to their polarization towards the M1 state. A complementary *in vivo* study showed that CAR-Ms produced from the arterial hemogenic endothelium (HE) have much greater antitumor activity than wild-type macrophages.¹³⁹

Focusing on the ACOD1 gene (as a potent regulator of the pro-inflammatory state), human ACOD1 knockout macrophages were developed using an induced pluripotent stem cell-derived CAR macrophage (CAR-iMAC) platform. The engineered iMACs presented a more robust and enduring polarization towards the pro-inflammatory state. Moreover, elevated production of reactive oxygen species (ROS) as well as enhanced phagocytic and cytotoxic activities targeting cancer cells *in vitro* were observed. Finally, by transplantation into ovarian and pancreatic cancer mouse models, ACOD1-depleted CAR iMACs showed an improved capacity to suppress tumor cells, leading to a longer lifespan in the treated mice.¹⁴⁰ Three clinical trials were approved by the FDA to assess the efficacy of CAR-M-based approaches in solid tumors until the end of June 2023. One of these trials encompasses a cohort investigation aimed at ascertaining the antitumor efficacy of fresh CAR-macrophages in organoids derived from patients with breast cancer [NCT05007379]. The other trial used CAR-macrophages for the management of solid tumors, overexpressing HER2 [NCT04660929]. In the third trial, a phase 1 study of intra-peritoneal MCY-M11 (Mesothelin-targeting CAR) in peritoneal mesothelioma and ovarian cancer treatment was designed

[NCT03608618].

From the available evidence, we can infer that the clinical translation of CAR-M cells necessitates a reliable source of expansion that can be obtained through the use of PBMCs, HSCs, or iPSCs with the assurance of safety and efficacy.¹⁴¹ The iPSC origin may be an attractive source because of its ability to reproduce indefinitely and transform into almost any differentiated cell type. It is worth noticing that most CAR-M gene transfers have been via viral transfection that may lead to the occurrence of insertion mutations and have unforeseeable implications for therapeutic applications. Utilization of the safe and effective CRISPR-Cas9 technique in gene editing facilitates the refinement of M cell-based treatments, particularly by producing a universal "off-the-shelf" cellular product and redirecting effector cells to overcome resistance in various neoplasms, spanning from hematological to solid tumors.¹⁴²

Challenges of CRISPR-Cas9

Despite being effective in the treatment of different types of cancers, such as any genetic manipulation, the CRISPR/Cas9 system has some limitations. CRISPR/Cas9 systems suffer from the main limitation of having potential off-target effects. Therefore, the progress of CRISPR-Cas9-based cancer therapeutic strategies has been impeded due to their probability of causing cancer. Alternatively, the delivery systems, including viral and bacteriophage-derived vectors, have the potential to result in gene and cell toxicity (discussed in the CRISPR-Cas9 delivery section).^{143,144} These drawbacks can be hindered by the high precision of the 5' end sequence of sgRNA.¹⁴⁵ The modification of Cas9 activity is beneficial to decrease off-target effects, as the higher the Cas9 activity, the greater the number of off-targets due to non-specific cleavage. Generally, on-target effects can be provided by some parameters such as gRNA design (decrease the length of gRNAs to 20 bases), Cas9 structure (changing the polarity of Cas9's domains and usage protein form of Cas9), gRNA/Cas9 ratio, and target site originality.^{145,146}

Ethical concern

Due to promising advantages, such as easy handling, high accuracy, and affordability, CRISPR-Cas9 technology made a revolution in the genetic engineering era. Even though it has jumped rapidly into different fields of human diseases, including cancer therapy, several unsolved questions regarding its clinical applications and bioethical issues remain. Most concerns are related to its ability to genetically modify human germ line cells and embryos, which may create undesirable genetic alterations in the treated patient or influence the fate of future reproduction. Although scientists agree with the utilization of CRISPR-Cas9 for providing models of human diseases and comprehending the cellular and molecular processes of disease, its use for eugenics or enhancement purposes should be forbidden. Therefore, the scientific community

Review Highlights

What is the current knowledge?

- ✓ Advancements in conducting clinical trials for CRISPR-based cancer therapies.
- ✓ Therapeutic potentials of CRISPR-Cas9 system in treating solid tumors and leukemia.

What is new here?

- ✓ CRISPR/Cas9 revealed the mechanisms of oncogenes and tumor suppressor genes involved in cancer pathogenesis.
- ✓ Optimistic use of CRISPR-Cas9 in production of effective CAR-M, CAR-NK, CAR-T cells in cancer therapy.

and bioethical, social, legal, and governmental parties should be comprehensively informed of the advantages and disadvantages of this technology in clinical applications.

Conclusion

Tumor cells undergo various genetic alterations affecting proto-oncogenes, tumor-suppressing genes, DNA repair genes, and genes involved in apoptosis and cell survival. CRISPR-Cas9 technology has revolutionized the detection and treatment of genetic disorders as well as cancers. This technique showed efficient and precise editing/replacing of individual genes and reconstruction of targets in different types of cancer cells and significantly suppressed the activity of different oncogenes, leading to reduced tumor growth. The use of the CRISPR-Cas9 tool exhibited promises in the treatment of different solid tumors by effectively targeting oncogenes and tumor-suppressor genes in cancer therapies, including those associated with breast, lung, liver, prostate, and colon cancers. The application of CRISPR-Cas9 for generating feasible, effective, and safe CAR-T cells, CAR-NK cells, and CAR-M cells has already progressed to the clinical phase. While over a dozen of in vivo studies have been conducted to treat solid and hematologic cancers by using this technique, transition from the laboratory to the bedside still require further clinical trials.

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Competing Interest

The authors declare no conflict of interest.

Ethical Statement

Authors declare no ethical issues.

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