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REVIEW

CRISPR in Targeted Therapy and Adoptive T Cell Immunotherapy for Hepatocellular Carcinoma

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Abstract: Despite recent therapeutic advancements, outcomes for advanced hepatocellular carcinoma (HCC) remain unsatisfactory, highlighting the need for novel treatments. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene-editing technology offers innovative treatment approaches, involving genetic manipulation of either cancer cells or adoptive T cells to combat HCC. This review comprehensively assesses the applications of CRISPR systems in HCC treatment, focusing on in vivo targeting of cancer cells and the development of chimeric antigen receptor (CAR) T cells and T cell receptor (TCR)-engineered T cells. We explore potential synergies between CRISPR-based cancer therapeutics and existing treatment options, discussing ongoing clinical trials and the role of CRISPR technology in improving HCC treatment outcomes with advanced safety measures. In summary, this review provides insights into the promising prospects and current challenges of using CRISPR technology in HCC treatment, with the ultimate goal of improving patient outcomes and revolutionizing the landscape of HCC therapeutics.

Keywords: CRISPR, hepatocellular carcinoma, HCC, targeted cancer therapy, adoptive T cell immunotherapy, CAR T cell therapy

Introduction

Hepatocellular carcinoma (HCC) constitutes approximately 90% of primary liver cancers, a major global health concern, ranking as the third leading cause of cancer-related mortality worldwide.[1] For early-stage HCC, therapeutic options include surgical resection, ablation, and liver transplantation, while transarterial chemoembolization (TACE) or transarterial radioembolization (TARE) is preferred for intermediate-stage cases.[2] However, unresectable disease affects a substantial portion of patients, with recurrence impacting up to 70% of those who have undergone tumor resection or ablation within 5 years.[3–5] Consequently, over 50% of HCC patients ultimately require systemic therapies, typically during the advanced disease stages.[4,6]

HCC is resistant to conventional chemotherapy, leading to the use of targeted therapies such as sorafenib or lenvatinib (tyrosine kinase inhibitors, TKIs) as first-line treatments. Second-line therapy options include regorafenib (TKI), cabozantinib (TKI), or ramucirumab (anti-VEGFR2).[6,7] Notably, immunotherapy approaches utilizing immune checkpoint inhibitors (ICIs) have shown potent antitumor activity in recent studies.[8] Approved immunotherapy regimens now include first-line options like atezolizumab + bevacizumab (anti-PD-L1 + anti-VEGFA) or durvalumab + tremelimumab (anti-PD-L1 + anti-CTLA-4). For advanced HCC in the second-line setting, treatments consist of pembrolizumab (anti-PD-1) monotherapy or the combination of nivolumab + ipilimumab (anti-PD-1 + anti-CTLA-4). Additionally, numerous Phase III clinical trials are currently investigating novel agents and combination therapies.[6–9]

Despite these notable advancements, outcomes for advanced HCC patients remain suboptimal. For instance, atezolizumab + bevacizumab yields an objective response rate (ORR) of 27% and a median overall survival of 19.2 months, while the novel combination of camrelizumab (anti-PD-1) and rivoceranib (apatinib, a TKI) shows an ORR of 34% and a median overall survival of 22.1 months.[10,11] Thus, the exploration of novel targeted therapies and immunotherapeutic approaches remains crucial for further improving the effectiveness of treatment.

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CRISPR-Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins) systems serve as RNA-guided adaptive immune mechanisms utilized by prokaryotes to defend against invading nucleic acids like bacteriophages.[12] The effector complex of a CRISPR system comprises one or more Cas proteins and a CRISPR-RNA (crRNA) that guides the effector complex to find and cleave a specific DNA or RNA sequence through base pairing.[13] As a bioengineering tool, CRISPR effector complexes can be easily and inexpensively reprogrammed to target different DNA or RNA sequences by simply modifying the crRNA. Therefore, CRISPR-based tools have been widely adopted in biomedical research for various applications, including disease modeling,[14] diagnostics,[15] drug discovery,[16] and gene therapy.[17,18] Notably, CRISPR systems have been harnessed in the fields of cancer research, diagnosis, and therapy.[19–21] In early clinical trials, CRISPR technology has shown significant promise in the development of chimeric antigen receptor (CAR) T cells and T cell receptor (TCR)-engineered T cells for the treatment of various cancer types.[22–25]

Despite recent advancements in HCC treatment, current therapies often fail to provide durable remission and are limited by toxicity and the development of resistance.[6] The ability of CRISPR to enable precise genetic modifications, particularly through its multiplex targeting capacity, presents an opportunity to reduce the likelihood of resistance, potentially leading to more effective and durable therapeutic strategies either as a potent standalone intervention or in combination with existing treatments. In this review, we focus on the application of CRISPR systems in the management of HCC. We describe the applications of CRISPR systems in targeted cancer therapy and adoptive T cell immunotherapy approaches, including CAR T cells, TCR T cells, tumor-infiltrating lymphocytes (TILs), and cytokine-induced killer (CIK) cells for HCC treatment. We also discuss the potential of combining CRISPR-based therapeutics with current treatment options. Finally, we mention ongoing clinical trials and discuss the promise, potential applications, and challenges of CRISPR systems in HCC treatment, emphasizing their potential for clinical translation and enhanced therapeutic outcomes.

Outline of CRISPR-Based Gene Editing Technologies

Precisely editing human cell DNA is a significant advancement in understanding disease mechanisms and developing new therapies. Early genome editing methods, like meganucleases, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), relied on protein-DNA interactions. Therefore, adapting these methods for different DNA sequences was labor-intensive and time-consuming.[13] CRISPR revolutionized genome editing by using Cas9 nuclease guided by a programmable RNA for precise DNA cleavage, both in vitro and within human cells.[26–28] Various CRISPR tools have since emerged and are now widely used in vivo for therapeutic applications.[29,30]

CRISPR nucleases induce a double-strand break (DSB) at the target DNA site guided by a ~20-nt portion of the crRNA. This target DNA must be adjacent to a protospacer adjacent motif (PAM), typically NGG for commonly used Cas9 from *Streptococcus pyogenes* (SpCas9). In mammalian cells, DSBs are typically repaired through non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ), resulting in insertion/deletion (indel) mutations at the target DNA site. CRISPR nucleases like Cas9 or Cas12 can disrupt knockout protein-coding genes by introducing frameshift mutations or disrupt non-coding regulatory sequences within the genome (Figure 1A). Homology-directed repair (HDR) is another repair pathway requiring a DNA template, predominantly active in dividing cells and less efficient than error-prone pathways. CRISPR nucleases can use HDR with an external DNA template flanked by homology regions to introduce desired edits in actively dividing cells (Figure 1A).[31] To enhance specificity and minimize off-target effects, the double nickase strategy employs two Cas9 nickases (nCas9, an engineered Cas9 that cleaves a single DNA strand that each nick one DNA strand), enabling a controlled double-strand break with higher precision and reduced unintended modifications (Figure 1B). In addition, a catalytically inactive Cas9 (dCas9) fused with transcriptional activator or repressor domains can regulate gene expression transiently, resulting in gene activation (CRISPRa) or inhibition (CRISPRi) (Figure 1C). Combining dCas9 with a transcriptional repressor and DNA methyltransferases enables long-term gene silencing across multiple cell divisions, referred to as the CRISPRoff system (Figure 1D).[32] Using dCas9 fused with a DNA demethylase domain, along with gRNA-guided recruitment of transcriptional activators can have the opposite effect, known as CRISPRon.

Base editors comprise a nCas9, a dCas9, or a dCas12 protein combined with either cytidine deaminase and a uracil glycosylase inhibitor (cytosine base editors, CBEs) or adenosine deaminase domains (adenine base editors, ABEs).[33] When Cas9 or Cas12 binds to the target DNA site and the crRNA hybridizes with the targeted DNA strand, it forms an

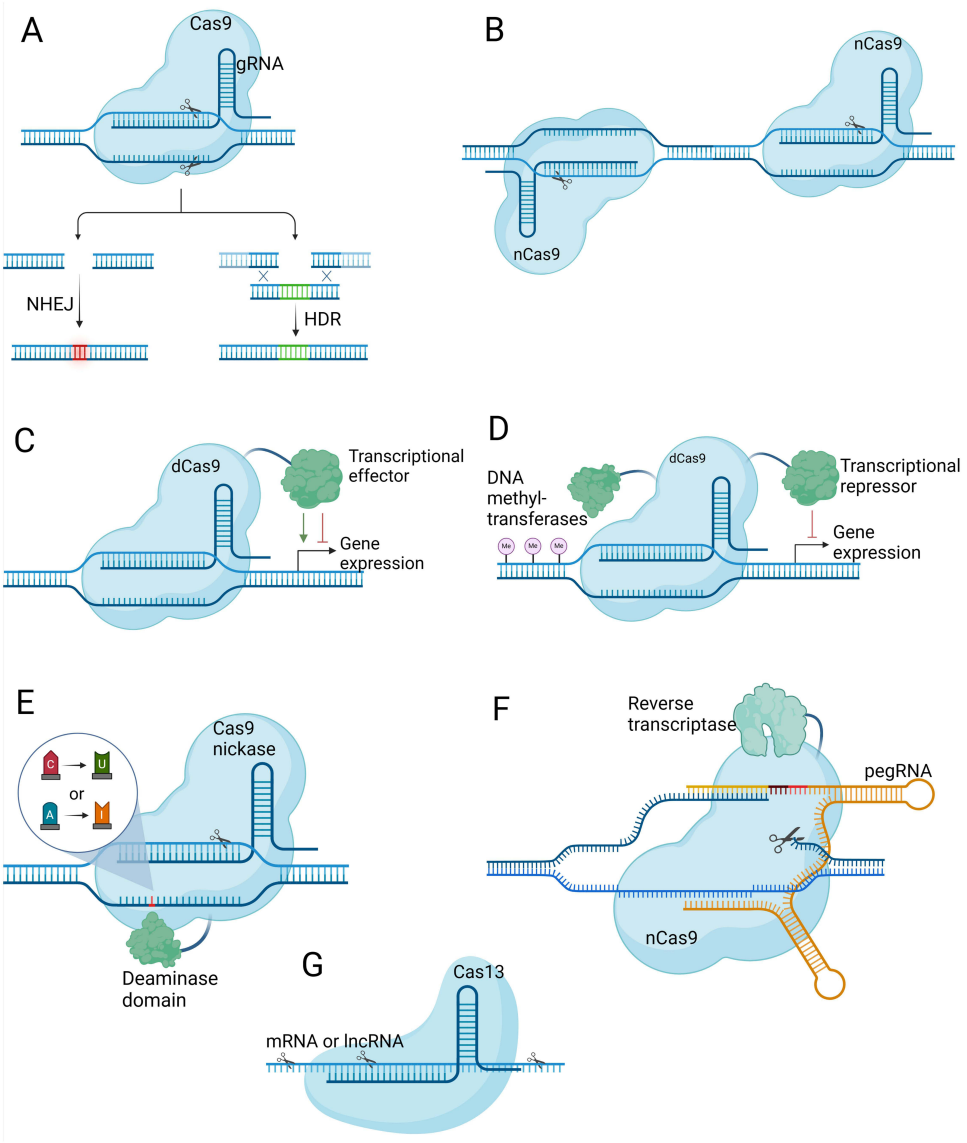

Figure 1 Overview of CRISPR-based gene editing technologies. Created with BioRender.com. (**A**) CRISPR-mediated approaches for gene disruption (NHEJ) and precise editing (HDR). (**B**) Double nickase strategy employing two Cas9 nickases for controlled and precise DSBs, minimizing off-target effects. (**C**) CRISPRa and CRISPRi systems for transient regulation of gene expression using deactivated Cas9 and transcription activator/inhibitor domains. (**D**) CRISPRoff system for durable gene repression by combining deactivated Cas9 with DNA methyltransferase domains and a transcriptional repressor domain. (**E**) Cytosine and adenine base editing systems that alter DNA bases without inducing double-strand breaks. (**F**) Prime editing method enabling precise installation of single base mutations and small indels using a Cas9 nickase and reverse transcriptase. (**G**) RNA-targeting Cas13 system for the manipulation of RNA molecules in cells.

R-loop, exposing the complementary DNA strand as single-stranded DNA (ssDNA). Within a small region of this exposed DNA strand, cytidine deaminase or adenosine deaminase catalyzes the deamination of C or A, resulting in the creation of U or I bases, respectively (Figure 1E). Subsequently, the U or I base is repaired to T or G using the cellular DNA repair machinery. Therefore, CBEs and ABEs perform C to T and A to G base editing. Importantly, CBEs can also generate premature stop codons in coding genes. For example, CGA, CAG, and CAA codons can be converted to TGA, TAG, and TAA stop codons.[22,34] This capability allows nuclease-free knockout of coding genes, avoiding undesired effects associated with DSB formation using Cas9 nuclease.[35] While base editing has facilitated the creation of transition mutations, the scope of the technology has expanded considerably with the advent of the CRISPR prime editing method, which enables the generation of any transition or transversion base conversions, small insertions and deletions, and combinations thereof. This advancement was achieved through the fusion of a Cas9 nickase with a reverse transcriptase

(RT) domain, giving rise to prime editors capable of precisely introducing all feasible point mutations and small insertions and deletions (Figure 1F).[36]

In addition to genomic targeting, CRISPR effectors like Cas13 can efficiently target RNA within human cells. Cas13 enzymes, guided by sequences of around 20–30 nucleotides in length, can locate and cleave various target RNAs, including mRNAs, long non-coding RNAs (lncRNAs), and circular RNAs, enabling effective gene knockdown (Figure 1G). RNA base editors have also been developed by fusing a catalytically inactive Cas13 with adenosine deaminase or cytidine deaminase domains. This enables the conversion of A to I (interpreted as G in translation) and C to U base editing.[33]

CRISPR-Based Targeted Therapies for HCC

Targeting HCC with CRISPR Demonstrates Antitumor Effects in vitro and in vivo

Numerous preclinical investigations utilizing HCC models have unveiled the potential of CRISPR-based strategies in eliciting various anti-cancer effects (Table 1).[37–79] In vitro studies have revealed compelling outcomes, including the inhibition of HCC cell proliferation,[73,74] suppression of invasion,[70,76] reduced migration,[71,72] and the induction of apoptosis.[75,78] Further demonstrating the adaptability of these methods, researchers have effectively employed dCas9, targeted at promoter regions to act as a transcriptional repressor by blocking the binding of RNA polymerase. This approach has been utilized to diminish the expression of the long non-coding RNA (lncRNA) *SNHG9* that promotes HCC development, thereby achieving significant reductions in cell proliferation, migration, and invasion.[72]

Furthermore, in vivo assessments have provided substantial evidence of CRISPR's therapeutic efficacy, with studies reporting inhibited tumor growth,[37–39] diminished angiogenesis,[41,65] reduced tumor-initiating capacity,[62,63] and suppressed metastasis.[41,56] Moreover, these interventions have been associated with increased survival rates among HCC-bearing mice.[41,47] A noteworthy study involved the use of hepatic stellate cell-derived exosomes as carriers to deliver Cas9-gRNA RNP complexes, which are predominantly distributed in the liver, particularly in hepatocytes. In an orthotopic HCC model, this method, utilizing KAT5-targeting gRNA, effectively reduced tumor volumes and significantly improved the survival of tumor-bearing mice.[47]

Combining CRISPR with Existing Treatment Options Improves Antitumor Effects

The integration of CRISPR-based approaches with conventional treatment modalities has emerged as a promising strategy for enhancing therapeutic outcomes in HCC models. CRISPR-based cancer-targeting methods exhibited synergistic effects when combined with established treatments like sorafenib or TACE, significantly improving treatment efficacy. In this regard, Qi et al devised a delivery system based on a lactose-derived biopolymer with a high affinity for asialoglycoprotein receptors, which are abundant on the surface of HCC cells. Nanoparticles loaded with sorafenib and/or plasmids encoding Cas9 and gRNA targeting *Survivin* (pCas9-gRNA-*Survivin*) were intravenously injected. In an orthotopic HCC model, the combination of CRISPR and sorafenib therapy exhibited superior antitumor effects compared to either CRISPR or sorafenib monotherapy.[37] Similarly, Nie et al employed nanocomplexes to deliver sorafenib and pCas9-gRNA-*Survivin* to HCC in an orthotopic tumor model. Upon IV injection of these nanocomplexes, the CRISPR and sorafenib combination therapy yielded the most potent antitumor effect, resulting in a significant reduction in tumor volume.[38] He et al utilized extracellular vesicles coated with antibodies specifically targeting glypican-3 (GPC3), a protein abundantly expressed on the surface of HCC cells. These vesicles delivered the Cas9 protein along with a dual gRNA expression plasmid designed to target two genes, *IQGAP1* and *FOXM1*. When combined with intraperitoneal sorafenib treatment, IV injection of extracellular vesicles led to a substantial decrease in the volume of xenograft tumors.[45] Another innovative approach involved hollow mesoporous silica nanoparticles coated with tumor-targeting DNA aptamers, specifically binding to epithelial cell adhesion markers (EpCAMs) on HCC cell surfaces.[40] These nanoparticles were utilized for the co-delivery of sorafenib along with a CRISPR plasmid targeting *EGFR*. Systemic administration of aptamer-coated nanoparticles carrying both sorafenib and CRISPR exhibited superior antitumor effects compared to other treatment groups in xenograft tumor-bearing mice.[40] Lu et al established orthotopic liver tumors utilizing HCC cells with miR-23a-3p knockout achieved using CRISPR. When combined with sorafenib treatment, their study demonstrated a significant reduction in tumor growth accompanied by an increase in apoptosis.[64]

Table 1 The Summary of Studies Using CRISPR Tools to Target HCC

CRISPR Method	Target Gene	HCC Model	Delivery route and method	Effects of CRISPR	Ref.
Cas9 nuclease	*Survivin*	Orthotopic liver tumor	IV injection of lactose-derived branched cationic biopolymer carrying pCas9-gRNA and sorafenib	CRISPR efficiently inhibited tumor growth. A synergistic effect was observed for the CRISPR + sorafenib combination.	[37]
Cas9 nuclease	*Survivin*	Orthotopic liver tumor	IV injection of nanocomplexes with cationic shell and heparin nanoparticle core carrying the pCas9-gRNA and sorafenib	Tumor growth substantially decreased. A synergistic effect was observed for the CRISPR + sorafenib combination.	[38]
Cas13a, RNA-targeting	*TERT* *EZH2* *RelA*	Subcutaneous xenograft	IV injection of AAVs coding for Cas13 and crRNAs. The Cas13 was expressed from a tumor-specific promoter.	The apoptosis rate of tumor cells increased, and cell growth decreased significantly. Cas13a expression was shown to be specific for the tumor tissues. The weight, volume, and markers of tumors decreased significantly.	[39]
Cas9 nuclease	*EGFR*	Subcutaneous xenograft	IV injection of aptamer-coated hollow mesoporous silica nanoparticles enabled the co-delivery of pCas9-gRNA and sorafenib	CRISPR efficiently inhibited tumor growth. A synergistic effect was observed for the CRISPR + sorafenib combination.	[40]
Cas9 nuclease	*HIF-1α*	Subcutaneous xenograft and orthotopic liver tumor	In the xenograft model, intratumoral injection of lentivirus coding Cas9 and gRNA was performed. Orthotopic tumor-bearing mice were generated using lentivirally injected HCC cells.	In the xenograft model, CRISPR caused a substantial decrease in *HIF-1α* in tumor cells. In the orthotopic tumor model, a synergistic effect was observed with the combination of CRISPR + hepatic artery ligation, which mimics transarterial chemoembolization (TACE). The combination therapy reduced tumor growth and angiogenesis, induced apoptosis, and significantly prolonged the survival of HCC-bearing mice.	[41]
Cas9 nuclease	*VEGFR2*	Xenograft tumor	IV injection of pH-responsive chitosan-based nanoparticles enabled the co-delivery of pCas9-gRNA and paclitaxel	CRISPR efficiently inhibited tumor growth. A synergistic effect was observed for the CRISPR + paclitaxel combination, which provided the best antitumor effect among all groups.	[42]
Cas9 nuclease	*WNT10B*	Subcutaneous xenograft; tumor organoids are also used ex vivo	IV injection of aptamer-coated extracellular vesicles carrying Cas9-gRNA RNP complexes	CRISPR halted tumor development in vivo, and widespread apoptosis was observed in tumor organoids.	[43]
Cas9 nuclease	*NFE2L2*	Subcutaneous xenograft	IV injection of lipid nanoparticles carrying Cas9-gRNA RNP complexes and hematoporphyrin monomethyl ether (HMME)	Ultrasound-controlled nanoparticles decreased tumor growth substantially. A synergistic effect was observed for the CRISPR + sonodynamic therapy combination.	[44]
Cas9 nuclease	*IQGAP1*, *FOXM1*	Subcutaneous xenograft	IV injection of anti-GLP3 antibody-coated extracellular vesicles containing Cas9 protein and dual gRNA expression plasmid	CRISPR efficiently inhibited tumor growth. A synergistic antitumor effect was observed for the CRISPR + sorafenib combination, which was given intraperitoneally.	[45]
Cas9 nuclease	*IQGAP1*	Subcutaneous xenograft	Intratumoral injection of extracellular vesicles containing Cas9 protein and gRNA expression plasmid	Tumor growth was inhibited in CRISPR-treated xenograft models. A synergistic effect was observed for CRISPR + sorafenib combination in vitro.	[46]
Cas9 nuclease	*KAT5*	Orthotopic liver tumor	IV injection of exosomes carrying Cas9-gRNA RNP complex	CRISPR-treated mice had smaller tumors and significantly prolonged survival.	[47]

(*Continued*)

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Table 1 (Continued).

CRISPR Method	Target Gene	HCC Model	Delivery route and method	Effects of CRISPR	Ref.
Cas9 nuclease	*PLK1*	Subcutaneous xenograft	Intratumoral injection of lipid nanoparticles carrying pCas9-gRNA	CRISPR efficiently induced apoptosis and inhibited tumor growth.	[48]
Cas9 nuclease	*ADSL*	Mice with IV–injected tumor-initiating vectors for c-Myc or CTNNB1-N90 overexpression, or *TP53* knockout	Mice were also IV–injected with CRISPR plasmid targeting *ADSL*.	While multiple liver tumors were observed in the control group, only microscopic tumor nodules developed in the liver of *ADSL*-targeted mice.	[49]
Cas9 nuclease	*NSUN2*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumors were smaller for the *NSUN2*-deficient group than the control groups.	[50]
Cas9 nuclease	Enhancer of *C/EBPβ*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumor growth was significantly lower in both homozygous and heterozygous *C/EBPβ* enhancer mutants.	[51]
Cas9 nuclease	*CXCR4*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Knockout of CXCR4 resulted in increased sensitivity of cancer cells to cisplatin in vitro. Tumors were smaller for the *CXCR4*-deficient group than for the control group.	[52]
Cas9 nuclease	*NSD1*	Subcutaneous xenograft developed from CRISPR-treated cells	—	In vitro studies showed suppressed cell proliferation, migration, and invasion. Tumor weight and volume were significantly lower in those generated from *NSD1* knockout HCC cells.	[53]
Cas9 nuclease	*TRRAP, KAT5*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumor weight and volume were significantly lower in those generated from *TTRAP* or *KAT5* knockout HCC cells.	[54]
Cas9 nuclease	*PTPMT1*	Orthotopic liver tumor developed from CRISPR-treated cells	—	Tumors were smaller and less pathologically aggressive for *PTPMT1*-knockout groups. Lung metastasis was also reduced.	[55]
Cas9 nuclease	*G9a*	Orthotopic liver tumor developed from CRISPR-treated cells	—	Tumor development and lung metastasis were substantially lower for orthotopic tumors derived from *G9a*-knockout HCC cells compared to the control group.	[56]
Cas9 nuclease	*SEPT11*	Mice with IV–injected CRISPR-treated cells	—	Tumor metastatic nodules were not observed in the lungs of mice injected with *SEPT11*-knockout HCC cells. The group also had reduced angiogenesis in histology.	[57]
Cas9 nuclease	*SQSTM1*	Mice with IV–injected CRISPR-treated cells	—	In mice with CRISPR-treated HCC cells, no metastatic lesions were seen in the liver, and tumor cells were much lower in the lung.	[58]
Cas9 nuclease	*CDK5*	Mice with IV–injected CRISPR-treated cells	—	Cdk5 knockout by CRISPR impaired HCC cell dissemination in vivo.	[59]

Cas9 nuclease	*LMNA*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumors developed from *LMNA*-knockout HCC cells had lower tumor weight and volume.	[60]
Cas9 nuclease	*ZNF384*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumors developed from *ZFN384*-knockout HCC cells were smaller than those of the control group.	[61]
Cas9 nuclease	*GLS1*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumors developed from *GLS1*-knockout HCC cells had smaller volumes and reduced tumor-initiating capacity.	[62]
Cas9 nuclease	*ZIC2*, *BPTF*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumors developed from *ZIC2*-knockout HCC cells had smaller volumes and reduced tumor-initiating capacity. Knockout of *BPTF* impaired sphere formation in vitro.	[63]
Cas9 nuclease	miR-23a-3p	Orthotopic liver tumor developed from CRISPR-treated cells	—	Tumors developed from *miR-23a-3p*-knockout HCC cells were significantly lower when combined with sorafenib therapy.	[64]
Cas9 nuclease	*SOX4*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumors developed from *SOX4*-knockout HCC cells had lower growth rates, improved PET results, and reduced extracellular matrix support and angiogenesis.	[65]
Cas9 nuclease	*BOLA2*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumor development was halted when *BOLA2*-knockout cells were used. Also, intracellular iron levels were lower in these tumors.	[66]
Cas9 nuclease	*FAM122A*	Subcutaneous xenograft developed from CRISPR-treated cells	—	Tumors developed from *FAM122A*-knockout HCC cells had reduced cell proliferation and tumor growth.	[67]
Cas9 nuclease	Loci with cancer-specific mutations	Tumor organoids transfected with CRISPR-encoded lentivirus	—	Organoid number was substantially reduced when cell-line specific mutations were targeted via CRISPR, co-treated with DNA repair inhibitors.	[68]
Cas9 nuclease	*ASPH*	—	—	Knockout of *ASPH* significantly reduced cell proliferation and colony formation, and induced tumor cell senescence.	[69]
dCas9-KRAB transcriptional repressor	*GRN*	—	—	Silencing of *GRN* reduced cell proliferation, invasion, and tumor sphere formation abilities of HCC cells.	[70]
dCas9-VPR transcriptional activator	*HHIP*, *MT1M*, *PZP*, and *TTC36*	—	—	Epigenetic activation of tumor suppressor genes decreased cell proliferation, viability, and migration ability of HCC cells.	[71]

(*Continued*)

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Table 1 (Continued).

CRISPR Method	Target Gene	HCC Model	Delivery route and method	Effects of CRISPR	Ref.
dCas9 transcriptional repressor	lncRNA *SNHG9*	—	—	Knockdown of *SNHG9* inhibited the proliferation, migration, and invasion of HCC cells, and caused cell cycle arrest.	[72]
Cas9 nuclease	*GPC1*	—	—	The proliferation of HCC cells was significantly attenuated.	[73]
Cas9 nuclease	*PHGDH*	—	—	Knockout of *PHGDH* significantly suppressed HCC cell proliferation in the presence of sorafenib.	[74]
Cas9 nuclease	*TXNDC9*	—	—	Cell proliferation inhibition, cell cycle arrest, and induction of apoptosis were observed in *TXNDC9*-knockout HCC cells.	[75]
Cas9 nuclease	*MDM2*	—	—	Knockout of *MDM2* reduced HCC cell growth and invasion.	[76]
Cas9 nuclease	*EEF2*	—	—	Knockout of *EEF2* reduced HCC cell proliferation and altered the cell morphology.	[77]
Cas9 nuclease	*HGF*	—	—	Knockout of *HGF* decreased HCC cell proliferation, invasion, and migration while inducing apoptosis.	[78]
Cas9 nuclease	*NCOA5*	—	—	CRISPR inhibited proliferation, tumor microsphere formation, and migration abilities of HCC cells by suppressing epithelial to mesenchymal transition.	[79]

While the current treatment strategy for intermediate-stage HCC includes chemoembolization, ongoing clinical trials actively explore avenues to enhance patient survival through the integration of TACE with TKIs or immune checkpoint inhibitors.[8] In their study, Liu et al demonstrated the efficacy of combining CRISPR with hepatic artery ligation (HAL), which mimics TACE, in orthotopic tumor-bearing mice. This innovative approach resulted in a substantial reduction in liver tumors developed from *HIF-1α* knockout cells, leading to improved survival rates in mice receiving the combination therapy compared to those treated with HAL alone. Similar synergistic effects have also been observed in studies combining CRISPR with paclitaxel or cisplatin.[42,52] These cumulative findings underscore the potential of combining CRISPR with existing treatment modalities to achieve enhanced antitumor efficacy and ultimately improve patient survival.

Various CRISPR Tools Can Be Used to Target HCC

While the majority of studies employ CRISPR tools for targeted gene knockout, diverse CRISPR approaches have demonstrated effectiveness in targeting HCC. These versatile CRISPR technologies offer precise interventions for tailored HCC treatment. For instance, in one study, the RNA-targeting CRISPR-Cas13 system enabled multiplex knockdown of *TERT, EZH2*, and *RelA* mRNAs in HCC cells (Figure 1G), leading to reduced xenograft tumor growth upon IV injection of adeno-associated virus (AAV) carrying Cas13 and crRNAs.[39] Furthermore, CRISPR-based transcriptional activators and repressors were applied to target HCC in vitro (Figure 1C and D). Targeting the *Granulin (GRN)* promoter with dCas9-DNMT3A (for DNA methylation), dCas9-EZH2 (for histone 3 lysine 27 methylation), or dCas9-KRAB (for transcriptional repression) resulted in a substantial reduction in *GRN* mRNA levels in HCC cells.[70] It was observed that These CRISPR-based epigenetic modulators induced *de novo* CpG DNA methylation in the *GRN* promoter and histone modifications, leading to gene suppression and subsequently decreased cell proliferation, invasion, and tumor sphere formation ability. Notably, dCas9-KRAB exhibited the most potent antitumor efficiency among the tested epigenetic suppressors.[70]

The epigenetic silencing of tumor suppressor genes plays a pivotal role in the development of HCC. Sgro et al leveraged the CRISPR activation (CRISPRa) system, which involves dCas9 fused with transcriptional activation domains VP64, p65, and Rta, in combination with MS2 aptamer-containing sgRNA. This combination recruits multiple MS2 coat proteins fused with p65 and HSF1 activator domains to reactivate tumor suppressor genes.[71] Targeting *HHIP, MT1M, PZP*, and *TTC36*, genes that are significantly downregulated in HCC, the CRISPRa technology enabled highly specific and potent reactivation of these tumor suppressors, surpassing the effectiveness of epigenetic-modifying drugs. As a result, this approach led to a substantial reduction in HCC cell proliferation, viability, and migration.[71] These findings underscore the versatility of various CRISPR-based approaches in effectively targeting HCC and hold promise for innovative therapeutic interventions in the field of liver cancer research and treatment.

Reprogrammability and Multiplex Targeting Capacity of CRISPR Enables Personalized and More Potent Cancer Targeting

Utilizing multiple gRNAs, CRISPR systems have the capability to simultaneously target several DNAs or RNAs, potentially leading to more robust antitumor effects and reducing the likelihood of resistance. In HCC cells, the multiplex targeting of *TERT, EZH2*, and *RelA* mRNAs using Cas13 resulted in a higher rate of apoptotic cells compared to single mRNA targeting.[39] Additionally, the reprogrammability and multiplexing capabilities inherent to CRISPR technology open the door to personalized treatment strategies for HCC. In this context, Jiang et al introduced an innovative CRISPR-based personalized cancer therapy approach. Customized gRNAs were designed to target unique mutations present in cancer cells, identified through DNA sequencing.[68] Whole-genome sequencing performed on an HCC cell line defined single nucleotide variations and indels. Multiplex CRISPR targeting, combined with DNA repair inhibitors, effectively suppressed cell proliferation. Notably, the highest rate of apoptosis was observed when CRISPR targeted 8 loci, as opposed to targeting only 4 loci. Another group utilized multiplex CRISPRa to reactivate several epigenetically silenced tumor suppressor genes within HCC cells, achieving potent antitumor effects.[71] These findings underscore the potential

of CRISPR-based multiplexed and tailored targeting for personalized therapeutic strategies aimed at maximizing antitumor efficacy and minimizing tumor escape.

Tumor-Specific Delivery, Expression, and Activation Methods Enable More Effective and Specific Targeting of HCC

Ensuring the precision and safety of CRISPR applications in vivo is crucial. Tumor-specific delivery methods play a pivotal role in achieving efficient targeting of CRISPR tools to the tumor microenvironment, thereby enhancing antitumor activity, elevating local concentrations of CRISPR components, and ensuring the safety of genome editing. In this regard, Zhang et al harnessed EpCAM-targeting aptamer-coated silica nanoparticles loaded with sorafenib and an *EGFR*-targeting CRISPR plasmid to specifically target HCC cells in vivo. It was shown that the nanoparticles predominantly accumulated in tumor tissue with significantly higher concentrations compared to control groups, contributing to the safety and efficacy of the treatment.[40] Similarly, Zhuang et al employed extracellular vesicles coated with the TLS11a DNA aptamer, which has a specific affinity for HCC cells,[80] to deliver Cas9-gRNA ribonucleoprotein (RNP) complexes to tumor tissue. This approach facilitated highly efficient and specific delivery of CRISPR cargo to HCC cells within tumor organoids and xenograft tumor-bearing mice in vivo. It induced widespread apoptosis in tumor organoids and resulted in a significant reduction in tumor size in mice.[43] Another strategy involved the use of extracellular vesicles containing anti-GPC3 antibodies, enabling the specific delivery of Cas9 protein and dual sgRNA-encoding plasmids to HCC cells in xenograft tumor-bearing mice. Systemic administration of these extracellular vesicles successfully demonstrated their accumulation within xenograft tumors.[45]

The overexpression of the asialoglycoprotein receptor (ASGPR) on the surface of HCC cells provides an opportunity for selective binding to galactose residues, facilitating endocytosis. Researchers have leveraged this receptor to improve the delivery of therapeutic agents, including CRISPR plasmids. To achieve efficient and specific delivery, researchers conjugated β-galactose-carrying lactobionic acid to chitosan nanoparticles. These nanoparticles served as carriers for paclitaxel and CRISPR plasmids targeting *VEGFR*.[42] Moreover, the water solubility of chitosan is increased at acidic pH, promoting the controlled release of nanoparticle content within the tumor microenvironment. This two-stage control system facilitated the accumulation of nanoparticles in xenograft tumor tissue, leading to a significant reduction in tumor volume upon systemic administration. This approach highlights the effectiveness of tumor-specific delivery methods.[42] Another ASGPR-specific targeting approach involved the use of lactose-derived branched cationic biopolymer. This enabled the efficient delivery of CRISPR plasmids to HCC within an orthotopic liver tumor model upon systemic administration.[37] Nie et al utilized nanocomplexes consisting of a cationic shell with high transfection efficiency and a negatively charged heparin core to deliver CRISPR plasmid to orthotopic liver tumors. After systemic administration, the nanocomplexes exhibited prolonged enrichment within the liver of mice, demonstrating the safe and efficient targeting of the liver in vivo.[38]

Cancer-specific promoters represent a valuable strategy for ensuring tumor-specific CRISPR targeting. In this regard, Jiang et al employed an AAV vector for in vivo Cas13 and crRNA delivery in xenograft-bearing mice. A cancer-specific promoter known as the decoy minimal protomer was used, which enables Cas13 expression only in the presence of high levels of NF-κB, a cancer-associated transcription factor overexpressed in many cancer types.[39] After systemic administration, while AAVs were detected in several organs, Cas13 expression was exclusively observed in xenograft tumor tissue derived from HCC cells. This approach exemplifies the capability of tumor-specific expression methods to enhance safety and precision in CRISPR-based therapies.[39]

Sonodynamic therapy (SDT) relies on the generation of reactive oxygen species (ROS) to induce cancer cell death and represents a promising approach for HCC treatment due to its low cost, non-invasiveness, and high tissue-penetrating depth. In a study by Yin et al, it was observed that *NFE2L2* expression is activated immediately after SDT, leading to tumor growth promotion and reduced treatment efficiency. To address this issue, the researchers utilized an FDA-approved lipid nanoparticle system for the delivery of a sonosensitizer called hematoporphyrin monomethyl ether (HMME) and a Cas9-gRNA RNP complex targeting the *NFE2L2* gene. Tumor-localized ultrasound application facilitated the production of abundant ROS by HMME, which also induced endosomal rupture and the release of Cas9-gRNA complexes into the cytoplasm. These complexes subsequently translocated into the nucleus to knock out *NFE2L2*.

Systemic administration of the lipid nanoparticle system resulted in a high concentration of ROS combined with CRISPR-mediated *NFE2L2* knockout within xenograft tumors. This dual approach led to a significant reduction in tumor volume. Importantly, spatial control of ultrasound stimulation ensured the specific action of CRISPR within tumor tissue, minimizing off-target effects in unrelated tissues.[44] These studies underscore the importance of tumor-specific delivery, activation, and expression methods in enhancing the efficiency and safety of CRISPR-based targeting for HCC treatment.

CRISPR-Based Adoptive T Cell Therapies in HCC and Ongoing Clinical Trials

The complex immune landscape of the liver plays a dual role in both immune surveillance and immune tolerance. It contains various immune cell types, such as Kupffer cells, natural killer (NK) cells, dendritic cells, CD4+ T cells, and CD8+ T cells, which collectively serve to detect and combat pathogens circulating in the bloodstream. However, the liver also maintains an immunosuppressive environment to tolerate harmless substances like food antigens and baseline microbial products, which is crucial for normal liver function and immune homeostasis. This immunosuppressive state also plays a role in facilitating organ transplantation.[81] In cases of chronic inflammatory conditions like hepatitis B (HBV) or hepatitis C (HCV) infections, excessive alcohol consumption, and Metabolic dysfunction-Associated Fatty Liver Disease (MAFLD), continuous recruitment of immune cells and chronic inflammation can lead to the initiation of tumorigenesis in the liver. During this process, immunosuppressive mechanisms may come into play, leading to the inhibition and exhaustion of T cells, resulting in the failure of halting *de novo* tumorigenesis events or metastases from different organs.[82] As cancer cells proliferate, a process known as epithelial-to-mesenchymal transition occurs, contributing to the creation of a tumor microenvironment that further suppresses the immune response. This suppression occurs through various mechanisms, including the production of anti-inflammatory cytokines and the overexpression of immune checkpoint molecules like PD-L1. Given these challenges, the use of immunotherapy approaches for enhancing the function of existing host immune cells and targeting HCC by utilizing ex vivo activated and expanded T cells hold great promise to halt cancer progression.[7]

Adoptive cancer immunotherapy is a potent approach based on enhancing the ability of immune cells to target cancer, with significant improvements have been achieved over the last decade. This method involves the ex vivo sensitization and expansion of autologous or allogeneic lymphocytes, which are then infused into patients. Key strategies within adoptive immunotherapy include the use of chimeric antigen receptor (CAR)-expressing T cells, tumor antigen-specific T cell receptor (TCR)-engineered T cells, cytokine-induced killer (CIK) cells, tumor-infiltrating lymphocytes (TILs), lymphokine-activated killer (LAK) cells, and NK cells. While adoptive T cell therapies have primarily been employed for hematologic malignancies, researchers have increasingly focused on their application in solid tumors, with liver cancer, including hepatocellular carcinoma (HCC), being a prominent target.[83] Recent clinical trials in this context have shown promising results.[9,84,85]

While adoptive T cell therapies have shown promise in treating solid tumors like HCC, there are significant challenges that need to be addressed to enhance their safety and efficacy. One major challenge is T cell exhaustion, which can occur due to the immunosuppressive nature of the tumor microenvironment. This exhaustion is often characterized by the overexpression of immune checkpoint proteins like PD-1 on T cells, which can render them ineffective in targeting cancer cells.[86] Another concern in adoptive T cell therapies, particularly those involving T cell receptor (TCR)-engineered T cells, is the potential for unintended dimeric complex formation between endogenous and recombinant TCRs. This can result in unpredictable antigen-binding specificities and potential safety issues.[87] Additionally, the delivery of chimeric antigen receptors (CARs) and TCRs to T cells using lentiviruses can lead to random integration into the genome. This randomness can result in variable CAR and TCR expression levels and may raise safety concerns.[88] CRISPR-based tools offer promising solutions to address these challenges. For instance, CRISPR can be used to knock out the PD-1 gene in T cells, preventing T cell exhaustion and enhancing their effectiveness in targeting cancer cells. It can also be employed to knock out endogenous TCR-coding genes, eliminating the risk of unintended dimeric complex formation (Figure 2). Furthermore, CRISPR can enable the precise insertion of TCR or

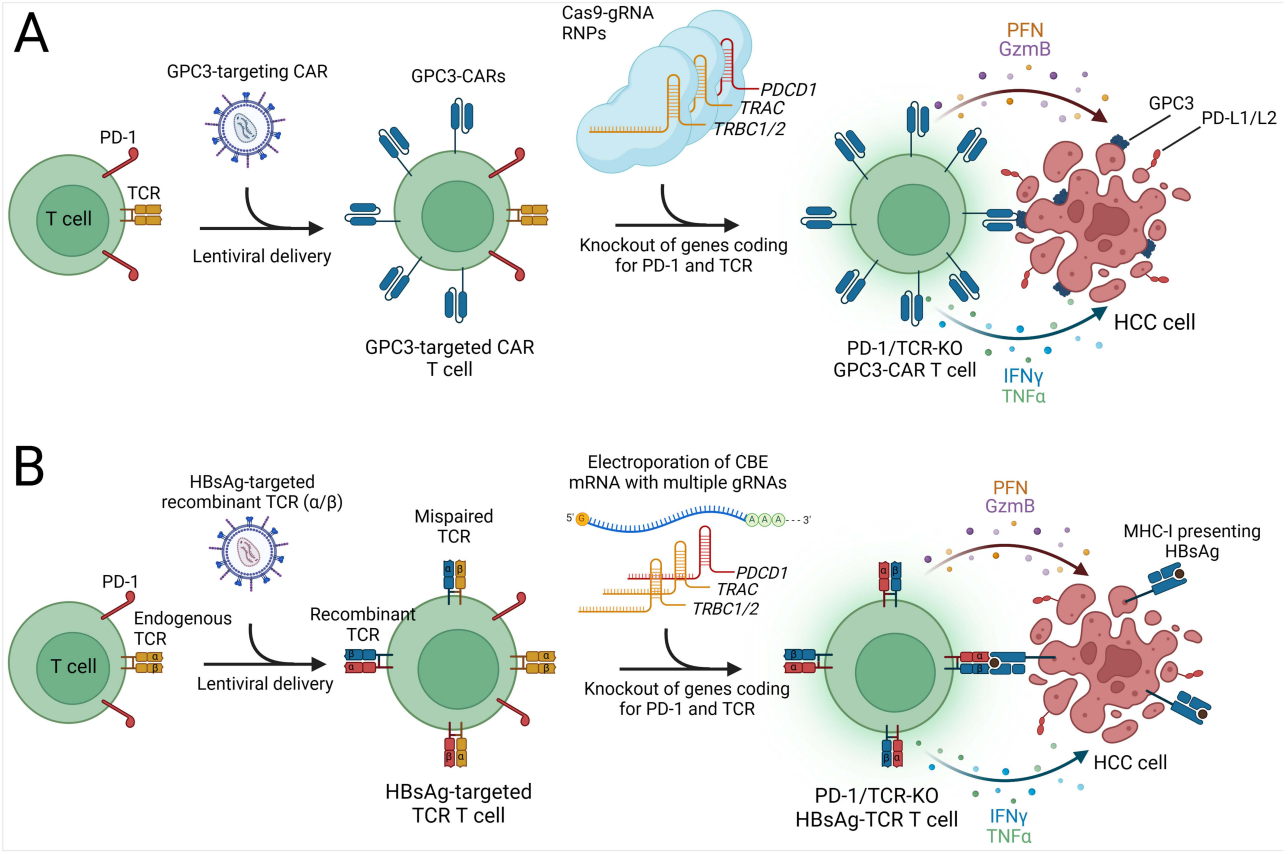

Figure 2 CRISPR-based enhancements for CAR T and TCR T cell therapies in HCC. Created with Biorender.com. (**A**) Autologous T cells are transduced with a lentivirus carrying a GPC3-targeting CAR expression cassette, followed by electroporation of Cas9-gRNA RNPs to knockout genes encoding the PD-1 and TCR α and β chains. These modifications prevent T cell exhaustion and enhance specificity. The engineered CAR T cells effectively bind GPC3 on HCC cells via GPC3-targeted CARs, triggering a release of cytotoxic proteins (perforin, granzyme B) and pro-inflammatory cytokines (IFN-γ, TNF-α), which collectively contribute to the targeted destruction of tumor cells. (**B**) Development of Hepatitis B surface antigen (HBsAg)-targeted TCR T cells involves lentiviral delivery of recombinant TCR α and β chains to T cells. The presence of endogenous TCR chains can lead to the formation of mispaired TCRs with unpredictable antigen specificity, posing a potential safety risk. For enhancing safety and efficacy, the cytosine base editor (CBE) is introduced as mRNA to perform knockout of endogenous TCR genes and PD-1. The specific interaction between the HBsAg-targeted TCR and HBsAg presented by MHC-I on HCC cells activates the TCR T cells, promoting robust antitumor activity.

CAR expression cassettes into defined loci in the T cell genome. This approach ensures safer and more stable expression of recombinant receptors, potentially improving the overall safety and efficacy of adoptive T cell therapies.[19]

Knockout of Immune Checkpoint Proteins Alleviates T Cell Exhaustion and Enhances the Efficacy of CAR T Cell Therapy

To improve the effectiveness of CAR T cell therapy for HCC, researchers have explored CRISPR-based strategies to disrupt the *PDCD1* gene, which codes for PD-1, in CAR T cells, thereby mitigating T cell exhaustion and enhancing antitumor activity. In a study, researchers observed a significant upregulation of PD-L1 in HCC cells when cocultured with glypican-3 (GPC3)-targeted CAR T cells. To counteract T cell exhaustion, PD-1 was disrupted in CAR T cells using Cas9 with two gRNAs targeting *PDCD1*.[89] This intervention resulted in the potent killing activity of GPC3-targeted CAR T cells against PD-L1-expressing HCC cell lines in vitro. Additionally, PD-1 disruption augmented the antitumor efficacy, persistence, infiltration, and pro-inflammatory cytokine production of GPC3-CAR T cells in a subcutaneous HCC xenograft model, leading to reduced tumor volume, prolonged presence of peripheral blood T cells post-infusion, increased CAR T cell infiltration in tumor tissue, and elevated serum levels of IFN-γ and IL-2. However, a more comprehensive approach involved the disruption of both PD-1 and endogenous TCR to ensure the safe and efficient use of GPC3-CAR T cells, as PD-1 knockout CAR T cells could potentially express autoreactive TCRs, posing a risk of autoimmune adverse effects (Figure 2A).[90] In another study, researchers harvested CIK cells from the peripheral blood of HCC patients, and electroporated Cas9-gRNA RNPs into CIK cells to knockout PD-1 followed by lentiviral delivery of

Table 2 CRISPR-Mediated Adoptive T Cell Immunotherapy Studies in HCC

Production of Adoptive T Cells	Resulting T Cells	Results of the Study	Ref.
T cells with lentiviral expression of GPC3-targeted CAR were electroporated with Cas9 protein and 2 gRNAs targeting *PD-1*	*PD-1*-knockout, GPC3-targeted CAR T cells	PD-1 disruption enhanced antitumor efficacy, persistence, infiltration, and pro-inflammatory cytokine production of GPC3-CAR T cells in vivo.	[89]
CIK cells were electroporated with Cas9-gRNA RNP complex targeting *PD-1* followed by lentiviral transduction of *hTER*	*PD-1*-knockout, *hTERT*-expressing CIK cells	PD-1 knockout improved IFN-γ secretion capacity and antitumor efficacy of *hTERT*-transduced CIK cells.	[91]
T cells with lentiviral expression of HBsAg-targeted TCR and a sgRNA targeting *TRBC1/2* were electroporated with cytosine base editor (BE3) mRNA	*TRBC1/2*-knockout, HBsAg-targeted TCR T cells	Knockout of the TCR β chain eliminated endogenous TCR expression and prevented mispairing of endogenous TCR with recombinant TCR chains.	[93]
T cells were electroporated with 2 vectors: one for expression of Cas9 and gRNA targeting AAVS1 locus; and the other carrying AAVS1 homology arms and CD105-targeted CAR cassette	CD105-targeted CAR T cells	CRISPR-based knock-in of anti-CD105 CAR expression cassette into AAVS1 locus enabled potent and stable expression of anti-CD105 nanobody.	[94]

the human telomerase reverse transcriptase (*hTERT*) gene.[91] In vitro investigations demonstrated that *hTERT* expression in CIK cells led to an increase in telomere length and significantly improved the persistence of these engineered CIK cells. Furthermore, the knockout of PD-1 enhanced the IFN-γ secretion capacity and antitumor efficacy of CIK cells (Table 2). Adjuvant immunotherapy using autologous CIK cells has demonstrated a significant increase in recurrence-free and overall survival rates among patients with HCC.[92] Leveraging CRISPR-based methods presents an exciting opportunity to enhance CIK cell therapies further, potentially contributing to improved survival outcomes in HCC patients.

In an ongoing Phase I clinical trial (NCT04417764) initiated in 2019, researchers are investigating a combination therapy approach for advanced HCC. This trial combines transarterial chemoembolization (TACE) with infusions of autologous T cells that have undergone PD-1 knockout using CRISPR-Cas9 technology. The treatment regimen involves one TACE procedure followed by three or more cycles of CRISPR-Cas9-mediated PD-1 knockout T cell infusions at 4-week intervals. During each cycle, a total of 1 to 3×10^9 engineered T cells are administered via percutaneous infusion into the peripheral tumor.[95] Another phase I clinical trial (NCT04842812) focuses on patients with advanced solid tumors, including liver cancer. In this trial, tumor-infiltrating lymphocytes (TILs) are harvested from patients and subjected to CRISPR-Cas9-mediated knockout of the PD-1 gene. Additionally, the TILs are engineered to express single-chain fragment variables (scFvs) targeting immune checkpoint proteins PD-1 and CTLA-4, as well as chimeric antigen receptors (CARs) against various antigens, including GPC3. The resulting PD-1 knockout CAR/TILs, which secrete anti-PD-1/CTLA-4 scFvs and carry CARs against multiple antigens, are administered systemically or locally at a dose of 1 to 10×10^8 cells per kilogram for each treatment, with at least three cycles of treatment planned.[96] Furthermore, researchers have identified an intracellular immune checkpoint protein Cytokine-induced SH2 (CISH) protein, which negatively regulates T cell function. Knockout of the *CISH* gene has been shown to enhance the efficacy of adoptive TIL therapy for gastrointestinal cancers.[97] In an associated phase I/II clinical trial (NCT04426669), investigators aim to utilize *CISH*-knockout (CISH-KO) TILs as a therapeutic approach for patients with metastatic gastrointestinal epithelial cancers that have not responded to any first-line therapy (Table 3).[98] These studies and ongoing clinical trials represent promising developments in the field of adoptive T cell therapy for HCC and underscore the potential of CRISPR-based strategies to enhance the safety and efficacy of these treatments.

Targeting Endogenous TCRs for Recombinant TCR T Cell Therapy

Cytosine base editors offer a valuable tool for generating premature stop codons via single-base editing (Figure 1E).[22,99] This approach offers several advantages, including the ability to achieve gene knockout without inducing DSBs, thereby preventing the formation of large insertions or deletions and complex genomic rearrangements that can occur with traditional Cas9-based gene disruption methods.[35] In the context of adoptive T cell therapy, preventing the expression of endogenous T cell receptor (TCR) chains is crucial to avoid unpredictable pairings with exogenously delivered

Table 3 Clinical Trials Using CRISPR in Adoptive Cell Therapy for HCC

Immunotherapy Approach	Clinical Trial Identifier	Phase	Current Status	Co-Treatment	Estimated Participants	Estimated Primary Completion	Ref.
Autologous *PD-1*-knockout T cells to treat advanced HCC	NCT04417764	I	Recruiting	TACE	10	Dec 2024	[95]
Autologous *PD1*-knockout TILs/CAR-TILs that secretes anti-PD-1/CTLA-4 scFvs and CARs* to treat advanced solid cancers including liver cancer	NCT04842812	I	Recruiting	N/A	40	Dec 2025	[96]
Autologous *CISH*-knockout TILs to treat metastatic gastrointestinal malignancies	NCT04426669	I/II	Recruiting	Aldesleukin, Cyclophosphamide, Fludarabine	20	Sep 2023	[98]

Notes: *Against various antigens, including HER2, Mesothelin, PSCA, MUC1, Lewis-Y, GPC3, AXL, EGFR, Claudin18.2/6, ROR1, GD1, or B7-H3.

recombinant TCR α and β chains, which can lead to mispairing of TCRs, which reduces the cell surface expression of recombinant TCR and potentially generates self-targeting TCRs. To address this challenge, Preece et al utilized cytosine base editing to disrupt the endogenous TCR β chain, encoded by the *TRBC1/2* genes (Figure 2B). Recombinant TCR and gRNA expression cassettes targeting hepatitis B surface antigen (HBsAg) were introduced via lentivirus delivery, followed by the electroporation of Base Editor 3 (BE3) mRNA into T cells. This approach resulted in a significant reduction in the number of T cells expressing the endogenous TCR, with the majority of cells carrying the recombinant TCR. To further purify the cell population, a magnetic bead-mediated depletion process was conducted to remove any remaining endogenous TCR-expressing cells, resulting in a homogeneous population in which approximately 95% of the T cells expressed the recombinant TCR while lacking the endogenous TCR. In vitro analyses using a 3D microfluidic device, which included HBsAg-expressing HCC cells, demonstrated that T cells equipped with the recombinant TCR effectively killed cancer cells and exhibited elevated levels of inflammatory cytokines, including IFN-γ, TNF-α, and IL-2, compared to control groups. Although the incorporation of a disulfide bond between recombinant TCR chains reduced the likelihood of mispairing with endogenous TCR chains in this study, targeting multiple genes, including *TRAC* (TCR α chain) and *PDCD1* (PD-1), in addition to *TRBC1/2* (TCR β chain), represents a promising and comprehensive strategy to further improve the safety and efficacy of TCR T cell therapy for HCC (Figure 2B).[23]

CRISPR Enables Site-Specific Genome Integration of CAR in T Cells

CD105, also known as endoglin, is highly expressed on the surface of cancer cells and endothelial cells within the tumor microenvironment, playing a key role in angiogenesis. Mo et al utilized CRISPR-Cas9-mediated HDR to precisely insert the anti-CD105 nanobody expression cassette into the AAVS1 locus of T cells (Figure 1A). This approach allowed for the stable and controlled expression of the anti-CD105 CAR on the surface of T cells. The engineered CD105-CAR T cells, administered twice to mice with xenograft HCC tumors, significantly reduced tumor size and improved the survival of the mice. Furthermore, CD105-CAR T cells effectively inhibited the growth of HCC patient-derived xenografts in vivo. Importantly, these engineered T cells were well-tolerated, with no fever, bleeding, or increased IL-6 levels observed in the mice. The CAR cassette can also be inserted into the *TRAC* locus using CRISPR-Cas9, enabling stable expression of CAR with the simultaneous knockout of *TRAC*.[100]

Discussion and Future Perspectives

The emergence of CRISPR-based therapies in the context of hepatocellular carcinoma represents a significant advancement in the field of cancer treatment. Promising results have emerged from preclinical studies, and early-phase clinical trials are currently in progress. However, as we move toward practical implementation, it is essential to acknowledge and address important considerations and challenges.

In translating CRISPR-based cancer therapies from bench to bedside, several critical challenges must be addressed to realize their potential in clinical settings. Regulatory hurdles are particularly significant, as current frameworks are not fully adapted to the novel complexities introduced by gene editing technologies.[101] Additionally, the development of efficient and safe delivery methods remains a pivotal concern. These delivery systems must ensure targeted delivery to cancer cells while minimizing systemic exposure to reduce adverse effects.[102] More research is also needed to develop more precise CRISPR systems that can enhance targeting accuracy, thereby reducing the likelihood of off-target effects and improving therapeutic outcomes.[103] Furthermore, the long-term safety and efficacy of these therapies need to be rigorously evaluated through extended follow-up in clinical trials to monitor for potential delayed adverse effects and ensure sustained therapeutic benefits.[25] Addressing these challenges is essential for the successful integration of CRISPR therapies into routine clinical practice for treating cancers, including HCC.

Addressing the immunogenicity of CRISPR-based therapeutics is imperative for their successful translation into clinical applications. The development of adaptive immune responses against CRISPR-associated proteins, such as Cas9, which often originate from common bacterial pathogens, represents a significant hurdle.[104] This immunogenicity can compromise the safety and efficacy of CRISPR-based interventions, potentially leading to adverse outcomes similar to those observed in past gene therapy trials. Strategies to circumvent these immune responses include engineering CRISPR components to reduce immunogenicity and utilizing delivery tools with limited immune visibility.[105] Furthermore, leveraging in silico methods combined with empirical testing can help predict and mitigate potential immunogenic hotspots within the CRISPR proteins.[106] Continuous monitoring and evaluation of immune responses in preclinical and clinical settings are crucial. This approach includes assessing both the cell-mediated and humoral immune responses to CRISPR components and their delivery vectors.[107] Regulatory frameworks should also adapt to these evaluations, ensuring that immunogenicity assessments are an integral part of the development pipeline for CRISPR-based therapies.

CRISPR-based therapies hold a pivotal promise in the context of HCC, primarily due to their precision in targeting genes directly involved in cancer progression and in modulating immune checkpoints in adoptive T cells. This allows for highly tailored therapeutic strategies. However, the use of CRISPR technology also rises some ethical and safety concerns, such as the risk of inadvertently editing germline cells, which could lead to heritable genetic changes.[108] To mitigate this risk, it is essential to implement rigorous control measures and utilize specific delivery methods that exclusively target cancer cells while avoiding germline cells during the development and execution of in vivo CRISPR treatments.[109] Additionally, nuclease-mediated genome editing, a key component of CRISPR, may unintentionally create large indels at targeted DNA sites, edit off-target DNA regions, and cause chromosomal translocations, inversions, and truncations, presenting significant safety concerns.[35,110–112] However, the field has made notable progress in addressing these issues. One approach to enhance precision involves the utilization of engineered high-fidelity Cas9 variants and improved gRNAs that exhibit reduced off-target editing, thereby mitigating the risk of unintended genetic alterations.[113,114] Alternatively, optimized cytosine base editors (CBEs) with reduced off-target and genotoxic effects can be employed to introduce premature stop codons, effectively knocking out coding genes (Figure 1E).[115–117] This method is regarded as a safer alternative to Cas9-mediated gene disruption, as CBEs introduce a significantly lower level of DNA double-strand breaks (DSBs). This approach has been recently applied in a preclinical study for TCR T cell development for HCC and in a clinical study for CAR T cell therapy in T-cell acute lymphoblastic leukemia.[22,93] Additionally, the CRISPRoff system, capable of establishing a robust and enduring epigenetic memory spanning hundreds of cell divisions, can be harnessed to suppress the expression of specific genes in T cells, including immune checkpoint regulators (Figure 1D).[32] Moreover, the rigorous implementation of unbiased, genome-wide methods for assessing off-target effects is imperative to ensure the safe application of CRISPR technologies before their translation into clinical practice.[118,119] These advancements underscore the ongoing efforts to mitigate the ethical and safety concerns associated with CRISPR-based interventions in the HCC treatment landscape.

The combination of CRISPR-based therapies with established treatments like sorafenib and TACE in both preclinical investigations and clinical trials for HCC offers exciting prospects.[37,38,41,95] Encouragingly, these studies have demonstrated synergistic effects, suggesting the potential for improved therapeutic outcomes in HCC patients. However, the intricacies of integrating diverse modalities, including considerations related to timing, dosage, and patient selection, necessitate rigorous exploration through clinical trials to ensure both safety and efficacy. Future research endeavors may

emphasize the integration of CRISPR-based targeted cancer therapy approaches with immune checkpoint inhibitors, such as atezolizumab (anti-PD-L1) or pembrolizumab (anti-PD-1). Furthermore, researchers might prioritize the development of CRISPR-engineered CAR T and TCR T cell immunotherapies for HCC, given the highly promising preclinical results (Table 2). These therapies are more likely to transition into clinical practice compared to CRISPR-based strategies that directly target tumor cells (Table 3).

To optimize the accessibility and effectiveness of CAR T cell immunotherapy, advancing CRISPR technology for the development of universal CAR T cells is essential. Through multiplex gene editing, targeting specific genes such as *TRAC* and *beta-2 microglobulin* (*B2M*), T cells can be engineered for allogeneic use, substantially reducing the risk of graft-versus-host disease (GvHD).[99,120,121] Such advancements could not only decrease production costs and enhance the availability of CAR T cell therapies but also expedite their integration into clinical practice, thus extending this advanced treatment to a wider range of patients.[120–122] Successfully implementing these strategies for HCC treatment has the potential to dramatically transform the therapeutic outlook for this challenging cancer, markedly improving patient outcomes.

One of the most important challenges in HCC treatment lies in the immunosuppressive microenvironment, which hampers the infiltration and activation of T cells within the tumor.[123] CRISPR-based immunotherapies aim to overcome this obstacle by targeting immune checkpoints like PD-1 and CISH. Additionally, an innovative approach involves engineering CAR T cells to secrete single-chain variable fragments (scFvs) that bind and inhibit immune checkpoint proteins, such as PD-1 and CTLA-4.[124] This strategy prevents the suppression of both CAR T cells and endogenous T cells in the tumor microenvironment and is currently undergoing clinical evaluation for advanced solid tumors, including HCC.[96] While these approaches have shown promise, the complex interplay of immune cells, cytokines, and tumor cells within the microenvironment necessitates further investigation.[125] In addition, it is imperative to conduct thorough investigations into resistance mechanisms against adoptive T cell therapies and potential undesired outcomes, including antigen modulation in cancer cells and CAR-related toxicities.[126]

Delivery tools for CRISPR components to tumor tissues in vivo and T cells ex vivo are another critical area of development.[109,127] In the context of targeted cancer therapy, the critical objective is to ensure that these components effectively reach their designated targets within the liver, particularly at the tumor site, as this is fundamental for both safety and therapeutic efficacy. Innovative delivery systems designed specifically for tumor targeting, such as nanoparticles and extracellular vesicles, demonstrate promise in this regard (Table 1). However, it is imperative to subject these technologies to comprehensive assessments of their clinical feasibility and safety profiles.

CRISPR-based functional genomic screens conducted both in vitro and in vivo have paved the way for the discovery of novel genes and gene regulatory elements that hold essential roles in cancer development, tumor immunology, and immune cell function.[128–130] CRISPR screens are useful for investigating tumor-specific processes, including hypoxia, immune evasion, effects of cytokines, and DNA damage. Notably, several studies performing CRISPR screens in HCC cells have identified potential therapeutic targets to impede tumor progression.[49,54,55,74] Similarly, CRISPR screens in T cells have unveiled novel genes associated with T cell proliferation, activation, and antitumor activity.[131–133] These discoveries can be leveraged through CRISPR-based interventions to enhance adoptive T cell therapies. Therefore, future research focusing on CRISPR screening in HCC cells or organoids to identify novel therapeutic targets, alongside investigations in T cells to unveil factors that can enhance adoptive T cell immunotherapy, represent areas of significant interest and potential advancement.

In addressing the transition of CRISPR therapies from bench to bedside, it is imperative to consider the regulatory challenges inherent in such innovative treatments. The evolving regulatory landscape requires developers to navigate complex approval processes that ensure safety and efficacy while adapting to the rapid advancements in gene editing technologies.

The field of CRISPR-based therapies for HCC holds tremendous potential. As ongoing clinical trials generate more data and regulatory approvals are pursued, these therapies may emerge as valuable additions to the repertoire of liver cancer treatments. However, it is essential to approach these advancements with caution, prioritizing patient safety and ethical considerations. In conclusion, CRISPR-based therapies for hepatocellular carcinoma are poised to bring significant improvements to cancer treatment. While challenges do exist, the application of scientific rigor, collaborative efforts, and continuous research endeavors will undoubtedly shape the promising future of HCC therapy. The incorporation of CRISPR technology into clinical practice has the potential to enhance patient outcomes and represents an exciting frontier in the fight against HCC.

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