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# RNA-based therapies in hepatocellular carcinoma: state of the art and clinical perspectives

**Le Xing[1,2], Zhen-Kai Wang[3], Dong-Mei Li[2], Jing Li[2], Ming Liu[1,2]**

[1]Affiliated Cancer Hospital and Institute of Guangzhou Medical University, Guangzhou, Guangdong 510095, China.
[2]Guangzhou Municipal and Guangdong Provincial Key Laboratory of Protein Modification and Degradation, School of Basic Medical Science, Guangzhou Medical University, Guangzhou, Guangdong 511436, China.
[3]The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510260, China.

**Correspondence to:** Dr. Ming Liu, Affiliated Cancer Hospital & Institute of Guangzhou Medical University, Key Laboratory of Protein Modification and Degradation, School of Basic Medical Sciences, Guangzhou Medical University, Guangzhou, Guangdong 510095, China. E-mail: liuming@gzhmu.edu.cn

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## Abstract

Liver cancer is a heterogeneous disease and is one of the leading causes of cancer deaths worldwide. Hepatocellular carcinoma, comprising approximately 90% of cases, presents a formidable challenge with a less than 20% 5-year survival rate despite recent treatment advancements. The impediments of drug resistance and off-target effects underscore the critical need for innovative and efficacious therapies. Harnessing the growing understanding of RNA function offers a promising avenue to address previously "undruggable" proteins, transcripts, and genes. Various RNAs demonstrate the potential to selectively act on these targets, expanding the scope of therapeutic interventions. With diverse regulatory roles in cancer pathways, RNAs emerge as valuable targets and tools for anticancer therapy development. This article provides an in-depth exploration of current RNA-based therapies, elucidates their mechanisms of action, and discusses their combinations with chemo-/immunotherapies in clinical trials for hepatocellular carcinoma.

**Keywords:** RNA-based therapies, drug resistance, mRNA vaccines

## INTRODUCTION

Primary liver cancer is one of the most prevalent cancers worldwide. With a total of 905,677 newly

registered cases and 830,180 deaths from liver cancer--the third highest number of cancer-related deaths--liver cancer was the sixth most common entity tumor in the world in 2020[1]. Of the various rare forms of primary liver cancer, hepatocellular carcinoma (HCC) accounts for 75%-85% of cases, while intrahepatic cholangiocarcinoma accounts for 10%-15% of cases[2]. Patients with early-stage hepatocellular carcinoma can benefit from radical interventions such as surgery, ablation, and liver transplantation.

The currently approved treatments for HCC include sorafenib, levatinib, regorafenib, and cabozantinib. These medications target tyrosine kinase growth factor receptors and/or serine/threonine kinases associated with angiogenesis and cell proliferation. Patients treated with sorafenib showed a significant improvement in both overall and progression-free survival, according to the study[3]. Median overall survival was 10.7 months in the sorafenib group and 7.9 months in the placebo group (hazard ratio in the sorafenib group, 0.69; 95% confidence interval, 0.55 to 0.87; $P < 0.001$). There was no significant difference between the two groups in the median time to symptomatic progression (4.1 months *vs.* 4.9 months, $P = 0.77$). Nonetheless, a noteworthy segment of patients encountered toxic reactions, leading to the cessation of treatment for 10%-15% of the population. Immunogenic features are usually present in HCC, but the intratumoral environment is usually immunosuppressive. A portion of this immunosuppression is linked to acquired immune dysfunction brought on by viral infections, cirrhosis, or environmental insults that exacerbate the disease. In addition, the intratumoral milieu's immunosuppressive properties are influenced by intrinsic hepatic tolerance[4]. Patients with advanced hepatocellular carcinoma experience constrained survival advantages when treated with immune checkpoint inhibitors (ICIs). Although anti-PD-1/PD-L1 therapy demonstrates a higher rate of complete and lasting responses compared to sorafenib, the effectiveness of anti-PD-1/PD-L1 therapy is hindered by the absence of dependable predictive markers[5,6]. In phase 3 studies of treatment, remission rates were associated in the range of 15% to 20%, but they did not significantly improve overall survival[7]. Atezolizumab and Bevacizumab Combination Therapy Provides Significant Therapeutic Benefits in Multiple Cancer Types and Significantly Outperforms Sorafenib in Overall Survival and Progression-Free Survival in Patients with HCC, Making It One of the First-Line Therapeutic Options for HCC. The hazard ratio for death with atezolizumab-bevacizumab as compared with sorafenib was 0.58 [95% confidence interval (CI), 0.42 to 0.79; $P < 0.001$]. Overall survival at 12 months was 67.2% (95%CI: 61.3 to 73.1) with atezolizumab-bevacizumab and 54.6% (95%CI: 45.2 to 64.0) with sorafenib. Median progression-free survival was 6.8 months (95%CI: 5.7 to 8.3) and 4.3 months (95%CI: 4.0 to 5.6) in the respective groups (hazard ratio for disease progression or death, 0.59; 95%CI: 0.47 to 0.76; $P < 0.001$)[8]. Although combination therapies have shown significant efficacy, drug resistance remains a challenge. Further research is needed to understand the mechanisms of resistance and develop strategies to combat it.

To address the constraints of current treatments, there is a growing interest in RNA-based therapies within the scientific community. In comparison to conventional drugs targeting proteins or DNA, RNA-based therapies show promise owing to their distinctive physicochemical and physiological properties[9-12]. ASOs, siRNAs, and microRNAs are RNA molecules that specifically interact with mRNA and non-coding RNAs (ncRNAs) through Watson-Crick base pairing. This interaction allows them to directly target and regulate the activity of these RNA molecules[12]. Theoretically, RNA can selectively target any specific gene by identifying the appropriate nucleotide sequence on the target RNA. RNA interference (RNAi) serves as a cellular mechanism for the downregulation of gene expression in response to genetic abnormalities and infections[13]. The current consensus regards it as a universally applicable and straightforward biological tool for conducting gene silencing studies. This tool holds promise for developing therapeutic approaches aimed at treating various diseases, including cancer[14,15]. *In vitro* transcribed (IVT) mRNAs have the potential to be employed in protein replacement therapy or immunization. Upon entering the cytoplasm, they can achieve these therapeutic goals without inducing irreversible genomic alterations or posing genetic risks, in contrast

to DNA-based therapies[16]. Furthermore, the clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing technique enables the precise modification of target RNA sequences for the targeted treatment of specific diseases[17]. RNA aptamers possess the ability to impede protein activity, akin to the functionality exhibited by small molecule inhibitors and antibodies[13]. Consequently, RNA-based treatments are thought to be the most appealing therapeutic targets because they can increase the number of druggable targets[18]. Currently, RNA-based therapies in combination with chemotherapy and immunotherapy have become a hot research topic for developing therapies for different types of cancers. This review provides an overview of the classification and use of RNA-based therapies, as well as the development trends and practical directions of RNA-based therapies for the treatment of HCC. It also covers the advancements made in clinical studies involving RNA as drugs combined with immunotherapy and chemotherapy.

## RNA INTERFERENCE

Andrew Fire and Craig C. Mello's 1998 paper introduced the concept that the suppression of homologous gene expression through positive-sense RNA was initiated by the presence of minute amounts of dsRNA in RNA obtained through *in vitro* transcription. This phenomenon, termed RNA interference (RNAi), brought about a revolutionary shift in the domain of gene silencing[19]. RNAi-based therapeutic strategies involve the silencing of genes by harnessing the inherent cellular machinery of the targeted cells. The RNA-induced silencing complex (RISC), which binds to single-stranded 20-30 nt RNAs in the miRNA and siRNA pathways, is the minimal effector of RNAi. Through pre- or post-initiation translational repression and mRNA complementary degradation, RISC can be activated to produce gene silencing effects through the catalytic inactivation of Argonaute's or partial complementation with the target genes[13]. After cutting, the newly produced siRNA fragment can be utilized as a primer to create a new dsRNA using mRNA as a template. This dsRNA can then bind with Dicer to form an RISC, which will mediate the subsequent round of homologous mRNA degradation. This allows for the degradation of a large number of homologous mRNAs, which amplifies the cascade amplification effect and amplifies the inhibition effect on gene expression [Figure 1][20]. Post-transcriptional gene silencing by RNAi has a high degree of sequence specificity, closely follows the base complementary pairing principle, attaches to the target mRNA, and only destroys the homologous mRNA, effectively silencing the target gene alone. Moreover, the RNA interference effect can be stably inherited to the next generation[19].

siRNA is a class of double-stranded non-coding RNA molecules, 20-27 base pairs in length. These molecules are generated when RNase III endonuclease Dicer cleaves long dsRNA precursors into smaller pieces. siRNAs are incorporated into the structure of a complex consisting of RISC and AGO2[20]. The antisense strand of the siRNA binds to complementary sequences on specific target mRNAs, triggering the cleavage pathway, and the AGO2 ribonucleic acid endonuclease cleaves the target mRNAs into small fragments, blocking their translation into functional proteins [Figure 1][20-22].

miRNAs are endogenous small non-coding RNAs with a length of approximately 19-25 nucleotides. Primary miRNAs (pri-miRNAs) have a stem-loop or short hairpin structure of ~33 nucleotides and are processed into precursor miRNAs (pre-miRNAs) of nearly 70 nucleotides[23-26]. Pre-miRNAs translocate to the cytoplasm by Exportin5. Activated by Dicer, it is processed into a mature miRNA that is loaded into the RISC-AGO2 complex [Figure 1]. There, the antisense strand binds complementarily to the target mRNA's 3′-untranslated region (3′-UTR), cleaving the mRNA and preventing translation of the mRNA into protein[23-26]. miRNAs can target multiple mRNAs, and a single mRNA can contain multiple miRNA recognition signals[27].

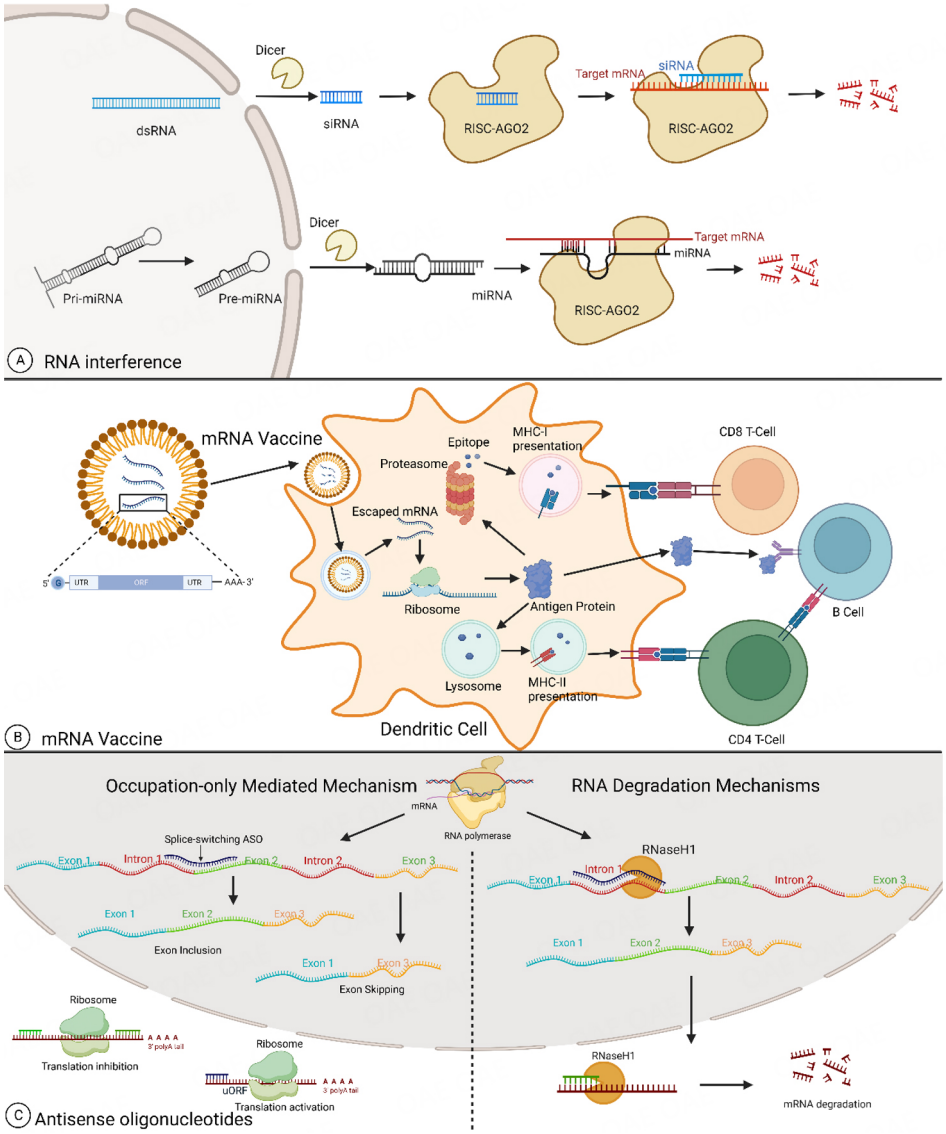


**Figure 1.** Types and mechanisms of RNA-based therapies (Created with BioRender.com). (A) RNA interference. Long double-stranded RNA and precursor microRNA are processed by Dicer into siRNAs and mature miRNAs, which are loaded into RISCs for RNA targeting, degradation, or translational repression; (B) mRNA vaccines. mRNAs are delivered to dendritic cells by lipid nanoparticles, released into the cytoplasm, and translated into antigenic proteins by ribosomes. Some antigenic proteins are degraded into small peptides by the proteasome and are presented to the surface of CD8+ T cells by MHC I. CD8+ cytotoxic T cell-mediated immunity kills infected cells by secretion of perforin or granzyme. Other antigenic proteins are degraded in lysosomes and displayed on the surface of CD4+T cells by MHC II. B cell-mediated humoral immunity uses antibodies to neutralize pathogens; (C) Antisense oligonucleotides. ASO can regulate the expression of target genes by two mechanisms: (1) an occupancy-only mechanism and (2) an RNA degradation mechanism.

## MRNA AND MRNA VACCINES

In 1978, scientists used lipid-based membrane structures known as liposomes to deliver mRNA into both mouse organisms and human cells, causing the expression of proteins[28]. mRNAs are translated into the cytoplasm instead of the nucleus, which eliminates the possibility of them becoming integrated into the host genome, in contrast to conventional vaccines[16]. Moreover, it is possible to produce RNA vaccines quickly, cheaply, and uniformly[10]. These mRNAs, which are produced outside of the normal cellular environment, encode tumor antigens, cytokines, tumor suppressors, chimeric antigen receptors, or genome editing

proteins. They are essential to a number of therapeutic applications, such as cancer vaccines, immunotherapy, tumor suppression, engineered T-cell therapies, and gene therapy. Similar to naturally occurring mRNAs in eukaryotic cells, conventional IVT mRNAs consist of essential elements such as a 5' cap, 5' and 3' -UTRs, open reading frames (ORFs), and poly(A) tails[29,30]. To enhance the effectiveness of mRNA-based therapies, it is crucial to minimize immunogenicity and maximize protein expression. Because of its 5′cap structure, mRNA is stable and can initiate translation by preventing exonucleases from breaking it down. During translation initiation, the 5′cap structure recruits eukaryotic translation initiation factor 4E (eIF4E) to form a preinitiation complex. The ribosome then recognizes the preinitiation complex to start transcription[31]. There are three main cap structures: cap0, cap1, and cap2. Since the Cap1 structure has only been discovered in eukaryotic mRNAs thus far, its RNA can be labeled to reduce the stimulation of pattern recognition receptors (PRRs), thereby reducing the stimulation of the host's natural immune response and ultimately reducing the inflammatory response to improve the translation efficiency of mRNA *in vivo*, and is most commonly used in mRNA vaccines. *In vitro* transcription can be capped by enzymatic, chemical, or chemoenzymatic methods[32]. The 5′ and 3′ terminal UTRs do not directly encode proteins, but their chemical and sequence optimization improves mRNA stability and prolongs translation time[33,34]. To increase translation efficiency, a Kozak sequence (GCCACC) is frequently added after the 5′-UTR sequence[35]. The 3′-UTR mainly affects mRNA stability and half-life. In current studies, UTR sequences are mainly derived from highly expressed genes (e.g., human β-bead proteins), and studies have also been conducted to identify novel UTR sequences by high-throughput screening or deep learning, *etc.*, to improve mRNA expression[33]. Human mRNA contains naturally modified nucleosides. When natural uridine and cytidine are substituted with modified nucleosides, such as pseudouridine (Ψ), 1-methyl pseudouridine (m1Ψ), and 5-methylcytidine (m5C), during *in vitro* mRNA transcription, the mRNA's stability and translational efficacy are increased, and its immunogenicity is decreased[36,37]. Natural nucleosides are rarely used in nucleoside-modified mRNA vaccines; instead, 100% chemically modified nucleosides are used[38-40]. An optimal length of poly(A) tail improves translation efficiency and mRNA stability. IVT mRNAs are tailed by the addition of oligo(dT) to the coding transcription template vector or by enzymatic reactions using recombinant poly(A) polymerase. Poly(A) tails slow down the degradation of the RNA by RNA nucleic acid exonucleases, thus improving mRNA stability. Studies in dendritic cells have shown that the optimal length of the poly(A) tail is between 120 and 150 nucleotides[30].

Tumor-associated antigens (TAA) and cancer neoantigens are the primary targets of cancer vaccines. TAA refers to atypically expressed proteins in tumors relative to normal tissues, such as overexpression, distinct subcellular localization, and expression that is specific to the tumor (typically isolated at immune-privileged sites or expressed at specific stages of differentiation)[29]. Carrier-delivered mRNA enters dendritic cells through endocytosis, forming endosomes. Within the acidic environment of endosomes, the heads of ionizable lipids are protonated to the cationic state, which generates ion pairs with anionic phospholipids naturally occurring in the endosomal membranes. These ion pairs are smaller than the combined areas of the individual ionizable lipid heads before membrane fusion, thus facilitating membrane fusion and disruption and enabling the mRNA to escape from the endosome and enter the cytoplasm. Once in the cytoplasm, the mRNA is translated by ribosomes into antigenic proteins[41]. After that, the proteasome breaks it down into smaller pieces that are exposed to the cell surface and interact with MHC class I molecules on dendritic cells to activate CD8+T cells and cause cancer cells to undergo apoptosis [Figure 1 ][42]. As an alternative, mRNA translation produces antigens that are released into the extracellular space, broken down into peptide fragments in the endosome following absorption by antigen-presenting cells, and then transported as exogenous antigens to CD4+ T cells by MHC II. These cells then exert humoral immunity by releasing cytokines and stimulating the activation of B cells that are specific to the antigen, leading to their proliferation and differentiation into plasma cells, which in turn produces targeted antibodies [Figure 1][43]. Furthermore, exogenous IVT mRNA functions as an intrinsic immunostimulatory

factor and is recognized by a range of cytoplasmic, endosomal, and cell surface PRRs[16]. ssRNAs can be recognized by TLR7 and TLR8 in the endosomes. dsRNAs can be recognized by TLR3, RIG-I, and other molecules, resulting in the release of pro-inflammatory cytokines and the activation of the IFN-α pathway[44,45]. Anticancer immunotherapy may benefit or suffer from the activation of multiple PRRs and the synthesis of IFN-α[29]. It can promote antigen-presenting cell maturation and activation, which can be beneficial for vaccination; however, it can also result in antigen expression suppression, which can impede the immune response. Neoantigenic cancer vaccine antigens are produced by screening biopsy samples from cancer patients, identifying mutations in the genes encoding tumor-specific proteins in both tumor and normal tissues[46]. These mutated genes produce peptides or antigens, which are processed to predict whether they will interact with MHC I or II. Plasmids containing the genes encoding the identified neoantigens that bind to MHC I or II are used to transcribe the genes into mRNA *in vitro*[47-49].

Cytokines play a key role in regulating immune system function, with IFN-α and IL-2 being representative immune agents for tumors. However, there are many problems with previous agent approaches: short half-life of directly injected proteins, high doses leading to systemic toxicity, and the risk of uncontrollable gene integration in DNA. The mRNA encoding cytokines can effectively activate innate and adaptive immunity, effectively suppress tumors and prevent tumor recurrence[50].

The application of mRNA technology in the design of specific T cell receptor (TCR) or chimeric antigen receptor (CAR) retargeting T cells has become an important direction in the field of tumor immunotherapy in recent years. mRNAs can encode different TCR or CAR molecules, providing a variety of design choices for targeting different antigens. mRNAs can mediate the rapid and controllable expression of genes, avoiding the risks of insertional mutagenesis and genomic instability, and improving therapeutic safety. The rapid and controlled expression of mRNA-mediated genes avoids the risk of insertion mutations and genomic instability, improving the safety of the treatment, and also enables the design of personalized TCR or CAR T cells based on the patient's specific tumor antigens, improving the specificity and efficacy of the treatment. mRNAs can encode for specific TCR alpha and TCR beta chains, and can be introduced into T cells via electroporation or other delivery systems to cause them to transiently express specific TCRs. These T cells can recognize and kill tumor cells expressing the corresponding antigens[51,52].

## ANTISENSE OLIGONUCLEOTIDE

Antisense oligonucleotides are single-stranded nucleic acid synthesis polymers. ASOs can be divided into two subcategories according to their mode of action:

One mechanism by which ASOs downregulate gene expression involves the promotion of RNA cleavage and degradation through interactions with endogenous enzymes such as ribonuclease H1 (RNase H1) or argonaute2. The ASO binds to target RNAs at its binding site, facilitating the binding of endogenous enzymes that subsequently cleave the target RNAs. This process leads to the degradation of the target RNAs, and its effectiveness relies on the involvement of specific enzymes in the regulatory pathway[13,53]. RNase H1 represents a highly discerning nucleic acid endonuclease with a specific activity targeting the RNA component within RNA-DNA heteroduplexes[24,54]. RNase H1 is present in the nucleus, mitochondria, and cytoplasm of mammalian cells[54,55]. It participates in DNA repair, resolves R-loops, removes pre-mRNAs connected to chromatin, aids in transcriptional termination, maintains genome integrity, and gets rid of RNA linked to Okazaki fragments, among other diverse roles it plays in the genome[56-60]. The primary benefit of employing RNase H1-based ASO lies in its facilitation of targeting nuclear transcripts, such as pre-mRNAs and long-stranded non-coding RNAs[24,61].

The other is occupation-only mediated regulation, often termed steric block[62]. High-affinity ASOs are used in the occupation-only mediated mechanism to bind to target RNAs without directly degrading them[53,63]. ASOs are designed to prevent the establishment of nucleotide compositions within RNA-DNA duplexes. This strategy aims to minimize the risk of generating substrates for RNaseH1 or Ago2, thereby reducing the likelihood of undesired cleavage of target RNA [Figure 1][62]. Spatially blocking ASOs bind to specific sequences of target nucleotides and act by regulating RNA translation, processing, splicing, RNA-protein interactions, and the target RNA interactome[64]. The most common tactic is to modify RNA splicing[65]. Splicing-switching ASOs have the capability to alter splicing patterns by specifically targeting splicing regulatory cis-elements. Through the recruitment of trans-splicing factors, cis-acting elements are essential in either activating or inhibiting adjacent splice sites. These cis-elements include silencers and enhancers for splicing. Stimulated splicing factors have trouble binding to their corresponding enhancer binding sites when ASOs form base pairs with splicing enhancer sequences. Exon skips are caused by this inhibition of binding, which makes splicing more difficult. On the other hand, ASOs that target the splicing silencing sequence element prevent repressive splicing factors from binding. As a result, exon inclusion56 is eventually caused by the silencing element's negative regulatory effect on splicing activation at the splice site [Figure 1][62].

## DELIVERY VEHICLE

Successful RNA therapy depends on the ability to efficiently transport RNA to the cytoplasm through extracellular and intracellular barriers. RNA drugs must evade the large number of RNases present in blood and body fluids[66]. Moreover, RNA medications need to escape from endosomes and enter the cytoplasm in a nontoxic way by crossing the cell membrane and extracellular matrix through receptor-mediated endocytosis[67]. The limitations of RNA drug delivery have been overcome by delivery carriers, and advances in materials science and nanotechnology have also significantly improved drug usage and safety. Carriers can not only encapsulate the drug inside to prevent degradation but also cross the cell membrane to deliver RNA drugs into the cell. In addition, optimization of the carrier itself can play the role of specific targeting, reduce drug off-targeting, and improve the efficacy of RNA drugs while reducing toxic side effects[49,68-70].

Originally designed for the delivery of siRNA, lipid nanoparticles (LNPs) have recently been applied to deliver mRNA and have become the most clinically translatable non-viral delivery vector[29]. Cationic or ionizable lipids have the ability to form complexes with mRNA. Cholesterol enhances stability and facilitates membrane fusion, while polyethylene glycol (PEG)-lipids play a role in controlling size and stability to prevent aggregation. Phospholipids are involved in mediating mRNA encapsulation. By adjusting the ratios of these components, LNPs can be tailored to possess RNA delivery capabilities and target specific organs or cells[44,71-73]. Receptor-mediated endocytosis based on apolipoprotein E (ApoE) or albumin and nonspecific microcellular efflux are the two main mechanisms responsible for the renewal of RNA-loaded LNPs[74,75]. Moreover, LNPs employed for nucleic acid delivery must exhibit metabolic compatibility and swift clearance, thereby mitigating the risk of systemic toxicity attributed to the carrier. This facilitates consistent reproducibility in administration.

Polymeric nanoparticles, serving as non-viral carriers for gene delivery, exhibit promising potential as nucleic acid vectors. Key structural characteristics, such as molecular weight, surface charge, hydrophobicity, and chain compliance, make it easy to determine their fate. The goal of this strategy is to improve gene delivery efficiency[76-79]. Chitosan and natural chitosan nanoparticles, such as exosomes, have been employed as innovative carriers for delivering siRNA. This approach effectively silences crucial target genes *in vivo*, resulting in substantial antitumor activity[80,81]. The linear or branched polycations in the polyethyleneimine (PEI) polymer family can form nanoscale complexes with siRNAs or miRNAs. RNA

molecules are shielded by this interaction, which also makes it easier for them to enter cells[82,83].

## IMPLICATIONS OF RNA-BASED THERAPIES IN HCC

Through inhibition of the Notch3/Hes1/p27 cascade reaction, transcription factor C/EBP-α (CCAAT/enhancer-binding protein alpha) is a major regulator of liver and medullary function, as well as several oncogenic processes. It also suppresses tumor growth *in vitro* and *in vivo* and its expression is suppressed in several liver diseases, including HCC[84]. MTL-CEBPA, a liposomal nanoparticle-formulated small activating RNA (CEBPA-51) that upregulates hepatic C/EBP-α expression[85], is the first saRNA drug to enter clinical trials. It was tested in a phase I clinical trial in patients with advanced hepatocellular carcinoma in combination with sorafenib, and it demonstrated an acceptable safety profile in hepatocellular carcinoma as well as potential synergism with TKIs[86] [Table 1] [https://clinicaltrials.gov/study/NCT02716012]. A phase II study is recruiting patients with advanced HCC caused by hepatitis B or hepatitis C infection [Table 1] [https://clinicaltrials.gov/study/NCT04710641]. Additionally, a Phase I clinical trial combining MTL-CEBPA with bevacizumab and atilizumab, the standard of care, is being conducted to treat advanced hepatocellular carcinoma [Table 1] [https://clinicaltrials.gov/study/NCT05097911].

siRNA drugs efficiently silence the expression of target genes in a sequence-specific manner, providing an excellent opportunity to use this strategy in targeted cancer therapies. The MYC pathway is activated in 70% of hepatocellular carcinoma[87], which is involved in a variety of cellular processes that promote tumorigenesis, including cell cycle progression, apoptosis inhibition, cellular metabolism, and immune evasion[88]. MYC is therefore a very desirable therapeutic target. DCR-MYC is a lipid particle created by Dicerna Pharmaceuticals that binds to synthetic double-stranded RNA to target and silence the MYC oncogene, thereby preventing the spread of cancer. Patients with hepatocellular carcinoma who are either sorafenib-intolerant or sorafenib-refractory were evaluated for DCR-MYC in a Phase Ib/II clinical trial. Nevertheless, preliminary findings fell short of projections, leading to the termination of all DCR-MYC clinical trials, necessitating additional research [Table 1] [https://clinicaltrials.gov/study/NCT02314052]. The most well-known member of the serine-threonine kinase family, Plk1, is a Polo-like kinase (Plks) that controls cell mitosis and is overexpressed in many cancers[89]. miRNA targeting Plk1 is used to make TKM-080301 (TKM-PLK1), a lipid nanoparticle formulation that suppresses Plk1 expression, cleaves Plk1 mRNA, and exhibits strong antiproliferative activity in a variety of cancer cell lines in human cancer cells[90]. To investigate the safety, tolerability, pharmacokinetic parameters, and antitumor activity of TKM-PLK1 in patients with advanced hepatocellular carcinoma, TKM-080301 was assessed in a human dose-escalation Phase I study in patients with primary or secondary hepatocellular carcinoma. The drug was injected directly into the cancer blood supply in the hepatic circulation [Table 1] [https://clinicaltrials.gov/study/NCT01437007]. TKM-080301's toxicity evaluation at the tested doses showed a favorable profile. However, patients with advanced hepatocellular carcinoma did not show improved overall survival outcomes when a small interfering RNA mechanism was used to target PLK1. As a result, this lack of effectiveness does not justify additional thought for assessment as a treatment agent on its own[91]. Two siRNAs that specifically target kinesin spindle protein (KSP) and vascular endothelial growth factor (VEGF)-A are included in the therapeutic formulation ALN-VSP02. Stable nucleic acid lipid particles (SNALP) contain these siRNAs. Cancer patients have higher levels of KSP and VEGF expression, and it has been demonstrated that lowering these levels will prevent tumor cell proliferation and angiogenesis. The safety, tolerability, pharmacokinetics, and pharmacodynamics of intravenous administration of ALN-VSP02 in patients with advanced solid tumors involving the liver were evaluated in a phase 1 dose-escalation trial [Table 1] [https://clinicaltrials.gov/study/NCT00882180]. This phase has been completed and ALN-VSP25 was well tolerated with antitumor activity[92].

**Table 1. RNA-based therapeutics in clinical trials in liver cancer**

| RNA class | Target gene | Drug | delivery method | Administration route | Sponsor | Phases | References |
|---|---|---|---|---|---|---|---|
| siRNA | MYC | DCR-MYC | LNP | I.V. | Dicerna Pharmaceuticals, Inc., a Novo Nordisk company | Ib/II | [https://clinicaltrials.gov/study/NCT02314052] |
| siRNA | PLK1 | TKM-080301 | LNP | HAI | National Cancer Institute | I | [https://clinicaltrials.gov/study/NCT014337007] |
| siRNA | KSP, VEGF | ALN-VSP02 | SNALP | I.V. | Alnylam Pharmaceuticals | I | [https://clinicaltrials.gov/study/NCT00882180] |
| saRNA | CEBPA | MTL-CEBPA+Sorafenib | LNP | I.V. | Mina Alpha Limited | I | [https://clinicaltrials.gov/study/NCT02716012] |
| saRNA | CEBPA | MTL-CEBPA+Sorafenib | LNP | I.V. | Mina Alpha Limited | II | [https://clinicaltrials.gov/study/NCT04710641] |
| saRNA | CEBPA | MTL-CEBPA +Atezolizumab+Bevacizumab | LNP | I.V. | National University Hospital, Singapore | I | [https://clinicaltrials.gov/study/NCT05097911] |
| microRNA | MRX34 | MRX34 | Liposomal | I.V. | Mirna Therapeutics, Inc. | I | [https://clinicaltrials.gov/study/NCT01829971] |
| mRNA | CEA | CEA RNA-pulsed DC cancer vaccine | DC vaccine | I.V. | Duke University | I | [https://clinicaltrials.gov/study/NCT00004604] |
| mRNA | Neoantigen | Neoantigen mRNA vaccine with or without PD-1/L1 | _ | _ | Jianming Xu | I | [https://clinicaltrials.gov/study/NCT05192460] |
| mRNA | Neoantigen | Neoantigen mRNA vaccine + Stintilimab I | _ | _ | Shanghai Zhongshan Hospital | I | [https://clinicaltrials.gov/study/NCT05761717] |
| mRNA | HBV | HBV mRNA Vaccine | _ | I.M | West China Hospital | I | [https://clinicaltrials.gov/study/NCT05738447] |
| mRNA | Neoantigen | ABOR2014/IPM511 | _ | I.M | Peking Union Medical | I | [https://clinicaltrials.gov/study/NCT05981066] |
| mRNA | MYC | OTX-2002+Tyrosine | LNP | I.V. | College Hospital Omega Therapeutics | I/II | [https://clinicaltrials.gov/study/NCT05497453] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| mRNA | HBV | kinase inhibitor+Checkpoint | _ | _ | Lion TCR Pte. Ltd. | I | [https://clinicaltrials.gov/study/NCT03634683] |
| mRNA | HBV | Inhibitor LioCyx | _ | _ | Lion TCR Pte. Ltd. | I | [https://clinicaltrials.gov/study/NCT05195294] |
| mRNA | HBV | LioCyx-M+ Lenvatinib | _ | _ | Lion TCR Pte. Ltd | I | [https://clinicaltrials.gov/study/NCT04745403] |
| mRNA | HBV | mRNA HBV/TCR T-cells | _ | _ | Beijing 302 Hospital | I | [https://clinicaltrials.gov/study/NCT03899415] |
| ASO | XIAP | TCR redirected T cells AEG35156+ Sorafenib | _ | I.V. | Aegera Therapeutics | I | [https://clinicaltrials.gov/study/NCT00882869] |

• Mis-expression or mutation of certain factors in the miRNA machinery can lead to altered processing, stability, or targeting of miRNAs, which can lead to serious diseases including cancer[27]. The miR-34 family suppresses tumorigenesis and slows the progression of tumors by taking part in the epithelial-mesenchymal transition (EMT) through the EMT-transcription factor p53 and various key signaling pathways[93]. The phase I clinical trial of MRX34, the first tumor-targeting miRNA drug based on a miR-34a mimic, ended prematurely due to five immune-related adverse events; however, the proof-of-concept for miRNA-based cancer therapy was provided by the dose-dependent regulation of the associated target genes[94] [Table 1] [https://clinicaltrials.gov/study/NCT01437007].

There are currently very few studies specifically for HCC, and most clinical trials for cancer vaccines are in phase I or phase II. The field of cancer vaccines is still in its developmental stage. Developing or bolstering anti-cancer immunity is the main goal of cancer vaccines. Tumor antigens elicit an immune response against cancer, and developing new tumor antigens presents several difficult obstacles. Globally, around 350 million individuals are chronically infected with HBV, and chronic HBV infection is a major contributor, constituting at least 50% of hepatocellular carcinoma cases worldwide[1]. Anti-HBV represents a promising focus in addressing hepatocellular carcinoma. A phase I clinical study is currently in progress, evaluating an mRNA vaccine for treating individuals with HBV-positive advanced hepatocellular carcinoma. This study aims to enroll patients who have experienced failure with second-line standard therapy or are ineligible for standard therapy. The trial involves a dose-escalation phase to determine an effective dosage for subsequent fixed-dose trials. The investigation focuses on assessing the safety, tolerability, and efficacy of applying mRNA immunotherapy technology in the clinical treatment of advanced hepatocellular carcinoma [Table 1] [https://clinicaltrials.gov/study/NCT05738447]. Mutations occurring in cancerous cells lead to the creation of novel self-antigenic epitopes known as neoepitopes or neoantigens. These personalized neoantigens can elicit T-cell responses specific to the tumor, preventing unintended damage to non-tumor tissues. Because neoantigens emerge from somatic mutations, they have the potential to evade the central T-cell tolerance to their epitopes, thereby stimulating immune responses against tumors. A clinical trial is underway, recruiting patients with advanced hepatocellular carcinoma. Through intramuscular injection, participants will receive a fixed applicable dose of the ABOR2014 (IPM511) vaccine. The trial's objectives

are to evaluate ABOR2014 injection's preliminary efficacy, safety, and tolerability in patients with relapsed or recurrent hepatocellular carcinoma [Table 1] [https://clinicaltrials.gov/study/NCT05981066]. In many clinical trials, mRNA cancer vaccines are administered together with checkpoint modulators (PD-1, CTLA-4, and TIM3) or cytokine mixtures to boost the effectiveness against tumors. A dose-escalation clinical trial is currently enrolling participants to assess the safety and tolerance of a neoantigen tumor vaccine in individuals with advanced gastric, esophageal, and hepatocellular cancers. The trial also aims to provide preliminary insights into the efficacy of the neoantigen tumor vaccine in treating advanced cases of gastric, esophageal, and liver cancer when combined with PD-1/L1 therapy [Table 1] [https://clinicaltrials.gov/study/NCT05192460]. An open, single-arm study is also evaluating the safety and efficacy of an mRNA personalized tumor vaccine encoding the neoantigen in combination with sintilimab injection for the adjuvant prevention of postoperative recurrence of hepatocellular carcinoma[95].

Notably, mRNA-4157/V940 was created and produced based on a distinct mutational profile of the patient's tumor DNA sequence. This mRNA encodes a single mRNA that can contain up to 34 neoantigens. In this Phase IIb clinical trial, postoperative patients with intermediate to advanced melanoma who were at high risk of recurrence received an adjuvant treatment consisting of the mRNA-4157/V940 vaccine plus pembrolizumab [Table 1] [https://clinicaltrials.gov/study/NCT03897881]. According to data, patients who received both the mRNA vaccine and pembrolizumab had a 44% lower risk of dying or having their disease return than those who only received pembrolizumab[96]. In February 2023, the mRNA vaccine, in combination with a PD-1 inhibitor, was cleared by the FDA as a new adjuvant therapy for the treatment of melanoma with a high risk of recurrence. This certification marks the first-ever mRNA vaccine against cancer worldwide, and it represents a significant advancement for mRNA vaccines in the anti-tumor arena. It is anticipated that this vaccine's phase III clinical trials will begin in accordance with other cancer types.

OTX-2002 is an mRNA therapy given to patients via LNP. It downregulates MYC expression pre-transcriptionally through epigenetic regulation while overcoming MYC auto-regulation. OTX-2002 is currently being evaluated as a single agent and in combination with a TKI or PD-(L)1 inhibitor in Phase 1/2 trials[97]. The primary endpoints of this trial for relapsed or refractory HCC and other solid tumor types known to be associated with the MYC oncogene are to determine the maximum tolerated dose of therapy, dose-limiting toxicity, incidence of therapeutic-emergent adverse events, overall efficacy rate, and duration of response [Table 1] [https://clinicaltrials.gov/study/NCT05497453].

The mRNA of the antigen-specific TCR-expressing antigen is introduced into T cells by electrotransformation and transiently expressed on T cells. Such a protocol is able to strike a good balance between safety and efficacy, ensuring that the infused specific TCR-T cells reach the target site through the blood circulation, triggering a controlled local inflammatory response that effectively kills tumor cells. Targeting HBV antigens expressed on HBV-HCC cells by transfecting autologous T cells (HBV-TCR T cells) with mRNA encoding HBV antigen-specific TCRs is currently the most widely used strategy [https://clinicaltrials.gov/study/NCT05738447][51,52,97], and several studies have entered clinical trials [Table 1].

## DISCUSSION

Hepatocellular carcinoma, a highly aggressive form of cancer, has spurred ongoing exploration for novel therapeutic approaches due to the limitations of conventional treatments. RNA therapies have emerged as promising and innovative advancements in the field of hepatocellular carcinoma immunotherapy. The COVID-19 pandemic marked a significant milestone with the approval of two mRNA vaccines, namely mRNA-1273-P301 and BNT162b1. These vaccines were instrumental in initiating an immune response against SARS-CoV-2 and in spurring the mainstream drug development landscape to embrace RNA

interference (RNAi) and mRNA-based therapies[98,99]. The results of RNA-based treatments have opened up new directions for the study of RNA components in the creation of cancer treatments. A more focused approach to therapy is provided by the development of personalized therapeutic strategies. By analyzing an individual's genomic profile, a customized mRNA vaccine is formulated to effectively stimulate the immune system for attacking hepatocellular carcinoma. The execution of clinical trials highlights the substantial potential of this approach in enhancing patient survival and reducing recurrence rates. The prospect of personalized mRNA vaccine therapy emerges as a crucial direction for future research. Given that siRNA/miRNA presents a polygenic challenge, advancements in understanding these small RNAs are anticipated to contribute to the development of more effective "combinatorial approaches" to cancer treatment, leveraging the multi-targeting properties of these small RNAs. Integrating RNA interference technology with other therapeutic modalities (e.g., chemotherapy, radiotherapy) will enable the implementation of multi-targeted therapeutic strategies to enhance overall treatment efficacy. The development of small molecule RNA-targeted small molecules (RTMs) is in its early stages and shows great potential. These technologies not only provide highly specific and effective means of gene regulation, but also avoid the risk of genomic integration associated with traditional gene therapy. RTMs specifically bind RNA molecules through small molecule compounds, inducing their degradation or inhibiting their function[100]. RIBOTACs (Ribonuclease-targeting chimeras) can precisely target and degrade pathogenic RNAs, showing great potential in the treatment of cancer, genetic diseases, viral infections, *etc.* Disrupting RNA-protein interactions specifically interferes with RNA-protein interactions and prevents the function of pathogenic RNAs. The discovery and optimization of small molecule compounds using high-throughput screening and structural biology techniques to target RNA molecules with greater specificity and potency has shown great potential in the treatment of various diseases, including cancer, genetic diseases, and viral infections[12,101].

Furthermore, highly customized delivery platforms are made possible by optimizing targeting functionality and physical properties of nanoparticles. Increasing the effectiveness of RNA delivery will be largely dependent on the creation of innovative nanomaterials. By utilizing targeting molecules with specific affinity, RNA will be precisely directed to the surface of hepatocellular carcinoma cells to improve the precision of delivery. In addition, optimization of the physicochemical properties of the delivery system is also an important direction to ensure that RNA can be released stably and controllably *in vivo*.

RNA therapy also has some possible toxicity and safety issues. mRNA and siRNA, which may be recognized by the body's immune system as foreign substances, activate innate and adaptive immune responses, which may lead to inflammatory responses and cytokine storms, resulting in serious side effects[102,103]. Additionally, RNA molecular delivery systems may pose additional safety concerns. Lipid nanoparticles (LNPs) may be inherently toxic, possibly triggering an immune response or organ toxicity[72,73]. To reduce risk, researchers are developing safer delivery systems, optimizing RNA sequence design, and conducting exhaustive toxicology studies to ensure the safety and efficacy of RNA therapies in clinical applications.

RNA therapy has experienced notable advancements in the realm of cancer treatment, particularly due to its capacity to target diverse genetic materials within the body and the swift progress in drug development. It is plausible to anticipate an increased emphasis on research endeavors dedicated to the evolution of RNA-based therapies in the coming years. Within the domain of hepatocellular carcinoma immunotherapy, RNA therapies exhibit substantial potential for innovation. Future developments in RNA therapy will concentrate on enhancing precision and individualization, as well as exploring combination strategies. This approach aims to offer more efficacious and personalized options for the treatment of liver cancer in the future.

## DECLARATIONS

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The figures are created with BioRender.com and quoted with permission from BioRender.

### Authors' contributions

Initiated the study and finalized the manuscript: Liu M
Reviewed the literature and wrote the manuscript: Xing L, Wang ZK, Li DM, Li J
Reviewed the literature and drew the pictures: Xing L
Review and revised the manuscript: Liu M
Read and approved the final manuscript: Xing L, Wang ZK, L DM, Li J, Liu M

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

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