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Advances and applications of RNA vaccines in tumor treatment

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Abstract
Compared to other types of tumor vaccines, RNA vaccines have emerged as promising alternatives to conventional vaccine therapy due to their high efficiency, rapid development capability, and potential for low-cost manufacturing and safe drug delivery. RNA vaccines mainly include mRNA, circular RNA (circRNA), and Self-amplifying mRNA(SAM). Different RNA vaccine platforms for different tumors have shown encouraging results in animal and human models. This review comprehensively describes the advances and applications of RNA vaccines in antitumor therapy. Future directions for extending this promising vaccine platform to a wide range of therapeutic uses are also discussed.

Introduction

Immunotherapy has become an essential tool in treating malignant tumors [1]. Immune checkpoint inhibitors such as anti-CTLA-4 and anti-PD-1/PD-L1 prolong patient survival in various cancer types [2]. Adoptive T-cell therapy (ACT) and chimeric antigen receptor (CAR) therapy have shown that the anti-tumor response of T cells can be stimulated by recognizing mutant neoantigens [3]. These therapies bring new ideas for immunotherapy of malignant tumors.

Tumor vaccines can target tumor cells that overexpress antigens and achieve a long-term immune response due to immune memory. Thus, compared to other immunotherapies, tumor vaccines offer specific, safe, and tolerable treatment [4]. Conventional tumor vaccines target tumor-associated antigens (TAAs), a regular host protein that induces central and peripheral immune tolerance. However, T cells that recognize TAAs will likely be cleared during development, leading to poor efficacy of tumor vaccines targeting TAA [5]. Tumor-specific antigens (TSAs) can be identified by major histocompatibility complex (MHC) class I or II molecules as neoantigens not present in the host, offering new hope for tumor vaccines [6]. MHC I molecules recognize peptides mainly consisting of 8–10 amino acid fragments. MHC II molecules recognize 13–25 amino acid fragments, which impose stringent requirements on the length and immunogenicity of the neoantigens [7]. Tumor vaccines include cellular vaccines (modified tumor cells, dendritic cell vaccines loaded with tumor antigens), viral vector vaccines, DNA vaccines, RNA vaccines for protein, or synthetic peptide vaccines for tumor antigens [8]. The mechanisms of common tumor vaccines are summarised in Fig. 1.

However, DNA vaccines carry a risk of integration into the host genome and are inefficient at generating neoantigens [9]. Peptide or protein vaccines need to be cleaved into small peptides with the help of histone proteases in the endosome after entering the cell before they can be recognized by MHC molecules [10]. This results in its insufficiency in promoting antigen presentation and generating practical and durable anti-tumor immunity [11]. The high cost and difficulty of preparing personalized

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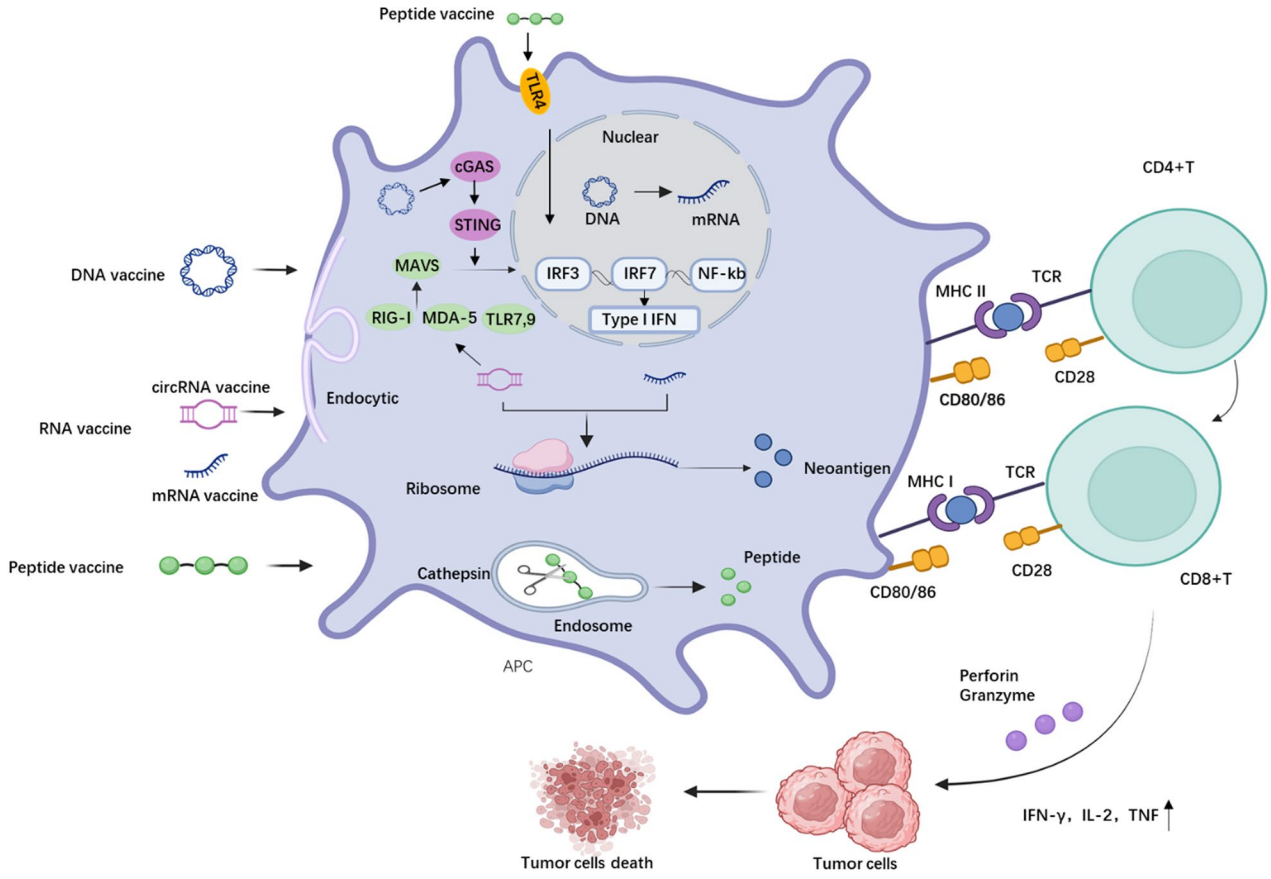

Fig. 1 Mechanisms of action of different vaccines. RIG-I: retinoic acid-inducible gene I; MDA5: melanoma differentiation-related gene 5; MAVS: mitochondrial antiviral signaling protein; cGAS: Cyclic GMP-AMP synthase. IRF: interferon-regulating factor APC: antigen-presenting cell; MHC: major histocompatibility complex; TCR: T-cell receptor. TLR: TLR: toll-like receptor

dendritic cells have hampered their clinical application [12].

Unlike DAN or peptide vaccines, RNA vaccines can simultaneously encode the entire length of tumor antigens, enabling antigen-presenting cells to present multiple epitopes specific to human leukocyte antigens (HLA) classes I and II. Therefore, they are less type-restricted by human HLA and more likely to stimulate a broader range of T-cell responses. There is no risk of integration into the host genome [13].

In 1990, Wolff et al. demonstrated for the first time that intramuscular injection of an mRNA vaccine in mice produced the target protein [14]. mRNA vaccines have the unique advantage of introducing exogenous mRNAs encoding antigens into the body and translating and synthesizing the antigens in the cells [15]. In addition, mRNA vaccines have the benefit of being easily accessible and inexpensive [16]. It has been demonstrated that mRNA tumor vaccines can induce stronger humoral and cellular responses, improving therapeutic outcomes [17]. Cafri et al. reported that an mRNA vaccine using tumor-infiltrating lymphocytes to recognize specific immunogenic mutations expressed in patients' tumors induced neoantigen-specific T-cell immunity in gastrointestinal cancer patients [4].

Another one that promises to maximize antigen production and yield is the self-amplified mRNA (SAM) vaccine. SAM is derived from positive single-stranded mRNA viruses, most commonly from alphaviruses [18]. The genes coding for the structural proteins of alphaviruses formed using viral particles have been replaced by genes coding for the target antigens. At the same time, the RNA replication mechanism remains in place.SAM is also expected to become an essential tumor treatment due to its long-lasting efficacy and lower injection dose [19]. circRNA has been regarded as a non-coding RNA with no biological effect. In recent years, it has been found that circRNAs can mediate the initiation of ribosome initiation and protein translation through the internal ribosomal entry site (IRES) element in the absence of the free 5′ ends to achieve antigen presentation, thus becoming a new research hotspot for tumor vaccine research. Protein translation without the free 5′ ends to present antigens, thus becoming a new research hotspot for tumor vaccines [20].

Significant technological innovations in recent years have made RNA more viable candidates for tumor vaccines. Various modifications to the main chain and untranslated regions of RNA have made them more stable and translatable. Combining RNA with delivery vectors allows for more efficient in vivo delivery and easier mRNA production using in vitro transcription (IVT) methods [16].

In this review, we discuss improvements made to the structure of RNAs to increase stability and translational efficiency, highlight the advantages and limitations of various RNAs as tumor vaccines, and summarise the progress and clinical applications of RNA tumor vaccines and a summary of the different types of adjuvants and delivery vehicles. And the current clinical applications and challenges of RNA tumor vaccines.

Screening for appropriate neoantigens

The discovery of suitable neoantigens is an essential factor in synthesizing tumor vaccines. Neoantigens include TSAs and TAAs.TSAs are produced by tumor-recognized cells as a result of specific alterations such as gene mutations, dysregulated RNA splicing, and aberrant post-translational modifications, and these can give rise to cancer-specific neoepitopes that are recognized by autologous T cells [21]. Since neoepitopes are not subject to central immune tolerance and are not expressed in healthy tissues, they are attractive targets for therapeutic tumor vaccines [22]. TSAs identify tumor-specific non-synonymous mutants in protein-coding genes by comparing next-generation sequencing (NGS) data from patient tumors and healthy tissues [23]. Multicomponent calculations assess the binding of mutant peptide regions to the patient's HLA allele, which helps T cells to induce practical anti-tumor effects [24]. These data enable the production of personalized tumor vaccines with unique components for each patient [25].

TAAs are antigens that are aberrantly expressed or produced only at specific stages of differentiation with limited expression in normal tissues, such as ERBB2, mucin1, and gp100 [26]. However, TAAs are unmutated autoantigens, and central T cell tolerance may lead to poor T cell responses [27].

Characteristics of RNA vaccines

mRNA vaccine

mRNA is a single-stranded macromolecule, and given that mRNA can be translated into protein, this principle can be used to produce any protein [28]. The basic principle of mRNA as a tumor vaccination is that it encodes one or more TAA or TSA and is delivered to the cytoplasm of antigen-presenting cells to express antigens, exerting anti-tumour effects [29].

Self-amplifying mRNA(SAM)vaccine

Self-amplifying mRNAs (SAMs) carry essential components capable of self-amplification and expressing the target protein. In Table 1, we summarise the different types of RNA vaccines. Genes encoding viral non-structural proteins (nsP) are critical elements for self-amplification of target antigens and can encode RNA polymerase [30]. SAM was found to be an autoadjuvant, stimulating plasma and endosomal pattern-recognition receptors (PRRs) and inducing an immune response [31]. After delivery to target cells, SAM can produce large amounts of antigen from tiny doses of the vaccine due to the presence of the nsP gene, which provides multiple RNA replication cycles [32]. Thus, the SAM vaccine has the advantage of more efficient antigen expression at lower doses (Fig. 2) [33].

Antisense oligonucleotides (ASOs) vaccine

Antisense Oligonucleotides (ASOs) consist of short single-stranded RNAs 18–21 deoxyribonucleotides in length that complement the pre-RNA or mRNA sequence of the target gene and prevent the formation of the 5'-mRNA cap, which cuts off the target RNA via RNase H, thus preventing the protein from being expressed [34]. The

Table 1 Comparison of different types of RNA vaccines

Vaccine types	Structures	Salient features	Disadvantages
mRNA	Linear	• Direct expression of target antigen. • Nucleotide modification is possible. • Shorter sequence length.	• Short expression durations. • Higher effective doses are needed for anti-tumor effects.
Self-amplifying mRNA	Linear	• Lower doses, higher antigen expression. • Safer immunization • Self-adjuvant effect	• Non-protein structures of viruses may undergo recombination with the host genome. • Reduced delivery efficiency due to larger molecular weights.
Antisense Oligonucleotides	Linear	• ASOs target the target RNA and cut it off via RNase H, preventing the protein from being expressed. • RNase H exists in the nucleus as well as outside the nucleus, so ASOs can regulate a wide range of RNAs.	• Delivery inefficiencies • High Costs
CircRNA	circle	• Circle structure for more excellent stability • Self-adjuvant effect	• Inefficient cyclisation • Higher preparation costs

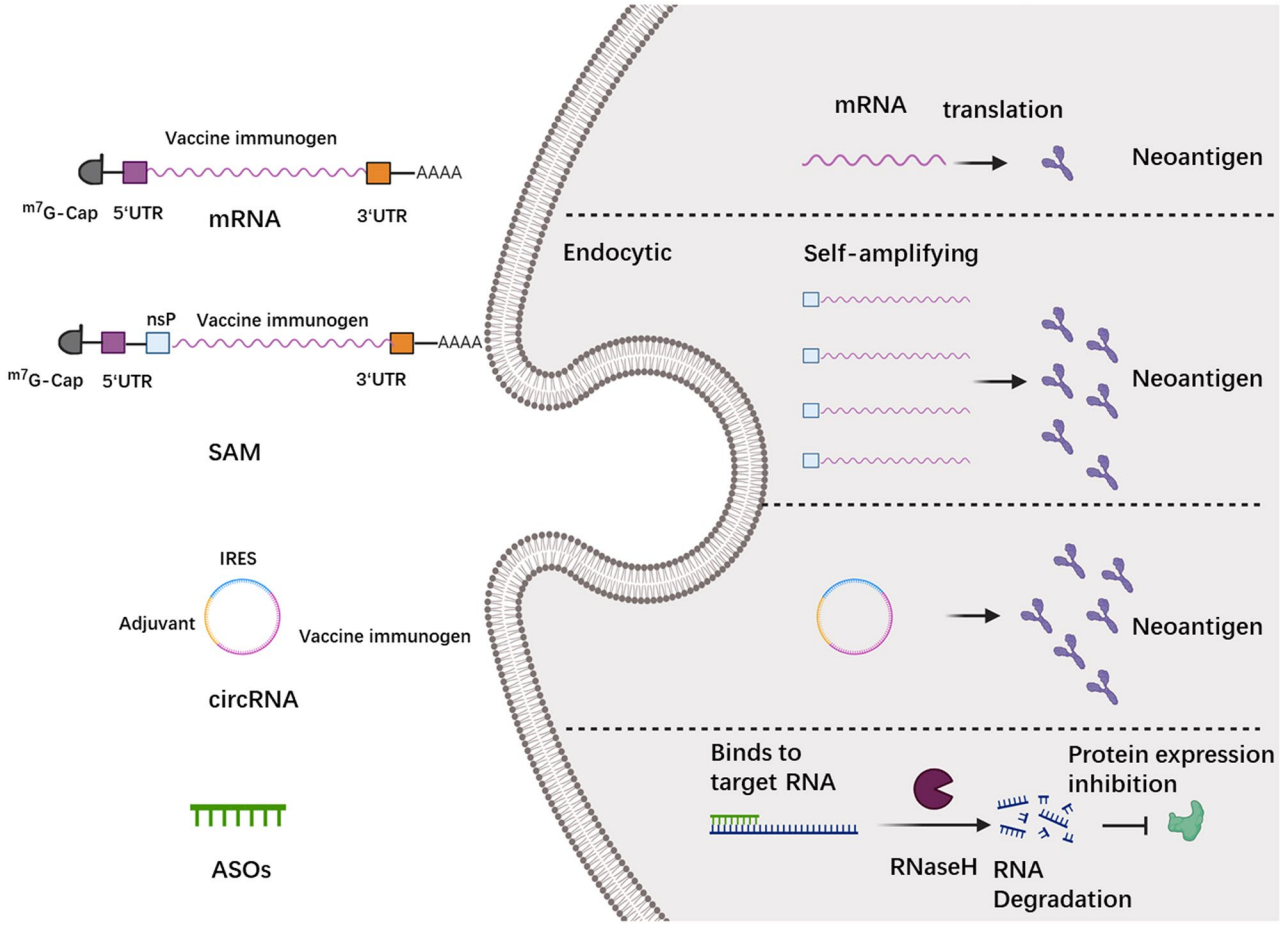

Fig. 2 The Mechanisms of different types of RNA vaccines. It compares the mRNA, self-amplifying mRNA, circRNA, and antisense oligonucleotides vaccine pathways for antitumor effect

free and open availability of human genome sequence information allows for obtaining specific synthetic ASOs designed against specific target genes.

circRNA vaccine

circRNAs are a class of natural or synthetic closed RNAs that do not have 5' or 3' ends [35]. Their unique covalent closed structure prevents RNases from degrading, making them more drug-stable and biologically stable than linear mRNAs [36]. Natural circRNAs can be non-coding RNAs with specific regulatory capabilities [37]. Recent evidence suggests that some circRNAs contain open reading frames (ORFs), IRES, and N6-adenylate methylation (m6A)-modified nucleotide sequences that enable natural circRNAs to encode and translate proteins and peptides [38]. Insertion order of IRES elements or modification of the m6A and 5′ untranslated region (UTR) has improved translation efficiency in a 5′ cap non-dependent manner [20]. CircRNAs, because of their circular structure, may allow ribosomes to readily re-engage after a translation cycle, resulting in a unique rolling loop translation, thus increasing translation efficiency [39]. These properties give circRNAs great potential. Thus, exploiting the tumor-specific and protein-coding capabilities of circRNAs would make themselves or their encoded products potential neoantigens and, thus, attractive targets for tumor vaccines. To improve the expression efficiency of the encoded neoantigen, adding a human tissue plasminogen activator (tPA) signal sequence to the encoded gene's N-terminus increases the neoantigen secretion [40]. CircRNAs can enhance antitumour immune responses by modulating immune cells and are somewhat immunogenic. This also provides the potential to use them as self-adjuvanted vaccines [41].

RNA vaccines in pre-clinical studies

mRNA vaccine

Increased stability and improved translational efficiency of the mRNA vaccine by modification have led to their great utility in vaccines for SARS-CoV-2 and infectious diseases and their great potential as tumor vaccines. The mRNA vaccine encoding OVA also showed improved protection and therapeutic efficacy in a B16F10 melanoma model carrying the OVA antigen [17]. In colorectal

cancer, mRNA vaccines encoding tumor neoantigens have been shown to induce effective T-cell responses and inhibit colorectal cancer tumor growth [42]. Similarly, Deng et al. found that an mRNA vaccine encoding OX40L triggered a robust immune response and boosted survival in mice with hepatocellular carcinoma [43].

However, effective therapeutic tumor vaccines rely on antigen presentation and DC activation of the immune system. Functional inhibition of DCs is a hallmark of tumor immune evasion [44]. Therefore, antigen-presenting cells (APCs) must first be activated to initiate the adaptive immune system. Activation of DCs with immunostimulatory adjuvants is a critical component of many tumor vaccine strategies [44] and can enhance the immunogenicity and efficacy of mRNA vaccines [45]. Adjuvant mainly targeted recognition of Toll-like receptors (TLRs) and RIG-I-like receptors typical in mRNA vaccines. The novel TLR agonist molecular adjuvant Poly (I: C) is a ligand for TLR3 and can activate other pattern recognition receptors (PRRs) in the APC, such as MDA5, which belongs to the retinoic acid-inducible gene I-like receptor (RLR) family and recognizes mainly RNAs [46], leading to a helper T-cell (Th) type 1 response and CTL production [47]. It has been found that bacterial-derived outer membrane vesicles (OMVs) combined with adjuvant Poly (I: C) significantly inhibit the progression of melanoma and colon cancer and induce long-term immune memory in mice [48].

Li et al. found that using C3d as an adjuvant for mRNA vaccines elicited a strong reaction in mouse T cells [49]. C3d is part of an immune response known as the complement system that binds to antigens and amplifies the antibody response to those antigens [50]. The mechanism by which C3d enhances the immune response involves binding and signaling via complement receptor 2 (CR2) [51].C3d binds to CR2, stimulates antigen presentation via DC, and contributes to maintaining immune B cell memory [52]. At the same time, the interaction of C3d with CR2 will collect molecules that trigger signaling cascades, leading to cell activation and proliferation-enhancing antigen uptake [53]' [54].

Similarly, Wang et al. found that mRNA antigen with MHC class I molecules (MITD) -associated mRNA enhances antigen presentation to trigger antigen-specific T-cell responses [55]. MITD significantly enhances the presentation of MHC class I and II epitopes in human APCs when bound to antigens [56]. There is no consensus on the optimal adjuvant for an oncology vaccine, representing a future avenue of research to further optimize vaccine design [57].

SAM and ASOs vaccine

As a SARS-CoV-2 vaccine candidate, SAM demonstrated safe and robust protective immunity in preclinical models in mice, guinea pigs, and rats, generating a potent and specific antibody response after vaccination [33]. Based on its powerful effects, the ARCT-154 vaccine became the first fully approved influenza vaccine [58].

SAM also plays a prominent role in tumor vaccines. Jamile et al. found that a SAM tumor vaccine encoding the target antigen gDE7 exhibited anti-tumor effects in a mouse model of HPV-16-associated tumors [59]. Luisi et al. reported the development of a Zika virus SAM vaccine, which demonstrated a potent neutralizing antibody response after two immunizations in mice and non-human primates, effectively protecting the animals against the Zika virus [60]. These data provide strong support for SAM as a tumor vaccine.

Vaccines for ASOs are also playing an active role in tumor therapy. Rubenstein et al. evaluated the effects of bispecific ASOs targeting BCL-2 and epidermal growth factor receptor (EGFR) on the in vitro growth and expression of prostate antigens in androgen-sensitive human prostate cancer (LNCaP) cells. Cell growth and up-regulation of interferon-gamma expression were significantly inhibited after treatment with bispecific ASOs. This suggests that a vaccine with bispecific ASOs formed by double strands enhances the expression of cell surface antigens and may enhance the efficacy of tumor vaccines [61].

circRNA vaccine

It has been shown that exogenously infused circRNA can successfully express neoantigens in vivo, which can activate innate immune genes that enhance the anti-tumor immune response, such as protein kinase R (PKR), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-related gene 5 (MDA5) [62]. CircRNA induces more robust RIG-I expression than linear mRNAs of the same sequence [63]. Activation of RIG-I provides a complete IFN pathway and produces many cytokines that act as an immune response to clear pathogens and even tumor cells [64]. It has been found that circRNAs exhibit higher translation efficiency and longer duration than their linear counterparts when coding for the same antigenic sequence [40]. Furthermore, exogenous circRNA activates both DCs and induces robust T and B cell immune responses [65]. None of the animals showed typical clinical signs throughout immunization with the circRNA SARS-CoV-2 vaccine [40]. It showed no significant change in body weight, temperature, heart rate, other factors from pre-treatment, no cytokine storm from immunotherapy, and almost undetectable TNF-a, IL-1β, and IFN-α [40]. Thus, circRNA vaccines have the outstanding advantage of being tolerable and safe.

Wang et al. found that circRNA encoding hepatocellular carcinoma neoantigen (Ptpn2_I383T) promotes dendritic cell activation and enhances tumor cell killing by

T cells, and superior tumor therapeutic and prophylactic effects in mouse tumor models [66]. Several studies have found that in vitro synthesized circRNA vaccines encoding OVA combined with adjuvant Poly(I: C)significantly inhibit melanoma growth [67, 68]. Tumour-specific circFAM53B combined with adjuvant Poly (I: C) generates cryptic peptides through non-classical translation that bind to HLA-I and HLA-II and activate anti-tumour immune responses. Mouse experiments demonstrated that vaccination with tumor-specific circRNAs or their encoded peptides effectively controlled tumors, suggesting that such vaccines may be an immunotherapeutic strategy against malignant tumors [69].

Therefore, circRNA neoantigen vaccine-induced antitumour immune responses may show better therapeutic efficacy than other vaccines. Combining circRNAs encoding proteins as neoantigens is an emerging tumor immunotherapy with great potential as a vaccine and therapeutic approach.

Anti-tumour role played by mRNA vaccines in the clinical setting

Recently, a few clinical trials have used mRNA vaccines to treat tumors. One study (NCT03480152) developed a method using tumor-infiltrating lymphocytes to identify specific immunogenic mutations expressed in patients' tumors [4]. Validated, defined neoantigens, predicted neoepitopes and mutations in driver genes were tandemly linked into a single mRNA construct to vaccinate patients with metastatic gastrointestinal cancer. Results showed that the vaccine was safe and stimulated antigen-specific T-cell responses [4].

In clinical trial NCT03394937, 20 patients with stage IIc, III, and IV resected melanoma received an intranodal mRNA vaccine (ECI-006), which consists of mRNA encoding melanoma TAA and the adjuvant TriMix, which encodes the DC activators: CD40L, CD70, and caTLR4. Patients with metastatic melanoma who had stable disease after 3–12 months of standard therapy received the ECI-006 vaccine in combination with standard anti-PD-1 therapy; patients well tolerated the dose of 600–1800 μg. ECI-006 was immunogenic in 40% of patients [70].

In addition to mRNA vaccines alone, combination immunotherapy is also an important therapeutic tool. Luis A et al. found that individualized mRNA neoantigen vaccines in combination with atelizumab (anti-PD-L1 immunotherapy) also showed great potential for pancreatic cancer patients with poor immunotherapy outcomes [71]. Its mRNA vaccine contains 20 MHC class I and MHC class II-restricted neoantigens, injected intravenously into the patient's body with lipid nanoparticles (LNPs), massively activating the patient's T-cells and delaying relapse [71]. A randomized phase 2b study (NCT03897881) using an mRNA tumor vaccine encoding 34 neoantigens (mRNA-4157/V940) with or without pembrolizumab (anti-PD-1 immunotherapy) to treat patients with surgically resected high-risk melanoma (stage III/IV) and found that Compared with pembrolizumab alone, recurrence-free survival (RFS) was 78.6% in the combination group, which was higher than the 62.2% in the pembrolizumab alone treatment group. Patients in the combination group had a 44% lower risk of recurrence or death [72].

Studies have shown that personalized xenotropic chimpanzee adenovirus (ChAd68) and SAM neoantigen vaccine in combination with navulizumab and ipilimumab were safe and well tolerated. An overall survival (OS) rate of up to 42.9% at 12 months was observed in seven patients with microsatellite-stable colorectal cancer (MSS-CRC), with multiple patients achieving durable benefit [73]. This suggests that SAM also has excellent potential in anti-tumor therapy.

DCs also play a significant role in mRNA vaccines. Using autologous tumors to prepare mRNA vaccines, DCs were simultaneously isolated from patients and induced to mature by the cytokines interleukin 4 (IL-4) and human macrophage colony-stimulating factor (M-CSF). The mRNA loaded with encoded tumor antigens is then transfected into dendritic cells and injected back into the patient. The ability to activate an effective anti-tumor immune response [74]. To better explore the use of mRNA vaccines in different tumors, we have summarised the clinical trials in various types of tumors registered with ClinicalTrials.gov in recent years, which are currently inconclusive (Table 2). There are currently no approved clinical studies of circRNA for antitumor therapy; therefore, they are not summarized in this paper.

Optimization of RNA vaccine

RNA is an unstable molecule easily degraded by various ribonucleases (RNases). As a result, the delivery efficiency is low, and the RNA neoantigen cannot be efficiently presented to the DCs [75]. To solve the current bottleneck in the development of RNA vaccines, in recent years, with the development of new technologies, RNA modification can be used to enhance translation efficiency and stability through various methods.

Five-prime cap (5'Cap) and 3'Poly (A) tail modification to enhance stability

Structural improvements have been the focus of mRNA vaccine design, including optimization of the 5' cap structure and 3' Poly(A) tail length and regulatory elements within the 5' and 3' untranslated region [16]. The 5' cap structure is essential for efficient protein production by mRNA. Since chemical modification of the 5' cap structure significantly affects RNA stability and

Table 2 ClinicalTrials.gov Registered mRNA-based Cancer vaccine trials

	Trial phase	Research status	Target antigen	Tumor type	Co-administration	Units
NCT03289962	1a/1b	Active, not recruiting	Autogene cevumeran (RO7198457)	Advanced NSCLC, TNBC, colorectal cancer, HNSCC, UC, RCC, Melanoma, and other advanced solid cancers	Atelizumab	Genentech, Inc.
NCT04526899	2	Active, not recruiting	BNT111	unresectable Stage III or IV melanoma	Cemiplimab	BioNTech SE
NCT03313788	1	Active, not recruiting	Individualised neoantigen therapy	Unresectable metastatic HPV-HNSCC	pembrolizumab	George Washington University
NCT03897881	2b	Recruiting	Personalized tumor vaccine mRNA-4157	Patients with completely resected melanoma (stage IIIB-IV)	pembrolizumab	ModernaTX, Inc.
NCT06389591	1	Not yet recruiting	pp65 RNA-loaded lipid particles	Recurrent glioblastoma	NA	University of Florida
NCT05202561	1	Unknown status	KRAS	Advanced solid tumors	Combined with PD-1 inhibitors	First Affiliated Hospital Bengbu Medical College
NCT05660408	2	Not yet recruiting	Pulmonary osteosarcoma	Recurrent pulmonary osteosarcoma.	NA	University of Florida
NCT00108264	1	Completed	Autologous cancer cells' RNA transfected with dendritic cells	Metastatic prostate cancer	NA	US Department of Veterans Affairs
NCT04963413	1	Terminated	CMV pp65 RNA-pulsed DCs	Newly Diagnosed Glioblastoma	Temozolomide adjuvant chemotherapy	University of Florida
NCT05264974	1	Recruiting	Autologous total tumor mRNA	Early Melanoma Recurrence	Adjuvant Anti-PD-1 Antibody Therapy	University of Florida
NCT04573140	1	Recruiting	Autologous total tumor mRNA and pp65 full-length lysosomal associated membrane protein mRNA	Adult MGMT non-methylated glioblastoma.	NA	University of Florida
NCT04335890	1	Unknown status	Autologous tumor RNA + RNA encoding specific antigens and driver mutations in IKKb mature dendritic cells	Metastatic uveal melanoma	NA	Hasumi International Research Foundation
NCT03418480	1/2	Completed	Therapeutic HPV vaccine (BNT113)	HPV16-driven tumours	NA	University of Southampton
NCT00672542	1	Completed	Dendritic cells transfected with proteasome siRNA and tumor antigen RNAs	metastatic melanoma	NA	Scott Pruitt, Duke University
NCT05456165	2	Terminated	Individualized neoantigen vaccines (chimpanzee adenovirus and SAM vectors)	Colorectal cancer	Atelizumab	Gritstone bio, Inc.

NA: Not available, NSCLC: Non-Small Cell Lung Cancer, TNBC: Triple-negative breast cancer, HNSCC: Head and neck squamous cell carcinoma, UC: Urothelial carcinoma, RCC: Renal cell carcinoma

translation efficiency, it is also a key factor in optimizing RNA vaccine design. The poxvirus capping system is the most widely used in vitro post-translational capping enzyme method, based on the poxvirus capping enzyme (VCE) [76]. In addition to the normal 5'-cap structure, some RNAs have "reverse" caps, where m^7G is adjacent to the RNA body. In mRNAs that are routinely synthesized in vitro, this reverse cap results in reduced translation efficiency [77]. To prevent reverse doping, Stepinski et al. designed two novel cap analogs, termed reverse cap analogs (ARCA) [78]. ARCA-capped mRNA increases and prolongs protein expression in vitro.

The poly (A) tail is present on almost every mRNA in eukaryotes and also plays a crucial regulatory role in mRNA translation and stability, further stabilizing mRNA and facilitating transfection [79]. The commonly used Poly(A) is 250 nucleotides in length [76]. In addition, shorter Poly(A) sequences can facilitate the formation of a closed-loop structure of Poly(A) binding protein (PABP) with the 5'cap, thereby improving translation efficiency [79].

Enhanced mRNA translation efficiency

Nucleotide modification can increase the stability of RNA. Recent research discovered that the delivery of

base-modified mRNAs significantly increased protein production while decreasing inflammatory responses compared to unmodified mRNAs, contributing considerably to the development of mRNA vaccines [80, 81]. Proteins encoded by replacing rare codons with synonymous codons can maintain proteins' original structure and function, improve the protein expression of exogenous genes in host cells, and enhance mRNA's stability [82].

Pseudouridine modifications are the most abundant RNA modifications that enhance mRNA stability and protein expression. It has been demonstrated that compared to unmodified mRNAs, mRNAs modified by pseudouridine can have up to 10-fold greater protein expression [80], Greatly increasing mRNA translation efficiency [83]. However, N6-methyladenosine (m6A) modification on circRNA suppresses innate immunity. m6A modification inhibits immune gene activation and paracrine activity through the m6A-reading protein YTHDF2, which fails to induce antigen-specific T-cell responses [84].

Besides, increased translational efficiency through codon optimization and functional modulation by adding 5′ and 3′ untranslated regions (UTRs). UTRs can interact with RNA-binding proteins to affect mRNA degradation rate and translation efficiency, and optimizing the 5'UTR sequence can improve the accuracy of mRNA translation [85]. Different 5' UTR features affect mRNA translation. Therefore, using canonical initiation codons (AUG) and non-canonical initiation codons (AUG and CUG) in the 5' UTR should be avoided. Secondly, highly stable secondary structures can negatively affect mRNA translation by preventing ribosome recruitment, scanning, and starting codon recognition. to increase their translational efficiency, 3' UTRs from human α- and β-bead proteins are often added to mRNA translation. To increase their translational efficiency, 3' UTRs from human α- and β-bead proteins are often added to mRNAs [86]. Protein expression can also be improved by adding 3' UTR sequences twice in tandem [87],

Delivery system

Efficient in vivo cyclic or linear RNA delivery is essential for tumor therapy. Suitable delivery systems allow RNA to escape the surveillance of the autoimmune system and facilitate the delivery of RNA-like vaccines to their target sites for expression [88]. Therefore, delivery systems play a critical role in effectively protecting exogenous RNA vaccines and their transport into the cell [16]. The development of LNPs achieves high encapsulation efficiency for RNA vaccines [89] and protects the RNA from degradation by RNase [90], It has been found that LNP-encapsulated RNAs exhibit better thermal stability than linear mRNA and can be stored for at least 28 days at 4 °C and 14 days at room temperature [40]. The charge alteration release transporter (CART) is a novel cationic ionizable LNP, mediating mRNA and circRNA delivery in mice [91]. Enabling neoantigen expression and immune response after injection [92]. and can be rapidly degraded in the spleen, accelerating the release of RNA vaccines [93]. LNPs significantly improve RNA stability and transfection efficiency [94].

In addition, LNP itself can activate the immune system. It has been found that LNP induces the activation of C5a and sC5b-9, which activates complement through an alternative pathway [95]. C5a recruits phagocytes such as neutrophils, macrophages, and eosinophils and promotes inflammation [96]. NP-attached proteins in blood or biological fluids can also activate T and B lymphocytes, and Goswami et al. found that LNP-encapsulated SAM vaccines exhibited higher antibody levels and antigen-specific CD8 T responses [97], which has been similarly demonstrated in mRNA and circRNA vaccines [17, 91]. The chemical structure of liposomes can trigger antibody production by B lymphocytes. In a mouse model of the center-influenza virus, the use of LNP enhances the immune response to the vaccine by inducing a robust T follicle helper, germinal center B cell, long-lived plasma cell, and memory B cell response, which in turn promotes the production of long-lasting and protective antibodies [98].

Overall, the LNP delivery system is a promising platform for RNA delivery. They are providing new directions and ideas for RNA vaccines [99].

Challenges and prospects for RNA vaccines

Although substantial progress has been made in RNA vaccines, there are still some limitations in vaccine development and application. The First challenge is to obtain relatively pure mRNA from IVT. IVT products contain not only the desired target mRNA product but also residual NTPs and DNA templates, as well as aberrant mRNA products formed during IVT. Laboratory-grade purification methods are based on DNase digestion to remove DNA, but these methods do not remove dsRNA and truncated RNA fragments. The impurities associated with these products can reduce translation efficiency, so there is a need to develop complete and efficient isolation and purification techniques [100]. Noteworthily, mRNA vaccines require low-temperature storage at around −70 °C. Harsh conditions increase transport costs, and poor temperature stability increases the variability of effectiveness in use, which calls for developing more stable mRNA vaccines [101].

Secondly, the challenge is to find the best way to deliver the vaccine. The administration route determines the RNA distribution and the vaccine's efficacy. Since intradermal and subcutaneous areas are rich in APCs, injected mRNA is quickly processed and presented by APCs in

the area. Still, this mode of administration can cause local injection site reactions [102]. Intranasal, intra-lymph node, and intra-tumor injections of mRNA reach APCs in the surrounding lymph nodes [103]. Local inflammation is induced by mRNAs encoding co-activating molecules, but this approach allows only small injections [105]. Muscle tissue is rich in blood vessels and various immune cells and causes fewer injection site reactions. Intravenous infusion allows the RNA to travel with the bloodstream to many lymphoid organs and has been shown to induce a robust CD8+T-cell response. The disadvantage is that residual toxic solvents remain from the preparation. They can cause greater side effects than other delivery methods [104]. The above modes of administration have their advantages and disadvantages, and using any of them should be analyzed on a case-by-case basis.

Besides, neoantigen expression and delivery specificity are key factors limiting RNA vaccine development. The most straightforward delivery method for RNA vaccines is naked mRNA. However, it can be taken up by the cell through scavenger receptor-mediated endocytosis; at the same time, only a tiny amount of the RNA is ultimately released into the cytoplasm in the presence of the RNase enzyme and is not efficiently internalized by APCs. Degradable after translation initiation and, therefore, may require multiple administrations to maintain the desired protein expression level, potentially leading to side effects and even "off-target effects," resulting in poor specificity and inefficiency of neoantigens delivered to the APCs [105]. The rapid development of technology in recent years has made LNPs the primary delivery vehicle for RNA. However, apolipoprotein E (ApoE) in serum can bind to intravenously injected LNPs, and the liver is the main organ to remove ApoE-bound lipoproteins and delivered LNPs-RNAs will preferentially enter the liver in combination with ApoE. At the same time, the reticuloendothelial system in the liver can provide ample space for these relatively large nanoparticles, allowing them to enter the liver [106]. Thus, intravenous or intramuscularly injected LNP-RNA showed extreme accumulation in the liver, which triggered severe liver damage after vaccination [107]. The current challenge of optimizing LNP design to reduce the inflammatory side effects of RNA vaccines is still unresolved.

Thirdly, the timing of vaccine immunization also needs to be investigated. Tumor vaccines take longer to produce results than other therapies. Early-stage patients have a low tumor load, so it was thought that initial treatment or non-metastatic tumors would be more efficacious [108]. In addition, for optimal efficacy, current RNA vaccines require multiple administrations or combination therapies (e.g., combined with immune checkpoint inhibitors, adjuvants, etc.) to induce immune response. The best way to combine remains to be explored in the future.

Eventually, the SAM vaccine faces the unique challenge of considering the ability of nsP to integrate with the host genome. SAM contains viral sequences and is less safe than circRNA and mRNA. Besides, SAM links the sequence encoding the RNA polymerase to the sequence expressing the target protein, resulting in a considerable molecular weight of the entire mRNA, which is too large and may lead to a decrease in delivery efficiency. CircRNA vaccines still have some limitations in the synthetic methods, such as low cyclization efficiency and high cost of reagents required for cyclization. Moreover, linear precursor RNA, nicked RNA, is generated during IVT affecting circRNA purity. It is difficult to obtain high-purity circRNA using RNase R alone, while electrophoresis-gel recovery or high-performance liquid chromatography (HPLC) can obtain circRNA with purity as high as 90%, which is the preferred solution for circRNA purification in large quantities [109].

We envisage that advanced technologies can be developed in the coming years to remedy the current problems and believe that RNA tumor vaccines have a bright future in tumor therapy.

Conclusion

Unlike traditional treatments, RNA vaccines can encode neoantigens, and activate T cells in the body to produce a strong immune response. At the same time, due to the inefficient expression and toxicity of other types of vaccines, RNA vaccines have emerged as an essential tool for treating tumors compared to traditional vaccines based on pathogens, DNA, and protein peptides.

In recent years, with the continuous development of technology, mRNAs have been modified to enhance stability and improve translation efficiency, enabling them to exert more durable anti-tumor effects. Because of their covalently closed structure, circRNAs may have a longer half-life than mRNAs, thus allowing longer-lasting antigenic expression.

However, the current RNA vaccines have limitations such as low purity and high transport temperature requirements. circRNA faces the challenge of low in vitro cyclization efficiency. In addition, how RNA vaccines better play an important role in the comprehensive treatment of tumors is also a direction explored in the future. Nevertheless, we firmly believe that RNA vaccines are realizing their potential as a critical strategy for future oncology treatments and could push immunotherapy and oncology into a new era.

Author contributions

YRH wrote the paper, edited the paper, and made the figures; CJW finally edited the paper. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Declarations.

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