mRNA-Based Cancer Immunotherapies

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Significant technological innovations have led to messenger RNA (mRNA) becoming a promising option for developing prophylactic and therapeutic vaccines, protein replacement therapies, and genome engineering. The success of the two COVID-19 mRNA vaccines has sparked new enthusiasm for other medical applications, particularly in cancer treatment. In vitro-transcribed (IVT) mRNAs are structurally designed to resemble naturally occurring mature mRNA. Delivery of IVT mRNA via delivery platforms such as lipid nanoparticles allows host cells to produce many copies of encoded proteins, which can serve as antigens to stimulate immune responses or as additional beneficial proteins for supplements. mRNA-based cancer therapeutics include mRNA cancer vaccines, mRNA encoding cytokines, chimeric antigen receptors, tumor suppressors, and other combination therapies.

messenger RNA modification delivery mRNA vaccine

1. Introduction

Immunotherapies are used to eliminate cancer cells by activating the innate and adaptive immunity, and various studies and strategies have been well tested. Owing to their efficacy and wide range of applications, immunotherapies are considered the most promising strategies for cancer treatment. There are a number of stepwise processes in the immune response to cancer. First, cancer cells release antigens that are taken up by DCs, presented on the major histocompatibility complex (MHC), and recognized by T cells to initiate proliferation and activation. Effector T cells then recognize and kill cancer target cells via T cell receptors (TCRs) and peptide–MHC-specific recognition, releasing more tumor antigens to expand the immune response. However, there are many reasons for the poor performance of the autoimmune response in patients with cancer. For example, (1) a low abundance of tumor antigens protects them from being presented by MHC; (2) DCs and T cells cannot recognize the antigens due to peripheral tolerance; (3) immunosuppression may be caused by the tumor microenvironment; and (4) immune suppression markers on the surface of cancer cells assist cancer cells in causing immune suppression [1][2]. To restore and strengthen cancer immunity, cancer immunotherapies that target different targets have been developed. Among these, cancer vaccines based on mRNA platforms have been rapidly developed. In particular, the recent FDA approval of two mRNA-LNP vaccines for COVID-19 prevention makes the clinical use of mRNA vaccines in cancer treatment promising.

2. Neoantigen mRNA Vaccines

The most critical aspect of tumor antigen mRNA vaccine design is the selection of antigens that are ideally expressed only by cancer cells and are immunogenic [3]. This class of cancer cell epitopes is referred to as

neoantigens or tumor-specific antigens (TSAs). In non-viral pathogenic human tumors, new epitopes are exclusively generated by tumor-specific DNA alterations caused by genetic instability [4]. These DNA changes include nonsynonymous mutations, frameshift mutations (insertions or deletions), gene fusions, post-translational modifications that alter the amino acid sequence, and intron retention [5][6][7][8][9]. In addition, post-translational modifications that alter the amino acid sequence and intron retention at the mRNA level can lead to the expression of non-autologous proteins. These new epitopes with individual specificity, called neoantigens [10], enable the immune system to recognize and destroy a tumor carrying these mutations. Epitopes from viral open reading frames (ORF) also contribute to neoantigens in virus-associated tumors, such as those caused by human papillomavirus (HPV). Thereafter, they undergo cytosolic degradation, are processed into short peptides (8–10 amino acid residues), and then transported to the endoplasmic reticulum to be loaded onto HLA molecules [11]. In contrast to autologous sequences, to which the immune system is tolerant, the 'foreign' peptide will be recognized by the T cell receptor (TCR) of $CD8^+$ T cells and activated cytotoxic T lymphocytes (CTLs), which are responsible for the killing of tumor cells [12][13]. However, although tumor cells have many mutations, few are recognized by the patient's own T cells, because neoantigen-specific T cell reactivity is generally limited to a few mutant epitopes [14]. One way to break the immune tolerance of T cells is to use mRNA to express neoantigen peptides to establish systemic DC targeting and neoantigen-specific T cell immunity.

Some neoantigens have high prevalence and conserved mutation profiles and are referred to as shared neoantigens, which have significant potential for use as broad-spectrum therapeutic cancer vaccines for patients with the same mutated genes. When the same neoantigen is present in a patient's tumor cell, the corresponding off-the-shelf neoantigen-targeted immunotherapy can be used for treatment, which can significantly shorten the development cycle. For example, BRAF V600E, ERBB2 S310F, KRAS G12D, PIK3CA E545K, etc., are all generated by somatic mutations that are common in cancer patients [15]. Approaches to predict and prioritize immunogenic shared neoantigens are becoming more comprehensive, opening up new opportunities to develop neoantigen-targeted therapies in a very general way. For example, researchers have used computational epitope prediction, biochemical analysis, and proteomic analysis to predict and identify an mKRAS G12 peptide with high stability and affinity to HLA-A and HLA-B in a specific race [16]. In 2018, Moderna and Merck developed a novel shared antigen mRNA vaccine formulated with lipid nanoparticles called V941 (mRNA-5671), which targets the four most common KRAS mutations (*G12D*, *G12V*, *G13D*, and *G12C*) in solid tumors. Preclinical data show that after vaccination in a mouse model, the neoantigen is translated to induce $CD8^+IFN^+$ T cells that specifically target KRAS mutant tumor cells. Phase I trials of mRNA-5671 were recently completed in two groups (NCT03948763), either as monotherapy (intramuscular injection) or in combination with the anti-PD-1 antibody pembrolizumab (intravenous injection) to assess the safety and tolerability, involving 100 patients with lung, pancreatic, and colorectal cancers (not published yet). Since all types of HPV encode "early proteins" (E proteins: E1, E2, E6, E7) and "late proteins" (L proteins: L1, L2), the development of mRNA vaccines for HPV-positive malignancies has also evolved rapidly [17]. BNT113 (HPV16 E7 mRNA), an intravenous cancer vaccine that efficiently matures and amplifies antigen-specific effector and memory $CD8^+$ T cells, was tested in mice using lipoplex (LPX) delivery. Its administration mediated tumor regression and prevented tumor recurrence in two HPV-positive mouse tumor models (TC-1 and C3) and showed a combined effect with PD-L1 inhibitors [18]. BNT113 in combination with anti-

CD40 (HARE-40) is currently being tested in a phase I/II vaccine dose-escalation study in patients with advanced HPV16$^+$ cancer (NCT03418480). Another phase II trial of BNT113 combined with pembrolizumab versus pembrolizumab alone as a first-line treatment in patients with HPV16$^+$ head and neck cancer expressing PD-L1 is also underway (NCT04534205).

However, most cancer mutations are unique to each individual patient and require a personalized medical approach; thus, a highly specific procedure has been developed. Surgically resected tumors, tumor biopsies, and healthy blood cells were collected, and the extracted DNA from the samples was subjected to whole-exome and RNA sequencing to identify nonsynonymous mutations. Whether a mutation can be used as a therapeutic target depends on several critical factors [19]: (1) the mutated sequence can be translated into a protein in tumor cells, and the expression level of the originating gene should be greater than 33 TPM; (2) the mutated protein can be processed into a peptide; immunogenic peptides usually have low hydrophobicity and mutations do not occur at the second amino acid site; (3) the peptide can be presented by MHC with a binding stability greater than 1.4 h; (4) the mutated peptide has high affinity, which is usually stronger than 34 nM for MHC molecules; and (5) the mutated peptide–MHC complex has high affinity, ranging from 30 nM to 26 pM, for the T cell receptor (TCR) [20]. Therefore, the prediction of neoantigens requires not only the identification of mutations expressed in the genome, but also data on the patient's MHC type [21]. A number of computational, biochemical, proteomic, and immunological assays have been used to predict the high affinity, immunogenicity, and expression efficiency of mutant peptides and HLA in tumors. Furthermore, a number of MS-based immunopeptidomic datasets such as IEDB [22], SysteMHC Atlas [23], and PRIDE [24] have been used in machine learning for neoantigen prediction. Tools such as NetMHC [25], MHCflurry [26], NetMHCpan [27], PSSMHCpan antigen-garnish [28], pVAC-Seq [29], and others have been widely used to predict peptide–HLA affinity based on various algorithms. The expression of mutated alleles and the processing and presentation of neoantigens can be confirmed by RNA-seq [30], HLA immunoprecipitation, and targeted mass spectrometry separately [31]. However, candidate neoantigens selected on a computer may not be recognized by T cells; therefore, it is necessary to verify the presentation and immunogenicity of neoepitopes [32] [33]. Biochemical assays were performed to characterize the affinity and stability of peptide–HLA (p-HLA). Immunological datasets were collected by co-culturing T cells with mature dendritic cells (mDCs) pulsed with candidate epitopes [16][30] or stimulating peripheral blood mononuclear cells (PBMCs) from patients with neoepitopes, followed by T cell activation assessment by IFN-γ-ELISPOT, flow cytometry, etc. [34]. Subsequently, a series of potentially immunogenic peptides can be selected based on their immunogenicity and protein-binding affinity.

A one-step procedure for the design and synthesis of neoantigenic mRNA has been developed. A patient-tailored DNA plasmid encoding a selected set of several neoantigens in tandem with minigenes (TMG^{NEO} plasmid) was developed. It has been reported that the combination of an N-terminal leader peptide with MITD bound to the C-terminus of the antigen significantly improves the presentation of HLA epitopes in DCs [35]. The TMG template design consists of the T7 promoter, sequences encoding the MHC-I signal peptide (SP), TMG^{NEO}, the trafficking domain of major histocompatibility complex class I (MITD), two consecutive 3'-untranslated regions of human β-globin, and 120 adenosine poly(A) tails [36]. To generate a tandem minigene, minigenes were linked with a non-

immunogenic glycine/serine linker [37][38][39]. After plasmid synthesis, in vitro-transcribed mRNA can be produced, which can be used for ex vivo loading of autologous DCs or LNP encapsulation to produce the final vaccine [40].

BioNTech SE has developed an iNeST platform for patient-specific cancer antigen therapy, including BNT121 and BNT122. BNT121, a vaccine containing 10 neoantigens, was tested by intranodal administration in 13 melanoma patients. It was found to induce T cell infiltration to kill tumor cells and to have recurrence-free disease activity (NCT02035956) [37]. Strong immunogenicity has also been observed in a number of tumor types following injection of BNT122 (RO7198457), which contains up to 20 patient-specific novel epitopes (NCT03289962). mRNA-4157 is another personalized mRNA cancer vaccine developed by Moderna, which contains 20 neoepitopes with strong immunogenicity selected according to the unique characteristics of the patient's immune system and specific mutations. The mRNA is encapsulated in the LNP, and the vaccine is injected intramuscularly. The drug is being tested for an acceptable safety profile and observed clinical responses in patients with solid tumors (NCT03313778) and melanoma (NCT03897881).

3. TAA mRNA Vaccines

In addition to neoantigen vaccines, another class of tumor antigen vaccines is also widely used, namely tumor-associated antigen (TAA) vaccines [41][42]. TAAs are autoantigens that are preferentially or abnormally expressed in tumor cells and can also be expressed at certain levels in normal cells. They can be classified into the following categories [43][44]: (1) cancer/germline antigens (or cancer testis antigens), which are normally expressed only in immune-privileged germline cells but are transcriptionally reactivated in tumor cells (e.g., melanoma antigen gene family (MAGE), B-M antigen-1 (BAGE), New York esophageal squamous cell carcinoma (NY-ESO-1), and synovial sarcoma X chromosome breakpoint-2 (SSX-2)) [45]; (2) cell lineage differentiation antigens, which are derived from normal tissues (e.g., tyrosinase, glycoprotein 100 (gp100), melanoma antigen recognized by T cells 1 (Melan-A/MART-1), prostate-specific antigen (PSA) and prostate acid phosphatase (PAP) in prostate cancer, and mammaglobin-A (MAM-A) in breast cancer) [46]; and (3) proliferation-, differentiation-, and antiapoptosis-related proteins with tumor-selective high expression contributing to the malignant phenotype (e.g., carcinoembryonic antigen (CEA), human telomerase reverse transcriptase (hTERT), human epidermal growth factor-2/neu (HER2/Neu), baculoviral inhibitor of apoptosis repeat-containing protein 7 (livin), baculoviral inhibitor of apoptosis repeat-containing 5 (survivin), and mucin-1 (MUC-1)) [47]. Despite significant differences in the expression of TAAs in normal tissues and cancer cells, TAAs are characterized by low tumor specificity and low immunogenicity [48][49]. Therefore, cancer vaccines using these antigens must be sufficiently effective to break immune tolerance with several features. Incomplete peripheral tolerance of TAA-reactive T cells and very low expression of TAA in peripheral tissues are critical for restoring immunoreactivity via expression of the relevant TAA in APCs [50].

In 1995, the first TAA mRNA encoding the human carcinoembryonic antigen CEA was constructed, capped, polyadenylated, and stabilized by the 5′ and 3′ UTRs of human β-globin. After the injection of naked mRNA into mice, CEA antibody production was observed, which was the first proof of concept for TAA mRNA vaccines for cancer therapy [51]. A series of TAA mRNAs were then validated in a mouse cancer model, including gp100 [52],

melanoma antigen recognized by T cells 1 (MART1) [53], and tyrosinase-related protein 2 (TRP2) [54][55] in B16F10 melanoma tumors, cytokeratin19 mRNA in Lewis lung cancer [56], and CD133 mRNA in gliomas [57].

The main problem in the development of TAA mRNA vaccines is the achievement of immunogenicity from TAA. The use of multiple shared TAA mRNA has become the main trend in the development of clinical cancer vaccines, which have been verified in various clinical trials and show strong potential for the induction of antitumor immune responses [58]. Vaccination with DCs electroporated with mRNA encoding WT1 (NCT00965224) or WT1, PRAME, and CMVpp65 (NCT01734304) or CT7, MAGE-A3, and WT1 mRNA (NCT01995708) or WT1/PRAME (NCT02405338) mRNA was tested in acute myeloid leukemia (AML). An increase in antigen-specific T cells and induced antibody responses was observed [59][60], and overall survival (OS) improved [61].

Melanoma, a form of skin cancer, is a malignant tumor that is prone to metastasis. Because of the location of the lesion, which lends itself to the local injection of mRNA with a high degree of safety, melanoma mRNA vaccines have been tested in several clinical trials and have significantly advanced. BNT111, a mixture of RNA-LPX encoding four TAAs (NY-ESO-1, MAGE-A3, tyrosinase, and TPTE), has shown great therapeutic potential alone or in combination with the PD-1 inhibitor, inducing strong $CD4^+$ and $CD8^+$ T cell immunity and maintaining antitumor effects for months after vaccination was ceased [62]. Moderate flu-like symptoms (such as fever and chills), which were classified as grade 1-2 adverse events, occurred in 5% of the patients (NCT02410733). Based on these results, BNT111 was fast-tracked by the FDA for a phase II clinical trial with the anti-PD-1 antibody cemiplimab in patients with anti-PD-1 refractory or relapsed, unresectable stage III/IV melanoma (NCT04526899). The BNT112 cancer vaccine has also been tested as monotherapy or in combination with cemiplimab in patients with prostate cancer (NCT04382898). BNT114 (a mixture of TAA mRNAs encoding breast cancer antigens) and BNT115 (a mixture of three ovarian cancer antigen mRNAs) are being developed. Reinhard et al. described another strategy, called CarVac, in which TAA mRNA was used as a chimeric antigen receptor (CAR)-T therapy stimulator to achieve adjustable expansion of low doses of CAR-T cells. CLDN6-CAR-T cells gradually disappeared from the tumor microenvironment (TME) in the absence of a proliferation signal. Administration of CLDN6 mRNA-LPX (BNT211) effectively induced APCs to present antigens, and the number of CLDN6-CAR-T cells peaked 3-4 days after vaccination and then declined. Good safety and efficacy have also been demonstrated after multiple administrations [63].

Another mRNA drug company, CureVac AG, has developed a series of RNActive® vaccines that use chemically unmodified, sequence-optimized mRNA to encode TAAs for cancer treatment [64]. Specific cytotoxic T lymphocytes and antibodies can be induced by exposure to unmodified mRNA to produce self-adjuvants. CV9103, a prostate cancer vaccine containing protamine-stabilized mRNA encoding the antigens PSA, PSCA, PSMA, and STEAP1, was well tolerated in a clinical trial of 48 participants and induced immune responses that could lead to prolonged patient survival [65] (NCT00906243, NCT00906243). CV9201 is another mRNA-based cancer immunotherapy encoding five TAAs (NY-ESO-1, MAGE-C1, MAGE-C2, survivin, and 5T4). In 60% of the patients, there was more than a twofold increase in B cells directed against antigens after treatment with CV9201 [66]. CV9202 contains mRNAs encoding six different NSCLC TAAs (MUC-1, survivin, trophoblast glycoprotein, NY-ESO-1, MAGE-C1, and MAGE-C2) (NCT01915524). Following intradermal administration, antigen-specific immune responses increased in

84% of patients; 80% of patients had a 40% increase in antigen-specific antibody levels and functional T cell levels, and 52% of patients had multiple antigen specificities [64] (NCT01915524). Based on these studies, CV9202 has also been evaluated in phase I/II studies in combination with the anti-PD-L1 antibody durvalumab or the anti-CTLA4 antibody tremelimumab, administered subcutaneously with a needle-free injection device (NCT03164772).

Standardization of TAA mRNA construction is also possible; BNT111 is a good example. The addition of a 5′-cap analog, 5′ and 3′ UTRs, and a poly(A) tail can increase mRNA stability and translation efficiency. The full-length TAA-coding sequence was tagged with a signal peptide (SP), tetanus toxoid $CD4^+$ epitopes P2 and P16, and MITD for enhanced HLA presentation and immunogenicity [62].

In TAA mRNA vaccines, other strategies have been used to activate antigen-presenting cells, such as the electrical transfer of DC in adoptive therapy or administration of antigen mRNA targeting the spleen. These strategies have considerable therapeutic value in AML and offer potential treatment options for non-solid cancers that are difficult to treat.

Clinical trials with an mRNA cancer vaccine have shown that vaccination against mutant epitopes or TAAs was safe and well tolerated, with most of these conditions being early onset, transient, and manageable. When injected intramuscularly, the most common adverse events of mRNA-LNP were pain at the injection site, fatigue, headache, arthritis, and myalgias [67]. When the mRNA-based cancer vaccine was administered intravenously by LPX, the clinical adverse events were mild to moderate flu-like symptoms, such as pyrexia and chills [62]. Future preclinical and clinical studies should investigate potential safety concerns such as local and systemic inflammation.

4. mRNA Encoding Ab

Since the development of hybridoma technology for the production of monoclonal antibodies (mAbs) in 1975, antibodies have become the most rapidly developing cancer-targeted drugs [68]. A series of antibodies that mediate tumor cell killing by antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) activities, or by immunosuppressive signal blockade, are well used in clinical trials. Conventional antibodies consist of antigen-binding sites (Fabs) and constant region (Fc) fragments. Fab fragments bind to tumor antigens and the Fc region lyses cancer cells by interacting with Fc receptors (FcγRs) on effector cells (such as NK cells and macrophages) [69]. Many chimeric antibodies against cancer antigens (murine Fab and human Fc regions) have been approved for clinical use. In addition, many immune checkpoint inhibitors (ICIs) are also widely used in immunotherapies [69][70][71][72] and are often combined with other therapies (such as neoantigen mRNA and TAA mRNA). In addition to traditional antibodies, antibody fragments (including single-chain variable fragments (scFvs) and single-domain antibodies (sdAds)) and bispecific/multispecific antibodies have shown great potential in immunotherapies. Bispecific antibodies (bsAbs) have two antigen-binding arms and function to mediate immune cell killing by forming a T cell–bsAb–tumor cell complex, blocking two receptors of tumor cells [73].

However, customization of each antibody, quality control, and purification are challenges for mass antibody production. Therefore, the use of mRNA to generate intact mAbs in vivo was tested. Compared with protein antibody therapy, mRNA platforms have some unique advantages: (1) different antibodies can share the same design, production, and purification protocol of IVT mRNA; (2) optimized variants can be produced by changing the coding region of the IVT mRNA; (3) IVT mRNA uses the cells' own ribosomes to encode proteins and undergoes correct assembly and post-translational modification; (4) as the serum half-life of the mRNA-encoded Ab is determined by the half-life of both the Ab itself and the mRNA, the half-life of short-lived proteins can be extended [74]; and (5) in the study, there was no upper dose and no dose-limiting toxicity for antibody mRNA administration. mRNA therapy features easy quality control, rapid production, and good tolerance and safety, which makes it a better mAb protein alternative [75].

In 2008, CureVac attempted the expression of mRNA-encoded antibodies against HER2, EGFR, and CD20 in vitro. Nine years later, the CureVac team tried to use mRNA-LNPs encoding the anti-CD20 antibody rituximab in vivo and established high serum titers in mice with curative effects of significant inhibition of tumor cell growth in lymphoma models, demonstrating for the first time that mAb mRNA is effective in cancer immunotherapy [76]. Rybakova et al. tested the pharmacokinetics and pharmacodynamics of the mRNA-encoded anti-HER2 antibody, trastuzumab, and demonstrated its anticancer activity [77].

In addition to monoclonal antibodies, a series of mRNA-encoded bispecific antibodies (bsAbs) have been developed. Two chemokines, chemokine ligand 2 (CCL2) and CCL5, play major roles in the accumulation of tumor-associated macrophages (TAMs) and induction of immunosuppression in hepatocellular carcinoma (HCC). To prevent immune cell chemotaxis, a bsAb, BisCCL2/5i, which binds CCL2 and CCL5, was developed by Wang et al. The drug effectively promotes the differentiation of TAM into the antitumoral M1 phenotype and reverses immunosuppression in the TME. The use of BisCCL2/5i renders HCC sensitive to trimeric PD-1 ligand inhibitors (PD-Li) and prolongs survival in liver malignancy models [78].

Bispecific T cell engagers (BiTEs) are a class of bsAbs without the Fc region. They consist of two single-chain variable fragments (scFv) joined by a flexible linker. One scFv recognizes the T cell surface protein CD3, whereas the other scFv binds to a target antigen on cancer cells. This specific structure of BiTEs enables the localization of T cells to tumor cells and thus mediates tumor killing [79]. Stadler et al. generated a RiboMab platform with three BiTE mRNAs targeting three tumor-associated antigens (TAAs) (CD3 × tight-junction proteins claudin 6 (CLDN6), claudin 18.2 (CLDN18.2) × CD3, and epithelial cell adhesion molecule (EpCAM) × CD3). mRNA-encoded CD3×CLDN6 BiTE (which remained above half-maximum levels for up to 6 days) had a longer duration in serum than protein (which was barely detectable after 24 h). CD3×CLDN6 and EpCAM×CD3 IVT mRNA in a human ovarian cancer xenograft mouse model showed complete tumor regression without a systemic immune response [80]. CD3×CLDN6 mRNA (BNT142) is currently in phase I/II clinical trials (NCT05262530).

Although the number of clinical studies relying on mRNA antibody expression is still very limited, the applications of both mAb and bsAb have already been validated. Targeting cancer antigens, blocking immunosuppressive molecules on the surface of cancer cells, and mediating the antitumor effect of T cells through mRNA-encoded

antibodies demonstrate the great potential of mRNA antibody immunotherapy. The development of mRNA antibody platforms is expected to lead to more optimal antibody design, longer half-life, and more clinical product applications in the future.

5. Immunomodulator mRNA Vaccines

The TME is closely associated with tumorigenesis and development. Tumor cells mediate immune suppression by releasing signaling molecules into the TME. This explains the difficulty in activating immune responses in tumors and results in the failure of cancer therapies in some patients [81]. Therefore, it is important to restore the antitumor immune response environment by regulating immunosuppression with immunomodulatory agents [82]. Clinically, injecting cytokines into cancer patients has become a cancer treatment strategy. For instance, more than 140 clinical trials have been launched to test type I interferon (IFN-I), which can directly induce apoptosis of tumor cells, prevent angiogenesis of tumor blood vessels, activate mDCs, and promote the differentiation of effector T cells [83]. Cytokines that activate antitumor effector cells (IL-12, IL-23, IL-36, GM-CSF, and IFN-α), costimulators (OX40L (CD252), inducible costimulatory ligand (ICOSLG/CD275), tumor necrosis factor receptor superfamily 9 (TNFSF9/4-1BBL/CD137L)), pattern-recognition receptor (PRR) agonists (TLRs and RIG-I agonists), and others are commonly used in immunotherapy [81]. Commonly used antitumor cytokines include interferons, interleukins, lymphokines, and tumor necrosis factors with various functions. Some have proinflammatory functions (IL-23, IL-36γ, IFN-α), stimulate the proliferation and differentiation of immune cells (CD70, IL-15, GM-CSF), or activate lymphocyte functions (IFN-γ, IL-12, IL-27). Costimulatory molecules act as stimulatory immune regulators to enhance the magnitude of immunological responses against malignant cells by binding to T cell surface receptors [84]. PRR agonists activate the innate immunity and release various cytokines to activate the immune system [85]. Current immunomodulator therapies have some clinical limitations, such as severe dose toxicity due to their short half-life, repeated administration, and systemic delivery (such as IL-12). Therefore, intratumoral (i.t.) and intradermal (i.d.) injections are commonly used to induce local immune responses. The standout advantages of both transiently induced protein expression and delivery via the local route make mRNA therapy well suited to modulate the TME, and a number of preclinical studies have been performed.

IL-12 is a well-described cytokine important for the activation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. In 2018, the therapeutic effect of IL-12 mRNA-LNPs on *MYC* oncogene-driven hepatocellular carcinomas (HCC) was verified [86]. In this case, the liver-targeted delivery feature of LNP was used to target HCC, but this mode of administration is not applicable to many other cancers. Then, more intratumorally (i.t.) delivered mRNA was tested in mice. Furthermore, because of the unique functions of each cytokine, the use of a single cytokine has limited effects on tumor treatment. Therefore, multiple cytokines with different functions are often combined to achieve improved therapeutic effects. The efficacy of IL-12, IL-27, GM-CSF, and their combination encapsulated in di-amino lipid nanoparticles was tested in the B16F10 model. Administration of IL-12 and IL-27 mRNA appeared to induce NK and $CD8^+$ T cells in the TME and showed the best therapeutic effect [87]. Another preclinical study evaluated the intratumoral delivery of an mRNA mixture (IL-12, GM-CSF, IL-15, and IFN-α) in a B16F10/CT26 tumor model. mRNA expression increases the number of proinflammatory $CD4^+$ and $CD8^+$ T cells in

the TME and induces an immune response in distal tumors. The addition of anti-PD-1 antibodies further improved the survival rate of the mice [88]. In 2019, Haabeth et al. established a precedent for the combination of cytokines and costimulator mRNA to initiate global anticancer immunity. They utilized a charge-altering releasable transporter mRNA delivery platform to induce the local expression of cytokines (CD70, IL-12, and IFN-γ) and costimulators (OX40L, CD80, and CD86) individually and in combination in two tumor models (A20B-cell lymphoma and CT26 colon carcinoma). Mice treated with OX40L mRNA showed complete eradication of both local and distal tumors, whereas those treated with other mRNA showed only a partial response. Furthermore, the combination of OX40L with CD80 or CD86, or OX40L with IL-12 dramatically increased both survival and tumor growth delay [89].

These preclinical data suggest that some cytokines and costimulatory pathway molecules can be effective strategies to revitalize T cell responses in cancer, particularly when administered in combination or in combination with immune checkpoint antibodies. In 2006, the ability of the mRNA adjuvant to enhance the effect of the TAA mRNA vaccine was evaluated in a mouse model of prostate adenocarcinoma. GM-CSF mRNA co-delivery has been found to enhance the CTL response [90]. DC-activating FLT3 ligand mRNA further enhances the immunological efficacy of naked RNA vaccines [91][92]. More mRNA adjuvants have been used in clinical studies. One of the pioneers of mRNA adjuvants is eTheRNA AG, which contains three naked mRNA molecules (constitutively active TLR4 (caTLR4), CD40L, and CD70). It promotes the activation and maturation of DCs, ex vivo or in situ, to activate T helper cells and CTLs [93][94][95]. Administration of HPV/melanoma-associated TAA mRNA in conjunction with TriMix showed a promising clinical response without increased toxicity [96][97]. A phase I study on TriMix in breast cancer is also underway (NCT03788083). In the pipeline of Moderna, mRNA-2752, an OX40L/IL-23/IL-36γ cocktail mRNA drug, promotes tumor immune infiltration and tumor regression by inducing a broad immune response involving many DC types and lymphocytes. IL-36γ and IL-23 specifically interact to mediate antitumor efficacy, while the T cell costimulator OX40L significantly increases lymphocyte response rates. Notably, in an immunologically barren tumor mouse model (B16F10-AP3), the combination of the drug and ICIs increased survival to 85%, whereas tumor cells were insensitive to ICIs alone [98]. A dose-escalation study of mRNA-2752 in various advanced malignancies and an observational study of mRNA-2752 in combination with the anti-PD-1 antibody pembrolizumab in ductal carcinoma are also ongoing (NCT03739931, NCT02872025). Another mRNA adjuvant containing only OX40L (mRNA-2416) is also being tested for tolerability and safety in combination with the anti-PD-L1 antibody durvalumab in metastatic ovarian and lymphoma cancers (NCT03323398). Similarly, MEDI1191 (IL-12 mRNA) has also demonstrated excellent safety, tolerability, and efficacy in combination with durvalumab for the treatment of solid tumors (NCT03946800). Another IL-12 mRNA product, BNT151, developed by BioNTech, is currently in phase I testing for metastatic tumors (NCT03871348).

These studies suggest that local modulator mRNA therapy enables many immunosuppressed or immune-cell-deficient TMEs to remodel their function and elicit a global immune response from various DCs and lymphocytes, showing exciting therapeutic results in distal tumors and multidrug-resistant metastatic tumors. In particular, when combined with ICIs, they show enhanced antitumor responses.

6. Protein Replacement Therapy

Tumor suppressor genes (TSGs) play important roles in maintaining genome integrity and regulating cell proliferation, differentiation, and apoptosis. The loss of function of TSGs is usually associated with cancer development, progression, and treatment resistance [99]. In addition, several human cancer exome sequencing studies have uncovered a series of cancer driver genes, most of which are TSGs [100]. Several key signaling pathways and processes are associated with the most likely cancer-driving TSGs, including the Wnt/β-catenin pathway (adenomatous polyposis coli (*APC*), *AXIN1*, and cadherin-1 (*CDH1*)), the phosphoinositide 3-kinases (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway (phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*), phosphatase and tensin homolog (*PTEN*), and tuberous sclerosis proteins ½ (*TSC1/2*)), cell growth and differentiation (ras superfamily, hedgehog protein family), apoptosis/cell cycle (tumor protein P53 (*TP53*), RB transcriptional corepressor 1 (*RB1*)), chromatin modifications (CREB-binding protein (*CREBBP*), tet methylcytosine dioxygenase 2 (*TET2*), Wilms tumor 1 (*WT1*), and ubiquitin carboxyl-terminal hydrolase BAP1 (*BAP1*)), DNA damage repair (serine-protein kinase ATM (*ATM*), serine/threonine-protein kinase ATR (*ATR*), breast cancer 1/2 (*BRCA1/2*), DNA mismatch repair protein MLH1 (*MLH1*), and DNA mismatch repair protein MSH2/6 (*MSH2/6*)), and transcriptional regulation (transcription factor GATA-3 (*GATA3*) and runt-related transcription factor 1 (*RUNX1*)) [100][101]. Loss of function occurs in most TSGs, and the cancer phenotype is mediated by hyperactivation of the mentioned pathways. In this case, a possible therapeutic approach would be able to inhibit downstream pathways by replenishing TSGs. However, when DNA transfection is used to restore functional copies, difficulties in delivery, genome integration, and mutation risk have become major obstacles to gene therapy. mRNA has been shown to be advantageous as an alternative to genes and proteins, and several preclinical studies have been conducted.

In a 2018 study, PTEN mRNA was encapsulated in PEG-coated polymer lipid hybrid nanoparticles (NPs) and introduced into PTEN-null prostate cancer cells in vitro and in vivo. Treatment with PTEN mRNA-NPs significantly promoted cancer cell apoptosis by inhibiting the PI3K/Akt pathway, and the therapeutic effect was verified in a mouse model of prostate cancer (PCa) xenograft [102]. In 2021, the team further investigated whether PTEN mRNA-NPs restored protein expression and autophagy was induced in PTEN-null cancer cells (B16F10 melanoma and anti-PD-1 ineffective prostate cancer). In addition, combinatorial treatment with anti-PD-1 antibody resulted in upregulation of CTLs and proinflammatory cytokines (e.g., IL-6, TNF-α, TNF-β, and IFN-γ) in the TME and downregulation of myeloid-derived suppressor cells (MDSCs), which also triggered immunological memory [103]. p53, one of the most frequently altered TSGs that promote apoptosis, was also tested in mRNA therapy. Kong et al. used redox-responsive particles (PDSA added) to deliver p53 mRNA in models of hepatocellular carcinomas (HCCs) and non-small-cell lung cancers (NSCLCs) and showed an effect on tumor growth inhibition. In addition, combination therapy with the mTOR inhibitor everolimus showed the strongest therapeutic effect on in situ tumors [104]. Furthermore, the team added a CXCR4-targeted peptide to hybrid NPs to achieve selective HCC targeting and high mRNA transfection efficiency. The combination of p53 mRNA-NPs and PD-1 blockade significantly reduced bloody ascites, pleural effusions, and lung metastases and prolonged survival in HCC model mice [105]. Lung-targeting LNPs were effectively used to introduce TSC2 mRNA into TSC2-null cells and suppress the mTOR

pathway, resulting in improved control of tumor cell proliferation in a mouse model of pulmonary lymphangioleiomyomatosis [106].

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