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REVIEW

mRNA vaccination in breast cancer: current progress and future direction

Xiao-ting Jiang[1] · Qiang Liu[1]

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Abstract

Messenger RNA (mRNA) vaccination has proven to be highly successful in combating Coronavirus disease 2019 (COVID-19) and has recently sparked tremendous interest. This technology has been a popular topic of research over the past decade and is viewed as a promising treatment strategy for cancer immunotherapy. However, despite being the most prevalent malignant disease for women worldwide, breast cancer patients have limited access to immunotherapy benefits. mRNA vaccination has the potential to convert cold breast cancer into hot and expand the responders. Effective mRNA vaccine design for in vivo function requires consideration of vaccine targets, mRNA structures, transport vectors, and injection routes. This review provides an overview of pre-clinical and clinical data on various mRNA vaccination platforms used for breast cancer treatment and discusses potential approaches to combine appropriate vaccination platforms or other immunotherapies to improve mRNA vaccine therapy efficacy for breast cancer.

Keywords mRNA vaccination · Breast cancer · Cancer vaccine

Abbreviations

4-1BBL	4-1BB ligand
ADCC	Antibody-dependent cell-mediated cytotoxicity
APCs	Antigen-presenting cells
BC	Breast cancer
CAFs	Cancer-associated fibroblasts
COVID-19	Coronavirus disease 2019
CP	Cyclophosphamide
DCs	Dendritic cells
dsRNA	Double-stranded RNA
ECD	Extracellular domains
FAP-α	Fibroblast activation protein-alpha
HER2	Human epidermal growth factor receptor 2
i.d.	Intradermal
i.m.	Intramuscular
i.n.	Intranodal
i.t.	Intratumoral
i.v.	Intravenous
ICB	Immune checkpoint blockade
ISV	In situ vaccination
IVT	In vitro transcription
LABC	Locally advanced breast cancer
LNPs	Lipid-based nanoparticles
LNs	Lymph nodes
mAb	Monoclonal antibody
mRNA	Messenger RNA
MUC1	Mucin 1
MV-CEA	Measles virus-based RNA vaccine
ORF	Open-reading frame
PEG	Polyethylene glycol
PRRs	Pattern recognition receptors
s.c.	Subcutaneous
SAM	Self-amplifying mRNA
SFV	Semliki Forest Virus
SOI	Site of injection
ssRNA	Single-stranded RNA
TAAs	Tumor-associated antigens
TILs	Tumor-infiltrating lymphocytes
TM	Transmembrane domains
TMB	Tumor mutational burden
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TSAs	Tumor-specific antigens
VNTR	Variable number tandem repeats

✉ Qiang Liu
victorlq@hotmail.com

[1] Breast Tumor Center, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, 107 Yanjiang West Road, Guangzhou 510120, China

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Introduction

Breast cancer (BC) is the most prevalent form of cancer worldwide, surpassing lung cancer in 2020 as the most common cancer (WHO 2021). To reduce the global burden of breast cancer, there is an urgent need for more cost-effective treatments and strategies. Conventional treatments, such as surgical ablation, radiotherapy, chemotherapy, and hormone therapy, are often associated with side effects, resistance, and recurrence (Behravan et al. 2019).

Immunotherapy has revolutionized cancer treatment, offering specific tumor targeting with minimal cytotoxicity. This field of medicine seeks to stimulate an anti-tumor immune response, leading to tumor shrinkage and improved clinical outcomes. Recent advances have included several distinct classes of drugs, such as cytokines, immune checkpoint blockade (ICB), adoptive T-cell treatments, and vaccinations (Mellman et al. 2011; Pardoll 2012; Rosenberg et al. 2008). Trastuzumab's success in treating HER2-overexpression metastatic breast cancer at the start of this century can be attributed, at least in part, to immunotherapy (5), as well as the use of ICB agents (atezolizumab, anti-PD-L1) in treating advanced triple negative breast cancer (TNBC) (Schmid et al. 2018). However, its clinical benefits were limited to PD-L1-positive patients, so the pressing needs for BC immunotherapy are to accurately identify responders to current treatments, and to increase the number of responders through the development of new strategies (Solinas et al. 2020).

Breast cancer vaccines

Cancer vaccination is an active immunotherapy strategy designed to stimulate the patient's immune system and help recognize and destroy tumor cells by presenting tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). The analysis of pre-existing immune responses against tumors in cancer patients can provide important information to guide selection of cancer vaccine targets and determine reliable responders (Met et al. 2011).

Prophylactic and therapeutic vaccinations are two functional categories for cancer vaccines. The former, such as HPV in cervical cancer, targets viruses involved in malignant transformation. The latter, however, is the focus of this article. Therapeutic vaccination has many key advantages over other immunological therapies such as creating and amplifying a highly specific adaptive immune response and setting up immunologic memory with the potential to manage and remove residual disease. Solinas et al. conducted pre-clinical and clinical investigations of breast cancer vaccines in the past 2 decades, such as peptide vaccines, BC cell-based vaccines, bacterial or viral vector vaccines, dendritic cell-based vaccinations, and more (Solinas et al. 2020). A GP2 peptide vaccine showed promising results of 100% 5 year survival in a phase II clinical trial for $HER2^+$ BC (NCT00524277), but none of the BC vaccines has been approved for either treatment or prevention in clinical trials yet (Burg 2018). The author summarized the potential causes of negative results from BC vaccine clinical trials, and we briefly conclude the following:

1. Uncontrolled confounding factors in clinical trials, such as disease burden, extent of prior therapy, and tumor immune contexture.
2. Low immunogenicity of vaccination because of insufficient or improper selection of vaccine targets and delivery methods.
3. Mechanisms of immune escape, including the down-regulation of tumor antigen expression, alterations in antigen processing machinery, the loss of HLA class I expression, and the constitutive expression of ligands for immune checkpoints.

Therapeutic mRNA cancer vaccines

Messenger RNA (mRNA) vaccines are antigen/effector-encoding mRNA which can be easily produced in large quantities at low cost through in vitro transcription (IVT) of a template DNA using RNA polymerase, either with or without carriers. In 1990, a report on the successful use of IVT mRNA in animals showed that protein production was detectable when reporter gene mRNAs were injected into mice (Wolff et al. 1990). However, mRNA vaccines were unobtrusive compared to DNA and other cancer vaccines due to concerns about unwanted immune responses generated by high innate immunogenic mRNA structures or double-stranded RNA (dsRNA) contamination, and RNase-related instability and inefficiency in delivery in vivo (McNamara et al. 2015). However, the development of technology in mRNA vaccine manufacture and delivery has changed the situation significantly. HPLC purification of IVT mRNA, insertion of modified nucleosides into mRNA sequences, and complexing of mRNA with different carrier molecules can alter the immunostimulatory profile (reviewed in Pardi et al. (2018); Pardi et al. 2020)). The formula of conventional mRNA vaccines is based on the architecture of mature mRNA in eukaryotic cells, which encodes the target sequence of interest within an open-reading frame. On the other hand, a novel type of mRNA vaccine mimics the genomes of single-stranded RNA viruses, particularly alphaviruses. This is achieved by substituting the gene sequences responsible for coding the virus's structural

proteins with the desired gene sequence, while keeping intact the gene coding for non-structural proteins that form the RNA replication complex. Once these vaccines enter the cytoplasm of cancer cells, they can self-synthesize the replicase, allowing for self-amplification of the interest sequence, known as the "RNA replicon." This technology is highly attractive for cancer immunotherapy due to its continuous protein expression and long-lasting efficacy compared to conventional non-replicating mRNA vaccines. Additionally, this approach is safe as eliminating the risk of immune responses associated with viral particles. Furthermore, advancements in vector technology, such as lipid-based nanoparticles (LNPs), have demonstrated superior performance compared to other vectors. This is due to their high loading efficiency, large surface area, excellent bioavailability, improved cellular uptake, and endosomal escape. The unique properties of LNPs have reduced the amount of mRNA required for fabrication, resulting in improved therapeutic efficiency due to their higher stability, longer circulation time, and enhanced delivery efficiency (Guevara et al. 2019; Lundstrom and Self-Amplifying 2020). These manufacturing and delivery advancements make mRNA cancer vaccination a safe, viable, and successful strategy for inducing robust anti-tumor immune responses. In fact, mRNA vaccine technology has gained popularity recently as a result of the Coronavirus disease 2019 (COVID-19) vaccine. However, despite these successes, there is currently no specific summary for breast cancer, which is known as a "cold tumor" with a low response rate to immune therapy. mRNA vaccination may be a potential strategy to address the associated variables of unfavorable clinical trials for breast cancer vaccines.

1. mRNAs can easily transport multiple antigens with a single immunization, producing multi-specific attacking actions for personalized non-synonymous somatic mutations of cancer cells. Additionally, mRNAs can encode both the antigen and the viral replication apparatus, allowing for extensive protein expression and intracellular RNA amplification, reducing dosage needs.
2. Naked-mRNA is quickly destroyed by common RNases. Its large size and negative charge make it difficult to transport inside cells. Lipid-based nanoparticles (LNPs) help solve these problems, enhancing endosomal escape activities and facilitating molecule-targeted delivery to cells like antigen-presenting cells (APCs), boosting tumor-specific immunogenicity in the process.
3. Combination immunotherapy using mRNA vaccines and tumor microenvironment (TME) regulation techniques can simultaneously address two primary features of BC: pathological complexities (Behravan et al. 2019) and highly immunosuppressive TME (Lei et al. 2020; Gordon and Gadi 2020), while also preventing immune escape pathways and boosting anti-tumor immune responses.

To gain a deeper understanding of the potential of mRNA vaccination as a future therapy for breast cancer and to make informed choices for different cancer subtypes, this review summarizes existing pre-clinical (Table 1) and clinical (Table 2) trials of mRNA vaccines for breast cancer. Essential features are highlighted for vaccine design in terms of efficacy. Additionally, combining RNA vaccines with other therapies holds promise for improving BC treatment outcomes.

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DC dendritic cell, *HER2* human epidermal growth factor receptor 2, *hTERT* human telomerase reverse transcriptase, *IL-23* interleukin 23, *IL-36γ* interleukin 36 gamma, *ISV* in situ vaccine, *i.d.* intraductal, *i.m.* intramuscular, *i.t.* intratumoral, *i.v.* intravenous, *LNP* lipid-based nanoparticle, *NA* not applicable, *OX40L* Recombinant Human OX40 ligand, *SAM* self-amplifying mRNA, *TAA* tumor-associated antigen, *WT1* Wilm tumor gene1

Important aspects for rational vaccine design of breast cancer

Recent years have seen the development of various mRNA vaccine platforms, and scientists have identified four key aspects for designing the perfect vaccine: target selection, mRNA structure modification, delivery vectors, and injection routes.

Vaccine targets

Compared to traditional cancer vaccines, mRNA-based vaccines have more diverse target options, such as tumor-associated antigens (TAAs), tumor-specific antigens (TSAs), and tumor microenvironment (TME) antigens. These target options are not limited by the space limitation like peptide-based vaccines, allowing for whole nucleotide sequences of target tumor proteins or other protein genes, such as virus replicase genes, to be used in pre-clinical and clinical studies of breast cancer.

Tumor-associated antigens

TAAs are preferentially expressed in tumor cells and can be found to some extent in normal cells, including commonly overexpressed antigens (e.g., HER2/neu, MUC1), cancer testis antigens, and differentiation antigens. Although TAAs generally have a certain degree of self-tolerance, they

Table 1 Preclinical studies with mRNA vaccines against breast cancer models

Vaccine type	Target antigen or agonist	RNA modification	Dosage of mRNA	Route of administration	Combination therapy	Disease and treatment models	Results	References
SAM vaccine (alphaviral vector)	HER2	NA	1×10^7 particles/mouse	Footpad injection	NA	BALB/c mice immunized with vaccines before or after implantation of $hHER2^+$ tumor cells	1. Vaccination-induced HER-2 specific T cells and antibodies 2. Vaccination provided a long-lasting protective effect against tumors and was related to a systemic expansion of $CD8^+$ memory T cells	Crosby (2019)
LNP mRNA vaccine	MUC1	Modified nucleotides: 5-methylcytidine-5'-triphosphate, pseudouridine-5'-triphosphate RNA cap: 3'-O-Me-m7G (5') ppp (5') G	NA	s.b	Anti-CTLA-4 mAb	BALB/c mice were treated with a vaccine with CTLA-4 blockade after implantation with TNBC 4T1 cells	1. Vaccine modified with a mannose reached draining lymph node 2. Vaccination induced a strong, antigen-specific CTL response against 4T1 cells 3. Combination therapy significantly enhanced the anti-tumor responses with increased $CD8^+$ TIL	Liu (2018)
DC vaccine	HER-2/neu	NA	NA	NA	Cotransfection with 4-1BBL	In vitro induced HER-2/neu-specific CTLs incubated with DC vaccine	1. Vaccination increased tumor-specific CTL responses	Grunebach (2005)
DC vaccine	p53	NA	NA	NA	NA	Primary diagnosed BC patients treated with vaccine	1. Vaccination induced stronger p53-specific T-cell responses in $P53^{high}$ BC patients	Met (2011)

Table 1 (continued)

Vaccine type	Target antigen or agonist	RNA modification	Dosage of mRNA	Route of administration	Combination therapy	Disease and treatment models	Results	References
SAM vaccine (measle viral vector)	CEA (just as a marker)	NA	1.2×10^7 TCID50/mouse	i.v	NA	BALB/c nude mice immunized with vaccines after implantation of MDA-MB-231 cells	1. Vaccination brought significant cytopathic effects consisting of extensive syncytia formation and massive cell death 2. Vaccination significantly delayed tumor growth and prolonged survival	McDonald (2006)
ISV	Alphavirus vector expressing IL-12	NA	5×10^9 viral particles/mouse	i.t	Attenuated *Salmonella*	BALB/c mice were treated with vaccine before/after attenuated *Salmonella* after implantation with TNBC 4T1 cells	1. Vaccination inhibited tumor growth, prolonged survival, reduced angiogenesis, and lung metastasis 2. The combined therapy was markedly synergistic when the vaccine was administered previous to attenuated *Salmonella*	Kramer (2015)

4-1BBL 4-1BB ligand, *CEA* carcinoembryonic antigen, *CTLA-4* cytotoxic T lymphocyte-associated antigen-4, *DC* dendritic cell, *HER2* human epidermal growth factor receptor 2, *IL-12* interleukin 12, *ISV* in situ vaccine, *i.t.* intratumoral injection, *i.v.* intravenous injection, *LNP* lipid nanoparticle, *MUC1* mucin 1, *NA* not applicable, *SAM* self-amplifying mRNA, *s.b.* subcutaneous injection, *TCID* tissue culture infective dose

Table 2 Clinical trials with mRNA vaccines against breast cancer

Trial numbers (clinical phase)	Vaccine type	Target antigen or agonist	Dosage of mRNA	Combination therapy	Route of administration	Condition	Results or recruitment status	Sponsor/collaborators
NCT01526473(I)	SAM vaccine (AVX901)	HER2	4×10^8 IU given every 2 weeks for 3 injections total	NA	i.m	$HER2^+$ Breast Cancer	Completed, Safety and Toxicities	H. Kim Lyerly Susan G. Komen Breast Cancer Foundation Duke University
NCT03632941(II)	SAM vaccine (AVX901)	HER2	4×10^8 IU given every 2 weeks for three injections total	Pembrolizumab	i.m. + i.v	$HER2^+$ Breast Cancer	Recruiting	Herbert Lyerly Merck Sharp & Dohme Corp Duke University
NCT00978913 (I)	DC vaccine	Survivin, hTERT, and p53	Primary 6 biweekly injections with a minimum of 1×10^6 dendritic cells per treatment	Cyclophosphamide	i.d	Breast Cancer or Malignant Melanoma	Complete, safety, and toxicities	Inge Marie Svane Herlev Hospital
NCT00004604 (I)	DC vaccine	CEA	NA	NA	i.v	Metastatic Cancer with CEA expression (Breast Cancer, et al.)	Completed, Safety and Toxicities	Duke University National Cancer Institute (NCI)
NCT01291420 (I/II) PMID19530029	DC vaccine	WT1	4 biweekly injections with a minimum of 10×10^6 dendritic cells per treatment	NA	i.d	Solid Tumors (Breast Cancers, et al.)	Phase I study: vaccination with DC will be well-tolerated and will increase WT1-specific $CD8^+$ T-cell responses	BioNTech SE Seventh Framework Programme
NCT02316457 (I)	LNP mRNA vaccine	Neoantigens + 4 TAAs (2–3 variant RNAs + p53 RNA)	NA	NA	i.m	Triple Negative Breast Cancer	Active, not recruiting	BioNTech SE Seventh Framework Programme
NCT03739931 (I)	ISV (LNP encapsulated)	mRNA-2752 (Human OX40L, IL-23, and IL-36γ)	NA	Durvalumab	i.t. + i.v	Triple Negative Breast Cancer, Head, and Neck Squamous Cell Carcinoma, Non-Hodgkin Lymphoma Urothelial Cancer	Recruiting	ModernaTX, Inc AstraZeneca

Table 2 (continued)

Trial numbers (clinical phase)	Vaccine type	Target antigen or agonist	Dosage of mRNA	Combination therapy	Route of administration	Condition	Results or recruitment status	Sponsor/collaborators
NCT03788083 (I)	ISV	TriMix (caTLR4, CD40L and CD70)	NA	NA	i.t	Early stage Breast Cancer	Recruiting	Universitair Ziekenhuis Brussel eTheRNA immunotherapies

also induce specific T-cell responses in tumors (Hobo et al. 2013). BC mRNA vaccines that target TAAs are one of the most popular and simple options due to their roles in the oncogenic process (by either being involved in the oncogenic process or promoting cancer cell survival) and their relative presence in many cancer patients' spontaneous immunity. Studies have suggested a favorable clinical outcome for TAA-targeted therapies on patients with pre-existing TAA-specific immunity and have recommended the use of overlapping peptide pools instead of single epitopes when examining naturally occurring TAA-specific T cells in cancer patients (Met et al. 2011).

Human epidermal growth factor receptor 2 (HER2/neu) is overexpressed in 20–30% of breast cancers, leading to sustained HER2 signaling at the cell surface as an oncogenic mechanism that is associated with increased metastasis and poor prognosis. Due to its proven effectiveness in the metastatic setting, HER2 is becoming a popular target for BC immunotherapy. However, due to the loss of HER2-specific immunity, its modest adaptive immune response leads to drug efficacy limitation following disease progression, despite HER2 still being overexpressed (Ritter et al. 2007). Recent studies have revealed that mRNA vaccines present a higher number of immunostimulatory antigens compared to $HER2^+$ tumor cell-based vaccine and peptide-loading DCs. As a result, they induced the strongest cell lysis by the in vitro-induced HER2-specific cytotoxic T cell (CTL) (Grunebach et al. 2005). Crosby et al. indicated that a viral-based HER2 RNA vaccine (VRP-HER2 vaccine) could stimulate potent HER2-specific T cells in mice and significantly inhibit tumor growth. VRP-HER2 was well tolerated by patients, inducing HER2-specific T cells and, in particular, a cluster of perforin-expressed memory $CD8^+$ T cells, which were significantly correlated with improved PFS in BC patients (Crosby et al. 2019). Apart from T-cell responses, serum from VRP-HER2 vaccinated mice showed significantly more anti-HER2 antibodies than uninfected mice. While the total levels of HER2-specific antibodies were modest, they differed from HER2-targeting monoclonal antibody (mAb) medicines in that they were vaccine-induced polyclonal anti-HER2 antibodies with several effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC). These antibodies are more effective at mediating HER2 internalization, degradation, and signaling reduction than either mAbs (such as trastuzumab) or protein vaccine-induced HER2-specific antibodies (Ren et al. 2012). The dramatic reduction in plasma membrane HER2 expression and signaling resulting from these antibodies will benefit the clinical outcome of HER2 therapy-resistant disease (Ferrer-Soler et al. 2007; Blackwell et al. 2010).

Mucin 1(MUC1) is a transmembrane glycosylated mucin, normally found on the apical surface of breast ductal epithelia. However, in breast cancer patients, MUC1 is

overexpressed and typically hypo-glycosylated, leading to the production of cryptic epitopes that stimulate an immune response (Acres et al. 2017). Vaccines derived from two peptides that encode long non-glycosylated polypeptides from the variable number tandem repeats (VNTR) regions are currently in clinical development (Apostolopoulos et al. 2006; Powell and Chow 2008), but potential CTL epitopes exist outside of the VNTRs; thus, mRNA vaccines incorporating the entire MUC1 molecule may be a better option (Liu et al. 2018).

P53, a tumor suppressor protein, is essential for the maintenance of the non-tumorigenic phenotype of cells. Pre-existing T-cell responses to p53 have been observed in more than 40% of BC patients at the time of initial diagnosis, consisting of both polyclonal p53-specific $CD8^+$ and $CD4^+$ T cells (Met et al. 2011). This implies that a coordinated immune response is being elicited in BC patients; however, the response may be too limited to be effective. Wild-type p53 mRNA vaccines can serve as an effective immunotherapeutic approach for treating patients with $p53^{high}$ BC, promoting a stronger and more widespread (i.e., polyclonal) response (Met et al. 2011; Svane et al. 2007).

To summarize, the most fundamental and crucial aspect of producing a practical and effective mRNA BC vaccine is targeting selection. The process of introducing TAAs (and TSAs) into the body via mRNA vaccines is relatively more straightforward than using peptide vaccines. This is because mRNA vaccines are capable of delivering more antigenic information with a single immunization, and can encompass the entire gene sequence of interest without requiring additional selection of HLA-restricted epitopes as targets. In the active immunotherapy of breast cancer, selecting targets based on the distinct biological behaviors of different molecular subtypes of breast cancer is key to designing and producing mRNA for a BC vaccine. For HER2 overexpressed BC patients, HER2 is theoretically an effective and potent object as a vaccine, and even in advanced BC patients, it is applied to induce specific CTL responses (Crosby et al. 2019). On the other hand, TNBC, a subtype of BC, is associated with aggressive growth, a high rate of metastasis, and the poorest prognosis in patients (Ismail-Khan and Bui 2010). Because of the lack of shared tumor antigens in TNBC, selecting the right targets for vaccination is challenging and critical. MUC1, p53, and the neoantigens mentioned below have been or are currently being used in pre-clinical and clinical studies against TNBC and suggested to have effective anti-tumor activities.

Neoantigens/neoepitopes

Tumor-specific antigens include proteins of oncogenic viruses expressed by transformed cells such as HPV, as well as unique mutated proteins giving rise to neoantigens generated through somatic and frameshift mutations. Although a substantial fraction of cancer mutations are immunogenic (Kreiter et al. 2015), only a small fraction of them induce immune responses in the tumor-bearing host, limiting the efficacy of neoepitope-targeted immunotherapy (Matsushita et al. 2012). Therefore, personalized poly-neoepitope vaccination is needed to trigger immune responses against these "antigen pools" and reduce the risk of single neoantigen loss variants' outgrowth. For example, Sahin et al. pioneered the use of individualized neoepitope mRNA cancer vaccines by identifying mutanome (Sahin et al. 2017), which are mutations with high-affinity binding to autologous HLA-II and high expression of the mutation-encoding RNA, as well as predicted HLA-I binding. This enabled them to select dozens of high HLA-affinity and high expression epitopes as targets, which were engineered into several pentatope mRNAs, each encoding five linker-connected peptides containing the mutation in ORF. Upon intranodal injection of naked pentatope mRNAs into patients, $CD4^+$ T-cell responses were detected against the majority of the neoepitopes, and a low frequency of metastatic disease was observed after several months of follow-up (Sahin et al. 2017). Moreover, in a breast tumor model (implantation of 4T1 cell line), it was found that mutations were frequently immunogenic (21–45%), and poly-neoepitope mRNA vaccination effectively controlled advanced tumors in mice (Vormehr et al. 2015). Additionally, personalized vaccines targeting neoepitopes specific to each patient's tumor tissue have been used in early phase BC clinical trials without toxic effects (Table 2).

Accordingly, the clinical feasibility, safety, and anti-tumor activity of targeting individual cancer mutations through poly-neoepitope mRNA vaccination have been demonstrated, providing evidence in favor of increasing access to individually tailored medicines for a broader range of breast cancer patients

Microenvironment targeting vaccine

Utilizing TME antigens over tumor cell antigens is a novel approach to breast cancer vaccination. Due to their genomic stability, these antigens may stop the immune escape caused by antigen mutations and make immunotherapy more effective. Several prospective targets have been identified in preclinical models, such as whole-cell endothelial-based (38), EGFR- (Jin et al. 2017), CD105- (40), PDGFR-β- (Kaplan et al. 2006), and VEGF-targeting (Jin et al. 2017; Yan et al. 2013) BC vaccines, as well as DNA-based fibroblast activation protein-alpha (FAP-α)-targeting vaccines in 4T1 mouse models (Geng et al. 2019). To date, no RNA vaccines have been reported and it remains to be seen if pre-clinical studies will benefit patients.

Two types of mRNA structures in BC vaccination

According to the existing studies, there are two major structures utilized in breast cancer vaccination: conventional non-replicating mRNA (Fig. 1A) and virally derived, self-amplifying mRNA (SAM; Fig. 1B).

Conventional mRNA vaccine

Conventional mRNA vaccine products are engineered to resemble fully processed mature mRNA molecules found in eukaryotic cells and contain an open-reading frame (ORF), flanking UTRs, a 5′ cap, and a poly (A) tail. Optimizing these components, such as IVT mRNA pharmacology, decreases unwanted immune responses and enhances translation efficiency, making it useful for cancer therapeutics (McNamara et al. 2015).

Virally derived, self-amplifying mRNA, termed as "replicon"

Single-stranded RNA (ssRNA) viruses, such as alphaviruses, flaviviruses, measles viruses, and rhabdoviruses, are known for their ability to undergo spontaneous and highly efficient self-amplification of RNA in the host cytoplasm without entering the nucleus. Self-amplifying mRNA (SAM) or replicon vaccines, which are a popular category of mRNA vaccinations, are based on the structural characteristics of these viruses. However, they do not contain the viral particles and instead replace the gene sequence of virus structural proteins with the gene sequence of interest via gene recombination. This eliminates potential immune responses against the viral particles and the risk posed by viral packaging. Compared to conventional mRNA vaccines, the significant advantage of self-amplifying vaccines is the ability to administer doses that are 5–200 times lower, resulting in dose-sparing benefits. Several efficient expression systems have been developed and clinically applied (Lundstrom and Self-Amplifying 2020). Alphavirus-based vectors are the most used. Subgenomic RNA is the most common genome model of ssRNA viruses (Fig. 1B), with a 5' cap, poly(A) tail, and UTR structures similar to conventional non-replicating mRNA. It has two ORFs, the first encoding non-structural proteins of viruses that formed RNA replication complexes; and the second encoding structure proteins, initiated by the strong subgenomic promoter of ssRNA virus upstream. Replicons are used to insert interest genes in the place of the structural gene religion, which would be highly expressed by RNA replication complex (i.e., viral replication machinery) together with subgenomic promoters (Lundstrom and Self-Amplifying 2020).

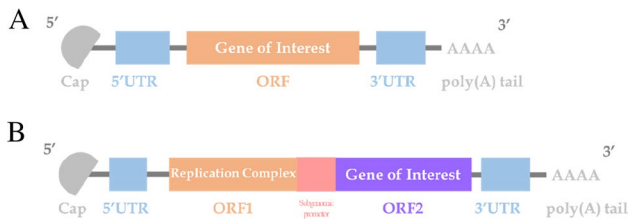

Fig. 1 Two types of mRNA structures. Messenger RNA is an intermediate step between DNA genome and protein expression. Eukaryotic pre-mRNA undergoes several maturation steps en route from the nuclear to the cytoplastic ribosome for translation, including 5' capping, 3' cleavage and polyadenylation, and splicing. **A** The structure of conventional mRNA vaccines is based on classical eukaryotic mRNA, containing a 5' cap, untranslatable regions (UTR), an open-reading frame (ORF), and a poly(A) tail. mRNA vaccines request further modifications to reduce undesired immune responses and improve the anti-tumor immunogenicity of the vaccines. **B** The general structure of alphavirus-derived SAM vaccine is characterized by its self-amplifying subgenomic structure, which permits two ORFs in a single mRNA strand to encode a replication complex and gene of interest in sequence, driven by ssRNA virus-derived subgenomic promoters

Virus-based vectors have various applications, including oncolytic effects, delivering targeting antigens to APCs or tumor targeting. BC is known to overexpress the measles virus receptor CD46, enabling the use of measles virus-based RNA vaccine (MV-CEA) for effective oncolysis (McDonald et al. 2006). However, replicon is limited by the size constraints, for instance, an alphavirus-like replicon only encoding the extracellular (ECD) and transmembrane (TM) domains of HER2 or a truncated gene (Crosby et al. 2019). Additionally, viral-based vectors can induce neutralizing antibodies and possibly block gene delivery (Crosby et al. 2019).

Studies in BC have shown that SAM vaccines elicited strong CTL, memory, and humoral immune responses against tumor antigens and limited tumor growth (Table 1). Alphavirus-like replicon models [e.g., Sindbis virus (SIN) replicon model and Venezuelan equine encephalitis virus (VEE) replicon model] encoding HER2/neu elicited strong neu-specific $CD8^+$ T-cell responses (Moran et al. 2007), $CD8^+$ memory T-cell differentiation (Crosby et al. 2019), and HER2/neu-specific antibody responses (Crosby et al. 2019; Moran et al. 2007; Lachman et al. 2001) in A2L2 or other BC cell lines. Those replicon models consequently inhibited A2L2 tumor cell growth (Wang et al. 2005), reduced tumor incidence, tumor mass (Moran et al. 2007; Lachman et al. 2001), and lung metastasis (Lachman et al. 2001). The survival rates of A2L2 tumor-bearing mice were considerably increased by a prime-boost regimen of SIN-neu immunization followed by Adenovirus-neu injection (Wang et al. 2005). These vaccines were proven safe and acceptable. Vasilevska et al. demonstrated that re-administration of RNA replicons can prolong gene expression in 4T1 tumor-bearing

animals without the risk of undesired effects related to viral delivery (Vasilevska et al. 2012).

In conclusion, SAM vaccination offers greater flexibility for using RNA in immunization studies compared to conventional mRNA vaccines. This results in robust immune protection against lethal doses of BC cells, as well as oncolytic and tracking functions. While clinical responses to SAM BC vaccination have been relatively modest thus far, further improvements in vector development, dosage optimization, and delivery are expected to lead to the production of more efficient vaccines in the near future (Lundstrom and Self-Amplifying 2020).

mRNA delivery methods in BC immunization

Two commonly used approaches for mRNA vaccines in BC therapy are loading mRNA into DCs ex vivo, followed by re-infusion of the transfected cells (Fig. 2A), and direct injection of mRNA with a protective carrier adjuvant (Fig. 2B). These approaches, together with efficient binding of mRNAs, protection against RNase, promoting cellular uptake and endosomal escape, and targeting vaccines to antigen-presenting cells (APCs, especially DCs) or tumor tissues (Guevara et al. 2019), constitute delivery methods of mRNA, and an ideal delivery requires all these factors.

Ex vivo loading of dendritic cells

Dendritic cells (DCs) are the most potent professional APCs and have been subject to intense investigation as a cellular adjuvant in cancer vaccination for the past 2 decades. These DCs can be collected from patients and transfected with mRNA ex vivo via electroporation, and adjuvants, such as TNF-α, IL-6, IL-12, IFN-γ, PGE2, LPS, and others, are used to induce a mature DC phenotype. Upon infusion into the autologous vaccine recipient, these matured DCs can initiate a predominantly cell-mediated immune response to activate $CD8^+$ and $CD4^+$ T cells by providing the first signal via pHLA-I and pHLA-II and the second signal via IL-12 and CD80/86 as costimulatory to bind CD28 on T cells (Gordon and Gadi 2020). Thus, DCs can be regarded as "nature's adjuvants".mRNA-loaded DC vaccines provide precise control of cellular target and transfection efficiency (Met et al. 2011; Grunebach et al. 2005), as well as mobilizing immunosuppressive iDCs in BC TME, which is a common pathological feature of primary BC (Treilleux et al. 2004). iDCs are not functional as nature adjuvants, but rather have immunosuppressive effects and angiogenesis. Studies show that electroporation of DCs with mRNAs encoding costimulatory molecules, such as CD83, TNFRSF4(OX40), and 4-1BB ligand (4-1BBL), increases immune stimulatory activity of DCs (Grunebach et al. 2005). The sequence of antigen transfection and DC maturation ex vivo is also an important factor for optimizing in vivo expression; antigen persistence in DCs has been shown to affect magnitude of the immune response. Met et al. observed 3–4 × fold higher p53 expression in mature electroporated DCs 2–6 h post-transfection, declining rapidly thereafter, while immature electroporated DCs showed more sustained expression over 12 h (Met et al. 2011). These results suggest introducing mRNA into mature DCs in a clinical vaccine regime for optimal T-cell stimulatory activity.

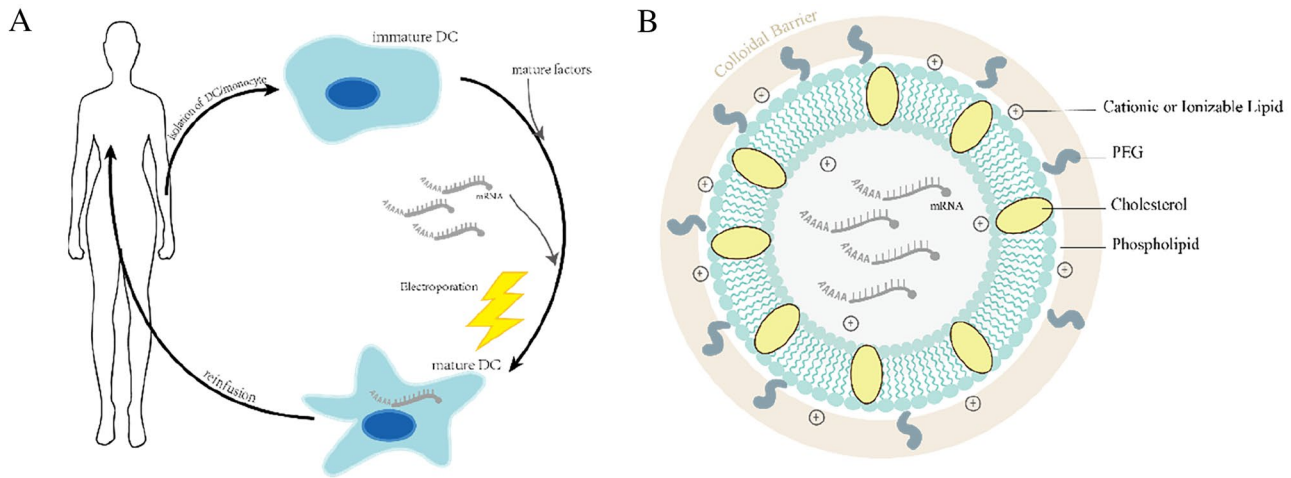

Fig. 2 Delivery platforms for vaccination. **A** Ex vivo loading of dendritic cells. Monocyte-derived DCs can be generated from patients' peripheral blood cells. Isolated immature DCs are cultured with maturation factors ex vivo, then loaded with antigen-encoding mRNAs via electroporation, and reinfused into the patient intravenously. **B** Lipid-based nano-vectors. Antigen-encoding mRNAs are complexed with cationic or ionizable lipids, cholesterol, and phospholipid to formulate a spheroid nanoparticle. Superficial lipids are then modified with PEG to form a colloidal barrier with a protective function. *PEG* polyethylene glycol

Lipid-based nanoparticles

Concerning the limitations of cell therapy, researchers have focused on developing high bioavailability and low-cost carriers with novel materials and formulations for several years. Only recently has there been rapid progress in the utilization of cationic or ionizable lipid-based nanoparticles (cLNPs, iLNP) in non-replicating mRNA and SAM delivery (Pardi et al. 2018, 2020; Guevara et al. 2019). LNPs have been found to be effective with adjuvant profiles in anti-tumor immunity, leading to efficient delivery of mRNA into APCs and induction of a strong cytotoxic T-cell response (Guevara et al. 2019).

Structures of lipid-based vectors for therapeutic mRNA-based anti-cancer vaccines have been well reviewed (Guevara et al. 2019) (Fig. 2B), and typically consist of four components: cationic or ionizable lipids, which promote self-assembly into nanoparticles, bind efficiently with RNAs' negatively charged phosphate groups, and interact with anionic endosomal lipids to facilitate endosomal release of mRNA to the cytoplasm (Liu and Huang 2010); lipid-linked polyethylene glycol (PEG), which forms a colloidal barrier on the surface of lipid nanoparticles to enhance stability and circulation time (Pasut et al. 2015); cholesterol, acting as a stabilizing agent; and naturally occurring phospholipids, which provide structure to the lipid bilayer (Pardi et al. 2018). Active targeting to APCs or tumor cells of lipid surfaces with targeting molecules is a distinct approach, and it has been demonstrated that modulating size and charge of LNPs can improve passive APC/tumor targeting and in vivo intratumoral accumulation of RNA. LNPs of sizes ranging from 20 to 50 nm can directly diffuse to draining lymph nodes (LNs) (Manolova et al. 2008), while larger ones (> 50 nm) stay at the site of injection (SOI) awaiting tissue-resident DC uptake; positively charged NPs are taken up by APCs more quickly than negative or neutral ones (Foged et al. 2004). Additionally, PEG can mask the surface charge and increase clearance from the injection site while accumulating in LNs (Kaur et al. 2012), and thus, a combination of active and passive targeting has the potential to improve immunological responses against tumor tissues.

Liu et al. first utilized a lipid-coated calcium phosphate (LCP) NP-based MUC1 mRNA vaccine for TNBC treatment (Liu et al. 2018); the vectors containing CaP core, DOPA, DOTAP (cationic lipid), and DSPE-PEG with an encapsulation efficiency of 50%, a size of 25 nm, a surface charge of 38 mV, and the surface was modified with mannose to target the mannose receptor highly expressed on DCs (Sallusto et al. 1995). It was demonstrated to be effective in draining into the lymph node and allow MUC1 settled in DCs. In vivo studies showed a significantly stronger MUC1-specific CTL response than with naked mRNA (Liu et al. 2018). Ionizable lipid cKK-E12-based LNPs have been formulated for highly selective delivery to the liver (Paunovska et al. 2019). The cKK-E12 delivery system offers protection for trastuzumab mRNA from degradation, enabling expression of full-size therapeutic antibodies in the liver. This approach has resulted in sustained high serum concentrations for up to 14 days after injection, significantly delaying the growth of HER2-positive breast cancer and improving animal survival (Rybakova et al. 2019).

In summary, LNPs have displayed great immunogenicity, stability, targeting ability, and therapeutic efficacy in BC pre-clinical phases. Currently, only BioNTech has ongoing a clinical trial assessing cationic lipoplex loading mRNA of TAA and neo-Ag as vaccination to treat TNBC (NTC02316457, an aforementioned clinical trial). Since LNP is a designable carrier, studies on better structures, components, and surface modifications of LNPs in this field are still urgently needed.

Delivery routes and in situ vaccination

Subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.), intravenous (i.v.), or intranodal injection (i.n.) of mRNA vaccination provides various delivery options. To improve effectiveness and overcome extracellular hurdles, delivery mechanisms or carriers should be considered for each administration route. For instance, direct injection into the lymph node was found to be superior to other routes such as s.c., i.d., and near nodal vaccinations in terms of the growth of antigen-specific T cells (Kreiter et al. 2010). Moreover, i.v. injection of LNPs encapsulated mRNA vaccines ensures systemic distribution specifically in APCs, particularly significant DCs, with protection from degradation and optimization of bioavailability (Kreiter et al. 2015).

Orthotopic breast cancer, an accessible tumor that can be examined through imaging, such as ultrasound, is suitable for intratumoral (i.t.) mRNA vaccination [also called in situ vaccination, ISV, and in vivo modulating of TME (Locy et al. 2018)]. The use of immune adjuvants in mRNA therapy is a double-edged sword, as their recognition by the innate immune system may impede mRNA translation and complicate their clinical application. Nevertheless, if the sequence is carefully selected, the inherent immunogenicity of mRNA can be advantageous as it provides self-adjuvanticity to immune-desert tumors like breast cancer. This self-adjuvanticity can stimulate pattern recognition receptors (PRRs) expressed by dendritic cells (DCs) that recognize RNA of viral features, resulting in start-up of inflammation and immunogenic cell death of tumor cells (Pastor et al. 2018). Most of the in situ vaccines for breast cancer do not introduce mRNA-encoding TAAs, but code for effectors like immune stimulatory molecules and immune-activating cytokines. ISVs also activate PRRs like TLRs, and the trait of mRNA allows for multiple agents

to be expressed together, such as the TriMix strategy (ISV including mRNA of CD70, CD40 ligand, and the constitutively active form of TLR4), which can increase tumor-specific T-cell responses (Lint et al. 2015). Examples of these immune-activating cytokines include IL-12, which is an important DC stimulator for producing effective anti-tumor T-cell responses (Garris et al. 2018). Kramer et al. applied a replicon of an alphavirus vector-Semliki Forest Virus (SFV)-expressing IL-12 (SFV-IL-12) as a neo-adjuvant in a locally advanced breast cancer (LABC) model. Intratumoral administrations of SFV-IL-12 inhibited tumor growth and achieved long-term survival (up to 200 days after lethal doses of 4T1 implantation) in 20% of mice after surgical resection (Kramer et al. 2015). Additionally, depletion of Treg, suppression of T-cell "stop signals", and imitation of an infectious molecule such as an oncolytic virus have also been used as in situ strategies to circumvent the BC TME problems (Gordon and Gadi 2020).

Combination immunotherapy of mRNA cancer vaccines in breast cancer

Pathological complexities (Behravan et al. 2019) and immunosuppressive TME (Lei et al. 2020; Gordon and Gadi 2020) are major factors in breast cancer immunotherapy. mRNA vaccination can provide multiple targets to manage the disease burden, while TME antigen targeting, or ISV may address the TME predicament. Researchers have called for combinations of mRNA vaccine with other therapeutics to be validated (Liu et al. 2018). However, BC vaccines are ineffective without effective and healthy T cells. Unfortunately, tumor-infiltrating lymphocytes (TILs) often express an exhausted phenotype (i.e., $CD39^+$ $CD8^+$ T cells) (Savas et al. 2018), which stimulate tumor growth. To get around these hurdles, strategies such as BC vaccine adjuvant with checkpoint-blocking antibodies and depletion of immunosuppressive cells can be used.

ICBs as adjuvants to BC vaccines

Recent pre-clinical and clinical progress testing ICBs in BC has compelled researchers and clinicians to combine these approaches with therapeutic vaccines to improve the activity and effectiveness of ICB by expanding tumor-specific T cells while simultaneously preventing the activation of inhibitory immune checkpoint pathways expressed by the vaccine-induced activated T cells (Zahm et al. 2017). Several ongoing trials are using pembrolizumab (anti-PD-1) (NCT03632941), durvalamab (anti-PD-1) (NCT02643303, NCT03199040, NCT03739931), avelumab (anti-PD-L1) (NCT03387085), and tremelimumab (anti-CTLA-4) (NCT02643303) as adjuvants or boosters to various BC vaccines (Gordon and Gadi 2020); these drugs can significantly enhanced the anti-tumor responses and revive TIL (Liu et al. 2018; Crosby et al. 2020). Liu et al. found that the combination of MUC1-based mRNA vaccine with an anti-CTLA-4 monoclonal antibody induces a potent CTL response against TNBC, and the combination can greatly enhance T-cell immune response significantly better than treatment with either the mRNA vaccine or the anti-CTLA-4 monoclonal antibody alone (Liu et al. 2018). Another ongoing clinical trial using RO7198457 (individualized mRNA vaccine) + atezolizumab (anti-PD-L1) targeting TNBC(NCT03289962) HER2 overexpressing tumors have been reported with lower response rates to ICB (Dirix et al. 2018), but treatment with HER2-specific mRNA vaccines may enhance the responsiveness of tumor cells toward immune checkpoint inhibitors by reviving T cells (Crosby et al. 2020).

Treg depletion

$CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells are major immunosuppressive cells in the breast cancer TME, a significant hurdle for effective BC vaccine response. Two vaccine-adjuvant strategies that block Treg cells are cyclophosphamide (CP, NCT03066947) or Treg blocking antibodies (NCT01660529). CP is a commonly used alkylating chemotherapy for patients with BC and given at routinely low doses can significantly impacts Tregs and endothelial cells (antiangiogenic), perhaps by downgrading the TGF-β receptor or via ATP-dependent proliferation-dependent cytotoxicity (Madondo et al. 2016). In patients with advanced breast cancer, metronomic CP administration transiently decreases Tregs while increasing effector CTLs (Ge et al. 2012). Anti-FoxP3 and anti-CD25 are two studied antibodies. Anti-FoxP3 treatment with a neutralizing peptide (p60) in a 4T1 model with DC-based vaccines improved survival outcomes and decreased lung metastasis more than p60 or vaccine alone (Moreno Ayala et al. 2017). This treatment works by suppressing IL-10 and reducing TME immunosuppression. Anti-CD25 treatment (daclizumab) has been used as an adjuvant to BC vaccines in clinical trials, and the treatment was well tolerated, with a statistically significant decline of $FoxP3^+$ CD4 Treg cells at multiple time intervals (Rech et al. 2012). Combination of all three with mRNA vaccines should be further explored in human trials. Ultimately, CP, anti-FoxP3, and anti-CD25 have not yet been combined with mRNA vaccines and needed to be further explored in human pre-clinical and clinical trials as vaccine adjuvants.

The foundation of immunotherapy and cancer vaccines lies in a thorough comprehension of the immune evasion mechanisms of tumors (Saxena et al. 2021). While many researchers and doctors have believed that generating an

activated anti-BC response through vaccination alone may not be sufficient to demonstrate tumor killing of bulky BC tumors, combining it with ICB is a more promising option in clinical applications. As our understanding of the TME advances, we may discover novel combinations.

Summary and outlook

Significantly, the anti-tumor immune response mediated by $CD8^+$ T cells constitutes a crucial aspect of our multistep approach to combating cancer. Any failure in T-cell priming, trafficking, infiltration, survival, as well as recognition and elimination of the tumor can result in the immune-cold phenotype of breast cancer. While passive immunotherapy, such as ICB, is an option, active immunotherapy is a more direct means of stimulating the host immune system. Therefore, we emphasize the importance of mRNA vaccination in the treatment of breast cancer.

In conclusion, TNBC and $HER2^+$ breast cancer are currently the two major subtypes of breast cancer with active research of immunotherapy. Nevertheless, research on mRNA breast cancer vaccines should also expand to HR^+ subtypes, which consists of majority of breast cancer. The total level of BC tumor mutational burden (TMB) is only poor to modest (Zehir et al. 2017). Among all subtypes of breast cancer, TNBC has higher response rates to ICB and generally contains higher TMB and TILs (Li et al. 2021) compared to HR^+ and $HER2^+$ subtypes (Narang et al. 2019). Additionally, the high somatic mutational burdens of TNBC may make neoepitopes more suitable as TNBC vaccine targets. HER2 as the target for overexpressed BC is almost uncontested, while some researchers further identified the oncogenic isotype of HER2, HER2Δ16, as the target of vaccines, and generated oncogene-specific TILs enable sustained anti-tumor responses (Crosby et al. 2020). The success of HER2-ADC in HER2-low breast cancer implies the possibility to target HER2 as an immunotherapy target in HER2-low breast cancer patients. SAM structures and LNP delivery vectors have made low-dose injections feasible. Immunosuppressive TME in BC patients may be resolved through ISV or combinational therapies. However, pre-clinical and clinical trials using mRNA BC vaccines have yet to deliver significant improvement. It is hopeful that target selection and combination therapy will eventually yield a breakthrough in BC vaccination, in light of the multiple advances in mRNA vaccine delivery and immunogenicity.

Combination therapy with mRNA vaccines can be informed by mechanistic insights into tumor cells' resistance to immunotherapy, which can be divided into "intrinsic" and "extrinsic" mechanisms (Saxena et al. 2021). Intrinsic causes include T-cell recognition prevention, alterations in antigen processing pathways, and loss of HLA expression. Tumor cells also can constitutively express the ligands for immune checkpoints (for example, PD-L1) and interferes with the activation of effector functions in T cells (Yarchoan et al. 2019), like TNBC. Besides, by increasing the expression of β-catenin, tumor cells can repress infiltration of effectors, such as T-cell exclusion (Luke et al. 2019). Extrinsic causes are mediated by immunosuppressive cellular components, such as Treg cells, MDSCs, TAMs, cancer-associated fibroblasts (CAFs), and protumor N2 neutrophils (Welters et al. 2016), reducing anti-tumor immunity through immunological checkpoint interactions and immunosuppressive cytokines. TME components, including CAFs, can remodel the extracellular matrix to form a thick fibrotic stroma, inhibiting DC proliferation and migration, blocking T-cell infiltration, and recruiting MDSCs. Several studies have established the presence of these pathways in refractory BC (Gordon and Gadi 2020; Li et al. 2020), suggesting future therapeutic methods, such as ISV, ICB, and oncolytic virus combinations for BC recurrence and metastasis. Combination therapy with next-generation mRNA vaccines could improve global women's health care and reduce recurrence and metastasis of breast cancer.

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