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siRNA in Breast Cancer Treatment: Targets, Efficacy and Safety

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Abstract. Breast cancer is a malignant tumor with a high incidence in the world. The treatment of breast cancer is faced with the dual dilemma of unavailability of molecular targets and chemotherapy resistance. New intervention strategies are urgently needed. Small interfering RNA (siRNA) therapeutic drugs have the ability for sequence-specific gene silencing. These drugs can inhibit key targets such as the anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) and survivin, as well as the oncogenic transcription factor signal transducer and activator of transcription 6 (STAT6) at the messenger RNA (mRNA) level. This inhibition effectively restores the chemosensitivity of tumor cells and inhibits the malignant phenotype. This effect provides a feasible way to overcome the limitations associated with traditional targeting dependence and drug resistance. The clinical transformation of siRNA is still hampered by two core challenges including low efficiency of in vivo delivery and safety risks. This recognition triggers unexpected toxicity and reduces the therapeutic effect. Current studies have partially alleviated the above-mentioned obstacles through nanocarrier design, chemical modification, and targeted ligand coupling. Nevertheless, sufficient data on long-term administration, in situ tumor models, and systems toxicology are still lacking. The strategy of combined gene silencing or combined therapy remains to be further explored. This review covers key molecular targets, extracellular and intracellular delivery disorders, immune stimulation and target mechanisms. The prospect of multifunctional nanoplatforms and combined therapy is discussed, which provides a theoretical reference for the optimization and clinical transformation of siRNA-based breast cancer therapeutic drugs.

Keywords: siRNA, breast cancer, off-target, Bcl-2

1. Introduction

Breast cancer is one of the most common malignant tumors in the world. According to statistics, there were about 2.3 million new cases of breast cancer worldwide in 2022. This disease has caused a huge burden of disease. The treatment of modern breast cancer is mainly based on molecular subtypes. Treatment methods generally include chemotherapy and targeted therapy. Some molecular subtypes such as triple-negative breast cancer lack operable therapeutic targets and rely only on chemotherapy. This reality highlights the limitations of targeted therapy in the absence of targets. Tumor cells develop drug resistance during chemotherapy. This phenomenon requires repeated

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administration and causes cumulative damage to normal tissues. Therefore, new treatments are urgently needed in clinical practice.

Traditional small molecule drugs usually act on specific proteins in cancer cells, but it is often difficult to find effective targets in the face of non-pharmaceutical proteins. Antibody drugs, especially antibody drug conjugates, can accurately deliver highly active cytotoxic drugs to tumor cells by virtue of the targeting characteristics of monoclonal antibodies, thereby achieving the effect of 'targeted chemotherapy'. However, although antibody drugs can achieve precise delivery, their effects depend on the expression level of tumor cell surface antigens, and heterogeneous expression often leads to different efficacy. In contrast, if the intervention target is moved from the protein level to the messenger RNA (mRNA) level, gene silencing can be achieved through sequence complementation, and protein synthesis can be inhibited from the source, which may bypass the limitations caused by differences in antigen expression. At the same time, the heterogeneity of tumor antigen expression may also limit the complete elimination of all tumor cells by antibody drugs [1].

Small interfering RNA (siRNA) therapy provides a feasible solution for this situation. The specified siRNA molecules inhibit genes associated with drug efflux pumps, deoxyribonucleic acid (DNA) repair enzymes, anti-apoptotic regulators, and epithelial-mesenchymal transition. Tumor cells restore their sensitivity to chemotherapeutic drugs through this inhibition. siRNA silences the drug-resistant gene in a sequence-specific manner. By shifting the therapeutic target from the protein structure to the mRNA sequence, siRNA therapy greatly reduces the dependence on traditional therapeutic targets. Bare siRNA has poor stability in vivo. Bare siRNA is easily degraded and quickly cleared by the kidney. The uptake efficiency of siRNA by tumor cells is still low. Therefore, siRNA delivery requires nanomaterials. Nanocarriers can protect siRNA molecules, enhance tumor accumulation, and achieve targeted release. This review summarizes the knowledge of key therapeutic targets, delivery-related challenges, and safety. This review provides a theoretical basis and reference for the future development of siRNA-based breast cancer treatment strategies.

2. Targets of siRNA for breast cancer therapy

In response to the dilemma of chemotherapy resistance and target loss in breast cancer, siRNA therapy can restore drug sensitivity by silencing specific genes. This chapter focuses on three representative molecular targets: the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), survivin, which has the function of inhibiting apoptosis and promoting division, and the cancer-promoting transcription factor signal transducer and activator of transcription 6 (STAT6). The former two mainly affect the tolerance of cells to chemotherapeutic drugs, while the latter is involved in the regulation of tumor metastasis and immune response; together, these three targets constitute important candidates for siRNA intervention. This chapter reviews the biological functions, siRNA delivery strategies and effect verification of each target.

2.1. Bcl-2

B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein overexpressed in breast cancer. Its main function is to stabilize the mitochondrial membrane and prevent the release of apoptotic factors such as cytochrome C, thereby inhibiting caspase activation and apoptosis. Bcl-2 prevents cancer cells from apoptosis during chemotherapy and radiotherapy, allowing cancer cells to continue to survive. Therefore, inhibition of Bcl-2 expression can sensitize tumor cells and enhance drug effects (Table 1).

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Au-S covalent bonds can be used to directly assemble 3-thiolated Bcl-2 siRNA on the surface of gold nanoparticles (AuNPs). After reaching Bcl-2 mRNA, siRNA can degrade the full-length Bcl-2 mRNA, and doxorubicin (Dox) has the ability to directly embed nucleic acid base pairs, which can load drugs onto AuNPs for simultaneous delivery. Among the tested AuNP formulations, Bcl-2-AuNPs that only delivered Bcl-2 induced apoptosis in 26% of cells, and co-delivery induced apoptosis in 35% of cells. Bcl-2 gene silencing and Dox exposure lead to cytoplasmic contraction or shedding in many cells.

After successful delivery, the Dox-Bcl-2-AuNP co-delivery group reduced the colony formation ability of breast cancer cells by 98%, and Bcl-2 silencing and Dox delivery inhibited wound closure. Dox-Bcl-2-AuNP treatment had the strongest inhibition of wound closure, with a closure rate of about 45%. The treatment also caused the largest inhibition of cell migration [2].

2.2. Survivin

Survivin is a member of the inhibitor of apoptosis protein (IAP) family. Its mechanism of action has two aspects: one is to inhibit the apoptosis of cancer cells; the other is to regulate cell division. Survivin can promote cell proliferation. This survivin is a key cytokine that causes human tumor cells to develop resistance to chemotherapeutic drugs and radiotherapy. Inhibition of survivin can also sensitize tumor cells, making it easier for human tumor cells to be killed. Making chemotherapy and radiotherapy more efficient also reduces their side effects (Table 1).

The nanocarrier binds to the N-terminal baculovirus IAP repeat (BIR) domain of survivin in breast cancer cells, and the center of the BIR domain binds to a zinc ion, which is essential for the stability and function of the survivin protein structure. The researchers found that the nanocarriers they studied had a high physical affinity for the BIR domain (binding energy -10.3 kcal/mol). The target of siRNA is survivin mRNA in breast cancer. After siRNA enters the cell, RNA-induced silencing complex (RISC) is formed. Through the principle of complementary base pairing, survivin mRNA in breast cancer cells is recognized and bound, and it is cleaved and degraded.

After targeted therapy, real-time polymerase chain reaction (PCR) showed that the mRNA expression level of survivin in MCF-7 human breast cancer cells decreased significantly after siRNA delivery. Western blot showed that the expression of survivin protein was significantly decreased. After treatment, the apoptosis of MCF-7 breast cancer cells was significantly induced and the sensitivity of cells to the drug was significantly increased [3].

2.3. STAT6 signaling pathway

STAT6 is highly expressed in tumors such as colorectal cancer, breast cancer, pancreatic cancer, prostate cancer, and lymphoma. Four proprietary STAT6-specific siRNA sequences were used to knock down the expression of the STAT6 gene. Among them, sequence 1 (STAT6.1) and sequence 4 (STAT6.4) had the best effect, which could effectively silence the gene expression of STAT6 and significantly inhibit the proliferation and induce apoptosis of colorectal cancer and breast cancer cells. The principle is that the synthesized STAT6 siRNA is a 21-23 nucleotide double-stranded RNA. After entering the cell, it assembles with the protein to form a RISC complex, and then one is degraded and another is accurately identified and bound to the messenger RNA transcribed by the STAT6 gene. The key protein Argonaute 2 (Ago2) in the RISC complex cleaves mRNA in the cytoplasm, and the mRNA cannot be translated into STAT6 protein, thereby achieving gene knockdown (Table 1) [4].

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3. Practical challenges for siRNA application

Although siRNA therapy provides a new idea for gene intervention in breast cancer, its clinical transformation is still subject to two core challenges: first, the delivery efficiency is insufficient, including multiple barriers such as serum nuclease degradation, rapid kidney clearance, limited tumor tissue penetration, and difficulty in endosomal escape, making it difficult for sufficient siRNA to reach the cytoplasm to play a role; second, there is a potential safety risk, mainly reflected in off-target silencing and immune stimulation effects, which may lead to unexpected gene regulation or inflammatory response. These factors not only restrict the treatment window of the existing delivery system, but also largely determine the necessity of joint strategy and modification optimization. The following will be discussed from the above two aspects.

Table 1. siRNA targets, molecular features, and silencing outcomes in breast cancer

Target	Main features	Key effects after siRNA silencing	Ref.
Bcl-2	Stabilize the mitochondrial membrane, stop the release of apoptosis factors, and block caspase activation	Protein expression decreased by about 40% (up to 55% with doxorubicin); induced apoptosis rate of about 26%~35%; significantly inhibited colony formation and cell migration	[2].
Survivin	Double action: inhibits cell death and controls cell division	mRNA and protein levels dropped significantly; increased sensitivity to mitoxantrone, and combined treatment further boosted cell death	[3].
STAT6	Regulates cell differentiation, growth and survival; promotes proliferation and invasion	mRNA reduced by about 50%, protein knockdown >50% (maintained for 7 days); cell proliferation decreased by about 50%	[4].

3.1. Efficacy-related concerns

In order to reach the cytoplasm of breast cancer cells and exert gene silencing function, siRNA needs to overcome multiple extracellular and intracellular barriers. The extracellular barrier mainly includes the degradation by serum nuclease, rapid renal clearance, and the limited vascular permeability in tumor tissues and the distribution disorder caused by interstitial hypertension. These factors together limit the ability of the nano-delivery system to carry sufficient siRNA to the tumor area. Even if some vectors successfully enter the cells, the lack of endosomal escape efficiency is still a key factor restricting cytoplasmic delivery. The above barriers are described separately below.

The blood contains a large amount of ribonuclease, which can rapidly hydrolyze naked siRNA. The half-life of free siRNA in serum is usually only a few minutes. One of the core design goals of lipid nanoparticles (LNPs) is to protect siRNA from enzymatic degradation. However, even if it is encapsulated in LNP, siRNA still faces challenges before reaching the target cells. The surface of LNP adsorbs a variety of proteins in the serum to form a protein corona. The protein corona may not only mask the targeted ligand of LNP, but also change the particle size and surface charge of LNPs, and even lead to the aggregation of LNPs in plasma or non-specific uptake by the reticuloendothelial

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system, thereby reducing the number of LNPs that can completely reach the tumor blood vessels, and indirectly reducing the probability of siRNA entering the cytoplasm.

The glomerular basement membrane of the kidney has a pore size cutoff value of about 5−10nm . The molecular weight of naked siRNA is about 13−14kDa, and the hydrodynamic diameter is much smaller than the glomerular filtration threshold, so it is easily filtered by glomeruli and quickly excreted with urine. If LNP is dissociated in the systemic circulation, the released free siRNA will be immediately cleared by the kidney. In addition, fluorescence imaging in the paper showed that if even intact LNPs are intercepted by a large number of reticuloendothelial system organs such as the liver and spleen, the absolute number of particles that can penetrate tumor blood vessels is further reduced, which indirectly limits the total amount of siRNA that can eventually be used for cytoplasmic delivery [5, 6].

The main pathway for the delivery of siRNA nanoparticles into cells is the endocytosis pathway, but after these nanocarriers enter the endosome, if they cannot escape in time, the endosome will mature into a lysosome, and the siRNA will be completely degraded under the action of the acidic environment and various enzymes. The RNA-induced silencing complex (RISC) in the cytoplasm is destroyed [7]. This endosomal escape problem is also one of the key factors to solve the problem of effectiveness in the future.

3.2. Safety-related concerns

The clinical use of siRNA still faces another problem: siRNA treatment is not necessarily safe. In the face of siRNA treatment, the human body may undergo sequence-dependent off-target gene silencing and sequence-independent immune response initiation [8].

The off-target effect refers to the fact that because of the large number of similar genes in the human body, siRNA fails to hit the designated genes, but mistakenly inhibits the expression of other normal genes, leading to cell death or dysfunction. This can cause unexpected side effects or toxicity. For the off-target effect, there is still a huge space for the development of nanoparticle systems to reduce the off-target effect by coupling targeted ligands [9]. siRNA can induce an off-target effect through a microRNA (miRNA)-type interaction between the antisense strand seed region and off-target mRNA. α-L-threofuranosyl nucleic acid (TNA) modification within the seed region reduces the thermodynamic stability of double-stranded siRNA duplexes because of spatial constraints between TNA molecules. The affinity between the seed region and non-target mRNA declines, and off-target activity decreases. Introduction of a TNA modification at position 7 of the antisense strand maintains on-target potency while substantially suppressing off-target activity in relevant investigations [10].

The innate immune system recognizes systemically delivered siRNA as exogenous material. Recognition proceeds via Toll-like receptor (TLR)-dependent or non-TLR-dependent pathways. siRNA is misidentified as viral RNA. Large quantities of inflammatory cytokines are subsequently secreted. Major secreted mediators include type I interferons such as interferon-α (IFN-α) and pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α).

Immune-stimulatory siRNA molecules exhibit distinct length and sequence signatures. These signatures drive toxic reactions and reduce therapeutic efficacy [8, 11, 12]. Long double-stranded RNA exceeding 30 nucleotides induces robust interferon responses and undergoes rapid degradation. Standard 21-23-nucleotide siRNA largely avoids such strong immune recognition. Certain nucleic-acid motifs such as GU-rich sequences are preferentially recognized by TLR7/8 to produce sequence-dependent immune stimulation [8, 12].

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Multiple strategies can mitigate TLR7/8-mediated recognition. These strategies include U/A base substitution, elimination of GU-rich motifs such as 5'-GUCCUUCA-3', introduction of 3' overhangs instead of blunt ends, and removal of 5'-triphosphate groups to diminish retinoic acid-inducible gene I (RIG-I)-mediated detection [12]. Chemical modifications such as 2'-O-methyl (2'-OMe), 2'-fluoro (2'-F), and 2'-O-methoxyethyl (2'-MOE) substantially reduce recognition by TLR7/8 and RIG-I [10, 11]. Concrete implementation approaches must be adjusted according to practical experimental requirements.

4. Conclusion

In summary, siRNA therapy has entered the stage of strategy integration from target verification in the field of breast cancer treatment, and has formed a more systematic body of research results around the two core pain points of chemotherapy resistance and target loss. At the level of target intervention, a large number of experiments have confirmed that specific siRNA can induce mitochondrial apoptosis pathway restart for anti-apoptotic protein Bcl-2, interfere with cell cycle progression for survivin with both apoptosis inhibition and division promotion functions, and weaken the cancer-promoting transcription network for transcription factor STAT6 involved in metastasis and immune regulation, respectively. Therefore, specific siRNA can effectively reverse chemotherapy tolerance and inhibit tumor malignant phenotype, and the potential synergistic effect between the three has begun to emerge. In terms of delivery carriers, platforms such as gold nanoparticles, lipid nanoparticles, and polymer complexes have shown the ability to protect siRNA from serum enzymatic degradation, enhance tumor tissue accumulation, and achieve co-delivery with chemotherapeutic drugs. The integrated strategy of gene silencing combined with chemotherapy sensitization has been preliminarily verified. However, most of the existing studies are limited to in vitro transient transfection or single administration, and in situ tumor models and long-term medication data are still relatively weak. Two major obstacles have been clearly defined in this field. Extracellular barriers such as nuclease degradation, rapid renal clearance, limited tumor penetration and insufficient intracellular endosomal escape lead to low cytoplasmic delivery efficiency, while off-target silencing and TLR / RIG-I pathway-mediated immune overactivation constitute significant safety risks. Current chemical modifications such as base substitution, sugar ring modification, and targeted ligand coupling have initially reduced immune recognition and reduced nonspecific uptake, thus broadening the therapeutic window.

In the future, the overall development direction of this technology will focus on the construction of intelligent responsive nanosystems, and achieve accurate space-time delivery by integrating multiple microenvironment-sensitive release mechanisms, active crossing of vascular barriers, and efficient endosomal escape functions. At the same time, the combined treatment strategy is going beyond chemotherapy sensitization alone and extending to the organic integration with immune checkpoint inhibitors, epigenetic regulatory drugs, and small molecule targeted drugs to cope with tumor heterogeneity and multidrug resistance. In addition, the use of multi-omics and bioinformatics tools to carry out individualized target combination and administration timing optimization under the guidance of molecular typing is expected to further improve the accuracy of efficacy prediction. Long-term toxicological evaluation, large-scale preparation processes and in situ preclinical model verification are the necessary cornerstones to promote clinical transformation. In general, siRNA breast cancer therapy has evolved from concept validation to engineering and systematization, and its ultimate success depends on the synergistic balance of delivery efficiency, safety and treatment accuracy. The deep integration of materials science, molecular pharmacology and clinical oncology will be the core engine driving this process.

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