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Research on Single-cell Sequencing of Tumor Stem cells in the Nano-Targeting of Triple-Negative Breast Cancer

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Abstract:

Primarily attributed to its profound intratumoral heterogeneity and the lack of actionable molecular targets for therapy, triple-negative breast cancer (TNBC) exhibits the most unfavorable clinical treatment prognosis. In recent years, the emergence of single-cell sequencing (SCS) technology has provided unprecedented resolution for analyzing the internal heterogeneity of TNBC, especially showing unique advantages in analyzing the heterogeneity of tumor stem cells (CSCs). At the same time, the development of nanotechnology has provided new opportunities for targeted therapy of CSCs. Nanotargeted drugs based on the key targets identified by SCS have shown good potential in anti-resistance and anti-metastasis in preclinical studies. However, the dynamics of the tumor microenvironment, the immunogenicity of nanomaterials, and individual molecular differences remain significant challenges currently. Future research should focus on the dynamic monitoring function of SCS and the optimization of the biocompatibility of the nanomedicine delivery system to achieve the clinical translation of individualized treatment strategies.

Keywords:-Cancer stem cells; single-cell sequencing; tumor heterogeneity; nanomedicine; triple-negative breast cancer

I. Introduction

Constituting 15 to 20% of breast cancer incidences, TNBC is distinguished by the lack of actionable therapeutic targets. Clinical validation confirms HER2 negativity and immunohistochemical results showing estrogen receptor (ER) and progesterone receptor (PR) expression <1%. Characterized by medium/high-grade differentiation, highly proliferative cancer cells, and restricted treatment options, this biologically invasive subtype exhibits the worst prognosis among breast cancer types [1].

Characterized by its ability to measure molecular changes at the single-cell level, single-cell sequencing technology (SCS) surpasses traditional bulk sequencing methods in resolving cellular heterogeneity.

The sequencing data obtained by traditional high-throughput sequencing techniques merely mix the average gene measurements of the collected cells into a large number of tissue samples.Compared with traditional sequencing techniques, SCS not only can accurately measure gene expression levels and detect trace expressions of non-coding RNA, but also can fully utilize the advantages of sequencing special samples and make up for the disadvantages of small sample sizes and difficulty in obtaining special samples [2]. With the development of SCS technology, scientists can resolve the cellular heterogeneity of TNBC at an unprecedented resolution, precisely identify the cancer stem cell (CSC) population and its key signaling pathways. These discoveries provide a new molecular basis for developing targeted therapeutic strategies against CSCs.

Concurrently, the advancement of nanotechnology offers promise for addressing TNBC treatment challenges. Nanoparticulate drug delivery systems can achieve precise tumor tissue targeting via passive or active mechanisms, even enabling direct engagement with CSCs, thereby enhancing therapeutic efficacy while minimizing systemic toxicity.

II. Molecular Markers and Classification of TNBC-CSCs

The identification of TNBC-CSCs often relies on the combined detection of specific molecular markers. Among the numerous markers, CD44, CD24, and aldehyde dehydrogenase 1 (ALDH1) constitute the most commonly used identification system.

A. CD44+/CD24-: Core Marker of CSCs

Studies on solid tumors have revealed that the breast cancer stem cell population exhibits unique surface marker characteristics: high expression of CD44 and nearly absent CD24, while lacking the typical lineage differentiation markers of white blood cells, endothelial cells, mesothelial cells, and fibroblasts [3]. It is a remarkable fact that there are significant differences in the CSCs phenotypes among different breast cancer subtypes and even among patients with the same subtype, which poses special challenges for targeted therapy.

B. ALDH1: Regulates the Antioxidant Defense Function of CSCs

The ALDH protein family, comprising 19 cytoplasmic enzymes, primarily functions in detoxifying cytotoxic reactive oxygen species (ROS). Beyond its role in aldehyde oxidation during early stem cell differentiation, this enzyme family is critical for converting retinol to retinoic acid. Notably, the ALDH1 subtype specifically generates NADP, which facilitates ROS scavenging [4].

III. Molecular Signaling Pathway

A. Wnt/β-Catenin Pathway: Promotes self-renewal of CSCs

In TNBC, the abnormal activation of the Wnt/β-catenin signaling pathway is closely associated with the poor prognosis of breast cancer. The excessive activation of Wnt/b-catenin signal transduction and the upregulation of Wnt receptor expression in TNBC/BLBC indicate that the Wnt/b-catenin pathway may be an attractive new therapeutic target for TNBC/BLBC [5].

B. STAT3-FAO Pathway: Influences Stemness through Regulating Lipid Metabolism

STAT3, as a central regulatory factor within the inflammatory factor signaling pathway, plays a crucial role in cellular proliferation and differentiation. Wang and his research team demonstrated that the JAK/STAT3 pathway exerts significant regulatory effects on lipid metabolism dynamics. This function is vital for maintaining the self-sustaining capacity and chemoresistant properties of breast cancer stem cells (BCSCs). Inhibition of the JAK/STAT3 pathway effectively disrupts the self-renewal process of BCSCs and downregulates the expression levels of several key genes involved in lipid metabolism. These genes include carnitine palmitoyltransferase 1B (CPT1B), which encodes the rate-limiting enzyme for fatty acid β-oxidation (FAO). Analysis data derived from human breast cancer tissues further reveal that the STAT3-CPT1B-FAO signaling axis is a key driver in the formation of cancer cell stemness characteristics and chemoresistance. Disrupting FAO can restore the chemosensitivity of tumor cells, and animal experiments have confirmed that this strategy can significantly reduce the proportion of BCSCs in mouse transplanted tumors [6].

C. HIF-1αpathway: Activating the Characteristics of CSCs in Hypoxic Microenvironments

Researchers such as Sarah J used the Aldefluor assay to identify the population rich in CSCs, and determined that the population of human breast cancer cells with enhanced growth would increase under mild hypoxic conditions. It was also proved that this effect was mediated by HIF-1α. The hypoxic environment can significantly increase the proportion of such stem cells through the HIF-1 signaling pathway [7].

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The above studies have shown that CD44+/CD24- and ALDH1 are the core surface markers for identifying TNBC-CSCs. In terms of the molecular mechanism, the abnormal activation of the Wnt/β-catenin pathway maintains the stemness of CSCs; the STAT3-FAO pathway affects the maintenance of stemness and chemotherapy resistance by regulating fatty acid metabolism; and the further activation of the HIF-1α pathway in the hypoxic environment enhances the survival and invasion ability of CSCs. These findings not only reveal the heterogeneity basis of TNBC-CSCs, but also provide a key direction for subsequent targeted intervention. Notably, molecular marker expression and pathway activity vary significantly across TNBC subtypes, so future research can combine SCS for classification to develop more precise targeted treatment strategies.

IV. SCS Analyzes Stem Cell Characteristics

A. SCS Reveals Characteristics of Stem Cell Subpopulations

The core challenge currently faced in the treatment of TNBC lies in the lack of validated effective targets, which makes it a difficult point in clinical treatment. The existing molecular classification system mainly relies on the overall tumor expression profile rather than single-cell level analysis. The use of SCS has opened up a new way to identify potential markers of TNBC stem cells. By identifying specific markers, it is possible to precisely distinguish different subgroups within mixed cell samples. This breakthrough progress is particularly crucial for clarifying the cell lineage and heterogeneity of TNBC [8].

The Nguyen team used SCS to analyze breast tissue from clinical healthy donors in a cell state context, developing a breast single-cell atlas that described 23 epithelial cell states: including 8 basal-myoepithelial (BM) cells, 3 intermediate basal-luminal (BL) progenitor cells, 8 luminal progenitor cells (luminal adaptative secretory precursor (LASP) cells), and 4 mature luminal cells. This study also provided precise guidance for stem cell targeting strategies [9].

B. Single-cell Technology for Analyzing the Tumor Microenvironment (TME)

The Sunny Wu team used SCS to analyze approximately 24,300 single cells from 5 TNBC persons. They identified two cancer-associated fibroblasts (CAF) and two perivascular-like (PVL) cell subpopulations. Through conducting deeper studies, they gradually revealed the mechanisms of microenvironmental changes that might regulate tumor progression or treatment response. Notably, the investigators demonstrated that inflammatory cancer-associated fibroblasts (iCAFs) secrete the chemokine CXCL12, which exerts immunosuppressive effects on T cells. This discovery provides a potential target for enhancing the efficacy of immunotherapy for TNBC [10].

The Mihriban team conducted a detailed analysis of over 1,500 cells from 6 female patients with primary triple-negative breast cancer (TNBC) using SCS. The aim was to decipher the potential biological characteristics of TNBC. The researchers analyzed the tumor cells and their subgroups of each patient one by one, observing significant differences in the gene expression programs among the tumor cells, and finding that this heterogeneity was closely associated with the inferred clonal genomic copy number variations (CNV). This correlation indicates the hypothesis that the genotype largely drives the gene phenotype expression of individual subgroups. This study not only clarified the heterogeneity of TNBC and its intrinsic connection with genomic evolution, but also suggested unexpected biological mechanisms that lead to poor disease prognosis [11].

C. Important Discovery: Stem Cell Resistance Characteristics after Chemotherapy

To explore the pronounced chemotherapy resistance observed in TNBC, the Charissa team employed whole exome sequencing (WES) combined with SCS technology to longitudinally track the molecular evolution process of 20 TNBC patients during their chemotherapy. They observed that 50% of the patients (10/20) achieved tumor clone clearance after chemotherapy, while the drug-resistant clones persisted in the remaining 10 patients during the treatment. Focusing on 8 representative cases, the team performed deeper SCS-based analyses. These detailed examinations demonstrated that chemotherapy-resistant cancer cells pre-existed prior to treatment initiation and underwent selective expansion during chemotherapy exposure. Additionally, the transcriptional profiles of these chemotherapy-resistant cells experienced substantial rewiring following chemotherapy treatment. Crucially, by developing predictive models leveraging extended clinical follow-up data, the research team identified a method to potentially forecast which patients are most likely to derive positive outcomes from chemotherapy, aiming to enhance therapeutic precision [12].

The Mei-Chong Wendy Lee team used SCS to explore the MDA-MB-231 cell line and deeply analyzed the transcriptional heterogeneity of TNBC after chemotherapy. The researchers treated metastatic human breast cancer

cells with paclitaxel (a microtubule-targeted chemotherapy agent) and conducted whole-transcriptome analysis at both single-cell and population levels. This experiment demonstrated that in untreated, stressed, and drug-resistant cell subpopulations, specific gene expression patterns were activated, and individual cells within and between populations exhibited significant heterogeneity differences. The researchers further discovered that drug-resistant cells contained unique RNA variants, which were concentrated in genes related to microtubule assembly stability, cell adhesion, and surface signal transduction. Therefore, SCS suggested the dynamic mechanism of stress response, including specific RNA variants driving cell heterogeneity, promoting the survival of a few cell subpopulations under stress, and allowing stress-tolerant cells to efficiently recover to a normal state [13].

V. Existing Strategies for Targeting CSCs with Nanoparticles

A. Dual-Drug Co-Delivery System: Paclitaxel Combined with PKM2 Inhibitor

As the core drug of the basic chemotherapy regimen for TNBC, the clinical efficacy of paclitaxel (PTX) is often limited by drug resistance. This resistance mechanism mainly involves the activation of CSCs, the formation of vascular mimicry (VM), and the effects of the immunosuppressive TME. Studies have shown that the overexpressed pyruvate kinase M2 (PKM2) in TNBC may become a new therapeutic target. Based on this, Siqi Wu and his team developed a biomimetic co-delivery system - using albumin nanoparticles (S/P NP) to simultaneously load PTX and the natural PKM2 inhibitor shikonin (SHK). SHK effectively inhibits the heterogeneous characteristics of CSCs and blocks the formation of VM by blocking the β-Catenin signaling pathway regulated by PKM2.

The experiment has confirmed that the S/P NP system not only has tumor targeting properties, but also can significantly inhibit the growth of the primary tumor and the formation of lung metastasis foci. Its mechanism of action includes: reversing the epithelial-mesenchymal transition (EMT) process, eliminating the characteristics of tumor stem cells, inhibiting the construction of the VM network, and reconstructing the TIME components. This synchronous targeted strategy against CSCs, VM and the immune microenvironment provides new therapeutic ideas for overcoming PTX resistance and inhibiting tumor metastasis [14].

The dual-drug delivery system achieves a synergistic therapeutic strategy by simultaneously targeting the heterogeneity characteristics of CSCs. In the aforementioned studies, the combined delivery of PTX and the PKM2 inhibitor not only inhibited the self-renewal ability of CSCs, but also enhanced the anti-tumor immune response by reshaping the immune microenvironment.

B. Specific Targeting Vector: 89Zr-labeled MLP, Selectively Delivering Drugs to CSCs

The Rui Yang team innovatively used radioactive nuclide zirconium-89 (^{89}Zr) to label multifunctional liposomes (MLPs), achieving specific targeted tracking of $CD44^+$ TNBC-CSCs. This study first analyzed the expression characteristics of CD44 in TNBC-CSCs and tumor tissues to elucidate the biological mechanism of chitosan (CS) targeting CD44 therapy. Subsequently, by combining molecular docking and kinetic simulation techniques, the team further explored the binding mechanism and conformational stability of CS and the CD44 active site. The team discovered that CS could precisely anchor the active domain of CD44 through hydrogen bond networks, forming a stable complex. Based on this mechanism, the researchers constructed CS-modified MLP carriers (CS-MLPs) and further labeled them with ^{89}Zr to form ^{89}Zr@CS-MLPs composite systems. In the experiment, the researchers loaded the anti-tumor drug ginkgoic acid (GA) onto ^{89}Zr@CS-MLPs (i.e., ^{89}Zr-CS-GA-MLPs), and further evaluated its anti-tumor effect in animal models.

This study found that the CD44 protein was abnormally highly expressed in TNBC-CSCs and tumor tissues. Through computer simulation analysis, CS was able to precisely bind to the active domain of CD44 in a stable spatial conformation. Experimental data showed that ^{89}Zr@CS-GA-MLPs not only had specific binding ability to $CD44^+$ TNBC-CSCs, but also could achieve targeted aggregation at the tumor site in the transplanted tumor mouse model while maintaining excellent radiochemical stability. Notably, the drug delivery system ^{89}Zr@CS-GA-MLPs exhibited a significant tumor growth inhibition effect in vivo experiments [15].

The nanocarriers targeting CD44 achieved efficient tracking and precise drug delivery to CSCs through ligand-receptor specific binding. Meanwhile, this drug-loaded system achieved targeted aggregation at the tumor site in animal models and significantly inhibited tumor growth.

C. Combination Therapy: JAK/STAT Inhibitors Combined with Nanoparticles

The complete absence of ER/PR/HER2 in TNBC is the key factor limiting the existing treatments. To break through this limitation, the research adopted an integrated treatment approach - combining the JAK/STAT signal-

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ing pathway inhibitor mometinib (MMB) with a targeted CD44 nanocarrier system. This strategy demonstrated significant clinical potential in the treatment of TNBC and could simultaneously enhance patient survival benefits and treatment efficacy. In the specific experiments, MMB enhanced the sensitivity of TNBC cells to the apoptosis inducer CFM-4.16, achieving a significant synergistic effect with a combined index (CI) of ≤0.5 in the MDA-MB-231 and MDA-MB-468 cell lines.

The researchers further combined MMB with CD44-targeted polymer nanoparticles carrying CFM-4.16 (CD44-T-PNPs), which enabling selective drug delivery to the TNBC lesions with overexpressed CD44. The experiments confirmed that the combined treatment not only significantly reduced the viability of cancer cells and optimized the dose requirement index (DRI). The underlying mechanism of this synergistic effect was the simultaneous inhibition of STAT3 phosphorylation (P-STAT3↓) and activation of CARP-1 expression (↑), thereby inducing ROS-dependent apoptosis through the mitochondrial pathway, triggering a series of effects such as cell contraction, DNA damage, and migration inhibition [16].

This multi-pathway intervention effectively inhibits the maintenance of CSC heterogeneity and the development of drug resistance by simultaneously blocking the STAT3 signal and inducing ROS apoptosis. However, its ultimate efficacy may be affected by the dynamic changes of the tumor microenvironment.

VI. Conclusion

The breakthrough progress in SCS technology has provided a brand-new perspective for analyzing the heterogeneity of tumor stem cells in TNBC. This article mainly reviews the analysis of the heterogeneity of TNBC-CSCs based on SCS, such as the markers CD44+/CD24- and ALDH1+ can effectively identify CSC subgroups, while the abnormal activation of signaling pathways such as Wnt/β-catenin, STAT3-FAO and HIF-1α is closely related to the maintenance of their stemness. Based on these findings, nanoscale targeted drugs can precisely deliver drugs to stem cells and show potential in overcoming chemotherapy resistance and inhibiting metastasis in preclinical studies. However, the dynamic changes of the TME, the immunogenicity of carrier materials, and significant molecular differences among individuals are still the key factors limiting its clinical translation. Therefore, future research still needs to develop dynamic monitoring and tracking of SCS, and further improve the biocompatibility of nanobiological materials to address individualized adaptive treatment strategies.

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