Dean&Francis
ISSN 2959-409X

Single - cell Sequencing in Stem Cell Nanotargeted Therapy for Triple - negative Breast Cancer

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Abstract:

Triple-negative breast cancer (TNBC) lacks the expression of three Targeted hormone receptors, namely Estrogen receptor (ER), Progesterone receptor (PR), and Human epidermal growth factor receptor 2 (HER2), leading to difficulties in implementing Targeted therapy. Its high Heterogeneity is primarily regulated by the Interaction between the Tumor microenvironment (TME) and Cancer stem cell subpopulations (CSCs). The Immunosuppressive microenvironment within the TME promotes the survival and Metastasis of tumor stem cells, while CSCs, by secreting Immune checkpoint molecules, in turn promote the immunosuppressive microenvironment and simultaneously remodel the TME.This study, by investigating their interactions and the integration of single-cell RNA sequencing (sc-RNAseq) technology, aims to identify the Heterogeneity of Triple-negative breast cancer stem cells (TNBC-CSCs) and their surface markers, to elucidate the molecular characteristics, regulatory mechanisms, and functional properties of CSCs, and to seek prognosis-related therapeutic targets for different subtypes of TNBC. Simultaneously, novel nanomaterials, such as nCOF, will be utilized to deliver drugs to overcome the difficulties in drug delivery for TNBC, thereby achieving precise drug release.

Keywords: Triple-negative breast cancer; Single-cell RNA sequencing; Nanotargeted therapy; Immune micro-environment; Cancer Stem Cells.

1. Introduction

Breast cancer is more widespread in women. Nonexpression of all three hormone receptors in neoplastic cells is a trait of TNBC, and this type of breast cancer makes up about 10% - 15% of all. Due to this trait, hormone and anti-HER2 Targeted therapy are limited [1]. Furthermore, its high distant metastasis rate and high recurrence risk make it a highly aggressive subgroup of breast cancer. Thus, chemotherapy remains

the most suitable current treatment strategy. sc-RNAseq technology reveals cell Heterogeneity by investigating gene expression per cell [2].This technology can determine therapeutic targets for TNBC and identify subtle differences among various tumor cell subpopulations. The Heterogeneity of TNBC-CSCs is the basis for studying the Tumor microenvironment and its associated T cell subpopulations, for identifying tumor cell subpopulations with high metastatic potential and their molecular characteristics through cellular Heterogeneity analysis based on sc-RNAseq technology, and for determining therapeutic targets, all of which form the foundation for subsequent nano-Targeted therapy. In nano-Targeted therapy, nanoscale covalent organic frameworks (nCOFs) are used, which utilize their stable porous structure to enhance drug loading capacity, achieve precise drug release, and augment the effect of nanoparticles (NPs) on tumor cells [3-5].Despite nanotechnology showing significant promise in the treatment of TNBC, its clinical application still needs to overcome multiple challenges. This survey is intended to elucidate three aspects: the Non-uniformity and biomarker identification of TNBC-CSCs, the interaction mechanisms between the tumor immune microenvironment and stem cell subpopulations, and the strategies and challenges of covalent organic frameworks (nCOF) nanocarrier drug delivery technology in targeted therapy of TNBC-CSCs, in an effort to address the current scarcity of research on the combined therapy of TNBC using sc-RNAseq identification and nanotargeting.

2. TNBC-CSCs Heterogeneity and Marker Identification

TNBC is mainly characterized by the insufficient expression of ER, PR and HER2 [1]. Owing to its absence of Targeted therapy receptors and highly aggressive nature, treatment options are limited, and the likelihood of recurrence remains elevated. CSCs are formed by the proliferation derived from normal stem cells that have undergone gene mutation. Current research has revealed types of CSCsHeterogeneity,namely phenotypic Heterogeneity,molecular Heterogeneity, and functional Heterogeneity. Phenotypic Heterogeneity refers to the presence of different cell surface markers, morphological characteristics, etc., within the CSCs population; molecular Heterogeneity refers to CSCs exhibiting differences in molecular characteristics at the genomic, epigenetic variations, metabolic, and cell cycle regulation levels; whereas functional Heterogeneity refers to differences in biological behaviors such as self-renewal, differentiation, and metastasis among CSCs subpopulations [5,6].

Interactions between components of the Tumor microenvironment (TME), genomic variants, epigenetic modifications (such as DNA methylation, etc.), and dysregulation of signaling pathways are the main reasons for the extensive proliferation and differentiation of CSCs in most malignant neoplasms [5,7]. Self-renewal and heterogeneous differentiation of TNBC-CSCs lead to tumor Heterogeneity. Traditional RNA-seq can provide a large number of tumor immune gene expression profiles, but cannot provide intercellular signaling pathways, interactions, along with interplay between different immune cells and the immune microenvironment, etc.

Compared to traditional RNA-seq, sc-RNAseq technology,through single-cell high-resolution and high-sensitivity detection, has broken through the limitations of traditional RNA transcriptome research, thereby individually analyzing cell expression profiles, inferring cell developmental trajectories, and dissecting the immune microenvironment. Its technical workflow includes single-cell capture and sorting, multi-omics library preparation, high-throughput sequencing, and bioinformatics analysis. It not only optimized the limitations of RNA-seq but also provided a basis for cell trajectory inference and cell communication network construction [3].Based on Tumor microenvironment and its related T cell subgroups, by sc-RNAseq technology, we analyze the differentially expressed gene traits of various cell clusters in single-cell transcriptome data, quantify the differences in activity of predefined functional pathways through the Gene Set Variation Analysis (GSVA) algorithm, reveal the molecular regulatory mechanisms of cellular Heterogeneity, and through high-resolution analysis of individual cells' gene expression, uncover different subgroups of TNBC-CSCs, which contributes to studying the Heterogeneity of TNBC-CSCs and localizing tumor biomarkers. Through single-cell RNA sequencing and other traditional methods, common biomarkers of TNBC-CSCs have been summarized [2,3,8], see Table1.

ISSN 2959-409X

Table 1. Common Markers of TNBC-CSCs

Drug resistance and metastasis marker	Cell surface marker	Metabolic-related markers	Epigenetic regulatory marker	Signaling pathways and related markers
The ABC transporter family MRP-1/ABCC1, BRCP/ABCG2, MRP-8/ABCC11 P-gp1 MDR-1/ABCB1	CD44 CD20 CD90 CD133 CD200	ALDH1 GLUT1 LDH HK2 FASN	EpCAM SUV420H2 H4K20me3 microRNAs BCL11A	Notch pathway-related markers: CD44, CD24, CD8 Wnt/β-catenin pathway: CD44, CXCR4, DLL Hedgehog(Hh)pathway: CD44, CD133, CD24, ALDH1, Sca-1 TGF-βpathway: CD44, CD24, SOX2, OCT4, NANOG, ALDH1

Previous studies have identified six TNBC subtypes, including basal-like subtypes (BL1 and BL2), mesenchymal subtype (M), mesenchymal stem-like subtype (MSL), immunomodulatory subtype (IM), and luminal androgen receptor subtype (LAR) [9]. Meanwhile, sc-RNAseq analyzes gene expression (GE) profiles, calculates the similarity of gene expression between cells, and groups single cells with similar characteristics, which can further reveal TNBC molecular subtypes, thereby guiding treatment more precisely. For example, by histopathology quantification and laser capture microdissection (LCM) technology, it was found that the transcription of the immunomodulatory (IM) subgroup primarily derived from tumor-infiltrating lymphocytes (TILs), whereas the transcription of the mesenchymal stem-like (MSL) subgroup derived from tumor-associated stromal cells (e.g., fibroblasts). This indicates that the IM and MSL subgroups in the original six-subgroup classification were not solely the effect of the tumor cells themselves, while were confounded by microenvironmental components. Therefore, the TNBC molecular classification was revised from 6 types to 4 types, retaining only the subgroups that reflect the intrinsic molecular characteristics of tumor cells [10]. The Fudan classification, applying gene ontology (GO) and pathway profiling, divided TNBC into four subtypes through co-expression network analysis: namely, immunomodulatory subtype (IM), luminal androgen receptor subtype (LAR), mesenchymal-like subtype (MES), and basal-like immunosuppressed (BLIS) subtype [11]. For the different molecular subtypes of TNBC, several distinct treatment approaches have been proposed, such as combined targeted pathway therapy, combined immunotherapy, and platinum-based chemotherapy, etc. [12].

3. The Interaction Mechanism between Tumor Immune Microenvironment (TME) and Stem Cell Subpopulation (CSCs)

In TNBC, TME exhibits significant Heterogeneity. It is composed of a complex regulatory network, including cancer-associated fibroblasts (CAFs), M2-type tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and vascular endothelial cells (TECs), among others [13]. This network, by secreting pro-carcinogenic factors and matrix remodeling—namely, the dynamic changes in the extracellular matrix (ECM) occurring under physiological or pathological conditions—synergistically drives the epithelial-mesenchymal transition (EMT) of tumor cells, angiogenesis, immune evasion, and chemotherapy resistance, collectively promoting tumor initiation, progression, immune evasion, and metastasis [13,14].Tumorigenesis, progression, metastasis, and the formation of Heterogeneity are all related to Cancer stem cells (CSCs), and signal transduction occurring in the TME is associated with the development, self-renewal, and differentiation of CSCs in TNBC. Therefore, the proliferation of CSCs can be inhibited by suppressing some cellular signal transduction pathways, thereby achieving the goal of tumor inhibition. In other words, TME 'nourishes' CSCs through signal pathway activation, hypoxia protection, and immune sanctuary, maintaining stemness, and ultimately producing biological effects. Conversely, CSCs 'remodels' TME by releasing pro-tumor factors and through metabolic regulation, creating a favorable environment for tumor growth.

The rapid proliferation of solid tumors (such as breast carcinoma, etc.) prevents the vascular system from effectively compensating, thereby leading to a hypoxic microenvironment within tumors, and driving the Heterogeneity, invasiveness, and metastasis of CSCs through the

hypoxia-inducible factor (HIF) pathway.Moreover, under hypoxic conditions, prolyl hydroxylase (PHD) modulates HIF stability through oxygen sensing: by reducing its own activity, PHD inhibits HIFα degradation, thereby facilitating HIFα's assembly with HIFβ into a functional complex. This complex subsequently regulates downstream gene production, ultimately driving cancer cells survival, proliferation, and metastasis. More precisely, deletion of the PHD2 subgroup suppresses tumor growth and metastasis through reduced activation of cancer-associated fibroblasts, mitigated matrix stiffening, and normalized vascular function [15,16].The HIF pathway can modulate the function of immune activation within the tumor microenvironment through two mechanisms, and these mechanisms are closely related to the immune escape, metabolic adaptation, and therapeutic resistance of CSCs. On the one hand, deletion of HIF-1α in TAMs can inhibit M2 polarization and angiogenic responses, while overexpression of HIF-2α is associated with poor patient prognosis [17]. On the other hand, during hypoxia, glucose is required to maintain cellular energy supply and the synthesis of biomacromolecules, and HIF-1α adapts to various mechanisms to remain stable to drive glycolysis and lactic acid secretion. Lactic acid is transferred to the extracellular environment via monocarboxylate transporter 4 (MCT4), forming an acidic TME, activating IL-23/IL-17, driving chronic inflammation, inhibiting immune cells, and promoting angiogenesis, thereby forming a "pro-carcinogenic microenvironment" that promotes the survival of highly invasive tumor cells, and further inhibits tumor immunity [18,19].

Studies have also suggested that metabolites such as mitochondrial ROS and α-ketoglutarate (α-KG) can directly regulate HIF activity, forming a "metabolic reprogramming-immune evasion" positive feedback loop, that is, the process by which cancer cells alter their own metabolic patterns, remodel TME, and thus elude detection and attack by the immune network [15]. This process not only promotes tumor progression but also accelerates metastasis by weakening immune surveillance and is one of the core mechanisms of malignant tumor progression. CSCs transmit signals through the HIF pathway, release cytokines, and produce some metabolites. These mechanisms allow them to interact with the surrounding TME, jointly promoting tumor initiation and progression.

Additionally, cells take up lactic acid via lactic acid transporter MCT1, and when intracellular lactic acid levels rise, receptor GPR81 is activated. This receptor regulates cell metabolism by preventing ATP from converting to cAMP. GPR81 can regulate the expression of MCT1 based on lactic acid and glucose levels to promote lactic acid uptake, and regulate the expression of MCT4 to promote lactic acid excretion, thereby controlling the lactic acid metabolic balance of tumor cells. Experiments show that inhibiting GPR81 can reduce tumor cell dependence on lactic acid, thereby inhibiting their growth and proliferation [20].

Tregs, through CTLA-4, inhibit CD8+ T cell-mediated clearance of CSCs. In the TME, Treg accumulation is closely related to the reduction of CD8+ T cells. Tregs directly inhibit CD8+ T cell activation through highly expressed CTLA-4 and inhibit their function through mechanisms such as competitive consumption of IL-2, leading to a decrease in the types of CD8+ T cells capable of recognizing different antigens in the body and a weakened anti-tumor capacity. This dysfunctional CD8+ T cell phenotype promotes tumor progression. After reducing Tregs in tumors through gene knockout or antibody targeting, the ability of CD8+ T cells to acquire IL-2 is restored, their proliferative capacity and clonal diversity are improved, thereby enhancing the anti-tumor immune response [21].

4. Strategies of nCOF Nanodrug Delivery Technology in Targeted TNBC-CSCs Therapy

Nanomaterials include lipid-based nanoparticles, polymer nanoparticles, inorganic and organic nanoparticle and cell-based nanoparticles, etc. Nanoparticles reduce immune recognition by optimizing surface properties (such as PEG modification), thereby prolonging circulation half-life and inhibiting reticuloendothelial system (RES)-mediated clearance. In addition, nanocarriers targeted therapy also reduced the associated side effects of chemotherapy treatment. As a crystalline covalent organic polymer, nCOF's crystalline nature, namely the use of covalent bonds, gives it a fixed spatial structure and a fixed pore size. Since 2005, nCOF has received widespread attention.

In the treatment of TNBC, nCOF, due to its unique structure, can, through surface modification, precisely connect to TNBC-specific markers (such as special surface proteins) to target cancer cells and their cancer stem cells, thereby reducing damage to healthy tissues; its abundant internal nanopores can simultaneously load various drugs such as chemotherapeutic agents and immunomodulators, and slowly release them through the pore structure, extending the drug's retention time at the tumor site, thus avoiding insufficient efficacy or side effects caused by rapid metabolism. Additionally, nCOF can "sense" specific Tumor microenvironments (such as acidity, hypoxia, or enzymes unique to cancer cells), releasing drugs only when the required "signal" appears, ensuring that the

ISSN 2959-409X

drugs act precisely within cancer cells. Furthermore, it can also carry enzymes that degrade the dense matrix or molecules that activate immune cells, loosening the cancer cells' "protective net" and enhancing the body's own anti-cancer capabilities.

nCOF can also address drug delivery challenges. A prominent feature of TNBC is a dense matrix composed of extracellular matrix components such as tumor-associated fibroblasts, fibrous structural proteins, and hyaluronic acid. This dense tumor matrix significantly hinders the transport of therapeutic drugs to the tumor parenchyma through physical barriers and interstitial hypertension and limits the penetration and accumulation of nanomedicines in the deep part of the tumor [22]. According to current research progress, the delivery of nanoparticles to tumor cells can be achieved through post-synthetic modification of COFs to target membrane receptors, such as COFs synthesized with the assistance of p-toluenesulfonic acid (PTSA) and folic acid-functionalized nanoscale COFs, which act as specific targeting ligands.Nanodelivery has many advantages over traditional therapies, such as high bioavailability in pharmacokinetics and the ability to penetrate biological barriers [23,24]. In pharmacodynamics, it can target specific tumor markers and has reduced immunogenicity. The surface of nCOFs can achieve directional functionalization through surface chemical modification, allowing for the customization of specific delivery systems in cancer treatment. This system can also be specifically stimulated by the chemical environment within the human body, achieving precise drug release and reducing systemic toxicity.

nCOF structures are stable and easily processed and can be combined with various treatment modalities such as photodynamic therapy, radiotherapy, and chemotherapy, to provide TNBC patients with more personalized and efficient treatment options. This material is widely applied in the domain of oncology treatment, promoting the formation of drug delivery systems in the cancer field [4]. For example, in the treatment of TNBC, when TRIPTA-COF and Cisplatin are present simultaneously, it can maximally reduce cell migration, effectively transfer Cisplatin into cancer cells, and enhance Cisplatin's effect on EMT [25].

5. Discussion

While sc-RNAseq offers numerous advantages in clinical application, some limitations persist. For example, it can magnify non-biological variants introduced during experimental procedures, amplification, or sequencing, thereby masking true gene expression signals and affecting data reliability and the accuracy of biological conclusions. Additionally, due to technical barriers and resource limitations, the number of sc-RNAseq sequences is small, and even the single-cell atlas of TNBC-CSCs remains incomplete, indicating insufficient research on TNBC [26]. Although nanodelivery systems have developed rapidly in cancer treatment, they still face many technical bottlenecks, including insufficient toxicity assessment, complex and expensive production processes, low reproducibility in large-scale production, and poor storage stability.

The treatment of TNBC is achieving therapeutic breakthroughs through multidisciplinary integration, which, due to its complexity, requires integrating knowledge and technologies from multiple fields such as oncology, immunology, molecular biology, imaging, and artificial intelligence. Currently, with the development of AI, its applications have expanded to multiple fields, including medical diagnosis, treatment, and healthcare. The identification and classification of different subgroups of TNBC are crucial for treatment, and researchers can utilize AI to integrate multi-omics data to improve typing consistency. Liu's team developed an AI framework integrating mRNA and long non-coding RNA expression, which screened for key features through gene selection algorithms, addressed sample processing differences and platform data conflicts, and classified TNBC [27].Chen's team, based on immunophenotyping analysis, used a random forest model to identify 11 immune-related genes and classified TNBC into high immune infiltration (SI type) and low immune infiltration (S2 type), providing a basis for personalized immunotherapy [28]. These studies utilized AI through multi-dimensional data analysis to promote the precise subtyping and optimization of treatment strategies for TNBC. Furthermore, AI can also predict the prognosis of cancer patients through histopathology images and predict the disease-specific survival rate of TNBC through digital scoring of stromal tumor lymphocytes and tumor-associated stroma, among others.

6. Conclusion

The breakthrough in single-cell sequencing technology has provided a crucial instrument fordissecting the Heterogeneity and therapeutic targets of Triple-negative breast cancer stem cells (TNBC-CSCs). Through its high-resolution transcriptome analysis, it precisely identified stemness markers, drug resistance-related pathways, and Heterogeneity expression of microenvironment-regulating factors in TNBC-CSCs, revealing the core driving role of CSCs in the progression of TNBC research. Tumor cells, by secreting cytokines (such as VEGF, ILs, TGF-β, etc.), interact with tumor-associated macrophages (TAMs), fibroblasts (CAFs), T cells, etc., thereby regulating key processes in the Tumor microenvironment such as cell

differentiation, drug resistance, hypoxia adaptation, metabolic reprogramming, immune evasion, and angiogenesis. In the treatment of TNBC, drug delivery has become a challenge, while nCOF (covalent organic framework) nanodrug delivery technology, by virtue of its high specific surface area, functionalizable modification, and Tumor microenvironment-responsive characteristics, has become an emerging strategy for targeting TNBC-CSCs. In cancer research, sc-RNAseq and spatial transcriptomics (ST) technologies are increasingly used. Integrated analysis of single-cell and spatial transcriptomics has elucidated how the "stem cell-microenvironment" bidirectionally promotes the vicious cycle of drug resistance and metastasis. sc-RNAseq can analyze the gene activity of individual cells in detail but loses the positional information of cells within tissues; ST can retain the positional information of tissues but cannot identify the details of individual cells. Currently, two technologies are often combined to record gene expression in different regions and to magnify details. During the treatment of TNBC, the combination of the two technologies can more clearly identify important CSCs in TNBC and their spatial relationship, helping doctors pinpoint key therapeutic targets. The combination of sc-RNAseq and nanotargeting is expected to drive a transformation in TNBC treatment throughout the entire chain of "molecular analysis - precise delivery - dynamic regulation". In the future, with the interdisciplinary integration of single-cell multi-omics, nanobiomaterials, and artificial intelligence, TNBC is expected to shift from "difficult-to-treat" to "personalized cure". Although still facing challenges such as technological translation, standardization, and drug resistance management, this direction has already demonstrated immense clinical application potential and will become the core breakthrough point for TNBC precision medicine.

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