Advances in Single-Cell Sequencing Technology and its Applications in Triple-Negative Breast Cancer

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Research Article

Keywords: TNBC, SCS, Drug resistance, Metastasis

Posted Date: November 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1034157/v1

Abstract

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer and is mainly treated with chemotherapy-based combination therapy. In recent years, the increasing development of single-cell sequencing (SCS) has become one of the most promising technologies in the field of biotechnology. The study of the heterogeneity of TNBC tumor cells using SCS will expand our current knowledge of metastasis, drug resistance mechanisms, mutations, and cloning in these cells; this will further guide clinical chemotherapy, targeted therapy, and immunotherapy. Relevant studies shown that SCS exactly plays an important role in clinical diagnosis and treatment. To highlight the role of SCS in the study of TNBC, we elaborate on the progress of research and the applications of SCS in TNBC.

Introduction

Breast cancer (BC) occurs in the epithelial tissue and ductal epithelial cells of the breast under the action of various oncogenic factors, including human epidermal growth factor receptor 2 (HER2). BC can be classified into four pathological subtypes: Luminal A, Luminal B, HER2-positive, and triple-negative breast cancer (TNBC) [1]. Among these, TNBC is characterized by the absence of estrogen and progesterone receptors and overexpression of the HER2 gene; it accounts for approximately 12–17% of all pathological BC subtypes [2]. The 5-year survival rate of patients with TNBC is only 77%, whereas it is 93% for patients with other BC subtypes [3].

TNBC is characterized by extensive inter- and intra-tumor heterogeneity, as well as frequent P53, PIK3CA, and PTEN somatic mutations, which result in increased angiogenesis and epithelial-mesenchymal transition (EMT), making it more aggressive [4-6]. The outcome of common chemotherapy in the treatment of TNBC is often unsatisfactory and the prognosis is generally poor because of its high malignancy and tendency towards recurrence and metastasis [7]. Therefore, there is an urgent need to discover novel therapies; our research focuses on the use of single-cell sequencing (SCS) in the treatment of TNBC. In recent years, owing to the development of high-throughput sequencing technology, SCS has been widely applied in various research fields and various cell types. SCS provides a comprehensive understanding of the genomic, transcriptomic, and epigenetic properties of individual cells; aids identification of the unique phenotypes generated by individual cells; and serves as a powerful tool for addressing tumor cell heterogeneity and detecting associated subtypes[8, 9]. Thus, information obtained through SCS can form the basis for improved TNBC treatment.

1 Background Of Scs Technology

The first single-cell cDNA amplification method was reported in 1990. Then, in 2009, Tang et al. [10]reported single-cell transcriptome sequencing (scRNA-seq) technology for high-throughput sequencing. In 2011, Cold Spring Harbor Laboratory, in collaboration with the University of Texas

Anderson Cancer Research Center in New York, developed and reported SCS [11]. Finally, in 2013 SCS was selected as the technology of the year [12]; it has undergone continuous development since then.

2 Scs Technology

The sequencing data obtained by traditional high-throughput sequencing technology is only the average gene determination of a mixed collection of cells from a large number of tissue samples. SCS not only accurately measures gene expression levels and detects trace expression of non-coding RNA, but it also allows the sequencing of unusual samples and makes up for the deficiencies associated with small sample size due to limited availability of these samples [13, 14]. Single-cell isolation and acquisition, DNA or RNA sequence amplification, gene sequencing, and data analysis are the steps involved in SCS.

2.1 Single cell sorting acquisition

Unlike traditional sequencing techniques, SCS requires the efficient isolation of individual cells in good condition from tumor tissue or other large tissues. Currently, the methods for obtaining single cells include infinite dilution, single-cell microscopy, flow cytometry cell sorting, laser capture microdissection, and microfluidics[15]. Among them, microfluidics, as an emerging technique for manipulating microfluids, is mainly applicable to the isolation of single cells from sample tissues [16]. Because each method requires different tissue sections, reagents, and equipment; incurs varying expenditure; and is based on unique experimental principles, researchers must choose the appropriate method for their specific experimental purposes.

2.2 Nucleotide sequence amplification

Traditional high-throughput sequencing technology cannot directly extract micro-nucleic acids from individual cells to reveal the differences in cell groups. Therefore, it is necessary to use both whole genome amplification and whole transcriptome amplification techniques to obtain enough DNA and RNA to construct relevant libraries. Because the final sequencing results are largely dependent on the quantity and quality of single-cell nucleic acid amplification, this method is particularly important.

2.2.1 Single-cell genome sequencing

Single-cell genome sequencing is performed by whole-genome amplification and sequencing analysis of DNA from isolated individual cells. It is mainly applied to chromosome structure variation, single nucleotide copy number variation, and nucleotide mutations [17, 18]. The main methods currently applied to single-cell genome sequencing are simplex oligonucleotide primer PCR, multiplexed strand replacement amplification, these have proved to be efficient in terms of amplification, gene coverage, uniformity, reproducibility, and detection of single-nucleotide variants and copy number variants[19, 20].

2.2.2 scRNA-seq

scRNA-seq is the process of extracting RNA from single cells and then reverse transcribing the captured mRNA into cDNA for transcriptome amplification and sequencing analysis. Transcriptome sequencing not only precisely measures levels of gene expression in cells, but also detects differential genes in terms of non-coding RNA and low abundance[21]. It directly responds to gene expression and allows for a more comprehensive response to cell type, status, subtype, and mechanism of action[10, 20] .

2.2.3 Single-cell epigenome sequencing

Single-cell epigenome sequencing focuses on various epigenetic modifications of DNA, such as DNA hydroxylation, methylation, and histone modification. The most commonly used sequencing method is single-cell methylation sequencing (scMseq). Epigenetic modification plays an essential role in gene regulation. For example, modification of normal methylation can regulate cell growth and metabolism, while modification of abnormal methylation can induce malignant tumors. Thus, single-cell epigenome sequencing can provide a deeper understanding of the growth and development of individual organisms and can be beneficial in the treatment of diseases [22].

2.3 Gene sequencing

Various sequencing methods are used to analyze the base sequences of DNA or RNA and epigenetic modifications of genes. The main genome sequencing techniques include multiple displacement amplification, degenerate oligonucleotide-primed PCR, multiple annealing loop amplification, multiplex amplification PCR, and multiple annealing and looping-based amplification cycles. Genome sequencing is followed by second-generation DNA sequencing to enable accurate detection of changes in the different types of genes.

2.4 Data analysis

Data analysis involves the collection and screening of sequencing results by various computational methods. The results are processed using calculations and simulations to extract useful information.

3 Application Of Scs In Tnbc

3.1 Exploring the efficacy of combination therapy and immune response

Because of the heterogeneity of TNBC and the poor efficacy of general chemotherapy, the current research explores the combination of chemotherapy with immunotherapy. Deng et al. [23] used scRNA-seq to sequence approximately 4000 single cells from each collected live sample to examine the therapeutic efficacy of albumin paclitaxel in combination with pembrolizumab in advanced TNBC. After scRNA-seq, serial biopsies of tumor tissue from the same site were performed in two patients; the samples were analyzed for infiltrating immune cells using SCS technology. A comparison between baseline and post-treatment samples revealed a significant difference in tumor immune cell infiltration between responders and non-responders. Furthermore, high expression of PD-1 T cells were present in patients who responded

to treatment with albumin paclitaxel combined with T cells, which were significantly reduced after pembrolizumab immunochemotherapy. In addition, there were IFN + and GZMB + CD8 + T cells, tissue-resident T cells (TRM), and significantly increased tumor-infiltrating leukocytes in the tissues. Related studies have shown that TRM in CD8+ TRM is significantly associated with improved survival in TNBC[24]; in non-responders, there is a significant myeloid infiltration and no higher presence of PD-1 T cells. Once again these findings demonstrate that the presence of TRM in BC tissue is associated with improved prognosis in patients [24]. In addition, it was verified that infiltrating lymphocytes can predict the response associated with immunotherapy[25].

The previously discussed study not only provides a reliable basis for the study of therapeutic changes in advanced TNBC during immunochemotherapy, but also demonstrates the feasibility of performing single-cell analysis after serial biopsies of tumor tissue and the variability of therapeutic changes in advanced TNBC.

It has been shown that high levels of HSP90 and HDAC6 expression are present in TNBC cells. In addition, while increasing levels of HDAC6 expression were found in TNBC cell lines, HSP90 expression levels remained almost uniform [26]. In this study, we further confirmed that the heat shock protein 90 inhibitor 17-AGG combined with the histone deacetylase 6 inhibitor belinostat had a synergistic effect in TNBC. Upon comparison with 17-AGG or belinostat alone, the migration and invasion of combined TNBC tumor cells were significantly inhibited, while ROS levels were significantly increased after the combination of both treatments, in which regulatory signaling pathways related to EGFR, COX5B, and UBA52 were significantly affected. This once again demonstrated that the combination of drugs could inhibit tumor cell proliferation and further suppress the migration and invasion of TNBC tumor cells. Thus, it provided a reliable reference value for the clinical treatment of TNBC.

From the evaluation of several studies, the therapeutic effect of combination drugs on TNBC was found to be remarkable. The latest relevant findings can further provide new directions and strategies for clinical combination therapy. This will ensure that TNBC patients can benefit significantly from the therapeutic efficacy in the clinical treatment process.

3.2 Exploring the heterogeneity of TNBC cells

Heterogeneity is one of the characteristics of malignant tumors, which are tumors that show molecular biological or genetic alterations in their daughter cells after multiple divisions and proliferation during growth, resulting in differences in various aspects such as growth rate, invasiveness, drug sensitivity, and disease prognosis. Wu et al. [27]performed a human transcriptome analysis of SUM149 TNBC cells at the single cell level and identified three cancer stem cell (CSC) populations: EMT, mesenchymal-epithelial transition (MET), and Dual EMT-MET CSCs, demonstrating the heterogeneity of TNBC cells. In addition, Bao et al.[28]not only identified a significant correlation between tumor cells at the single-cell level by SCS technology between tumor stem cells, angiogenesis, and EMT, but also identified cellular subtypes of all three, which may lead to a high incidence of recurrent TNBC metastasis. M2-like tumor-associated

macrophages (TAMs) were found to be the major component of macrophages in tumor-infiltrating cells, promoting tumorigenesis by inducing angiogenesis and exhibiting a distinct immunosuppressive profile. In that study, after sequencing data from several regions of interest, it was found that non-tumor cells, such as lymphocytes in TNBC, exhibited a high rate of infiltration and that the degree of lymphocyte infiltration varied between patients [29]. After studying the functional status of B immune cells, it was found that B immune cells showed a heterogeneous activity distribution between patients with TNBC and those with the same type of TNBC cells. It was found that there are differences in the composition of the tumor immune population between individuals[30], a phenomenon consistent with the presence of immune subpopulations and overall immune infiltration. The type of immune infiltration proves the heterogeneity of tumor cells within the immune cell population. Sebastian et al.[31]investigated the heterogeneity of cancer-associated fibroblasts (CAFs) in TNBC and identified six CAF subtypes: Ly6c1high, α-SMAhigh, dividing/cycling, Cd53high, Crabp1high, and Cd74high. Among them, α-SMAhigh CAFs and inflammatory CAFs have highly similar transcriptional profiles in BC and pancreatic cancer. Furthermore, α-SMAhigh CAFs may promote tumor development and progression and may play an important role in tumor control [32]. Cancer progression can be prevented by studying CAF heterogeneity in TNBC using SCS technology to effectively target its subtypes.

TNBC is characterized by intra- and inter-tumor heterogeneity[4]. This heterogeneity considerably reduces the efficacy of chemotherapy; thus, the use of chemotherapy alone cannot completely eliminate tumor cells in tumor tissues and effectively inhibit the progression of TNBC [33]. The subpopulation structure of highly metastatic TNBC cells can be identified using SCS; this not only helps to deepen our understanding of TNBC heterogeneity, but also facilitates targeted treatment of TNBC to improve clinical outcome and prognosis.

3.3 Mechanism of recurrence and metastasis of TNBC

In recent years, although great progress has been made in the treatment of BC, among the molecular subtypes of BC, the majority of patients with TNBC develop to the metastatic stage. Some studies have shown that this recurrence is mainly characterized by a significant increase in recurrence rate in the first 2 years after definitive diagnosis, with a peak at 2-3 years, followed by a gradual decrease in the risk of recurrence over the next 5 years[2]. The time from diagnosis to tumor recurrence and death was significantly shorter due to the presence of high levels of vascular endothelial growth factor in TNBC patients [34]. Exploring the mechanisms of recurrence and metastasis in TNBC to find new therapeutic targets is a goal constantly pursued by researchers in this field, and SCS may become a powerful tool to achieve this goal. TNBC cells excessively and frequently express mutated somatic cells, such as PIK3CA, p53, and PTEN [5], characterized by invasion of tissue boundaries, central necrotic areas, or compressed sites [2], thus exhibiting metastatic features. In addition, it has been shown that the recurrence and metastasis of TNBC are closely related to tumor stemness, EMT, and angiogenesis [35]. By further analyzing the characteristics of all three, at the single-cell level, the stemness of tumor cells, angiogenesis, and EMT are more strongly correlated in TNBC than in other types of BC, which may lead to

a high incidence of TNBC recurrence. Abnormal activation of EMT and TAM, which account for a large proportion of immune cells, are important factors for metastasis and recurrence in TNBC.

It has been shown [36] that polyclonal inoculation of single clones in TNBC tissues can lead to multisite metastasis. This may provide strong evidence to guide tumor heterogeneity and lead to tumor progression. Whether the CD44 molecule in CSC is associated with metastasis and recurrence is highly controversial, with some studies suggesting that the presence of CD44 is associated with distant metastasis [37] and others showing that CD44 is not predictive of distant metastasis[38]. The existence of this contradiction may be due to the fact that the CD44 gene has multiple isoforms. In CD44v6+ and CD44v6- groups, the levels of MCL1 and JUN were significantly elevated, particularly in the CD44v6+ group in a correlation gene analysis. Furthermore, and it has been shown that MCL1 is required for tumorigenesis and metastasis[39, 40]. Similarly, JUN is associated with metastasis and stem cell expansion in tumor cells[41] [42]. Overall, according to relevant studies, CD44v6 is closely associated with tumor cell proliferation and genesis. The risk of tumor cell progression includes lymphatic metastasis and distant metastasis, which remains one of the most important issues in modern oncology. Tumor metastasis occurs not only in individual cells but also through tumor cell clusters, which pass through capillaries and then metastasize to distant sites [43, 44].

Through various studies, the molecular mechanisms related to TNBC recurrence and metastasis have been continuously confirmed and explored, which can effectively improve the treatment strategy for TNBC, thus reducing the recurrence and metastasis rate of TNBC patients, prolonging their survival time.

3.4 Determination of TNBC cell types

Human breast tissue is not composed of a single type of breast cell, and by understanding the cell types of each subtype of BC, further clarity can be obtained regarding the relevant types of breast cells. Peng et al. [45] used SCS to analyze 3193 cells in postmenopausal breast tissue and identified 10 different cell clusters, seven of which did not express cytokeratin. To further clarify which types of cluster cells in these cell clusters could promote BC, a gene set variation analysis was derived from the differential gene expression profile of each cell cluster. From this study, it was concluded that ductal epithelial and fibroblastic cells were prevalent in ductal BC, with basal and other epithelia predominantly present in TNBC. In addition, it was shown that more than 20% of TNBC tumor cells may originate from the basal epithelium, and other epithelia are significantly associated with poor survival in TNBC, suggesting that other epithelial types may give rise to subtypes of invasive TNBC. Using SCS technology, single cell clusters of BC subtypes were further characterized, and in addition, four intrinsic molecular subtypes of TNBC were identified based on gene expression characteristics [46]or six TNBC types[47]. It has been shown that highly infiltrative immune cells and inflammatory gene expression are present in TNBC tumor cells [48, 49]. SCS not only provides further insight into the cell types of BC, but also provides monitoring of disease status after analysis of cells contributing to TNBC, providing a basis for clinical response to treatment and seeing individual differences.

3.5 Revealing resistance to chemotherapeutic agents

TNBC lacks the corresponding hormone receptors [2], and there is significant intra-tumor and inter-tumor cell heterogeneity [4]. It has been shown that the key to tumor drug resistance is mainly untreated intra-tumor heterogeneity[50-53], which is highly susceptible to drug resistance during chemotherapy and targeted therapy. For TNBC patients receiving chemotherapy, the proportion of patients who develop resistance is approximately 30–50%, leading to a decrease in the overall survival of TNBC. Studies on the development of bacterial resistance to antibiotics have been conducted for many years, but the mechanisms associated with the development of resistance in tumor cells have only attracted the attention of relevant researchers in recent years, and this clinical problem of vulnerability to drug resistance has not been effectively addressed.

The analysis of studies at the single-cell level revealed that intra-tumor heterogeneity plays a very important role in the evolution of tumor development. Resistance is also responsible for the vulnerability of TNBC to drug resistance. To further investigate this issue, Kim et al. [54] performed a longitudinal analysis of 900 single-cell DNA and 6862 single-cell RNA using SCS in 20 patients undergoing neoadjuvant therapy (NAC) in which resistance genes were already present in tumor cells prior to chemotherapy and were adaptively selected by NAC during chemotherapy. CAFs are the major stromal component in solid tumors, and CAF-producing cells may include tissue fibroblasts, hematopoietic stem cells, adipocytes, and endothelial cells. Alternatively, CAFs may be produced directly from cancer cells via EMT[55]. Factors released from CAFs into the tumor microenvironment may play an important role in drug resistance to therapy.

By revealing the drug resistance mechanism of TNBC at the single-cell level, we can provide clinicians with relevant treatment plans so that more TNBC patients can obtain the best therapeutic efficacy.

3.6 Revealing mutations and clonal evolution of TNBC

Wang et al. [56] examined individual cells and tumor nuclei of TNBC ductal carcinoma using SCS technology. Their findings showed that aneuploidy rearrangements already existed at an early stage of tumor progression, and such rearrangements remained highly stable during tumor progression. In contrast, point mutations evolve progressively during tumor progression and subsequently generate a wide diversity of clones. Mathematical modeling showed an increase in the mutation rate in TNBC, but not in estrogen receptor-positive tumor cells [57, 58]. Expression profiles during the study showed that among the subtypes of BC, TNBC was the pathological type with the largest number of mutations, whereas the lowest frequency of mutations occurred in the Luminal A subtype[5, 59, 60]. The data from this study suggest that TNBC may have evolved in terms of clonal diversity and mutations; however, this conclusion is only an analysis performed at the single-cell level, and it is difficult to make such inferences from large portions of tissue[51, 61]. The study of TNBC mutation and cloning at the single-cell level allows further diagnosis of BC resistance during chemotherapy and has important clinical implications in terms of treatment and disease evolution.

4. Conclusion

SCS technology has served as the foundation for studies on intra- and inter-tumor heterogeneity of TNBC, mechanisms of clinical drug resistance, recurrence and metastasis of tumor cells, mutations, and cloning. These studies provide a basis for the diagnosis and treatment of TNBC. Although the application of SCS technology in TNBC has played an important role in clinical studies and the development of biomedicine, the technology itself, along with its derived technologies, still has certain limitations and requires further improvement. Ensuring the integrity of individual cells throughout the sequencing process is a key focus area for subsequent technology. Currently, most tumor samples are frozen for experimental manipulation, therefore the integrity of the cell membrane cannot be fully guaranteed; in addition, the molecules on the cell surface may change during the cell thawing and lysis processes. Therefore, if tumor-related mechanisms are studied at the single-cell level, the techniques related to sample production need to be further improved. Further, the considerable cost involved in SCS limits the development of this technology to an extent.

In addition to the technical shortcomings, it is unclear whether TRM infiltration in TNBC is related to the response to anti-PD-1 therapy during immune-related responses. The mode of action of the combinatorial analysis of single cells, or even a large number of single cells, requires further exploration to better understand the molecular mechanisms related to immunotherapy. Similarly, CAF is one of the causes of TNBC heterogeneity, however there are no studies that focus on the different types of CAF subgroups. Therefore, we were unable to study the structure of the CAF subgroups and understand the molecular mechanisms related to heterogeneity for the development targeted clinical treatment.

Although SCS still has limitations and TNBC-related molecular mechanisms are not yet fully understood, we believe these shortcomings will soon be overcome with the rapid development of new technologies, and this will translate to improved outcomes for patients with clinical TNBC.

Abbreviations

TNBC: Triple-negative breast cancer; SCS: Single-cell sequencing; BC: Breast cancer: HER2: epidermal growth factor receptor 2; EMT: Epithelial-mesenchymal transition; scRNA-seq single-cell transcriptome sequencing; scMseq: Single-cell methylation sequencing; TRM: Tissue-resident T cells; CSC: Cancer stem cell; MET: Mesenchymal-epithelial transition; TAMs: Tumor-associated macrophages; CAFs: cancer-associated fibroblasts; NAC: Neoadjuvant therapy;

Declarations

Authors' contributions

ML, TY, and MW contributed to the conception and the drafting of manuscripts. YX, YL are responsible for coordinating and participating in the article revision. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have declared no conflicts of interest.

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Acknowledgements

Not applicable.

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