

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

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**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, with the increasing development of global precision medicine, single-cell sequencing (SCS) has become one of the most promising technologies in the field of biotechnology. Moreover, the related application of this technology in TNBC has been applied and developed. By using SCS to study the heterogeneity of TNBC tumor cells, metastasis, drug resistance mechanisms, mutations, and cloning; it can further guide clinical chemotherapy, targeted therapy, and immunotherapy. To further reflect the importance of SCS in TNBC, this paper elaborated on and summarized the research and application progress of SCS in TNBC.

**Keywords:** TNBC, SCS, heterogeneity, drug resistance, metastasis

Breast cancer (BC) can be classified into four subtypes according to pathology: Luminal A, Luminal B, HER2-positive, and TNBC.<sup>1</sup> Among these, TNBC is characterized by the absence of corresponding hormone receptors, and this type accounts for approximately 12–17% of all BC pathological subtypes.<sup>2</sup> Studies have shown that the 5-year survival rate of patients with TNBC is only 77% and 93% for other types, which is lower compared with other types of BC.<sup>3</sup> TNBC is a disease entity characterized by extensive inter- and intra-tumor heterogeneity, and likely represents multiple clinically and biologically distinct subgroups that have not been clearly defined.<sup>4,5</sup> The majority of TNBCs share common histological and molecular features including frequent p53 mutations, a high proliferation index, and frequent expression of basal-like gene expression signature.<sup>6</sup> These signatures make TNBC exhibit significant angiogenesis and epithelial-mesenchymal (EMT), which makes tumor cells more aggressive, and prone to recurrence and metastasis, with a relatively poor recovery.<sup>7</sup> Therefore, there is an urgent need to use emerging technologies to discover new strategies regarding the treatment of TNBC, in which the study of TNBC from the single-cell level is the focus of our research. In recent years, with the continuous development of high-throughput sequencing technology, SCS has been widely used in various research fields and various cell types. Using SCS at the cellular level on the target analysis provides a comprehensive understanding of the genome, transcriptome, and epigenetic property. Thus, we can fully and effectively recognize the unique phenotypes produced by individual cells and provides a powerful tool to address the heterogeneity of tumor cells, detect related subtypes and reconstruct evolutionary lineage, Therefore, which can provide a reliable basis for the precise treatment of TNBC.<sup>8,9</sup>

## Development and Principles of SCS Technology

SCS is a technique that reveals the genome, transcriptome, or epigenetic changes of cells at the level of single cells, revealing the functions and characteristics of cells from different perspectives. Information of hundreds or thousands of single cells can be measured through database construction at a time. Human research on single cells is mainly based on sequencing technologies (first-generation sequencing, second-generation sequencing, and third-generation sequencing). The first-generation sequencing technology, namely the Sanger sequencing method, developed by Frederick Sanger et al in 1997, focuses on the calibration of bases of single nucleic acid molecules for sequencing purposes, and

the result of this technology draws a preliminary sketch of a human gene sequence in 2003, laying the foundation for decoding of life. In 2000, second-generation sequencing (high-throughput parallel sequencing) was developed to further decode cellular and molecular changes from single-cell transcriptome, genome, epigenetics, and combined multi-omics single-cell sequencing technologies, thus providing a good platform for medical research progress. 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. Compared with traditional sequencing technology, SCS not only accurately measures gene expression levels and detects trace expression of non-coding RNA, but also can give full play to the advantages of sequencing special samples and make up for the shortcomings of special samples due to small sample size and not easy to obtain.<sup>10,11</sup> The technical process of SCS mainly includes single-cell isolation and acquisition, genome/transcriptome/epigenome library construction, gene sequencing, and data analysis.

## Single-Cell Sequencing Technology

### Single-Cell Isolation and Extraction

Single cells isolated from tissues are lysed to obtain pg-level nucleic acids, which are subsequently amplified to ng or ug level and finally used for subsequent sequencing. According to the sample state, the number of cells needed, and the purpose of analysis, a variety of cell isolation methods have been developed, mainly including limited dilution method, micromanipulation, laser capture microdissection (LCM), fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), and microfluidic control (Table 1).

**Table 1** Single-Cell Isolation Technology

Single-Cell Isolation Technology	Advantages	Disadvantages	Classification
Laser capture microdissection	Live tissue cells are extracted and formalin-fixed, paraffin-embedded, and cryoprecipitate. Combined with immunohistochemistry for single-cell analysis	It is easy to destroy the integrity of single cells, and single cells will mix with adjacent cells, reducing accuracy	Low throughput
Limited dilution method	Simple process, good repeatability, low cost	Not easy for cell identification and difficult to avoid DNA contamination	Low throughput
Micromanipulation	Simple process, low cost, and suitable for suspension of cells	Cell identification is prone to error, and DNA is easily contaminated	Low throughput
Microfluidic control	High throughput separation and sorting of up to thousands of cells per second; A large number of cells operate in parallel. Fluid dynamics capture can even be integrated into manual aspiration of single cells without requiring micropipette manipulation under the microscope	Higher cost	High throughput
Fluorescence activating Cell sorter (FACS)	It can screen for rare cells with less than 1% occupancy in highly heterogeneous cell populations.	Samples must be single cell suspensions, and some information on cell function and intercellular interaction will be lost; overlap of fluorescent dye spectra of subpopulations with similar marker expression; low-intensity fluorescent samples could not be detected; It is difficult to achieve aseptic operation, etc.	High throughput
Magnetically activated cell sorting (MACS)	The advantage is similar to FACS, Compared to FACS, MASS requires less equipment and time.	The disadvantage is similar to FACS, MACS lacks the sensitivity and cell specificity provided by fluorescent labeling.	High throughput

## Single-Cell Sequencing

### Single-Cell Genome Sequencing

Single-cell genome sequencing is a process that uses precise separation techniques to isolate individual cells, and extract and amplify their DNA by whole genome amplification (WGA) techniques to obtain a whole-genome map of a single cell, which is mainly used to reveal differences and evolutionary relationships of the cell population. The most widely used single-cell genome sequencing techniques are shown in [Table 2](#).

### Single-Cell Transcriptome Sequencing

Single-cell gene transcriptome sequencing is the analysis of transcriptome amplification and sequencing after reverse transcription of captured mRNA into cDNA, which not only accurately measure the high and low levels of gene expression in cells, but also detect non-coding RNA and low abundance differential genes.<sup>12</sup> Single-cell transcriptome sequencing can reflect cell type, status, subtype, and mechanism of action more comprehensively.<sup>13,14</sup> [Table 3](#) summarizes the current major single-cell transcriptome sequencing technologies.

### Single-Cell Epigenome Sequencing

Single-cell epigenetic sequencing focuses on heritable information other than DNA sequence, including DNA methylation, RNA methylation, histone modifications, chromatin remodeling, and three-dimensional spatial conformation. If the epigenetic level of a single cell is changed, cell function will also be changed. At present, a relatively mature technology in this field is DNA methylation ([Table 4](#)). Sequencing the epigenetics of single cells can provide a deeper understanding of the growth and development of individual organisms, and can be beneficial in the treatment of diseases.<sup>15</sup>

### Single-Cell Multi-Omics Combined Analysis

Single-cell multi-omics combined analysis is the integration of multi-omics information from isolated single cells by obtaining genome, transcriptome, and epigenome information, which can provide a comprehensive analysis of individual cell information. [Table 5](#) summarizes the current representative single-cell multi-omics combined analysis.

**Table 2** Single-Cell Genome Sequencing Technology

Genome Sequencing Technology	Advantages	Disadvantages	Classification
PEP-PCR	Compared with the traditional WGA method, it is a random primer method based on PCR Sequence selective shifts occur less	Non-specific expansion of high, and expansion efficiency is not high, the application is limited	Low throughput
LA-PCR		Non-specific expansion of high, and expansion efficiency is not high, the application is limited	Low throughput
DOP-PCR	It is suitable for quantification of CNV of chromosomes	Low genome coverage, non-specific amplification, and low amplification efficiency	Low throughput
MDA	High amplification efficiency and simple experimental method	Prone to sequence-dependent bias, uneven genome-wide coverage, high allele loss rate, unsuitable for CNV analysis	Higher throughput
MALBAC	Simple operation, high yield, and high homogeneity. It can improve the accuracy of single cell whole genome sequencing and discover genetic differences between individual cells	The possibility of false positive rate was higher and the amplification homogeneity was better, but the amplification efficiency was relatively low	Higher throughput
LIANTI	Greatly reduce the sequence bias during amplification, improved high gene coverage, and accuracy of CNV	Higher false positives for C-T base pairs	Higher throughput

**Abbreviations:** WGA, whole genome amplification; PEP-PCR, primer extension preamplification PCR; LA-PCR, ligation anchorage PCR; DOP-PCR, degenerate oligonucleotide primer PCR; MDA, multiple permutation amplification of MDA; MALBAC, multiple annealing loop amplification method malbac; LIANTI, linear amplification of transposon insertion.

**Table 3** Single-Cell Transcriptome Sequencing Technology

Transcriptome Sequencing Technology	Advantages	Disadvantages
Tang RNA-seq	The full length of the transcript can be measured, and gene expression detection is more sensitive and accurate	The cell flux is low and the price is expensive
Smart-seq	The sequence coverage is good.	Sequences larger than 4 KB cannot be efficiently transcribed, and priority amplification of high-abundance transcripts and purification will lead to material loss
Smart-seq 2	No purification step is required, greatly improving yields and high levels of localizable sequences	Non-chain specific amplification and only sequencing poly(A) + RNA, the cell flux is low and the price is expensive
CEL-seq	Contamination between samples is greatly reduced, and reading preference is very low	The error rate was low, but sequence preference existed in both amplification and PCR
Quartz-seq	Greatly reduce PCR by-products, and reduce pollution in small pieces	It is easy to cause amplification bias due to GC content difference
STRT-seq	Cell flux is high and the price is relatively cheap	Only one end of the transcript is measured, and the sensitivity of gene expression was low
Drop-seq	Low-cost, rapid library preparation, single-cell high-throughput analysis, and the possibility of multi-omics analysis	The sensitivity of single-cell genes is low

**Table 4** Summary of Single-Cell Epigenome Sequencing Technology

Single-Cell Epigenome Sequencing Technology	Advantages	Disadvantages	Classification
scTTBS	Detection of single bases in genome-wide coverage of CpG islands at the single-cell level	Presence of DNA loss	High throughput
scATAC-seq	Chromosomes analysis of single cell to achieve genome-wide open chromatin sequencing at the single-cell level	-	High throughput
scCOOL-seq	Better coverage of the whole genome, solving the problem of over-enrichment of mitochondrial fragments in scATAC-seq studies, resulting in too little effective data	-	High throughput
CoBATCH	It significantly improves the efficiency of ChIP and enables high-throughput labeling of single cells, which is the most advanced and efficient ChIP-seq technology available at present	-	High throughput

**Abbreviations:** scRRBS, single-cell reduced-representation bisulfite sequencing technology; scATAC-seq, single-cell sequencing assay for transposase-accessible chromatin; scCOOL-seq, single-cell multi-omics sequencing; CoBATCH, combinatorial barcoding and targeted chromatin release, for single-cell profiling of genomic distribution of chromatin-binding proteins in cell culture and tissue.

## Gene Sequencing

Various sequencing methods are used to analyze the base sequences of DNA or RNA and epigenetic modifications of genes.

## Data Analysis

Data analysis is a method to obtain useful information from sequencing results by sorting and screening the sequencing results through various algorithms. At present, there are still many technical errors in single sequencing technology, and improving bioinformatics analysis technology is the key.

**Table 5** Multi-Omics Co-Sequencing Technology (High Throughput)

Multi-Omics Co-Sequencing Technology	Technology Introduction	Advantages
DR-seq	After single cell lysis, DNA and RNA are amplified simultaneously and the amplified products are used for genome sequencing and transcriptome sequencing	The amplification process keeps DNA and RNA together and minimizes acid loss
G&T seq	The mRNA and DNA are physically separated using magnetic beads coated with short oligonucleotide sequences that bind mRNA, they are physically separated and then amplified and sequenced separately.	To achieve automation, the throughput is relatively high. Avoid potential cross-contamination
scM&T-seq	Genomic DNA was treated with bisulfite to convert unmethylated cytosine to uracil, and then amplified and sequenced to determine the methylation groups	Parallel analysis of DNA methylation and RNA information in the same single cell. Provides information on the relationship between DNA methylation heterogeneity and differences in expression of specific genes in single cells
scTrio-seq	The nucleus is separated by centrifugation, and the membrane is selectively cleaved to separate the mRNA in the cytoplasm from the genomic DNA in the intact nucleus. Genomic DNA was detected using a modified sulfite treatment and sequencing method to detect methylation groups	Genomic copy number variation (CNV), DNA methylation group, and transcriptome of individual cells can be analyzed simultaneously

**Abbreviations:** DR –seq, DNA-mRNA sequencing; G&T Seq, genome and tranome sequencing; scM&T-seq, simultaneous single-cell methylome and transcriptome sequencing; scTrio-seq, single-cell genome, DNA methylome, and transcriptome sequencing methods.

## Application of SCS in TNBC

### Application of SCS at the Level of TNBC Cells

#### Determination of TNBC Cell Types

Human breast tissue is not composed of a single breast cell, and by understanding the cell types associated with each subtype of breast cancer, we can further understand the related cell types of breast cells. Sen Peng et al<sup>16</sup> used SCS to analyze 3193 cells in postmenopausal breast tissue and identified 10 different cell clusters, seven of which did not express cytokeatin and the others expressed cytokeatin. To further clarify which types of cluster cells in these cell clusters could promote the occurrence of BC, gene set variation analysis was derived from the differential gene expression profile of each cell cluster and finally concluded that ductal epithelial and fibroblastic cells are prevalent in ductal BC, with basal and other epithelia were predominantly present in TNBC. Studies have shown that more than 20% of TNBC tumor cells may originate from the basal epithelium, and another epithelium is significantly associated with poor survival in TNBC, suggesting that other epithelial types may give rise to subtypes of aggressive TNBC. SCS technology was used to further accurately identify the characteristics of single-cell clusters of BC subtypes and identify four inherent molecular subtypes of TNBC<sup>17</sup> or six TNBC types according to gene express characteristics.<sup>18</sup> Through SCS, we not only have a further understanding of the cell types of breast cancer but also analyze the cells that contribute to TNBC and monitor the disease status, providing a basis p for clinical response to treatment and individual differences.

#### Revealing Mutations and Clonal Evolution of TNBC

Wang et al<sup>19</sup> used SCS to detect the single cells and tumor nuclei of TNBC ductal carcinoma, and their results showed that aneuploidy rearrangements existed in the early stage of tumor progression, and this rearrangement remained highly stable in the progress of tumor progression. In contrast, point mutations evolve progressively during tumor progression and subsequently generate a wide diversity of clones. Navin et al<sup>20</sup> studied the tumor population structure and evolution of BC by single-cell genome sequencing, precisely quantifying the genome copy number within an individual cell nucleus, and found that three distinct clonal subpopulations may represent sequence amplification of different clones. Further analysis of one of the primary tumors revealed that a single clonal amplification formed the primary tumor and sowed the seeds of metastasis. In addition, a special subpopulation of genetically diverse “pseudodiploid” cells was

identified that, in contrast to the progressive model of tumor progression, did not migrate to the tumor site and demonstrated that breast tumors grow continuously in the form of uninterrupted clonal expansion. It is the uninterrupted growth of different clonal subpopulations of breast cancer that allows the differentiation of different subtypes of breast cancer, with TNBC having the highest number of mutations in these cancer subtypes.

By studying the mutation and cloning of TNBC at the single-cell level, the drug resistance of TNBC during chemotherapy can be further diagnosed, and it plays a role in clinical implications in terms of treatment and disease evolution.

### Exploring the Heterogeneity of TNBC Cells

Tumor heterogeneity refers to the changes in molecular biology or genetic alteration of tumor daughter cells after multiple divisions and proliferation during tumor growth, resulting in differences in various aspects such as growth rate, invasiveness, drug sensitivity, and disease prognosis. Wu et al<sup>21</sup> identified three cancer stem cell (CSC) populations, epithelial-mesenchymal transition (EMT), mesenchymal-epithelial transition (MET), and Dual EMT-MET CSC, after analyzing cell SUM149 of TNBC at the single-cell level, which further demonstrated the heterogeneity of TNBC cells. One study found that lymphocytes exhibited a high rate of infiltration by sequencing multiple regions of TNBC, and the degree of lymphocyte infiltration varied between patients,<sup>22</sup> based on the B lymphocytes function status of related research showed that the cells exhibited a heterogeneous distribution of activity between different TNBC patients and in the same TNBC patients. Sebastian et al<sup>23</sup> investigated the heterogeneity of cancer-associated fibroblasts (CAFs) in TNBC, and further identified six isoforms of CAFs: Ly6c1high CAFs,  $\alpha$ -SMA high CAFs, dividing/cycling CAFs, Cd53high CAFs, Crabp1high CAFs, and Cd74high CAFs, among which  $\alpha$ -SMA high CAFs may promote tumor development and progression, and may play an important role in tumor control.<sup>24</sup> Cancer progression can be prevented by studying the CAF heterogeneity of TNBC using SCS and effectively targeting therapy its subtypes. Karaayvaz et al<sup>25</sup> performed a scRNA-seq analysis of more than 1500 cells from six primary TNBCs, revealing the functional heterogeneity of TNBC and its relationship with genomic evolution. Chiu et al<sup>26</sup> used a multi-omics dataset to classify TNBC patients by combining gene expression, miRNA expression, and copy number variation into three distinct patient groups, in which most TNBC patients were classified as the basal subtype, but one subgroup was enriched with non-basal subtypes that exhibited more aggressive clinical features, and unique characteristics of oncogenic mutations, miRNAs and expressed genes. This analysis based on multiple histological datasets provides insight into the heterogeneity of TNBC at the molecular level.

From the relevant studies mentioned above, it is shown that TNBC is a tumor characterized by intra- and inter-tumor heterogeneity.<sup>25</sup> Because of the existence of heterogeneity, the subpopulation structure of highly metastatic cells can be identified by using SCS, which not only helps deepen our understanding of TNBC heterogeneity but also enables precise treatment of TNBC to achieve the expected clinical outcome and improve patient prognosis.

### Mechanism of Recurrence and Metastasis of TNBC

The characteristics of recurrence and metastasis in TNBC patients are mainly characterized by a significant increase in the recurrence rate in the first 2 years after the definite diagnosis, with a peak in 2–3 years, and the risk of recurrence decreases gradually in the next 5 years.<sup>2</sup> Because TNBC patients have high levels of vascular endothelial growth factor (VEGF) and significantly shorter recurrence-free survival time (RFS), the time from diagnosis to tumor recurrence and death is significantly short.<sup>27</sup> Exploring the mechanisms of recurrence and metastasis in TNBC and seeking new therapeutic targets is a constant pursuit of researchers in this field, and SCS may become a powerful tool to achieve this goal. TNBC cells are characterized by overexpression of somatic cells with mutations such as PIK3CA and p53<sup>6</sup> and are characterized by invasion of tissue boundaries, central necrotic areas, or compressed sites,<sup>2</sup> thus exhibiting metastatic features. In addition, studies have shown that recurrence and metastasis of TNBC are closely related to tumor stemness, EMT, and angiogenesis,<sup>28</sup> and further analysis the characteristics of them at the single-cell level shows that compared with other types of breast cancer, the stemness of tumor cells, angiogenesis, and EMT of TNBC cells are more strongly correlated with them, which may lead to a high incidence of TNBC recurrence. Meanwhile, abnormal activation of EMT and the proportion of TAM in the majority of immune cells are important factors for metastasis and recurrence in TNBC.

Whether the CD44 in CSC is associated with metastasis and recurrence has been controversial, some studies have shown that the presence of CD44 is associated with distant metastasis,<sup>29</sup> while others have shown that CD44 is not predictive of distant metastasis.<sup>30</sup> Such a contradictory conclusion is likely to the fact that there are multiple isoforms of the CD44 gene, The level of MCL1 and JUN in the CD44v6+ group were significantly increased, and relevant studies have shown that MCL1 is necessary for tumor cell genesis and metastasis,<sup>31,32</sup> Similarly, JUN is associated with metastasis and stem cell amplification.<sup>33,34</sup> According to the current relevant findings, CD44v6+ is closely associated with the recurrence and metastasis of tumor cells.

Continuously confirming and exploring molecular mechanisms related to TNBC recurrence and metastasis through various studies, can effectively improve the treatment strategy for TNBC and reduce the recurrence and metastasis rate of TNBC patients and prolong the survival time of patients.

## Application of SCS in the Treatment of TNBC

### Exploring the Efficacy of Combination Therapy and Immune Response

Because of the heterogeneity of TNBC and the poor efficacy of general chemotherapy, exploring chemotherapy combined with immunotherapy has become the focus of current research. Deng et al,<sup>35</sup> after investigating the therapeutic efficacy of albumin-paclitaxel combined with pembrolizumab in the treatment of advanced TNBC, conducted a continuous biopsy of tumor tissues in the same part of the two patients with SCS and analyzed infiltrated immune cells, and found that patients who responded to the treatment had high expression of PD-1 T cells. After treatment with albumin-paclitaxel combined with pembrolizumab, T cells were significantly reduced, and the presence of GZMB + CD8 + T cells, TRM, and a significant increase of tumor-infiltrating leukocytes (TIL). Related studies showed that TRM in CD8+ TRM was significantly correlated with TNBC survival improvement, for non-responders, there was no higher presence of PD-1 T cells, and such findings once again demonstrate that TRM in breast cancer tissues is related to the improvement of prognosis of patients.<sup>36</sup> The above study not only provides a reliable basis for studying the therapeutic changes in advanced TNBC during immunochemotherapy but also demonstrates the feasibility of single-cell analysis after continuous biopsies of tumor tissues and the difference in the therapeutic changes of advanced TNBC. A study<sup>37</sup> showed that the expression of heat shock protein 90(HSP90) and histone deacetylase 6(HDAC6) were significantly increased in TNBC cells, and in this study, a synergistic effect of the HSP90 inhibitor 17-AGG combined with the HDAC6 inhibitor Belinostat was further confirmed in TNBC. The migration and invasion of TNBC tumor cells were significantly inhibited by the combination treatment compared with 17-AGG or Belinostat alone, and it was confirmed that ROS levels were significantly increased in patients. In this study, EGFR, COX5B, UBA52, and other regulatory signaling pathways were significantly affected, which once again demonstrated that the combination of drugs could inhibit tumor cell proliferation, migration, and invasion.

A few studies have confirmed that combination therapy has a significant effect on the treatment of TNBC, among which the latest relevant research results can further provide new directions and strategies for clinical combination therapy, this will ensure that TNBC patients significantly benefit from the therapeutic efficacy in the process of clinical treatment.

### Revealing Resistance to Chemotherapeutic Agents

TNBC is characterized by a lack of corresponding hormone receptors and intra-tumor and inter-tumor cell heterogeneity.<sup>2,25</sup> It has been shown that the key to tumor drug resistance is mainly untreated intra-tumor heterogeneity,<sup>38–41</sup> especially in the course of chemotherapy and targeted therapy where the rate of drug resistance is significantly increased. For TNBC patients receiving chemotherapy, the proportion of patients who develop resistance is approximately 30–50%, resulting in a decrease in the overall survival (OS) of TNBC patients.<sup>2,42</sup> Research on antibiotic resistance of bacteria has been conducted for many years, but the mechanisms associated with the development of resistance in tumor cells have only attracted the attention of researchers in recent years. At present, the clinical problem of vulnerability to drug resistance has not been effectively addressed. Studies at the single-cell level have revealed that the susceptibility of TNBC patients to drug resistance is not only related to tumor heterogeneity but also related to resistance. To further investigate this issue, Kim et al<sup>43</sup> showed by longitudinal analysis of 900 single-cell DNAs and

6862 single-cell RNAs using SCS in 20 patients undergoing neoadjuvant therapy (NAC) that the drug-resistant genotype was preexisted, but the drug-resistant expression profile of tumor cells was acquired by transcriptional recoding after chemotherapy, as shown by TNBC patients can adaptively undergo genome mutations and copy number aberrations after chemotherapy and evolve drug-resistant phenotypes by transcriptional recoding, which can further conclude that the drug resistance model of TNBC patients is established by a combination of adaptive and acquired drug resistance. In studies related to tumor microenvironment,<sup>44</sup> fibroblasts (CAF) are the main stromal component in solid tumors, and CAF-producing cells may include tissue fibroblasts, hematopoietic stem cells, adipocytes, endothelial cells, etc. In addition, CAF may also be directly produced by cancer cells through EMT. Factors released from CAF into the tumor microenvironment may play an important role in drug resistance to therapy.

These findings suggest that SCS can be used to trace the lineage of tumor drug resistance cells and help to explore tumor heterogeneity and the tumor microenvironment in the mechanism of drug resistance, as well as to uncover drug resistance genes and provide a theoretical basis for targeted therapy of tumor drug resistance.

## Conclusion

SCS technology has served as the foundation for studies on intra- and inter-tumor heterogeneity of TNBC, drug resistance, recurrence and metastasis, mutations, and cloning. These studies provide a basis for the diagnosis and treatment of TNBC. However, the technology itself and its derivatives still have certain defects, which need to be further improved. In the whole process of sequencing technology, ensuring the integrity of single cells is a key step in the subsequent technology. Currently, most tumor samples are frozen for experimental manipulation, so the integrity of the cell membrane cannot be fully guaranteed, and the molecules on the cell surface may be changed during the cell thawing and lysis. Therefore, if tumor-related mechanisms are studied at the single-cell level, further improvement of sample production techniques is needed. At the same time, due to the high cost and price of SCS, patients bear a huge financial burden, which limits the development of this technology to some extent.

In addition to the technical defects, it is unclear whether TRM infiltration in TNBC is associated with the response to anti-PD-1 therapy during the study of immune-related responses, which requires us to further explore the mode of action from the combinatorial analysis of single cells or even large numbers of single cells to understand the molecular mechanisms associated with immunotherapy at a deeper level. Similarly, in the study of TNBC heterogeneity, CAF is one of the causes of TNBC heterogeneity, but there are no studies to show the specific subgroup and structure of CAF, so the relevant molecular mechanism of heterogeneity cannot be accurately understood, and the purpose of clinical precision treatment cannot be achieved. Although SCS and TNBC-related molecular mechanisms still face technical and related challenges, it is believed that with the rapid development of emerging technologies and the continuous improvement of derivative technologies, the deficiencies related to SCS will be overcome one by one, and bring benefits to more patients with TNBC.

## Abbreviations

TNBC, Triple-negative breast cancer; SCS, Single-cell sequencing; BC, Breast cancer; HER2, epidermal growth factor receptor 2; WGA, whole genome amplification; EMT, Epithelial-mesenchymal transition; siRNA, 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; OS, overall survival; RFS, recurrence-free survival time.

## Funding

Popularization and application of breast cancer early diagnosis and treatment technology in Qinghai, 2022ZY009.

## Disclosure

The authors have declared no conflicts of interest.

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