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Deciphering Treatment Resistance in NSCLC with Single-Cell Sequencing Technology

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Abstract

Acquired resistance to targeted and immune therapies severely limits the success of non-small cell lung cancer (NSCLC) treatment. Single-cell sequencing technologies now empower researchers to dissect this resistance at unprecedented resolution, moving beyond the averaging limitations of bulk genomics. This review highlights how single-cell and spatial multiomics approaches reveal key mechanisms of NSCLC resistance, from rare drug-tolerant subpopulations and cellular plasticity to immunosuppressive niches and metabolic adaptation within the TME. We also discuss emerging strategies-such as liquid biopsy and AI-driven data integration-that hold promise for translating these insights into more effective therapeutic interventions.

Keywords

single-cell RNA sequencing, tumor heterogeneity, drug resistance, tumor microenvironment, NSCLC

1. Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) accounting for the vast majority of cases and posing a significant public health challenge[1]. In recent years, breakthrough advances have been made in NSCLC treatment strategies, particularly with tyrosine kinase inhibitors (TKIs) that target specific driver genes (e.g., EGFR) and immune checkpoint inhibitors (e.g., PD-1/PD-L1), which have significantly improved outcomes for patients with advanced disease[2, 3].

However, acquired resistance is almost inevitable, ultimately leading to treatment failure and disease recurrence, representing a major obstacle to long-term patient survival[4]). Traditional views hold that resistance primarily arises from preexisting clones or new mutations selected under therapeutic pressure. However, bulk sequencing technologies based on population-level analysis provide only average signals and fail to capture the high degree of intratumoral heterogeneity. This limitation obstructs the understanding of rare resistant subpopulations, nongenetic adaptive mechanisms, and interactions within the tumor microenvironment (TME)[5]. In reality, treatment resistance is a dynamic process driven by the intrinsic plasticity of tumor cells (e.g., cell state transitions), clonal evolution, and remodeling of the TME[6, 7].

The emergence of single-cell sequencing technologies has provided a revolutionary tool for in-depth dissection of this complex system[8]. Techniques such as single-cell RNA sequencing (scRNA-seq) enable unbiased identification of all cell types within the tumor ecosystem, revealing previously unknown resistant

cell subpopulations[6, 9]. Integrated multiomics approaches (e.g., combining scRNA-seq with scATAC-seq) further reveal upstream transcriptional regulation and epigenetic networks underlying resistant phenotypes[10, 11]. More advanced spatial transcriptomic and genomic technologies preserve the spatial context of cells within native tissue, helping to address critical questions such as "where are the resistant cells located?" and "with whom do they interact?", thereby elucidating specialized niches of resistance such as those facilitating immune evasion[12, 13]. Together, these technologies are shifting NSCLC resistance research from a "static, population-level" perspective to a "dynamic, high-resolution, ecosystem-level" understanding.

This review aims to systematically outline how single-cell sequencing technologies profoundly transform our understanding of treatment resistance in NSCLC. First, we introduce the fundamental methodologies of single-cell technologies[14, 15]. We then highlight their applications in uncovering cell-intrinsic mechanisms of resistance (e.g., identifying resistant subclones and tracking state transitions)[6, 9, 13], dissecting the regulatory roles of the tumor microenvironment [16, 17], and leveraging multiomics and spatial technologies to map the resistant ecosystem [11-13]. Finally, we discuss current challenges in computational analysis and clinical translation and envision future directions, such as integrating liquid biopsy with single-cell sequencing and employing artificial intelligence for multidimensional data integration.

2. The Technological Landscape and Methodological Foundations of Single-Cell Sequencing

2.1 Development History of the Technology and Core Workflow

Single-cell RNA sequencing (scRNA-seq), a revolutionary technology for resolving cellular heterogeneity, has significantly advanced precision medicine research, particularly in the study of treatment resistance mechanisms in non-small cell lung cancer (NSCLC)[14]. scRNA-seq enables the detection of rare cell subpopulations and transcriptional states that are undetectable by conventional bulk sequencing, providing unprecedented resolution for understanding tumor heterogeneity and therapy resistance[15].

The technical workflow of scRNA-seq primarily involves the following key steps: single-cell isolation, cell lysis and mRNA capture, reverse transcription and cDNA amplification, library preparation and high-throughput sequencing, followed by bioinformatic analysis[14]. Methods for single-cell isolation include fluorescence-activated cell sorting (FACS), laser capture microdissection (LCM), and microfluidic technologies, with the choice of method depending on sample type and research objectives[15].

In terms of whole-transcriptome amplification (WTA) methods, different technological platforms exhibit distinct characteristics. The SMART-seq2 method, which is based on the switching mechanism at the 5' end of the RNA template (SMART) principle, is suitable for alternative splicing and mutation analysis. In contrast, droplet-based systems employing 3'-end enrichment (e.g., 10x Genomics) are better suited for large-scale cell atlas construction[14, 15]. These technical features allow researchers to select the most appropriate platform on the basis of specific research needs in NSCLC, such as identifying rare resistant cell populations or comprehensively profiling tumor heterogeneity.

The characteristic high sparsity and technical noise (e.g., dropout events) of single-cell data pose unique challenges for analysis. Current approaches primarily employ unique molecular identifier (UMI)-based normalization methods and specially designed statistical models (e.g., negative binomial distributions) to address these technical variations[14]. Furthermore, advancements in dimensionality reduction techniques (e.g., PCA, t-SNE, and UMAP) and clustering algorithms have greatly enhanced the ability to identify cell subpopulations, providing powerful tools for discovering therapy-resistant clusters in NSCLC.

The continued maturation of scRNA-seq technology and the widespread adoption of commercial platforms have established it as a core tool for investigating tumor microenvironment heterogeneity, clonal evolution mechanisms, and treatment resistance in NSCLC, offering critical technical support for the development of novel therapeutic strategies.

2.2 Technical and Biological Principles of scRNA-seq

Single-cell RNA sequencing (scRNA-seq) fundamentally aims to resolve heterogeneity in gene expression among individual cells, overcoming the averaging effect of traditional bulk sequencing and providing key insights into drug resistance mechanisms within the tumor microenvironment[8].

The scRNA-seq workflow begins with efficient isolation of single cells. Commonly used methods include fluorescence-activated cell sorting (FACS), microfluidic platforms (such as Fluidigm C1), and droplet-based high-throughput systems (e.g., 10x Genomics). Microfluidic systems perform cell lysis and reverse transcription in nanoliter-scale reaction volumes, significantly improving sensitivity but requiring specific cell size criteria. Droplet systems, on the other hand, enable massively parallel processing and are particularly suitable for identifying rare cell types, such as drug-resistant subpopulations in NSCLC[8].

At the molecular level, different amplification strategies offer distinct advantages: full-length transcript methods (e.g., SMART-Seq2) are suitable for alternative splicing and mutation analysis, whereas 3′ end tagging methods (such as CEL-Seq and Drop-seq) improve quantification accuracy and throughput, making them more appropriate for large-scale cell atlas construction. To correct for technical noise, unique molecular identifiers (UMIs) and external RNA controls (e.g., ERCC spike-ins) are widely used to achieve absolute quantification of RNA molecules and accurately distinguish technical variation from true biological heterogeneity[8].

The biological value of scRNA-seq is most prominently demonstrated in its ability to uncover previously unknown cell subtypes and resolve dynamic processes. Through cluster analysis, principal component analysis (PCA), and pseudotime trajectory inference, researchers can identify critical regulatory genes and cell fate branch points, revealing tumor evolutionary pathways and drug resistance mechanisms[8]. This technology has become a core tool for studying NSCLC heterogeneity, clonal evolution, and treatment resistance, providing critical evidence for developing combination therapies.

2.3 From Single-Cell to Multiomics and Spatial Resolution

Single-cell RNA sequencing (scRNA-seq) can reveal cellular heterogeneity in NSCLC, yet transcriptomic data alone are insufficient to fully elucidate the mechanisms underlying treatment resistance. In recent years, advances in single-cell multiomics and spatial omics technologies have enabled the integration of multidimensional information at single-cell and spatial resolutions, providing new avenues for understanding NSCLC resistance.

Multiomics technologies such as iscCOOL-seq allow simultaneous detection of transcriptomic and epigenomic information within the same cell. Studies indicate that epigenetic preregulation in NSCLC may be associated with early activation of drug resistance genes [18]. Spatial multiomics approaches such as BaSISS (base-specific in situ sequencing) enable quantitative analysis of subclonal distribution across entire tumor sections, integrating transcriptomic and protein data to reveal spatial expansion patterns of clones and their interactions with the microenvironment. Research suggests that NSCLC exhibits significant spatial segregation of subclones and heterogeneous transcriptional states, with evolutionary trajectories that are not synchronized with histological progression, providing clues to the evolution of resistant subpopulations (Dressler et al., 2022). Furthermore, technologies such as multiplex immunofluorescence (mIHC) and in situ sequencing (ISS) allow simultaneous analysis of RNA and protein within spatial contexts and identification of immune composition and cell–cell communication in the tumor microenvironment, thereby helping to decipher the architecture and regulatory networks of resistance niches in NSCLC [12].

In summary, the integration of multiomics and spatial technologies is advancing NSCLC research from cell classification toward mechanistic exploration and spatial ecosystem reconstruction; significantly enhancing the understanding of tumor heterogeneity, the microenvironment, and treatment resistance; and offering new perspectives for targeted therapies and overcoming drug resistance.

3. scRNA-seq Decodes the Cell-intrinsic Mechanisms of Drug Resistance

3.1 Identification of Drug-Resistant Cell Subpopulations

Single-cell RNA sequencing (scRNA-seq) has significantly advanced our understanding of resistance mechanisms to tyrosine kinase inhibitors (TKIs) in EGFR-mutated non-small cell lung cancer (NSCLC) because of its high-resolution capabilities. By revealing tumor cellular heterogeneity and identifying key resistant subpopulations, this technology provides critical insights into the cellular basis of treatment resistance [7].

Kim et al. performed scRNA-seq on over 200,000 cells from 44 lung adenocarcinoma patients and identified a cancer cell subpopulation (tS2) that dominated in the metastatic stage. This subpopulation exhibits a transcriptomic profile completely divergent from the normal epithelial differentiation trajectory, with upregulated genes related to cell motility, aberrant proliferation, and apoptosis escape. The tS2 subpopulation was significantly enriched in advanced and metastatic samples, and its presence was strongly associated with reduced overall survival, suggesting that the tS2 phenotype may be a key driver of lung cancer progression and treatment resistance [7].

In another study focused on EGFR-TKI resistance, Kashima et al. integrated scRNA-seq with single-cell ATAC-seq (scATAC-seq) to perform multiomics analysis on resistant cell lines and clinical samples. Their work revealed highly heterogeneous transcriptional and epigenetic regulatory patterns in drug-tolerant persistent (DTP) cells. In addition to known resistance-related genes such as AURKA, VIM, and AXL, CD74 was identified as a novel candidate gene that was significantly upregulated in DTP cells. It mediates osimertinib tolerance by inhibiting apoptosis and promoting BCL-XL expression. Differential activation of AURKA, VIM, or CD74 has been observed not only among different patients but also within individual tumors, highlighting the diversity of resistance mechanisms and the clinical importance of intratumoral heterogeneity [7].

These studies demonstrate the powerful ability of scRNA-seq to identify treatment-resistant cell subpopulations and underscore its value in identifying potential therapeutic targets and prognostic biomarkers.

3.2 Revealing Non-Genetic Functional Plasticity

In addition to genetic alterations, the nongenetic functional plasticity exhibited by tumor cells under the pressure of targeted therapy serves as a crucial mechanism mediating drug resistance. For the first time, Maynard et al. performed scRNA-seq on 49 biopsy samples from 30 patients with advanced NSCLC collected at three stages: before targeted therapy (treatment-naïve, TN), at residual disease (RD), and at progressive disease (PD), systematically revealing therapy-induced tumor cell state evolution in clinical samples [4].

The study revealed that cancer cells in the RD state highly express gene signatures associated with alveolar regeneration (e.g., SFTPB/C/D, *NKX2-1*, AQP4, AGER), suggesting that they may adapt to therapeutic pressure through dedifferentiation or acquisition of a stem-like state, entering a slowly proliferating but reparative “persister cell” state [4]. This alveolar signature was significantly associated with better overall survival in patients and was validated in preclinical models [4]. Further mechanistic studies revealed the activation of the WNT/β-catenin pathway in RD cells (e.g., the upregulation of SUSD2 and CTNNB1), and the combined use of a WNT inhibitor significantly increased the efficacy of TKIs [4].

In contrast, cancer cells at the PD stage exhibited a distinctly different transcriptional state, with upregulated genes related to immunosuppression (e.g., kynurenine pathway genes IDO1 and KYNU), cell invasion (plasminogen activation pathway), and intercellular communication (gap junction proteins) [4]. These changes collectively promote a more aggressive and immune-evasive drug-resistant phenotype.

This study provides the first in vivo evidence in humans that targeted therapy can drive reversible transcriptional reprogramming in lung cancer cells, enabling escape from drug-induced cell death. This state-dependent resistance does not rely on genetic mutations but is achieved through the activation of developmental or damage repair-related pathways, offering a theoretical foundation for novel treatment strategies targeting “cell states” rather than “genetic alterations” [4].

3.3 Tumor Heterogeneity and Clinical Outcomes

Tumor heterogeneity is not only a core feature of cancer progression but also a critical determinant of variations in treatment response and clinical outcomes. Single-cell RNA sequencing (scRNA-seq) technology provides unprecedented resolution for deciphering the complexity of the cellular composition within tumors, enabling researchers to identify specific cell subpopulations associated with poor prognosis and to elucidate their functional states and interaction networks at the single-cell level.

Hu et al. [6] systematically revealed a high degree of heterogeneity in clear cell renal cell carcinoma (ccRCC) through scRNA-seq analysis. The study identified 15 major cell types and 39 cell subpopulations, including various immune and stromal cells derived from both tumor and nonmalignant tissues [6]. Importantly,

the existence of these cell subpopulations was validated via immunofluorescence on tissue microarrays. Furthermore, the CIBERSORTx algorithm was used to deconvolve the cellular composition of 533 patients from the TCGA-KIRC cohort, and the researchers stratified patients into three subgroups with significantly distinct prognoses. One subgroup, characterized by a lower proportion of activated $CD8^+$ T cells and a higher proportion of exhausted $CD8^+$ T cells, was significantly associated with poorer overall survival [6]. This finding not only confirms the central role of T-cell exhaustion in the immunosuppressive microenvironment of ccRCC but also suggests that the ratio of exhausted T cells could serve as an important prognostic biomarker.

Additionally, metabolic dysregulation is not confined to tumor cells but is also widespread among stromal cells within the tumor microenvironment. For example, cancer-associated fibroblasts (CAFs) exhibit significant upregulation of genes related to lipid metabolism (such as FABP5), which cooccur in a tumor tissue-specific manner [6]. Through SCENIC analysis, researchers further revealed transcription factor networks regulating these aberrant metabolic states, including altered expression of PPAR signaling pathway members in tumor cells and activation of CEBPB and KLF6 in fibroblasts [6]. These findings underscore the pervasiveness and cell-type specificity of metabolic reprogramming in the tumor microenvironment, providing new insights into the functional consequences of tumor heterogeneity.

In summary, scRNA-seq technology not only enables detailed characterization of intratumoral cellular heterogeneity but also directly links specific cell subpopulations to clinical outcomes, thereby revealing key cell states and molecular mechanisms driving disease progression. These studies provide a theoretical basis for developing precise therapeutic strategies targeting specific cell subpopulations and highlight the great potential of single-cell technologies in prognostic assessment and personalized cancer treatment.

4. Multiomics and Spatial Technologies for Deciphering the Drug Resistance Ecosystem

4.1 Integrated Multiomics: Bridging Phenotype and Regulation

Single-cell RNA sequencing (scRNA-seq) can reveal heterogeneity in cellular states, yet its limitations in deciphering regulatory mechanisms have prompted researchers to integrate multiomics data to comprehensively understand the molecular basis of drug resistance. Although the study by Sathe et al. focused on gastric cancer, its research paradigm offers valuable insights for understanding drug resistance in lung cancer. Using scRNA-seq, 56,167 cells from tumor tissues, paired normal tissues, and peripheral blood mononuclear cells (PBMCs) from seven gastric cancer patients and one patient with intestinal metaplasia were analyzed, and the remodeling of the cellular composition and transcriptional reprogramming within the tumor microenvironment (TME) were systematically characterized [11]. A previous study revealed that tumor epithelial cells exhibit distinct copy number alterations (CNAs) and unique gene expression programs, along with significant intratumoral heterogeneity [11]. More importantly, the research extended beyond the transcriptome by computationally inferring CNAs [19] and validating key protein expression via multiplex immunofluorescence, demonstrating the value of multiomics integration in verifying findings and enhancing the reliability of conclusions [11].

Furthermore, the study employed regulatory network analysis (e.g., SCENIC [20] to identify key transcription factors (regulons) that drive state transitions across different cell types within the TME. For example, noncanonical M1/M2 regulators such as NFKB1 and ETS2 were identified in tumor-associated macrophages [11]. This integrated approach combining transcriptomics and regulatory network analysis provides a methodological foundation for understanding the dynamic regulation of immune and tumor cells under therapeutic pressure in lung cancer. Similarly, in lung cancer research, multiomics approaches integrating scRNA-seq with chromatin accessibility (scATAC-seq) or proteomics (CITE-seq) can more precisely identify key regulatory pathways driving resistant phenotypes, such as the epithelial–mesenchymal transition (EMT)-associated transcriptional program observed in resistance to EGFR inhibitors [4, 9].

In summary, the study by Sathe et al. demonstrated how scRNA-seq combined with computational methods for inferring CNAs and regulatory networks can link transcriptional phenotypes to upstream regulatory mechanisms at single-cell resolution [11]. This strategy provides an important reference for systematically dissecting the multiomics basis of drug resistance in lung cancer.

4.2 Core Role of the Tumor Microenvironment

The tumor microenvironment (TME) is a highly complex and dynamically evolving ecosystem composed of cancer cells, immune cells, stromal cells (such as cancer-associated fibroblasts (CAFs) and endothelial cells), and noncellular components such as the extracellular matrix (ECM). In the development of treatment resistance in non-small cell lung cancer (NSCLC), the TME not only provides a physical barrier but also actively mediates immune evasion and drug tolerance through diverse cell–cell interactions and signaling pathways [18]. The application of single-cell sequencing technologies now enables the dissection of functional states and interaction networks among cellular components within the TME at unprecedented resolution, revealing its central role in drug resistance.

4.2.1 Formation of Immunosuppressive Cell Populations and the Drug-Resistant Niche

The immune cell infiltration status in the tumor microenvironment (TME) is a critical determinant of the response to immunotherapy. Studies have demonstrated that non-small cell lung cancer (NSCLC) tissues harbor abundant regulatory T cells (Tregs), M2-type tumor-associated macrophages (TAMs), and exhausted T cells, which collectively establish an immunosuppressive microenvironment [16]. Hu et al. [6] used scRNA-seq to show that anti-PD-1-resistant NSCLC patients have increased levels of exhausted $CD8^+$ T cells (which express checkpoint molecules such as LAG3 and TIM-3), Tregs, and TAMs in the TME [6]. These cells suppress antitumor immunity either by secreting inhibitory cytokines such as TGF-β and IL-10 or through direct interaction with T cells via surface molecules such as PD-L1, ultimately leading to immunotherapy failure.

Single-cell studies have further revealed that immune cells in the TME do not exist as discrete static entities but rather exhibit a continuous phenotypic spectrum. Through scRNA-seq analysis of 45,000 immune cells from the breast cancer TME, Azizi et al. reported that both T cells and myeloid cells in tumors displayed significant phenotypic expansion, occupying a "phenotypic space" far broader than that in normal tissues [16]. This continuity was particularly evident in T cells, where the activation status-defined by the expression of activation signature genes-followed a broad continuous distribution rather than falling into discrete intermediate states [16]. This suggests that traditional cell classification (e.g., naïve T cells, effector T cells, exhausted T cells) may oversimplify the actual states of immune cells in the TME. This continuous phenotypic diversity is partly driven by T-cell receptor (TCR) diversity, but more importantly, it reflects T-cell responses to diverse combined environmental cues within the TME, such as varying levels of inflammatory signals, hypoxia, and nutrient deprivation [16]. Therefore, therapeutic strategies aimed at reversing immunosuppression by targeting the TME may need to address multiple continuous functional states simultaneously rather than target a single cell subset.

4.2.2 Metabolic Reprogramming and Immunosuppression

Metabolic dysregulation in the tumor microenvironment (TME) represents another critical factor contributing to therapy resistance. Metabolic competition between tumor cells and stromal cells can lead to microenvironmental acidosis and nutrient depletion (e.g., tryptophan, arginine), thereby impairing the function of T cells and NK cells [21]. Single-cell analysis enables simultaneous capture of the metabolic states of both cancer and stromal cells. For example, in clear cell renal cell carcinoma (ccRCC), Hu et al. utilized scRNA-seq to reveal not only significant lipid metabolic abnormalities in cancer cells but also distinct metabolic reprogramming features in tumor-infiltrating stromal cells (including macrophages and fibroblasts). This widespread metabolic alteration may contribute to the establishment of an immunosuppressive TME [6]. Similar mechanisms of metabolic competition are likely present in NSCLC and may mediate resistance to both targeted therapy and immunotherapy.

4.2.3 Dynamic Evolution of the TME and Therapeutic Intervention

Notably, the TME is not static but rather undergoes dynamic evolution under therapeutic pressure. Conventional chemotherapy, radiotherapy, targeted therapy, and even immunotherapy itself can reshape the cellular composition and functional state of the TME [22]. For example, studies in breast cancer have revealed that residual tumors following neoadjuvant chemotherapy often exhibit more pronounced immunosuppressive features, including increased proportions of Tregs, elevated levels of M2-type TAMs, and functional exhaustion of CD8+ T cells [23]. Single-cell technologies enable tracking of such dynamic changes. Research

by Azizi et al. demonstrated that immune cell states in the TME are highly plastic and shaped by both TCR signals and local environmental cues [16]. These findings suggest that combination therapies should fully account for the dynamic nature of the TME. For example, concurrently targeting TAMs or CAFs alongside immunotherapy may reverse drug resistance.

In summary, the TME actively contributes to the development of therapeutic resistance in NSCLC through complex interactions among diverse cellular and noncellular components. Leveraging single-cell and spatial multiomics technologies to deeply dissect the cellular heterogeneity, interaction networks, and dynamic evolution of the TME will provide critical insights for developing novel combination strategies to overcome resistance.

4.3 Spatial Multiomics: Mapping the Drug-Resistant Niche

While traditional single-cell sequencing technologies can reveal heterogeneity in tumor cells and the microenvironment, they miss the spatial context of cells within tissues-information critical for understanding the spatial distribution of tumor clones, cell–cell interactions, and the formation of drug-resistant niches. Recently, emerging technologies such as spatial transcriptomics and multiplexed fluorescence in situ hybridization (multiplexed FISH) have enabled high-throughput molecular profiling while preserving the spatial context.

In a study published in Nature (2022), Dressler et al. developed a spatial genomics workflow called Base-Specific In Situ Sequencing (BaSISS), which allows simultaneous quantitative mapping of multiple cancer clones while integrating transcriptomic and microenvironmental data[13]. An analysis of multifocal breast cancer samples revealed that distinct clones present significantly different spatial distributions, transcriptional profiles, and immune microenvironment compositions across stages, including ductal carcinoma in situ (DCIS), invasive carcinoma, and lymph node metastasis. For example, in lymph node metastases, different subclones (e.g., P2-blue and P2-orange) not only display distinct histopathological growth patterns but are also associated with B-cell-enriched regions or hypoxic lymphatic sinus areas, suggesting clone-specific immune editing and adaptive evolution[13].

Similarly, Wu et al. (2020) identified two major cancer-associated fibroblast (CAF) subtypes-myofibroblast-like CAFs (myCAFs) and inflammatory CAFs (iCAFs)-as well as two perivascular-like (PVL) cell subtypes-differentiated PVL (dPVL) and immature PVL (imPVL)-through single-cell transcriptomic analysis of triple-negative breast cancer (TNBC)[21]. These stromal subtypes have distinct spatial distributions: myCAFs are located predominantly at the tumor invasive front and are closely associated with collagen deposition and stromal remodeling; iCAFs are distributed farther from the tumor boundary and highly express chemokines (e.g., CXCL12 and CXCL13) and growth factors (e.g., IGF1 and HGF), potentially regulating immune cell recruitment and angiogenesis via long-range signaling, whereas dPVL cells are significantly correlated with the exclusion of cytotoxic T cells (CTLs), forming an “immune desert” phenotype[21]. Further spatial protein marker analysis (e.g., CD34, α-SMA, and CD146) confirmed the highly specific tissue distribution of these stromal subtypes and their significant correlation with immune cell infiltration[21].

Andersson et al. (2021), using imaging mass cytometry, demonstrated the diversity of cellular neighborhood structures in breast cancer tissues and identified spatial niches associated with poor prognosis[17]. By simultaneously quantifying 35 biomarkers, the authors constructed high-dimensional pathological images that revealed spatial interaction patterns between tumor and stromal cells. They further defined “single-cell pathology (SCP) subgroups” that were significantly associated with clinical outcomes. These subgroups not only reflect the phenotypic composition of tumor cells but also capture their spatial organization, providing a new dimension for understanding drug resistance[17].

Collectively, these studies demonstrate that drug resistance does not develop uniformly but is confined to specific spatial niches composed of distinct clones, stromal cells, and immune cells. Spatial multiomics technologies enable precise mapping of these regions, offering unprecedented insights into resistance mechanisms and informing the development of niche-targeted therapeutic strategies.

5. Discussion

While single-cell sequencing has significantly advanced our understanding of treatment resistance in NSCLC, its inherent limitations continue to challenge the robustness of research conclusions. Tissue dissociation may induce transcriptional stress responses, distorting the in vivo cellular state [14], but the low capture efficiency of rare resistant subpopulations limits the comprehensive identification of key resistance events. Moreover, high data sparsity and batch effects significantly hinder cross-time-point and cross-sample comparisons, and current annotations of cell clusters remain heavily reliant on prior assumptions, introducing considerable subjectivity.

Single-cell studies are inherently observational-they can reveal correlations between resistance and molecular phenotypes but cannot establish causality. Therefore, any inferred resistance-associated cell subpopulation or biomarker must undergo rigorous functional validation [9], a process that is both complex and costly, substantially limiting its clinical translation.

Although this technology shows promise for translational applications, it still faces multiple bottlenecks. For example, dynamic monitoring based on circulating tumor cells (CTCs) remains constrained by detection sensitivity and insufficient technical standardization. Candidate biomarkers derived from single-cell data-such as intermediate EMT-state cells or specific TAM subtypes-require further validation through spatial multiplex fluorescence techniques and large clinical cohorts before they can serve as clinically useful indicators. Similarly, most mechanism-informed combination therapies (e.g., those that target AXL or the CCL2/CCR2 axis) [6] remain in the preclinical stage, with their actual efficacy and applicable patient populations still awaiting confirmation.

Future progress will rely heavily on technological innovation and multidimensional data integration. Although artificial intelligence and generative models hold promise for predicting clonal evolution or virtually screening drug combinations, their predictive reliability depends critically on data quality and algorithmic interpretability. Multiomics integration (e.g., transcriptomic, epigenomic, and proteomic analyses) could theoretically uncover multilayered regulatory networks underlying resistance [6], yet major challenges remain in terms of technical compatibility and analytical harmonization. Furthermore, functional validation platforms such as organoids combined with CRISPR screening offer new pathways for target verification [4], although their throughput and clinical relevance still require improvement.

In summary, single-cell sequencing has increased resistance to single-cell resolution. However, only by systematically addressing multiple bottlenecks-including technical noise, data analysis limitations, and experimental validation-can we deepen our mechanistic understanding of the resistance ecosystem and closely integrate these insights with those of clinical practice to realize the full potential of this technology in precision medicine.

6. Conclusion

Single-cell sequencing technology has increased research on drug resistance in NSCLC to single-cell resolution, profoundly revealing the critical roles of tumor heterogeneity, cellular plasticity, and the TME in treatment resistance [4, 7]. By identifying rare drug-resistant subpopulations, deciphering nongenetic functional state transitions, and characterizing immunosuppressive niches, this technology provides unprecedented insights into the mechanisms underlying drug resistance [9, 12].

However, the technology still faces multiple challenges. Technically, issues such as tissue dissociation-induced stress responses, low capture efficiency of rare cell subpopulations, high data sparsity, and batch effects constrain the accuracy and reproducibility of the data [14]. Analytically, studies remain largely observational and unable to establish causality directly, and cell type annotation still relies heavily on empirical knowledge, introducing subjectivity. From a translational perspective, most candidate biomarkers and mechanism-informed combination therapies remain in the preclinical validation stage, and their clinical utility urgently requires confirmation through large-scale cohorts and functional experiments [6].

Future breakthroughs will depend on synergistic progress in three key areas: first, technological innovation and integration, necessitating improvements in sample processing protocols; second, the development of highly sensitive liquid biopsy techniques; and third, the convergence of single-cell multiomics with spatial technologies [13]. Second, intelligent computing and data analysis, leveraging artificial intelligence and generative models to enhance the reliability of predicting clonal evolution and virtually screening drug combinations, while also improving algorithmic interpretability. Third, functional validation systems should be strengthened by the use of platforms such as organoid models and CRISPR screening to efficiently verify candidate targets and mechanistic hypotheses [4].

In conclusion, only by systematically addressing the bottlenecks ranging from technical noise and data analysis limitations to clinical validation can the profound insights generated by single-cell sequencing be translated into precise diagnostic and therapeutic strategies that guide clinical practice, ultimately realizing its full potential for improving outcomes in NSCLC patients.

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Conflicts of Interest

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