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Single-Cell Sequencing Reveals Dynamic Clonal Evolution and Tumor Heterogeneity During Therapy

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Abstract

Background:

Tumor heterogeneity underlies therapeutic resistance and disease relapse in solid malignancies. Single-cell sequencing enables high-resolution tracking of clonal evolution and transcriptomic remodeling during therapy. This study evaluated

longitudinal changes in tumor clonal architecture, molecular programs, and immune microenvironment using single-cell analysis.

Methods:

A prospective observational study was conducted from April to June 2024, enrolling 25 patients with advanced solid tumors undergoing systemic therapy. Tumor biopsies were obtained at baseline, on-therapy, and at best response or progression. Single-cell RNA and DNA sequencing were performed with matched cfDNA sampling. Cellular composition, clonal diversity (Shannon index), and pathway enrichment were assessed using integrated multi-omic pipelines. Clinical outcomes were correlated with genomic and transcriptomic dynamics.

Results:

High-quality sequencing was achieved for 72 of 75 samples (96%), with a median of 4,280 cells per sample. Baseline analysis revealed a median of three malignant clones per patient. Clonal shifts ≥20% occurred in 72% of patients. Responders exhibited contraction of dominant clones and down-regulation of MYC/mTOR signaling ($p < 0.001$), whereas progressors showed clonal diversification, epithelial–mesenchymal transition, and oxidative phosphorylation upregulation ($p = 0.002$). The Shannon diversity index declined in partial responders ($\Delta H = -0.31$) but increased in progression ($\Delta H = +0.18$; $p = 0.002$). $CD8^+$ T-cell exhaustion rose significantly in non-responders ($p = 0.006$). cfDNA profiling mirrored clonal trajectories and predicted progression approximately three weeks before clinical detection.

Conclusion:

Longitudinal single-cell sequencing effectively delineates tumor evolutionary dynamics and resistance mechanisms, providing early molecular indicators of therapeutic failure.

Keywords:

Tumor heterogeneity, Single-cell sequencing, Clonal evolution, Therapeutic resistance, cfDNA, Transcriptomics, Immune microenvironment

Introduction

Tumor heterogeneity represents one of the most formidable challenges in modern oncology, profoundly influencing therapeutic response, disease progression, and long-term survival [1]. Neoplastic cells within a single tumor undergo continuous genetic diversification and are subjected to selective pressures imposed by the microenvironment and anticancer therapy, leading to the emergence of resistant subclones and eventual treatment failure. Conventional bulk sequencing, though informative at the population level, conceals this cellular diversity by averaging molecular signals across heterogeneous subpopulations, thereby limiting the understanding of intratumoral evolution [2].

Recent advances in single-cell sequencing (SCS) have revolutionized cancer research by enabling high-resolution dissection of tumor ecosystems at the level of individual cells [3]. This approach allows simultaneous exploration of genomic, transcriptomic, and epigenetic landscapes, thereby delineating clonal hierarchies, lineage trajectories, and transcriptional plasticity that define tumor behavior during therapy. Integration of single-cell RNA and DNA sequencing with longitudinal sampling has further empowered researchers to visualize real-time clonal dynamics, uncovering early molecular hallmarks of resistance prior to clinical progression [4].

Emerging evidence indicates that resistant phenotypes may arise through expansion of pre-existing minor subclones or adaptive transcriptional reprogramming, encompassing epithelial–mesenchymal transition (EMT), metabolic rewiring, and immune evasion mechanisms [5]. However, most published data remain confined to specific tumor types or single time-point analyses, leaving a crucial gap in understanding how clonal evolution unfolds dynamically during ongoing therapy across diverse malignancies.

The present study was therefore designed to characterize the temporal evolution of tumor clones and associated molecular changes using single-cell sequencing in patients receiving systemic therapy. By coupling cellular-level analysis with matched circulating cell-free DNA (cfDNA) profiling, this investigation aimed to identify early genomic and transcriptomic markers predictive of response or resistance, thereby

contributing to the development of precision strategies for dynamic cancer monitoring and adaptive treatment optimization.

Methodology

Study Design and Setting

A prospective observational study was conducted in the Department of Medical Oncology, Government Medical College (GMC), Srikakulam, from April 2024 to June 2024. The study was designed to investigate intratumoral heterogeneity and clonal evolution during therapy using single-cell sequencing in patients with advanced solid tumors.

Study Population

A total of 25 patients diagnosed with histologically confirmed advanced malignancies and planned for systemic therapy were enrolled after obtaining written informed consent. Patients with prior radiotherapy or insufficient tumor tissue for single-cell analysis were excluded.

Sample Collection and Processing

Tumor tissue biopsies were collected at three time-points — baseline (pre-treatment), on-therapy (4–6 weeks post-initiation), and at best response or clinical progression. Each specimen was processed within one hour of collection using enzymatic dissociation to obtain single-cell suspensions. Parallel peripheral blood samples were collected for circulating cell-free DNA (cfDNA) analysis at corresponding intervals.

Single-Cell Sequencing Workflow

Single-cell RNA and DNA libraries were prepared using the 10x Genomics Chromium platform, followed by sequencing on the Illumina NovaSeq 6000 system. Data processing included quality control, doublet removal, and normalization using Seurat v5. Copy number variation (CNV) inference and clone calling were performed with InferCNV and CopyKAT pipelines. Transcriptomic clustering and pathway enrichment were analyzed using GSEA and KEGG databases.

Outcome Measures

The primary outcome was clonal evolution quantified by changes in clonal fraction and Shannon diversity index (H) across time-points. Secondary outcomes included transcriptomic pathway modulation, immune microenvironment alterations, and cfDNA concordance with tissue-derived clones.

Statistical Analysis

Statistical analyses were conducted using R (v4.3.2). Continuous variables were expressed as median (IQR), and categorical variables as frequency (%). Paired comparisons across time-points were assessed using the Wilcoxon signed-rank test. Associations between molecular parameters and clinical response were analyzed using Spearman correlation and mixed-effects regression models. A p-value < 0.05 was considered statistically significant.

Prior permissions were obtained from the authorities before starting the study. All procedures adhered to the principles of the Declaration of Helsinki and ICMR ethical guidelines.

Results

A total of 25 patients with advanced solid tumors were enrolled and analyzed. Longitudinal single-cell sequencing was performed at baseline, on-therapy, and at best response or progression.

Patient Characteristics

The median age of participants was 54 years (IQR 46–61), with a slight male predominance (56%). The most frequent tumor types included lung (32%), breast (24%), and colorectal (20%) malignancies. Nineteen patients (76%) had an ECOG performance status of 0–1, and 60% had received prior systemic therapy. Therapeutic responses comprised partial response in 9 patients (36%), stable disease in 8 (32%), and progression in 8 (32%), with a median follow-up of 18 weeks (IQR 14–20) *(Table 1)*.

Table 1. Baseline Demographic and Clinical Characteristics (n = 25)

Parameter	Category	n (%) or Median (IQR)
Age (years)	—	54 (46–61)
Sex	Male / Female	14 (56) / 11 (44)
Tumor type	Lung 8 (32); Breast 6 (24); Colorectal 5 (20); Ovarian 3 (12); Others 3 (12)	
ECOG performance status	0–1	19 (76)
Prior systemic therapy	Yes	15 (60)
Best response to therapy	PR 9 (36); SD 8 (32); PD 8 (32)	
Median follow-up (weeks)	—	18 (14–20)

Sequencing Quality and Cellular Metrics

High-quality single-cell libraries were obtained from 72 of 75 intended samples, representing a 96% success rate. The median number of cells captured per sample was 4,280 (IQR 3,670–5,120) with an average 1,850 genes and 28,900 UMIs per cell. The tumor cell fraction averaged 54% (IQR 41–66), and the doublet rate remained low at 3.2%. Overall sequencing depth and cell yield were consistent across all time-points, confirming technical reproducibility *(Table 2)*.

Table 2. Sequencing Quality and Cellular Metrics

Variable	Median (IQR)	Range
Cells captured per sample	4,280 (3,670–5,120)	2,950–5,800
UMIs per cell	28,900 (24,200–33,800)	19,600–38,700
Genes detected per cell	1,850 (1,620–2,040)	1,300–2,400
Tumor cell fraction (%)	54 (41–66)	30–79
Doublet rate (%)	3.2 (2.6–3.9)	2.0–5.0
Libraries passing QC	72 / 75 (96%)	—

Clonal Architecture and Evolutionary Dynamics

Baseline analysis revealed a median of three distinct malignant clones per patient (IQR 2–4). A dominant clone (≥50% of malignant cells) was detected in 68% of patients at baseline, declining to 44% on therapy and 40% at progression. Significant clonal shifts (≥20% change in clonal fraction) occurred in 72% of patients during therapy.

The Shannon diversity index (H) increased from 0.84 on therapy to 1.07 at progression, indicating diversification of resistant subclones, whereas partial responders exhibited a decline (ΔH = −0.31). In contrast, patients with disease progression demonstrated an increase in clonal diversity (ΔH = +0.18; $p = 0.002$). Emergent resistance subclones were identified in 50% of progressive disease cases, mainly harboring alterations in *KRAS*, *TP53*, or *CDKN2A/B (Table 3)*.

Table 3. Clonal Architecture and Evolution Dynamics

Parameter	**Baseline (n = 25)**	**On-therapy**	**Progression / Best Response**
Median no. of clones per patient	3 (2–4)	—	—
Dominant clone ≥50%	17 (68%)	11 (44%)	10 (40%)
Patients with ≥20% clonal shift	—	18 (72%)	—
Mean Shannon diversity index (H)	0.92 (0.64–1.15)	0.84	1.07
ΔH PR vs PD	PR: −0.31 (↓)	PD: +0.18 (↑)	*p = 0.002*
Emergent resistance subclone	—	—	4/8 (50%) in PD

Molecular and Microenvironmental Remodeling

Functional pathway analysis revealed divergent transcriptional programs between responders and non-responders. In partial responders, MYC and mTOR signaling were significantly down-regulated (89%, $p < 0.001$), corresponding with contraction of dominant clones (89%). In contrast, epithelial–mesenchymal transition (EMT) and

oxidative phosphorylation pathways were upregulated in 88% of progressive disease cases ($p = 0.002$).

Immune profiling demonstrated that $CD8^+$ T-cell exhaustion scores increased markedly in progression (+0.55 SD, $p = 0.006$), whereas responders exhibited relative preservation of progenitor-like $TCF7^+$ subsets. Drug-tolerant persister (DTP)-like states were identified in 56% of responders and 75% of progressors (ns, $p = 0.21$).

Among 19 patients with matched cfDNA, clonal concordance between tissue and plasma sequencing exceeded 80%, and in 55% of progressors, cfDNA detected expansion of resistance subclones approximately three weeks before radiological progression *(Table 4)*.

Table 4. Molecular and Microenvironmental Changes During Therapy

Feature	**Partial Response (n = 9)**	**Progressive Disease (n = 8)**	***p-value***
Clonal contraction (dominant clone ↓)	8 (89%)	1 (12%)	0.001
EMT/oxidative phosphorylation upregulation	1 (11%)	7 (88%)	0.002
MYC/mTOR signaling down-modulation	8 (89%)	0	<0.001
Increase in $CD8^+$ exhaustion score	+0.12 SD	+0.55 SD	0.006
DTP-like (drug-tolerant persister) program	5 (56%)	6 (75%)	0.21
Concordant cfDNA clone tracking	15/19 (79%)	16/19 (84%)	—
Early cfDNA detection before clinical PD	—	6/11 (55%)	—

Discussion

The present study demonstrates that single-cell sequencing can effectively delineate tumor heterogeneity and trace clonal evolution during systemic therapy in patients with advanced solid malignancies. Conducted within an Indian tertiary-care setting, this investigation contributes valuable real-world evidence complementing the global literature on precision oncology [6].

Our analysis revealed that most tumors comprised multiple subclones at baseline, aligning with prior findings that underscore the intrinsic complexity of clonal hierarchies within solid cancers [6,7]. The detection of dynamic clonal shifts in 72% of patients emphasizes the remarkable adaptability of tumor architecture under therapeutic pressure. Similar to the observations of Vázquez-García et al. (2022), resistant subclones often pre-exist at low frequencies and expand following exposure to selective stress, reflecting the evolutionary plasticity characteristic of malignant disease [7].

The observed reduction in clonal diversity among responders ($\Delta H = -0.31$) contrasted sharply with the diversification seen in progressive disease ($\Delta H = +0.18$; $p = 0.002$), reinforcing the concept that therapeutic efficacy correlates with clonal contraction, while resistance is driven by subclonal diversification [6,8]. Consistent with previous genomic studies, resistant subclones in our cohort predominantly exhibited KRAS, TP53, and CDKN2A/B alterations—well-recognized mediators of therapy escape [7,9].

At the transcriptomic level, disease progression was characterized by upregulation of epithelial–mesenchymal transition (EMT) and oxidative phosphorylation pathways, suggesting metabolic and phenotypic plasticity in resistant cells. Such adaptive transcriptional reprogramming parallels prior studies in lung and breast cancers, where EMT activation and metabolic rewiring were linked to therapeutic tolerance [8,11]. Conversely, responders exhibited marked down-regulation of MYC and mTOR signaling, indicating effective suppression of proliferative and translational programs—molecular signatures often associated with favorable therapeutic response [11].

The evolving immune microenvironment also mirrored these molecular transitions. A significant rise in $CD8^+$ T-cell exhaustion markers and the depletion of $TCF7^+$ progenitor subsets among non-responders highlight therapy-induced immune dysfunction. This observation aligns with the view that tumor evolution and immune escape proceed in tandem, reinforcing the co-evolutionary nature of cancer and host immunity [6,8].

Importantly, cfDNA analysis closely paralleled tissue-derived clonal trajectories, detecting emergent resistant clones approximately three weeks prior to clinical or radiological progression. This lead time underscores the potential of cfDNA as a non-invasive, real-time biomarker for early relapse detection [10]. Such molecular surveillance, as emphasized in prior translational studies, can significantly enhance adaptive treatment strategies and clinical monitoring precision [10,12].

Limitations

The study was limited by its modest sample size and short follow-up period, inherent to a three-month observational design. Tissue availability and sequencing costs constrained broader multi-omics integration. Nevertheless, the high-quality longitudinal data provide valuable insights into clonal behavior under therapy.

Conclusion

This prospective study from Government Medical College, Srikakulam, demonstrates that single-cell sequencing enables precise visualization of tumor evolution during therapy, capturing both genetic and transcriptional adaptations underlying therapeutic response and resistance. Responders exhibited marked clonal contraction and suppression of MYC/mTOR signaling, whereas progressors showed diversification of resistant subclones with EMT and metabolic activation. Concurrent cfDNA monitoring closely mirrored intratumoral dynamics, detecting molecular progression several weeks before clinical relapse. These findings highlight the translational potential of longitudinal single-cell and cfDNA profiling as complementary tools for early detection of resistance, real-time treatment adaptation, and advancement of personalized oncology in heterogeneous solid tumors.

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