

Longitudinal spatial multi-omics delineates tumor microenvironment remodeling across sequential EGFR-TKIs in EGFR-mutant NSCLC

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Abstract

Background

Resistance to therapy is a frequent occurrence in patients with epidermal growth factor receptor (EGFR)-mutant non-small-cell lung cancer (NSCLC) who are treated with EGFR tyrosine kinase inhibitors (EGFR-TKIs). However, spatial information on how the tumor microenvironment (TME) changes within the same patient from baseline to resistance to first- and then third-generation EGFR-TKIs is scarce. Here, we used rare consecutive re-biopsies to build a longitudinal, compartment-resolved spatial atlas that captures within-patient TME remodeling across sequential EGFR-TKIs.

Methods

A patient-matched biopsy cohort with serial samples obtained at baseline (T0), after resistance to first-generation EGFR-TKI (T1), and after resistance to third-generation EGFR-TKI (T2) was created. Using GeoMx Digital Spatial Profiling (DSP), tumor-enriched and stroma-enriched areas of interest (AOIs) were segmented, and paired RNA and protein profiles were quantified. We tracked temporal changes in compartment-specific heterogeneity and immune remodeling, and explored associations between early remodeling and subsequent T790M acquisition.

Results

We analyzed the data from 15 samples of 6 patients. Tumor- and stroma-enriched compartments were not only transcriptionally but also protein-wise consistently distinct. Spatial heterogeneity was almost entirely stable from T0 to T1 and increased substantially from T1 to T2, with changes in the stroma-enriched compartments accounting for most of the increase. Early remodeling was characterized by loss of T cell activation programs, reduced neutrophil signatures, increased myeloid remodeling, and impairment of antigen presentation. Longitudinal analyses pointed to a biphasic immune trajectory, with early myeloid remodeling and late stromal checkpoint reprogramming. Importantly, early spatial remodeling patterns were different in samples, which later acquired T790M.

Conclusions

This study shows how the TME changes dynamically during sequential EGFR-TKI therapy in EGFR-mutant NSCLC. It reveals a late, stroma-driven expansion of spatial heterogeneity and stage-dependent immune remodeling, and provides a longitudinal reference that supports testable, time-aware hypotheses for future validation and functional studies.

Background

Treatment of epidermal growth factor receptor (EGFR)-mutated non-small-cell lung cancer (NSCLC) has advanced rapidly with successive generations of EGFR tyrosine kinase inhibitors (TKIs) improving outcomes.^[1–3] Yet acquired resistance remains nearly inevitable, and despite substantial progress in characterizing resistance, its dynamic evolution and determinants are still poorly defined.^[4, 5]

An intractable clinical problem is pronounced inter-patient heterogeneity: progression tempo, lesion behavior, and subsequent therapeutic benefit vary widely across individuals, implying that resistance is often a trajectory rather than a single endpoint.^[6–8] Cross-sectional sampling across different patients inevitably conflates baseline differences with treatment-associated remodeling, obscuring true time-dependent trends.^[9, 10] Collecting longitudinal, within-patient samples is the best way to address this confounding, but is inherently hard to execute in practice because sequential EGFR-TKI courses span long intervals, necessitating prolonged follow-up and multiple biopsy windows under real-world constraints.

In parallel, some studies have highlighted that the TME in EGFR-mutant NSCLC is spatially heterogeneous, with compartment-specific cellular and molecular programs that are not captured by bulk profiling.^[11, 12] Spatially resolved platforms enable high-plex RNA and protein quantification in morphologically defined compartments from FFPE biopsies, providing a pragmatic approach to interrogate tumor–stroma ecosystems in situ.^[13] Emerging Digital Spatial Profiling (DSP) studies further suggest that EGFR-TKI exposure can drive compartment-specific remodeling, the full spatial evidence remains cross-sectional or limited to two time points; thus, the full sequential trajectory remains unclear.^[14] To address this gap, we made a rare, patient-matched three-time-point biopsy series and applied compartment-resolved DSP profiling to explore the evolution of tumor–stroma ecosystem under EGFR-TKI therapy.

Methods

Patient and sample collection

We retrospectively screened patients who were initially diagnosed with EGFR-mutant NSCLC at Shanghai Pulmonary Hospital from 2015 and had longitudinal follow-up documenting subsequent systemic therapies. Patients were eligible if they had archived lung biopsy tissues at three matched clinical time points obtained at our institution: baseline biopsy at initial diagnosis, re-biopsy at acquired resistance to first-generation EGFR-TKIs, and re-biopsy again at acquired resistance to third-generation EGFR-TKIs. All three biopsies were obtained from the lung and had sufficient material for spatial profiling.

Using these criteria, three patients met the requirements. All three patients developed the EGFR T790M mutation after first-generation EGFR-TKI resistance, received third-generation EGFR-TKI therapy, and eventually developed resistance, enabling collection of the third time-point biopsy. To explore stratification by T790M acquisition, we additionally included three T790M-negative patients who had paired baseline and post–first-generation EGFR-TKI resistance lung biopsies, but they did not proceed to

third-generation EGFR-TKI treatment, therefore, only two time points were available for these patients. In total, 15 lung biopsy specimens from 6 patients were included for subsequent DSP protein and RNA analyses.

Sample preparation for Nanostring GeoMx DSP protein assay

The formalin-fixed paraffin-embedded (FFPE) slides were placed in a 60°C drying oven and baked for 1.5 h. Then placed the slide in a rack and performed the following wash steps to deparaffinize and rehydrate the slides: 3 washes (5 min each) in CitriSolv, 2 washes (5 min each) in 100% ethanol, 2 washes (5 min each) in 95% ethanol, and 2 washes (5 min each) in double-distilled water. For antigen retrieval, the slides in a staining jar with 1 × freshly prepared Citrate Buffer pH 6 at room temperature. The staining jar was placed into a preheated pressure cooker and run on high pressure and temperature for 15 min. When the timer reached zero, released the pressure. The staining jar was transferred to a lab bench at room temperature. The lid of the staining jar was removed, and the slides were allowed to stand for 25 min. 1 × Tris-buffered saline with Tween-20 (TBS-T) was used to wash the slides for 5 min. After antigen retrieval, the slides were placed into a humidity chamber and incubated with Buffer W (NanoString) for 1 h at 25°C to perform the blocking. Next, the slides were stained with morphological markers CD45 and panCK as well as the mixture of UV-photocleavable oligo antibody sets (Immune Cell Typing Module, IO Drug Target Module, Immune Cell Profiling Core, and Pan-Tumor Module) (NanoString, 121300301) in the humidity chamber at 4°C and incubated overnight. The slides were then washed 3 times (10 min each) in TBS-T, covered with 4% paraformaldehyde and incubated for 30 min in a humidity chamber at 25°C. When the incubation completed, the slides were washed for 2 times (5 min each) in TBS-T. Then the slides were stained with SYTO 13 (NanoString, 121300301) at 25°C for 15 min and rinsed with 1 × TBS-T. After staining, the slides were loaded onto the GeoMx instrument.

Sample preparation for Nanostring GeoMx DSP RNA assay

FFPE slides (4 µm) were placed in a 60°C drying oven and baked for 1.5 h. Then placed the slides in a rack and underwent the following washes to deparaffinize and rehydrate the slides: 3 washes (5 min each) in CitriSolv, 2 washes (5 min each) in 100% ethanol, 1 wash (5 min) in 95% ethanol, and 1 wash (5 min) in phosphate-buffered saline (PBS). To perform target retrieval, the slides were placed in diethylpyrocarbonate-treated water for 10 s to bring the slides temperature up to 99°C. Then the slides were transferred to the preheated 1 × Tris-Ethylene Diamine Tetraacetic Acid with a temperature of 99°C. The slides were incubated for 20 min. When the retrieval time was up, the slides were immediately moved to 1 × PBS with a temperature of 25°C and washed the slides in 1 × PBS for 5 min. For RNA target exposure, the slides were incubated in proteinase K solution with a concentration of 1 µg/mL at 37°C for 15 min and washed in 1 × PBS for 5 min.

When RNA target exposure was completed, the slides were postfixed as follows: 1 wash (5 min) in 10% neutral buffered formalin (NBF), 2 washes (5 min each) in NBF Stop Buffer, and 1 wash (5 min) in 1 ×

PBS. The slides were removed from 1 × PBS and set in hybridization chamber to perform in situ hybridization. The hybridization solution was added into UV-photocleavable oligo probes (Cancer Transcriptome Atlas, CTA) to the slide to detect the target RNA. The hybridization chamber was closed and inserted into the hybridization oven. Then, the slides were incubated at 37°C overnight. After incubation, the slides were dipped in 2 × Saline Sodium Citrate (SSC) buffer and performed stringent washes to remove off-target probes as follows: 2 washes (25 min each) in Stringent Wash at 37°C, 2 washes (2 min each) in 2 × SSC. Then, buffer W was used to cover the tissue at room temperature for 30 min which was protected from light. After removing the Buffer W, the slides were stained with morphological markers CD45 and panCK as well as nuclear stain SYTO 13 (NanoString, 121300310) for 1 h in a humidity chamber at room temperature. After staining, the solution was removed and the slides were washed in 2 × SSC, 2 times for 5 min each. Finally, the slides loaded onto the GeoMx DSP instrument.

Digital spatial profiling

Prepared slides were scanned to capture fluorescent images. The region of interest (ROI) selection was independently determined by two participating authors and a pathologist based on immunofluorescence images. For each specimen, 1–10 ROIs were selected. A sequential mask strategy was used to divide the ROI into stromal compartment-specific areas of interest (AOIs) (panCK-negative staining) and tumor compartment-specific AOIs (panCK-positive staining). AOIs were treated as repeated measurements within each specimen; primary inference was performed at the specimen level using aggregation to avoid pseudo-replication. After segmentation, the GeoMx DSP instrument (NanoString) was used to cut and collect the spatially indexed barcode. To quantify protein expression, the collection plate was removed from the instrument. In the collection plate, the aspirate containing the barcode was dried and then rehydrated. For hybridization, the samples were transferred to a new plate and combined with GeoMx Hyb Code Pack reagents. The hybridization reaction took place overnight. After hybridization, the products were pooled into the strip tube. Then the strip tubes were loaded on the nCounter Analysis System for counting the probes collected with GeoMx DSP. When counting was completed, the reporter code count (RCC) files were generated with the nCounter system and uploaded onto the GeoMx DSP system.

In the GeoMx DSP system, the oligonucleotide counts with spatial data were integrated and proceeded to data analysis. To quantify gene expression, the collection plate was removed from the instrument and was dried down by incubating on a thermocycler at 65°C for 1 hour. Adding nuclease-free water to rehydrate the sample. The PCR setup was performed as follows: aliquoted 5 × polymerase chain reaction (PCR) Master Mix to each well of a new PCR plate; transferred Primer Mix from each well of the Seq Code Primer Plate to the corresponding well of the PCR plate; transferred DSP aspirate from each well of the DSP collection plate to the corresponding well of the PCR plate. When the PCR was completed, the PCR products were pooled and purified. After purification, the library quality and quantity were assessed. Next, the sequencing was performed on an Illumina NextSeq 550AR instrument. After the sequencing,

the FASTQ files were generated. Then the FASTQ files were converted to digital count conversion (DCC) files in the NanoString GeoMx NGS Pipeline. In the GeoMx DSP system, selecting and uploading the DCC files. Finally, the oligonucleotide counts with spatial data were integrated and proceeded to data analysis.

Spatial proteomic data analysis

Uploading the RCC files onto the GeoMx DSP system. In the GeoMx DSP Control Center, the Data Analysis module (Version 2.4.0.421) was used to perform quality control (QC) checks and data analysis. There were three steps in the QC process for the nCounter readout: the percentage of fields of view (FOVs), binding density, minimum nuclei and surface area count. At least 75% of FOVs should be successfully counted to obtain robust data. The binding density was a measurement of the concentration of barcodes detected by the nCounter instrument. AOIs were retained when the binding density ranged from 0.1 to 2.25. For each AOI, the cut-off value for the number of nuclei and the surface area were 20 and 1600 square microns, respectively. In order to avoid the variations in the different ROIs, the sizes of these ROIs were adjusted through two steps: area normalization and different cell count. Furthermore, the background of immunoglobulin G was used to normalize all targets that met QC across samples. The normalized counts were used to compare the differences between different groups.

Spatial transcriptomic data analysis

The generated FASTQ files were converted to DCC files with the GeoMx NGS Pipeline Software (version 2.2.0.2). The DCC files were then uploaded onto the GeoMx DSP system. In the GeoMx DSP Control Center, the Data Analysis Module (Version 2.4.0.421) was used to perform QC checks and data analysis. There were four steps in the QC process for the transcriptome data: technical signal QC, technical background QC, probe QC, and normalization QC. Technical signal QC assesses the quality of sequencing for each segment. When the percentage of the raw reads that align to a target sequence was less than 80%, the corresponding ROI was removed. Technical background QC was a GeoMx DSP run control. There were three indicators for technical background QC control: negative probe count, no template control (NCT) count, minimum nuclei and surface area count. The negative probe count establishes the level of technical noise expected and flags segments with signal below that level. The cut-off value for the negative probe count was 4 counts. The NTC count establishes the level at which counts in the NTC would be flagged. This QC was used to detect contamination in the library preparation. ROIs would be removed when the NTC count was more than 1000. The parameters for the minimum nuclei and surface area count for the ROI were 100 and 8000 square microns, respectively. In order to avoid variations in the different ROIs, the sizes of these ROIs were adjusted through two steps: area normalization and different cell counts. Furthermore, the Quantile 3 (Q3) normalization was used to normalize all targets that met QC across samples.^[15]

The gene expression matrix was used to compare the differences between different groups and calculate the different signature scores with single sample gene set enrichment analysis (ssGSEA), including immune response, cell function, signaling pathways, and metabolism.^[16] The edgeR (3.34.0) package was used to analyze the differences between different groups, and the significant difference was determined with a False Discovery Rate (FDR) value < 0.05, and the absolute value of log2 (Fold Change) > 1.^[17] KOBAS-i web tool was used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment with a *p*-value < 0.05.^[18]

Statistical analysis

The R (version 4.1.0) package of ComplexHeatmap (version 2.8.0) was used to draw the heatmaps.^[19] Umap (version 0.2.8.0) was used to perform the dimension reduction analysis. The difference between the two groups was compared by Wilcoxon test. Pearson's correlation analysis was used to conduct the correlation analysis. For high-dimensional comparisons, multiple testing was controlled using the Benjamini–Hochberg FDR. For analyses involving multiple AOIs per specimen, results were summarized at the specimen/patient level where appropriate to avoid inflation from within-patient correlation; two-sided *P* < 0.05 was considered statistically significant unless otherwise stated.

Results

Establishment of a spatially resolved, patient-matched longitudinal cohort to track microenvironment remodeling during EGFR-TKI therapy

We constructed a patient-matched longitudinal cohort specifically designed along the therapeutic timeline and key molecular evolutionary landmarks (Fig. 1A). All enrolled patients had lung adenocarcinoma harboring classical sensitizing EGFR mutations (L858R, exon 19 deletion, or G719X) and received first-generation EGFR-TKIs (Gefitinib or Icotinib) as first-line treatment. Upon disease progression, patients were stratified according to the acquisition of the T790M mutation, T790M-positive patients subsequently received the third-generation EGFR-TKI (Osimertinib). Tumor samples were categorized into three clinically defined time points: baseline before treatment (T0), post–first-generation EGFR-TKI at first progression (T1), and post–third-generation EGFR-TKI at second progression (T2). Importantly, longitudinal sampling was performed on lung tissues from the same individuals across time points, enabling within-patient comparisons under a consistent host background.

To preserve tissue architecture while quantifying immune and tumor programs, we performed GeoMx DSP for both proteins and transcripts at each time point (Fig. 1B). Each section was segmented into tumor-enriched and stroma-enriched AOIs (Fig. 1C) for compartment-resolved analyses. In total, we profiled 60 tumor and 43 stromal AOIs for proteins, 60 tumor and 46 stromal AOIs for RNA (Fig. 1D). In addition, QC showed comparable AOI area, cellularity, detection metrics, and background across

samples (Fig. S1–S2), supporting consistent segmentation and assay performance across the longitudinal dataset.

Longitudinal spatial dynamics reveal a late, stroma-driven increase in heterogeneity during EGFR-TKI treatment

To place subsequent analyses in a global spatial context, we first quantified compartment structure and spatial similarity across the longitudinal series. The same AOIs were profiled at both the protein and RNA levels (Fig. 2A). In UMAP, tumor- and stromal AOIs formed two well-separated clusters in the protein data (Fig. 2B), and the same separation was seen in the RNA data (Fig. 2C), showing that tumor and stroma remained distinct in both readouts.

Next, protein and RNA-based correlations across different spatial distances were further compared. At the protein level, within-compartment correlations (tumor–tumor and stroma–stroma) were higher than cross-compartment correlations (tumor–stroma; $p < 0.001$) (Fig. 2D). Cross-compartment similarity also showed a clear gradient: tumor–stroma correlations within the same ROI were higher than tumor–stroma correlations across different ROIs (Fig. 2D), consistent with visual inspection of the tissue images (Fig. S3A), indicating shared local programs at short distances despite overall compartment separation.

On this basis, we then explored whether spatial heterogeneity changed with treatment stage. Global expression patterns became more dispersed at T2 in both protein and RNA spaces (Fig. 2F and Fig. S3C). Statistical analysis found that heterogeneity did not increase from T0 to T1 ($p = 0.5$) but rose significantly from T1 to T2 ($p = 0.021$) (Fig. 2G), which indicates that the pronounced increase in spatial complexity may not occur during the early period, but is more concentrated in later stages under sustained accumulation of therapeutic pressure. When assessed by compartment, this increase was confined to stromal AOIs (T1 to T2, $p = 0.008$) (Fig. S4A), with no significant change in tumor AOIs ($p = 0.5$) (Fig. S4B). Consistent with these results, stromal AOIs were more similar to each other than tumor AOIs in the baseline correlation analysis ($p < 0.0001$) (Fig. S3D), and this relative stromal uniformity was progressively lost at later stage.

Compartment identity was supported by expected molecular features. Tumor

AOIs showed higher epithelial markers, whereas stromal AOIs were enriched for extracellular matrix and immune-related molecules (Fig. S4C), with concordant trends at the RNA level (Fig. S4D). Pathway enrichment further showed that tumor regions were more biased toward tumor signaling and resistance-associated modules, whereas stromal regions were significantly enriched for immune–stromal response networks such as chemokine signaling, complement and coagulation cascades, and cytokine–cytokine receptor interactions (Fig. S4E). Together, these data indicate that the increase in spatial complexity emerges late during sequential EGFR-TKI therapy and is driven primarily by stromal remodeling.

Early EGFR-TKI exposure induces stromal immune reprogramming and tumor intrinsic adaptation

To identify early events that precede the later rise in heterogeneity, we compared baseline (T0) with post–first-generation TKI resistance (T1). In DSP protein data, T1 showed lower T-cell activation and checkpoint markers [tumor necrosis factor receptor superfamily member 9 (TNFRSF9/4-1BB), programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte–associated protein 4 (CTLA4), V-domain Ig suppressor of T cell activation (VISTA)], reduced CD66b, increased CD163, and decreased β 2-microglobulin (B2M) with a lower major histocompatibility complex class II (MHC-II) antigen presentation score (Fig. 3A–B, Fig. S5A), indicating a shift toward myeloid remodeling and impaired antigen presentation. To clarify the spatial origin of these immune signals, we further found that PD-1 and VISTA declined significantly in stromal areas, whereas no significant changes were observed in tumor areas (Fig. S5B), indicating that early immune remodeling may primarily be stromal driven.

Tumor-side transcriptomic signatures at T1 pointed to a stress-and-repair response, with higher cell cycle ($p < 0.001$), nucleotide synthesis ($p = 0.022$), and DNA damage repair scores ($p < 0.001$), no overall increase in energy modules, and decreased lipid metabolism ($p = 0.045$) (Fig. 3C). In parallel, pathways linked to outward signaling and microenvironment communication were dampened, including vascular endothelial growth factor (VEGF) ($p = 0.010$), platelet-derived growth factor (PDGF) ($p = 0.028$), and G protein–coupled receptor (GPCR) signaling ($p = 0.030$) (Fig. 3D). Among major bypass programs, only Notch signaling rose selectively ($p = 0.017$) (Fig. 3E). Therefore, first-generation EGFR-TKI exposure might already be associated with measurable stromal immune changes and tumor intrinsic adaptation, providing an early background for later spatial evolution.

Longitudinal profiling reveals a biphasic shift from early innate remodeling to late stromal checkpoint reprogramming

To test whether the early changes observed after first-generation EGFR-TKI evolve into a more stable state under continued selective pressure, we extended the analysis to the post–third-generation EGFR-TKI resistance (T2). The abundances of immune cells showed clear stage-dependent shifts, with greater overall variability at T2 (Fig. 4A). From T0 to T1, the dominant change involved innate immunity. Neutrophil abundance decreased sharply ($p < 0.001$) and did not decline further at T2 (Fig. 4B). In contrast, fibroblasts and NK cells increased from T1 to T2 ($p = 0.004$ and $p = 0.001$), consistent with late stromal expansion and reconfiguration of innate components (Fig. 4B). Notably, the overall cytotoxicity score did not increase, suggesting a dissociation between cell composition and functional state at late stage.

Protein-level longitudinal comparisons further indicated a directional switch in immune regulation. Most T cell and checkpoint markers declined from T0 to T1, whereas several exhaustion-associated axes rebounded at T2 (Fig. 4C). CTLA4 and VISTA increased after an initial decline, and Tim-3 also rose

significantly from T1 to T2 ($p = 0.02$) (Fig. 4D). This down-then-up trajectory suggests that T1 represents a transitional reset, followed by renewed checkpoint remodeling under sustained treatment pressure, in line with the more complex immune landscape at T2.

Transcriptomic signatures supported this staged remodeling at the functional program level. Nucleotide synthesis was already increased from T0 to T1 ($p = 0.022$) (Fig. S5C), while GPCR signaling decreased from T0 to T1 ($p = 0.029$) and remained low thereafter (Fig. 4E). At T2, tumor state modules shifted further. Differentiation signatures decreased from T1 to T2 ($p = 0.006$) (Fig. 4E), mitochondrial metabolism and tricarboxylic acid cycle (TCA) also declined ($p = 0.026$) (Fig. S5C). In contrast, canonical bypass and growth pathways showed no consistent cohort-level increase (Fig. S5D), supporting a late-stage phenotype dominated by reorganization of the microenvironment and immune regulatory networks rather than broad activation of classic bypass programs.

T790M stratification reveals distinct early remodeling trajectories

To assess whether early remodeling already diverges into distinct evolutionary directions, we stratified patients by T790M acquisition, a hallmark mechanism after first-generation EGFR-TKI resistance. From T0 to T1, T790M-positive patients showed a predominant downward shift in immune regulation and antigen presentation signals, because CD66b, CTLA4, VISTA, and B2M all trended lower (Fig. 5A). In contrast, T790M-negative patients showed a more prominent increase in effector and tissue-response signals, including Granzyme B (GZMB) and fibronectin (Fig. 5A). These results suggest that early stage remodeling is not uniform across patients, but differs by resistance background.

Cell abundance estimates and functional scores aligned with these patterns. In the T790M-positive group, the most pronounced change from T0 to T1 was a sharp decline in neutrophil abundance ($p < 0.001$), consistent with reduced CD66b. In the T790M-negative group, the dominant change was an increase in the cytotoxicity signature ($p = 0.0051$) (Fig. 5B–C). Along antigen presentation and immune regulation axes, B2M decreased with treatment in both groups, suggesting a shared impairment in a key antigen presentation component. However, the more specific MHC class II antigen presentation module showed stronger reduction in the T790M-negative group ($p < 0.001$), with no clear change in the T790M-positive group (Fig. 5D). Checkpoint proteins like CTLA4 and VISTA decreased significantly in T790M-positive cases ($p = 0.00034$ and $p = 0.01$) (Fig. S5E), whereas they remained largely stable in T790M-negative cases (Fig. S5F).

We next compared the two T790M strata at the group level, independent of treatment time. Differential features showed consistent between-group shifts in representative immune and stromal molecules (Fig. 6A). The T790M-negative group had higher cytotoxicity scores ($p = 0.0014$), higher estimated fibroblast ($p = 0.012$) and neutrophil ($p = 0.027$) abundance (Fig. 6B). In contrast, antigen presentation modules were higher overall in the T790M-positive group (Fig. 6C). Compartment-resolved comparisons of checkpoint expression indicated that most checkpoints were higher in the T790M-negative group (Fig. 6D). At the program level, metabolic modules particularly mitochondrial metabolism/TCA and fatty

acid oxidation—differed between groups (Fig. 6E), whereas migration/stromal and growth factor–related modules showed weaker separation; among canonical bypass pathways, only Wnt signaling differed significantly ($p = 0.042$) (Fig. 6F–G). Together, T790M stratification revealed broad, coordinated differences in immune–stromal state rather than isolated marker changes, providing group-level evidence that early remodeling trajectories vary with resistance context.

Discussion

In EGFR-mutated NSCLC patients, the resistance to EGFR-TKI does not seem to be a one-time point event, but rather an adaptive process under the treatment pressure.^[20] Identifying when this process begins to shift organizationally and distinguishing between changes that are treatment-induced rather than patient-specific in a real disease course is a significant challenge in clinical practice. Most of the previous evidence of EGFR-TKI resistance comes from cross-sectional comparisons of different patients or re-biopsy at a single point of progression.^[21, 22] These studies have revealed different kinds of resistance mechanisms, but they are also inevitably affected by the effects of inter-patient differences, sampling site, and intratumoral heterogeneity, which makes it difficult to capture the longitudinal dynamic changes of patients from baseline to the final resistance. At the same time, spatial omics studies further suggest that the spatial structure of the TME has significant regional characteristics, and many key changes are not globally consistent, but are limited to specific compartments.^[23, 24] If we ignore compartment discrimination, bulk sequencing can easily dilute or even mask these important local signals. Therefore, in our study, we integrated spatial transcriptomics and proteomics to construct a longitudinal cohort spanning the entire clinical process from the baseline to the acquisition of T790M-mediated resistance after first-generation EGFR-TKI treatment to re-resistance after third-generation EGFR-TKI treatment. Within a unified spatio-temporal framework, we systematically explored the dynamic trajectory of TME under therapeutic pressure. Our study suggests that early adaptive reprogramming lays the foundation for the subsequent expansion of complex spatial heterogeneity, and there is a clear co-evolution relationship between the intrinsic genotype evolution of tumors and the phenotypic remodeling of the microenvironment, which jointly drives the formation of end-point drug resistance environment.

In our study, the tumor and stromal regions maintain a clear separation at the protein and RNA levels, suggesting that compartmental differences are stable, and therefore longitudinal changes should be explained in the framework of compartmentalization. In the dynamic observation of evolution under EGFR-TKI treatment, bulk sequencing may easily overlap compartmental differences with temporal changes, thereby diluting or even masking the true signals.^[25] Many previous spatial studies suggested that TME is not a homogeneous mixture, but is full of different cellular compositions, signaling networks, and immune-related pathways.^[13, 23, 24, 26] For example, in a spatial single-cell map of lung adenocarcinoma, different immune cell activation states show reproducible spatial organization and relate to clinical outcomes, so the spatial information itself is part of the biological characteristics.^[27] Similarly, another study about cancer-associated fibroblasts (CAFs) has shown that such effects are

often no longer significant after the overall average is taken.^[28] Therefore, to explore dynamic tracking, spatial information is a point that cannot be ignored, which is also a necessary prerequisite for our subsequent time comparison.

Next, we found that spatial heterogeneity increased significantly after the third-generation EGFR-TKI treatment, and the heterogeneity mainly originated from the stromal region. A more reliable explanation for this phenomenon is that under the early treatment pressure, the overall TME may still be in a relatively consistent adaptive situation, showing a more consistent direction of change. However, under longer time and more complex drug selection pressures, the stromal region is more prone to form many different niches, which drives the spatial microenvironment to become more complex. This is also consistent with recent evidence of resistance microenvironment: in EGFR-mutated NSCLC, the intrinsic differences of tumor cells together with TGF β -related secretory programs, CAF recruitment, and immunosuppression together lead to a drug-tolerant TME.^[22] Meanwhile, previous clinical trials suggest that the resistance mechanism of Osimertinib are highly diverse, and a number of cases cannot be explained by a single classical bypass pathway, which also provides clinical support for our conclusion.^[21] Therefore, we prefer to view the stroma-driven heterogeneous expansion in the late stage of EGFR-TKI treatment as a framework conclusion that can be further tested by subsequent studies. The key feature of the terminal progression stage may not be the general enhancement of a pathway in the tumor region, but rather a stronger spatial differentiation of the microenvironment.

Previous studies on re-biopsy after EGFR-TKI resistance have shown that targeted therapy can cause changes in PD-L1, tumor-infiltrating lymphocyte (TIL) density, and other immune-related markers, suggesting that the effect of EGFR-TKI is not limited to the intrinsic properties of tumor cells, but will simultaneously reshape the TME.^[29, 30] In addition, existing evidence indicates that patients with EGFR mutations generally have a weak response to immune checkpoint inhibitors (ICIs), and the immunosuppressive microenvironment after EGFR-TKI resistance is considered to be one of the important reasons.^[31] Moreover, single-cell data show that the samples after EGFR resistance indeed exhibit an immunosuppressive microenvironment dominated by tumor-associated macrophages.^[32] In addition, a meta-analysis of the EGFR-mutated subgroup also suggested that the overall survival of ICIs was more limited in the mutant population than in the wild-type ones, and pointed out that the low proportion of PD-L1 expression and CD8 + TIL could partly explain the clinical phenomenon.^[33] The evidence closer to spatial partitioning came from the recent DSP study. Kim et al. used the spatial analysis of samples before and after EGFR-TKI treatment to stress that EGFR-mutated NSCLC has an immunosuppressive TME, and showed that the proportion of immune cells and related pathways changed at the spatial level after treatment.^[12] However, our study provided further evidence that immune-related remodeling is a phased transition during the whole treatment process. The axis of immunosuppression and exhaustion began to appear significantly after the resistance of the third generation EGFR-TKI, and the changes were more concentrated in the stroma, which provides more specific dynamic evidence from the time and space localization.

There has been found that different resistance pathways lead to different immune ecologies, and T790M stratification is the most clinically significant explanation. Haratani et al. performed a comparison by T790M status in NSCLC patients who received Nivolumab after EGFR-TKI progression and found that T790M-negative patients were more likely to benefit from Nivolumab, suggesting that this difference may be related to immune-related factors such as PD-L1.^[34] This also provides reasonable circumstantial evidence for the observation of different directions of microenvironment remodeling in the early window of our study. However, most of the previous studies were retrospective, with non-uniform sampling of individuals and biopsy locations. There may be differentiation trends at an early stage that are accompanied by subsequent molecular outcomes of drug resistance.

Our study still has some limitations. First of all, complete three-stage paired re-biopsy chains are exceptionally rare, and therefore, the cohort is small. This limits statistical power, especially for subgroup comparisons, and the means of the results should be considered as a map of longitudinal changes rather than population-level estimates. Secondly, the study is retrospective and observational. Hence, we can only describe how tumor and stromal programs change over time, but we cannot determine what causes those changes or prove any cause-effect relationships. Thirdly, we have not been able to perform the same analysis in an independent validation cohort due to the extreme difficulty in obtaining comparable longitudinal biopsy chains. However, the consistency between spatial RNA and protein measurements provides confidence in the observations. Nevertheless, independent replication will be required to confirm their wider applicability.

Conclusions

This study outlines a more realistic roadmap for the dynamic evolution of EGFR-mutated NSCLC patients during targeted therapy based on extremely scarce consecutive biopsies from the same patient, combined with spatial multi-omics technology. It was observed that the tumor and the stroma region were continuously clearly separated through mutual verification at the protein and RNA levels, emphasizing the importance of spatial information. The significant increase in spatial heterogeneity mainly occurred in the late stage and was mainly driven by the stroma side, suggesting that the late evolution was more likely to reflect the reorganization and diversification of the TME rather than the synchronous amplification in cancer cells. In addition, immune remodeling was non-linear and stage-dependent, different T790M statuses in the early stage may map onto different spatial ecological trajectories (Fig. 7). Although this study was an observational analysis, the longitudinal spatial evidence provided key clues and testable hypotheses for understanding the evolution under the pressure of EGFR-TKI treatment, and laid the data foundation and conceptual framework for subsequent independent cohort validation.

Declarations

Ethics approval and consent to participate: This study was approved by the Institutional Review Board of Shanghai Pulmonary Hospital (Approval No.L24-404-1). The study was conducted in accordance with

the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from all participants.

Consent for publication: Not applicable.

Availability of data and materials: The processed GeoMx DSP CTA and protein outputs supporting the findings of this study have been deposited in Zenodo (DOI: 10.5281/zenodo.18603267).

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: XS was responsible for designing the experiments, analyzing the data, and writing the paper, under the overall coordination of HL and CZ. PH and XZ assisted with the analysis of DSP data. The interpretation of tissue sections was independently guided by XS, ZL and pathologist ZZ. XS and AX assisted with the collection of clinical samples. All authors reviewed the paper. All authors read and approved the final manuscript.

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Figures

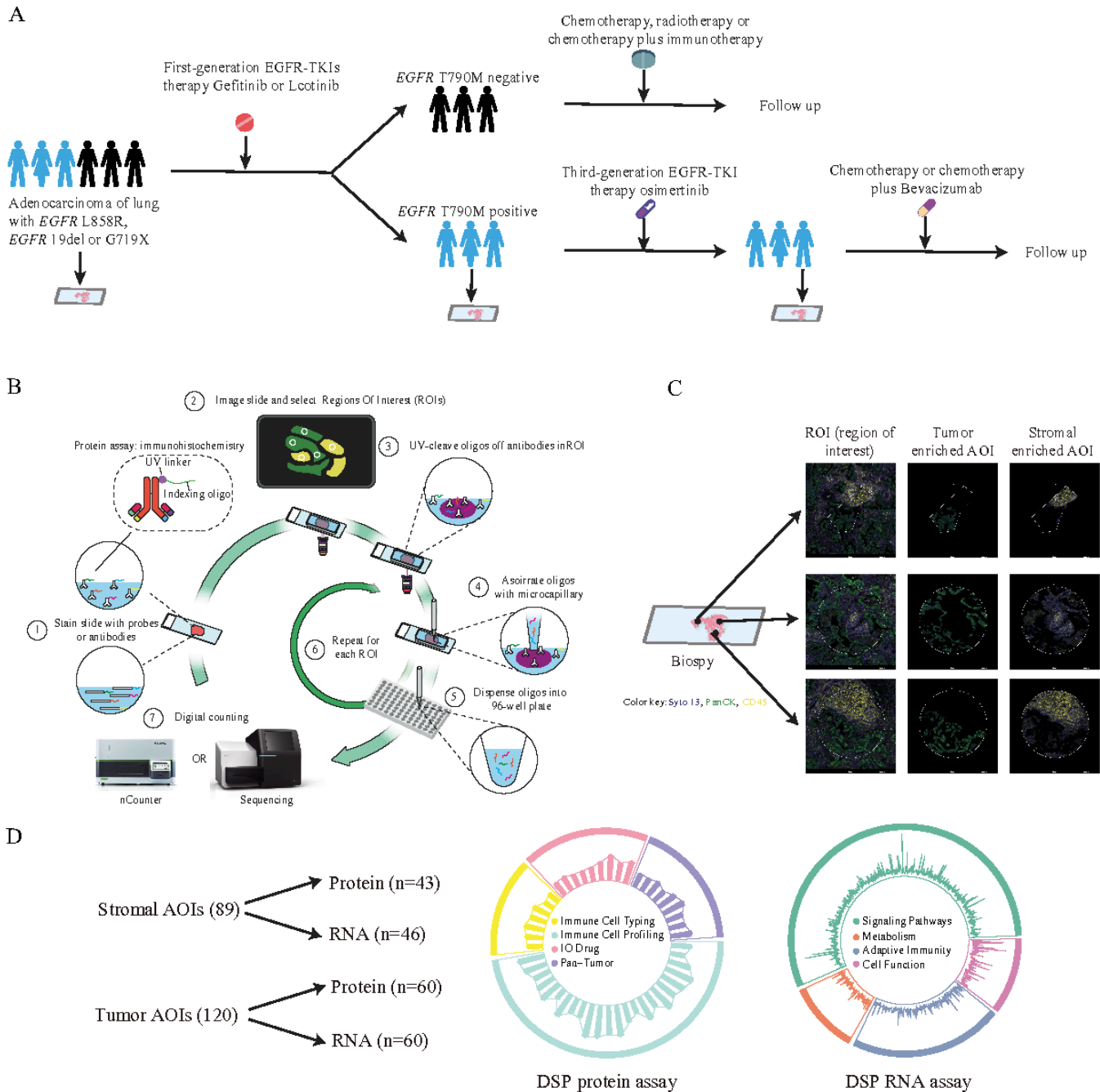
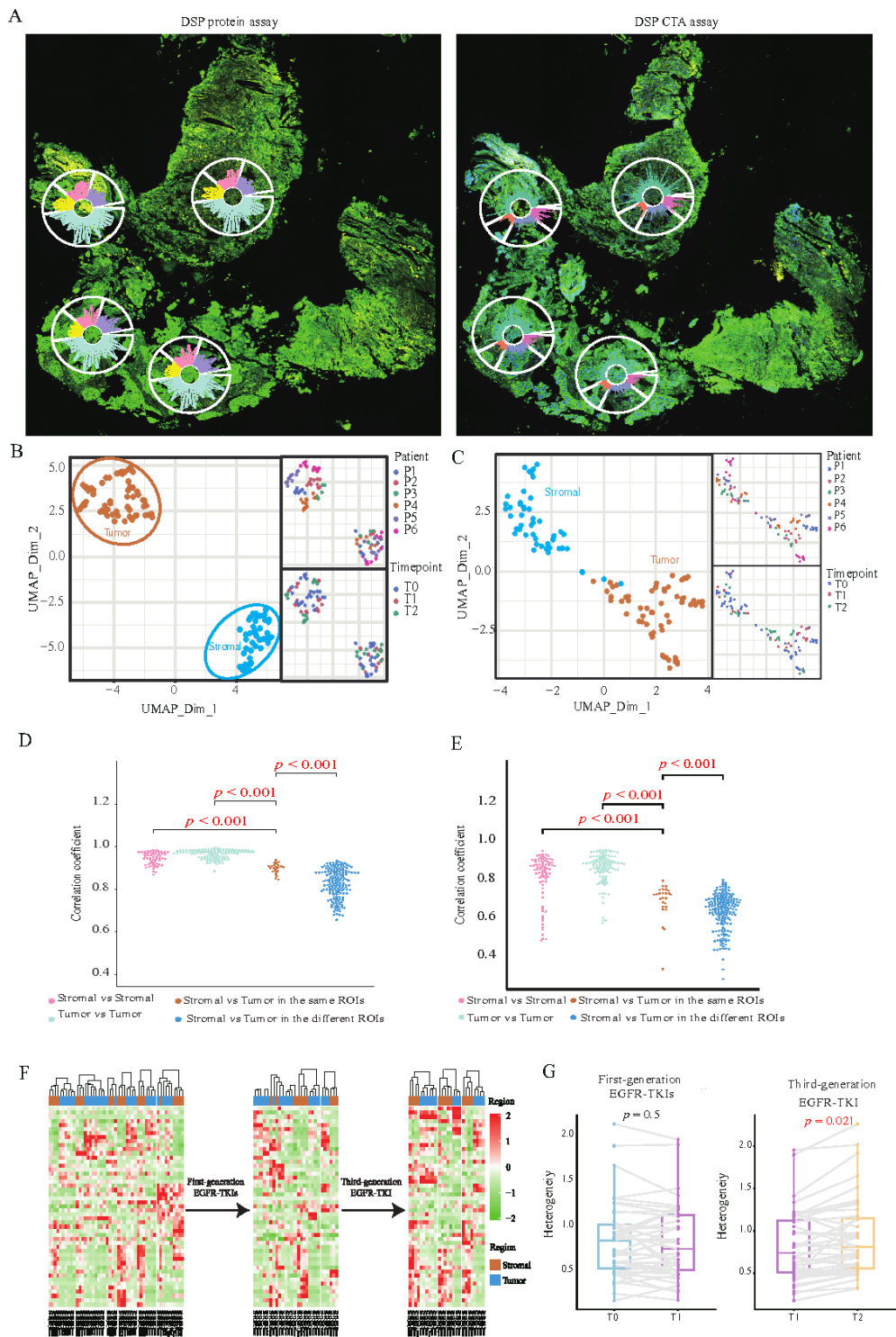


Figure 1

A spatially resolved, patient-matched longitudinal cohort to track microenvironment remodeling during EGFR-TKI therapy

(A) Study design of the longitudinal cohort. (B) Overview of GeoMx DSP for parallel high-plex protein and RNA profiling. (C) Representative segmentation of ROI into tumor-enriched and stroma-enriched AOIs.

(D) AOI numbers profiled for protein and RNA and the corresponding module coverage.



(A) The representative images of AOIs selected from the same location for the DSP protein and RNA assay. (B-C) UMAP for dimensionality reduction analysis based on protein (B) and RNA (C) expression data. (D-E) Comparison of stromal or tumor AOIs from the same or different ROIs performed with DSP protein (D) and RNA (E) assay. (F) Heatmap of protein data in different regions for each patient at different timepoint. The top track showed the AOIs region for each sample. The central heatmap displayed the expression of proteins for each AOI. The region and sample information corresponding to AOIs are displayed at the bottom. (G) The change of heterogeneity pre- and post-first and thied generation EGFR-TKIs treatment.

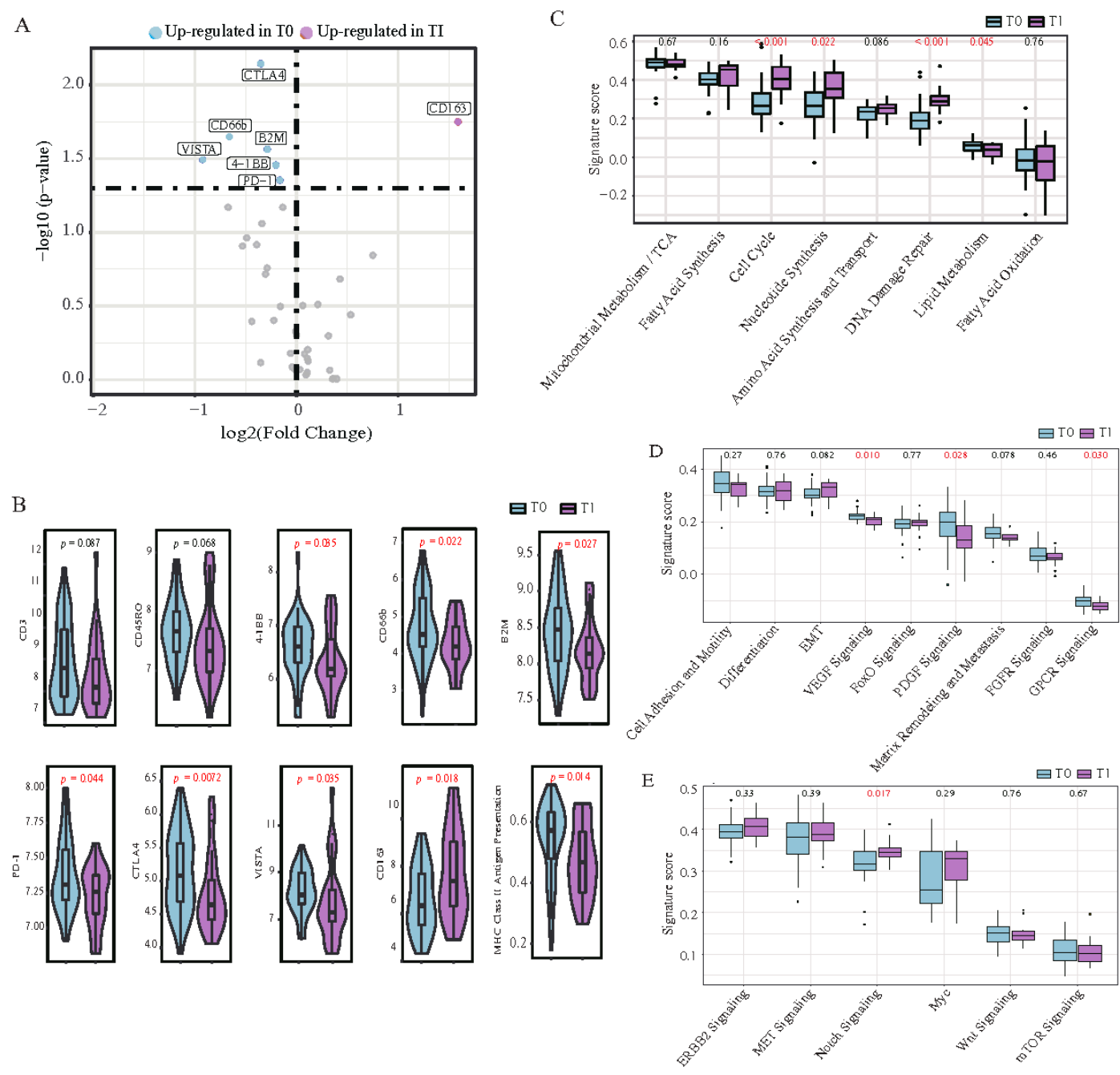


Figure 3

Early EGFR-TKI exposure induces stromal immune reprogramming and tumor intrinsic adaptation.

(A) Volcano map of differentially expressed proteins pre- and post-treatment of first-generation EGFR-TKIs. (B) Box plot of differentially expressed proteins pre- and post-treatment of first-generation EGFR-TKIs. (C) Comparison of the transcript signature scores of core metabolic and biosynthetic programs pre- and post-treatment of first-generation EGFR-TKIs in the tumor region. (D) Comparison the scores of cell state and microenvironment-associated programs. (E) Comparison the scores of oncogenic bypass and plasticity-related signaling pathways.

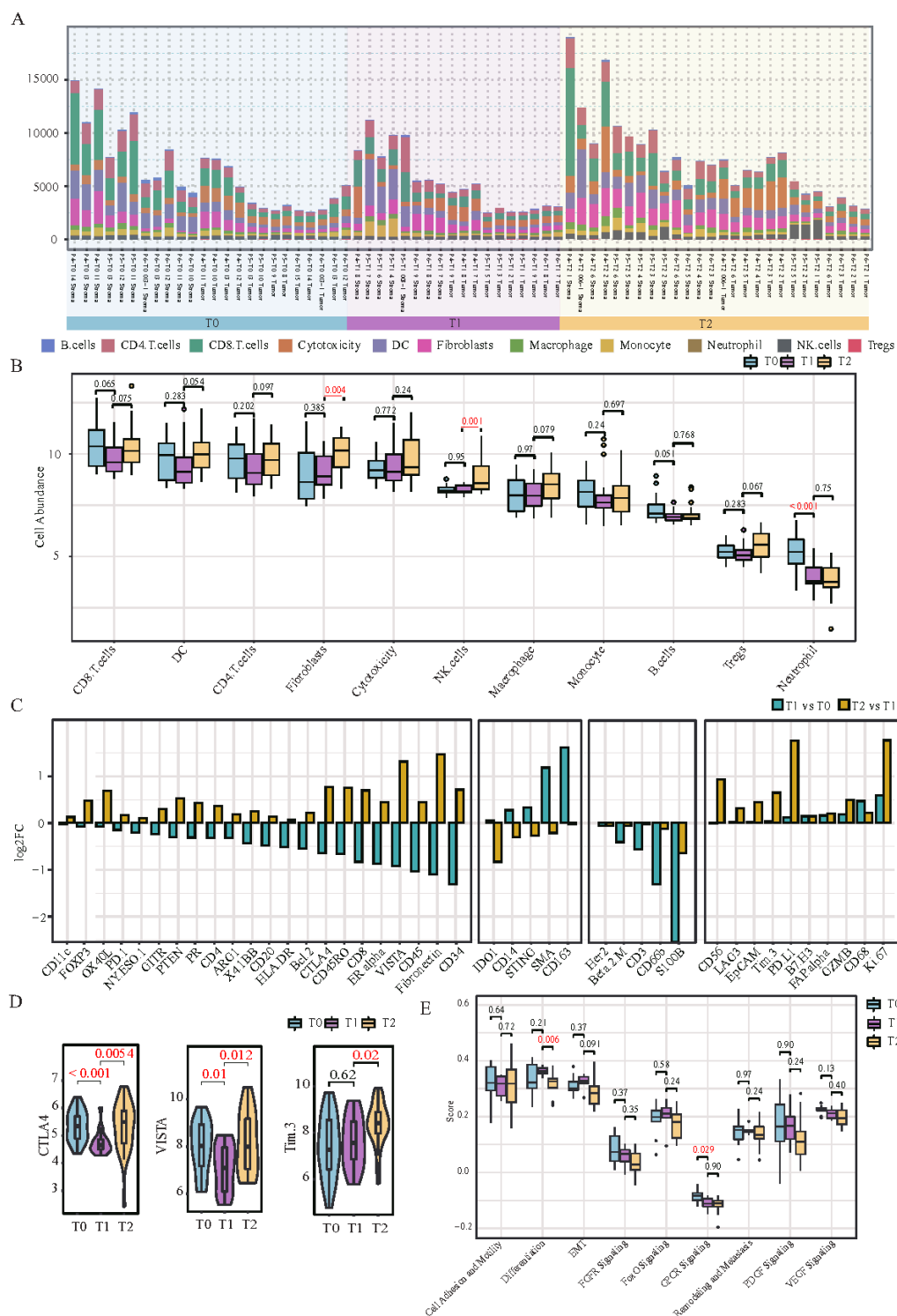


Figure 4

Longitudinal profiling reveals a biphasic shift from early innate remodeling to late stromal checkpoint reprogramming.

(A) The lymphocyte infiltration abundance estimated by protein expression of AOIs at different timepoint. (B) The longitudinal changes of lymphocyte infiltration with treatment stage. (C) Bar chart of the protein

fold change pre- and post-first-generation as well as third-generation EGFR-TKIs treatment. (D) The longitudinal changes of the expression of CTLA4, VISTA, and Tim-3 as the treatment progresses. (E) Comparison of some signature score for the growth, invasion, metastasis of tumor cells and the related cellular signal regulation mechanisms.

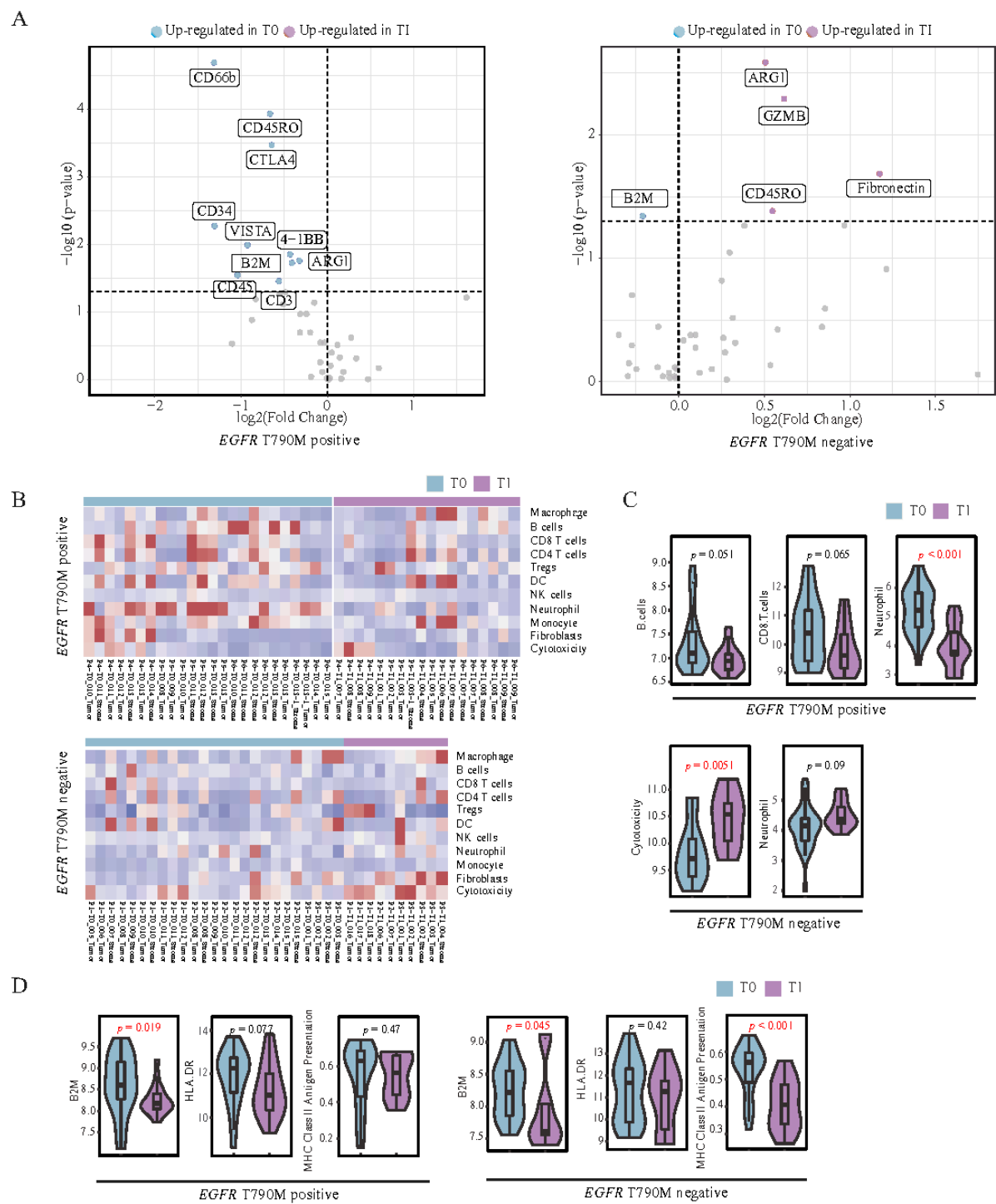


Figure 5

Early on-treatment immune remodeling diverges by subsequent T790M acquisition.

(A) Volcano map of differentially expressed proteins for *EGFR* T790M positive (left) and negative (right) NSCLC patients pre- and post-first-generation EGFR-TKIs treatment. (B) Heatmap of estimated abundance of infiltrating lymphocytes for *EGFR* T790M positive (top) and negative (bottom) NSCLC patients. (C) Box plot of major infiltrating lymphocytes for *EGFR*T790M positive and negative NSCLC patients. (D) Box plot of antigen presentation-related markers for *EGFR* T790M positive and negative NSCLC patients.

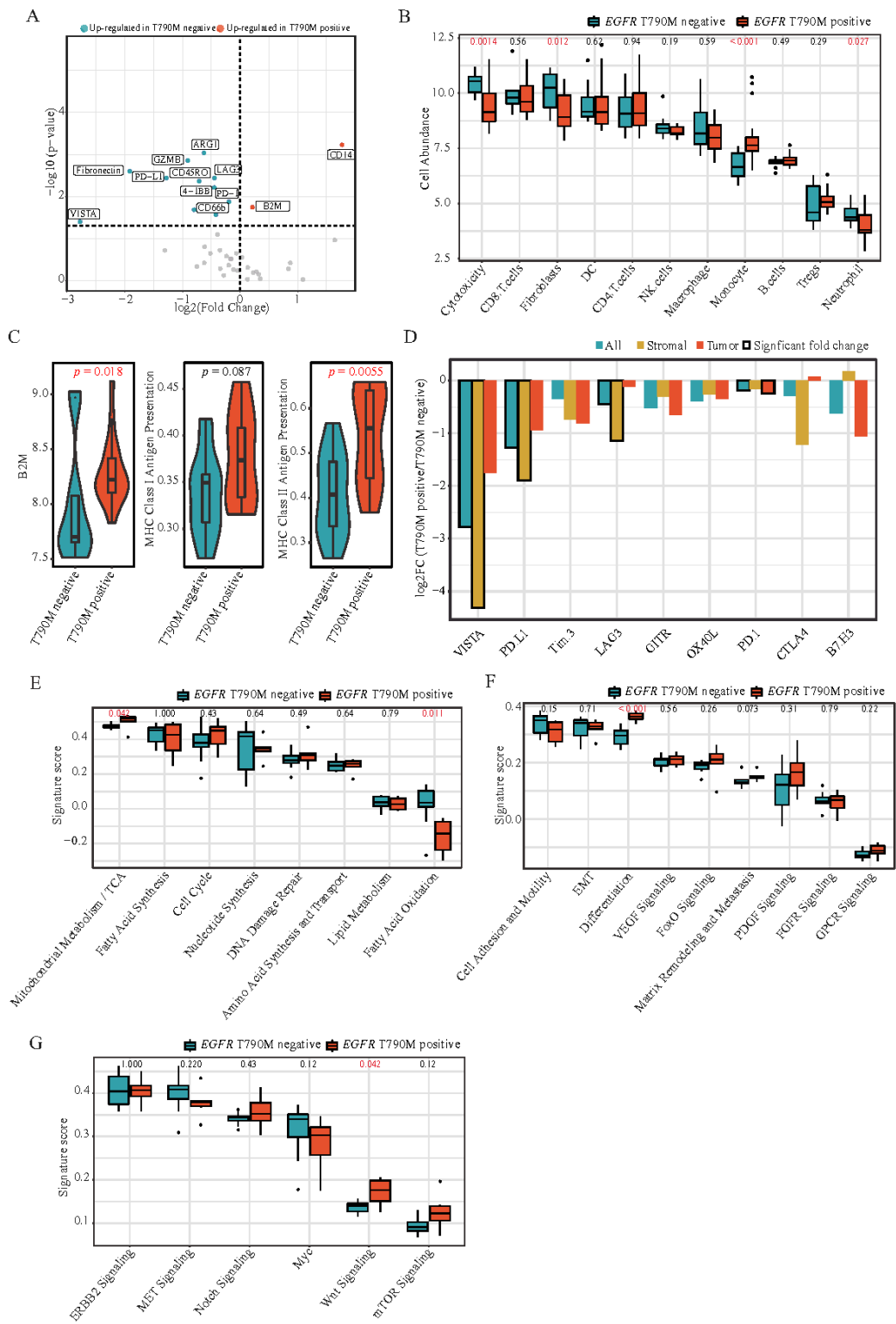


Figure 6

T790M-associated immune–stromal features at the comparable timepoint complement early on-treatment remodeling.

(A) Volcano map of differentially expressed proteins for *EGFR* T790M positive and negative NSCLC patients. (B) Heatmap of estimated abundance of infiltrating lymphocytes for *EGFR* T790M positive and

negative NSCLC patients post-first-generation EGFR-TKIs treatment. (C) Box plot of antigen presentation for *EGFR* T790M positive and negative NSCLC patients. (D) Expression of major immune checkpoints in all AOs, stromal AOs and tumor AOs. (E) Comparison of metabolic and biosynthetic program signature scores between *EGFR* T790M–negative and positive groups. (F) Comparison of differentiation, EMT/motility, and growth factor related signaling signature scores between *EGFR* T790M–negative positive groups. (G) Comparison of canonical oncogenic/adaptive signaling pathway signature scores between *EGFR* T790M–negative and positive groups.

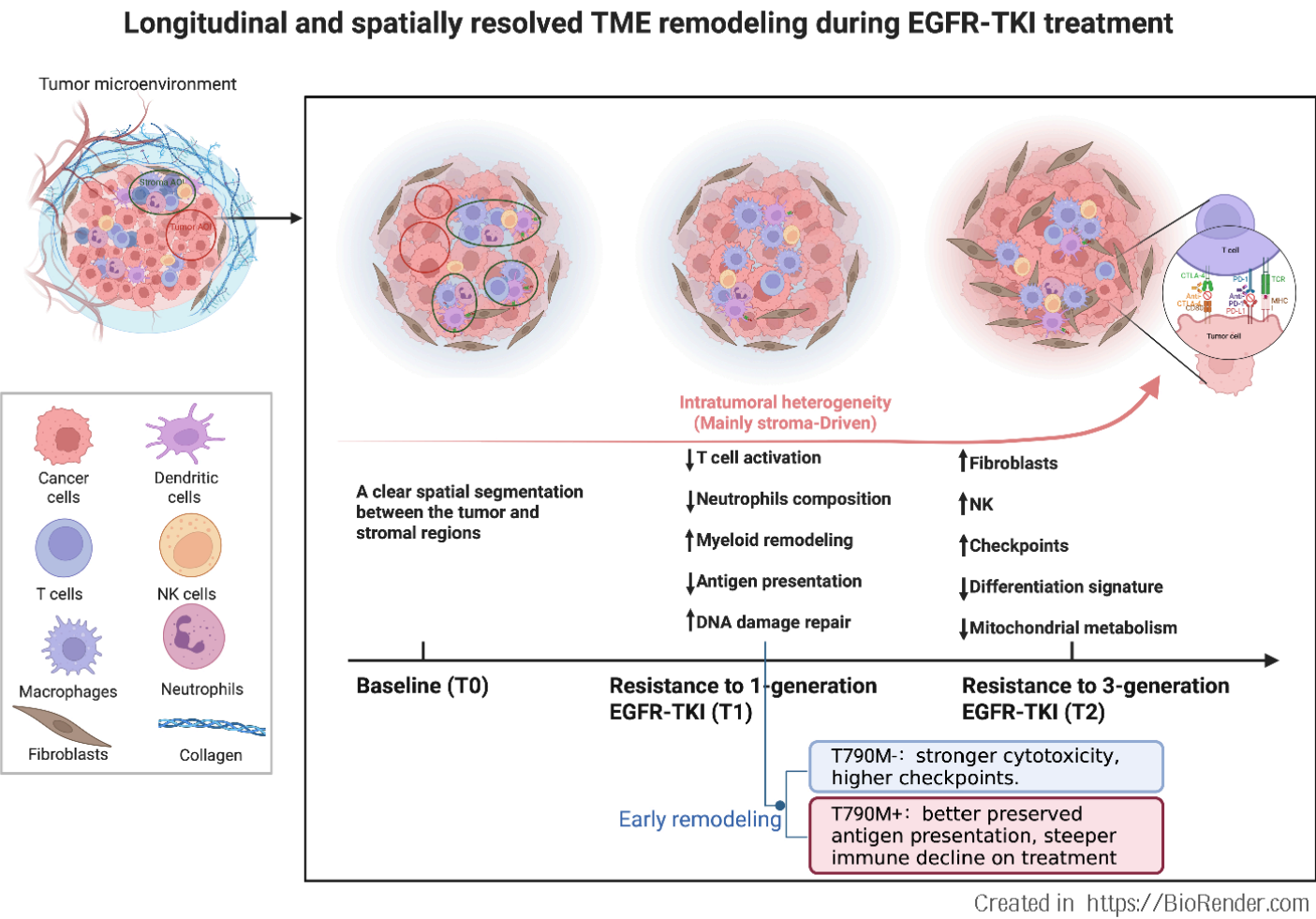


Figure 7

Graphical summary of compartment-resolved, longitudinal TME remodeling across EGFR-TKI treatment.

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