Single-Cell Spatial Proteomics Analyses of Head and Neck Squamous Cell Carcinoma Reveal Tumor Heterogeneity and Immune Architectures Associated with Clinical Outcome

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ABSTRACT

Recent research has provided compelling evidence that the spatial organization of cells within the tumor-immune microenvironment (TiME) of solid tumors correlates with survival and response to therapy in numerous cancer types. Here, we report results of a quantitative single-cell spatial analysis of the TiME of primary and recurrent human head and neck squamous cell carcinoma (HNSCC) tumors, that builds upon our initial longitudinal study of these same HNSCCs that annotated immune complexity at near single cell resolution. Herein, we extended multiple spatial algorithms to quantify spatial landscapes of immune cells within TiMEs. Most notably, we report that spatial compartmentalization, rather than mixing, between neoplastic tumor cells and immune cells is associated with longer patient survival, as well as revealing mesenchymal spatial cellular neighborhoods and their association with improved patient outcomes. Results reported herein are concordant with studies in other tumor types, thus indicating that cellular heterogeneity within tumors trends with spatial TiME features, and are likely prognostic for patient survival.

INTRODUCTION

Tumor microenvironments, comprising both neoplastic tumor cells and recruited stromal cells of various lineages, including a diverse assemblage of immune, mesenchymal, and vascular cells, play a key role in both de novo progression of tumors and regulating response to therapies (1-3). Numerous studies have reported that the spatial organization of the tumor-immune microenvironment (TiME), beyond just the types and quantities of cells present in the TiME, is prognostic for survival and response to therapy in multiple cancer types (4-13). Measures that quantify this spatial organization can range from simple density ratios within specific tumor regions (14), such as the Immunoscore (15), a now commonly used biomarker for tumor staging, to more complex measures that account for the precise locations of specific cells relative to other cells, such as mixing scores(4) and cellular neighborhood measures (7). These more advanced spatial quantifications are a result of emerging single-cell multiplex tissue imaging modalities (4, 6, 7, 16-18), that provide detailed phenotypic and effector statuses for each cell, while maintaining the spatial context of the tissue assayed. Knowing the precise locations of cells in the TiME enables a deeper understanding of how cells interact within the tumor, as both direct and indirect cell signaling mechanisms require cells to be near, if not directly adjacent to one another (19). This understanding can then aid treatment decisions, as many therapies require spatial proximity of specific cell types for efficacy (10). Given that single-cell imaging technologies are still quite new, there is much to be discovered regarding how the spatial organization of cells within the TiME relates to clinical outcome, and may be used for patient stratification decisions for therapy.

We previously developed a multiplex immunohistochemistry (mIHC) imaging platform to aid studies investigating the immune contexture of solid tumors and their response to therapies, at the single-cell level (16, 17). Using a sequential antibody staining protocol, detection of 25-50 proteins can be enumerated at single-cell resolution across a single

formalin-fixed paraffin-embedded (FFPE) tissue section. This enables single-cell phenotyping of discrete leukocyte lineages, capturing their phenotypic effector statuses, and importantly, reveals their spatial relationships to other cells in the tissue section. Utilizing this mIHC approach on a small cohort of primary and recurrent human head and neck squamous cell carcinomas (HNSCCs), we found immune contextures associated with disease recurrence, most notable that macrophage to CD8⁺ T cell ratios within tumor tissues correlate with outcomes (16, 17). HNSCC is the sixth leading form of cancer worldwide (20), and it accounts for more than 10,000 deaths per year in the US alone (21). While patients harboring human papilloma virus (HPV) within neoplastic cells tend to exhibit a better prognosis, their HPV-negative [HPV(-)] counterparts typically exhibit T cell suppressive TiMEs and have a significantly greater risk of recurrence and shorter 3-year survival (22-24). There is a critical need to improve the understanding of HNSCC TiMEs to enable better patient stratification for therapy, as well as identify new targets that could be leveraged for therapeutic intervention to improve outcome, particularly for patients with HPV(-) tumors who currently lack promising therapeutic options.

Here, we built upon our initial study that annotated FFPE sections of nine HPV(-) matched primary newly diagnosed versus their recurrent HNSCCs via our mIHC proteomics imaging platform for TIME leukocyte lineage contexture (17). We measured tumor heterogeneity and compositional changes from primary to recurrent tumors and, importantly, deployed multiple spatial algorithms to quantify the spatial organization of the TiMEs. Finally, we correlated these spatial features with survival and identified TIME architectures associated with survival that could be used to stratify future patients for therapy. Overall, our results are concordant with those from other studies, indicating that the features identified herein are likely shared and prognostic across cancer types.

METHODS

Multiplex immunohistochemistry data generation

mIHC is an immunohistochemical-based imaging platform that evaluates sequentially stained immune lineage epitope-specific antibodies for immunodetection on FFPE tissue sections (16, 17). Using our computational image processing workflow along with a hierarchical gating strategy, we are able to phenotype single cells while maintaining the Cartesian coordinates of each cell on the tissue section. We applied the mIHC pipeline to analyze matched primary and recurrent FFPE tissue specimens from nine HPV(-) HNSCC patients. We sequentially stained for 29 antibodies and gated cells as neoplastic tumor, stromal (mesenchymal), or one of seven different leukocyte subtypes spanning both lymphoid and myeloid lineages (**Table 1**). One to three regions of 2500² μm² each were assayed with mIHC per tumor resection. Patient characteristics and parameters of patient consent are as reported in Banik et al (17). Briefly, study subjects included nine patients with recurrent HNSCC after definitive surgery, chemotherapy, and/or radiation therapy. Matched FFPE tissue specimens from the newly diagnosed primary and recurrent primary tumors for each patient were obtained from the Oregon Health and Science University Knight Biobank. Tissue specimens were deidentified

and coded with a unique identifier prior to analysis. Demographic and clinical data including HPV status, tobacco and alcohol use, treatment regimens, and survival outcomes was collected. All HNSCC tumors were staged according to the 8th edition AJCC/UIC TNM classification and cohort characteristics are shown in **Table 2** (17). All studies involving human tissue were approved by institutional IRB (protocol #809 and #3609), and written informed consent was obtained.

Tumor heterogeneity analyses

The Kullback-Leibler divergence was calculated using the entropy function from the Scipy Python package (25). The distribution of each individual tumor region's cellular composition was compared to the tumor's average cellular composition distribution. Log base 2 was used for the calculation. Unsupervised hierarchical clustering of each tumor region was performed using the normalized percent contribution of each cell type present as the features. Euclidean distance was used to determine distances between observations.

Mixing score analysis

Keren et al. developed a mixing score to quantify the ratio of neoplastic and immune cell spatial interactions (4). This score is defined as the number of interactions between neoplastic tumor cells and immune cells divided by the number of interactions between immune cells and another immune cell within a tumor region. We defined there to be an interaction between two cells if their centers [the (x,y) coordinates provided by mIHC] were within 15 μm from one another. We used the median mixing score value (0.1034) for all primary tumors as the threshold to distinguish between mixed and compartmentalized spatial organization groups. Tumor regions with a mixing score of greater than 0.1034 were defined as mixed. Tumor regions with a mixing score of less than 0.1034 were defined as compartmentalized. We used a density threshold of less than 250 immune cells per $800^2 \mu\text{m}^2$ to define tumor regions as cold. We chose this threshold to match that used by Keren et al.

Cellular neighborhood clustering analyses

Cellular neighborhoods were defined by drawing a circle of a specified radius around all seed cells of a given phenotype. Cells whose centers were inside the circle were considered neighbors of that seed cell and contributed to that cell's neighborhood. All neighborhoods across all tumor regions were then clustered using scikit-learn's MiniBatchKMeans function (26) to perform K-means clustering according to their normalized cellular composition. The elbow method was used to determine the number of clusters to form. Each resulting cluster was comprised of neighborhoods with a similar cellular makeup.

Statistics

Independent t-tests were used to determine statistically significant differences for independent samples whose differences followed a normal distribution. Mann-Whitney U tests were used to determine statistically significant differences for independent samples whose differences did not follow a normal distribution. Paired t-tests were used to determine statistically significant differences for paired samples whose differences followed a normal distribution. Wilcoxon signed rank tests were used to determine statistically significant differences for paired samples whose differences did not follow a normal distribution. P-values less than 0.05 were considered statistically significant. All statistical calculations were performed with the Scipy package using Python software (25).

Survival analyses

Kaplan-Meier curves were generated and a log-rank test was performed using the lifelines package with Python software (27). P-values less than 0.05 were considered statistically significant.

Data and software availability

All of the data produced by our mlHC computational image processing pipeline, including protein abundance, cell phenotype, and cell location information saved in the form of a matrix, in addition to survival data, is available for download at Zenodo (<https://doi.org/10.5281/zenodo.4584441>). All computational analyses in this study were performed using Python software, version 3.6.5. The code created to produce the results of this study is available at https://github.com/kblise/HNSCC_mlHC_paper.

RESULTS

Immune contexture of HNSCC

All mlHC data for this study was generated using the protocol described in Banik et al (17). Single cells were phenotyped in 47 tumor regions from nine primary and nine matched recurrent tumors according to the gating strategy shown in **Table 1**, and their locations within the tumor region were reported as Cartesian coordinates. Patient demographic and clinical data is shown in **Table 2** (17). With this data, we investigated tumor heterogeneity both within and across patient samples, quantified the spatial architecture of the TIME using a mixing score and a neighborhood clustering method, associated spatial architectures with clinical outcome, and performed additional analyses to explain these prognostic features.

Single-cell analyses reveal varying degrees of tumor heterogeneity

To address the question of how cellular composition varies across tumor regions, we assessed tumor heterogeneity at multiple levels, including intra-tumoral, inter-tumoral, and inter-patient cellular heterogeneity by calculating the Kullback-Leibler (KL) divergence for each region, performing hierarchical clustering, and conducting a

principal component analysis (PCA). The fraction of each cell type present per region was first computed across all eighteen tumors (**Figure 1A,B**). Within individual tumors, there was less variability in the number of immune cells present across regions as compared to the number of neoplastic and non-immune cells present. Our interpretation of this finding was that the neoplastic cells and non-immune cell populations are generally the main drivers of intra-tumoral heterogeneity. To further assess this intra-tumoral heterogeneity in a more quantitative way, we calculated the KL divergence of each region's distribution of cell types present, as compared to its tumor's average cell type distribution. KL divergence is a measure of how similar two distributions are; this approach has been used previously to quantify tumor heterogeneity (6). We found that all regions had minimal KL divergence values ($KL < 0.35$; **Figure 1C**), signifying a low degree of intra-tumoral heterogeneity.

To investigate inter-tumoral heterogeneity, we performed unsupervised hierarchical clustering on the 47 tumor regions based on their normalized cellular composition (**Figure 1D**). We found that only one patient (in green at the bottom of the heatmap) contained all five tumor regions clustering together, independent of primary or recurrent state. All other patients' tumors exhibited a greater degree of inter-tumoral heterogeneity, as demonstrated by the distance between primary and recurrent tumor regions in the heatmap's accompanying dendrogram (**Figure 1D**). Finally, we assessed inter-patient tumor heterogeneity, and found that regions evaluated from the same patient tended to cluster together more than regions evaluated from different patients (**Figure 1D**), indicating increased tumor heterogeneity between patients as compared to between samples from the same patient. PCA results also supported these observations (**Figure 1E**).

TiME cellular composition altered by therapy

Multiple studies have reported differences in TiME cellular makeup (28, 29) and tumor clonal diversity (30) between primary and recurrent tumors. To detect whether any immune contexture changes existed following therapy in our cohort, we analyzed the cellular composition of primary tumors as compared to their recurrent tumors. All patients received combination therapy of cisplatin or cetuximab accompanied by radiation following surgical resections of their primary tumors. We used the average fraction of each cell type present across regions for a given tumor and compared primary tumor composition to their matched recurrent tumor composition. We observed a significant difference in the fraction of B cells present in recurrent tumors following therapy for all patients ($p=0.004$, **Figure 2A**), with no other common differences found across patients for any other immune cell type (**Figure 2B**). Further, hierarchical clustering and PCA results did not yield discrete clusters of regions grouped by their tumor status (primary or recurrent) (**Figure 1D, 2C**). We then questioned whether subsets of patients contained similar compositional changes to their TiMEs. To address this question, we performed unsupervised hierarchical clustering on the normalized average change in cellular composition across all cell types. This resulted in two distinct groups of patients; these appear to be most different from one another in the relative degree of change in their CD8⁺ T cell, CD4⁺ T helper, and dendritic cell populations

(**Figure 2D**). Interestingly, the two patients that received cetuximab (orange and purple), rather than cisplatin, clustered together within one of these groups and had similar overall changes to their tumor compositions following therapy. Together, these results indicate that there were shared trends in TiME composition changes for subsets of patients specific to therapy, and regardless of therapy, all patients saw a significant decrease in B cells from primary to recurrent tumors.

TiME cellular composition associated with survival

Prior research has revealed that increased CD8⁺ T cell abundance is associated with better outcomes in HNSCC (31-34). Using the average fraction of CD8⁺ T cells present in primary tumors, no significant association with survival was found ($p=0.423$, **Figure 2E**). However, patients with a smaller fraction of macrophages present in their primary tumors had better progression free survival (PFS) ($p=0.017$, **Figure 2F**). These findings are supported by other studies that also identified higher levels of macrophages associated with more aggressive tumors and decreased survival (35, 36). These findings expand on the results of our prior study, which reported that patients with myeloid-inflamed tumors demonstrated decreased PFS than those with lymphoid inflamed tumors (17). Here we report that this difference can be attributed to a single tumor feature—the fraction of macrophages present. We also found that patients with a greater fraction of other non-immune cells (PANCK⁻, CD45⁻) correlated with improved PFS ($p=0.003$, **Figure 2G**), with no other cell types evaluated demonstrating an association with survival.

Quantifying the spatial organization of tumor and immune cells

To investigate if TiME cellular spatial relationships were prognostic in our cohort of primary and recurrent HNSCC as has been reported in other cancer types (5-13), we deployed a mixing score, which was used previously to analyze immune cell spatial compartmentalization in triple negative breast cancers (4). To do this, we quantified the number of immune and neoplastic tumor cells within 15 μm of each other, then divided by the number of immune cells within 15 μm from another immune cell. We labeled each region as mixed or compartmentalized using the median mixing score value for all primary tumors as the threshold (**Figure 3A,B**; see Methods). This threshold categorized tumor regions as mixed when at least one neoplastic tumor cell was within 15 μm from an immune cell for approximately every ten immune cells within 15 μm from another immune cell. Tumor regions were compartmentalized when this ratio was smaller. Regions with fewer than 250 CD45⁺ immune cells per 800² μm^2 present were labeled as cold. Of the 47 total tumor regions, 23 were mixed, 20 were compartmentalized, and four were cold.

After calculating the mixing score for each region, we explored how cellular composition related to spatial organization. Regions with more mixing between neoplastic cells and immune cells often demonstrated higher presence of neoplastic cells than those with higher compartmentalization (**Figure 3C**). This is unsurprising given the equation for the mixing score, as more neoplastic cells will inevitably only contribute to the numerator of

the score, and thus will increase the overall score. However, regions with more compartmentalization between neoplastic cells and immune cells often also contained increased counts of alpha smooth muscle actin (α SMA)⁺ mesenchymal cells as compared to those with higher mixing (**Figure 3D**). This result was intriguing because these cells have no effect on the mixing score, yet there is a clear association between composition and organization. This association is further demonstrated by PCA, where regions of similar cellular composition cluster together and also tend to contain similar spatial organizations (**Figure 3E**).

We next examined associations between biomarker expression reflecting on bioeffector phenotypes of leukocytes and mixing scores for each tumor region. Mixed regions contained a greater proportion of neoplastic cells expressing immunoregulatory proteins, such as PD-1 and PD-L1, than compartmentalized regions ($p=0.0003$, $p=0.015$, **Figure 3F**). We also found that compartmentalized regions contained a greater proportion of lymphocytes (CD8⁺ T cells, CD4⁺ T cells, and B cells) expressing the proliferation marker Ki-67 ($p=0.020$, **Figure 3G**).

Spatial compartmentalization associated with longer survival

To investigate how spatial mixing correlated with PFS or patient outcome, we averaged the mixing score across regions and assigned a final mixing score and spatial label for each tumor. Patients with more compartmentalization between neoplastic cells and immune cells in their primary tumors exhibited longer PFS than those with more mixing between these cell types ($p=0.027$, **Figure 3H**).

In addition to quantifying TiME spatial organization using a mixing score, we also computed cellular neighborhoods, that were then clustered to identify common spatial features across tumors. Neighborhoods represent physical groupings of cells within a set distance threshold from a seed cell (**Figure 4A**). Each cell within the distance threshold was deemed a neighbor of the seed cell, contributing to that seed cell's neighborhood's composition. After identifying all neighborhoods for each seed cell present across all tumor regions, neighborhoods were grouped with K-means clustering, using the fraction of each cell type present in the neighborhoods as the clustering features. This revealed clusters of neighborhoods with similar cellular makeups. We then used the average neighbor composition of each cluster to perform survival analyses.

Given the relationship between mixing score and α SMA⁺ mesenchymal cell abundance, we applied our neighborhood clustering analysis with α SMA⁺ cells as the seed cells. We used a distance threshold of 22.5 μ m, as this produced neighborhoods with an average of approximately ten neighbors present. A recent study involving cellular neighborhood analyses employed a method that selected the ten nearest spatial neighbors of the seed cell, regardless of the distance between the seed cell and its neighbors (7). By setting a distance threshold of 22.5 μ m, our method required the cells be close, if not directly touching, while still capturing enough neighbors to cluster on. Clustering results yielded six groups, each different in their composition (**Figure 4B**). α SMA⁺ cell neighborhoods

in cluster 1 contained a majority of α SMA⁺ cells as the neighbors. Cluster 2 contained both α SMA⁺ cells as well as other non-immune (CD45⁻), non-neoplastic cells (panCK⁻) as neighbors. Cluster 3 was defined by its large proportion of panCK⁺ neoplastic cells, whereas Cluster 4 consisted of roughly half CD45⁺ immune cells and half other non-immune, non-neoplastic cells. Cluster 5 contained the highest proportion of CD45⁺ immune cells, and cluster 6 was dominated by other non-immune and non-neoplastic cells.

We found that patients with a greater proportion of their α SMA⁺ cells in clusters 2 and 6 in their primary tumors demonstrated longer PFS (cluster 2: $p=0.059$, cluster 6: $p=0.003$, **Figure 4C,D**), thus indicating that patients whose α SMA⁺ cells were predominantly surrounded by α SMA⁺ cells or other non-immune, non-neoplastic cells in their TiMEs were associated with better outcome. In addition, patients with a greater proportion of their α SMA⁺ cells belonging to cluster 3, which included a large number of neoplastic cells, in their primary tumors demonstrated shorter PFS ($p=0.047$, **Figure 4E**).

DISCUSSION

The significant role that TiME cellular composition plays in tumor progression and response to therapy has been accepted for over a decade (37). However, recent findings powered by single-cell proteomics imaging technologies have found that the spatial organization of the cells present in the TiME also plays a critical role (4-13). It is still unclear exactly which spatial features are important in dictating response to therapy or clinical outcome, as well as whether these features are shared across cancer types, and how they could be leveraged for therapeutic decisions and patient stratification. Here, we leveraged single-cell spatial proteomics data generated by our mIHC immunoassay-based imaging platform to quantitatively assess the TiME of nine matched primary and recurrent HPV(-) HNSCCs in order to identify possible prognostic spatial features. Our results provide novel insights into the heterogeneity and spatial landscape of HPV(-) HNSCCs, and highlight possible TiME spatial layouts that may impact clinical outcome across cancer types.

Despite the fact that HPV(-) HNSCCs often contain limited immune infiltrates (22), our analyses revealed significant differences in the immune cell spatial organization within these tumors that were associated with survival, highlighting the importance of considering the spatial context of the TiME. Most strikingly, patients whose primary tumors contained more compartmentalization between their tumor cell and immune cell populations demonstrated longer PFS. This correlation was also identified in a similar analysis of triple negative breast cancer patients (4), indicating that a compartmentalized spatial architecture may play a favorable role in survival across cancer types. Our analyses of functional marker expression indicate that the immune cells in regions of compartmentalization are proliferating and are perhaps subject to reduced direct regulation by neoplastic tumor cells (38-42) than those in regions of mixing. This increased proliferation and decreased regulation of immune cells in compartmentalized tumors may contribute to improved survival. We also found that the

presence of spatial cellular neighborhoods consisting of α SMA⁺ cells and other non-immune and non-neoplastic cells surrounding α SMA⁺ cells was associated with longer survival. On the contrary, the presence of cellular neighborhoods consisting of neoplastic tumor cells surrounding α SMA⁺ cells was associated with shorter survival. Given that α SMA is a common marker for cancer-associated fibroblasts (20, 43), whose presence in tumors tends to be associated with tumor growth, metastasis, and resistance to therapy (44-47), our results indicate that beyond just the abundance of these cells, their spatial organization may play a crucial role in determining how a tumor progresses, and/or how it responds to standard therapy. Our cellular neighborhood analyses highlight the importance of considering how multiple cells can work in tandem with one another, beyond single cell-cell interactions, to impact TiME organization, and ultimately, clinical outcome.

We also found concordance between the results from our heterogeneity and composition analyses and those from several other studies. Our results are similar to a study that clustered HNSCC biopsies based on their neoplastic and immune gene signatures as determined by RNA-sequencing, and reported that samples from the same patient were more similar to each other than samples from different patients (48). This trend has also been observed across cancer types, including melanoma (49), hepatocellular carcinoma (50), pancreatic ductal adenocarcinoma (51), and breast cancer (52). Finally, we found that decreased macrophage abundance was associated with longer survival, which has previously been reported in head and neck cancers (35, 36). Collectively, these results demonstrate concordance across multiomic analyses and cancer types.

This concordance provides orthogonal support for our conclusions and indicates that single-cell heterogeneity and spatial organization of tumors may share similarities across different types of cancer and across different molecular assays. Our spatial analyses demonstrate the use of various algorithms to quantify the spatial landscape of tumors using single-cell imaging data. Further, our computational methods provide a framework for future single-cell imaging analyses, as they are applicable to any multiplex imaging assay, including mIHC, co-detection by indexing technology (CODEX) (7), cyclic immunofluorescence (18), and imaging mass cytometry (6). Our results highlight several spatial architectures that may help guide precision medicine approaches for HPV(-) HNSCC patients, including architectures that may help predict which patients will have shorter survival and thus warrant more aggressive therapy and clinical follow-up versus those that predict which patients have an improved survival outlook, where therapy and follow-up could be modulated. While our results provide evidence that the spatial organization of HPV(-) HNSCC tumors plays a role in clinical outcome, future studies on larger cohorts are needed to assess the full prognostic capabilities of the spatial features identified in our analyses.

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Blise et al. Table 1

Cell Phenotype	Antibody Markers
CD8 ⁺ T Cell	CD45 ⁺ CD20 ⁻ CD3 ⁺ CD8 ⁺
CD4 ⁺ T Helper	CD45 ⁺ CD20 ⁻ CD3 ⁺ CD8 ⁻ FOXP3 ⁻
CD4 ⁺ Regulatory T Cell	CD45 ⁺ CD20 ⁻ CD3 ⁺ CD8 ⁻ FOXP3 ⁺
B Cell	CD45 ⁺ CD20 ⁺
Macrophage	CD45 ⁺ CD20 ⁻ CD3 ⁻ CD66B ⁻ CD68 ⁺
Granulocyte	CD45 ⁺ CD20 ⁻ CD3 ⁻ CD66B ⁺
Dendritic Cell	CD45 ⁺ CD20 ⁻ CD3 ⁻ CD66B ⁻ CD68 ⁻ MHCII ⁺
Other Immune	CD45 ⁺ CD20 ⁻ CD3 ⁻ CD66B ⁻ CD68 ⁻ MHCII ⁻
αSMA ⁺ Mesenchymal	CD45 ⁻ PANCK ⁻ αSMA ⁺
Other Non-Immune	CD45 ⁻ PANCK ⁻ αSMA ⁻
Neoplastic Tumor	CD45 ⁻ PANCK ⁺
Additional Functional Markers: PD-1, PD-L1, Ki-67	

Table 1. mIHC cell phenotype classification. Antibody markers used to hierarchically phenotype cells into one of eleven phenotype classifications. Additional functional markers stained are listed at the bottom.

Blise et al. Table 2

Characteristic	n (%)
Gender	Female: 7 (78%) Male: 2 (22%)
Race	Asian: 1 (11%) White: 8 (89%)
Average Age at Primary Surgery	61 years
Alcohol History	Yes: 7 (78%) No: 2 (22%)
Smoking History	Yes: 5 (56%) No: 4 (44%)
HPV Status	Positive: 0 (0%) Negative: 9 (100%)
Tumor Site	Larynx: 3 (33%) Oral Cavity: 4 (44%) Oropharynx: 2 (22%)
TNM Stage	Stage 1: 2 (22%) Stage 2: 1 (11%) Stage 3: 1 (11%) Stage 4: 5 (56%)
Therapy Following Primary Resection	Cisplatin + Radiation: 6 (67%) Cetuximab + Radiation: 3 (33%)
Average Progression Free Survival	504 days (1.4 years)

Table 2. Patient and tumor characteristics. Adapted from Banik et al (17).

Figure 1. Heterogeneity across patients and tumor regions. **A**, Fraction (out of 1.0) of each cell type present per individual primary (P) and recurrent (R) tumor. Each dot represents a single tumor region (n=47). **B**, Fraction (out of 1.0) of each immune cell type present per individual primary (P) and recurrent (R) tumor. Each dot represents a single tumor region (n=47). **C**, Dot plot of the Kullback-Leibler divergence from a single tumor region's cellular distribution compared to the tumor's average cellular distribution. **D**, Heatmap of cellular composition across tumor regions. Rows are individual tumor regions that are ordered by the hierarchical clustering of their cellular composition. Columns are the cell types used as clustering features. Composition was normalized [0,1] before clustering. Leftmost column is color coded by patient. Next column is color coded by tumor type. **E**, Principal component analysis on cellular composition. Each dot (n=47) represents one tumor region and is colored by patient.

Figure 2. Tumor cellular composition changes following therapy and composition

associations with survival. A, Box plot showing the percent that each cell type contributes to each tumor's cellular makeup. Each dot represents the percent contribution of that cell type for one tumor, averaged across regions (n=9 primary tumors, n=9 recurrent tumors). Statistical significance calculated using parametric paired two-tailed t-tests for cell types whose differences follow a normal distribution and non-parametric paired two-tailed t-tests (Wilcoxon signed rank test) for cell types whose differences do not follow a normal distribution. **B**, Line plots showing the change in cellular composition from primary to recurrent tumors for each cell type. Each dot represents the percent contribution of that cell type for one tumor, averaged across regions. Lines are colored by patient. **C**, Principal component analysis on cellular composition. Each dot (n=47) represents one tumor region and is colored by its primary or recurrent designation. **D**, Heatmap of average change in cellular composition from primary to recurrent tumors for each patient. Rows are individual patients that are ordered by the hierarchical clustering of their change in TiME cellular composition (averaged across regions). Columns are the cell types used as clustering features. Compositional change was normalized [0,1] before clustering. Leftmost column is color coded by patient. **E**, Kaplan-Meier curve of progression free survival for patients split by the percentage of CD8⁺ T cells in their primary tumors. Patients were split on the median value. P-value calculated using the log-rank test. **F**, Kaplan-Meier curve of progression free survival for patients split by the percentage of macrophages in their primary tumors. Patients were split on the median value. P-value calculated using the log-rank test. **G**, Kaplan-Meier curve of progression free survival for patients split by the percentage of other non-immune, non-neoplastic cells in their primary tumors. Patients were split on the median value. P-value calculated using the log-rank test.

A

B

C

D

E

F

G

H

Figure 3. Mixing score quantifies the spatial organization of tumors. **A**, Mixed tumor region (top) versus compartmentalized tumor region (bottom). Leftmost panel is the mIHC image of the tumor region (red = neoplastic tumor; blue = non-neoplastic, non-immune; other colors = immune). Remaining panels show scatterplot reconstructions of the mIHC image, excluding the non-immune, non-neoplastic cells. Each dot represents one cell, and is colored by cell type. **B**, Box plot showing the mixing scores across all primary and recurrent tumors (n=18). Each dot (n=47) represents the mixing score for one tumor region and is colored according to its spatial architecture. The average spatial architecture designation for the overall tumor is printed above each box. **C**, Bar chart showing the raw count of neoplastic tumor cells present per tumor region. Bars are ordered by neoplastic cell count and are colored according to the region's spatial architecture. **D**, Bar chart showing the raw count of α SMA⁺ mesenchymal cells present per tumor region. Bars are ordered by α SMA⁺ cell count and are colored according to the region's spatial architecture. **E**, Principal component analysis on cellular composition. Each dot (n=47) represents one tumor region and is colored according to the region's spatial architecture. **F**, PD-1 and PD-L1 expression on neoplastic tumor cells by spatial architecture. Each dot represents the percentage of neoplastic cells positive for that immunoregulatory protein for a single tumor region (n=43, excluding cold regions). P-values calculated using a one-tailed Mann-Whitney U test. **G**, Ki-67 expression on lymphocytes (CD8⁺ T cells, CD4⁺ Regulatory T cells, CD4⁺ T Helper, and B cells) by spatial architecture. Each dot represents the percentage of lymphocytes positive for Ki-67 for a single tumor region (n=43, excluding cold regions). P-value calculated using a one-tailed t-test. **H**, Kaplan-Meier curve of progression free survival for patients split by the mixing score of their primary tumors. Patients were split on the median value. Results exclude cold tumor regions. P-value calculated using the log-rank test.

Figure 4. α SMA⁺ mesenchymal cellular neighborhood clustering. **A**, Cellular neighborhoods were defined by drawing a circle of a specified radius around each seed cell (green) of a designated phenotype. Cells whose centers were inside the circle were considered neighbors of that seed cell. This figure was created using BioRender.com. **B**, Stacked bar chart showing the average cellular composition of each α SMA⁺ mesenchymal cell neighborhood cluster (n=6). Bars are colored by cell type and represent the average fraction (out of 1.0) of each cell type present in the neighborhoods belonging to each cluster. **C**, Kaplan-Meier curve of progression free survival for patients split by the percentage of α SMA⁺ mesenchymal cell neighborhoods belonging to cluster 2 in their primary tumors. Patients were split on the median value. P-value calculated using the log-rank test. **D**, Kaplan-Meier curve of progression free survival for patients split by the percentage of α SMA⁺ mesenchymal cell neighborhoods belonging to cluster 6 in their primary tumors. Patients were split on the median value. P-value calculated using the log-rank test. **E**, Kaplan-Meier curve of progression free survival for patients split by the percentage of α SMA⁺ mesenchymal cell neighborhoods belonging to cluster 3 in their primary tumors. Patients were split on the median value. P-value calculated using the log-rank test.