

Single Cell Expression Analysis of PIK3 Genes to Direct Solid Tumor Treatment with the Dual PI3K- δ,γ Inhibitor Duvelisib

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BACKGROUND

- Duvelisib (DUV), a dual PI3K- δ,γ inhibitor, is US FDA approved at 25 mg twice daily (BID) for the treatment of R/R chronic lymphocytic leukemia or small lymphocytic lymphoma after ≥ 2 lines of prior therapy and R/R follicular lymphoma after ≥ 2 prior systemic therapies. Accelerated approval for R/R follicular lymphoma was based on overall response rate and continued approval may be contingent upon confirmatory trials. (Duvelisib US Package Insert).
- Duvelisib targets malignant B and T cells in hematologic malignancies, but also has a demonstrated role in modulating the tumor microenvironment (TME) by effecting key non-malignant immune cells including immunosuppressive Tregs (Ali, Nature, 2014) and immunosuppressive myeloid cells (Kaneda, Nature 2016; De Henau, Nature, 2016).
- Immunomodulation by tumor infiltrating myeloid, T cells, and B cells is an important feature of solid tumors and it is expected that PI3K- δ,γ genes (PIK3CD & PIK3CG) are involved in these cell types. However, it is poorly understood whether PI3K- δ,γ may also play a role in the resident cancer cells of the tumor.

METHODS

PIK3 Expression in tumors

- Levels of PI3K- δ (PIK3CD) and PI3K- γ (PIK3CG) were analyzed in The Cancer Genome Atlas (TCGA) using Firebrowse to evaluate the relative expression in many tumor types compared to normal tissue.
- To segment tumors into 'Hot' and 'Cold' subgroups, an inflammatory/immunosuppressive gene signature for immune cell function (Givechian, npj Genomic Medicine, 2018) was used. 'Hot' clusters contain more inflammatory genes indicating these tumors have more inflammatory infiltrates.
- CIBERSORT deconvolution algorithms, the 'Hot' and 'Cold' clusters were estimated for immune cell abundance (Newman, Nat. Methods 2015) (Figure 1).

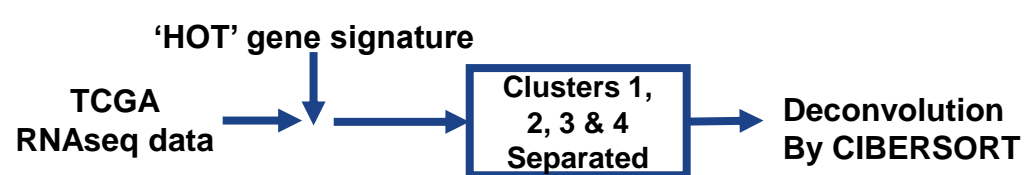


Figure 1: Workflow for Gene Expression and Deconvolution

Single Cell Datasets

- To investigate PIK3C gene expression in Head and Neck Squamous Cell Carcinoma (HNSCC) across cell types, a single-cell RNA expression dataset (Puram, 2017) was examined
- 18 primary HNSCC tumors, and 5 matched pairs of lymph node & tumor samples were evaluated
- For 5,902 cells analyzed, 2,215 malignant and 3,363 non-malignant cells were identified (Copy number variation (CNV) and averaged expression profile approaches used)

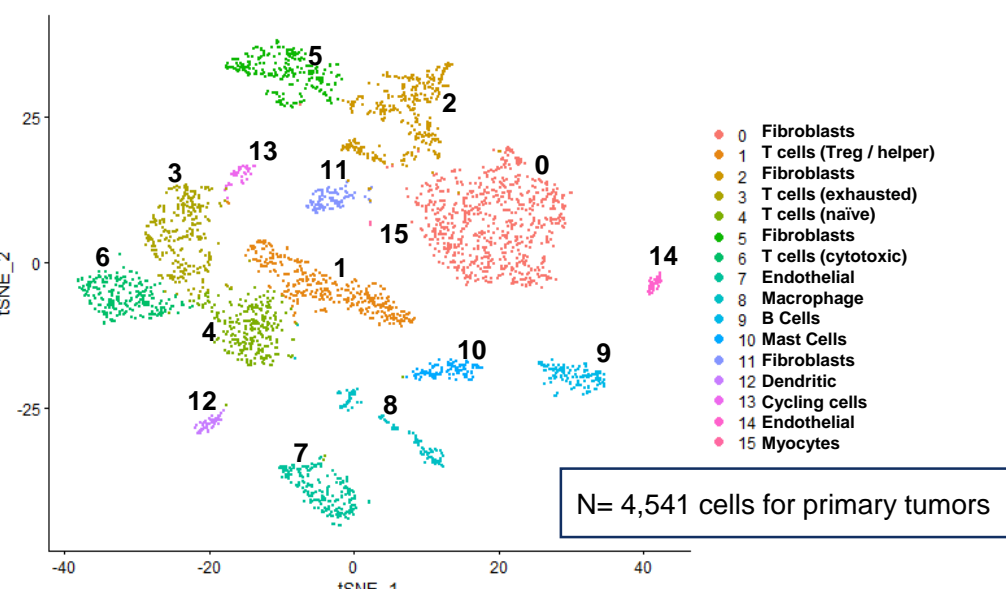


Figure 2: tSNE Plot of Tumor Microenvironment (TME) Cell Clusters from 18 Primary HNSCC Tumors

- tSNE** HNSCC cell type assignments were carried out: TME by performing unsupervised clustering of non-malignant cells using a Seurat workflow. Cell types were assigned to tSNE-identified clusters by manually reviewing cell type marker expression (Figure 2).
- Cancer cells were identified by gene expression signature according to Puram
- PIK3C isoform genes A (α), B (β), D (δ) & G (γ) analysis in HNSCC single cells**
 - A 0.3 Log₂ Transcripts per Million (TPM) threshold was applied to measure distinct expression patterns between PIK3C genes in single cells

Differential Expression (DE) of genes in cells expressing PIK3C isoform genes A (α), B (β), D (δ) & G (γ)

- We performed unsupervised hierarchical clustering of cells on unscaled gene expression of four PI3K isoforms from cancer and TME cells.
- Resulting clustering dendrograms were 'cut' to yield a set number of cell clusters chosen empirically to capture major clusters of interest expressing each isoform and their combinations, when possible.
- MAST method was used as implemented in Seurat to run DE comparison between the given group of cells (e.g. PIK3CA expressing cluster vs the rest of the cells of the same cell type).

RESULTS

1: PIK3CD and PIK3CG Expression in Tumors

- PIK3CD median expression is higher than PIK3CG across nearly all of the cancer types (Firebrowse) (Figure 3).
- PIK3CD and PIK3CG expression are higher in HNSCC tumor compared to normal tissue (median 9.51 vs 8.40 RSEM log₂ and 5.56 vs 5.38 RSEM log₂, respectively) (Firebrowse) (Figure 3).

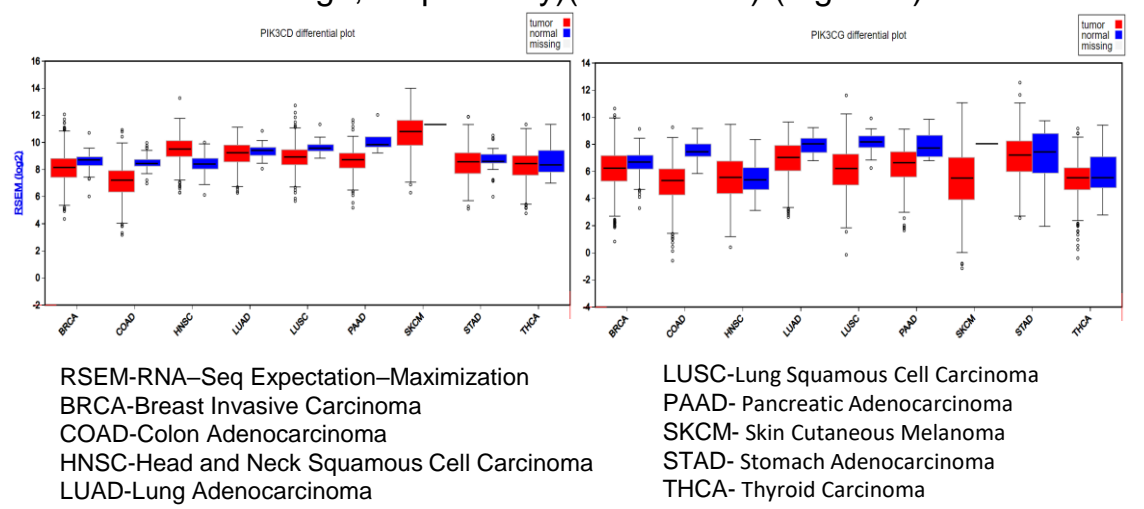


Figure 3: PIK3CD & PIK3CG RSEM Differential Plots [Firebrowse]

2: PIK3 Expression in Cancer Cells & DE Gene Analysis

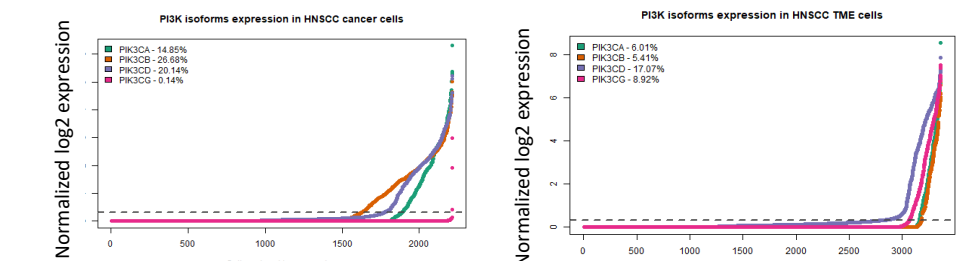


Figure 4: Gene expression thresholds for PIK3CD, PIK3CG, PIK3CA and PIK3CB. A 0.3 log₂ TPM threshold (dotted line) was applied for both TME and cancer cells, and cells were ranked by log₂ expression level.

- The percentages of HNSCC cancer cells with >0.3 Log₂ TPM gene expression were: PIK3CB (27%), PIK3CD (20%), PIK3CA (14%), and PIK3CG (0.1%)
- PIK3CD expression at >0.3 Log₂ TPM occurred in 17% of TME cells, compared to 5-10% of TME cells for PIK3CA, PIK3CB, and PIK3CG (Figure 4).
- Most individual tumors (with 50 or more cells sequenced) had cancer cells expressing PIK3CD (6%-63%); >50 cells/tumor while very few expressed PIK3CG (Table 1).

Table 1: Frequency of PIK3C gene expressing Cancer Cells in Individual HNSCC Tumors

HNSCC Tumors	Total*	PIK3CA High%	PIK3CB High%	PIK3CD High%	PIK3CG High%
HN16	56	19.64	23.21	28.57	0
HN17	330	13.33	34.85	9.39	0
HN18	140	12.14	15	6.43	0
HN20	662	17.07	25.08	16.31	0
HN22	119	10.92	17.65	17.65	0
HN25	209	8.13	29.19	63.16	0
HN26	267	18.35	24.34	10.11	0.37
HN28	138	7.97	34.78	31.16	0
HN5	132	18.94	24.24	18.94	0.76
HN6	123	18.7	32.52	16.26	0.81

* Tumors with > 50 cells were counted

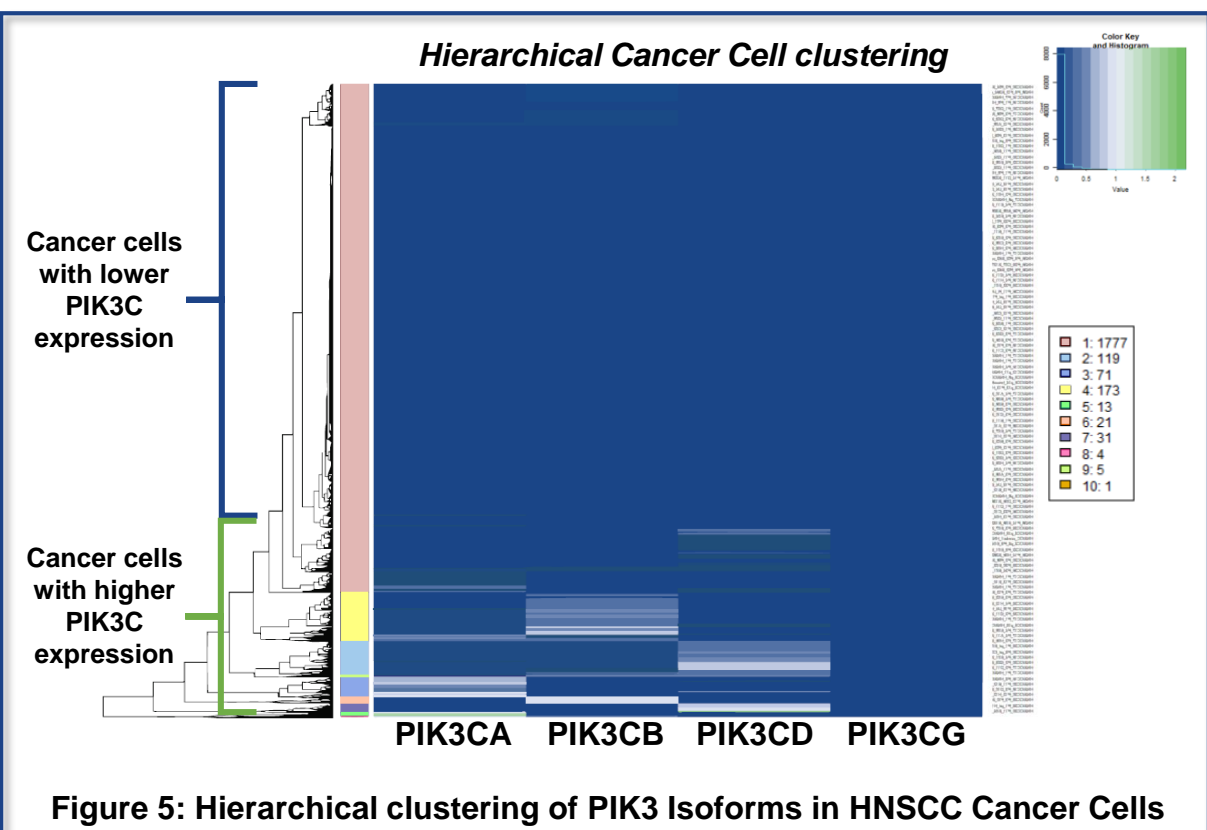


Figure 5: Hierarchical clustering of PIK3 Isoforms in HNSCC Cancer Cells

- Cancer cells were ranked by PIK3C expression and hierarchical clustering applied
- PIK3C expression was predominantly mutually exclusive to the expression of PIK3CG, PIK3CA, and PIK3CB in the cancer cells (Figure 5)

Table 2: Top 25 Differentially Expressed genes for PIK3CD Expressing HNSCC Cancer Cells

Gene	pct.1	pct.2	P-value Adjusted	Average Expression	Average logFC
PIK3CD	1	0.538	0	0.06203	0.5677
IF1B	0.987	0.946	1.62E-10	1.809	0.6399
PRDX2	0.94	0.918	4.02E-11	1.704	0.6787
GLUL	0.587	0.905	2.99E-19	1.586	-0.7157
CALR	1	0.995	1.59E-13	1.566	0.6419
IER2	0.953	0.883	4.06E-13	1.406	0.9112
JUNB	0.927	0.85	6.10E-14	1.337	1.166
WDR83OS	0.933	0.887	3.75E-13	1.184	0.5488
CRIP2	0.653	0.369	6.29E-12	0.5328	0.6787
STX10	0.733	0.529	4.43E-21	0.475	0.7571
TRMT1	0.68	0.456	1.73E-26	0.4452	1.025
DHPS	0.713	0.48	9.82E-12	0.4415	0.5256
VIM	0.553	0.257	1.35E-20	0.3385	1.241
SPHK1	0.667	0.367	8.17E-13	0.3114	0.5622
TPM2	0.587	0.323	1.59E-13	0.3071	0.6386
PLEC	0.94	0.834	4.39E-13	0.2802	0.2534
MAN2B1	0.613	0.441	2.55E-16	0.2603	0.4315
THBS2	0.587	0.259	3.45E-12	0.2372	0.468
MMP1	0.373	0.127	9.77E-11	0.2348	1.064
BGN	0.513	0.21	4.32E-15	0.2196	0.7238
GUS2	0.32	0.062	9.16E-17	0.1381	1.732
PXDN	0.64	0.296	3.13E-15	0.1204	0.2762
CSPG4	0.573	0.256	6.69E-20	0.08268	0.2743
ANPEP	0.4	0.076	7.33E-21	0.06472	0.4153
CLDN14	0.2	0.025	2.48E-11	0.02819	0.3622

pct.1 and pct.2 denote the proportion of cells in each group (delta high and low respectively) with non-zero expression of a given gene. Average expression and logFC show averaged log₂ TPM value across all cells and its difference between the groups being compared

- Cancer cells expressing 'high' versus 'low' levels of PIK3CD identified differentially expressed genes. (Table 2). For PIK3CD-'High' expression cancer cells, the DE genes identify GO processes for cell proliferation, cell adhesion, regulation of cell proliferation, tissue development, cytokine-mediated signaling pathways, extracellular organization, response to stress and immune system process (Top 10 identified).

3: Tumor Microenvironment Cell Type Abundance & PIK3 isoform expression

Table 3: Cell Type Abundance from single cell analysis of HNSCC Tumors

Cell Type	Cell Number	% of Total Cells
T Cell	1149	25.30%
Endothelial	197	4.34%
Mast	113	2.49%
Macrophage	92	2.03%
B Cell	72	1.59%
Dendritic	47	1.04%
Cancer Cell*	1427	31.42%
Fibroblast	1112	24.49%
Myocyte	19	0.42%

*separate algorithm for malignant cells

- Across all the primary HNSCC tumors combined, 57% of the cells were non-malignant, and ~25% of the total cells were T cells. This analysis excludes samples collected from draining lymph nodes (Table 3)

Table 4: Percentage of Cells Expressing PIK3 Isoforms in HNSCC tumors

	T Cell	Macrophage	B Cell	Dendritic	Mast	Cancer Cell	Fibroblast	Endothelial	Myocyte
N	1149	92	72	47	113	1427	1112	197	19
% Cells PIK3CD	32.4	23.9	12.5	14.9	11.5	19.6	7	5.6	10.5
% Cells PIK3CG	11.1	29.4	55.6	40.4	15	0.1	0.6	6.6	0
% Cells PIK3CA	3.0	9.8	4.2	21.3	11.5	14.4	6.8	8.6	5.3
% Cells PIK3CB	4.2	17.4	2.8	17.0	9.7	27.3	4.0	10.2	5.3

N=Number of cells

- PIK3CD and PIK3CG expression was observed in T cells (32% & 11%, respectively), B cells (13% & 56%), dendritic cells (15% & 40%) and macrophages (24% & 29%) in HNSCC primary tumors. PIK3A and PIK3B were less frequently expressed in macrophages (10% & 17%) and T cells (3% & 4%) than PIK3D (Table 4)

4: PIK3 isoform expression in TME

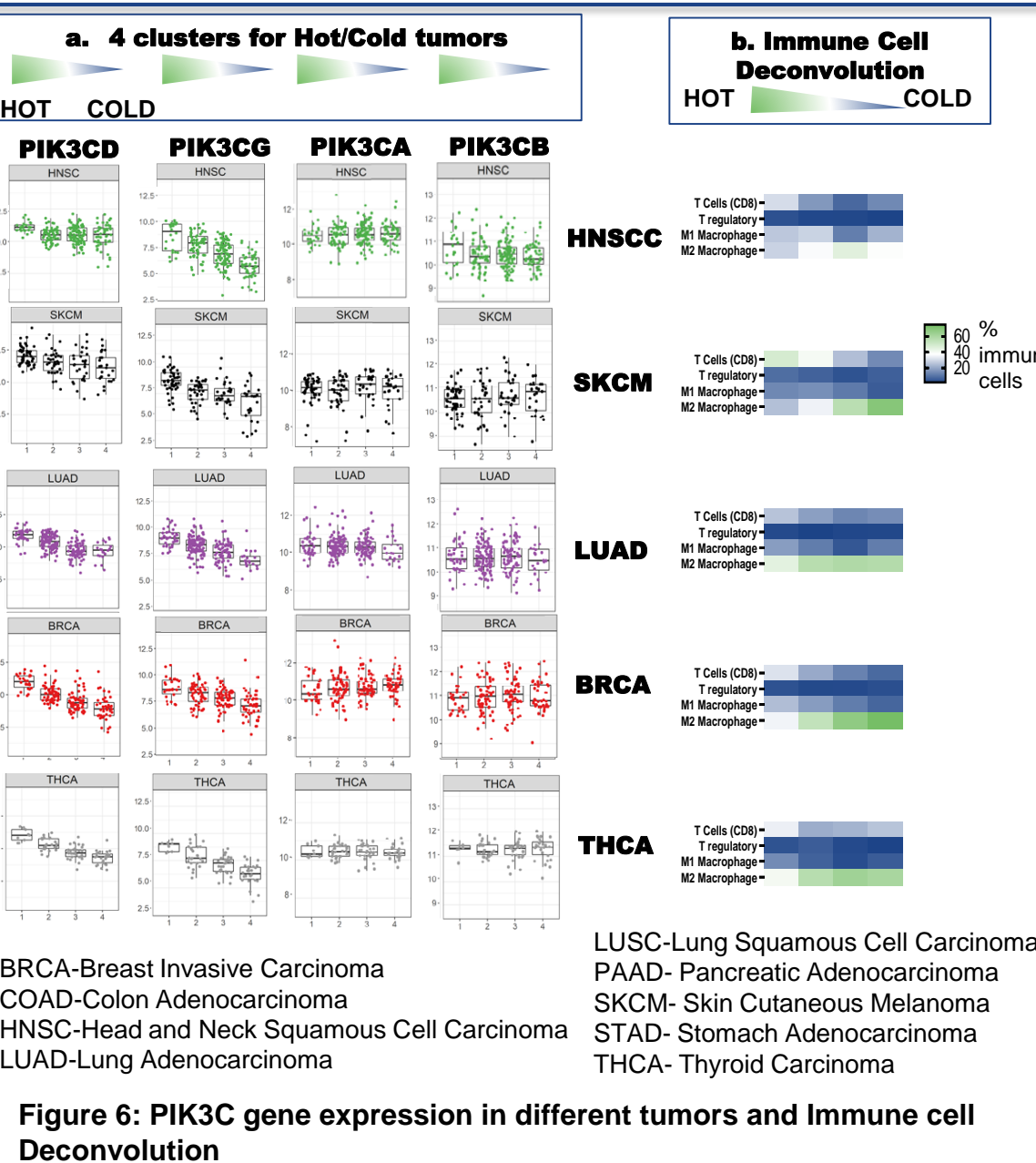


Figure 6: PIK3C gene expression in different tumors and immune cell deconvolution

- A correlation was observed between higher PIK3CD and PIK3CG expression in 'Hot' (median ≈ 11 log₂ TPM & ≈ 9.2 log₂ TPM) compared to 'Cold' tumors (median ≈ 9.4 log₂ TPM & ≈ 6.6 log₂ TPM) (Figure 6a).
- PIK3CA and PIK3CB expression levels are not associated with 'Hot' or 'Cold' clustering (Figure 6a)
- Most 'Hot' cluster tumors contained higher fractions of CD8+ T cells, M1-type macrophages, and lower M2-type macrophages; with Tregs at similar abundances in both 'Hot' and 'Cold' clusters (CIBERSORT deconvolution, Figure 6b)
- PIK3CD & PIK3CG expression is mostly mutually exclusive in T cells, with very few PIK3CA or PIK3CB expressing T cells (Figure 7 a).

- T Cells were further clustered into 4 tSNE clusters based on expression of markers. Naive T cells expressed neither CD4 nor CD8. CD4 Th (inc. Treg) cells expressed CD4 and markers such as FOXP3 & IL2RA. The remaining two clusters expressed CD8 and various markers of cytotoxic T cells that was split into cytotoxic T and exhausted T cells due to higher exhaustion markers (TIGIT, CTLA4 etc.)
- PIK3CD expressing cells are in a higher percentage of CD4+ T cells, exhausted, naive and cytotoxic T cells compared to PIK3CG. However, there is no notable difference in expression for the T cell subtypes except PIK3CG which is less commonly expressed in naive T cells.

- B Cells, Macrophages, and Dendritic cells have mostly mutually exclusive expression of the PIK3C genes (Figure 7 b, c & d)

- Except for PIK3CD expressing cancer cells we did not see any populations with high expression of a specific PIK3 isoform with robust differential expression (DE) signatures. PIK3 isoforms expression is detectable in a minority of other cell types thus there is a lack of sensitivity of the assay to detect DE genes

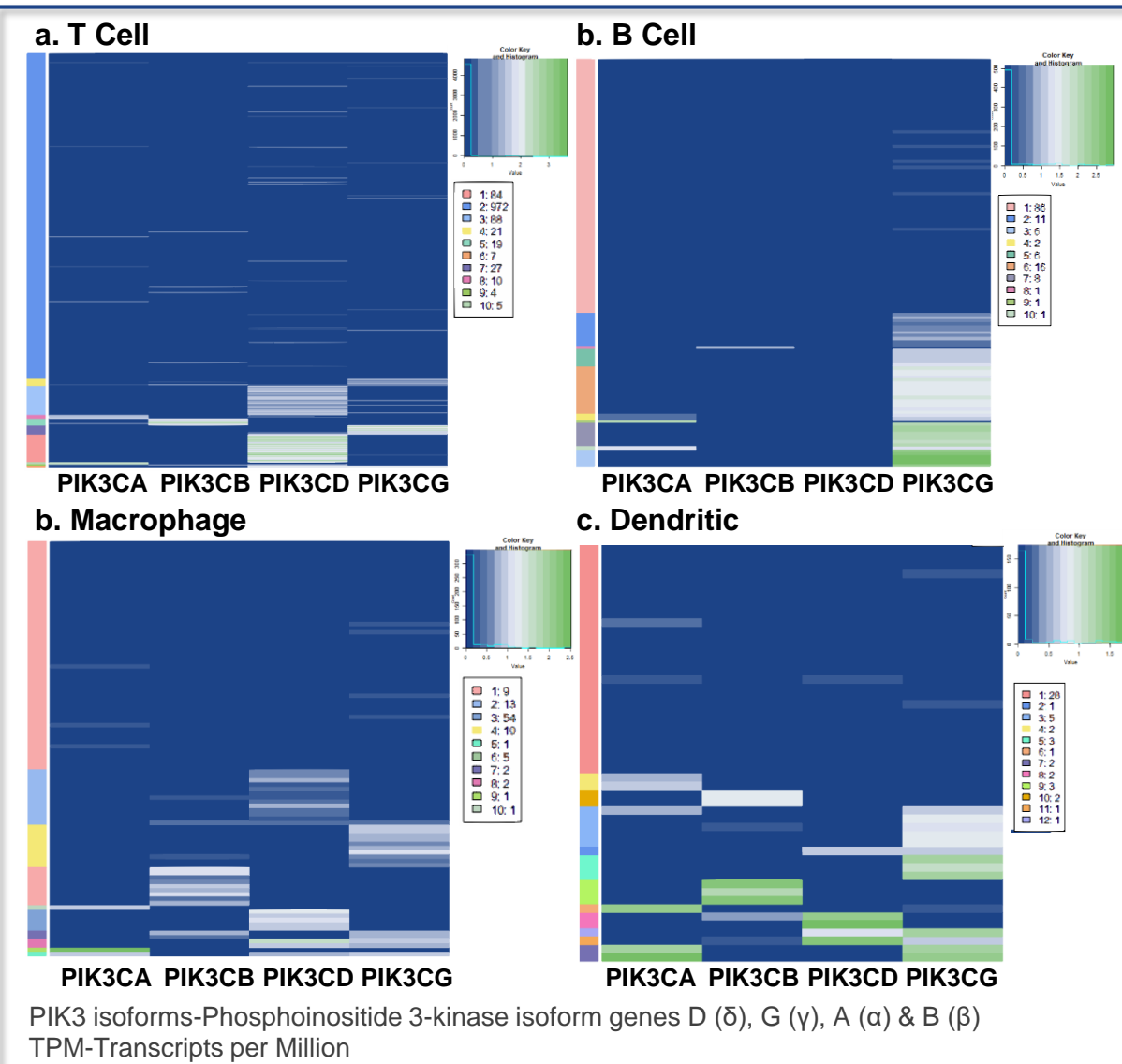


Figure 7: Hierarchical clustering of PIK3 Isoforms in HNSCC TME Cells

CONCLUSIONS

- PI3K δ (PIK3CD) gene expression is found in both head and neck cancer cells and in the immune cells of the suppressive microenvironment
- Individual cancer cells, immune macrophages, T, B, and dendritic cells most frequently have a dominant expression of one PIK3 gene
- The PIK3CD 'high' expression cancer cells have uniquely associated differential expression genes that are identified in cell proliferation and metastasis GO processes
- At AACR, in Abstract #3534, we show in pre-clinical research that duvelisib inhibits PIK3CD and PIK3CG expressing tumor cells derived from HNSCC and melanoma cancers. These findings suggest that tumor treatment by PIK3-isoform specific inhibitors may be guided by cell type expression of PIK3 genes
- This suggests a potential strategy for duvelisib use as monotherapy and in combinations with immunomodulators, such as PD-1 checkpoint inhibitors. The NCT04193293 clinical trial of duvelisib and pembrolizumab is currently open in Head and Neck Cancer

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DISCLOSURES

Hidy: Verastem Oncology; Employment. **Pachter:** Verastem Oncology; Employment. **Weaver:** Verastem Oncology; Employment, Equity Ownership, Patents & Royalties; Inventor; Hillstream Biopharma; Consultancy, Equity Ownership; FemtoDx; Consultancy, Equity Ownership, Membership on an entity's Board of Directors or advisory committees, Patents & Royalties; Inventor.

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