Gene expression and biological pathways associated with differential responsiveness to anti-PD1 immunotherapy in preclinical HPV+ tumors

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Background

- Human papillomavirus induced (HPV+) oropharyngeal cancer is quickly rising in the United States, accounting for as much as 70% of oropharyngeal cancers[1].
- Understanding the genetic determinants in the tumor microenvironment (TME) is important for rational designing of therapeutic strategies.
- The mouse tonsil derived epithelial tumor cell line expressing HPV-16 E6 and E7 genes is well-established as a surrogate preclinical oral HPV cancer model.
- In a recent study using this model, it was observed that mEER tumors implanted in the orthotopic site (tongue) were responsive to anti-PD1 immunotherapy, while those implanted in the flank (non-orthotopic) were resistant to treatment[4].

Hypothesis

- Differences in the expression of genes important for cytotoxic function of innate and adaptive antitumor effector cells (CD8 T cells and NK cells) are associated with response to immune checkpoint therapy
- There is higher expression of cytokines, signaling molecules that mediate and regulate immunity, in the sample of mEER tumors implanted in tongue.

Methods

RNA Extraction and Sequencing

- RNA was extracted from preclinical mEER tumor samples, alongside with immune cell infiltrates, for both the tongue and flank models.
- RNA data was then subjected to quality control testing, leaving 3 samples of the flank tumor and 2 samples of tongue tumor for further sequencing.

- **Identification of Differential Gene Expression**
- DESeq2 and Q-value were determined with the R software and employed to evaluate differential gene expression between the groups.
- The gene expression data is calculated based on the ratio of CPM (counts per million) values, normalized between samples, and principal component analysis (PCA) was performed to ensure that tumor location difference accounts for the majority of variance between gene expression data (Fig. 1).
- A sample of the flank-implanted mEER tumor group is removed due to PCA results highlighting it as an outlier relative to the remaining samples.
- Log fold change values of gene expression were calculated by taking the difference between the average expression of each individual gene from the two groups using the Flank group as baseline, and only genes with an absolute value of log2 ratio ≥ 2 were used for ingenuity's pathway analysis.

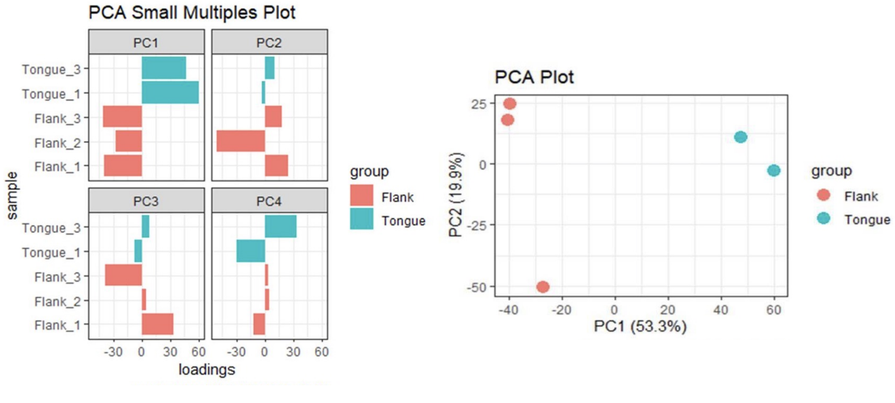

Figure 1. Principle Component Analysis results displaying that (A) tongue and flank tumor accounts for positive and negative loadings in the first principal component (PC1) and that (B) the first principal component accounts for 53.3% of the variability. Additionally, the second Flank sample accounts for majority loading of PC2 which accounts for 19.9% of variability. Hence, this sample is removed as an outlier.

Methods (Continued)

Biological Pathway Analysis via IPA

- The set of significant differentially expressed genes were further analyzed using Ingenuity Pathway Analysis (IPA) to obtain insights on potential pathways associated with treatment responsiveness.
- The p-value for canonical pathways were calculated using Fisher's T-test and the cutoff threshold was set to -log(p-value) ≥ 1.5.

Results

Identification of Differential Gene Expression

- 485 differentially expressed genes altered by >2 log2 fold change in tongue-implanted mEER tumors relative to flank tumors were identified.
- We observe significant upregulation in the expression of immune function related genes encoding such as IFNγ, Cxcl9, Chil1, and Gzmb in mEER oral tumors (Fig. 2).

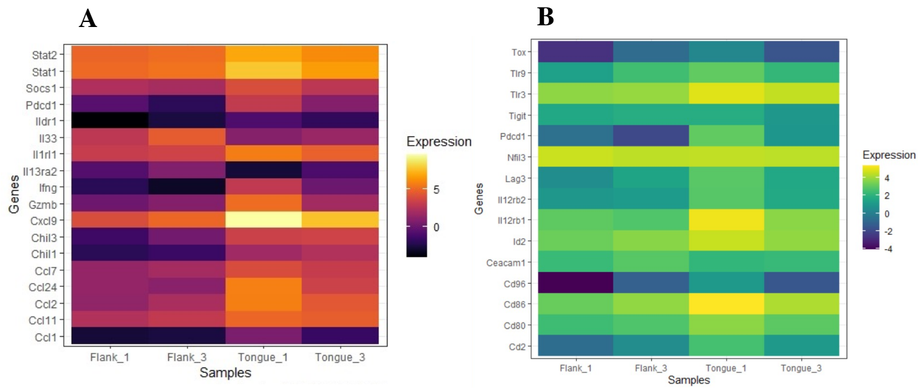

Figure 2. Heatmaps of normalized gene expression values (in CPM) of (**A**) the top differentially expressed cytokines and chemokines[6] and (**B**) NK cell relevant activators, inhibitors[2,3,5].

Biological Pathway Analysis via IPA

- 28 statistically significant canonical pathways were identified (Fig.3).
- Of which, it consists of activated immunological pathways such as interferon signaling and crosstalk between dendritic cells and natural kills cells.

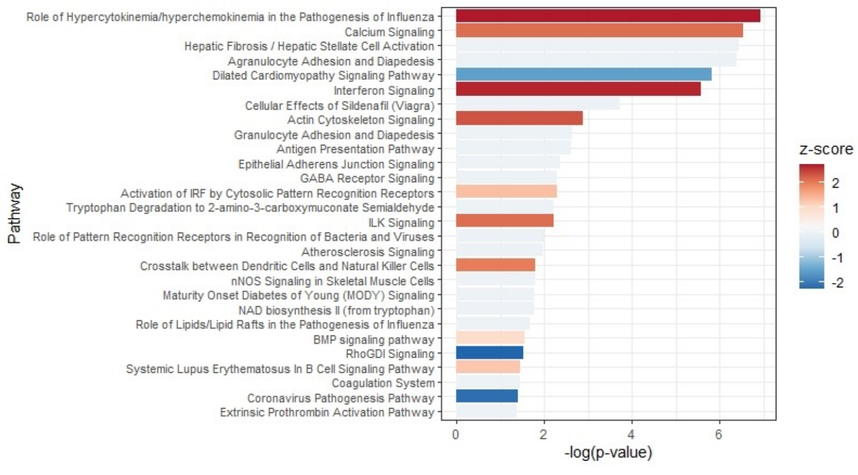

Figure 3. Statistically significant canonical pathways identified by IPA. Higher z-score corresponds to pathways that are predicted to be activated, while lower z-score correspond to inhibited pathways.

- Upstream Analysis highlights two networks related to cytokine expression that may impact immunotherapy response (Fig. 4).

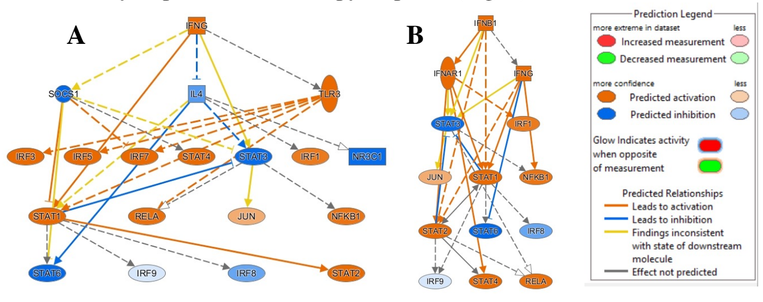

Figure 4. Mechanistic networks of (**A**) IFNγ and (**B**) IFN-β1, two upstream regulators crucial for anti-tumor immune response.

Results (Continued)

- The downstream function analysis predicted an increase in natural killer cell movement (z-score of 2.380, overlap p-value of 1.03E-03) activation of antigen presenting cells (z-score of 1.738, overlap p-value of 3.80E-04), and cytotoxic T-cell activity.

- These activities are consistent with upregulation of various gene encodes such as IFNγ, Cxcl9, Ccl2, etc. (Shown in Table 1)

GeneID	Log2FC	GeneID	Log2FC
Cxcl9	4.18	Clec7a	3.50
Ifng	4.10	Ccl2	3.33
Chil3	4.03	Cxcl10	3.21
Chil1	3.94	Pdk4	3.09
Ccl2	3.33	Ido1	2.77
Cxcl10	3.21	Ccl11	2.11
Ccl1	2.15	Stat1	2.02
Fasl	2.08		

Table 1.

Natural Killer Activators and Inhibitors

- Upon further examination of natural killer activator and inhibitory molecules and NKDC, we identified overexpression in CD96 and PDCD1 (PD1) in mEER tongue tumors, as potential targets for immunotherapy using anti-CD96 and anti-PD1 checkpoint antibodies (Fig. 2, Table 2).

- Importantly, these gene expression analyses data are validated in experiments where tongue implanted tumors showed better response to anti-CD96 (unpublished) and anti-PD1 immunotherapies[4].

geneID	log2FC	classification
Pdcd1	3.28	Inhibitor
Cd96	2.37	Inhibitor
Cd2	1.89	Activator
Il12rb1	1.52	NKDC
Cd86	1.30	NKDC
Lag3	1.28	Inhibitor
Il12rb2	1.25	NKDC
Tox	1.09	Transcription
Tlr3	0.88	Activator
Tlr9	0.76	Activator
Cd80	0.75	NKDC
Id2	0.72	Transcription
Nfil3	-0.08	Transcription
Tigit	-0.32	Inhibitor
Ceacam1	-0.39	Inhibitor

Table 2.

Conclusion

- Cytokines and chemokines crucial to immune response is observed to be overexpressed In the tongue implanted mEER tumor samples, which are molecular signatures indicative of an immunologically 'hot' tumor relative to the flank[1].
- The lack of significant differential expression for most natural killer cell activators and inhibitors such as TIGIT, Lag3, and Tlr3 alongside the abundance of cytokines overexpression and evidence of activated interferon signaling in the tongue tumor suggests that while the immune system may recognize the presence of the tongue tumor, there is blockade in effective attack against the tumor itself whereas the immune system may not be recognizing the tumor in the flank implanted tumor sample[5].
- The overexpression of CD96 and PD1, despite their role as natural killer cell inhibitors, in the tongue implanted mEER tumor suggests that immunotherapy efficacy is higher for the tongue implanted tumors due to an abundance of checkpoint molecules identified for targeting.
- Further studies should be conducted to determine the exact effects of the overexpressed chemokines in the tongue implanted model.

References

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