Profiling Exhausted T Cells using Vectra® Polaris™ Multiplex Immunofluorescence Assay in HNSCC

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Introduction

Background: Head and neck squamous cell carcinoma (HNSCC) is a cancer with the ability to modulate the immune system to evade detection. It is the sixth most frequently diagnosed cancer with 550,000 new cases and 300,000 lives lost worldwide per year. New treatments for HNSCC are urgently needed as patients continue to experience a high mortality rate and low response to surgery and chemotherapeutic treatments. Part of the reason why HNSCC is difficult to treat is it upregulates the expression of immune-checkpoint signaling molecule TIGIT (**T** cell **i**mmunoreceptor with **I**g and **IT**IM domains) to inhibit T cell activation in vivo. Emerging evidence shows TIGIT overexpression in the CD8+ and CD4+ T cells that infiltrate the tumor cells of HNSCC patients. TIGIT expression is also associated with up-regulation of immune-checkpoint ligands PD-1 (programmed cell death protein 1) and LAG-3 (lymphocyte-activation gene 3 aka CD223), markers of T-cell exhaustion. Altogether, activation of the TIGIT/PD-1/LAG-3 axis correlates with an immunosuppressive microenvironment as well as cancer development and progression. Although there is ample evidence that the upregulation of TIGIT decreases the immune response in HNSCC, only limited studies have been published that address the location, expression and co-expression of TIGIT, LAG-3 and PD-1 in the HNSCC microenvironment.

Methods: In this study, we sought to establish a robust report of immune cells in the tissue of patients with HNSCC. Using Vectra Polaris multiplex immunofluorescence (IF) assays, we studied T-cell expression and T-cell exhaustion in HNSCC patient tissue using a total of 9 markers essential in cancer immunology. Sequential tissue sections were stained in two panels, an exhausted T cell panel comprised of TIGIT, PD-1, LAG-3, CD4 and CD8 and a T cell panel including CD3, FOXP3, CD68, pan-cytokeratin, CD4 and CD8.

Results Multiplexing IF staining revealed a HNSCC histologic landscape characteristic of immune suppression in this study. The data demonstrated abundant T cells in the tissue microenvironment of HNSCC samples. Using Indica Halo algorithms, we quantified T helper cells (CD3+CD4+), T cytotoxic cells (CD3+CD8+), T regulatory cells (CD3+CD4+FoxP3), and different subtypes of exhausted T cells, within the tumor and the stromal regions.

Conclusions: Currently AB154, a fully humanized immunoglobulin G1 monoclonal antibody targeting human TIGIT is in phase ! clinical trials in HNSCC patients and BGB-A1217, an anti-TIGIT monoclonal antibody in combination with anti-PD-1 monoclonal antibody Tislelizumab is in a Phase 1/1b clinical trial in patients with advanced solid tumors. The Vectra Polaris imaging reported in this study identifies T cell composition in the tumor microenvironment of patients facing high mortality and the findings in this study can be used to identify the additional opportunities for combination immunotherapy.

Assay Development & Panel Specifications

A

Staining
- Automated staining workflow
- Opal TSA-based amplification reagent kit
- **Polaris 7-Color**: 480/ 520/ 570/ 620/ 690/ 780

Imaging
- **Polaris Vectra**
- Whole slide multispectral imaging up to 7 colors
- Fully automated continuous loading (80 slides)

Analysis
- **InForm**: Spectrally unmixing, Autofluorescence removal
- **Indica Halo**: Tissue seg, cell seg, phenotyping, spatial analysis

B

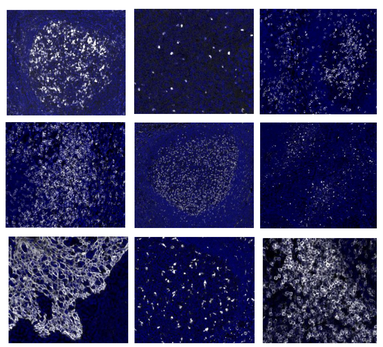


Table 1. Panel Composition

Panel 1	
CD3	panCK
FOXP3	CD4
CD68	CD8

Panel 2	
TIGIT	CD4
PD1	CD8
LAG3	

Table 2. Phenotyping of T cells and exhausted T cells

Co-expression	Phenotypes
CD3+CD4+	T helper cells
CD3+CD4+FOXP3+	T regulatory cells
CD3+CD8+	T cytotoxic cells
PD-1+CD4+	Exhausted T cells
PD-1+CD8+	
LAG3+CD4+	
LAG3+CD8+	
TIGIT+CD4+	
TIGIT+CD8+	
PD-1+LAG3+	
PD-1+TIGIT+	
LAG3+TIGIT+	
PD-1+TIGIT+LAG3+	

Figure 1. OPAL Assay Development. **A. Demonstration of Polaris Workflow** For panel staining, optimized antibody conditions were applied to a 4 µm section of tissue, followed by tyramide amplification and secondary OPAL dye. Heat retrieval enabled a subsequent round of staining with another marker and OPAL fluorescent antibody. HALO algorithms generate precise identification for every cell. **B.** Representative monoplex images.. Markers comprising each T cell panel are listed in Table 1 and the phenotypes are summarized in Table 2.

Characterization of Immune Suppressive Cells in HNSCC

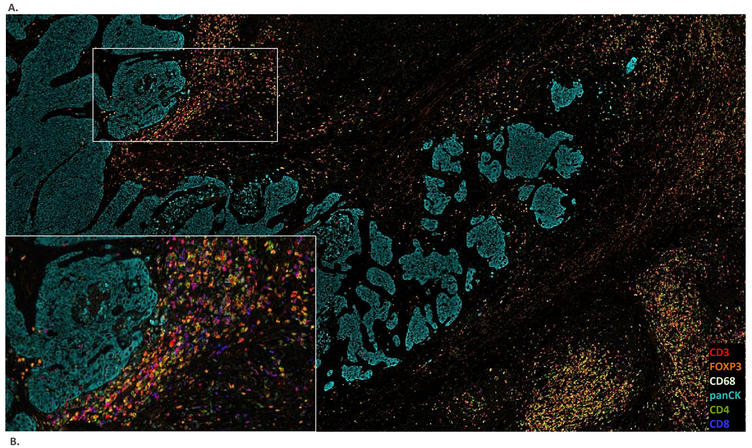
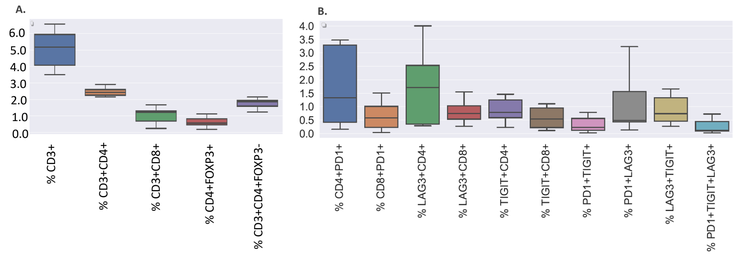


Figure 2. Characterization of Immuno-suppressive microenvironment in HNSCC. A. Vectra Polaris imaging of HNSCC tissue stained with T cell panel comprised of CD3, FOXP3, CD68, panCK, CD4 and CD8 markers. B. Sequential slide of HNSCC tissue in **A** stained with exhausted T cell panel including TIGIT, LAG3, PD-1, CD4 and CD8. **C.** PD-1+LAG+, PD-1+TIGIT+, TIGIT+LAG3+ staining in HNSCC. White arrows indicate co-expressing cells.

HALO Analytics



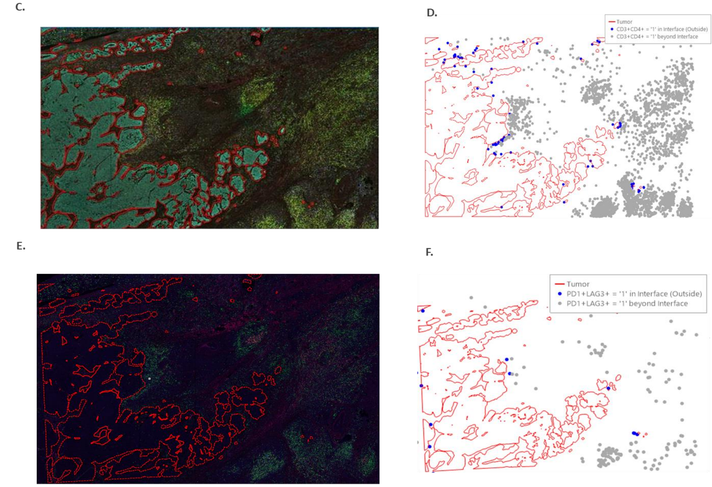


Figure 3. HALO Image Analysis.
A & B: Boxplots of classification results from Indica HALO analysis. Percent of positive cells reported for co-expressions in T cell panel(A) and Exhausted T cell panel(B).
C –F: Spatial analysis of phenotypes with respect to Tumor
C. Color overlay of panCK (cyan), T cells (red), killer T cells (blue), helper T cells (green) markers from the T cell panel. PanCK region is outlined in red.
D. HALO spatial plot: tumor region outlined in red. Helper T cells within 50 microns of tumor are shown in blue. Helper T cells more than 50 microns away from tumor shown in grey.
E. Exhausted T cell panel image(serial section) was registered to the T cell panel image and the panCK area is transferred to Exhausted T cell panel image. There is a slight rotation between the two serial sections. **.** Color overlay of PD1(red), LAG3(blue) and CD4(green).
F. HALO spatial plot: tumor region outlined in red. PD1+LAG3+ cells within 100 microns of tumor are shown in blue. PD1+LAG3+ cells more than 100 microns away from tumor shown in grey.

Key Findings

- Two panels were used in this study to characterize the immune landscape of FFPE samples from HNSCC patients.
- Multiplexing IF staining revealed a HNSCC histologic landscape characteristic of immune suppression in this study. Overall, helper T cells were more prevalent than cytotoxic T cells in this study.
- Different subtypes of exhausted T cells were observed in the study. Co-expression of PD-1+TIGIT+, PD1+LAG3+, TIGIT+LAG3+ and PD1+TIGIT+LAG3+ in T cells were also present in the TME of HNSCC samples in this study.
- Halo algorithms were used to study the spatial correlation of T cells with respect to tumor cells.

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