Characterizing viral mRNA and immuno-protein expression in head and neck squamous cell carcinoma using a novel automated RNAscope™/Polaris™ integrated assay

Sara G. Pollan • Arezoo Hanifi • Mate Nagy • Nicholas Stavrou • Erinn Parnell• Courtney Todorov • Maricel Gozo • Nickolas Attanasio • Josette William • Qingyan Au
NeoGenomics Laboratories, Aliso Viejo, CA

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Introduction

Background: Chronic viral infection can generate inflammatory microenvironments leading to neoplastic growth and cancer development. Cancer patients with chronic viral infection have been shown to exhibit worse outcomes compared to non-infected individuals. One such cohort, head and neck squamous cell carcinoma (HNSCC) patients infected with chronic human cytomegalovirus (CMV), have increased risk of death when receiving radiotherapy or radiochemotherapy. This is clinically significant as HNSCC already carries a high mortality rate and low response to surgery and chemotherapeutic treatment. The ability to detect and localize viral infection and immune response in the same patient tissues has been historically under-developed and may assist in stratifying patients for therapeutic intervention.

Methods: Immune infiltrate to tumor in CMV infected samples was assessed in HNSCC patient tissue using RNAscope in-situ hybridization (ISH) probe to detect CMV mRNA and Vectra® Polaris™ automated multiplex protein detection of CD8, PD-L1, CD68 and panCK with OPAL dyes.

Results: We developed a novel automated RNAscope™/Vectra® Polaris™ integrated multiplex immunofluorescence (IF) assay with OPAL detection and quantification of signal using Indica HALO® algorithms. The results include quantitative and reproducible RNAscope fluorescent ISH counting, cell-by-cell expression profiles, multiplex protein quantification and whole-slide image analysis.

Conclusion: NeoGenomics Laboratories RNAscope™/Polaris™ integrated assay detects and quantifies CMV viral infection and protein expression of PD-L1 positive and negative cytotoxic T cells and macrophages within and adjacent to HNSCC tumor regions.

Assay Workflow & Panel Specifications

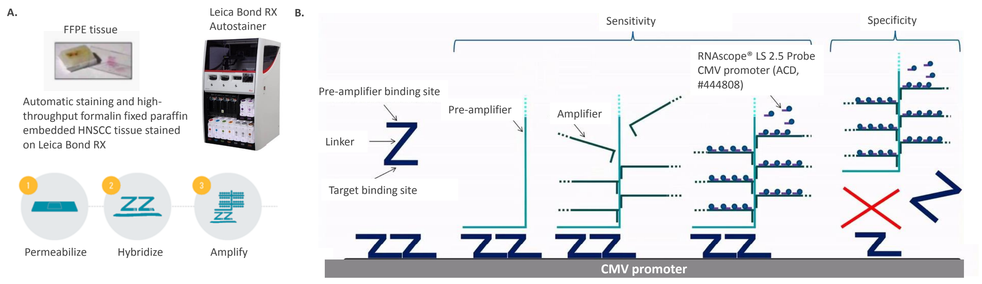

Figure 1. RNAscope™/Vectra® Polaris™ integrated assay staining protocol. **A. Step 1: RNAscope™.** FFPE HNSCC tissue is stained on a Leica Bond RX Autostainer. Tissue is first permeabilized then hybridized with CMV RNAscope probe which is amplified for detection of CMV RNA. **B. RNAscope™ on CMV promoter. C. Step Two: Vectra® Polaris™** In the same automated protocol as RNAscope™, tissue is stained for protein markers using Vectra® Polaris™ workflow from Akoya Biosciences. This includes rounds of optimized antigen retrieval, primary antibody, Opal HRP and secondary fluorophore binding. Proprietary cell segmentation algorithms using Indica HALO® software allow positive identification of each marker.

Table 1

5-Marker Panel	
1	CMV
2	CD8
3	PD-L1
4	CD68
5	panCK

Table 2

Expressions	Phenotypes
CMV+	Cytomegalovirus RNA
CD8+	T cytotoxic cells
CD68+	Macrophage
PD-L1	Programmed death ligand-1
panCK	Tumor segmentation

Table 3

Co-Expressions	Phenotypes
CMV+CD8+	Cytomegalovirus infected T cytotoxic cell
CMV+CD68+	Cytomegalovirus infected macrophage
CMV+PD-L1+	Cytomegalovirus infected programmed death ligand-1 expressing cell
CMV+panCK+	Cytomegalovirus infected tumor region

Table 1. 5-Marker panel RNAscope™/Vectra® Polaris™ integrated assay used in the study.

Table 2. Phenotyping of human tumor-associated RNA and protein in the study. RNA and protein markers analyzed in the tumor samples. **Table 3. Co-expression phenotypes in the study.** Co-expressing phenotypes analyzed in the tumor samples.

Characterization of CMV RNA and Immune Cells in HNSCC

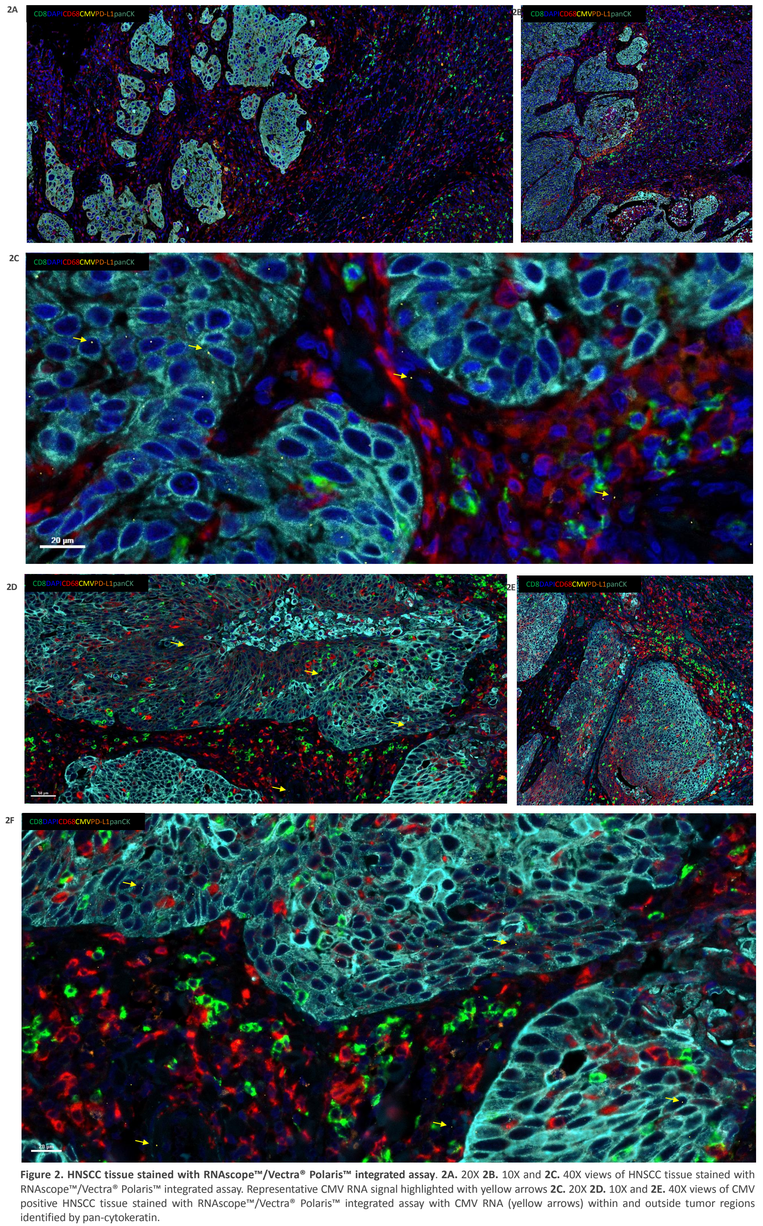

Figure 2. HNSCC tissue stained with RNAscope™/Vectra® Polaris™ integrated assay. **2A.** 20X **2B.** 10X and **2C.** 40X views of HNSCC tissue stained with RNAscope™/Vectra® Polaris™ integrated assay. Representative CMV RNA signal highlighted with yellow arrows **2C.** 20X **2D.** 10X and **2E.** 40X views of CMV positive HNSCC tissue stained with RNAscope™/Vectra® Polaris™ integrated assay with CMV RNA (yellow arrows) within and outside tumor regions identified by pan-cytokeratin.

HALO® Analytics

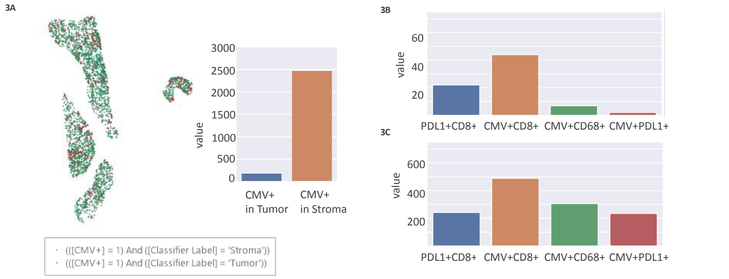

Figure 3. Quantification and Localization of HNSCC cells with one CMV+ dot identified by HALO® Analytics in a HNSCC sample. 3A. A CMV+ HNSCC sample stained with RNAscope™/Vectra® Polaris™ integrated assay. DAPI used for nuclei and panCK for tumor segmentation. Each nuclei with one CMV+ dot is identified, localized and quantified in tumor (red dot, blue bar) and stroma regions (green dot, orange bar).**3B.** Co-expressing cells indicated on x-axis quantified within Tumor area **3C.** Co-expressing cells indicated on x-axis quantified within Stroma

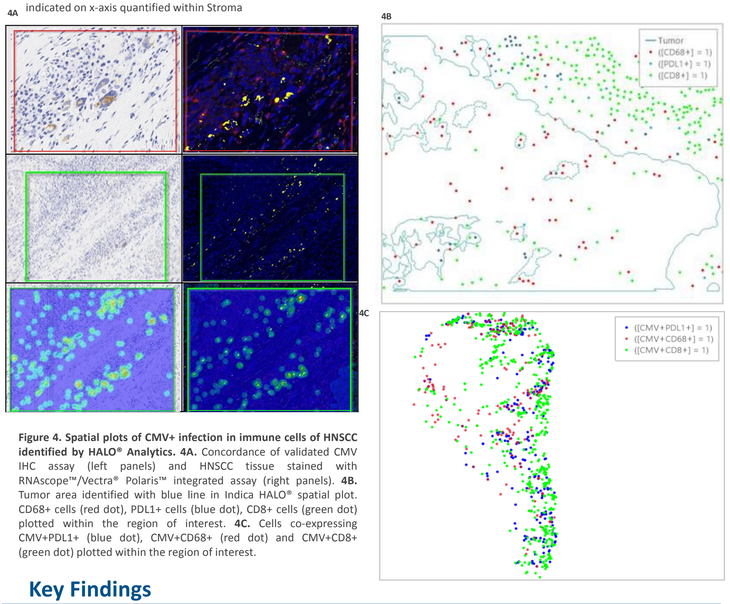

Figure 4. Spatial plots of CMV+ infection in immune cells of HNSCC identified by HALO® Analytics. 4A. Concordance of validated CMV IHC assay (left panels) and HNSCC tissue stained with RNAscope™/Vectra® Polaris™ integrated assay (right panels). **4B.** Tumor area identified with blue line in Indica HALO® spatial plot. CD68+ cells (red dot), PDL1+ cells (blue dot), CD8+ cells (green dot) plotted within the region of interest. **4C.** Cells co-expressing CMV+PDL1+ (blue dot), CMV+CD68+ (red dot) and CMV+CD8+ (green dot) plotted within the region of interest.

Key Findings

- A novel RNAscope™/Vectra® Polaris™ integrated assay was used in this study to characterize the immune landscape of FFPE samples from head and neck squamous cell carcinoma (HNSCC) patients.
- Cytomegalovirus (CMV) RNA was detected along with CD8, PD-L1, CD68 and pan-cytokeratin protein expression. Co-detection of RNA and protein in the same tissue provides critical information including cell type specific RNA transcript levels.
- The integrated panel is shown to spatially correlate with a validated IHC assay and reveal distribution of RNA expression in immune cell subsets within the tumor microenvironment of HNSCC samples.
- In this study, CMV RNA co-localized with CD8+ cytotoxic T cells, CD68+ macrophages or PD-L1+ cells in HNSCC tissue.
- Custom integrated panel generation techniques used in this panel may be applied to detect other viral MRA transcripts in cancer tissue and the immune landscape within the tumor microenvironment.

sara.pollan@neogenomics.com