Immune profiling of tumor infiltrating lymphocyte subsets, myeloid cells and HPV status of HNSCC biopsies using multiplex immunofluorescence

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ABSTRACT: Head and neck squamous cell carcinoma (HNSCC) has both human papilloma virus (HPV) positive and negative etiologies. It is one of the growing number of tumors for which an anti-tumor immune response is implicated to play a role in the course of the disease as shown by responsiveness to immune-modulation using PD-1/PD-L1 inhibitors. Profiling of the tumor microenvironment including specific infiltrating lymphocyte subsets, antigen presenting cells, expression of regulatory molecules on both tumor and immune cells and spatial relationships between cells could lead to predictive signatures for responsiveness to both conventional and immune modulatory therapies. However, such profiling has previously been hindered by the need to identify such cells "in-situ" in tissue sections using multiple cell markers per cell, and limitations in available technology. The current study demonstrates Cell IDx's novel UltraPlex technology, based on a simple two-step protocol using hapten labeled primary antibody cocktails followed by fluor labeled anti-hapten secondary cocktails, allowing simultaneous detection of 4 cellular markers on the same cell. Analysis of HNSCC HPV positive and negative tissue-microarrays combining UltraPlex detection of multiple markers per cell together with serial sections stained with individual phenotyping panels demonstrates a powerful technique for multiplex analysis.

UltraPlex: Proprietary Modified-Hapten-based Technology

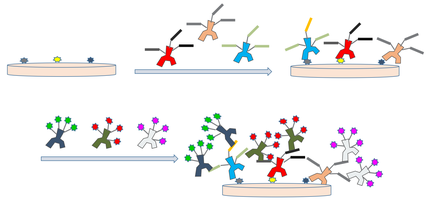


Figure 1: Schematic showing staining of tissue sections using modified hapten-labeled primary antibodies and detection using fluor-labeled anti-hapten secondaries.

METHODS: Primary antibodies were conjugated to 5-7 haptens/antibody. Anti-hapten antibodies were conjugated to fluorophors, Dy490, Dy550, Dy650 and Dy750. Antigen retrieval on 4-5 µm FFPE sections was performed by incubation of the slides in 0.1 mM citrate, pH 6.0 in a pressure cooker for 15 min after standard deparaffination treatment. Tissues were incubated with a cocktail of hapten-modified primary antibodies for 1 hour followed by washing and incubation with fluor-labeled anti-hapten antibodies. Following washing, the slides were imaged on a IC2000 imager (www.valasciences.com, San Diego, CA). Images were processed using ImageJ/Fiji software and image analysis was performed using CyteSeer Image Analysis software (Vala Sciences).

HNSCC Tumor Phenotyping

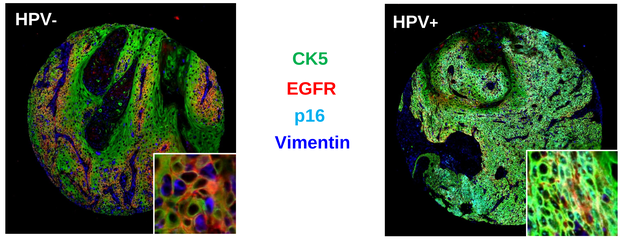
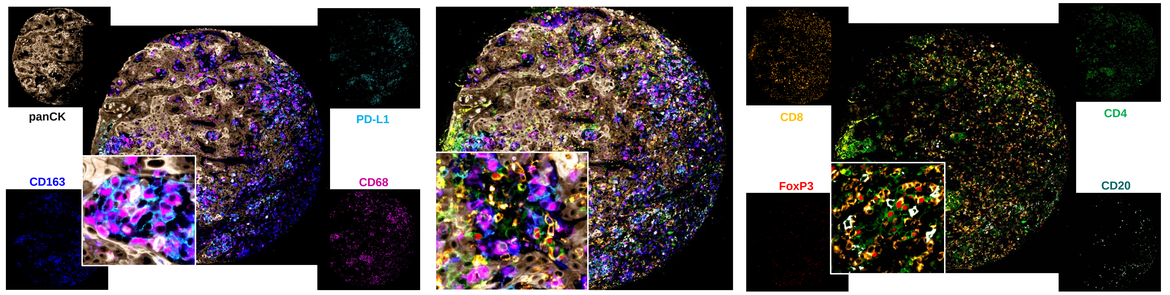


Figure 2: Tumor phenotyping - staining of HPV negative (left) and HPV positive (right) TMA cores for CK5 (green), EGFR (red), HPV p16 (cyan) and Vimentin (blue).

HPV Negative HNSCC



HPV Positive HNSCC

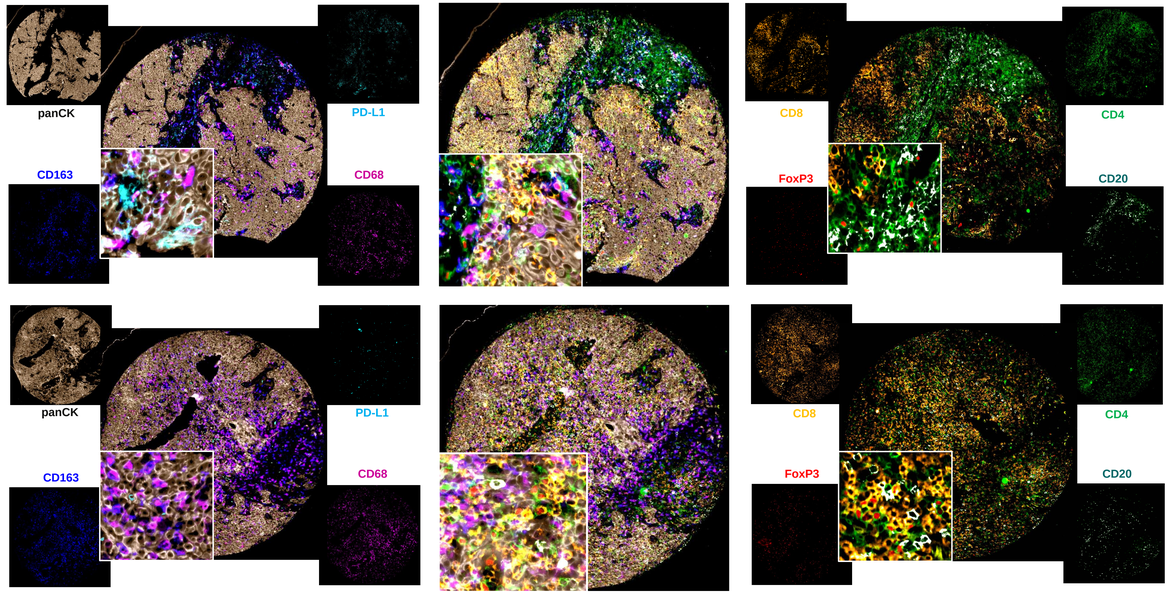


Figure 3: Staining of one HPV negative and two HPV positive HNSCC TMA cores with macrophage panel to determine PD-L1 expression on tumor (pan-CK) versus M1 (CD68+) and M2 (CD163+) macrophages (left panel) and Immune cell panel to analyzeCD4+ cells, CD4+FoxP3+ T regs, CD8+ cells and CD20+ B cells (right panel). Overlay of the two serial sections is shown in the center.

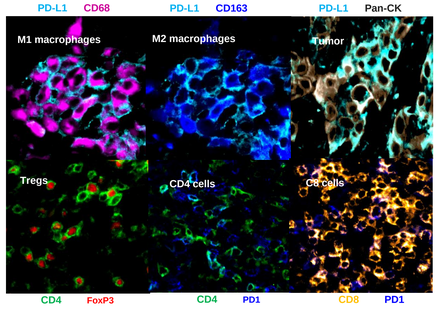


Figure 4: High-power views showing immune subset identification by multiplex staining

UltraPlex mxIF: A Paradigm Shifting and Disruptive Technology

- Primary antibody species independent
- Standard two-step staining protocol employed
- Signals equivalent to fluorescent secondary antibodies
- Significant time savings
- High throughput compatible
- Ability to detect multiple co-localized markers
- Minimal non-specific staining
- Minimal sample amounts required
  - *e.g. core biopsies and TMAs*
- Data readily transferrable to image analysis software
- Detection technology independent
- Potential for higher multiplexing
- Platform technology applicable to all immunoassays

CONCLUSION: A HNSCC TMA composed of duplicate samples of 36 HPV positive patients tumor samples (81% male, age range 45-81 years, 19% female, age range 42-90 years) and 31 HPV negative patient tumor samples (68% male, age range 25-75 years, 32% female, age range 28-80 years) were stained with multiple four-plex panels and images from serial sections overlaid. Multiplex panels included tumor phenotyping, identification of CD8, CD4 T cells, Treg cells, B cells and M1 and M2 macrophages and analysis of expression of PD-L1 on both tumor cells as well as macrophages. Multiple different patterns of expression were observed including samples in which most PD-L1 was expressed by the tumor and others where it was predominately macrophage restricted. Levels of CD8 T cells versus Treg cells also varied. This multiplex approach permits HPV status determination, full immune subset profiling, quantitative marker expression, relative cellular localization and potential for a correlative signature determination associated with disease outcome and response to treatment.

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