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Profiling Cytokeratins in the Tumor Microenvironment Using Multiplex Immunofluorescence

#1475

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Background

Intermediate filaments are a vastly diverse group of proteins responsible for the structure and function of the cytoskeleton. Among these filaments, the keratins comprise the largest portion of the group and provide mechanical and functional support to epithelial cells. Cytokeratins have been regarded as a predictive disease marker and provide important clinical information such as tumor growth, potential metastases, and response to therapeutic agents. For clinical purposes, it's important to capture the presence/absence of the correct keratins in a tumor microenvironment, as each has distinct diagnostic purposes (i.e., CK5/6 positivity is specific for bladder condyloma acuminatum versus basal/patchy staining in papillary noninvasive urothelial carcinoma). This study profiled Cytokeratins (types I and II) and the overall immune profile within the tumor microenvironments of numerous cancer tissues to showcase the importance individually and collectively as tumor biomarkers.

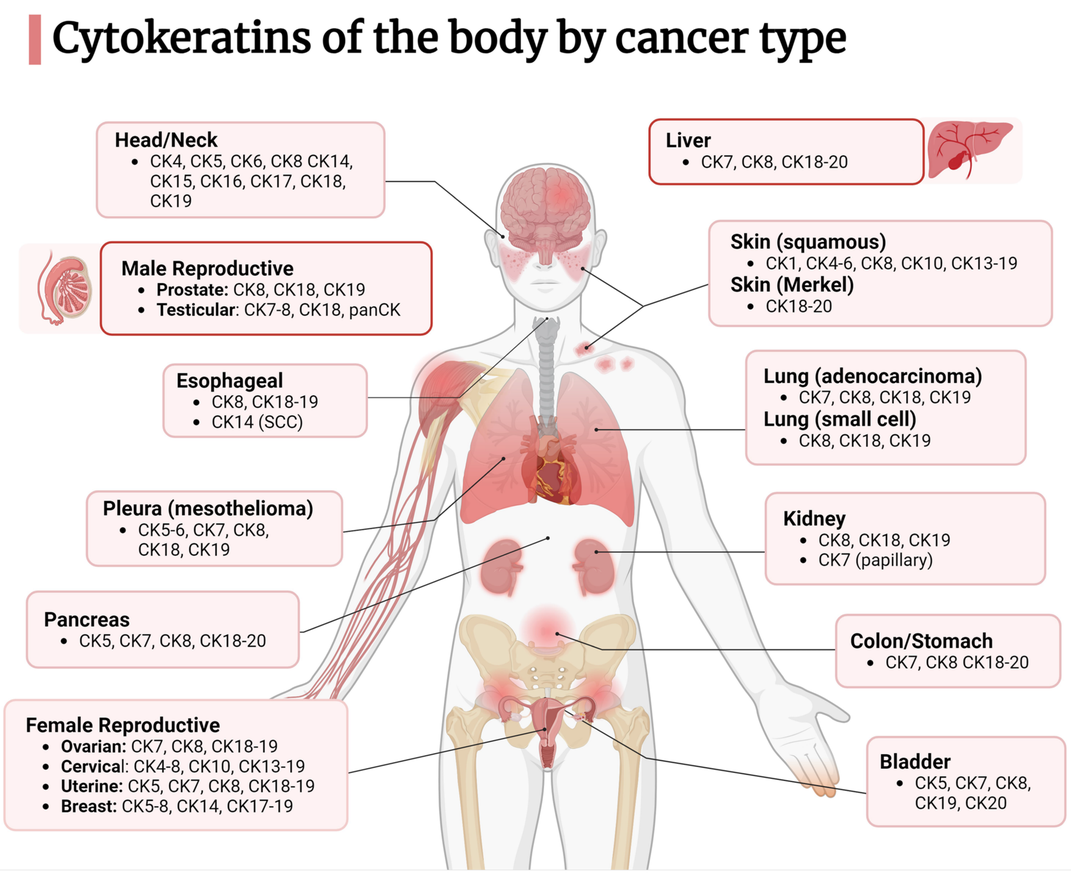

Figure 1. Cytokeratins of the human body. Each organ system and cancer type has cytokeratin profiles that are associated with it of prognostic significance. Additionally, the absence or presence of these markers within normal tissue of the same organ system can be differentially expressed. Image created with BioRender.com.

Methods

Tissue microarrays containing multiple (80+) FFPE human tissue types (cancerous and normal) were stained with Bethyl Laboratories IHC-validated primary antibodies. A panel consisting of ten immune targets (CD3e, CD20, FoxP3, CD8a, PD-L1, panCK, PD-1, CD68, CD45, PCNA), three epithelial to mesenchymal transitionary (EMT) targets (Vimentin, E-cadherin, Zeb1), and six Cytokeratins (CK7, CK8, CK14, CK18, CK19, CK20) was used to profile the tumor microenvironment. Optimization of antibody concentration, elution temperature, and organization of each targeted pair was performed for each biomarker prior to composite multiplex staining. Positive and negative control tissues were first utilized to validate each antibody prior to utilizing microarrays Antigen retrieval was done in the Epredia PT Module using Tris-EDTA ph9 solution, at 100°C, for 1 hour incubation. Automated immunofluorescent staining and imaging of the samples was performed on the Lunaphore COMET™ system. Subsequent analysis of images for distinct phenotypes was done using the HORIZON™ software.

Antibody	Host	Catalog #	Panel Significance
CD3e	Rb	A700-016	Total T-cell count
CD20	Ms	A500-017A	B-cell marker
FoxP3	Rb	A700-281	Regulatory T-cells
CD8a	Ms	A500-021A	Cytotoxic T-cell marker
PD-L1	Rb	A700-020	Checkpoint inhibitor
CD45	Rb	A700-012	Leukocyte marker
PD-1	Rb	A700-076	T-cell activation
CD68	Ms	A500-018A	Macrophage marker
PCNA	Ms	A500-024A	Cell proliferation
Vimentin	Rb	A700-100	EMT - mesenchymal
Zeb1	Rb	A700-217	EMT - transcription factor
E-cadherin	Rb	A700-088	EMT - epithelial
panCK	Ms	A500-019A	AE1/AE3 – broad CK marker
CK7	Rb	A700-186	Simple Epithelial – Type II
CK8	Rb	A700-299	Simple Epithelial – Type II
CK14	Ms	A700-293	Complex Epithelial – Type I
CK18	Ms	A500-035A	Simple Epithelial – Type I
CK19	Ms	A500-036A	Simple Epithelial – Type I
CK20	Rb	A700-105	Simple Epithelial – Type I

Table 1. Selection of immune (solid), EMT (dashed), and intermediate filament (dots) markers used in the study.

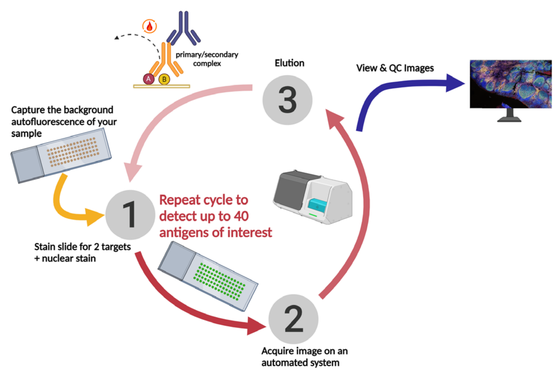

Figure 2. Cyclic immunofluorescent staining and imaging method designed for use with the Lunaphore COMET™ instrumentation and workflow. Antigen retrieval methods prior to automated staining is independent of this process.

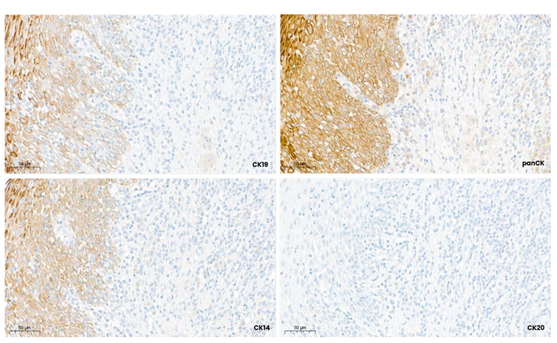

Figure 3. Chromogenic (DAB) stains performed on human tissue to ensure optimized concentration, correct localization of the target, and serve as quality check for the antibodies used. Pictured above are serial sections of a tissue microarray (tonsil in focus) stained with CK14, CK19, CK20, and panCK.

Results

Cytokeratin profiles were examined in conjunction with immune phenotypes. Any samples that experienced a significant loss of tissue due to technical procedures were excluded from analyses. An increase in CK19 coupled with a decrease in CK14 points to enhanced potential for malignancies, whereas the reverse or relatively similar levels trend towards benign disease status. In addition to this signature, the relationship between malignancy and/or metastasis and high expression of CK7 in was observed. Tissues that exhibited a CK8+/CK18+ phenotype, particularly breast cancers (invasive ductal carcinomas), were linked to malignancies. Finally, high expression of Vimentin (EMT) was correlated with metastasis, and this has been shown to be a target indicative of poor prognosis.

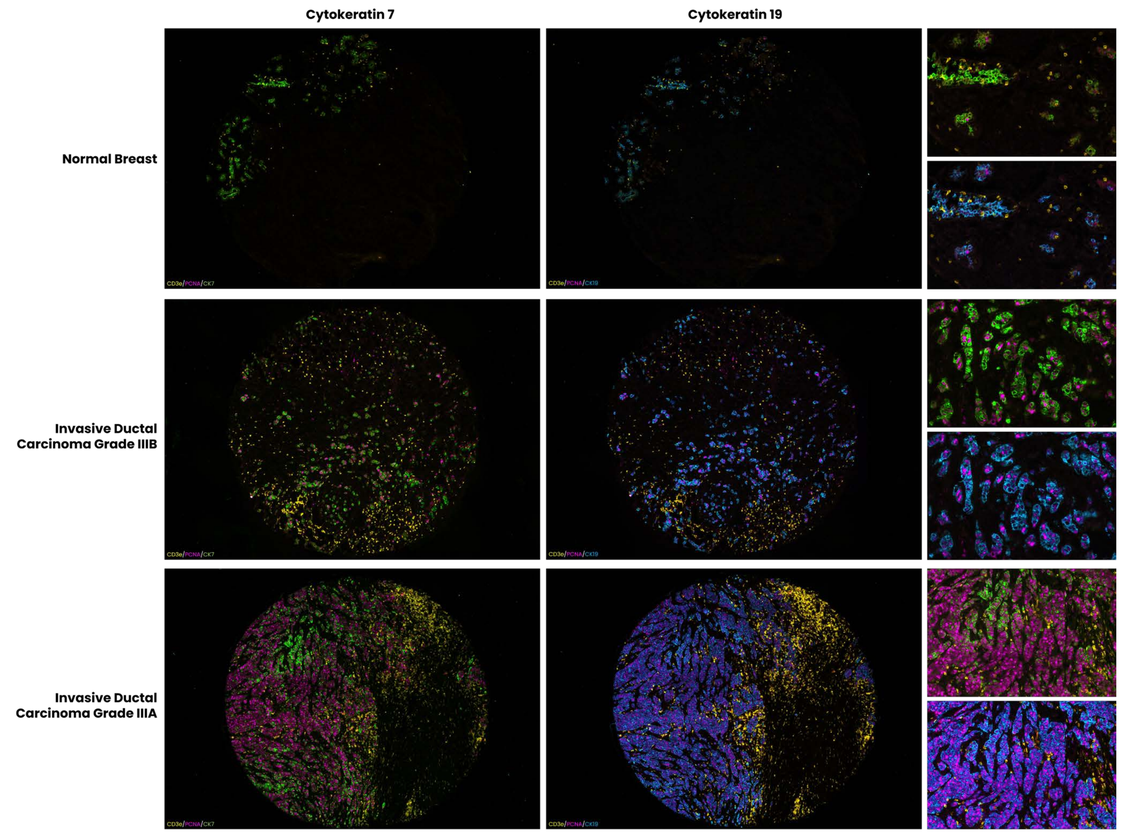

Figure 4. Comparison of Cytokeratins 7 and 19 in normal breast tissue and invasive ductal carcinomas. CD3e and PCNA were observed in conjunction with the keratins to track proliferation and t-cell counts. Cytokeratin 19 is slightly more prevalent than CK7 in grade IIIA breast carcinoma tissue which highlights the incomplete overlap in the two proteins.

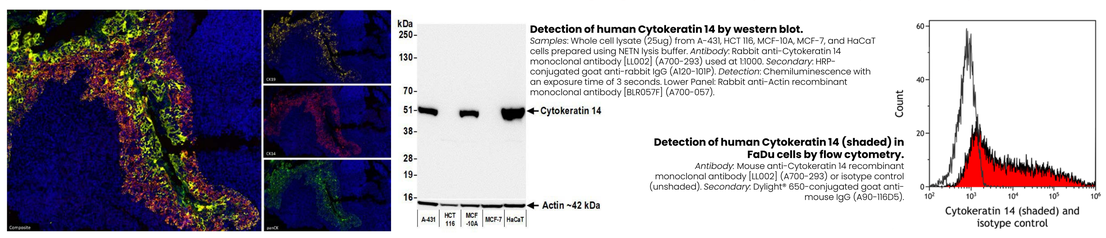

Figure 5. Fluorescent staining of panCK, CK14, and CK19 in conjunction with Western blot and flow cytometry data of Cytokeratin 14. Biomarker specificity was tested in various applications prior to panel design and optimization.

Conclusions

Examination of keratins in the tumor microenvironment provides data of prognostic significance. Current diagnostic tests for these proteins are often inexpensive and yield fast presence/absence information to aid in the treatment of a variety of conditions. However, patients often have unique circumstances that require a more individualized approach. Pathologists utilize the entire family of keratins to ascertain differences in disease state (i.e., CK7 and CK20 are used to distinguish primary lung carcinoma (CK7+/ CK20-) from metastatic colonic carcinoma to lung (CK7-/ CK20+). Historically, panCK has been utilized as a generalized tumor marker, but individual keratins provide key differential information.

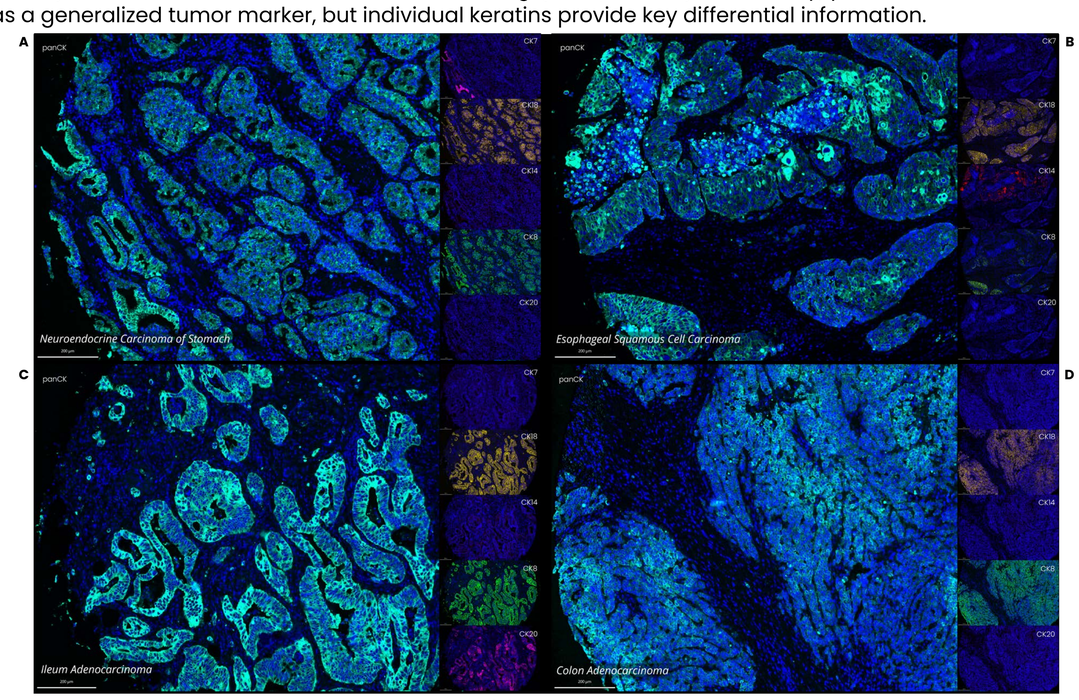

Figure 6. Cytokeratin profiles of stomach carcinoma (a), esophageal carcinoma (b), ileum carcinoma (c), and colon carcinoma (d) tissues. The absence or presence of each keratin is indicative of malignant status in that tissue type.

Acknowledgements

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