Spatial analysis of the tumor microenvironment in pancreatic neuroendocrine tumors

Junxiang Xu[1], Joakin O. Mori[1], Veronica Jarzabek[1], Kenel Dufort[1], Carlo Lanza[1], Katelyn Smith[2], Aatur D. Singhi[2], Ruben Dries[1], Christopher M. Heaphy[1]

[1]*Department of Medicine, Boston University Chobanian & Avedisian School of Medicine and Boston Medical Center, Boston, MA, USA*

[2]*Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA*

BACKGROUND

- PanNETs are the 2nd most common malignant neoplasm of the pancreas.
- The **incidence and prevalence has risen dramatically** over the last 3 decades.
- Patients with clinically and pathologically indistinguishable PanNETs can have marked differences in **disease progression, therapeutic response, and survival**.
- For example, while some patients with this disease present with a slowly growing, indolent tumor, others develop an infiltrative tumor that ultimately leads to widely metastatic disease.

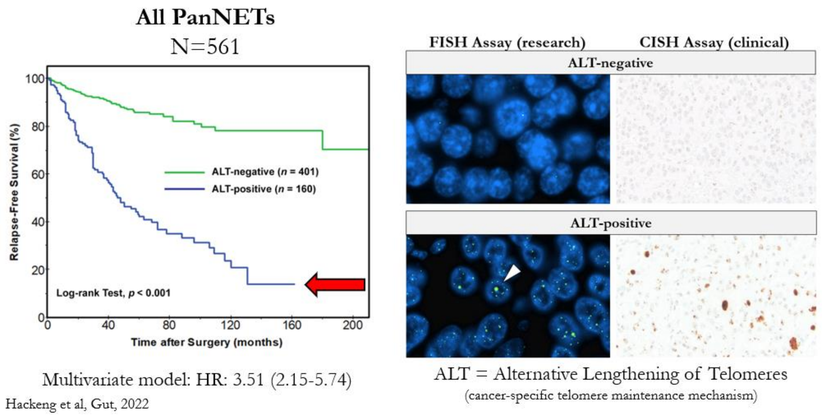


Fig. 1. Kaplan-Meier curves comparing relapse-free survival (RFS) after surgical resection for patients with NF-PanNETs. RFS for patients with ALT-positive NF-PanNETs was significantly shorter than patients with ALT-negative NF-PanNETs.

- PanNETs are characterized by complex topographic architectures; however, **spatial information** in the **tumor and tumor microenvironment** (TME) are not yet fully characterized.
- These biomarkers capture cancer cell specific alterations and **do not provide a systematic assessment of the TME**, including the role of the tumor architecture and the spatially organized biological and immunological processes.

OBJECTIVE

Comprehensively profile PanNETs by **integrating spatial transcriptomics** with established ***in situ* and immunolabelling-based biomarkers** to accurately map the molecularly-defined subgroups of PanNETs and explore the underlying biology.

MATERIALS AND METHODS

Study cohort: Cases were cross-referenced with clinical and follow-up data. The study inclusion criteria consisted of a solitary, well-differentiated PanNET (confirmed with positive immunolabelling for neuroendocrine markers: synaptophysin and/or chromogranin A) within the pancreas from an adult patient; absence of a confirmed or suspected genetic syndrome associated with pancreatic neuroendocrine neoplasms; and have sufficient tissue material for study. Patients were excluded if the PanNET that was removed incidentally for another malignancy (e.g. pancreatic ductal adenocarcinoma).

Spatial transcriptomics on Visium platform: FFPE sections were generated and prior histopathological information helped to guide the identification of the tumor-normal interface. To uncover spatial gene expression patterns and biological processes in a genome-wide manner, we used the Visium technology, which is based on spatially barcoded oligos that are printed within unique spots (Ø = 55 µm) on a glass slide. For each TMA, we mounted a single section on each imaging window on the 10X Visium slide, which was imaged at a final resolution of 0.74 µm/pixel and stained with H&E to identify both individual nuclei and morphological structures in the tumor and TME. Spatially barcoded transcripts were extracted, libraries constructed, and sequenced.

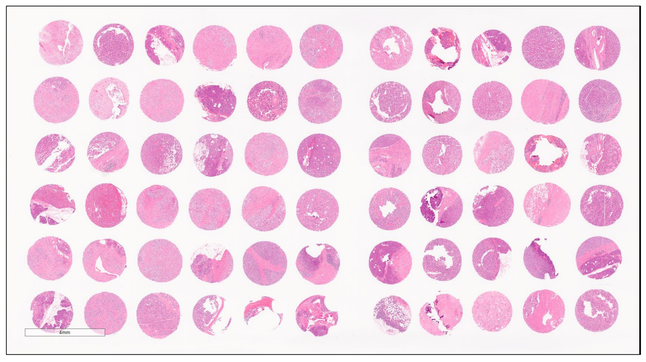
Lunaphore COMET platform: In collaboration with the Spatial Technology Platform group (Broad Institute), this platform was employed to perform staining and imaging on the FFPE TMA sections. The samples were microwave heated to dewax, rehydrate, and retrieve the antigens. Upon completion of heating, the slides were cooled and then loaded into the COMET and covered and sealed by a Fast-Fluidic Exchange microfluidics chip for staining and imaging in the instrument. The COMET performs a sequential immunofluorescence-based staining and imaging followed by an elution of the 17 primary and secondary antibodies.

ALT analysis: Using previously established criteria, ALT-positive cases were identified by the presence of large, ultrabright intranuclear foci consistent with telomere FISH signals in at least 1% of tumor nuclei and the total signal intensity for individual foci >10 fold than telomere signals from adjacent benign cells. ALT was determined by assessing at least 250 nuclei for each case, and areas of necrosis were excluded from evaluation.

Multi-modal analyses: All analyses and visualizations were conducted using Giotto Suite.

RESULTS

N=37 (all non-functional with Visium & multiplex IF data)		
Year of surgery: 2016-2023		
Gender		
Female	18	
Male	19	
Mean age (years)	58.5	
Mean tumor size (cm)	3.47	
WHO Grade		
G1	19	
G2	17	
G3	1	
ALT		
Presence	15	**73% (11 of 15) metastasized
Absence	22	**18% (4 of 22) metastasized
Developed metastasis		
Presence	15	
Absence	22	



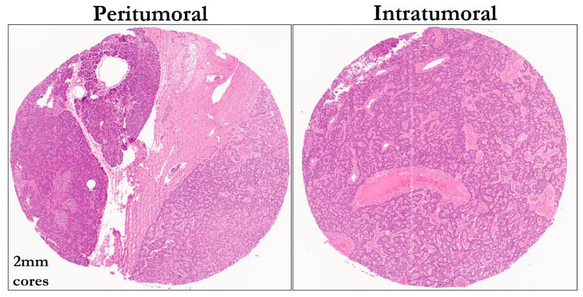


Fig. 2. Representative H&E images for the constructed tissue microarray (TMA) with regions sampled from peritumoral and intratumoral areas.

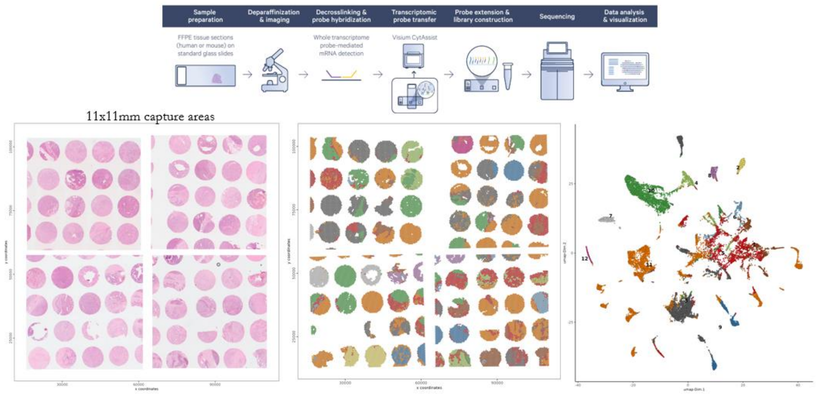
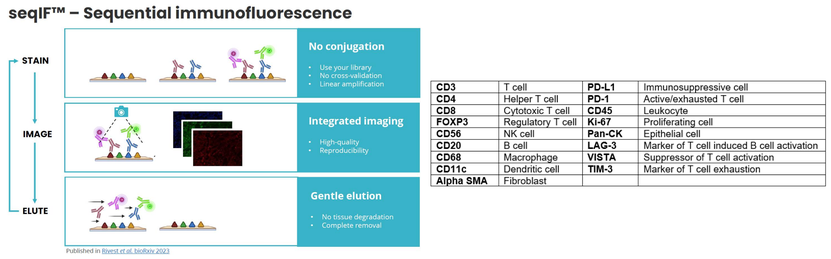


Fig. 3. Visium spatial transcriptomic analyses on TMA sections and cluster analysis.



CD3	T cell	**PD-L1**	Immunosuppressive cell
CD4	Helper T cell	**PD-1**	Active/exhausted T cell
CD8	Cytotoxic T cell	**CD45**	Leukocyte
FOXP3	Regulatory T cell	**KI-67**	Proliferating cell
CD56	NK cell	**Pan-CK**	Epithelial cell
CD20	B cell	**LAG-3**	Marker of T cell induced B cell activation
CD68	Macrophage	**VISTA**	Suppressor of T cell activation
CD11c	Dendritic cell	**TIM-3**	Marker of T cell exhaustion
Alpha SMA	Fibroblast		

Fig. 6. Outline of the 17 protein markers used to establish the sequential immunofluorescence staining protocol.

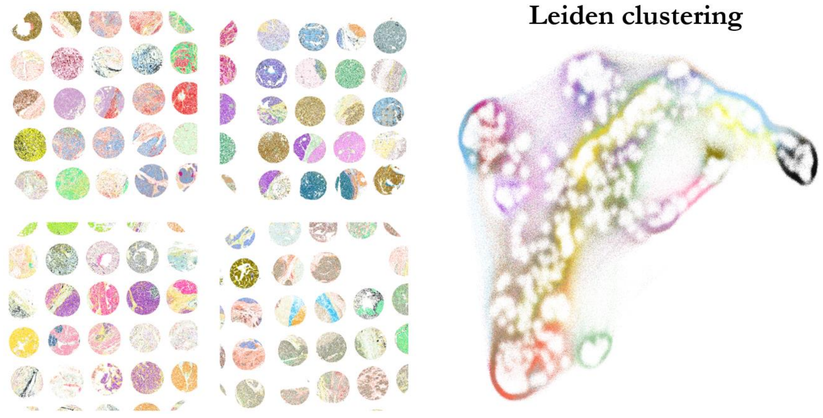


Fig. 7. Leiden clustering schematic of the multiplex IF data demonstrating differential protein expression across the tissue microarrays.

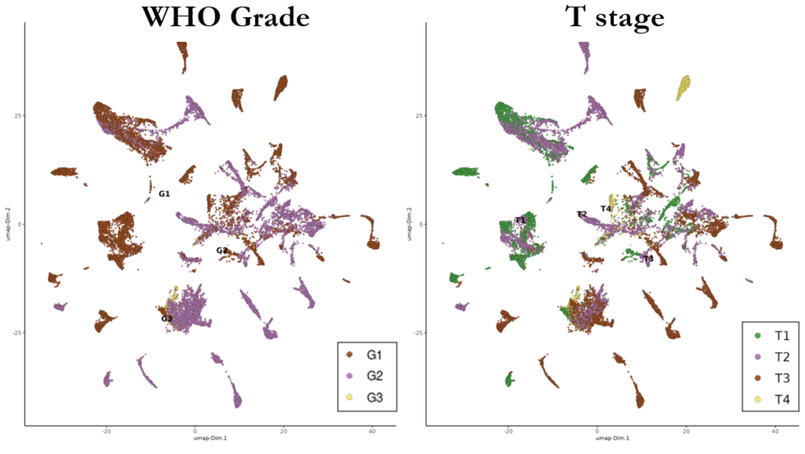


Fig. 4. Cluster analysis for WHO grade and T stage.

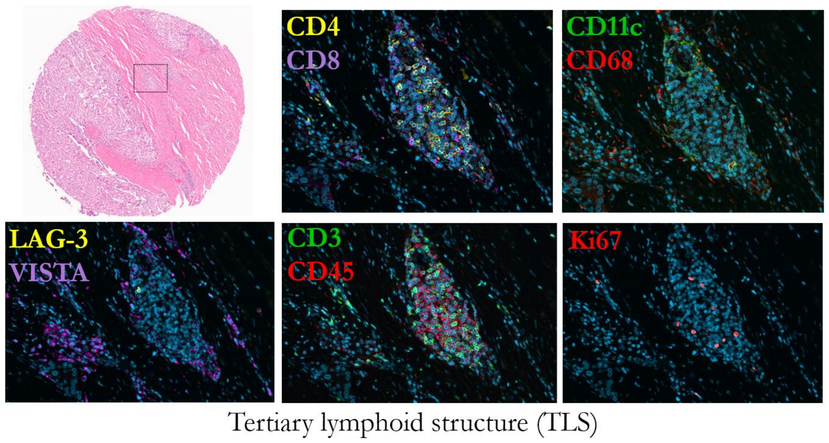


Fig. 8. An example of a tertiary lymphoid structure adjacent to a ALT-positive PanNET.

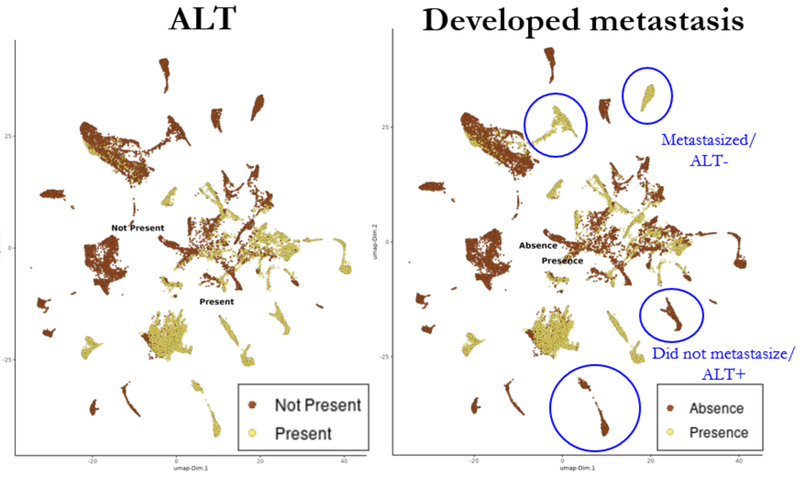


Fig. 5. Cluster analysis of ALT status and development of metastasis.

FUTURE DIRECTIONS

We will document the genetic, cellular, and structural components that create an **immunosuppressive microenvironment** causing lack of response to immuno-oncology therapies, which is commonly observed in patients with this disease.

This work will **delineate the spatial characteristics of the tumor and TME** and, by coupling new biological insights with biomarker discovery, we envision further **improving prognosis** and **uncovering potential therapeutic targets** for the treatment of patients with PanNETs.