Spatial Transcriptomics of Multifocal Ileal Neuroendocrine Tumors Reveals Tumor Heterogeneity based on Tumor Microenvironment and New Biomarkers

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Background

Ileal neuroendocrine tumors (i-NETs) are characterized by a high incidence of multiple primary tumors (>30-40%) and production of serotonin/other hormones.

Recent whole genome sequencing analyses revealed an absence of shared somatic variations among synchronous primary tumors, focusing on genomic alternations in bulk specimens (Nat Commun. 2021;12(1):6367., Genome Med. 2022 ;14(1):82.).

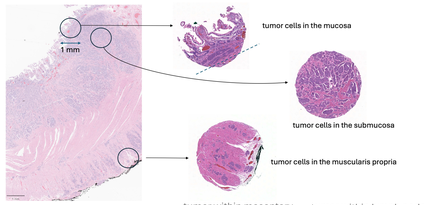
In this study, we evaluated gene expression patterns of multifocal i-NETs with spatial resolution to characterize subpopulations of i-NET cells depending on the tumor microenvironment and develop new hypotheses about multiple tumorigenesis focusing on the tumor microenvironment.

Material and Methods

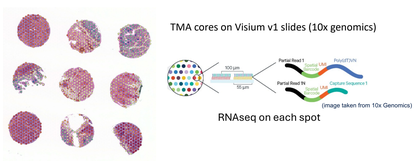
FFPE blocks of surgically resected specimens from 4 patients with multifocal i-NETs were used. Tissue microarrays (TMA) were constructed from 72 cores (18 one-mm cores per patient). Spatial gene expression libraries were constructed using Visium v1 (10x Genomics). R packages including Seurat, clusterProfiler, and monocle3 were used for data analysis.

TMA cores

Patient	total core numbers	non-tumoral tissues	primary tumor #1	#2	#3	lymph node metastasis	mesenteric mass
Patient 1	18	5	5	5	-	-	3
Patient 2	18	6	4	3	-	-	5
Patient 3	18	4	7	5	-	1	1
Patient 4	18	4	2	5	3	4	-



Spatial gene expression libraries

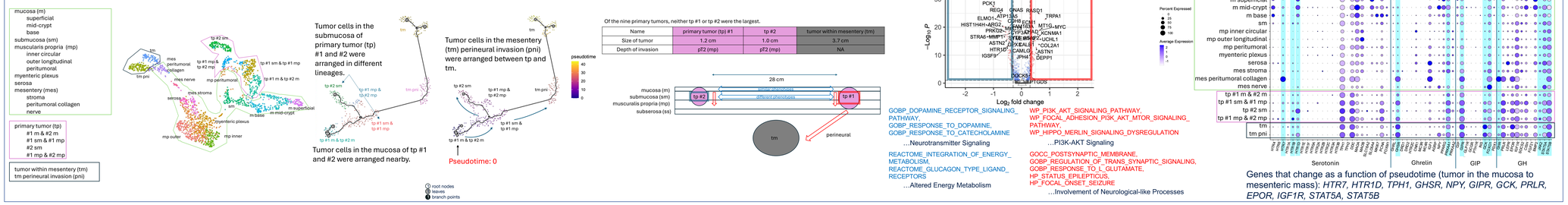


Results

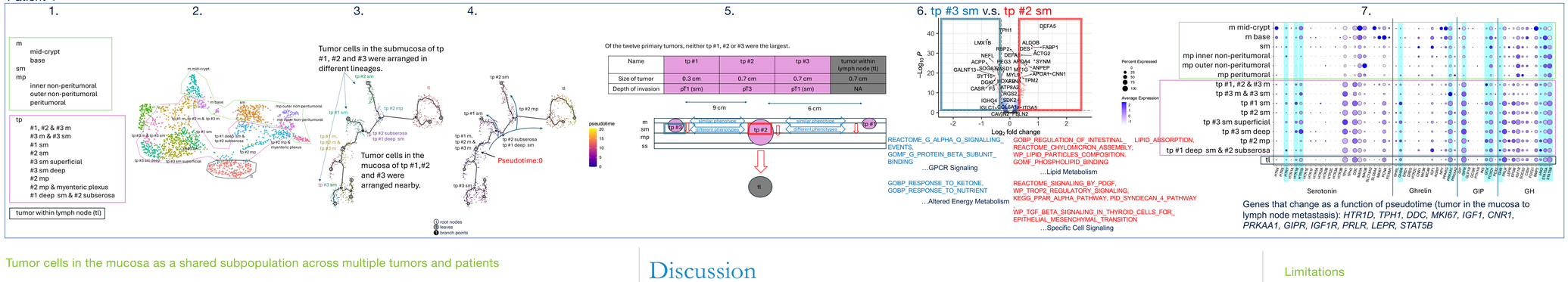
Tumor heterogeneity, similarity and biomarkers

1. Clusters detected by unbiased clustering
2. Clusters visualized in UMAP
3. Trajectory analysis with tumoral spots
4. Pseudotime analysis with tumoral spots
5. Suggested tumoral development
6. Over-representation analysis
7. Serotonin and other hormone-related genes

Patient 1

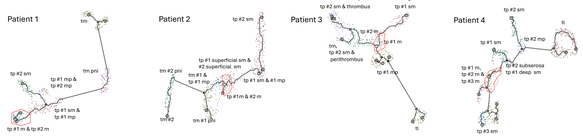


Patient 4

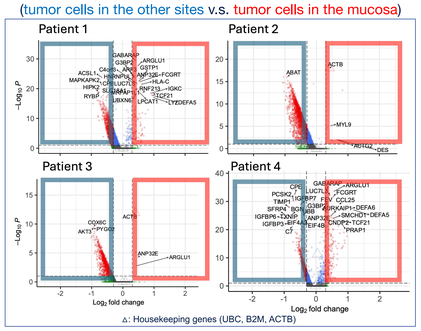


Tumor cells in the mucosa as a shared subpopulation across multiple tumors and patients

In all 4 patients, gene expressions of tumor cells in the mucosa were similar across multifocal tumors while tumor cells in other microenvironments clustered separately.



Differentially expressed genes of tumor cells in the mucosa (tumor cells in the other sites v.s. tumor cells in the mucosa)



Shared gene sets by tumor cells in the mucosa across patients

REACTOME_RHO_GTPASE_CYCLE
REACTOME_CLATHRIN_MEDIATED_ENDOCYTOSIS
REACTOME_RAB_GERANYLGERANYLATION
GOCC_TRANSPORT_VESICLE
GOBP_VESICLE_LOCALIZATION
…Reduced Cellular Transport and Signaling
REACTOME_NEUTROPHIL_DEGRANULATION
WP_PROTEASOME_DEGRADATION
…Impaired Immune Response
GOMF_DNA_BINDING_TRANSCRIPTION_FACTOR_BINDING
GOMF_RNA_POLYMERASE_II_SPECIFIC_DNA_BINDING_TRANSCRIPTION_FACTOR_BINDING
GOMF_TRANSCRIPTION_COACTIVATOR_ACTIVITY
GOMF_TRANSLATION_INITIATION_FACTOR_ACTIVITY
GOBP_REGULATION_OF_MRNA_METABOLIC_PROCESS
…Downregulated Transcription and Translation Activity
WP_VEGFA_VEGFR2_SIGNALING
REACTOME_PTEN_REGULATION
GOMF_CADHERIN_BINDING
GOCC_FICOLIN_1_RICH_GRANULE
GOCC_FICOLIN_1_RICH_GRANULE_LUMEN
GOBP_RESPONSE_TO_ENDOPLASMIC_RETICULUM_STRESS
HP_HYPERTRICHOSIS
HP_ABNORMAL_CHEEK_MORPHOLOGY
HP_FULL_CHEEKS

WP_SEROTONIN_HTR1_GROUP_AND_FOS_PATHWAY (shared by Patient 1,2)
…Reduced Neurotransmitter Signaling

GOBP_RETINA_HOMEOSTASIS
GOMF_PHOSPHOLIPID_BINDING (shared by Patient 1,3,4)
GOBP_REGULATION_OF_INTESTINAL_LIPID_ABSORPTION
GOMF_FATTY_ACID_BINDING (shared by Patient 1,4)
…Upregulated Lipid Metabolism

Discussion

Spatial transcriptomics analysis reliably captured spatial information of malignant and non-malignant cells in distinct tissue compartments.

Activated PI3K-Akt signaling might be associated with perineural metastasis to the mesentery. Upregulated lipid metabolism might be associated with metastasis to the lymph nodes. Reduced neurotransmitter or GPCR signaling and altered energy metabolism might be associated with both metastasis.

Tumor cells heterogeneously expressed serotonin receptors: *HTR7* (Gs coupled), *HTR1B* and *HTR1D* (Gi/o coupled). *HTR1B* could be specific to tumor cells than normal cells.

Tumoral expressions of hormone receptors: *GHSR* (and *PRKAA1*), *GIPR* (*GCK,* and *FOXO1*), and *GHR* (and JAK-STAT) were heterogeneous and well reproducible across patients.

Tumor cells in the mucosa could form a single phenotypic group across multiple tumors in one patient, with shared genomic and/or non-genomic backgrounds. They could exhibit reduced cellular transport and signaling, impaired immune response, downregulated transcription and translation activity, reduced neurotransmitter activity, and upregulated lipid metabolism.

Upregulated lipid-metabolism genes in tumor cells are reported to be associated with their adaption to the lipid-rich environment and potential for lymphatic metastasis (Cells. 2021;10(3):627.). The microenvironment of the small intestine, the site of the absorption and transportation of lipids, should contribute to the reprogramming of lipid metabolism in some i-NET subpopulations (Dev Cell. 2021;56(10):1363-1393.).

Tumoral expression of serotonin and other hormone receptors suggests that endocrine, paracrine, and autocrine signaling may play a role in tumorigenesis and progression, depending on the subpopulations with varying expressions. Specific biomarkers could be applied to future subpopulation-targeted therapies.

Limitations

The current study was exploratory and hypothesis-seeking. The theories or biomarkers suggested here should be verified by other hypothesis-proving studies.

Our analysis was based on gene expressions, which reflect tumor cell-intrinsic (genomic or epigenomic alternations) and extrinsic (microenvironmental modifications) factors.

Each spot may have contained 1-10 cells. Analyzed spots were selected using gene modules, which minimized the potential bias of contamination of tumoral and normal cells.

Patient 2, 3, and 4 had used somatostatin analogues, which could affect the gene expressions.

Conclusion

Spatial transcriptomic analysis of multifocal i-NETs reveals intratumoral and intertumoral heterogeneity depending on the microenvironment.