Discovery of G Protein Coupled Receptor Tumor Signatures and Identification of Positron Emission Tomography Imaging Targets in Ileal and Pancreatic Neuroendocrine Tumors

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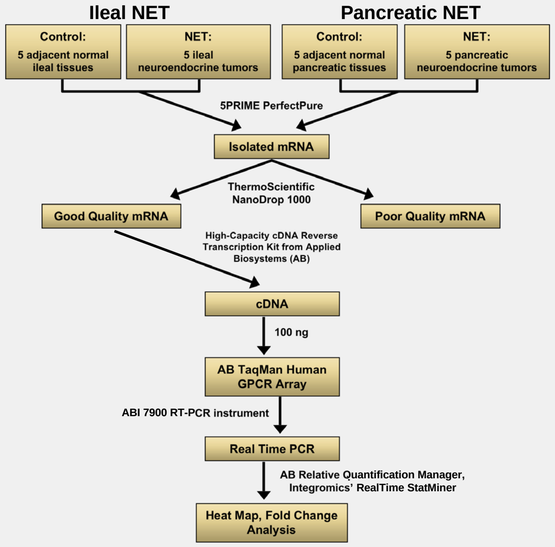
Introduction

Neuroendocrine tumors (NETs), comprised of carcinoids and pancreatic endocrine tumors, are a rare form of cancer that occur in approximately 1 in 100,000 people per year.[1,2] Early detection of NETs can be difficult due to their vague symptoms. This delay in diagnosis can give tumors the opportunity to metastasize, making surgical resection of the tumor impossible.[3] G protein coupled receptors (GPCRs) have emerged as candidates for molecular targeting in NETs, with potential uses in both tumor imaging and therapy. GPCR characteristics that account for their success as drug targets include cell surface overexpression, exquisite specificity of ligand-receptor interactions, and key roles in NET signal transduction pathways. **We hypothesize that pancreatic and ileal NETs have distinct GPCR signatures that can be exploited to develop tumor specific Positron Emission Tomography (PET) imaging agents.** To test the hypothesis, we isolated RNA from specimens of ileal and pancreatic NETs and compared GPCR expression to adjacent normal tissue. Based on these findings we synthesized multiple peptide ligands targeting the upregulated GPCRs specific to the ileal and pancreatic NETs. Following conjugation of the metal chelator DOTA, the peptides were radiolabeled with 68-gallium for *in vitro* and *in vivo* studies.

Methods

RNA isolation and GPCR expression



Solid phase peptide synthesis and DOTA conjugation Peptides were synthesized by standard Fmoc solid phase peptide synthesis at a 0.1 mmol scale on an AAPPTEC Apex 396 automated multiple peptide synthesizer. Rink amide resin was used to prepare peptides with *C*-terminal amides, while 2-chlorotrityl chloride resin was used to synthesize peptides with *C*-terminal alcohols. DOTA-tris (*t*-Bu ester) (Macrocyclics, San Diego, CA) was coupled to the *N*-terminus of each peptide. After conjugation with DOTA, the peptides were deprotected and cleaved from the resin, precipitated with ice-cold diethyl ether, isolated by centrifugation, and resuspended in water. Purification was achieved by RP-HPLC using a Vydac C18 semipreparative column (1.0 x 25 cm) eluted at 5 mL/min with 0.1% TFA and a 20-35% gradient of acetonitrile over 30 minutes. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, and lyophilized. The purified DOTA-VIP peptides were then reconstituted in water and characterized on an Agilent 1100 LC-ESI-ion trap by injecting 10 nmol onto a Vydac C18 analytical column (0.46 x 25 cm) eluted at 0.7 mL/min with 0.1% TFA and a 5 – 45% gradient of acetonitrile over 30 minutes with mass spectral data collected in the positive mode.

Radiolabeling of DOTA-peptides with ^{68}Ga or ^{111}In DOTA-conjugated peptides were radiolabeled according to the procedure previously reported by Breeman et al.[4]

Imaging Octreoscan whole body scintigraphy and SPECT CT were performed 24 hours following injection of 6 mCi ^{111}In-DTPA-Octreotide. All scans were performed on the GE Hawkeye instrument.

Results

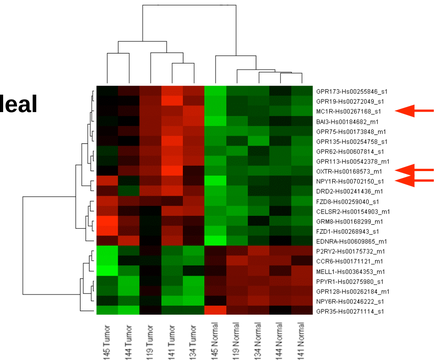


Figure 1. GPCR expression in ileal NET compared to normal adjacent tissue. Red color indicates low CT on qPCR, corresponding to high RNA expression; black is intermediate CT and intermediate expression; green is high CT and low RNA expression. Each gene is compared against itself for signal intensity. Genes shown are those for which differences in expression between ileal NET tumor and normal tissue are significant with p<0.01. Arrows point to the candidate genes significantly (p<0.01) overexpressed in ileal NET.

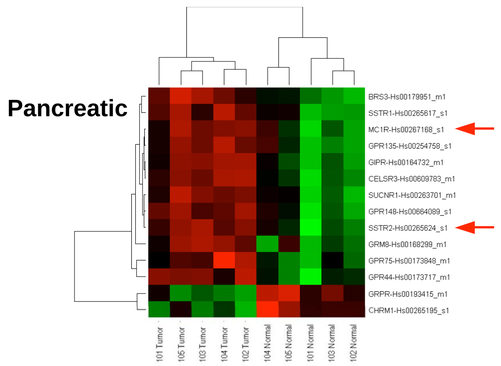


Figure 2. GPCR expression in pancreatic NET compared to normal adjacent tissue. Red color indicates low CT on qPCR, corresponding to high RNA expression; black is intermediate CT and intermediate expression; green is high CT and low RNA expression. Each gene is compared against itself for signal intensity. Genes shown are those for which differences in expression between pancreatic NET tumor and normal tissue are significant with p<0.01. Arrows point to the candidate genes significantly (p<0.01) overexpressed in pancreatic NET.

	Ligand	Ileal NET	P-value	Pancreatic NET	P-value
OPRK1	Opiate	117	0.037	2	0.642
GHSR	Ghrelin	116	0.023	0.4	0.224
CALCR	Calcitonin	86	0.040	5	0.449
NPY1R	Neuropeptide Y	26	0.006	0.7	0.578
OXTR	Oxytocin	26	0.006	11	0.081
MC1R	Melanocortin	10	0.001	10	0.009
VIPR1	Vasoactive Intestinal Peptide	0.3	0.152	19	0.017
HTR1D	Serotonin	0.5	0.641	27	0.050
SST5	Somatostatin	6	0.022	20	0.076
SST2	Somatostatin	13	0.047	52	0.008

Table 1. Gene expression fold changes between ileal or pancreatic neuroendocrine tumors and normal tissue for selected candidate GPCRs. GPCRs highlighted in yellow indicate good candidate receptors for PET imaging agents of ileal NETs, since the GPCR has a high fold change and is significantly overexpressed compared to normal tissue. GPCRs highlighted in orange indicate good candidate receptors for PET imaging agents of pancreatic NETs. The fold change and p-values were calculated from the statistical analysis program, Stat Miner.

Results Continued

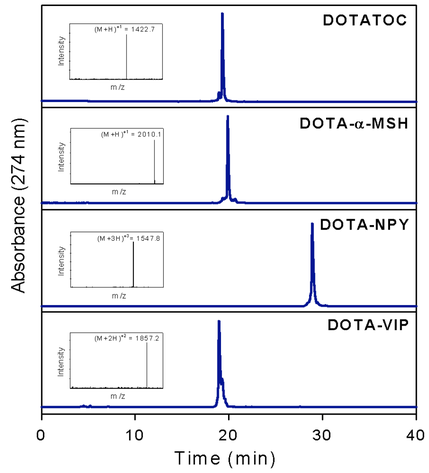


Figure 3. LC-MS characterization of DOTA-peptides. The purified DOTA-peptides were analyzed by LC-MS eluted with 0.1% TFA and an acetonitrile gradient of 5-45% over 30 min. The chromatograms were monitored by absorbance (274 nm) and ESI-MS (inset).

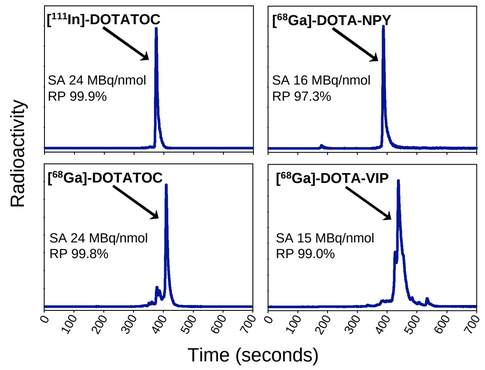


Figure 4. HPLC characterization of radiolabeled DOTA-peptides. Representative RP-HPLC traces showing specific activity (SA) and radiochemical purity (RP) of 111-Indium and 68-Gallium labeled DOTA-peptides.

		Pancreastatin	Chromogranin	Serotonin	Octreoscan Positivity	
					Primary Lesion	Metastatic Lesion
Ileal NET	Patient 145	62	<5	134	Positive	No Mets
	Patient 144	607	340	1140	Negative	Positive
	Patient 119	464	211	2091	Positive	Positive
	Patient 141	503	366	1626	Negative	Positive
	Patient 134	175	21	329	Positive	Positive
Pancreatic NET	Patient 101	225	125	154	Positive	Positive
	Patient 105	117	19603	248	Positive	Positive
	Patient 103	69	68	610	Positive	Positive
	Patient 104	86	45	174	Negative	Positive
	Patient 102	71	85	47	Positive	Positive

Table 2. Patient data for neuroendocrine tumor samples. Note that NET was not visible in 2 of 5 ileal primary lesions while 4 of 5 pancreatic primary lesions were visualized by Octreoscan. Metastatic lesions were positive in both ileal and pancreatic tumors.

Results Continued

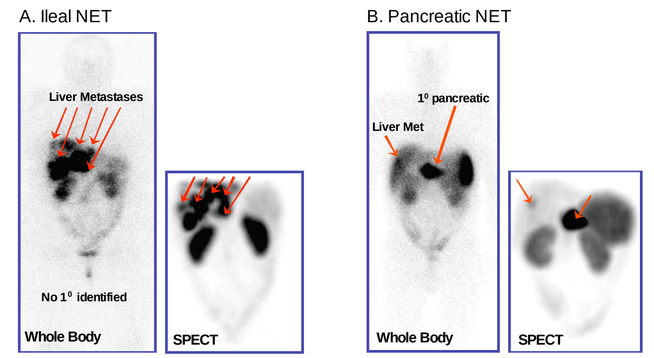


Figure 5. Whole body and SPECT imaging of ileal and pancreatic NETs. A. Ileal NET with multiple positive metastatic lesions in liver but no primary tumor identified. Surgical exploration of the bowel revealed a 1 centimeter ileal lesion pathologically confirmed as poorly differentiated neuroendocrine cancer. B. Primary pancreatic NET and single liver metastasis identified on initial Octreoscan.

Conclusions

- **Tumor specific GPCR signatures were identified in ileal and pancreatic NETs**
  - **Ileal → OPRK1, GHSR, CALCR, NPY1R, OXTR**
  - **Pancreatic → SST2, SST5, VIPR1, HTR1D**
  - **Both → MC1R**
- **Peptide ligands targeting receptors specific to each NET were synthesized, conjugated with DOTA, and radiolabeled with high specific activity**
  - **Ileal → DOTA-NPY targeting NPY1R**
  - **Pancreatic → DOTA-VIP targeting VIPR1, DOTATOC targeting SST2**
  - **Both → DOTA-α-MSH targeting MC1R**
- **Somatostatin (SST) receptors shown to be an excellent imaging target in pancreatic primary tumors, new agents targeting other GPCRs may be more sensitive in identifying ileal tumors**

Work Cited

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