2022 NETRF Symposium (November 16-18)
Poster Abstracts

Session 1: NET Models

A Human Pluripotent Stem Cell-based Model for Lung Carcinoid

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Presenting Author: Huanhuan Joyce Chen
Net Type: Lung; *Research Type:* Basic; *Keywords:* Cell lines Mouse model Single cell -omics

Background: Pulmonary carcinoids are rare neuroendocrine tumors, representing around 2% of all primary lung neoplasms, however, their incidence has rapidly increased within the past three decades. Surgical removal is an effective treatment for the early stage disease, however, no standardized therapy has been established for the advanced unresectable pulmonary carcinoids. This reflects limitations in precisely understanding of the disease mechanisms on oncogenic transformation, proliferation and progression of malignant cells. Current research progress has been hindered by the low frequency of pulmonary carcinoid, and scarcity of disease model and research resources for in-depth mechanistic or functional studies. In this proposal, we aim to develop a novel human cell-based pulmonary carcinoid model using the pulmonary neuroendocrine cells (PNECs) derived from human pluripotent stem cells (hPSCs). Although certain high-frequency gene mutations were previously reported, few oncogenic driver genes have been identified for pulmonary carcinoid. Therefore, we propose here to test the high-frequency mutations for their capacity to transform the hPSC-derived PNEC to pulmonary carcinoid, and to develop a new disease model. Moreover, we seek to establish cancerous cell lines from the hPSC-derived pulmonary carcinoid tumor tissues. If successful, these studies will generate foundational knowledge for identification of driver mutations and for functionally dissecting cancer pathways in pulmonary carcinoid. The models and cell lines created in this project are expected to serve as an innovative research platform for testing new targeted agents and for more intensive studies of these rare diseases.

Methods: I. Generation of genetically engineered human pluripotent stem cell (hPSC) lines Two main methods to insert a genetic mutation: 1. Doxycycline inducible mutations which could be induced in lung lineage stage 2. Pulmonary neuroendocrine cell (PNEC) marker controlled expression of gene insertion or deletion. Both will be added to the protocol at the hPSC stage in the form of lentivirus-infected cells II. Differentiation of hPSC lines into PNEC III. Characterization of successful genetic mutations in PNECs: Experimental group: hPSC differentiation line induced to form PNECs and with the putative mutations induced by doxycycline at PNEC stage Control group: hPSC differentiation line induced to form PNECs and with the putative mutations not induced by doxycycline at PNEC stage 1. With fluorescence microscope: The expression of fluorescent reporter genes that the infecting plasmids carry (e.g YFP or GFP) will be observed. In both doxycycline inducible cell line and PNEC marker controlled cell line, there will be a fluorescent gene sequence added after the genes of interest to ensure their visualization. 2. With western blot: We will use antibodies against specific proteins for their level of expression compared with the cell lines without genetic mutation of this protein. 3. Single-cell RNA profiling of the hESC

differentiated cell line Goals: To confirm the successful introduction of genetic mutation into the system before further characterizations. IV.Evaluation of PNEC in proliferation, apoptosis, and oncogenic transformation Experimental group: hESC differentiation line induced to form PNECs and with putative mutations of pulmonary carcinoids Control group: hESC differentiation line induced to form PNECs and with mutations to transform into SCLC-like cells 1. Proliferation: We will test the expression level of KI67 and TOP2A with western blot or FACS, both of which are markers of cell proliferation. An abnormally high index of proliferation markers indicates the possibility of cancer. Or we will use the BrdU/EdU-based cell analysis kit to evaluate cell cycle. 2. Replication: We'll test the ATP level of the cell with luciferin, which could react with ATP and form oxyluciferin. Cells with high replication behavior are considered ATP consuming. 3. Mitosis: We'll test the index of mitotic marker, phosphor-histone H3,

Results: Aim I. To establish the in vitro cell model of pulmonary carcinoid. Aim II. To develop the xenograft model and cell lines for pulmonary carcinoid. With the great support by Neuroendocrine Tumor Research Foundation (NETRF), we have successfully generated six hPSC lines with inducible over-expression or deletion of the candidate genes that are commonly mutated in pulmonary carcinoids. These include the familial mutation MEN1, and sporadic mutations IGF1R, ARID1, EGFR. We used two main methods to insert the genetic mutation, inducible mutations which could be induced specifically in PNEC differentiation stage or mutation expression controlled by a PNEC marker. After validation of the gene expression at protein level, these hPSC cell lines are differentiated into lung lineage including PNECs. The PNECs can be purified by a yellow fluorescent protein (YFP) marker and characterized for their capacity to transform to pulmonary carcinoids. A series of experiments like counting cell numbers, calculation of mitotic index and colony formation were then performed to evaluate the proliferation, apoptosis, and oncogenic transformation of PNECs in culture. In addition, we set up a PNEC culture with genetic mutations commonly found in small cell lung cancer (SCLC), another type of lung neuroendocrine tumor. Since SCLC and pulmonary carcinoids have quite different disease phenotypes and progression in clinic, the control group here is to compare the pulmonary carcinoid inducing lines to SCLC inducing lines as both derived from hPSC-PNECs. We hope to observe differences in the above mentioned markers and demonstrate the significance of these markers in distinguishing those two tumors.

Discussion: We now propose to exploit the novel experimental approach to develop human cell-based models of pulmonary carcinoid. By manipulating the genetic factors or signaling pathways recurrent in lung carcinoids, we design a series of experiments to convert the hPSC-derived normal PNECs into cancerous cells. We will further define the similarities between the genetic and physiological features of the lung carcinoids from hPSCs and the ones in patient samples. Through these studies, we expect to establish an innovative pulmonary carcinoid model with human cell origin that recapitulates the clinical features of these tumors. Once succeed, the feasible and trackable model system will pave a new avenue for studying a broad range of questions about lung carcinoids.

Patient-Derived Organoids and their Potential for Precision Medicine in Neuroendocrine Tumors

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Net Type: Multiple NET Types; *Research Type:* Translational; *Keywords:* Cell lines Organoids Chemoresistance Drug resistance

Background: Neuroendocrine tumors (NETs) are a heterogeneous group of malignant neoplasms arising from neuroendocrine cells distributed throughout the body. The most common sites of NETs are the gastrointestinal tract, pancreas and lungs. The clinical management of NETs is not standardized, with few FDA-approved therapies. Moreover, drug development has been challenging for NETs due to limited pre-clinical models. To address this unmet need, the NCI Natural History Study of Children and Adults with Neuroendocrine Neoplasms (NCT03739827 and NCT05237934) aims to

develop preclinical models, such as in vitro 3-dimentional tissue organoids, to develop more personalized therapies for NET patients.

Methods: From February 2020 – July 2022, 17 surgical specimens were collected for the development of patient-derived organoids. We selected 3 NET organoids (NET16, NET17 and NET18) to test the activity of select drugs: dovitinib (VEGFR inhibitor), vistusertib (mTOR inhibitor), cobimetinib (mitogen-activated protein kinase 1 inhibitor) and TAK243 (ubiquitin activating enzyme inhibitor). NET16 was derived from a 72-year-old male with a grade 1 (Ki-67 <3%) small bowel NET. NET17 was derived from a 36-year-old female with grade 2 liver segment metastasis. NET 18 was derived from a 66-year-old male, with grade 2 (Ki-67=3%) liver segment metastasis. Cell viability assays were performed using Cell Titer Glo after 3 days of drug testing. Chromogranin A, synaptophysin, and Ki67 biomarkers will be assessed in the parental tissues as well as the organoids.

Results: Overall, the activity of the drugs tested was significantly higher in NET16 than NET17 and NET18. TAK243 was the most potent drug in both NETs but had a greater effect in NET16 (IC50=0.39 nM) than NET17 (IC50=43.17 nM) and NET18 (IC50=6.02 nM). Dovitinib and vistusertib were more potent in NET16 (dovitinib IC50=1.46 μM; vistusertib IC50=0.17 μM) than NET17 (dovitinib IC50=11.18 μM; vistusertib IC50=16.45 μM) and NET18 (dovitinib IC50=9.36 μM; vistusertib IC50=7.77 μM). Cobimetinib had modest activity in NET 17 (IC50=12.02 μM) and NET18 (IC50=13.39 μM).

Discussion: We have developed an assay for in vitro drug testing in well-differentiated patient-derived NET organoids that will allow for further, large scale drug screening to help predict patient drug responses. Tumor heterogeneity may be contributing to the differences seen in the drug response between the three NET organoids and requires further evaluation. Replication of these studies in a larger subset of patient samples and drug combination studies will be important for the advancement of therapeutics in NETs.

An in vitro/in vivo platform to test the anti-tumor activity of tyrosine kinase inhibitors in medullary thyroid cancer

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Net Type: ; *Research Type:* Translational; *Keywords:* Patient-Derived Xenografts Tumor microenvironment Tumor vasculature Cell lines

Background: Medullary thyroid carcinoma (MTC) is a rare neuroendocrine tumor, originating from calcitonin-producing parafollicular C cells of the thyroid gland. The clinical course of patients with MTC is variable, ranging from indolent to extremely aggressive forms. RET proto-oncogene alterations represent the most frequent events that lead to the development of both sporadic and inherited forms. Surgery is the only curative treatment for MTC, but advanced MTC are often unresectable and unresponsive to radiotherapy and chemotherapy. Cabozantinib (CAB) and Vandetanib (VAN) are two multi-target tyrosine kinase inhibitors (TKIs) currently used as first-line treatment for unresectable, progressive and symptomatic MTC. Although these drugs increased progression-free survival, drug discontinuation due to either disease progression or toxicity has been reported in about 40-55% of patients. Therefore, new therapeutic strategies are urgently required. In the last decades, zebrafish (Danio rerio) has emerged as a powerful alternative model for the preclinical study of several human diseases, including cancer. Its intrinsic peculiarities, such as high fecundity, external fertilization, transparency, rapid development, embryo permeability to small molecules, together with easy genetic manipulation and low maintenance cost, make zebrafish an essential tool in biomedical research. The aim of this study was to adopt an in vitro and in vivo platform to investigate the antitumor activity of TKIs in MTC, comparing the effects of CAB and VAN, with SPP86, a novel RET specific inhibitor.

Methods: In vitro experiments were performed in two human MTC cell lines, TT and MZ-CRC-1, characterized by C634W and M918T RET mutations respectively. After six days of incubation, the effects of VAN, CAB and SPP86 on cell viability, cell cycle, and apoptosis of TT and MZ-CRC-1 cells were evaluated in vitro using MTT assay, DNA flow cytometry with propidium iodide, and Annexin V-FITC/propidium iodide staining, respectively. Our in vivo preclinical model was based on the implantation of human MTC cells in transgenic Tg(fli1a:EGFP)y1 zebrafish embryos at 48 h post-fertilization (hpf), that express the fluorescent protein EGFP in the endothelium, allowing the in vivo visualization of the entire vascular tree. MTC cells were labeled with a fluorescent viable dye and grafted into the sub-peridermal space of 48 hpf Tg(fli1a:EGFP)y1 embryos. The tumorigenic potential of MTC implanted cells was evaluated by the quantification of tumor-induced angiogenesis as early as 24 h post-injection (hpi). Due to the zebrafish embryo permeability to small molecules, CAB, VAN, and SPP86 were directly dissolved into fish medium, at the concentration of 2.5 µM, and their anti-angiogenic effect on MTC cell grafted embryos was evaluated. As control we considered injected embryos incubated in the fish medium and the vehicle in which the experimental substance was dissolved (DMSO).

Results: MTT assays showed a significant inhibition of cell viability in both MTC cell lines after incubation with all TKIs. In TT cells the maximal inhibition after SPP86 (-100%) was significantly higher than that of CAB (-91.2%, $p<0.001$) and VAN (-92.7%, $p<0.001$), while in MZ-CRC-1 cells the maximal inhibition of SPP86 (-82.5%) was higher than CAB (-74.9%, $p<0.01$) and comparable to VAN (-83.7%). Cytofluorimetric analyses showed that SPP86 and CAB similarly decreased the fraction of TT cells in the S and G2/M phases, while the effect of VAN was less prominent. SPP86 e VAN significantly decreased the fractions of MZ-CRC-1 cells in the S and G2/M phases. We observed a significant pro-apoptotic activity of all compounds in both cell lines. In zebrafish model the impact of SPP86 (-49%) in inhibiting TT-induced angiogenesis was comparable with that observed after VAN (-37%) and less potent than CAB (-86.4%, $p<0.05$).

Discussion: Our in vitro/in vivo platform resulted particularly reliable to easily and quickly test the effects of TKIs. This study revealed a significant anti-tumor activity exerted by SPP86, suggesting a good efficacy of this new RET-specific inhibitor with potentially less adverse effects than multi-target TKIs, such as CAB and VAN. Future studies are necessary to compare the antitumor activity of SPP86 with selpercatinib and pralsetinib, two RET-specific inhibitors recently approved by the FDA for the therapy of RET-mutant MTC. The possibility to implant a small number of cells makes the tumor xenograft in zebrafish embryos a procedure particularly suitable for cells derived from MTC patients, whose tumor cells availability is often limited, because of the small size of post-surgical samples. In this context, our platform may open a promising scenario in the field of precision medicine, promoting the identification of the most appropriate and personalized therapies for MTC patients.

Session 2: Genetics

Multiple endocrine neoplasia 4: genotype-phenotype association of germline CDKN1B variant type and site

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Net Type: Multiple; *Research Type:* Clinical; *Keywords:* Clinical Studies, Correlative Studies

Background: Multiple endocrine neoplasia 4 (MEN4) is a rare autosomal dominant multiglandular endocrine neoplasia syndrome clinically hallmarked by primary hyperparathyroidism (PHPT), pituitary adenoma (PitAd), and neuroendocrine

tumors (NET), clinically overlapping MEN1. The underlying mutated gene- CDKN1B, encodes for the cell-cycle regulator p27, a tumor suppressor. Possible genotype-phenotype correlation in MEN4 have not been thoroughly assessed.

Materials and Methods: Prompted by the findings in three Israeli MEN4 kindreds, we performed a literature review on published and unpublished data from previously reported MEN4/CDKN1B cases. Univariate analysis analyzed time-dependent risks for developing PHPT, PitAd, or NET by variant type and position along the gene.

Ressults: Overall, 74 MEN4 cases were analyzed. PHPT risk was 53.4% by age 60 years (mean age at diagnosis age 50.6±13.9 years), for PitAd - 16.2% and NET - 23.2% (34.4±21.4 and 52.9±13.9 years, respectively). The frameshift variant p.Q107fs was the most common variant identified (4/41 [9.7%] kindreds). Patients with Indels had a higher risk for PHPT vs. point mutations (Log-Rank, p=0.029). Variants in codons 94-96 were associated with a higher risk for PHPT (p<0.001) and PitAd (p=0.031).

Conclusions: MEN4 is clinically distinct from MEN1, with lower risk and older age for PHPT diagnosis. We report recurrent CDKN1B frameshift variants and possible genotype-phenotype correlations.

Loss of epigenetic repression of retrotransposons in pancreatic neuroendocrine tumors

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Net Type: Pancreas; *Research Type:* Basic; *Keywords:* Epigenetics Genomics

Background: Pancreatic neuroendocrine tumors (PNETs) are rare neoplasms and represent about 1-2% of all pancreatic tumors. They are a heterogeneous group of tumors, with most being non-functional and exhibiting variable degrees of malignancy. MEN1 (multiple endocrine neoplasia I), DAXX (death domain associated protein), and ATRX (ATRX chromatin remodeler) are the most frequently mutated genes occurring in up to 70% of PNETs. While MEN1 mutations can be inherited or somatic, ATRX and DAXX mutations are exclusively somatic, detected in about 40% of PNETs. ATRX and DAXX loss-of-function (LOF) mutations in PNETs are associated with metastatic disease, increased risk of recurrence, and poorer survival. Despite this recognized association, it is unclear how ATRX/DAXX mutations promote aggressive tumor behavior in PNETs. ATRX and DAXX are part of a critical pathway that transcriptionally silences retrotransposons (RTEs). Therefore, abrogation of this pathway has the potential to lead to the activation of RTEs. Expression of RTE-encoded proteins is a hallmark of many human cancers. When expressed, it indicates active retrotransposition that might drive the process of tumorigenesis. However, whether the RTEs are expressed in PNETs and their association with ATRX/DAXX mutation status is not known.

Methods: We used whole-genome sequencing, RNA-seq, and exome sequencing data from published datasets as well as from tissue specimens of de-identified PNET patients. We categorized them based on the mutation status of MEN1, DAXX, and ATRX and compared their transcriptomes and the extent of retrotransposon expression. We also performed knockdown and CRISPR knockout experiments in QGP1 cell line to test the role of ATRX/DAXX binding for retrotransposon suppression. We performed RNA-seq and H3K9me3 ChIP-seq in these knockout cells to determine the RTE expression changes and the extent of H3K9me3 loss. We plan to perform whole-genome bisulfite sequencing and H3K9me3 ChIP-seq for specimens with and without loss of function mutations of ATRX, and DAXX to profile the DNA methylation and heterochromatin status.

Results: We have analyzed published RNA-seq data from 49 specimens with known gene mutation status. In addition, RNA-seq (tumor) and exome-seq data (matched tumor and germline from blood) from 22 additional de-identified specimens were obtained from retrospectively collected PNETs. Exome-seq was processed using standard pipelines to determine the mutation status of each tumor. We quantified the expression of genes and RTEs in these samples. We further categorized them into different groups based on the genotype of the tumors and quantified the expression of all human ERVs. We found that tumors containing MEN1 mutation with or without ATRX/DAXX mutations were more similar in terms of their ERV expression profile. The groups containing PNETs with ATRX/DAXX mutations were clustered together indicating a

similar expression profile of ERVs. We found that the groups containing ATRX/DAXX mutation had a higher proportion of reads aligning to the HERV9 subfamily whereas the ERVL family had a similar number of reads across all groups. This suggests that the HERV9 ERVs are targeted by ATRX/DAXX complex for suppression. We are performing CRISPR KO studies in QGP1 cell line to determine the mechanistic details of ERV regulation by ATRX/DAXX and potentially MEN1. We have also confirmed the expression of retroviral proteins in PNET cell lines using available commercial antibodies. We are currently evaluating the protein-coding potential of HERV-derived transcripts and chimeric transcripts whose expression will be validated in PNET cell lines and PNET tumors. We will further perform DNA methylation profiling and immunohistochemistry in PNET specimens to check the expression of retroviral proteins and correlate with other clinical parameters.

Discussion: We believe these studies will define the role of RTE expression in PNET progression and provide mechanistic insights into how ATRX/DAXX mutation promotes PNETs. Furthermore, it will help identify clinically actionable genes and pathways upregulated by RTEs. Identifying RTE-encoded proteins that are aberrantly expressed and frequently tumor-specific antigens will help develop immunotherapeutic strategies to target PNETs.

Single cell RNA-seq analysis of neuroendocrine tumors

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Net Type: Gastrointestinal; *Research Type:* Basic; *Keywords:* Single cell -omics

Background: NETs reflect complex ecosystems that remain poorly understood. To improve our understanding of their cellular composition and diversity, we profiled NET patient samples by single nuclei RNA sequencing.

Methods: Due to difficulties in obtaining fresh NET samples we focused on analysis of frozen patient samples by single nuclei RNA-seq. Following optimizations and exclusion of low-quality datasets, we obtained comprehensive single cell data for 7 NETs, including five small-intestine, one pancreatic and one lung tumor, and we are continuously working to expand this dataset. In each tumor, we assigned cells to distinct cell types and evaluated the diversity of cell states within each cell type.

Results: We find that NETs have a rich microenvironment that includes four main cellular components - neuroendocrine, epithelial, immune (T-cell, B-cell and macrophage) and stromal (fibroblasts and endothelial cells). For each of those components, we find diversity within individual tumors, as well differences across tumors. For example, among the neuroendocrine cells, we find cellular diversity within an individual tumor with respect to metabolic, neural and stress-related expression programs. Across different tumors, we find marked differences in the degree and identity of proliferating cells. In the low-grade tumors, we do not observe proliferation of neuroendocrine cells but instead find aberrant proliferation of epithelial cells and lymphocytes, possibly reflecting the abnormal tumor microenvironment. In contrast, in a high-grade lung neuroendocrine tumor we find proliferation of both neuroendocrine and epithelial cells, as well as a population of proliferating cells that seem to reflect previously undescribed progenitor cells.

Discussion: The limited proliferation of neuroendocrine cells in low-grade NETs along with the higher proliferation of epithelial cells and lymphocytes within those tumors raises questions about the mode of growth of NETs and the function of microenvironment cells.

Session 3: Tumor Microenvironment and Immunology

No Posters

Session 4: Tumor Biology and Rare NETs

Mapping the gut microbiome in patients with small intestinal neuroendocrine tumors

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Background: The carcinoid syndrome (CS) is a debilitating endocrine complication accompanying the majority of metastatic small intestinal neuroendocrine tumors (SI-NETs). Patients with this malignancy experience long-term survival, but their quality of life is often limited due to hormonal symptoms of severe diarrhea, vasomotor flushes and bronchospasms. While incidence of SI-NETs has vastly increased over the past decades, very little progress has been made in the discovery of (epi)genetic drivers and treatment options. Recent microbiome research has provided novel treatment targets among various cancer types, yet SI-NETs have not been studied. Our aim was therefore to map the gut microbiome in SI-NET patients.
Methods: Fecal samples were collected from patients with SI-NETs and matched controls. Relevant variables were extracted from questionnaires and electronic health records. Next generation 16S sequencing was performed on the fecal samples to analyze between and within group variability and the differential abundance of microbiota.
Results: 182 participants were included: 87 patients, of whom 53 with the CS, and 95 controls. When comparing CS with non-CS patients, no differences in microbial richness and distribution were found. Moreover, no differentially abundant microbiota were found in either group. SI-NET patients, however, had a less rich and diverse microbiome compared to their controls. A total of 42 differentially abundant species were found, 28 were more abundant in controls and 14 were more abundant in SI-NET patients.
Discussion: No association between the gut microbiome and the CS was found. However, the microbiome of SI-NET patients and controls differed, suggesting a role in NEN development and providing novel targets for microbiome-based diagnostics and therapeutics.

Evaluation of Cases with Neuroendocrine Cell Hyperplasia for Classification as Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH) and Subsequent Whole Exome Sequencing Analysis

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Background: Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH) is a condition characterized by widespread occurrence of non-invasive neuroendocrine cell hyperplasias (NECHs) usually in the medium or small airways that eventually progress to invasive carcinoid tumors and tumorlets generally at multiple foci throughout the lung. NECH can be seen in other, often non-neoplastic settings, and therefore, "classic DIPNECH" is often described as including key clinical and radiographic findings such as chronic cough and dyspnea along with radiographic presence of multiple nodules and mosaic air trapping in a diffuse pattern of involvement. We hypothesize that DIPNECH results from a somatic mutation affecting pulmonary neuroendocrine cells resulting in proliferation and wide dispersal within the bronchial epithelium. In order to select informative cases for whole exome sequencing (WES) analysis, we will perform histologic and clinico-radiographic review of 60 cases of potential DIPNECH for classification prior to WES.

Methods: A cohort of 60 patients has been assembled under a newly approved secondary use institutional review board approved protocol. Where available, all hematoxylin and eosin (H&E) stained slides and matching formalin fixed paraffin embedded (FFPE) blocks of tissue were collected. Histologic review to assign classification of lesions as NECH, carcinoid tumorlets and carcinoid tumors as well as enumeration of lesions and quantification of lesion size are being performed. Also included is tallying the presence of airway obstruction by NECH. The presence of multiple lesion types, a NECH density of >/= 0.5 NECH per section and associated airway obstruction are used to indicate histology suggestive of DIPNECH. Protocols for optimal DNA extraction from small lesions such as NECH have been evaluated for impact on DNA yield, quality and adequacy for downstream WES. Fifty clinical and radiographic data elements have been defined and will be collected for all cases by chart review.

Results: Full sets of H&E slides and matching FFPE tissue blocks were retrieved for 58 of 60 potential DIPNECH cases. Assessment of an interim set of 15 cases indicates expected rates of potential cases with histology suggestive of DIPNECH of 60%. Two-thirds of these cases also demonstrate carcinoid tumorlets and relatively promising abundance of robust NECH lesions with at least two or more NECH lesions of adequate size to support WES in 7 of 9 cases with histology suggestive of DIPNECH. A protocol has been established for pre-sequencing tissue characterization and optimized DNA extraction that has been demonstrated to produce accurate and reproducible WES data from formalin-fixed, paraffin embedded (FFPE) limited biopsy tissues that approximate the size of some smaller NECHs by number of lesional cells. In limited tissues, inclusion of carrier RNA (cRNA) during extraction doubles yield. We have demonstrated in matched tissues with and without cRNA that the cRNA derived DNA yields WES data with reduced PCR duplicates, increased depth of coverage and equivalent variant calls without introducing sequencing noise. We are ready to begin chart review and expect this to be carried out on the whole cohort over the next few months.

Discussion: Completion of classification of cases as "classic DIPNECH" will establish the size of this part of the study cohort and we will then compliment these with cases of sporadic carcinoid tumors and non-DIPNECH associated NECH lesions. For DIPNECH cases, two or more foci of NECH, the associated carcinoid tumor and a tumorlet, if present, will be microdissected. This tissue will undergo DNA extraction employing our cRNA optimized protocol and be submitted for WES. The data will be reviewed to identify potential somatic alterations underlying the development and progression of DIPNECH associated premalignant lesions to invasive carcinoid tumors.

Investigating the Role of Pseudohypoxia-related Metabolites in GEP-NET

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Net Type: Multiple; *Research Type:* Basic; *Keywords:* Metabolomics

Background: Von Hippel-Lindau (VHL) disease is a multi-neoplasm inherited disorder caused by a germline mutation in the *VHL* tumor suppressor gene. Among other neoplasms, patients with VHL are at high risk of developing pancreatic neuroendocrine tumors (PNET). VHL-related PNETs (vPNET) have different genetic and clinical phenotypes compared to sporadic PNET (sPNET). VHL protein (pVHL) is critical for cellular oxygen sensing. In pVHL absence, hypoxia-inducible factor 1 (HIF1) accumulates, resulting in a metabolic shift towards glycolysis. Considering the literature supporting pseudohypoxic state in a subset of neuroendocrine tumors, we use vPNET as an extreme model of NET characterized by pseudohypoxia, aiming to study pro-tumoral factors and possible targeted interventions. We compared VHL-related and sporadic PNET. We found a higher adenosine 3´-monophosphate (3´-AMP) level in vPNET vs. sPNET, with a consistently elevated level of 3´-AMP across all VHL-related neoplasms. Hence, we propose to study the potential oncogenic impact of 3´-AMP in vPNET compared to sPNET. Adenosine concentration in biological fluids and extracellular spaces (eADO) is low but increases dramatically in hypoxic, injured, or inflamed tissues and in neoplasms. eADO is metabolized through several pathways. In the canonical pathway, adenosine triphosphate is metabolized to adenosine 5' monophosphate (5'-AMP) eADO is also metabolized via an alternative pathway: Initially, intracellular adenine to 2',3'- cyclic adenosine monophosphate, to intracellular and extracellular 3´-AMP and 2´-AMP. eADO acts through four G-protein coupled receptors, particularly the

A2$_A$ and A2$_B$ receptors that trigger intracellular cAMP accumulation and its dependent pathways. A2$_A$ and A2$_B$ receptors (A2$_A$R, A2$_B$R) are overexpressed in a hypoxic environment, and their stimulation activates the phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway. Hence, we study the role of A2$_A$R and A2$_B$R activation as a pro-neoplastic factor in GEP-NET and the expression of adenosine receptors on the various cells in various cell subtypes in NET tissue samples.

Materials and Methods: Our study is based on a metabolomic assessment of pancreatic and small intestine NET and VHL-related pancreatic NET (vPNET). In addition, we use the BON1 pancreatic NET cell line, in which we induced *VHL* gene silencing, as a novel *in vitro* model for pseudohypoxic PNET. The pseudohypoxic phenotype was compared by specific gene expression (*EPO* and *VEGF*) using real-time polymerase chain reaction, and A2$_A$R expression on BON1^{WT} and BON1^{PH} cells was assessed by enzyme-linked immunosorbent assay (ELISA).

Results: Metabolomic profiling of patient-derived samples processed for polar and lipid metabolites quantified 316 polar metabolites and 992 lipid metabolites. Heatmap and PCA plots revealed polar distinct metabolic signature between vPNET and sPNET. The volcano plot indicates significant high levels of 3´-AMP in vPNET vs. sPNET, in addition to five more metabolites. Pathway analysis based on the metabolites detected identified the purine metabolism pathway as significantly enriched, including guanosine diphosphate, xanthine, 3',5'-Cyclic AMP, adenosine triphosphate, adenosine diphosphate, and other metabolites significantly different between the groups. The *in vitro* model was established, and in the BON1^{PH} cells, the VHL protein expression was lower in the BON1^{PH} vs. BON1^{WT} cells, with a consequent increased expression of *VEGF* and *EPO* compared with BON1^{WT}. ELISA assay demonstrated A2$_A$R expression in BON1^{WT} and BON1^{PH} cells.

Conclusions: The high levels of 3'-AMP and purine pathway in vPNET may drive tumorigenesis via activation of A2$_A$R/A2$_B$R and may influence vPNET development. Validation of A2$_A$R expression on BON1 cells enables us to further test its role in tumor cells' neoplastic parameters. Further accurate characterization of the tumor cells is in progress.

"Neuron-specific enolase" revisited: a new drug target in SDH-deficient paraganglioma?
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Net Type: Pheo/Para; *Research Type:* Basic; *Keywords:* Metabolomics
Background: Pheochromocytomas and paragangliomas (PCPG) are rare neuroendocrine tumors that arise respectively from chromaffin cells in the adrenal medulla or developmentally related cells in paraganglia associated with paraxial sympathetic nerves throughout the body or branches of the vagus and glossopharyngeal nerves in the head and neck. A pheochromocytoma is an intra-adrenal paraganglioma. Approximately 40% of PCPG have a hereditary predisposition, and loss-of-function mutations in genes encoding subunits of the TCA cycle enzyme succinate dehydrogenase (SDH) are a frequent cause. PCPG with SDHB mutations are particularly prone to metastasize, and development of treatments for metastatic disease has historically been hampered by lack of valid experimental models. At the 2020 NETRF symposium the Tischler laboratory introduced a cell line and xenograft model of SDH-deficient pheochromocytoma called RS0 (for rat Sdhb-null) from rats with a heterozygous germline *Sdhb* mutation. RS0 closely resembles SDHB-mutated human PCPG with respect to genome, transcriptome, catecholamine production and metabolism (1). It therefore provides a valuable tool for pre-clinical testing of new drugs targeting vulnerabilities conferred by SDH deficiency, and for developing treatment strategies to improve patient care.
Materials and Methods: Sdh-deficient paragangliomas are essentially a metabolic disease characterized by altered metabolism that includes the shunting of TCA cycle intermediates away from their normal pathways and increased utilization of glycolysis to replenish molecules needed for cell proliferation while at the same time producing enough energy for cell survival. The altered metabolic state presents highly selective drug targets including Enolase, the penultimate enzyme in the glycolysis pathway. Enolase is a dimer consisting of subunits encoded by 3 separate genes ENO1, ENO2 or ENO 3, respectively encoding subunits alpha, gamma or beta. Enolase 2, also known as "Neuron-specific Enolase" (NSE) is a

predominantly gamma-gamma dimer selectively expressed in neurons and neuroendocrine cells and has been recognized for decades as a marker for those cell types and corresponding tumors. A novel selective enolase 2 inhibitor called POMHEX has recently already proven effective against ENO1 deficient human cancers in rodent xenografts (2). We therefore hypothesized that POMHEX would also be effective against RS0 cells. We tested the cytotoxicity of POMHEX in cell cultures both against RS0 cells and RS1/2 cells, a control SDH-intact cell line developed from the same rat lineage. In parallel, mechanisms of antitumoral effects were tested by querying changes in metabolism and protein expression relevant to tumor growth.

Results: POMHEX was cytotoxic to RS0 cells but not RS1/2 cells at nanomolar concentrations compatible with in vivo use. Cell death was preceded by ATP depletion and decreased incorporation of u-13C glucose into lactate, pyruvate and other metabolites. These findings establish "NSE" as a new potential drug target that could selectively target SDH-deficient tumors while minimizing bystander toxicity to SDH-intact cells.

Conclusions: Our findings in cell culture establish "NSE" as a new potential drug target and POMHEX as a prototype drug. The next steps are to test whether this proof of principle applies to in vivo xenografts. In addition, drug stability should be improved. To accomplish these steps we have established a collaboration the team at MD Anderson Hospital that developed POMHEX and is working on new analogs.

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Session 5: Clinical and Theranostic Studies

PAK4/NAMPT inhibitor KPT-9274 synergizes with Sunitinib in Pancreatic Neuroendocrine Tumor Cellular Models

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Net Type: Pancreas; *Research Type:* Translational; *Keywords:* Combinatorial drug treatments

Background: Pancreatic neuroendocrine tumors (PanNETs) are rare islet cell tumors. Although slow growing in early stages, the overall survival rates of metastatic PanNETs is dismally low at 25%. The main treatment option includes surgery followed by chemotherapy or targeted therapy. Unfortunately, advanced PNETs show minimal response to FDA approved therapies suggesting an urgent need for the identification of novel and effective treatments. In the present study, we have tested the combination effect of PAK4/NAMPT inhibitor KPT-9274 with FDA approved multiple RTK autophosphorylation inhibitor sunitinib (Sutent) in PanNET.

Methods: Cellular proliferation or growth inhibition was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cell titer glo (Promega) assay. Inhibitory concentrations were calculated using GraphPad Prism software. For the determination of combination indexes (CI values) and generation of isobologram, Calcusyn 2.1 software were utilized. Formation of colony and spheroid in 2D or 3D culture respectively was determined by clonogenic and spheroid formation assays. Apoptosis was determined by flow cytometric analysis (annexin V-propidium iodide) and western blotting techniques.

Results: The IC50 for KPT-9274 and sunitinib compound were determined as 0.056 μM and 6.6 μM respectively for BON1 PanNET cells. We also tested other kinase inhibitor sulfatinib (surufatinib) that specifically that targets VEGFR and FGFR1

and is in phase III clinical trial for PanNETs. However, we did not observe any response up to 6.4 µM dose. Further experiments assessed the combination of PAK4/NAMPT inhibitor with multiple RTK inhibitors. The combination of KPT-9274 and sunitinib showed synergy in PNET cell lines (CI value was calculated as low as 0.27 suggesting strong synergy between these two inhibitors). Both single agents alone or in combination were reduced the number and area of the colonies significantly. Similar trends observed in spheroid formation assay. The combination showed enhanced apoptosis as well.

Discussion: Taken together, this is the first study to reveal the therapeutic potential of novel PAK4/NAMPT inhibitor KPT-9274 and RTK inhibitor Sunitinib combination for the treatment of PanNETs. The in vivo evaluations of this novel combination in cell derived xenograft (CDx), patient derived xenograft (PDx) models are underway.

Mesenteric fibrosis in small bowel neuroendocrine neoplasms is related to IgG4 expression

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Net Type: Gastrointestinal; *Research Type:* Translational; *Keywords:* Biomarkers

Background: Mesenteric fibrosis in patients with small bowel neuroendocrine neoplasm (SB-NEN) might eventually lead to ischemia or bowel obstruction. The aim of this study was to investigate the relationship between IgG4 expression, the extent of mesenteric fibrosis and other clinicopathological features.

Methods: This retrospective study included patients who underwent resection of a SB-NEN. Imaging data from preoperative scans were assessed, and mesenteric fibrosis was quantified. Formalin fixed paraffin embedded resection material of these patients was selected for additional IgG4/IgG immunostaining. Subgroup analyses were performed for patients with high and low mesenteric fibrosis scores using a novel tool specifically designed for this purpose.

Results: A total of fourteen patients with a mean age of 64 years were included. The median (interquartile range) mesenteric fibrosis score was 54 (39-62). Ten out of fourteen samples had IgG4 positive plasma cells surrounding the tumour cells. The mean IgG4/IgG ratio was lower in the group with mesenteric fibrosis score <54 (24%) compared to the =54 group (36%). Tumours were grade 2 in 60% of patients with IgG4/IgG ratios over 40%, and in 22% with IgG4/IgG ratios less than 40%. Higher mean IgG4/IgG ratios were seen in stage IV vs. stage III patients (34 vs. 21%) and in symptomatic vs. asymptomatic patients (32 vs. 21%).

Discussion: There is a trend towards a higher IgG4/IgG ratio in patients with more extensive mesenteric fibrosis, higher grade tumours, higher stage and symptomatic disease. Further research is warranted to translate these findings to clinical practice and to further validate the mesenteric fibrosis score.

Proximity to healthcare provider, bankruptcy and relationship status within neuroendocrine cancer patients

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Net Type: Multiple NET Types; *Research Type:* Translational; *Keywords:* Biomarkers Clinical studies Metastasis Tumor immunology Tumor microenvironment

Background: Neuroendocrine cancer is a rare malignancy. Variables that weigh into patients living many years with the diagnosis likely contribute to years to decades of unplanned medical expenses. According to the American Journal of Managed Care (AJMC), the economic burden of rare diseases is 10 times higher than more common conditions. The purpose of this study was to quantify social and financial metrics in neuroendocrine cancer patients in order to better understand barriers to specialized care and potentially apply the lessons learned during the pandemic to develop strategies to survey and treat patients closer to home.
Methods: Deceased neuroendocrine cancer patient were identified from a large patient cohort treated at a subspecialty neuroendocrine center. Demographics (gender, race, relationship status, and zip code) were cross-referenced against public records for bankruptcy, and judgements/liens (N=908). AJCC stage at diagnosis time interval from diagnosis to death was also evaluated.
Results: Distance to provider: Median distance traveled for in-person consultation/treatment was 224±5.7miles (range 0.1-4070miles). Distance to provider and overall survival: Overall survival was shorter for patients who traveled >100miles (1776±93days) when compared to NET patients traveling <100miles (2445±451days, p=0.03). Bankruptcy rates: Bankruptcy rates in NET patients was 10.4%. The average age for filing a bankruptcy petition was 52yrs. Bankruptcy rates were highest in African American (15%), divorced (35%) and widowed (33%) patients. Bankruptcy rates and tumor grade: Bankruptcy rates were higher in G3 NET patients (24%) when compared to G2 (12) and G1 (9%). Bankruptcy rates and stage: In stage I-III NET patients the bankruptcy rate was 7.14%. In stage IV NET patients the bankruptcy rate increased to 15.8% Bankruptcy rates and proximity to NET specialist: Rates increased with distance to clinic up to 150 miles (max = 24%) after which levels decreased to baseline of 10%. Bankruptcy and overall survival: We were unable to show a significant difference in OS associated with bankruptcy. This may be due to confounding variables. Divorce rates in NET: Divorce rate in NET patients was 12.5%. Divorce rates were highest for female NET patients (15%) when compared to male NET patients (10%). There was no significant difference in overall survival between divorced/single/widowed NET patients when compared to married/significant other NET patients.
Discussion: Bankruptcy rates were increased in NET patients and linked to proximity to NET subspecialty care. Reduced proximity to specialist care was linked to worse overall outcomes. The burdens of bankruptcy appear to be in younger patients who may have unplanned medical expenses earlier in their life than anticipated. The data didn't show any significant association between bankruptcy and OS, potentially due to confounding variables. In combination, these data argue for improved access to specialist care, including but not limited to the use of telemedicine and engagement of localized medical oncologists as partners in the care of patients with a rare cancer. Other socioeconomic factors may also impact NET diagnosis and outcomes. Including but not limited to education, poverty, and payor medical policies.

Survival differences of lung neuroendocrine tumors in California by sociodemographic, clinicopathologic, and treatment factors

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Net Type: Lung; *Research Type:* Clinical; *Keywords:* Clinical studies

Background: Lung neuroendocrine tumors (NETs) are a rare, heterogeneous group of well-differentiated cancers with varying clinical behavior. Little is known about lung NET epidemiology or predictors of survival. We investigated associations between sociodemographic, clinicopathologic, geographic, and treatment factors with survival for patients with lung NETs in California.

Methods: We conducted a population-based prospective study of mortality among individuals with an incident lung NET diagnosis (typical or atypical histology) in the California Cancer Registry (CCR) from 1992 through 2017. We used Kaplan-Meier time-to-event survival analysis and compared univariate survival among demographic and disease factors by the log-rank test. Sequential multivariable Cox proportional hazards models were used to estimate independent associations of sociodemographic, clinicopathologic, geographic, and treatment-related factors with all-cause mortality.

Results: There were 5,127 cases diagnosed with lung NETs in the CCR from 1992-2017, including 4,784 typical carcinoid and 343 atypical carcinoid cases. Women were a majority of the lung NET diagnoses (70%), as were non-Hispanic White Californians (74%). We found that sociodemographic factors and social determinants of health were independently associated with survival, with better survival for women compared with men (hazard ratio [HR] 0.63, 95% confidence interval [CI] 0.57-0.70, p<0.001), married compared with unmarried Californians (HR 0.80, 95% CI 0.72-0.89, p<0.001), and cases living in high socioeconomic status (SES) neighborhoods compared with low SES neighborhoods (HR 0.65, 95% CI 0.59-0.76, p<0.001) in our fully adjusted multivariable model. There was also worse survival for cases with Medicare (HR 1.27, 95% CI 1.11-1.45, p=0.001) or Medicaid, military, or other public health insurance (HR 1.54, 95% CI 1.29-1.84, p<0.001) compared to cases with private health insurance only. Non-Hispanic Black Californians had worse survival than other racial/ethnic groups in our univariate model, but racial differences in survival attenuated after accounting for other social determinants of health, disease characteristics, and treatment variables. Localized stage, typical histology, and surgical resection were also independently associated with improved survival. In contrast, rural versus urban county of residence did not impact survival in any model.

Discussion: Beyond disease-related and treatment factors, we found that sociodemographic factors were independently associated with overall survival in lung NETs. Future strategies to improve outcomes for patients with lung NETs should include efforts to extend support to socially isolated and economically disadvantaged individuals to reduce survival disparities.

Economic disparities prevent neuroendocrine cancer diagnosis
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Net Type: Multiple NET Types; *Research Type:* Translational; *Keywords:* Correlative studies Biomarkers Clinical studies Tumor immunology Tumor microenvironment

Background: Background/Significance to NETs: The real incidence of neuroendocrine cancer is probably much higher than that observed or reported. This is probably due to barriers that prevent diagnosis. The purpose of this study was to examine the impact of personal bankruptcy on NET diagnosis. Our long-term goal is to better understand obstacles that impede access to specialist care and clinical trials. Using this data we can design/advocate countermeasures that will improve diagnosis, quality of life, and overall outcomes. This study is not focused on medical bankruptcy, or debt arising from medical bills resulting in bankruptcy. Rather we are investigating the effect of bankruptcy on outcomes and diagnosis. Specifically, we are asking: 1) does bankruptcy effect NET outcomes, and 2) does bankruptcy effect NET diagnosis.

Methods: Methods: To avoid confidentially issues this study was limited to deceased NET patients. Deceased NET patients were identified from medical records between 2006 and 2022 and were cross referenced against public records for bankruptcy to create a REDCap database for analysis (n=1236). From this database, bankruptcy rates were calculated and correlated against overall survival, age at bankruptcy, and age at death.

Results: Results: Of the 1236 NET patients, 10.4% had filed for bankruptcy (chapter 7 or chapter 13) during their life. The average age of first-time bankruptcy filing (a small subset of patients filed for bankruptcy multiple times) was 52±12yrs. In response to the specific aims of the project: 1) Bankruptcy had no effect on overall survival. There was no statistical difference in overall survival between NET patients who filed for bankruptcy (1853±171days) vs controls (1906±61days p=0.38). 2) The data suggests that bankruptcy reduced NET diagnosis and there are a cohort of NET tumors that are never diagnosed. This observation is implied by differences in the age of death and age of NET diagnosis between NET patients that had filed for bankruptcy vs controls. The average age of death for patients who had filed for bankruptcy was significantly lower (60±1yrs) when compared to controls (64±0.4, p<0.01). In a similar manner, the average age for NET diagnosis was earlier in patients who had filed for bankruptcy (56±0.9yrs) when compared to controls (59±0.6, p=0.01). These differences are an anomaly of the data set. We know of no reason why bankruptcy would accelerate the onset of NET resulting in younger diagnosis and younger death. The most logical explanation is that the data sets for bankrupt NET patients are incomplete, particularly due to missing older NET patients that never received a diagnosis. There are numerous statistical modeling methods that can be used to address missing data by imputing missing values. To correct for missing data, the bankrupt NET patient age at death data was subjected to maximum likelihood modeling based on data from non-bankrupt NET patients. The percentage of imputed missing values was calculated to be 37.5%. Consequently, the corrected bankruptcy rate in NET patients would be increased to 12.3%.

Discussion: Bankruptcy does not affect overall survival in patients that are diagnosed with NET. However, bankruptcy does impact the probability of diagnosis. It is plausible that an individual with an undiagnosed NET could endure financial hardship, file for bankruptcy, and lose health insurance and access to diagnostic resources. Failure to diagnose will skew resulting data sets. Based on NET incidence of 6/100000, US population of 332 million, bankruptcy rate of 12.3%, and 38% underreporting, we estimate that economic disparities in the USA leads to a failure to diagnose in approximately 927patients/year. Further correlative studies are required to strengthen the value of this data to facilitate medical or legislative initiatives to expand access to specialized care for NET patients. Future studies will monitor potential narrowing of the differences between bankrupt NET patients and controls with changes in health care access and affordability post Affordable Care Act expansion of health care.

Image-guided delivery of temozolomide to SSTR2 expressing cells

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Presenting Author: Ali Azhdarinia
Net Type: Multiple NET Types; *Research Type:* Translational; *Keywords:* Nuclear medicine Theranostics Chemoresistance

Background: Most chemotherapy regimens used to treat neuroendocrine tumors (NETs) include the DNA-alkylating agent temozolomide (TMZ) alone or in combination with other cytotoxic agents or targeted therapies. The cytotoxicity of TMZ is primarily mediated through DNA methylation at O6-guanine sites that lead to DNA mismatch, apoptosis, and cell death. Such methyl adducts can be directly repaired by the DNA repair enzyme methylguanine-DNA methyltransferase (MGMT), which specifically reverses this methylation, restores normal DNA architecture, and rescues tumor cells. Since MGMT is a suicide enzyme and is inactivated after each reaction, increasing the number of DNA adducts could saturate this repair mechanism and enhance sensitivity to TMZ. However, attempts to deplete MGMT with dose-dense TMZ have not demonstrated clinical benefit as systemic doses were necessarily reduced in the setting of intolerable myelotoxicity. Understanding whether TMZ resistance can be overcome by optimized dosing protocols would address a critical clinical need. Thus, new approaches that selectively increase intratumoral TMZ are required. Since nearly all NETs overexpress the somatostatin receptor subtype 2 (SSTR2), we hypothesized that a receptor-targeted TMZ analog could produce high intratumoral drug concentrations while avoiding systemic toxicity. Given the outstanding tumor-targeting properties of radiolabeled somatostatin analogs, we converted the clinically approved radiotracer 68Ga-DOTA-TOC into a radiolabeled peptide-drug conjugate (PDC) for SSTR2-targeted delivery of TMZ and report on the utility of the radioactive label for characterizing receptor-binding properties, pharmacokinetics, and tissue biodistribution.

Methods: The PDC was synthesized by (i) replacing DOTA with a multimodality chelator (MMC) to permit site-specific modification, (ii) attaching a modified TMZ analog to MMC, and (iii) conjugating the payload moiety to TOC on solid-phase. The resulting product, referred to as tumor-targeted TMZ (ttTMZ), was radiolabeled with 67Ga using cation exchange chromatography methods adapted from 68Ga. Retention of SSTR2 binding was examined in cell lines with different expression levels of SSTR2 with and without blocking doses of octreotide. A membrane acid wash was performed to evaluate internalization efficiency. The cytotoxicity of ttTMZ was tested in IMR-32 cells that endogenously express SSTR2, and potency was compared to free-TMZ (fTMZ). To do so, cells were exposed to a dose range of each agent for 72 h and cell viability was assessed with Cell-titer Glo. To evaluate DNA-damaging properties of the PDC, an alkaline comet assay was performed after exposure of the cells to the drug. Briefly, IMR-32 cells were treated with 100 µM of fTMZ or ttTMZ for 72 h in the presence or absence of 10x octreotide (blocking agent). To further assess the DNA damaging properties of ttTMZ, we investigated the formation of double-strand DNA breaks by ?H2AX immunostaining. IMR-32 cells were treated with 100 µM of fTMZ or ttTMZ with and without blocking agent for 48 h and stained with a ?H2AX antibody. To show the effect of the ttTMZ on the MGMT enzyme level, IMR-32 cells (MGMT+) were treated with increasing concentrations of ttTMZ, fTMZ, and O6-Benzylguanine (O6BG; a well-characterized pseudo-substrate for MGMT that has potent inhibitory effects) for 24 h and the total protein was extracted for western blot studies. To investigate SSTR2 targeting in vivo, positron emission tomography (PET) was performed 1 h after injection of 68Ga-ttTMZ in H69 xenografts in the presence and absence of blocking doses of octreotide. To further evaluate specificity and biodistribution at pharmacologically active drug concentrations, we performed a dose-escalation study with 67Ga-ttTMZ in (i) mice bilaterally implanted with HCT116-WT (SSTR2-negative) and HCT116-SSTR2 (transfected, high SSTR2) cells and (ii) mice implanted with IMR-32 cells (endogenous SSTR2). The xenografts were injected with different doses of radiolabeled ttTMZ (2, 5, 10, and 100 nmol) and euthanized at 3 h p.i. Resected tissues were weighed and gamma counting was performed to quantitatively measure drug biodistribution at 3h p.i.

Results: ttTMZ was efficiently produced with chemical and radiochemical purities >90% and >95%, respectively, which indicates the feasibility of using the MMC to directly label the drug conjugates. Cell-based experiments showed that the binding properties of 67Ga-ttTMZ was similar to 67Ga-DOTA-TOC and correlated with SSTR2 expression. In HCT116-SSTR2 cells that highly overexpress SSTR2, 14.8±4.8% of 67Ga-ttTMZ and 17.0±4.2% of 67Ga-DOTA-TOC were taken up by cells. Markedly less accumulation was observed in cell lines with lower SSTR2 expression. SSTR2 selectivity was further demonstrated in blocking studies where tracer binding was reduced by nearly 90% when co-incubated with octreotide. Acid-washing experiments demonstrated internalization of 67Ga-ttTMZ, indicating retention of agonist properties following chemical modification. The cell cytotoxicity results demonstrated that, similar to the free drug, ttTMZ was able to reduce the IMR-32 cell growth in a dose-dependent manner. The IC50s of fTMZ and ttTMZ were 81.6 and 75.6 µM, respectively. Comet assay results showed that ttTMZ caused DNA damage that was similar to fTMZ. This effect was significantly reduced (P<0.05) in the presence of octreotide blocking, further confirming that the DNA damage was receptor mediated. The results of ?H2AX immuno-staining showed that exposure of IMR-32 cells to fTMZ and ttTMZ caused 9 and 6-fold increase in the number of ?H2AX-positive cells, respectively, compared to non-treated cells. ?H2AX foci formation was significantly reduced in the presence of the blocking agent (P<0.05). Western blot analysis showed that ttTMZ reduces MGMT levels in a dose-dependent manner compared to untreated cells and was consistent with free TMZ treatment. PET imaging in H69 xenografts confirmed SSTR2 selectivity of the agent as indicated by notable accumulation of 68Ga-ttTMZ in tumors and a 1.3-fold reduction in signal in mice pre-injected with the blockade. Similar results were seen in the dual implant model as shown by >5-fold higher uptake in the SSTR2+ tumors compared to SSTR2- tumors. In these studies, we found that the tissue distribution profile of 67Ga-ttTMZ was analogous to 68Ga-DOTA-TOC: high tumor uptake, rapid elimination through the kidneys, and low signal in normal tissues. This observation was further supported by a dose escalation study with 67Ga-ttTMZ in IMR-32 xenografts, where off-target uptake did not increase as a function of dose whereas tumor uptake was maintained.

Discussion: Findings from this study support our central hypothesis and demonstrate the feasibility of tumor-targeted MGMT depletion with ttTMZ. We also demonstrate the use of the MMC as a linker to synthesize a bioactive TMZ analog that retains the receptor-targeting properties of DOTA-TOC. In addition, direct radiolabeling of ttTMZ via the MMC allowed us to quantify the binding and internalization and compare agent performance to the gold standard, 67Ga-DOTA-TOC which eventually can guide optimization strategies. We have also shown that ttTMZ produces DNA damage that leads to cell death, while also depleting MGMT levels in cells. Taken together, these observations suggest that it may be possible to increase the sensitivity of NETs to TMZ through tumor-targeted inhibition of MGMT.

3p-C-NETA-TATE: A versatile somatostatin analogue for Al18F-labeled and therapeutic SSTR2 targeting radiopharmaceuticals

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Net Type: Multiple NET Types; *Research Type:* Translational; *Keywords:* Nuclear medicine Theranostics

Background: Somatostatin-based radiopharmaceuticals (e.g. [68Ga]Ga-DOTATATE and [177Lu]Lu-DOTATATE) have been used to diagnose, monitor, and treat neuroendocrine tumor patients with great success [1]. However, widespread implementation of [68Ga]Ga-DOTA-SSA PET in clinical practice is often hampered by practical, regulatory and economic factors related to 68Ge/68Ga-generators, such as the high cost, limited availability, low activity yield per elution and regulatory and reimbursement barriers. The Al18F-method combines the advantages of a chelator-based radiolabeling method with the imaging and logistical advantages of fluorine-18. [18F]AlF-NOTA-octreotide, a promising 18F-labeled somatostatin analogue and potential alternative for 68Ga-DOTA-peptides, is under clinical evaluation [2]. A major drawback is that the most utilized chelator for the Al18F-method, 1,4,7-triazacyclononane-N,N',N''-triacetic acid (NOTA), is not compatible with therapeutic radionuclides such as the ß--emitter lutetium-177 (177Lu) and the promising a-emitter bismuth-213 (213Bi). Ideally, the same precursor (combination of chelator-linker-vector) can be used for production of both diagnostic and therapeutic radioprobes with very similar (e.g. Al18F/213Bi/177Lu) pharmacokinetic properties, which is possible with the promising chelator 3p-C-NETA [3], allowing accurate personalised dosimetry estimation, and radionuclide therapy of NET patients in a theranostic setting. In contrast to external high energy photon or proton therapy, targeted radionuclide therapy (TRNT) is a systemic cancer treatment allowing targeted irradiation of a primary tumor and all its metastases, resulting in less collateral damage to normal tissues. The single a-emitting radionuclide 213Bi (T1/2 = 45.6 min, Ea = 8.4 MeV, ? = 440 keV, a-particle range = 40–80 μm, corresponding to 2-10 cell diameter) has interesting properties and might be considered as a magic bullet for TRNT [4]. Further, it can be produced on site using a 225Ac/213Bi generator. In this study we evaluated 3p-C-NETA-TATE as theranostic precursor for NET imaging and therapy, and present first results of the preclinical evaluation of the diagnostic compound [18F]AlF-3p-C-NETA-TATE [5] and therapeutic ligands [213Bi]Bi-3p-C-NETA-TATE and [177Lu]Lu-3p-C-NETA-TATE.

Methods: 3p-C-NETA-TATE was synthesized using standard solid/liquid-phase peptide synthesis and purified using HPLC. [18F]AlF-3p-C-NETA-TATE was synthesized in an automated AllinOne® synthesis module. 3p-C-NETA-TATE (10 μM) was radiolabeled manually with 177Lu (NaOAc, 0.1 M, pH 4.1, 12 min) or 213Bi (Tris-HCl, 4 M, pH 8.5, 7 min) at 40 or 95 °C. The in vitro stability of the corresponding radiocomplexes was evaluated in formulation buffer, PBS and human serum at 37 °C using radioHPLC. In vitro cell binding and internalization was performed for [18F]AlF-3p-C-NETA-TATE, [213Bi]Bi-3p-C-NETA-TATE (185 kBq/well; A?: 1.23 GBq/μmol) and [177Lu]Lu-3p-C-NETA-TATE (53 kBq/well; 176.66 A?: GBq/μmol) using SSTR2 expressing cells (QGP1.SSTR2 and/or BON1.SSTR2) [4] and the pharmacokinetics of [18F]AlF-3p-C-NETA-TATE were evaluated in healthy rats and in xenograft (QGP1.SSTR2) bearing mice using μPET/MRI and μPET/CT, respectively. [18F]AlF-NOTA-Octreotide was used as benchmark. Cell viability and clonogenic assays were performed with [213Bi]Bi-3p-C-NETA-TATE and [177Lu]Lu-3p-C-NETA-TATE using BON.SSTR2 cells.

Results: [18F]AlF 3p-C-NETA-TATE was obtained in good RCY (56 ± 10%) and >98% radiochemical purity. [18F]AlF-3p-C-NETA-TATE displayed excellent in vitro stability with >95% intact tracer after 4 hours in all tested conditions. High SSTR2 specific cell binding and internalization (18.4 ± 2.1 % of which 78.3 ± 2.1 % is internalized) was observed after 60 min incubation. Finally, [18F]AlF-3p-C-NETA-TATE showed excellent pharmacokinetic properties (rats and mice) and tumor accumulation (SUVmean60 min:2.7 ± 1.1), which was comparable as for [18F]AlF-NOTA-Octreotide (SUVmean 60 min:3.2 ±

0.76). We were also able to block uptake in SSTR2 expressing organs and in tumors (>90%) by coinjection of 2.5 mg/kg octreotide acetate, indicating SSTR2 specific uptake. 3p-C-NETA-TATE efficiently sequestered 177Lu (RCC >95%) and 213Bi (RCC >90%) at 12 and 7 min respectively at 40 °C. [177Lu]Lu-3p-C-NETA-TATE showed excellent in vitro stability in both PBS and mouse serum (>90% intact complex at day 3). Starting with 94.3% radiochemical purity, [213Bi]Bi-3p-C-NETA-TATE demonstrated good stability (>90% intact radiocomplex) after 5 h in both PBS and human serum. High SSTR2 specific cell binding and internalization (11.4 ± 0.7 % of which 62.1% is internalized) was observed after 60 min incubation for [177Lu]Lu-3p-C-NETA-TATE whereas only 3.3 ± 0.5% cell binding (of which 39.2% is internalized) was observed for [213Bi]Bi-3p-C-NETA-TATE, probably due to blocking effects because of low apparent molar activity. >99% blocking after co-incubation with 100 μM octreotide was observed for both tracers at 60 min. Reduced viability of BON-1.SSTR2 was observed after 48 h incubation with [213Bi]Bi-3p-C-NETA-TATE (9.9 ± 0.9% viability at 0.1 MBq activity). Also, only 0.1 MBq activity of [213Bi]Bi-3p-C-NETA-TATE was required to achieve cell surviving fraction of 0.1 (SF0.1).

Discussion: 3p-C-NETA-TATE is an excellent and versatile agent that can be used for both targeted radionuclide therapy (177Lu, 213Bi) and diagnostic applications (Al18F) and has advantages as an alternative to the DOTA-SSA analogues in current clinical use. [18F]AlF-3p-C-NETA-TATE and [213Bi]Bi-3p-C-NETA-TATE/[177Lu]Lu-3p-C-NETA-TATE will be further evaluated as potential theranostic pairs in SSTR2 expressing tumor mice. 1. Pauwels, E., et al. (2018) AJNMMI, 8, 311-331 2. Pauwels, E, et al. , (2020). EJNMMI, 47 (13), 3033-3046 3. Chong, H.S. et al; . Bioorg. Med. Chem. Lett. 2008, 18, 3436–3439. 4. Ahenkorah, S et al. (2021). Pharmaceutics, 13 (5) 5. Ahenkorah et al. (2022) Theranostics 12(13): 5971-5985

Evaluation with DOTATATE-PET After Two Cycles of Peptide Receptor Radionuclide Therapy (PRRT) in Neuroendocrine Tumors (NET)

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Net Type: Multiple NET Types; *Research Type:* Clinical; *Keywords:* Clinical studies Metastasis Nuclear medicine Theranostics

Background: We aimed to evaluate the added information provided by DOTATATE-PET after two cycles of peptide receptor radionuclide therapy (PRRT) in patients with somatostatin receptor (SSTR)-expressing neuroendocrine tumors (NET).

Methods: In this retrospective study, 105 patients (54 women and 51 men, 62.5±10.5-year-old) with progressive NET treated with at least two cycles of 177Lu-DOTATATE were included. All patients had DOTATATE-PET (PET/CT or PET/MRI) at baseline, after two cycles, and upon completion of PRRT. RECIST and change in SSTR-density were used to evaluate the scans and assess treatment response. Change in tumor marker chromogranin A was recorded. Patients were surveyed regarding their stance on the additional scan midway through the treatment.

Results: All patients considered the additional DOTATATE-PET contributing to their quality of life as it provided awareness about the evolution of the therapy. After two PRRT cycles, 0/105 (0%) patients showed complete response (CR), 54/105 (51%) partial response (PR), and 40/105 (38%) had stable disease (SD) with agreement between RECIST and SSTR-density. In 11/105 (11%) patients RECIST and SSTR-density were discordant: progressive disease (PD) according to RECIST was seen in 11/11 patients, while evaluation of SSTR-density showed true progression in 4/11 and pseudo-progression in 7/11 patients. Follow-up imaging after completion of PRRT verified results from interim imaging: 4/11 had true progression while the other 7/11 patients showed PR. The pattern of pseudo-progression consisted in an up to 2 mm increase in size of known NET lesions, with or without central necrosis or new stranding, and no new lesions. The SSTR-density in these patients was stable or decreased when related to the liver based on Krenning score. Chromogranin A was available in 37/105 patients. The change in chromogranin A did not positively correlate with response to treatment. In addition, in two patients, the DOTATATE-PET/CT performed after two cycles was able to diagnose interval development of non-SSTR expressing lesions in the liver. Biopsy confirmed the lesions to be metastatic NET lesions grade 3.

Discussion: Our data show that a DOTATATE-PET (PET/CT or PET/MRI) after two cycles of PRRT provides important reassurance for the patient about the status of the disease and response to treatment. No patient showed CR after two cycles of PRRT, clearing concerns about possible overtreatment. DOTATATE-PET is more accurate in assessing treatment response of SSTR-expressing disease after two cycles than RECIST, allowing continuation of treatment in patients with pseudo-progression. Change in tumor marker did not correlate well with response to treatment after two treatment cycles.

SV2A PET Imaging for Noninvasive Assessment of Neuroendocrine Differentiation in Neuroendocrine Tumors
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Net Type: Multiple NET Types; *Research Type:* Translational; *Keywords:* Nuclear medicine Theranostics Biomarkers

Background: Neuroendocrine tumor (NET) remains a considerable diagnostic challenge even with the combination of circulating biomarker test, biopsy, and morphologic/functional imaging. Given the unique feature of NET cells closely related to both nervous and endocrine systems, investigating the contribution of nerves in the initiation and progression of NETs will lead to new strategies for its diagnosis and therapy. Somatostatin receptors (SSTRs) are good targets for specific PET imaging (e.g. 68Ga-DOTATATE) in NET diagnosis and targeted radionuclide therapy (e.g. 177Lu-DOTATATE), but there is a gap of no radiotracers to investigate the tumor innervation and target NE differentiation directly for NETs. Our initial studies have found that synaptic vesicle protein 2 isoform A (SV2A) has great potential to become a promising biomarker for neuroendocrine differentiation in NETs. Herein, we report our continuous efforts in evaluating and developing NET PET imaging by targeting SV2A.

Methods: De-identified human NET (19 lung, 15 pancreas, and 6 intestine) tumor tissues were identified from the Tissue Management Shared Resource at the UTSW Simmons Comprehensive Cancer Center. SV2A, CgA, SYP, GLUT1 (Sigma, 355A-1), and SSTR1–5 (Abcam: ab140945, ab271907, ab227601, ab272677, ab109495) were stained and interpreted based on the histochemical scoring (H-score) assessment incorporating both the staining intensity and a percentage of stained cells at each intensity level. The information we received for each sample includes gender, race, ethnicity, histology, prior treatment history, clinicopathologic features including known Gleason grade of the tumor, and site of primary or metastatic biopsy. Four new 19F-SV2A binding ligands were synthesized to pre-evaluate their SV2A specific binding affinity through a competition binding assay. The radiolabeling precursor for the -amino analog at the pyridine ring was prepared based on the binding assay, and the 18F-radiolabeling was tested afterward in an automated synthesizer.

Results: Due to the limit number of human NET tumor tissue samples, all samples were either at grade 1 or 2. Overall, SYP showed the highest H-score across all samples, with 262 ± 24, 270 ± 0, and 210 ± 46 for lung, pancreas, and intestine NETs, respectively. In contrast, SV2A showed the 2nd highest H-score in lung NETs (155 ± 75) and the 3rd highest H-score in the pancreas (134 ± 96) and intestine (132 ± 52) NETs. The SSTR2's H-scores were close to SV2A, with 147 ± 113, 187 ± 89, and 181 ± 124 for lung, pancreas, and intestine NETs, respectively ($p > 0.05$). Besides SSTR2, SSTR4 was also found with significant staining in some lung and pancreas NET samples. Due to the relatively low grades, the GLUT1 showed smaller H-scores as expected as 79 ± 40, 110 ± 47, and 91 ± 50 for lung, pancreas, and intestine NETs, respectively. Correlation analyses were tried for SV2A vs other biomarkers using the H-scores, but no conclusion could be made partially because of lack of higher grades NET samples. Additionally, the western blot method is expected to fit better for the correlation analyses than the IHC staining method based on the H-score.

Discussion: SV2A showed strong expressions in low-grade lung, pancreas, and intestine NET human tumor tissue samples, which is close to the level of SSTR2. Further correlation analyses are required to include more data in high-grade NETs and

conduct the experiments using the western blot method. A new 18F-SV2A tracer with higher hydrophilicity was successfully prepared, and it is ready for the following SV2A-PET imaging evaluation in NET xenograft models.

A novel anti-SSTR bispecific T-cell engager (BiTE)-like molecule for the treatment of neuroendocrine tumors

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Net Type: Gastrointestinal; *Research Type:* Translational; *Keywords:* Tumor immunology

Background: Bispecific T-cells engagers (BiTEs) are an emerging class of immunotherapeutic molecules that promotes the formation of a cytolytic immunological synapse between T cells and tumor cells. Well- differentiated NETs overexpress somatostatin receptors (SSTRs). We designed a novel BiTE targeting SSTR with an engager composed of 2 molecules of Somatostatin-14.
Methods: The optimized sequence of the BiTE was subcloned into a vector (pAcGP67a) designed for protein expression in insect cells using Baculovirus. Trichoplusia-ni (High Five) cells were used to express the recombinant protein, which was isolated from the supernatant using nickel affinity chromatography. Flow cytometry and confocal microscopy were used to determine the binding potential of the BiTE towards CD3 and SSTR2. CD3+ T cells isolated from the peripheral blood of healthy donors were co-incubated with 293T cells stably transduced to concurrently express SSTR2 and green fluorescent protein (GFP) in the absence or presence of the BiTE. The SSTR2- parental 293T cell line was used as negative control, while anti-CD3/CD28 beads were added as a positive control. The BiTE-induced T cell activation was evaluated measuring the secretion of IFN-gamma and Granzyme B by ELISA and OX40, 41BB, CD25 and CD69 by flow cytometry.
Results: At a concentration of 100 nM, the BiTE bound the CD3 receptor of approximately 85% of T cells and the SSTR2+ 293T cells were coated with BiTE by confocal microscopy. IFN-gamma and Granzyme B secretion was significantly higher when the T cells were co-cultured with SSTR+ 293T cells in the presence of the BiTE as compared with parallel preparations with SSTR- 293T cells or without the BiTE, suggesting that the BiTE-induced T cell activation is specific. At the same conditions, the percentage of T cells co-expressing OX40/41BB and CD25/CD69 increased compared with the controls.
Discussion: To our knowledge, this is the first BiTE to incorporate a hormone in one binding site, which efficiently engages SSTR2 and T cells enabling the formation of immune synapse.

Update on Clinical Evaluation of 3-[18F]fluoro-p-hydroxyphenethylguanidine ([18F]3F-PHPG) for Localization of Paraganglioma and Pheochromocytoma using Positron Emission Tomography (PET)

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Net Type: Pheo/Para; *Research Type:* Clinical; *Keywords:* Nuclear medicine Theranostics Tumor microenvironment

Background: We previously reported on our initial clinical findings with a new PET radiotracer, 3-[18F]fluoro-p-hydroxyphenethylguanidine ([18F]3F-PHPG), as an imaging agent for tumor localization in patients with paraganglioma (PGL) and pheochromocytoma (PCC). As an analog of norepinephrine, [18F]3F-PHPG is a good substrate of the norepinephrine transporter (NET) and the two isoforms of the vesicular monoamine transporter (VMAT1, VMAT2). It also has prolonged retention in norepinephrine storage vesicles. The primary goal of this study was to evaluate the diagnostic performance of [18F]3F-PHPG in patients with PCC and PGL, which often express NET and VMAT transporters. The [18F]3F-PHPG results have been directly compared with any additional clinical scans with [68Ga]DOTATATE (Netspot), [123I]MIBG or 2-[18F]fluoro-2-deoxy-D-glucose ([18F]FDG) in the same patients. Another goal of the study was to perform immunohistochemistry of NET, VMAT1 and VMAT2 expression levels in resected tumors from subjects to assess the relative importance of each transporter in driving tumor uptake of [18F]3F-PHPG.

Methods: [18F]3F-PHPG was prepared by a new radiosynthesis method that uses a spirocyclic iodonium ylide precursor, which provided consistently high radiochemical yields (50 to 190 mCi/batch). Whole body PET/CT scans with [18F]3F-PHPG were performed in n = 13 new subjects (7 females, ages 23-74, 6 males, ages 30-72) with PCC, PGL or mixed etiologies. Scans were acquired at 90 min following i.v. injection of 8.2 to 12.7 mCi of [18F]3F-PHPG. Images were interpreted by expert nuclear medicine physicians. [18F]3F-PHPG uptake in tumors and normal organs were quantified using standardized uptake values (SUVmax or SUVmean). If available, [18F]3F-PHPG scan results were directly compared to results from prior scans with [68Ga]DOTATATE, [123I]MIBG or [18F]FDG.

Results: Similar to our initial results, [18F]3F-PHPG was able to identify known lesions in most cases, and often exhibited diagnostic performance comparable to [68Ga]DOTATATE in those cases where clinical scans were available. Examples from PGL patients include study subject #7 (F, 53) who had a right paraganglioma. [18F]3F-PHPG uptake in the tumor near the liver had SUVmax = 7.1 compared to SUVmax = 8.5 for a recent [68Ga]DOTATATE scan. Subject #9 (M, 69) had long-standing metastatic PGL. [18F]3F-PHPG uptake was high in the many soft tissue lesions in the lungs, liver and abdomen (SUVmax from 38.5 to 98.2) and thoracic vertebra T12 (SUVmax = 72), comparable to SUVmax values from a prior [68Ga]DOTATATE scan (46 to 75, and 21, respectively). Subject #17 (M, 47) had a rare PGL originating from the prostate gland that had been resected, but a recent [123I]MIBG scan showed a pelvic lesion. [18F]3F-PHPG showed high uptake in the pelvic lesion, with SUVmax = 24. Among PCC patients, subject #8 (M, 60) had a sporadic right PCC. Tumor uptake of [18F]3F-PHPG had SUVmax = 24.1, compared to 37.2 for a prior [68Ga]DOTATATE scan. In subject #15 (M, 56), a large sporadic right PCC had good uptake of [18F]3F-PHPG, with SUVmax = 16.1 compared to values of 29.7 for [68Ga]DOTATATE and 10.8 for [18F]FDG. In four patients with head and neck PGL, three had low to moderate uptake of [18F]3F-PHPG. These included SUVmax = 2.2 in a patient with a left glomus jugulare tumor (F, 23), SUVmax = 2.2 in a patient with a right carotid body tumor (F, 49), and SUVmax = 6.6 in a patient with left carotid body tumor (F, 74). In the fourth head and neck PGL (F, 50), with SDHB-related hereditary paraganglioma syndrome, [18F]3F-PHPG had high uptake in the left glomus jugulare tumor, with SUVmax = 20.1, compared to SUVmax = 3.3 in a prior [68Ga]DOTATATE scan. Immunohistochemical staining studies of NET, VMAT1 and VMAT2 expression levels have been performed in n = 11 of 19 subjects scanned and are currently being analyzed to relate expression levels of these key adrenergic transporters to tumor [18F]3F-PHPG uptake levels.

Discussion: [18F]3F-PHPG continues to show clinical utility in localizing tumor sites in PGL and PCC patients. [18F]3F-PHPG offers comparable diagnostic performance with [68Ga]DOTATATE in most cases. Also, to date, [18F]3F-PHPG has detected all major lesions seen in [123I]MIBG scans in the same patients, but also identifies many smaller lesions lost in the quantum mottle of the planar scintigraphic [123I]MIBG images. [18F]3F-PHPG has several advantages over [123I]MIBG, including the higher spatial resolution and improved lesion contrast of PET imaging, and the ability to do same-day imaging. We plan to scan an additional n = 11 subjects in the coming months to reach a total cohort of n = 30 subjects.

Effect of Epigenetic Treatment on SST2 Expression in Neuroendocrine Tumor Patients

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Net Type: Multiple NET Types; *Research Type:* Translational; *Keywords:* Clinical studies Combinatorial drug treatments Epigenetics Cell lines

Background: Somatostatin receptor subtype 2 (SST2) expression is of eminent importance for the staging and treatment of neuroendocrine tumors (NETs). The benefit obtained by treatment with radiolabeled somatostatin analogues does not apply to patients with SST-negative tumors. Recent preclinical in vitro and in vivo data have uncovered the potential of upregulation of SST2 expression in NET cells by epigenetic treatment.

Methods: Clinical proof-of-concept trial in patients with advanced well-differentiated gastroenteropancreatic or pulmonary NETs with low SST expression, defined as tumor uptake on 68Ga-DOTATATE PET/CT below that or equal to that of the liver. Patients received 14-day treatment with histone deacetylase inhibitor valproic acid and DNA methyltransferase inhibitor hydralazine. The primary endpoint was the percentage of patients with an increase in 68Ga-DOTATATE uptake =1 point according to the predefined uptake scale. Epigenetic treatment effects were concurrently studied in the NET cell lines.

Results: Nine patients with lung NETs (n=4), pancreatic NETs (n=2), small intestinal NET, rectum NET and thymus NET completed the study. Following two weeks of epigenetic treatment with valproic acid and hydralazine, there was no change in 68Ga-DOTATATE uptake in tumor lesions. Epigenetic treatment did result in a 27% increase in kidney uptake of 68Ga-DOTATATE (p=0.02). In contrast to these results, increased SST2 expression and 111In-DOTATATE uptake were observed with valproic acid treatment in BON-1, GOT1 and NCI-H727 cells.

Discussion: A two-week epigenetic treatment with valproic acid and hydralazine did not lead to increased tumor-uptake of 68Ga-DOTATATE in NET patients with low baseline SST expression, contradicting the in vitro data.

Preclinical toxicity and therapy study of 225Ac-crown-TATE

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Net Type: Multiple NET Types; *Research Type:* Basic; *Keywords:* Nuclear medicine

Background: The overall aim of our study is to evaluate the treatment of mice inoculated with somatostatin-positive neuroendocrine tumors (NETs) using an alpha-emitting radiopharmaceutical known as 225Ac-crown-TATE. Due to the slow-growing nature of NETs and the lack of early symptoms, most NETs are diagnosed at a late stage and often involve metastatic spread, thus requiring systematic treatment. Currently, the treatment of disseminated NETs has been tested with 177Lu-DOTA-TATE (Lutathera™), a beta-emitting radiopharmaceutical. While this drug is relatively effective at treating low (G1) and intermediate (G2) grade tumors, only 26-55% of patients experience stabilization of disease, and 18-32% are refractory to the treatment. Consequently, we are proposing treatment of NETs with 225Ac-crown-TATE, since alpha-particles have lower tissue penetration and are more cytotoxic to cancer cells than beta-particles. The decision to use 'crown' as the chelator to bind 225Ac was made based on the high affinity and fast (<30 min) metal incorporation kinetics displayed toward 225Ac under ambient temperature conditions. This is in contrast to the more traditional chelator 'DOTA', which requires elevated temperatures and longer (hours) reaction times to produce a lower yield of the final drug product. Having established the labeling conditions for 225Ac-crown-TATE, we are now pursuing a study to demonstrate the rapid formulation and selective targeting of NETs in preclinical models. The results from this and upcoming studies will provide insight as to whether 225Ac-crown-TATE should move further into clinical development.

Methods: The overall experimental approach requires both the synthesis of 225Ac-crown-TATE (radiochemistry, formulation, and quality control), followed by a therapeutic response study of mice inoculated with murine NETs. Radiopharmaceutical preparation: We have established a procedure for the synthesis and quality control of 225Ac-crown-TATE, which is performed immediately prior to injection due to the radioactive decay and limited shelf-life of the drug. Quality control is achieved via a combination of radioHPLC, radioTLC, and gamma spectroscopy. Toxicity and therapy study:

We are currently conducting a dose-escalation study to establish the maximum tolerable dose (MTD) in AR42J tumor-bearing NRG mice. Therapy monitoring is being conducted with these same mice, with their appearance, tumor size, and weight being used as metrics to determine subject viability. Three groups of mice, each involving 4 to 6 mice, have been injected with a saline (PBS) solution, 30 kBq, and 60 kBq of 225Ac-crown-TATE, respectively. The mice are examined every day, with tumor size and weight being monitored three times in the first week, then twice a week until the endpoint; with the endpoint

determined to be any one of a tumor volume exceeding 3000 mm3, a loss of 15-20% of starting body mass, or 6 months post-injection, whichever comes first. Upon success, statistical relevance will be established using a final sample size of 8 to 12 animals per group. Furthermore, we will include two more groups; one for unlabeled crown-TATE and one for 225AcCl3 in buffer.

Results: We have shown that the drug can be produced within 30 minutes at ambient temperature, using micromolar concentrations of crown-TATE and as little as ~20 kBq of 225Ac in NH4OAc buffer solution (pH 7). The shelf-life of 225Ac-crown-TATE showed no degradation over 16 hours when sodium ascorbate was added. Despite there being no signs of radiolytic degradation, delivery and administration of 225Ac-crown-TATE was still done within five hours of synthesis to ensure radiopharmaceutical integrity. Toxicity and therapy monitoring study: All AR42J tumor-bearing NRG mice in the control group had to be sacrificed within 11 days due to reaching the tumor volume endpoint (2500-3000 mm3). Encouragingly, none of the mice treated with either 30 kBq or 60 kBq of 225Ac-crown-TATE were sacrificed at this stage, and their tumor sizes have decreased or stabilized. A loss in weight was noticed for several mice, though this was anticipated at this stage, with weight predicted to stabilize after two-three weeks.

Discussion: The synthesis of 225Ac-crown-TATE has been established, potentiating an important alternative to DOTA-TATE for 225Ac-based radiopharmaceutical drugs. Studies are ongoing, however, preliminary observations suggest all mice injected with 225Ac-crown-TATE (30 kBq or 60 kBq) remain viable when compared to mice injected with saline (PBS) solution. Tumor sizes in mice treated with the drug either stabilized or decreased over this period, while tumors in control mice reached 2500-3000 mm3 within 3 to 11 days, depending on the size of the tumor at the beginning of the study.