REACT
Attn: Donna LeBeau
Grant Proposal

Immune Profiling and Mutation Analysis in the Development of Combination Therapies for Advanced Medullary Thyroid Cancer.

Principal Investigator: Jena D. French, Ph.D.
Clinical Collaborator: Bryan R. Haugen, M.D.

Lay Summay: Novel therapies are necessary for patients with advanced medullary thyroid cancer (MTC). Current mono-therapies have proved ineffective in sustaining durable responses. For decades, researchers have hypothesized that the immune response could be enhanced to eliminate cancer. In the light of recent and growing successes of immune-based therapies in other cancers, we suggest that combination therapies that target both the tumor and the immune response could be effective in combating advanced cancers, including MTC. Our work is focused on characterizing the immune response in MTC, focusing on therapeutic targets that are currently under clinical investigation, approved for care in other types of advanced cancer, or in development specifically for MTC. In parallel, we plan to screen MTC cases for key mutations that could be targeted in such combination approaches. We expect that these studies will provide necessary data for the rational design of promising combination therapies for advanced MTC.

Research Proposal

Introduction: Medullary thyroid cancer (MTC) is a rare disease for which there is no cure. There are approximately 1000 confirmed cases of MTC in the United States.[1] Cytotoxic chemotherapeutic regimens have potential to induce transient therapeutic benefits in MTC, but their role as durable and/or curative therapies are limited.[2-5] Recent therapeutic strategies for MTC have included multi-kinase inhibitors (MKI) that largely target RET and VEGFR pathway. Vandetanib and cabozantinib achieved a partial response in 45% and 28% of patients with MTC, respectively, leading to the FDA approval of these drugs.[6, 7] However, the clinical effects of these therapies are temporary and patients eventually become resistant. Alternative or novel combination therapeutic approaches are necessary to improve clinical response. *We suggest that a paradigm shift is necessary in the treatment of advanced medullary thyroid cancer. We predict that novel combination therapies that include both TKIs and immune-based approaches will substantially benefit patients with aggressive MTC.*

Rationale for Immune-based Therapies in Advanced MTC: Immune-based therapies have long been considered for patients with MTC. A number of studies suggest that an MTC-specific T cell response is evident in patients with MTC.[8-10] Early attempts at dendritic cell-based vaccinations for MTC using tumor lysate, calcitonin, or carcinoembryonic antigen (CEA) have shown some promise.[11-13] A number of immune-based therapies for aggressive MTC are currently under investigation. A recent phase I trail investigating a recombinant yeast-CEA vaccine in patients with metastatic CEA^+ tumors included one patient with MTC who developed an impressive tumor-specific T cell response and disease stabilization.[14] Based on this study, a phase II trial is now evaluating this approach in patients with recurrent MTC (NCT01856920). Patients with progressive MTC are increasingly included in trials using immune checkpoint blockades (i.e., NCT03072160). Immune checkpoints (e.g., CTLA-4, PD-1, Lag-3, Tim-3) play an important role in the down-regulation of the immune response. Blockades of CTLA-4 and PD-1 have been shown to boost the immune response and reduce

tumor burden in a broad spectrum of cancers.[15-20] Furthermore, chimeric antigen receptor (CAR) T cell therapies are under investigation for MTC. This technology utilizes patient T cells that are engineered to recognize and target the tumor. Both GFRA-4, a receptor that is highly specific to MTC, and RET, which is expressed and commonly mutated in MTC, may be successfully targeted with this strategy. Surprisingly, little is known about the immune milieu in MTC. *Current and future immune-based therapeutic strategies would benefit greatly from a thorough characterization of the immune milieu in primary and metastatic MTC.*

Rationale for combination therapies that target both the tumor and the immune response: Despite the well-deserved excitement over recent successes in both MKI and immune-based therapies, these approaches, as mono-therapies, are unlikely to generate durable responses in all patients. The next phase of cancer treatment is focused on combination therapies that target both the tumor and multiple components of the immune response. *Successful development of optimal combination therapies will require a thorough characterization of the immune response within the tumor microenvironment (TME). In parallel, we must understand the biology of the tumor itself (e.g., targetable mutations or tumor antigens and sensitivity anti-angiogenic drugs or radiation).*

Patient Samples: Under our current protocol (Colorado Internal Review Board (IRB) #16-1461), we have recruited and consented over 30 patients from outside the University of Colorado, both United States and non-United States citizens. We have 19 patients that have been treated at the University of Colorado Hospital for MTC who will be given the opportunity to consent for this study. We conservatively expect to generate data from 40 patients in total. Consented patients will contribute archived surgical samples of both primary tumor and, when available, metastatic lymph node tissues for our analysis. The majority of these patients have advanced primary disease (pT3-pT4) and/or lymph node metastases. Twenty-two patients currently have either elevated calcitonin levels or confirmed metastases.

Specific Aims:

Specific Aim 1. To characterize the immune milieu in ATC and MTC. In the wake of successes in other types of advanced cancers, patients with MTC are now included in trials using immune-targeted therapies, including checkpoint blockades. Surprisingly little is known about the immune milieu and checkpoint molecule expression in MTC. We have developed a 7-color multispectral fluorescent immunohistochemistry (IHC) protocol assess *in situ* expression of key immune markers in tumor samples (Perkin Elmer; Vectra 3.0). Specifically, this technology allows us to analyze 5 immune markers in combination with a tumor marker (cytokeratin) and nuclear stain to characterize co-expression of key immune markers in the context of the tumor microenvironment. We have optimized 4 multispectral panels (Table 1) in tonsil tissue and are now beginning our analysis in medullary thyroid tumors, both primary tumors and metastatic lesions in the lymph nodes (Figure 1). The Vectra 3.0 microscope and analysis software (Perkin Elmer) will allow imaging of the entire tissue section and semi-quantitative analysis of key immune cells within and surrounding the tumor (Figure 2). As noted in Table 1, these analyses will further characterize the immune milieu in MTC, and will be the first to determine expression of therapeutically relevant checkpoint molecules. These studies will also investigate the potential for therapies that target immune suppressive cellular networks in the TME (i.e, regulatory T cells (Tregs), macrophages, and myeloid-derived suppressor cells) or co-stimulatory molecules (i.e., OX-40). These are the first studies to investigate the presence of B cells, NK cells, macrophages, and OX40 in MTC and may encourage future studies. Trials are currently underway to manipulate OX-40 (e.g.,

NCT02274155, NCT01644968, NCT02559024) and colony stimulating factor-1 receptor^{+} (CSF-1R) macrophages (e.g. NCT02777710, NCT02718911) in advanced cancer and could be applied to MTC in the future.

Table 1. 7-color IHC Strategy for Immune Profiling in MTC

	Panel 1	Panel 2	Panel 3	Panel 4
IHC markers	CD8, CD4, PD-1, Tim-3, FoxP3	CD8, PD-1, Lag-3, PD-L2, PD-L1	CD163, CSF1-R, MHCII, CD68, CD33	CD3, CD20, CD56, PD-1, OX-40
Immune Subsets	Regulatory T cells, classic T cell subsets	Cytotoxic T cells	Myeloid, macrophage subsets	T cells, B cells, NK cells
Research Application	PD-1 and Tim-3 checkpoint expression on T cells in MTC	Lag-3 and PD-1 co-expression; PD-L1 and PD-L2 expression in the TME	Macrophage phenotype and activation status, identification of MDSC	Expression of PD-1 on non-T cells, Role of B cells and NK cells in MTC, Expression of OX-40 in the TME
Clinical Application	Discovery of the appropriate checkpoint targets for future clinical trials		Investigate the potential for CSF-1R or other myeloid-targeted therapies	Investigate the potential for NK, B cell, and OX-40-based therapies

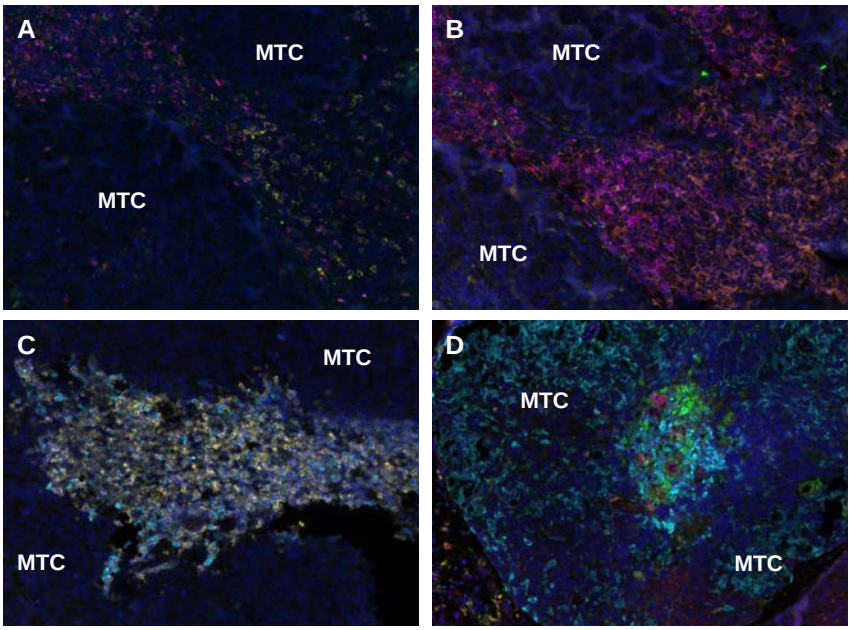

Figure 1. Multispectral Imaging in MTC. Representative images are shown from a metastatic lesion in a regional lymph node. A) Panel 1 imaging displays PD-1 (yellow) and Tim-3 (green) expression by CD4 (orange) and CD8 (red) T cells and nuclear FoxP3 (magenta) expression by regulatory CD4 T cells within the tumor (MTC). B) Panel 4 imaging displays CD20 B cells (orange) among PD-1+CD3+ T cells (magenta and red) with sparse expression of OX40 (green). C) Panel 4 imaging also identifies CD56+ NK cells (yellow) in close proximity with tumor cells (cyan). D) Panel 3 identifies CD68+ (magenta) and CD163+ (yellow) macrophages among tumor cells (cyan) and HLA-DR+ leukocytes and allows co-expression analysis with CD33 (orange) and CSF1-R (red).

Representative samples of primary tumor and grossly tumor-involved lymph node (TILN) will be screened using standard IHC for the general presence of leukocytes (i.e., CD45, a pan-leukocyte marker, and CD68, a macrophage marker). We expect that a small subset of patients will show and immune response in their primary tumor tissue and that all TILN will display leukocytes infiltrating the metastatic lesion to varying degrees. We also expect that some normal lymph node tissue will remain in these involved lymph nodes, providing a comparison of leukocyte phenotype surrounding and infiltrating the lesion. Immune profiles will be compared in primary tumors and TILN, when matched samples are available, and will be assessed for correlation to disease severity at the time of diagnosis and current disease status. We will also assess the association between immune milieu and mutation status (Aim 2). *These studies will provide a broader analysis of the immune milieu than was previously noted (R. Dadu, et al. ATA annual meeting 2016) and will more accurately assess immune phenotype by expanding the study to include TILN, where tumor-infiltrating leukocytes will be more plentiful.*

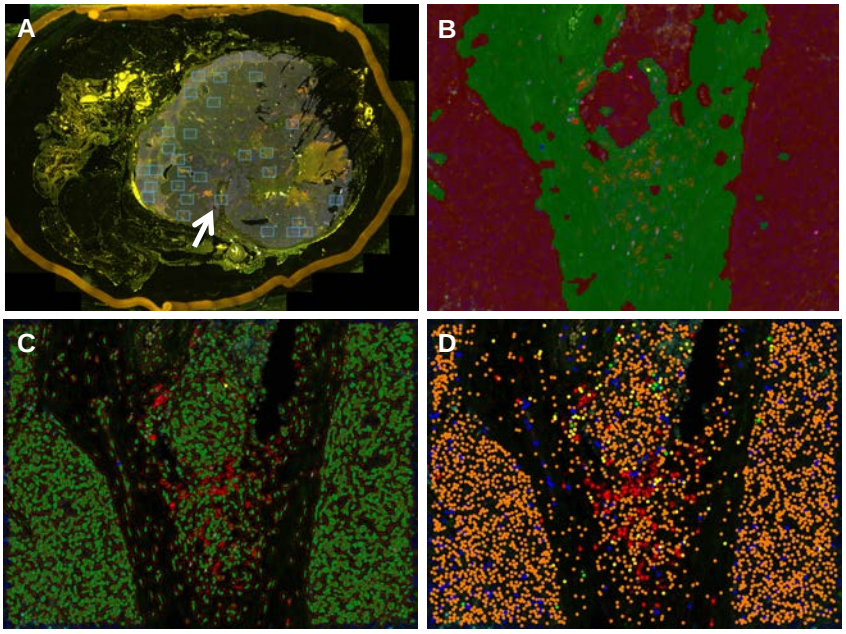

Figure 2. Quantification of Multispectral Imaging. A) A low-resolution composite image was generated following staining of a lymph node containing metastatic MTC. Areas of interests were chosen (blue rectangles) and scanned at high resolution for 7-color analysis (shown in Figure 1; the arrow designates the area shown in B-D). Inform software (Perkin Elmer) was used to assigned tumor (red) versus non-tumor (green) tissue areas (B), designate individual cells (green nucleus with red membrane, and assign a phenotype for CD8 T cells (red dots), CD4 T cells (green dots), regulatory T cells (yellow dots), tumor cells (orange dots), and "other" cells (blue dots) that were not assigned to any specific category (D). These data can be exported to quantify the cell types within each tumor sample.

Specific Aim 2. To characterize MTC samples for key targetable mutations. In parallel to the immune profiling studies in Aim 1, we plan to characterize each tumor sample using the targeted Next Generation Sequencing (NGS) approach, Thyroseq™.[21] This analysis was previously used to detect mutations in BRAF, RAS, PIK3CA, TP53, TSHR, PTEN, GNAS, CTNNB1, and RET genes using FFPE thyroid tumors and has now been expanded to analyze over 20 genes and 80 gene fusions.[21] This study will be performed in collaboration with Dr. Yuri Nikiforov at the University of Pittsburgh Medical Center. The type and frequency of mutations will be compared to our findings in Aim 1 to determine whether mutation status or frequency is associated with a specific immune signature (i.e., pro-inflammatory vs. immune suppressive). This study may also inform the development of novel combination therapies using clinically available TKIs to target the mutations that are driving tumor progression and immune-based therapies to boost tumor recognition and elimination for patients with advanced MTC.

Specific Aim 3. To assess expression of potential tumor antigens and immune-modulating proteins in MTC. To broaden the potential for novel immune-based approaches in MTC, we will used standard IHC techniques to investigate the expression of CEA, GFRA-4, and NY-ESO-1 in the primary or TILN samples, described above. CEA and NY-ESO-1 are well-characterized antigens in other tumor types, currently under investigation as tumor vaccine targets, and are expressed in a subset of MTC.[22-24] GFRA-4 is a co-receptor for RET and is known to be expression in calcitonin-producing C-cells and MTC tumors.[25-28] CAR-T cell therapies targeting GFRA-4 are currently in development and these studies would benefit from a larger analysis of GFRA-4 in MTC.

To determine the potential of MTC to present such tumor-antigens to tumor-specific T cells, we will assess expression of MHC-I molecules, which are commonly down-regulated in tumors. To investigate the ability of MTC to evade the immune response, we will assess expression of PD-L1, the major ligand for PD-1 and potential marker of responsiveness to PD-1-targeted therapies.[29] Finally, we will investigate whether MTC expresses CD47, a molecule that inhibits phagocytosis by macrophages and could be targeted to enhance immune-based therapies.[30] Current trials are investigating the effects of blocking CD47 in advanced cancers (e.g. NCT03013218, NCT02678338). These studies, in parallel to those describes in Aims 1

and 2, will shed light on the immunogenicity of MTC and will contribute to the optimization of rationale combination therapies for advanced MTC.

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