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14. ABSTRACT

Prostate cancer is not only heterogeneous among individuals, but tumors are heterogeneous on the molecular level within a given individual. One well-documented phenomenon is the presence of castrate-resistant prostate cancer with neuroendocrine features (CRPC-NE) that evolves over the course of anti-androgen therapy. Our group has discovered that CRPC-NE have a unique cell surface glycan composition enriched with fucose sugars. Therefore, the ability to image glycan fucosylation could be used to predict the emergence of lethal prostate cancer in men. This proposal tests the feasibility of a previously developed PET agent, [^{18}F] fluorofucose (Fuc-PET) as a functional method to quantify PCa glycan fucosylation, and thus, the burden of lethal disease using animal models. Our **hypothesis** is that Fuc-PET can quantify the amount of glycan fucosylation in tumors noninvasively and thus predict aggressive pathology in vivo. In the first year, we have made advancements in the characterization of fucose modifications to glycans through core glycan modifications and the synthesis of the Lewis-y antigen that are associated with more aggressive disease and shorter survival. We have also discovered that the ketogenic diet can inhibit the synthesis of these fucosylated glycans, suggesting that dietary modifications could enhance therapeutic efficacy in men with advanced prostate cancer.

15. SUBJECT TERMS

Prostate cancer, CRPC-NE, glycans, fucose

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INTRODUCTION

Prostate cancer is not only heterogeneous among individuals, but tumors are heterogeneous on the molecular level within a given individual. One well-documented phenomenon is the presence of castrate-resistant prostate cancer with neuroendocrine features (CRPC-NE) that evolves over the course of anti-androgen therapy. Our group has discovered that CRPC-NE have a unique cell surface glycan composition enriched with fucose sugars. Moreover, this enriched glycan fucosylation is not only present in CRPC-NE, but a subset of prostate adenocarcinomas at initial diagnosis and are associated with adverse clinical outcomes. Therefore, the ability to image glycan fucosylation could be used to stratify men and initial diagnosis as well as predict the emergence of lethal prostate cancer in men. This proposal tests the feasibility of a previously developed PET agent, [^{18}F] fluorofucose (Fuc-PET) as a functional method to quantify PCa glycan fucosylation. The degree of fucosylation will be proportional to the burden of lethal disease using animal models. Our hypothesis is that Fuc-PET can quantify the amount of glycan fucosylation in tumors noninvasively and thus predict aggressive pathology in vivo. Aim 1 will correlate Fuc-PET activity with tumor glycan fucosylation. Aim 2 will identify the effects of key fucosyltransferases (FUT's) on tumor glycan synthesis. Aim 3 will assess the effect of pharmacologic inhibition of tumor fucosylation on tumor growth and tumor immunity.

KEYWORDS

Prostate Cancer
Neuroendocrine Prostate Cancer
Castrate Resistant Prostate Cancer
Glycans
Fucose
Positron Emission Tomography
Fucosyltransferases
Fluorofucose
Immunotherapy

ACCOMPLISHMENTS

What were the major goals of the project?

For Year 1:

Major Task 1 (Aim 1): Imaging and biodistributions in tumor bearing mice with FucPET (Percent complete: 10%)

Major Task 2 (Aim 1): FucPET/MALDI imaging of xenografts (Percent complete: 20%)

Major Task 1 (Aim 3): Pharmacologic Study in TC2-sMIC-NE tumor model (Percent complete: 20%)

What was accomplished under these goals?

Although we have not been able to get the imaging agent injected into mice because of lab shutdown (described later), we have advanced the clinical characterization of fucosylated glycans as biomarkers for aggressive, lethal PCa.

Identification of fucosylated glycans as markers for small cell neuroendocrine prostate cancer. Because the overall goal is to identify glycomic signatures of lethal prostate adenocarcinoma, we started with a panel of primary and metastatic prostate cancers with phenotypic small cell neuroendocrine histology. These included 5 transurethral prostate resections, 2 lymph node metastases, and a liver metastasis. Two additional control specimens included 1 primary small cell lung

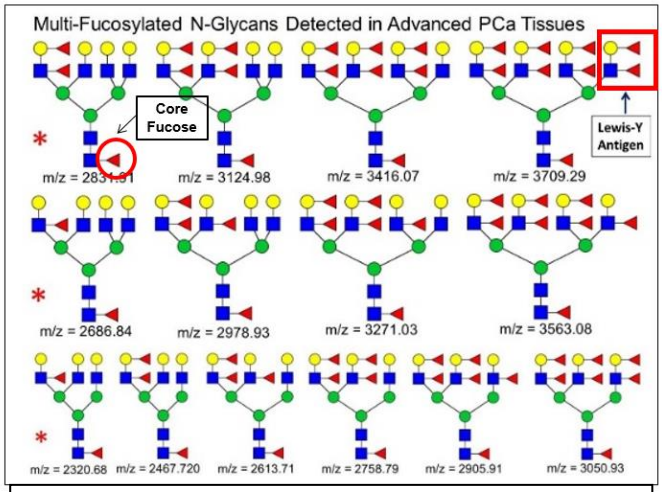

Figure 1. A panel of multi-fucosylated glycans as markers for lethal prostate cancer. Glycan structures composed of carbohydrate monomers (shapes) are listed along with their masses. The Lewis-y antigen (red box) and core fucosylation (red circle) are common features to this glycan signature. Fucose=red triangle.

carcinoma tumor, and 1 lymph node metastasis from a urothelial carcinoma. Conserved among all of these specimens were 14 glycans (**Figure 1**). There are two key components of the structures of these glycans that are relevant to this proposal. First, there is a core fucose (red triangle within the red circle) on the bases of all of these glycans. This core fucosylation is performed by fucosyltransferase 8 (discussed later) and has been implicated in as a regulator of castration resistance in prostate cancers [4]. Another intriguing feature of this glycan panel is the presence of the lewis-y antigen (red box, **Figure 1**) composed of two fucose sugars attached to a galactose (yellow circle) and a N-acetylglucosamine (blue square). Lewis-y antigen is implicated in tumorigenesis in numerous cancers including prostate and ovary and expression is associated with poor outcomes [5]. This is a significant finding, as Lewis-y is a target for clinical monoclonal antibody trials and could be potentially repurposed for imaging and therapy.

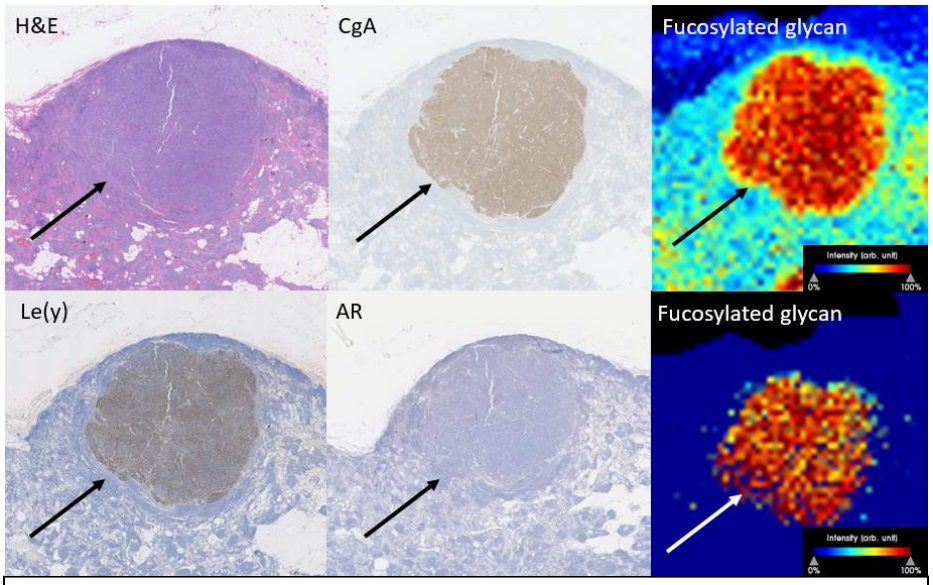

Figure 2. Lewis-y (Le(y)) expression in small cell prostate cancer. A lymph node metastasis (arrow) is positive for the neuroendocrine marker chromogranin A (CgA) and negative for the androgen receptor (AR) and positive for Le(y), further validating the presence of fucosylated glycans in lethal prostate cancer**.** This tumor is also positive high fucosylated glycans detected with MALDI.

To provide orthogonal validation of the presence of lewis-y in these specimens, we stained a small cell prostate cancer metastasis to a lymph node (Figure 2). As expected, this metastasis was positive for the conventional neuroendocrine marker chromogranin A (CgA) and negative for the androgen receptor (AR). This metastasis was also positive for Lewis-y that was superimposable with MALDI-detected fucosylated glycans (identified in Figure 1).

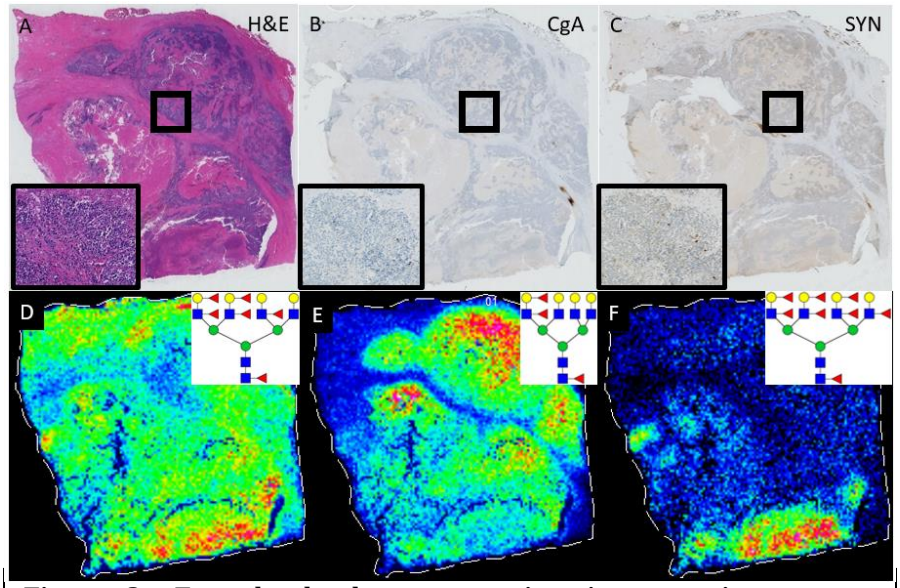

Figure 3. Fucosylated glycan expression in aggressive prostate adenocarcinomas is spatially heterogeneous. A. H&E demonstrating a poorly differentiated adenocarcinoma. B-C: Weak, but positive expression of the neuroendocrine markers chromogranin A (CgA) and synaptophysin (SYN). D-F. MALDI imaging of three selected fucosylated glycans showing spatial heterogeneity of glycan expression. Note the overall extent in expression of these glycans relative to conventional immunohistochemistry for neuroendocrine markers. Boxes in A-C represent the site for the magnified inset.

Expression of fucosylated glycans in prostate adenocarcinomas with adverse histologic features. Next, we wanted to identify if these fucosylated glycan biomarkers, expressed in lethal small cell carcinomas, were also present in adenocarcinomas and associated with poor outcomes and adverse histologic features. A series of 33 prostatectomy specimens diagnosed as adenocarcinoma were tested that ranged from Gleason scores 6-10. We identified that the presence of fucosylated glycans were enriched in the higher grade adenocarcinomas. An example of an invasive, metastatic (T4N1) adenocarcinoma with Gleason score 10 is provided in **Figure 3**. Expression of conventional neuroendocrine immunohistochemical markers chromogranin A (CgA) and synaptophysin (SYN) was positive, but among scattered cancer cells in the tumor. However, MALDI imaging

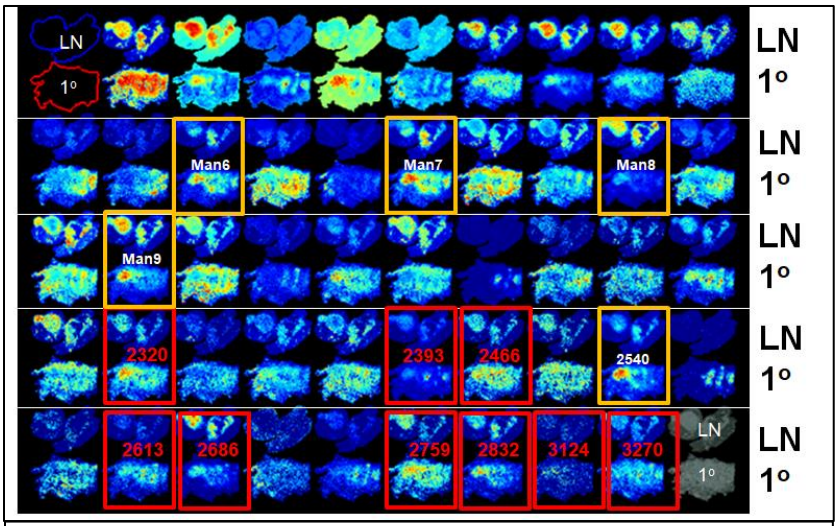

Figure 4. Heterogeneity of fucosylated glycans in primary and lymph node metastasis. Imaging mass spectrometry heat map of multiple glycans identified in a ISUP grade group 5 T3BN1 adenocarcinoma (1°) and corresponding lymph node metastasis (LN). Orange boxes denote conventional glycans enriched in adenocarcinoma. Red boxes denote fucosylated glycans.

4, a total of 9 fucosylated glycans were identified in the primary tumor and metastasis. Interestingly, not all glycans are uniformly enriched in both the primary tumor and nodal metastasis. However, the common presence of fucose in both the aggressive primary tumor and nodal metastasis support the use of our PET agent, fluorofucose as a way to not only detect aggressive primary prostate cancers, but their corresponding metastases.

The possibility that fucosylated glycan expression could correlate with adverse histologic features prompted us to form the basis of a putative "fucose score". An example of the application of a putative fucose score to prostatectomy specimens is described in **Figure 4.** We profiled a stage T2N0 Gleason 3+3, 3+4, and 4+4 tumor without invasive characteristics, metastasis, or recurrence (**Figure 4A**) and a tumor from a stage T3 Gleason 3+3 with extracapsular extension, lymphovascular and perineural invasion (**Figure 4B**). First, we profiled the abundant stroma glycans shown in blue and green colors to highlight the non-tumor regions of each tissue, and the red color to identify tumor regions. The first three panels in **Figure 4A and 4B** show these controls (denoted by "stroma" and "adeno"). Next, we determined how many of the multi-fucosylated glycans listed in **Figure 1** were identified in the region of the tumor and not present in adjacent stroma. The stage T2 Gleason 4+3 received a low fucose score of 3 compared the stage T3 Gleason 3+3 with a fucose score of 9. These findings suggest that non-invasive biomarker profiling in patient blood samples that can quantify the amount of fucosylated glycans or central carbon metabolites through a liquid biopsy could stratify individuals at diagnosis. Moreover, a recent extensive study of the N-glycan signature of plasma from 2144 middle-aged individuals indicated multiple glycan structural signatures associated with age, metabolic health, inflammation and

focused on the glycan signature in Figure 1 identified multiple fucosylated glycans (three represented in Figure 3) that were spatially heterogeneous I their expression. These data further validate MALDI imaging as a means to better molecularly characterize histologic samples that cannot be done with conventional immunohistochemistry. It further suggests that these fucosylated glycans could be better biomarkers than conventional CgA and SYN immunohistochemistry for neuroendocrine markers.

The molecular heterogeneity of aggressive prostate adenocarcinoma is also displayed in **Figure 4**. A Gleason 9 invasive, metastatic (T3BN1) prostate adenocarcinoma with corresponding lymph node metastasis was profiled with MALDI imaging. As seen in **Figure**

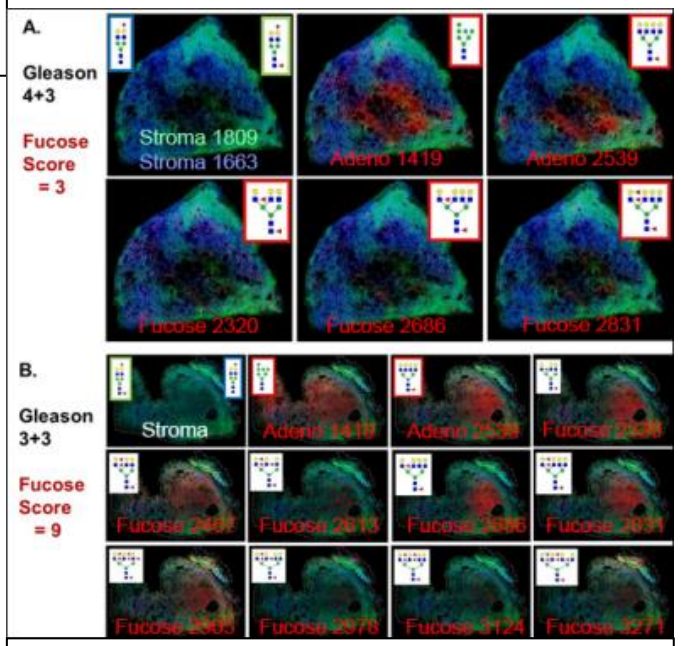

Figure 4. The "fucose score" may directly correlate with PCa stage. A. MALDI N-glycan imaging of G4+3 without invasion, metastasis, or subsequent recurrence (Stage T2) has a low fucose score of 3. B. G3+3 with extracapsular extension (Stage T3) has a higher Fucose Score of 9. The first three images in each panel show the distribution of the two abundant stroma glycans (in blue and green), and two common adenocarcinoma glycans (red). Other panels indicate the presence of a fucosylated glycan (red). Although the adenocarcinoma in panel A has a fucose score of 3 because of 3 fucosylated glycans, the overall intensity of these fucosylated glycans is much lower than the 9 fucosylated glycans (i.e. fucose score of 9) in the adenocarcinoma in panel B. Both of these tumors were negative for NE markers chromogranin A, synaptophysin, and dopa decarboxylase. Numbers refer to the mass of the glycan seen in Figure 1**.**

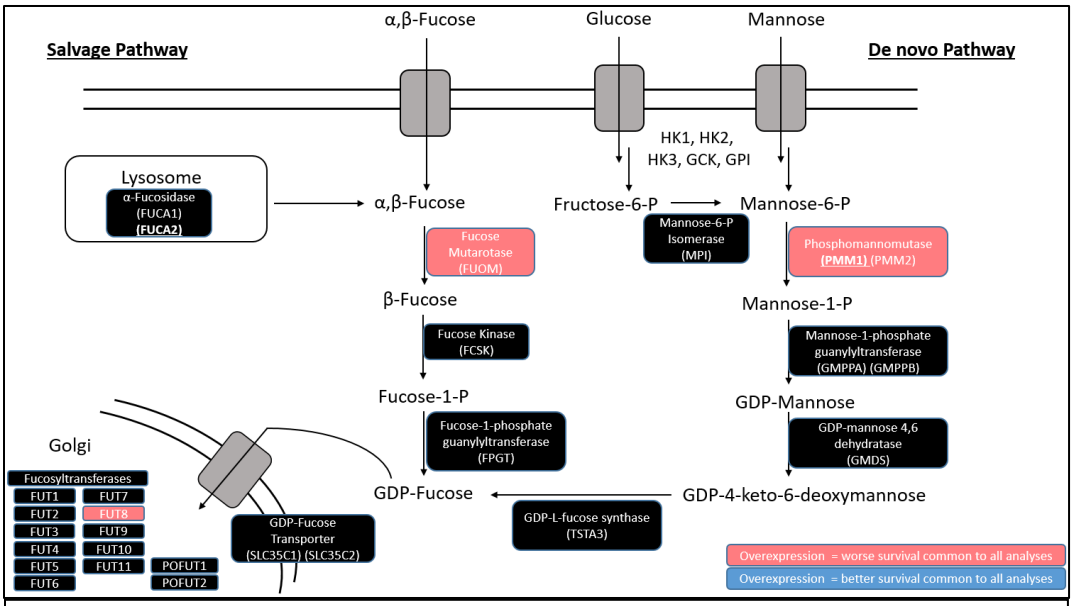

Figure 5. Overexpression of three genes involved fucose synthesis and glycan fucosylation are associated with worse OS in castrate resistant prostate cancer and hormone-naïve prostate cancer. OS analysis of the salvage and de novo pathways for fucose synthesis as well as glycan fucosylation were performed using a threshold biomarker optimization algorithm [1, 2]. Three enzymes in red (FUOM, PMM1 and FUT8) were identified in both hormone-naïve prostate cancer and castrate-resistant prostate cancer whose overexpression was associated with significantly worse OS ($p<0.05$). Expression and outcomes data obtained from [3].

smoking [6]. In these non-cancerous individuals, none of these glycans were multi-fucosylated or associated with Lewis-Y antigen. Therefore, we hypothesize the uniqueness of the multi-fucose signature (i.e., fucose Score) for aggressive PCa may be a critical step forward for PCa diagnostics. Thus, fluorofucose uptake may be directly proportional to the fucose score.

Glycan fucosylation gene expression is prognostic in castrate-resistant and hormone-naïve prostate cancer. Because the short observation period for this institutional cohort was not amenable to overall and progression free survival analyses, RNA expression of enzymes involved in (i) the de novo synthesis of fucose from other carbohydrates, (ii) the salvage synthesis pathway using imported fucose and (iii) the fucosylation of glycans (i.e. fucosyltransferases) were analyzed from The Cancer Genome Atlas (TCGA). First, the effects of these pathways on overall survival (OS) in metastatic CRPC patients were assessed [3]. A total of 7 genes were identified whose overexpression were associated with decreased OS. Two of these genes were associated with the salvage pathway (FUCA2, FUOM), four of these genes were associated with the de novo pathway (MPI, PMM1, GMDS, and TSTA3), and one of the genes was a fucosyltransferase (FUT8). Interestingly, there were 3 fucosyltransferases whose overexpression was associated with better OS (FUT3, FUT6, FUT7).

Next, the same methods were applied to OS in hormone-naïve prostate cancer at the time of prostatectomy. Similarly, overexpression of FUOM from the salvage pathway was associated with worse OS. PMM1 and GMPPB overexpression from the de novo pathway was associated with poor OS. Multiple fucosyltransferases (FUT2, FUT5, FUT6, FUT8, FUT9, POFUT2) including the GDP-fucose transporter SLC35C2 were also associated with worse OS. Overexpression of 1 gene in the salvage pathway (FUCA1) and 2 fucosyltransferases (FUT1, FUT10) were associated with better OS.

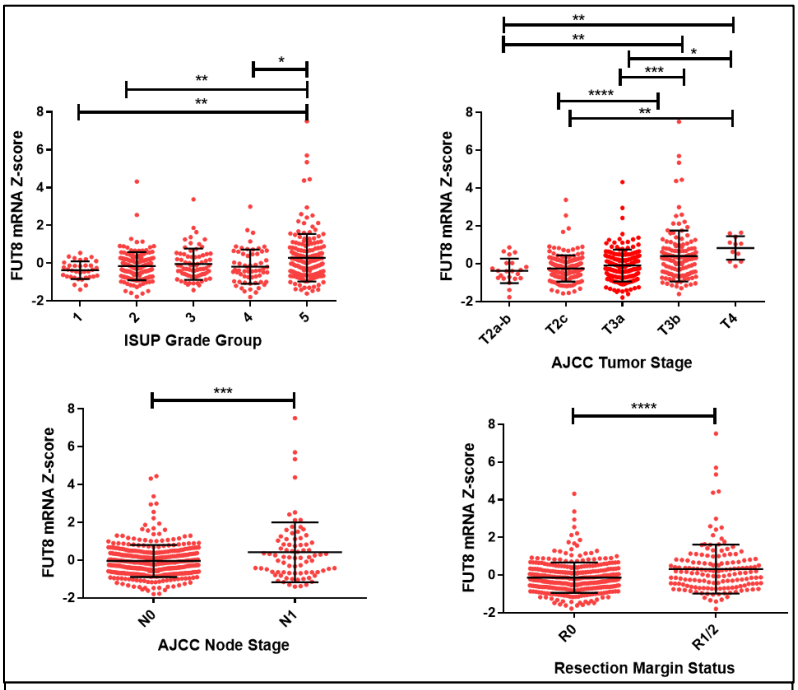

Figure 6. Overexpression of fucosyltransferase 8 (FUT8) is associated with asverse histologic features in hormone-naïve prostatectomy specimens. A. FUT8 expression increases with ISUP Gleason Group classification of the adenocarcinoma. B. FUT8 expression singificnatly increases with stage. C. FUT8 expression significantly increases with nodal metastasis. D. FUT8 expression is significantly higher with prostatectomies with positive margins. Data obtained from [3].

However, three enzymes were identified that were common to both the hormone-naïve prostatectomy and castrate-resistant prostate cancer datasets: fucose mutarotase (FUOM), phosphomannomutase 1 (PMM1), and fucosyltransferase 8 (FUT8), all of which whose overexpression was associated with worse OS (**Figure 5**). All three genes were tested for associations with adverse histopathologic features in light of the proposed fucose scoring system. Intriguingly, only FUT8 emerged of the three genes with significance. FUT8 mRNA expression increased with the ISUP grade group, tumor stage, was significantly increased in node positive cases, and increased with positive surgical margins (**Figure 6**). In contrast to other fucosyltransferases that target peripheral glycan fucosylation, FUT8 catalyzes core fucosylation [7, 8]. Upon correlation with the 14 glycans displayed in **Figure 1**, core fucosylation of the innermost N-acetylglucosamine by FUT8 activity is the common denominator to all glycans, further supporting the potential for the fucose score as potential marker for poor outcomes and aggressive disease. Moreover, this identifies FUT8 as the primary target for our CRISPR studies in Aim 2 as we move forward.

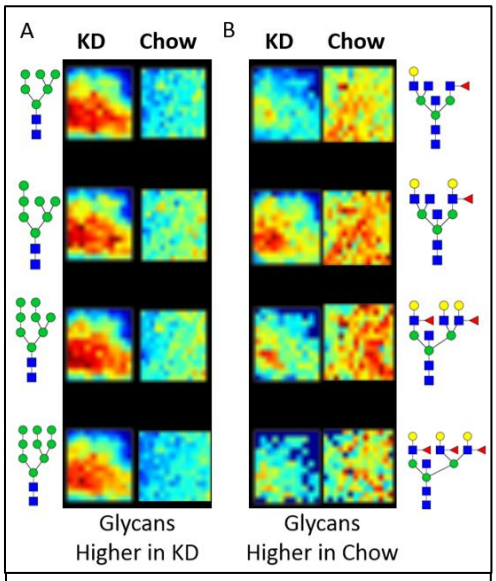

Figure 7. Dietary carbohydrate deprivation remodels the small cell prostate cancer glycome. Tumors from PNEC xenograft mice fed the KD for 4 weeks were imaged with MALDI. A square region of a tumor section on a slide was imaged (square regions) and images for selected glycans are displayed. **A**. Examples of glycans higher in tumors fed the KD and **B**. Examples of glycans higher in tumors fed chow. Intensity scale: blue (lowest) to red (highest).

Carbohydrate depletion from the ketogenic diet (KD) reduces fucosylated glycan content. The dependence of aggressive tumors on glucose is well known. Dietary carbohydrate restriction in the form of calorically-limited ketogenic diets (KD) has been investigated as a means to reduce tumor growth and enhance therapeutic efficacy in PCa through reduction in circulating carbohydrates [9-14].

Using MALDI imaging, the molecular effects of the KD on lethal PCa was assessed. The KD was fed to mice with *de novo* small cell prostate carcinoma (PNEC model; [15-19]). As expected, the KD can modulate the glycomes of small cell prostate carcinoma as noted by depletion of multiple fucosylated glycans that are part of the 14 fucosylated glycan signature (**Figure 7**). However, we *unexpectedly discovered* a cohort of glycans that were enriched from the KD. These glycans are characterized by the expansion of mannose sugars (green circles, **Figure 7**). This is significant because high mannose glycans are typically secreted [20, 21]. This further advances our rationale for noninvasive serum profiling of glycans, which will be described below. This is also significant, as high mannose glycans can be associated with activated macrophages [22] and has implications for using metabolism to modulate immune recognition of tumors. Therefore, we will be able to integrate this discovery into Aim 3 as a clinically-relevant strategy to decrease fucosylated glycans and therefore reduce uptake of fluorofucose PET tracer.

As we move forward with the fluorofucose PET tracer in animal models, we are starting to obtain data on the amounts of fucosylated glycans in cell lines. We have discovered that LNCaP and PNEC cell lines have measurable levels of fucosylated glycans and that TRAMP/MIC tumors from Dr. Wu's lab have the most robust expression of these fucosylated glycans. We will focus on these cells moving forward as we generate xenografts to perform fluorofucose PET Imaging.

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Opportunities for Training and Professional Development: Nothing to report.

How were results disseminated to communities of interest?: Because of multiple factors that have limited our progress (as will be discussed below), we have not yet disseminated any results to communities of interest.

What do you plan to do during the next reporting period to accomplish the goals? Now that our lab is ramping up to functionality again, we will work with our subcontractors and ramp up mouse studies to do the proposed experiments. I have hired a new technician that will be able to move the experiments forward.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

This project not only develops a new PET imaging agent to detect lethal prostate cancer noninvasively, but it characterizes the target molecule, i.e. fucosylated cell surface glycans, on the histopathologic level. Specifically, our group is correlating expression of fucosylated glycans with important clinical metrics such as grade, stage, and development of castrate-resistant disease. We are discovering that there are two specific fucose modifications to these cell surface glycans that are associated with the emergence of lethal neuroendocrine prostate cancer: (i) fucosylation of the core of the cell surface glycan by the fucosyltransferase 8 (FUT8) enzyme

and the synthesis of the fucosylated lewis-y antigen on the branches of the glycan.. It advances the idea of a "fucose scoring system", similar to the Gleason scoring system where the more fucoses a tumor has, the more aggressive it is. This has clear implications for the pathology field where both MALDI imaging mass spectrometry and immunohistochemistry could be used to assess these fucose scores on tumors from prostatectomies at initial diagnosis.

There is also an intriguing phenomenon that we are starting to identify: inhibition of the synthesis of fucosylated glycans through diet. Glycans are composed of various types of sugars, many of which are supplied by glucose. We are discovering that the ketogenic diet has the ability to effectively "prune" these fucosylated glycans and convert them to high mannose glycans that are implicated in inflammation and immune activation. Thus, it is possible that we might be able to use diet as a clinically actionable means to enhance tumor killing through immune recognition.

What was the impact on other disciplines?
Our clinical data demonstrate that there are two key biomarkers for lethal prostate cancers: (i) the presence of a fucosylated lewis-y antigen and (ii) a fucose attached to the core of the glycan by fucosyltransferase 8 (FUT8). These two biomarkers are readily measured with either MALDI imaging mass spectrometry and conventional immunohistochemistry. Therefore, this has clear impact on pathology and how specimens could be assessed on the clinical level.

What was the impact on technology transfer?
Our clinical data with MALDI and Lewis-y antigen staining are further advancing the potential efficacy of Lewis-y antigen and core glycan fucosylation by FUT8 as biomarkers for lethal, castrate-resistant prostate cancer. As these biomarkers are measurable with MALDI imaging mass spectrometry, this could advance the role of MALDI in histopathologic assessment.

What was the impact on society beyond science and technology? Nothing to report.

CHANGES/PROBLEMS

Changes in approach and reasons for change:
We have had a slight change in approach because of the multiple limitations described below. Because of lab shutdowns and facility closures, we have put more emphasis into the clinical research components of the project that characterize the role of fucosylated glycans in clinical outcomes in patients with prostate cancer.

Actual or anticipated problems or delays and actions or plans to resolve them:
We have had several problems with the laboratory component of the project. I searched for a technician to fill the position to perform the laboratory research and spent time training her on the basic conduct of the project including mouse handling and procedures. We had a mycoplasma outbreak in the lab involving the key TRAMP/MIC cell lines and had to shut down temporarily and acquire healthy cell lines. The COVID outbreak shut down our lab for a few months as well as the facilities where all mouse work had to be ramped down or shut down. Because of COVID, my department forced me to move labs in an attempt to reduce rent costs.

We are now ramping up in the new lab space. I have hired another technician to help out in lab that will increase productivity. We are starting to get data on the mouse models that we will use to test the new imaging agent and are also developing clinically-relevant therapies to change the glycan composition of the tumors. We hope to have a significant amount of data for the next reporting period. We are in the process of preparing a manuscript that characterizes the role of glycan fucosylation in patient outcomes and histopathologic assessment.

Changes that had a significant impact on expenditures:
COVID forced lab shutdown and expenses on the project. My technician was kept out of furlough, doing remote data analyses that moved the clinical aspects of the project forward.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
None

Significant changes in use or care of human subjects:
N/A

Significant changes in use or care of vertebrate animals:
None

Significant changes in use of biohazards and/or select agents:
None

PRODUCTS

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Joseph Ippolito (Washington University)
Project Role	PI
Researcher Identifier	eRA commons: ippolitoj
Nearest Person-month worked	1.2
Contribution to project	Overseeing entire project
Funding support	NA

Name	Richard Drake (MUSC subcontract)
Project Role	Co-PI
Researcher Identifier	eRA commons: RICHARD_R_DRAKE
Nearest Person-month worked	0.6
Contribution to project	Overseeing all MALDI analyses
Funding support	NA

Name	Jennifer Wu (Northwestern subcontract)
Project Role	Co-PI
Researcher Identifier	eRA commons: wumedd
Nearest Person-month worked	0.8
Contribution to project	Overseeing development of TRAMP/MIC model
Funding support	NA

Name	Dong Zhou (Washington University)
Project Role	Co-I
Researcher Identifier	eRA commons: D_ZZZZ
Nearest Person-month worked	1.2
Contribution to project	Synthesis, QI and QA of fluorofucose
Funding support	NA

Name	Elena Nunez (Washington University)
Project Role	technician
Researcher Identifier	NA
Nearest Person-month worked	12
Contribution to project	Cell culture, mouse handling, data analysis
Funding support	NA

Name	Grace Grimsley (MUSC subcontract)
Project Role	Technician
Researcher Identifier	NA

Nearest Person-month worked	1
Contribution to project	MALDI imaging and data analysis, tissue staining
Funding support	NA

Name	Ju Wu (Northwestern subcontract)
Project Role	Staff scientist
Researcher Identifier	NA
Nearest Person-month worked	2.4
Contribution to project	Cell culture, mouse handling, development of TRAMP/MIC
Funding support	NA

SPECIAL REPORTING REQUIREMENTS

N/A

APPENDICES

N/A