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14. ABSTRACT

Gastric cancer is a deadly disease that disproportionately impacts the African American community with decreased survival rates. It is largely unknown why this occurs but various proteins controlled by nuclear export protein 1 (XPO1/CRM1) are perturbed in African American gastric cancers at a higher rate. Although this is the case, there have been no conclusive studies identifying the connection between aberrant XPO1 activity and disparities. As part of our proposed Aim 1, we have obtained over 200 normal and gastric cancer patient tissues of African American and Caucasian American ethnicity and found a significant increase in XPO1 expression within the African American cohort (94% vs. 79%) but future investigation is underway to correlate XPO1 expression to overall survival in a retrospective analysis. We were one of the first groups to find XPO1 overexpression in gastric cancer compared to normal tissue and identify a molecular rationale that targeting this protein, with the small molecule inhibitor Selinexor (KPT-330), which is effective in targeting this disease. We have found that XPO1 inhibition targets the elicit perturbation of gastric cancer specific microRNAs that are responsible for disease progression, such as miR-7974 and miR-129-1-3p, and others which can be captured within the tumor and the serum. We have confirmed that up regulation of miR-7974 and downregulation of miR-129-1-3p leads to an increase in gastric cancer growth through targeting specific cancer related pathways including RASGEF1A (cell growth), TIMP2 (metastasis), DRAM1 (autophagy) and CCNG2 (cell cycle). This needs to be further confirmed in distinct gastric cancer cell line models. We anticipate within the upcoming months to perform an in *vivo* mouse study to utilize the perturbed microRNA expression profile, that specifically is altered after selinexor treatment, and identify a candidate miRNA that can be used as a as a predictive biomarker to monitor responses over time to this treatment.

15. SUBJECT TERMS
Gastric cancer, non-coding RNAs, selinexor, XPO1, nuclear export

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Introduction

Gastric cancer is an aggressive and deadly disease found throughout the world. There are three main subtypes of gastric cancer according to the Lauren classification, the well-differentiated (caused by environmental factors such as H. *Pylori*, poor diet and smoking), and poorly differentiated (genetic component), and mixed [1]. This disease is so deadly, with a 31% 5-year survival rate within the United States, because of the late detection, usually within Stage III-IV, as well a chemoresistance and lackluster treatment options.

We have previously found that Nuclear Export Protein 1 (XPO1) is a viable therapeutic target within gastric cancer due to its up regulation within gastric cancer tissues compared to normal mucosa and it can be targeted through the FDA approved compound Selinexor (XPOVIO) resulting in decreased cell viability and tumor burden in *vitro* and in *vivo* [2]. XPO1 is a critical cellular protein involved in transporting tumor suppressor proteins (TSPs), specific small noncoding RNAs and various oncogenes from the nucleus to the cytoplasm. In normal cells XPO1 maintains cellular homeostasis and cellular integrity but in malignant cells XPO1 over expression leads to aberrant cellular localization of critical TSPs such as TP53 and APC that are transported from the nucleus and degraded within the cytosol through the Ubiquitin-Protease system (UPS) [3]. Although we have shown targeting XPO1 is beneficial in treating gastric cancer it is unclear whether there is are specific patient populations that would benefit the greatest from XPO1 inhibitory treatment. To begin exploring this, we hypothesized there are differences between XPO1 expression between African American and Caucasian American gastric cancer patients. The rationale behind this is due to the literature suggesting there are differences in protein expression, such as TP53, which are directly controlled by XPO1 expression in African American gastric cancer patients [4]. Furthermore, it is known that there is disparates in African American cancer patients compared to Caucasians and this can be further observed specifically within gastric cancer [5].Unfortunately, there is limited patient data within publicly available databases to explore this question.

Although we have studied the preclinical efficacy of Selinexor within gastric cancer, to date there are no predictive or prognostic biomarkers that have been explored. XPO1 status may be predictive of whether a patient will respond to XPO1 inhibitor therapy, not all patients will respond within the same way due to the heterogenous nature of gastric cancer. Furthermore, there is no way to non-invasively assess XPO1 status and monitor treatment response over time. We have found with previous studies that cancer specific microRNA (miRNAs) expressions are altered after treatment with Selinexor [2].Through comparison to other small-noncoding RNA sequencing and RNA sequencing after treatment with Selinexor we have performed within our lab, we hypothesized that two miRNAs, miR-7974 and miR-129-1-3p, can be utilized as blood-based biomarkers that can predict responses to Selinexor over time. Selinexor (XPOVIO) has been FDA approved for the use in penta-refractory multiple myeloma as well as in combination with Non-Hodgkins lymphoma R-CHOP treatment. Not only will the work herein explore Selinexor efficacy within gastric cancer, this work may be expanded to other diseases where XPO1 inhibitory therapy is being utilized. The scope of this project is to (1) identify whether there are disparities in XPO1 expression between African American and Caucasian American gastric cancer patients and (2) to identify a predictive blood-based biomarker that can be used to monitor treatment responses to Selinexor.

KEYWORDS: *Gastric Cancer, Exportin 1 (XPO1), Predictive Biomarker, Disparities, MicroRNAs*

ACCOMPLISHMENTS

The major goals of this project are to understand (1) whether there are differences between XPO1 expression in African American and Caucasian American gastric cancer patients that can be linked to overall survival and (2) whether microRNAs can be utilized as a biomarker to Selinexor treatment for gastric cancer.

Major Task 1- Demonstrate the disparity of XPO1 overexpression between African American and Caucasian GC patients:

Subtask 1- Collect Bio banked Tissues and Stain for XPO1 expression (1-3 Months)- 100% Complete

The tissues were identified through the Wayne State University Pathology core, collected and sectioned for IHC staining (Date Began- 12/7/2020, Date Completed- 5/5/2021)

Table 1: Gastric cancer cases to be used for retrospective study partitioned by date of diagnosis and ethnic background

Malignant Stomach with Biopsy or Surgery to Primary Site		
Dx Year	**Black**	**White**
2009	13	36
2010	15	27
2011	25	30
2012	9	22
2013	16	24
2014	8	17
2015	10	17
2016	11	20
2017	12	11
Total	**119**	**204**

Table 2: Gastric cancer cases to be used for retrospective study partitioned by date of diagnosis and ethnic background

Stage	Black	White
0	**	**
I	23	35
II	15	29
III	31	35
IV	45	92
U	**	**
Total	**119**	**204**

Table 1 and Table 2 were our initial starting points for the study labeling the patient demographics. Out of all of these cases there were 107 African American and 183 Caucasian tumor blocks pulled. The remaining tissues were eliminated due to low tissue abundance within the blocks making a total of 221 total gastric cancer cases and we obtained 155 normal gastric tissue for a total of 358 blocks that were sectioned into slides for IHC analysis.

Subtask 2- Complete the Retrospective Analysis of XPO1 stained tissues- 50% Complete

The tissues are in the process of being scored and graded for XPO1 expression but preliminary results have shown that more XPO1 expression is found in tumor tissue than normal and is more abundant in the African American gastric cancer cohort (Table 3). Once a detailed scoring is complete the retrospective analysis will begin (Tentative start Date 10/15/2021).

Table 3: Preliminary baseline analysis of XPO1 expression based on IHC tissues, both gastric cancer and normal gastric tissues comparing African American and Caucasian American patients.

Racial Demographic	XPO1 +	XPO1 -
African American (Tumor Tissue)	94.05%	5.95%
Caucasian American (Tumor Tissue)	74.11%	25.89%

Major Task 2- Identifying a Suitable Pharmacodynamic Biomarker for KPT-330 treatment

Subtask 1- In *vitro* analysis of miR-7974 and miR-129-1-3p and their role in signaling for gastric cancer inducing processes such as suppression of Apoptosis, increase in oncogenic signaling pathways and alteration in cell cycle processes by focusing on critical cell pathways, such as DRAM1, TIMP2, CCNG2 and RASGEF1A- 50% Complete. More in depth studies are underway to confirm the oncogenic potential of these microRNAs including validation of microRNA expression in normal gastric cells (obtained from commercial source on 9/1/2021).

Subtask 2- In vivo analysis of selienxor serum based biomarker- 0%
Complete Projected Start Date for the Experiment in March 2022. IACUC approval complete.

What was accomplished under these goals?

Major Activities: Tissues were obtained from the Wayne State University Biobanking core and sectioned and processed on slides for further IHC analyses of XPO1. Some in *vitro* experiments were performed for Major Aim 2 including RT-qPCR.

Specific Objectives: (1) To identify if there is a disparity in XPO1 expression between gastric cancer patients of African American or Caucasian descent and (2) to identify the in *vitro* characteristics of two noncoding RNAs (miR-7974 and miR-129-1-3p) found to be perturbed in a small-noncoding RNA sequencing study and further identify a serum based noncoding predictive RNA biomarker using in *vivo* mice modeling.

Significant Results or Key Findings:
The tissue studies have shown XPO1 is overexpressed in gastric cancer patients. Further, I have found miR-129-1-3p downregulation or miR-7974 upregulation perpetuates gastric cancer growth and progression by up regulating various cancer related pathways such as cell growth (RASGEF1A), cell cycle (CCNG2), autophagy (DRAM1) and metastasis (TIMP2). We found that the up regulation of miR-7974 and downregulation of miR-129-1-3p enhances gastric cancer growth, which is statistically significant, in two gastric cancer models (SNU-1 and NCI-N87) (Figure 1). We are currently performing analyses on a third gastric cancer model, SNU-16, to assess whether this phenomena occurs globally in gastric cancer. These three models will represent the large majority of gastric cancer patients, ~80% having well-differentiated or poorly-differentiated, of differing stages of disease (primary and metastatic). Due to the adherent growth pattern of the NCI-N87 cell line a MTT cell viability assay was performed and due the floating nature of the SNU-1 cell line we performed a cell viability assay via counting with trypan blue. SNU-16 is also a floating cell line and will be assessed via cell viability assay. To ensure that miR-7974 and miR-129-1-3p were being upregulated or downregulated, respectively, we performed RT-qPCR utilizing primers specific to miR-7974 and miR-129-1-3p at the indicated concentrations used (Figure 1C).

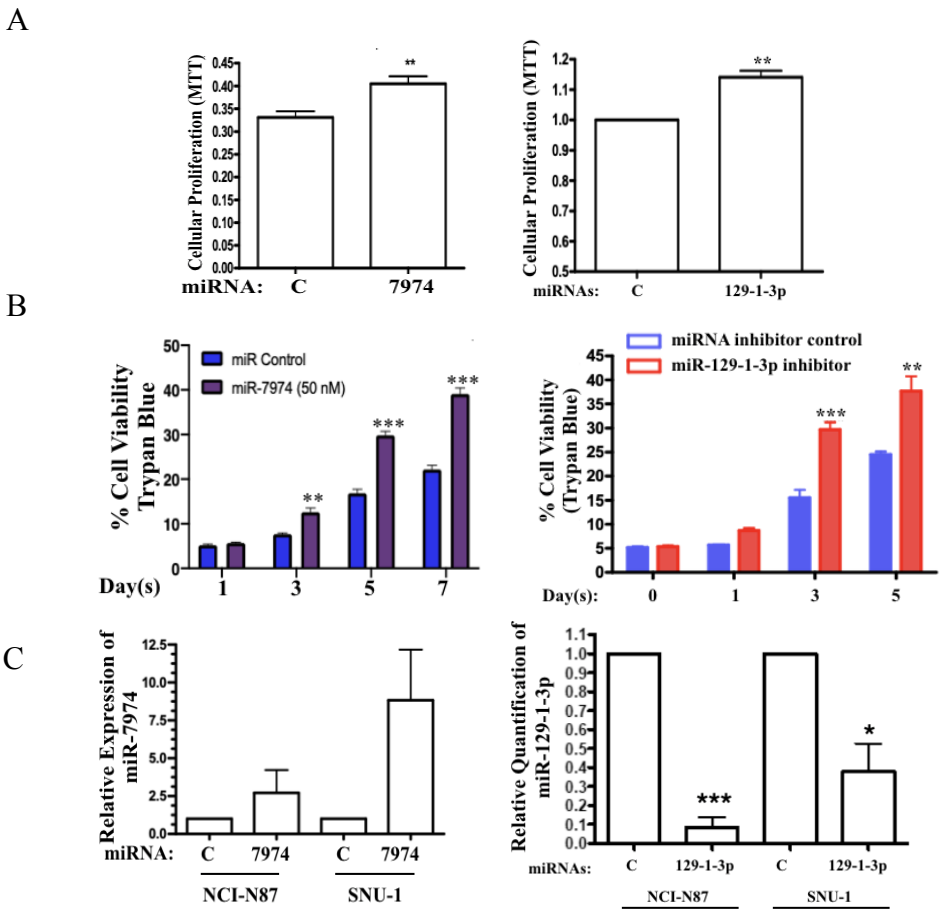

Figure 1: miR-7974 and miR-129-1-3p De-regulation Leads to Gastric Cancer Cell Growth. [A-B] Cells were transfected with mimic control, miR-7974 mimic or inhibitor control, miR-129-1-3p inhibitor at 50 nM and assessed for growth with trypan blue counting assay (**A**) or MTT (**B**). [**C**] Assessment of effect of 50 nM miR-7974 mimic or miR-129-1-3p inhibtor on two gastric cancer cell lines using Taq-man RT-qPCR. Figures include three distinct biological replicates and statistics were provided by GraphPad Prism.

One of the significant pathways that both miR-7974 and miR-129-1-3p were predicted to target is the RasGEF Domain Family Member 1A (RASGEF1A). RASGEF1A is a protein involved with activating the RAS signaling pathway, a major pathway involved in the progression GI malignancies including gastric cancer. As a GTPase, it adds a GTP molecule onto RAS allowing for the downstream phosphorylation cascade to occur. We found RASGEF1A was highly upregulated at the mRNA level after induction of our mimic (miR-7974) or inhibitor (miR-129-1-3p) in gastric cancer models (Figure 2A). Another predicted target of both miR-7974 and miR-129-1-3p was DNA Damage Regulated Autophagy Modulator 1 (*DRAM1*). We found downregulation after transfection with miR-7974 mimic or miR-129-1-3p inhibitor (Figure 2B). TIMP2, a metastasis suppressor, was predicted to be targeted by miR-129-1-3p and miR-7974. We found downregulation of *TIMP2* in miR-7974 upregulation or miR-129-1-3p downregulation differently between the two cell lines (Figure 2C). Finally, Cyclin G2 was predicted to be targeted by miR-129-1-3p and miR-7974 and at the RNA level *CCNG2* was downregulated after transfection of these two miRNAs (Figure 2D).

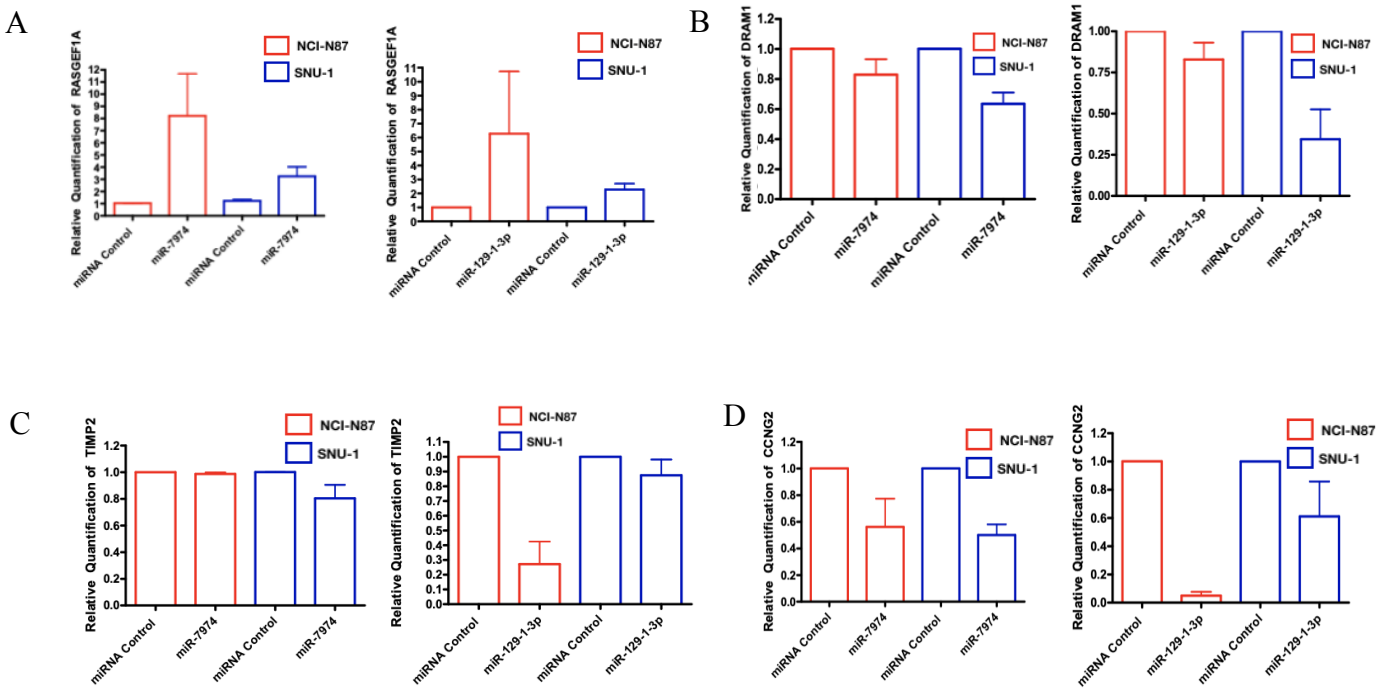

Figure 2: Critical cellular proteins are perturbed by miR-7974 and miR-129-1-3p in NCI-N87 and SNU-1 gastric cancer models [**A**] *RASGEF1A* is up regulated 24 hours after miR-7974 up regulation or miR-129-1-3p downregulation. [**B**] DRAM1 is downregulated 24 hours after miR-7974 up regulation or miR-129-1-3p downregulation. [**C**] TIMP2 is downregulated 24 hours after miR-7974 up regulation or miR-129-1-3p downregulation differently between the two cancer models. [**D**] CCNG2 is downregulated 24 hours after miR-7974 up regulation or miR-129-1-3p downregulation. Three individual replicates were performed.

What opportunities for training and professional development has the project provided?

There have been significant training opportunity within this project as a student learning and perfecting various laboratory techniques. I have learned the fundamentals of immunohistochemistry including scoring and how to process human tissues for these types of experiments. There was sufficient improvement and learning how to work with miRNAs and various assays that can be used to understand the relationship between miRNAs and the RNAs they target. I have presented my initial findings of the in vitro work for Major Task 2 at the AACR annual 2021 meeting (poster presentation) and at the Cancer Biology Symposium at Wayne State University March 26, 2021 as an oral presentation.

What do you plan to do during the next reporting period to accomplish the goals?

For Aim 1: I plan to begin the retrospective analysis of the tissues comparing XPO1 to overall survival with the help of Dr. Purrington (Wayne State University). I plan to report the findings within the next report.

For Aim 2: I plan to complete the in *vitro* studies of miR-7974 and miR-129-1-3p by confirming the initial findings across the gastric cancer cell lines mentioned. I also have obtained normal gastric tissues and will begin comparing the expression of these microRNAs between normal and cancer tissues. Finally, understanding the relationship between selinexor and these two microRNAs in vitro are underway and will be presented next report. In early 2022 the animal work will begin by ordering mice and starting the proposed experiments. The analysis will take a significant amount of time (~6 months) so beginning these experiments will be essential.

IMPACT

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

These results may help identify a specific patient population that may be more responsive to XPO1 targeted therapy. We have shown that both miRNA-7974 upregulation and miR-129-1-3p downregulation enhances gastric cancer growth and progression, a novel finding that has not been discovered previously, as well as its interaction with XPO1, a known protein involved with the perpetuation of gastric cancer. Future significance will include an understanding of how we can utilize these microRNAs as a predictive biomarker to monitor Selinexor effect on the tumor over time as well as understand whether there are cancer related disparates in gastric cancer patients that is related to XPO1 over expression.

What was the impact on other disciplines?

Overall, this information can be utilized to assist in other biomarker studies where Selinexor is being used clinically as a standard of care, specifically in multiple myeloma, Non-Hodgkins lymphoma and pancreatic cancer.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

These results will make an impact on not only furthering the science of gastric cancer but it can show the public that there is progress being made in rarer malignancies, such as gastric cancer, and that these results have the potential to be applied to other more common malignancies within the future. This may change the attitudes of the general public in a positive manner because (1) not only has a new therapeutic been introduced into the clinic that is being tested in gastric cancer patients but also (2) work into the identification of cancer related disparities related to XPO1 expression that can be correlated to overall survival and (3) ways in which this treatment efficacy has the possibility to be monitored over real time within the clinic to guide treatment decisions for future patients.

CHANGES/PROBLEMS

Changes in approach and reasons for change?
Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Delays have occurred since the grant start date with the most notable being the COVID-19 pandemic. This event was and continues to be an obstacle that we are working to overcome. There was mandatory closure of all Wayne State University laboratories between March-June of 2020, although this was before the start period of the grant, there were cuts to hourly work in the lab, mandated half days, which have begun since reopening of the lab in June 2020. Although there were these closures, we have optimized our time within the lab and worked diligently to obtain meaningful results. I am present within the lab as much as possible to work on this project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

We initially faced an issue with the approval for the IRB work for Aim 1 of this project. The IRB had to be revised in order to satisfy the requests of the DOD. I obtained necessary approval from Wayne State, Karmanos Cancer Institute and the DOD in November of 2020. This led to an almost 2-month delay in the start Aim 1. We have since resolved this issue and have obtained appropriate approval from all organizations to proceed with Aim 1 and have been working on this since we obtained all necessary approvals.
Approval date for IRB- 11/4/2020
IACUC approval dates were obtained- 7/8/2020
There were no significant changes in use or care of human subject
There were no significant changes in use or care of vertebrate animal
There were no significant changes in use of biohazards and/or select agents

PRODUCTS

Publications, conference papers, and presentations

- **Journal publications: Not Applicable**
- **Books or other non-periodical, one-time publications.**
  - Sexton, R; Al-Hallak, N; Tesfaye, A; Azmi, A.S; Elsevier; Noncoding ribonucleic acids in gastric cancer; Clinical applications of noncoding RNAs in Cancer, 2021; book chapter; awaiting publication; acknowledgement of federal support (**yes**/no)
- **Other publications, conference papers, and presentations.**
  - Cancer Biology Research Symposium Wayne State University- Oral Presentation (local)
  - AACR 2021 National Meeting- Poster Presentation (National)

- **Website(s) or other Internet site(s)**: Not Applicable
- **Technologies or techniques:** Not Applicable
- **Inventions, patent applications, and/or licenses:** Not Applicable
- **Other Products**
  - data or databases; Not applicable

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