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14. ABSTRACT
Purpose: We are investigating if sequestration of metabolically dysfunctional mitochondria by the autophagosomes (mitophagy) imparts anti-androgen resistance and if this phenomenon can be applied in circulating tumor cells in patient blood samples as a biomarker for development of drug resistance.
Method: Effects of the anti-androgen enzalutamide on the autophagy and mitophagy of androgen-dependent LNCaP and -independent C4-2 and CWR22v1 cells are studied first. Autophagy is monitored by fluorescence of cells with anti-LC3B antibody. Cellular fluorescence due to Mitosox dye oxidation is used to identify mitochondria producing high superoxide (O2-). Mitophagy is monitored using fluorescence resonance energy transfer (FRET) by visualization of FRET images and quantitation of FRET image intensities using a Leica Sp8 fluorescence STED confocal microscope and Image J software.
Results and Discussion: The degree of mitophagy is more in the surviving androgen-dependent LNCaP cells than in the -independent C4-2 cells, when grown in androgen-depleted media. Enzalutamide treatment induces mitophagy in both cell lines. However, the increase in mitophagy is significantly more in the enzalutamide-resistant C4-2 than in the sensitive LNCaP cells. Mitosox fluorescence and mitophagy in circulating tumor cells (CTCs) isolated from patient blood samples are being quantitated to identify drug resistance.

15. SUBJECT TERMS
Mitophagy, prostate cancer, drug-resistance, fluorescence resonance energy transfer (FRET), Circulating Tumor Cells

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Final Report

September 1, 2015 – October 31, 2019

1. INTRODUCTION

Clinical management of prostate cancer is challenging due to diverse clinical outcomes. Approximately 30% of prostate cancer patients progress to advanced, metastatic disease, which responds poorly to most cancer therapies. Despite intense research during the last decade, the mechanism of developing resistance to anti-androgens remains mostly unknown. This proposal was based on the hypothesis that cellular metabolic changes alter mitochondrial red-ox status and induce mitophagy that reduces apoptosis and imparts anti-androgen resistance.

2. KEYWORDS

1. AA = Antimycin A
2. ADT = Androgen-deprivation therapy
3. AR = Androgen receptor
4. ATP = Adenosine triphosphate
5. CK = Cytokeratin
6. CRPC = Castrate-resistant prostate cancer
7. CTC = Circulating tumor cells
8. ENZA = Enzalutamide
9. FRET = Fluorescence resonance energy transfer
10. ICC = Immunocytochemistry
11. LC3 = Light chain 3
12. Mito-O = MitoTracker orange dye
13. Mito-S = Mitosox red dye
14. MSR = Mitosox red dye
15. MTG = MitoTracker green
16. OCR = Oxygen consumption rate
17. PDX = Patient derived xenograft
18. TCA = Tricarboxylic acid

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1. Standardize confocal microscopy FRET analysis to quantitate mitophagy in androgen-dependent and castrate-resistant prostate cancer cells exposed to antiandrogens in the presence or absence of androgen.

Major Task 1. Standardize confocal microscopy FRET assay and compare degree of mitophagy in anti-androgen –sensitive and –resistant prostate cancer cells.

Subtask 1: Use 96-well plate based FRET assay to determine effect of anti-androgens on androgen-dependent LNCaP and androgen-independent C4-2 cells. (1-6 months)

Subtask 2: Use confocal microscopy FRET assay to confirm results of 96-well plate based assay. (3-9 months)

Subtask 3: Verify 96-well plated based and confocal microscopy FRET assay results using other androgen-dependent (LAPC-4) and androgen-independent (CWR22Rv1) prostate cancer cells. (6-15 months)

Specific Aim 2: Apply the standardized method to live circulating tumor cells (CTCs) for prostate cancer patients undergoing ADT to determine correlation of degree of mitophagy in CTCs with response to ADT/ development of resistance to ADT.

Major Task 2: Optimization of CTC isolation device protocols for fixation, permeabilization and staining of CTCs.

Subtask 1: Submit documents for HRPO approval. (1-2 months)

Subtask 2: Utilize CTCs from estimated 15 patients to test variations in fixation buffer/time, permeabilization buffer/time, and stain concentration/time. (4-9 months)

Major Task 3: Perform confocal microscopy FRET assay of mitophagy on CTCs from patients pre- and post- Enzalutamide therapy.

Subtask 1: Quantitate mitophagy in CTCs from 30 patients' pre-treatment and at the time of radiographic progression of the disease. (9-32 months)

Subtask 2: Analyze data for correlation with disease status. (33-36 months)

What was accomplished under these goals?

Anti-androgen resistant cells are less glycolytic than are anti-androgen sensitive cells. Cellular oxygen consumption rate (OCR) and pH changes in the media due to cellular acid efflux (mostly lactate from glycolysis, **Figure 1A**) by cultured cells can be measured by Seahorse assay at a high precision. Adding selected inhibitors of glycolysis (2-deoxy glucose, 2DG) and mitochondrial ETS inhibitors rotenone + antimycin A (AA) at specific time points, the rate of cellular glycolysis in terms of proton efflux rate (GlycoPER) can be accurately determined. This assay has now been accepted as a gold standard for measuring changes in glycolysis in cultured cells. The GlycoPER data from ADPC LNCaP, LNCaP derived CRPC C4-2 and CRPC MDA PCa 2b cells obtained from a patient derived xenograft (PDX) model incubated in androgen reduced media are shown in **Figure 1A**. The corresponding basal OCR data are shown in **Figure 1B**. All Seahorse data are normalized to

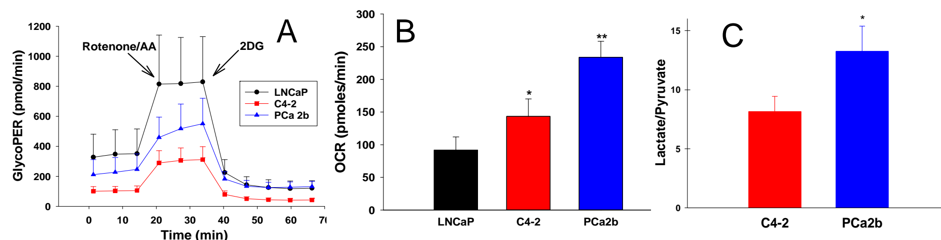

Figure 1. (**A**) Glycolysis (GlycoPER) and (**B**) oxygen consumption rate (OCR) in LNCaP, C4-2 and MDA PCa 2b cells grown in androgen reduced media for 48 h. All data are normalized to viable cells at the end of the assay. Each data point and error bar represent the mean and standard deviation of 45 data points from 3 repeats. *P<0.01, **P<0.001. (**C**) ^{13}C-labeled lactate/pyruvate peak has been determined in an IM-QTOF mass spectrometer in C4-2 and PCa2b tumor xenografts 30 minutes after 25 mM ^{13}C-pyruvate injection. Each data point and error bars are mean and standard deviation of 2 runs of each tumor repeated twice. * p<0.01.

viable cell counts (~85%-90% viable under these conditions) as determined by a propidium iodide dye fluorescence assay. The data show that C4-2 and PCa2b cells are significantly less glycolytic and depend more on mitochondrial ox-phos for their survival than do the LNCaP cells. As ox-phos generates 18 times more ATP than does glycolysis, a small increase in ox-phos (**Figure 1B**) can compensate for a large decrease in glycolysis (**Figure 1A**). We have also standardized a method of determining the rate of glycolysis in tumor xenografts *in vivo* following a protocol standardized in our laboratory by injecting ^{13}C-labeled pyruvate into tumor bearing animals before sacrifice and then estimating the ^{13}C-labeled lactate/pyruvate ratio in the tumors using an exact mass ion mobility quadrupole time of flight (IM-QTOF) instrument (**Figure 1C**). MDA PCa 2b cells produce more lactate and less pyruvate than do the C4-2 cells confirming the Seahorse data for the cell lines.

Anti-androgen enzalutamide (ENZA) reduces glycolysis in viable prostate cancer cells. The GlycoPER and OCR in ENZA-sensitive LNCaP and ENZA-resistant C4-2 cells treated for 24 h with graded concentrations of ENZA within achievable human serum level are shown in **Figure 2**. ENZA lowers the GlycoPER with small changes in OCR in both cell lines. These data have been further confirmed by determining the changes in total (cellular+media) lactate/pyruvate ratio using mass spectroscopy (**Figure 3**). LNCaP cells with higher glycolysis rates than those found in C4-2 cells (see **Figure 1A**) produce more lactate and less pyruvate than do C4-2 cells. ENZA significantly lowers lactate levels in the –sensitive LNCaP, but not in the –resistant C4-2 cells at or near their respective IC_{50} doses.

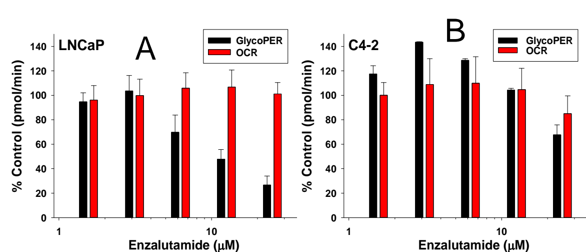

Figure 2. GlycoPER and OCR in LNCaP (**A**) and C4-2 (**B**) cells grown in androgen depleted media for 48 h and then treated with ENZA for 24 h. All data are normalized to viable cells at the end of the assay. Each data point and error bar represent the mean and standard deviation of 45 data points from 3 repeats.

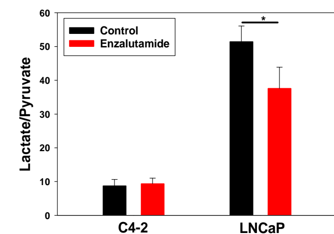

Figure 3. Lactate/pyruvate ratio in C4-2 and LNCaP cells incubated in androgen depleted medium for 48 h and then treated for 24 h with ENZA at respective IC_{50} doses. The metabolites are estimated in a QQQ mass spectroscope following a published method. Each data point and error bar are the mean and standard deviation of 2 parallel samples run at least twice.

Prolonged treatment with ENZA increases mitochondrial superoxide production. *Microscopy*−ENZA treatment for 72-96 hours at respective IC_{50} doses did not produce enough viable prostate cancer cells for reliable Seahorse assay results. To circumvent this problem, we standardized a confocal microscopy coupled with an image analysis assay to estimate mitochondrial ox-phos based on superoxide production. We incubated the cells with mitosox red (MSR) dye that specifically binds to the mitochondrial membrane and fluoresces upon oxidation only by the superoxide generated by the mitochondrial ox-phos activity. The representative fluorescence images of LNCaP and C4-2 cells after 96 h treatment with ENZA

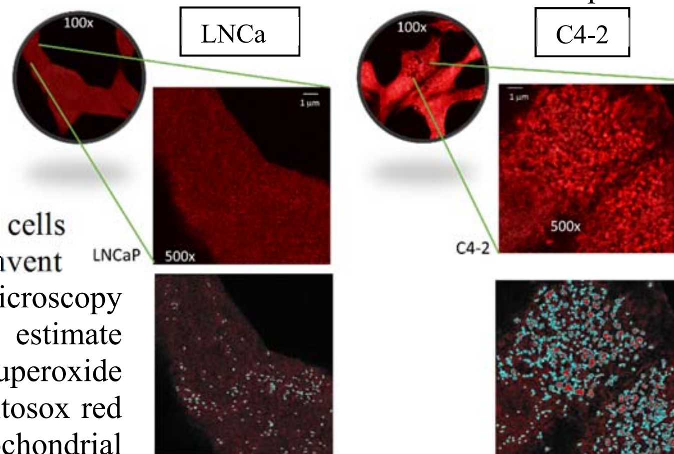

Figure 4. Representative confocal microscopy images of an optical section at 100x and 500x magnification each of LNCaP (**Left**) and C4-2 (**Right**) cells treated first 24 h with ENZA and then incubated with MSR dye for 4 hours and examples of Image J segmentation (**bottom**) for fluorescence intensity analysis.

(at or near the IC_{50} doses, see above) were obtained using a fluorescence confocal microscope (**Figure 4**). The fluorescence intensities of MSR dye are quantitated by Image J segmentation analysis. The mean pixel intensities in cells selected from 10 random areas of each slide are shown in **Figure 5**. The intensity is markedly higher in the ENZA treated CRPC C4-2 and CWR22v1 cells than that in the control untreated cells and also that in both ENZA-treated and -untreated LNCaP cells (data not shown).

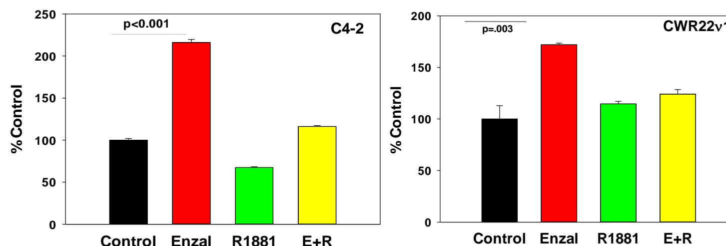

Figure 5. MSR dye intensities of mitochondria of prostate cancer cells treated with vehicle control, 20 μM ENZA (Enzal) (near IC_{50} dose), androgen R1881, and ENZA+R1881 (E+R).

Macroscale − To confirm that the microscopy image analysis data are true for most cells after prolonged exposure to ENZA, we standardized a macroscale assay to quantitate cellular ox-phos. The prostate cancer cells plated in each well of a 96-well plate were treated with desired concentrations of ENZA for 96 hours. Cells were then incubated with MSR for 4 hours to stain superoxide producing mitochondria followed by MitoTracker Green (MTG) dye for 30 min to stain all mitochondria. The non-overlapping fluorescence emission intensities of MSR and MTG were determined in a fluorescence plate reader. The MSR/MTG ratio represent the mitochondrial ox-phos normalized per mitochondrion. The increase in this ratio for LNCaP and C4-2 cells treated with ENZA compared with the ratio in untreated control cells is small but significant (**Figure 6**). This confirms the microscopic observation of relatively higher ox-phos in the cells that survive in the presence of ENZA (**Figures 4 and 5**). The relative increases in the MSR/MTG ratio is less than that observed in the microscopic images. This could be because this assay includes the MTG fluorescence from the mitochondria in dead cells that do not oxidize MSR.

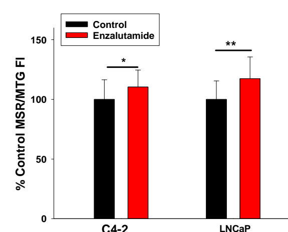

Figure 6. MSR/MTG in in C4-2 and LNCaP cells treated for 96 h with vehicle (control) and with IC_{50} dose of ENZA. Each data point and std. dev. is the mean of the readings from 6 wells run in triplicates and repeated at least 3 times. *$p<0.05$, **$p<0.005$.

We are now generating more cells under these treatment conditions to confirm the data using lactate/pyruvate ratio (see **Figure 3**) as well as estimation of other TCA cycle metabolites to confirm that prostate cancer cells switch mitochondrial metabolism from glycolysis to ox-phos while adapting to anti-androgen treatment.

ENZA changes mitochondrial organization. We stained functional mitochondria with MitoTracker orange dye (Mito-O) and applied high resolution microscopy to monitor mitochondrial organization in prostate cancer cells treated with ENZA (**Figure 7**). While most LNCaP cells show generally distributed mitochondria in the cytoplasm (**Figure 7**, left panels), C4-2 mitochondria are well organized and 20%-25% of them form long fused chains (**Figure 7**, white arrows in the right panel). Such mitochondrial reorganization has been reported to be associated with prostate cancer and

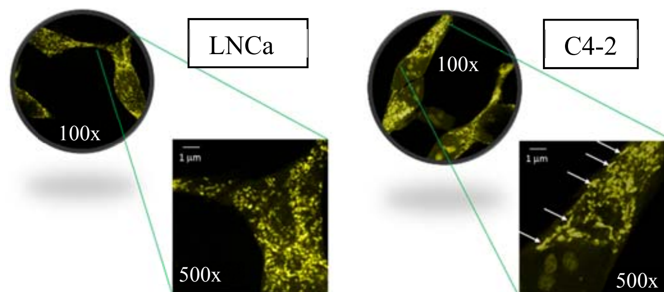

Figure 7. Confocal microscopy images at 100x and 500x magnification of LNCaP (**left**) and C4-2 (**Right**) cells treated for 24 h with 10μM and 20μM ENZA, respectively and then incubated with Mito-O dye for 30 min.

other cancer invasion and metastasis. We hypothesize that the mitochondrial metabolic switch that occurs in cells adapting to anti-androgen treatment alters mitochondrial organization to support cell survival, migration and metastasis.

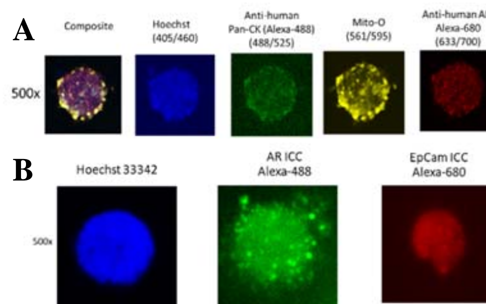

Figure 8. **(A)** Representative images of a human CTC from AR-positive MDA PCa 203 PDX carrying mouse blood sample; **(B)** a CTC isolated from a prostate cancer patient blood sample. The CTCs were identified from CD45 negative cells that are positive for antigens shown above the images.

Isolation of CTCs from PDX animal and prostate cancer patient blood samples and analysis of cellular ox-phos. We have standardized a method of isolating CTCs from blood samples in collaboration with James Reuben's laboratory at our Institution following a published procedure. Human prostate cancer CTCs are isolated from the blood samples of prostate cancer patients and from PDX animals after a certain stage of tumor development using the Parsortix microfluidic system (Angle, PLC, King of Prussia, PA). The system enriches tumor cells based on size and deformability without biasing the selection with any pre-determined surface antigen. After filtering blood through a 6.5 µm critical gap, reversing the flow of buffer allows for enrichment of live CTCs. Pan-cytokeratin (CK) positive and CD45 negative cells are tentatively identified as CTCs. AR-positive nucleated breast cancer CTC identification by ICC for AR and pan-CK have previously been published [PubMed ID 28957377]. We used the same method to identify AR-positive nucleated prostate cancer CTCs from the PDX MDA PCa 203 and also from a patient blood sample. Representative images are shown in **Figures 8A** and **8B**, respectively.

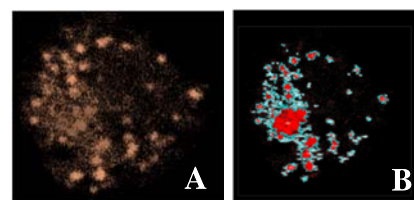

Figure 9. **(A)** A representative image of an optical section of MSR fluorescence due to oxidation by mitochondrial superoxide of a prostate cancer patient CTC from and **(B)** its image J segmentation for pixel fluorescence intensity analysis.

Increase in ox-phos in CTCs from patients developing resistance to AR-targeted therapies. We then followed the MSR dye oxidation *ex vivo* in the CTCs isolated from patient blood samples to estimate superoxide production by their mitochondria following the protocol standardized in this project (see **Figure 4**). The conditions for blood collection and storing in ice for less than 30 min before CTC isolation remain identical for all samples to control for any metabolic alterations during collection, transport and isolation. A representative image of an optical section of a CTC isolated from a prostate cancer patient blood sample and its corresponding Image J segmentation are shown in **Figure 9**. Thus far, we have collected 22 blood samples from 20 consented patients undergoing AR-targeted therapies under an IRB approved protocol (PA15-0956). From each 10 ml blood sample, we detected sufficient (~20-25) analyzable CTCs from 18 out of 20 patients. Mean intensities of a minimum of 15 CTCs per patient sample are shown in **Figure 10**. Patients, who contributed samples #14-20 (marked with a red line on top), progressed to therapy resistance within 3 to 6 months of the sample collection. Samples #6, #7 and #13, #14 (colored grey) were collected longitudinally from 2 patients within a time span of 6 months. At the time of

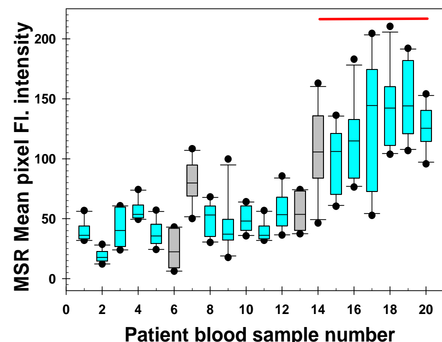

Figure 10. Mean MSR dye fluorescence intensities of 15+ CTCs obtained from prostate cancer patients undergoing androgen-signaling axis targeting therapies. Each sample is collected from an individual patient, except samples #6, #7 and #13, #14 (marked in grey). Samples marked with red line are from patients who developed resistance to anti-androgen therapy.

collection of samples #6 and #13, both patients were responding to treatment with androgen synthesis inhibitor abiraterone acetate. Within 3 months after sample #13, sample #14 was collected from the same patients. Shortly after collection of sample #14, the patient developed resistance to abiraterone acetate therapy and progressed. Sample #7 was collected recently from the patient who contributed sample #6 earlier this year. Sample #7 showed a small increase in ox-phos compared with sample #6. We are currently following this patient for disease outcome. An additional 12 patient samples have now been collected with another patient with pre- and during enzalutamide therapy. The analysis of these samples collected during the last 5 months are currently ongoing. This fulfilled our accrual goal of 30 patient samples as proposed. The complete dataset will be presented at the AACR annual meeting, San Diego, 2020 and a manuscript is now being prepared for publication in Clinical Cancer Research shortly after that presentation.

Mitophagy in anti-androgen resistance

Standardization of a super-resolution microscopy for co-localization of mitochondria with autophagosomes. High-resolution Leica STED-confocal microscope has been installed in our Department during this project and a whole new image analysis software at high resolution has been standardized to assay colocalization of mitochondria with autophagosomes has been visualized. At this resolution individual mitochondria and autophagosomes can be visualized and quantified (see **Figures 11 A and B**).

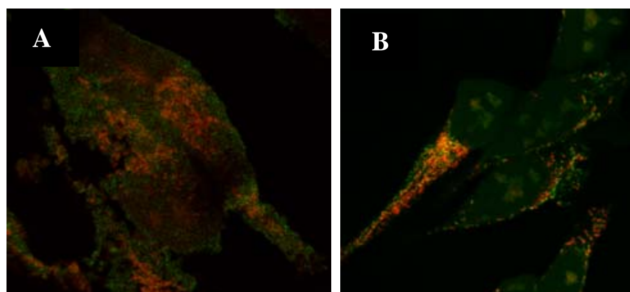

Figure 11. An optical section of a confocal microscopic image. LC-3B ICC autophagosomes (green) and Mitosox-red stained mitochondria (red) and co-localized (yellow). (**A**) C4-2 control; (**B**) C4-2 + 20 μM Enzalutamide.

For the confocal microscopy, LNCaP and C4-2 cells are seeded separately in 300 μL F1C4 in each chamber of 8-chamber slides. Anti-androgen-sensitive LNCaP cells have been treated with 1 μM (IC_{50}) and –resistance C4-2 cells have been treated with 10 μM enzalutamide for 96 h. Cells in different chambers are then treated with Mito-O alone or Mito-S alone and the slides are incubated for 4 h at 37°C in a CO_2/air incubator to allow for Mito-S oxidation. Cells are then fixed and stained with anti-LC3B antibody and a secondary Alexa488 conjugated antibody. The co-localization of the mitochondria with autophagosomes is observed at 1,600x magnification in 0.5 μm optical sections (**Figure 11**). The data show considerable separation between red and green stained organelles in control C4-2 cells. Appearance of yellow zones shows considerable co-localization of the two organelles in enzalutamide resistant C4-2 cells growing in the presence of enzalutamide.

Standardization of FRET analysis of the cells to quantify co-localization of mitochondria and autophagosomes (Mitophagy). The FRET images using 488 nm excitation/595 nm emission for LC-3B stained with Alexa488 and Mitosox red dye in the Leica Sp8 STED confocal microscope at 0.5 μm optical sections are shown in **Figure 12**. The fluorescence intensities of the FRET signals from all optical sections have been quantified and integrated using Image J software with appropriate threshold to contour map each cell and are analyzed. Similar

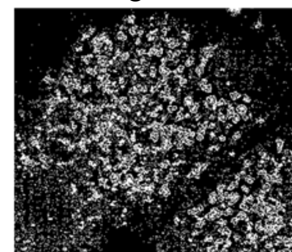

Figure 12. Representative Image J segmented FRET intensity pixels between Mitosox Red and LC-3B-Alexa 488 in an optical section of a C4-2 cell treated with 20 μM enzalutamide.

FRET signal intensities from LC-3B and Mito-O dye combination are also obtained for normalization (data not shown). The mean FRET intensities calculated from enzalutamide-treated and untreated LNCaP, C4-2 and CWR-22v1 cells are shown in **Figure 13**. Where there is no significant changes in FRET intensities for Mito-S and LC-3B and in LNCaP cells, in both enzalutamide-resistant cell lines, C4-2 and CWR22v1, an appreciable increase in FRET intensities has been observed in Enzalutamide treated cells as compared to that in the control vehicle treated cells. This increase in mostly reversed by androgen R1881 treatment suggesting that the mitophagy is a direct consequence of enzalutamide binding to androgen receptor.

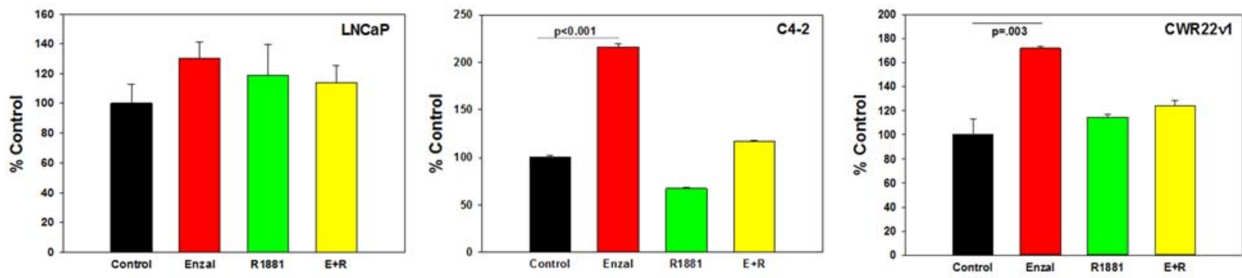

Figure 13. Mean of 2 repeat experiments determining the relative changes in mean pixel intensities of FRET image segments of 0.5 mm optical sections (10-12) in 10-15 cells/field. All data are normalized to FRET intensities from images of LC-3B and Mito-O with error bars representing standard deviations.

Standardization of FRET imaging of the isolated CTCs in a high-resolution microscope. The cells are plated immediately after isolation in 3 chambers of a lysine coated 8-chamber slide in RPMI1640 with 10% FBS. The cells are allowed to attach overnight. The next day, the cells in 2 chambers are treated with Mito-S for 4 hours. All cells are then fixed with paraformaldehyde and the cells in the third chamber are subject to immunocytochemical staining (ICC) with anti-CK, anti-AR, anti-EpCam and DAPI to identify nucleated epithelial cells of prostatic origin (data not shown), which we designate as prostate CTCs as mentioned in our grant application. The cells in the other 2 chambers are stained with both anti-AR (Alexa 647) and anti-LC3 (Alexa 488) antibodies. A representative FRET image (Ex 488/Em 595) between LC-3 (Ex 488/Em 535) and Mito-S (Ex 540/Em 595) is shown in **Figure 14**. The FRET signals from the CTCs analyzed thus far from 3 patients responding and 3 refracting from anti-androgen therapy are shown in **Figure 15**. There is a clear difference in FRET intensities between the two groups of patients. These data have also been collected from a total of 30 patients (see above) of which, 22 patients are responding and 8 patients have refracted from anti-androgen therapy at the time of collection. The data are currently being analyzed. These data will be statistically correlated with patient outcome to determine the threshold of FRET intensity values that may predict the therapy outcome.

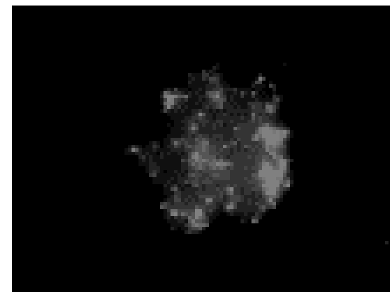

Figure 14. Representative FRET image (pseudo colored red) of a prostate cancer patient CTC stained with Mito-S and LC-3B antibody with Alexafluor-488 stain in a Leica SP8 confocal microscope.

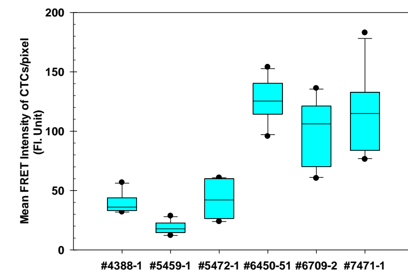

Figure 15. Mean FRET intensities of a minimum of 10 CTCs isolated from each of six prostate cancer patients (3 responders [blue line] and 3 (non-responders [orange line]) stained with Mito-S and then with LC3B antibody *ex vivo* following the method described (see text).

In summary, our data show that CRPC cells adapt to anti-androgen therapy by switching their metabolism from glycolysis to ox-phos. Such metabolic change increases the mitochondrial

superoxide production that can be monitored in patients by microscopic analysis of Mitosox dye intensities in CTCs isolated from blood samples. This may be applied to select patients for treatment with mitochondria targeting agents that are currently being developed for cancer therapy. The switch in metabolism also changes mitochondrial organization and enhances mitophagy that can be also monitored by FRET intensity measurement in the CTCs. The FRET intensity may also be used to predict development of therapy resistance in patients for therapy course correction as well as for treatment with mitophagy targeting agents that are currently under clinical development.

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

Nothing to report

How were the results disseminated to communities of interest?

An abstract has been submitted for presentation in the AACR annual meeting at San Diego in April, 2020 and a manuscript describing the standardization of the new methods and data collected thus far will be submitted for publication in an AACR journal shortly after that meeting.

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

No Applicable

4. IMPACT

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

The FRET method for detecting enzalutamide resistance in prostate cancer CTCs will be translated as one of the clinical biomarkers for development of enzalutamide therapy resistance as proposed. This may be used for selecting patients for treatment with mitophagy inhibitors currently under clinical development. Determination of mitochondrial oxidative stress in the CTCs may also be used to identify patients for treatment with the novel mitochondrial metabolism targeting agents that are also under clinical development.

During this project, the FRET technology standardized in this project has also been extended to patient derived mouse xenograft tissues as well as to live primary prostate cancer cells isolated from patient prostatectomy tissues under a separate NIH grant proposal (R01 CA185251). The contact PI of that multi-PI project Hirak Basu, who is a key personnel in this application will be analyzing patient prostate tissues to elucidate a role of mitochondrial metabolism, organization and metabolism in cell invasion and metastasis and thus, establishing a role of mitophagy in prostate cancer progression and drug resistance.

What was the impact on other disciplines?

We believe these studies will open a new avenue of research in the field of mitochondrial function (or dysfunction) in cancer progression, in general and may lead to application of this technology in the prognosis and prediction of drug resistance in other types of cancers in addition to

prostate cancer. In addition, accurate measurement of mitochondrial function and mitophagy in CTCs could be broadly adopted as a pharmacodynamic (PD) marker for the new mitochondrial metabolism and mitophagy-targeted therapies that are introduced in cancer treatment.

What was the impact on technology transfer?

Mitochondrial function and mitophagy in CTCs will be valuable in mitochondria-targeted drug development for patient selection as well as potential PD marker.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

No significant change of technology and/or goal was necessary.

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

This award experienced an initial delay when the PI Dr. Wilding and a Key Personnel Dr. Basu transferred from the University of Wisconsin to The University of Texas MD Anderson Cancer Center on 09/01/2015 and 03/16/2016, respectively. The award was transferred to MD Anderson and a revised agreement was provided to extend the effective period to 10/31/2019.

There was an initial delay in patient accrual due to change in prostate cancer therapy. Methodology was properly adjusted to account for adopting to the change. Those factors reduced the number of patients anticipated to be accrued early in this study, but we managed to accrue 20 patients by the end of the proposal period of 10/31/2019. The analysis of the data obtained from all accrued patients that was anticipated to be completed by the end of 2019 has now been projected to be complete by March, 2020.

Changes that had a significant impact on expenditures

Nothing to report

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

Nothing to report

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals.

Not applicable. No vertebrate animal work in this project.

Significant changes in use of biohazards and/or select agents

Not applicable. No use of biohazard or select agent in this study.

6. PRODUCTS

Journal publications

One manuscript for publication in Clinical Cancer Research is in preparation.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations

A poster summarizing the some of the data reported above was presented in 2018 AACR Prostate Cancer symposium in Orlando, Fl.

The complete patient CTC analysis data will be presented in the AACR Annual meeting, San Diego, CA, April, 2020. The manuscript will be submitted shortly thereafter.

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

FRET assay using high-resolution confocal fluorescence microscopy for prostate cancer cell and patient CTC has been standardized. The technology has been described in detail in the AACR poster and will be published in a peer-reviewed journal when all the data have been analyzed.

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

The following individuals worked on this project during the grant project period.

The University of Texas MD Anderson Cancer Center

Name:	**George Wilding**
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID ID):	n/a
Nearest Person Months Worked:	1.20 calendar months
Contribution to Project:	Dr. Wilding directed the research and supervised personnel in his laboratory. He was primarily responsible for data analysis and interpretation, troubleshooting, writing and editing all reports, and overall completion of the project.

Name:	**Hirak Basu**
Project Role:	Collaborator
Research Identifier (e.g. ORCID ID):	0000-0001-7733-8008
Nearest Person Months Worked:	Initially, 0 calendar months 0.60 calendar months starting 11/1/2017
Contribution to Project:	Dr. Basu's expertise in autophagy and metabolism was considered invaluable for the success of this project. He assisted the PI in troubleshooting and report writing.

Name:	**James Reuben**
Project Role:	Collaborator
Research Identifier (e.g. ORCID ID):	0000-0001-8972-2103
Nearest Person Months Worked:	0.12 calendar months Effective 7/1/2017
Contribution to Project:	Dr. Reuben collaborated with Dr. Zurita for the last several years in isolating and detecting CTCs in prostate cancer patients under an IRB approved protocol. His laboratory has a standardized protocol for prostate cancer CTC identification and isolation that has been adopted for the studies proposed in this project. He assisted Dr. Wilding with CTC isolation and with identification and troubleshooting and writing reports and publications.

Name:	**Grace T. Wu**
Project Role:	Lab Manager
Research Identifier (e.g. ORCID ID):	n/a
Nearest Person Months Worked:	Ms. Wu's worked 2.40 calendar months in FY17. She worked 5.40 calendar months in FY18. She worked 0.72 calendar months in FY19.
Contribution to Project:	Ms. Wu was responsible for maintenance of cell lines and assisted with cell culture studies in Aim 1 and microscopy in Aim 2. Additionally, she was responsible for the maintenance of laboratory

	supplies and solutions for this project and assisted the PI in ensuring compliance, coordinating data collection, data analysis, and data and financial management for the entire project.

Name:	**Amado Zurita**
Project Role:	Collaborator
Research Identifier (e.g. ORCID ID):	n/a
Nearest Person Months Worked:	0.12 calendar months Effective 11/1/2017
Contribution to Project:	Dr. Zurita and his clinical team were involved in patient identification, consenting and blood sample collection for CTC isolation. His clinical team de-identified samples and stored the link for pro-tected patient information to be used for outcome correlation at the end of the project in compliance with the IRB approved protocol.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No

What other organizations were involved as partners?

Not applicable

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS

Not applicable

QUAD CHARTS

Not applicable

9. APPENDICES

Not applicable