AWARD NUMBER: W81XWH-15-1-0353

TITLE: microRNA Biomarkers to Generate Sensitivity to Abiraterone-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Pheruza Tarapore

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, OH 45267

REPORT DATE: September 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE **September 2017**	**2. REPORT TYPE** Annual	**3. DATES COVERED** 15-AUG-2016 - 14Aug2017

4. TITLE AND SUBTITLE microRNA Biomarkers to Generate Sensitivity to Abiraterone-Resistant Prostate Cancer	**5a. CONTRACT NUMBER**
	5b. GRANT NUMBER W81XWH-15-1-0353
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Pheruza Tarapore, Sarah To, Dan Song, Shuk-mei Ho E-Mail: Pheruza.Tarapore@uc.edu	**5d. PROJECT NUMBER**
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Cincinnati 2600 CLIFTON AVE CINCINNATI OH 45220-2872	**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	**10. SPONSOR/MONITOR'S ACRONYM(S)**
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We plan to develop a combination therapeutic approach, employing Abiraterone (Abi) plus RNA therapy. For this, we will use an aptamer specific for PSMA (aptPSMA) to specifically target CRPC cells. The affinity and high specificity of aptPSMA for binding human CRPC cells expressing PSMA has already been reported, as has its utility as a drug delivery system for siRNAs. However, it has not been used to deliver pre-miRNA to cells. Identification of Abi-R markers is important for designing therapeutic interventions sensitizing PCas to combination therapies and for prognostic applications to monitor and predict for disease relapse (Abi-R). Additionally, we propose to use patient derived PCa xenograft animal model (PCa-PDX mice) to identify differentially expressed microRNA (miRNA) on castration and Abi dependent tumor regression followed by regrowth/relapse. *Our central hypothesis is that changes in miRNA expression underlie Abi-R mechanisms and that PCa-PDX mice will be excellent surrogates to identify markers for Abi-R. We further postulate that RNA therapy (restoring or targeting miRNA) should increase sensitivity of Abi-R tumors, allowing us to prolong treatments, and hence the life of a patient.* Our Aims: (1) To develop RNA aptamer therapy. We will test 8 of the recently identified Abi regulated miRNAs for therapeutic utility in vitro. We will design an aptPSMA-pre-miRNA therapeutic delivery vehicle for CRPC-tissue specific delivery. The best miRNA will be used for in vivo studies (2) To generate Abi-R PDX mice and identify the differentially expressed miRNA.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT UU**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT Unclassified	**b. ABSTRACT** Unclassified	**c. THIS PAGE** Unclassified	Unclassified	18	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. INTRODUCTION:

Prostate cancer (**PCa**) is the most frequent cancer occurring in men in the United States. While screening for elevated levels of prostate-specific antigen (PSA) has dramatically improved early detection of this disease (Cooperberg et al., 2004, Etzioni et al., 2002), PCa is still the second leading cause of cancer deaths in men. In 2014, it is estimated that 233,000 men will be diagnosed with and 29,480 men will die of cancer of the prostate (American Cancer Society, 2013). Of concern, a substantial proportion of patients develop an incurable disseminated disease after local therapy, even when the primary lesion was localized to the prostate when first diagnosed (Pendleton et al., 2007). Also, a large number of advanced PCas become androgen-independent, for which there is no known cure. Indeed, median survival for patients with metastatic hormone refractory PCa is still around 18 months (Hadaschik et al., 2007, Hadaschik and Gleave, 2007). Thus, there is an urgent need to come up with new treatment regimens for these patients.

In this proposal, we sought to develop a combination therapeutic approach to treat PCa, employing Abiraterone plus RNA therapy, as a promising methodological approach. A candidate cell surface receptor is PSMA (prostate specific membrane antigen). PSMA expression is associated with higher Gleason scores in tumors, and with increased aneuploidy. It is currently used as a tumor marker for diagnosis, monitoring and prognosis of prostatic carcinoma. Elevated levels of PSMA is also used as an independent marker for predicting disease relapse. We will use an RNA aptamer which binds specifically to PCa cells to deliver the miRNA. **miRNA** have an advantage over siRNA for gene silencing since they can target multiple components of the cellular networks / signaling pathways responsible for advanced disease progression.

We had identified miRNA induced by Abi treatment of castration resistant prostate cancer C4-2 cells using TaqMan Array Human MicroRNA Card Set v3.0 (Life Technologies). The top 7 candidates are shown in **Table 1**. From our literature search and data mining to determine known and potential gene targets for miRNA, we determined that most of these miRNA appear to be tumor suppressors (**Table 1**), inhibiting the expression of oncogenes, pro-angiogenic factors, or genes involved in cell proliferation, migration, or metastasis. The expression pattern of *miR-487a-3p, miR-492-5p, miR-510-5p and miR-623-5p especially suggest that these miRNAs maybe involved in Abi-R* (**Table1**, contrast the gene function with expression levels). <u>*Using mimics and inhibitors for each miRNA, we will determine which of the identified miRNA are best suited for further studies.*</u>

Since Abi targets androgen biosynthesis in the adrenals, testis, as well as has direct effects on the tumor tissues, an *in vitro* approach to identify the biomarkers of resistance to Abi can be imprecise. Our results and others (Kosaka et al., 2014) have shown that while Abi inhibits CYP17A1 activities and expression in C4-2 cells, it is not very effective in reducing cell proliferation *in vitro* at physiological levels of <14 µM. However, it is most effective at suppressing cancer properties *in vivo* in mouse xenograft models (Bruno et al., 2011, Mostaghel et al., 2011) and in patients with CRPC (Mostaghel, 2014), indicating that system-wide androgen suppression and inhibition of AR axis is important. **Patient-derived xenograft (PDX)** tumor models retain much of the biological diversity, heterogeneity, molecular characteristics and tissue architecture of the original patient tumor. Recent advances in the development of these models and their increasing sophistication have led to their escalating use for anticancer drug research and development, and as predictive clinical models. *Hence, for the correct identification of Abi-R* *markers, we propose using PCa-PDX animal model (PDX mice, Jackson Labs).* The identification of abi-R markers is important for designing therapeutic interventions sensitizing PCas to combination PCa therapies and for prognostic applications to monitor and predict for disease relapse (Abi-R).

Hypothesis: *We hypothesize that changes in miRNA expression underlie Abi resistance (Abi-R) mechanisms. We further postulate that a RNA therapy (restoring or targeting miRNA) should increase sensitivity of Abi-R tumors (Fig. 2), allowing us to prolong treatments (Table 2), and hence the life of a patient.*

2. KEYWORDS: RNA aptamer, PSMA, microRNA

3. ACCOMPLISHMENTS:

Table 1. Summary of miRNAseq data on Abi-treated C4-2 Cells

	Fold change	Functions	Target genes involved
Possible involvement in Abi-induced tumor regression			
hsa-miR-573-5p	4.8	Tumor suppressor	*Known Target:* Genes involved in cell adhesion (MCAM/ CD146/muc18). *Potential Targets :* SPARCL1, SLC25A26
hsa-miR-202-3p	4.2	Tumor suppressor	*Known Target:* Genes involved in tumor progression, epithelial–mesenchymal transition (EMT), bone metastasis (Gli2, LRP6, CALD1, GHR) *Potential Targets:* ARID3B, DICER1, MYCN, PPARGC1B, HMGA2
hsa-miR-1262-5p	1.5	?? Tumor suppressor	*Potential Targets : mRNA splicing* GEMIN5, ECM/proliferation: FGFR1, OCM2, ITGAV, MYB
Possible involvement in Abi-Resistance			
hsa-miR-487a-3p	0.45	Tumor suppressor	*Known Target:* Genes involved in mitoxantrone (MX) resistance (BCRP/ABCG2). *Potential targets:* SP1, SP3 which *upregulate PSA, AR, alpha integrin.*
hsa-miR-492-5p	0.65	Anti-angiogenic	*Known Target:* Genes involved in angiogenesis (restin, MCL1, DUSP3, BRAF, MAP3K1, MMP10, SP1) *Potential targets : ECM remodelling/metastasis* CCBE1, CD44, CLDN19
hsa-miR-510-5p	5.4	Oncomir	*Known Target:* Genes involved in inhibition of cancer progression/invasion PRDX1, prostate-derived Ets factor PDEF *Potential Targets :* SPOCK1, *RNA export* THOC2
hsa-miR-623-5p	0.03	?? Tumor suppressor	*Potential Targets: oncogene* SKI, cell cycle regulator CCND2

Specific Aims:

1. **To develop RNA aptamer therapy.** We will test 7 of the recently identified Abi regulated miRNAs (Table 1) for therapeutic utility *in vitro*. The best miRNA will be used for *in vivo* studies for inducing sensitivity to Abi, using the C4-2 and /or 22Rv1 CRPC cells which express PSMA. We will also identify (for miRNA), their downstream targets and pathways in CRPC cells to gain insights regarding the mechanism of resistance.

Task 1. Determine which miRNA (amongst miRNA miR202-3p, miR-487a-3p, miR-492-5p, miR-510-5p, miR-554-5p, miR-573-5p, miR-623-5p, miR-1262-5p) is best suited for PCa therapy. (months 1-9).

We had identified miRNA induced by Abi treatment of C4-2 cells using TaqMan Array Human MicroRNA Card Set v3.0 (Life Technologies). We first validated that expression of these miRNA was significantly changed by qPCR (**Figure 1**). We found that the expression of 4 of the miRNA changed; miR202-3p and miR-510-5p increased in expression and expression of miR-487a-3p and miR-623-5p decreased. We could not get good signals for miR-492-5p to quantitate it.

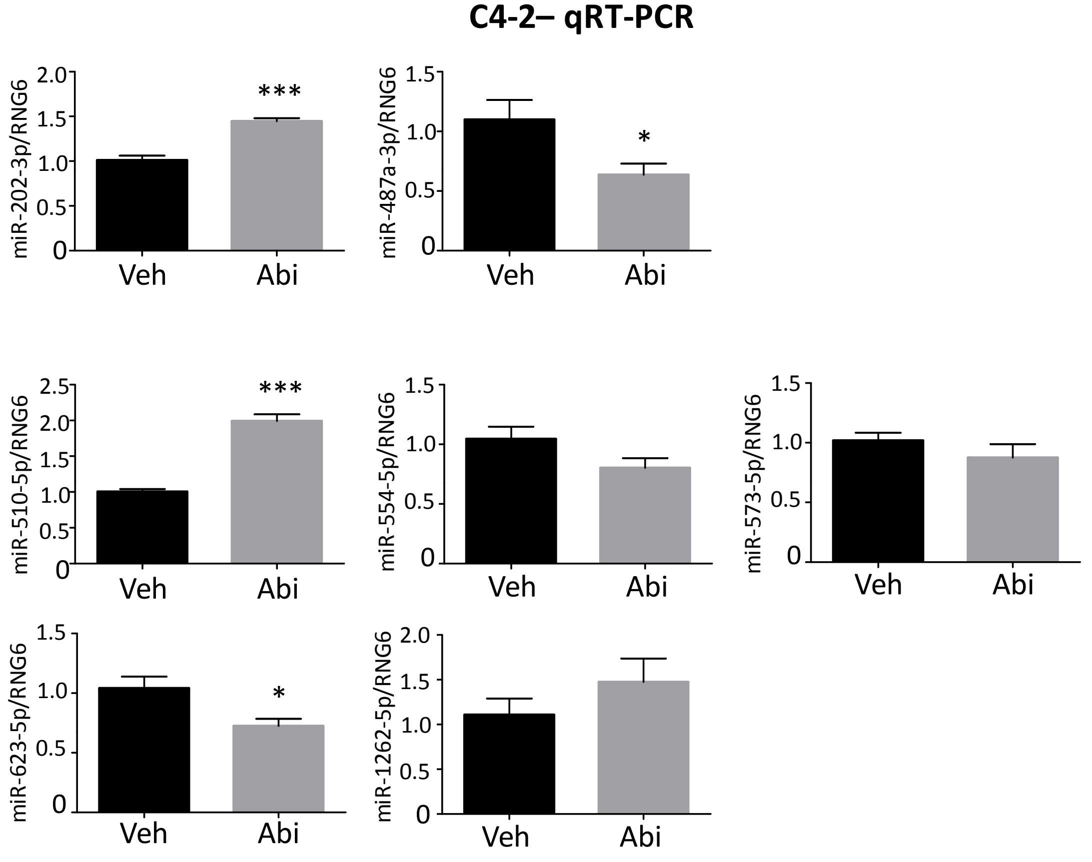

Figure 1. miR202-3p and miR-510-5p increased in expression and expression of miR-487a-3p and miR-623-5p decreased. C4-2 cells were treated with 10 µM Abiraterone for 48 h. miRNA isolated using RNAzol differential isolation method. RNA was reverse transcribed into cDNA using the NCode kit. Experiments were done in triplicate, three times. *$p<0.05$, **$p<0.01$, *$p<0.005$ by unpaired ttest.

At the same time, we ordered the miRNA mimics and inhibitors. These were transfected into two castration resistant prostate cancer (CRPC) celllines, C4-2 and 22rv1.

(1) We found that while transfection of miR202-3p, miR-573-5p and miR-623-5p into C4-2 cells inhibited cell growth (MTS assay, (**Figure 2**)), the same was not true for 22rv1 cells. While significance was not reached, miR-623-5p did decrease cell viability in 22rv1 cells.

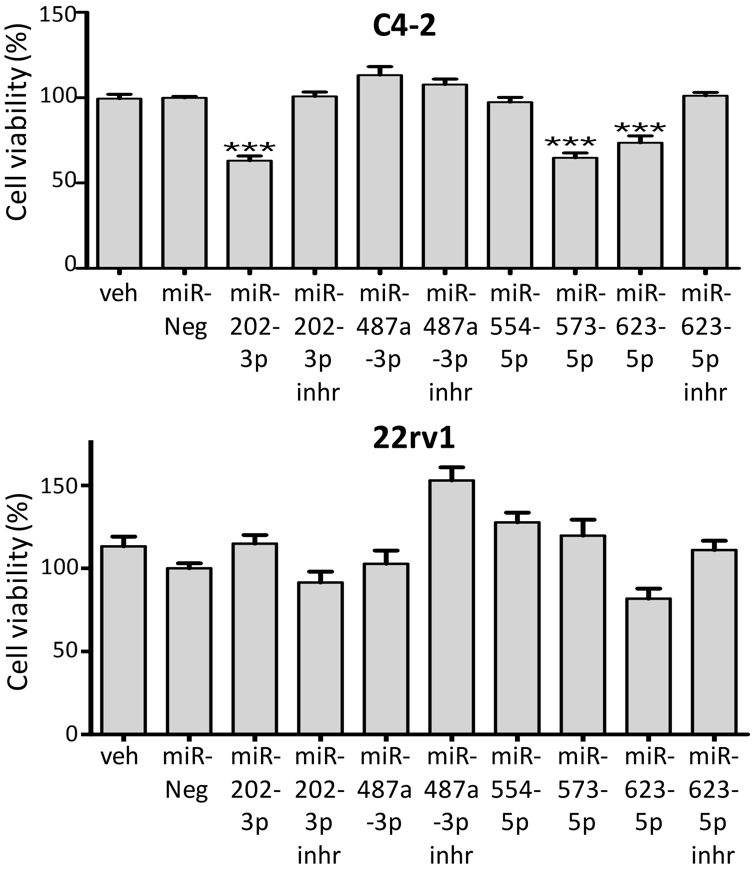

Figure 2. Transfection of miR202-3p, miR-573-5p and miR-623-5p into C4-2 cells inhibited cell viability or numbers significantly. miRNA mimics and inhibitors were transfected into C4-2 and 22rv1 cells. Cell viability was determined using the MTS assay (Promega) after 5 days. One-way ANOVA *p<0.05, **p<0.01, *p<0.005

(2) miR202-3p, miR-487a-3p, miR554-5p and miR-573-5p inhibit cell migration in C4-2 cells (scratch wound assay) but not in 22rv1 cells (**Figure 3**).

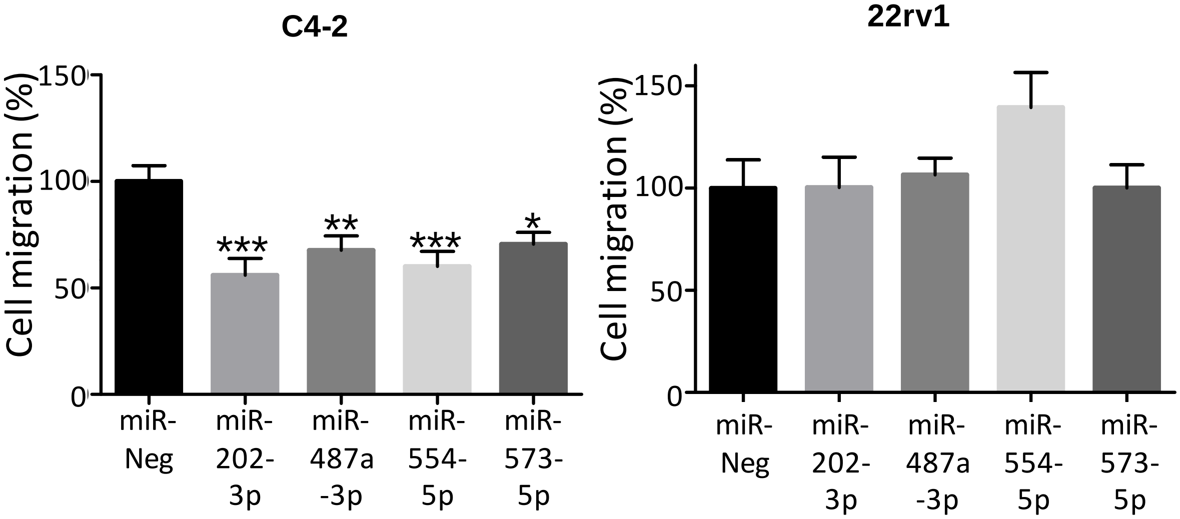

Figure 3. miR202-3p, miR-487a-3p, miR554-5p and miR-573-5p inhibit cell migration in C4-2 cells. miRNA mimics were transfected into C4-2 and 22rv1 cells. Scratch wound assay was performed to determine % migration compared to miR-Neg transfected cells at 48 hs post transfection. One-way ANOVA $*p<0.05$, $**p<0.01$, $*p<0.005$

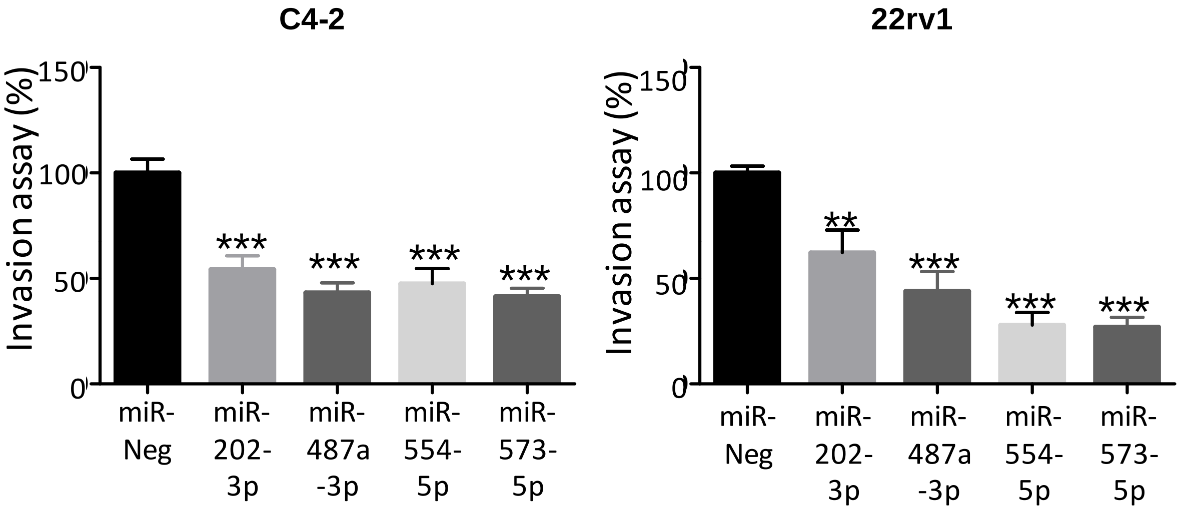

Figure 4. miR202-3p, miR-487a-3p, miR554-5p and miR-573-5p inhibit invasion potential in C4-2 and 22rv1 cells. miRNA mimics were transfected into C4-2 and 22rv1 cells. Boyden chamber invasion through matrigel was performed to determine % invasion (after the invasion matrix was fixed and stained with crystal violet, dissolved in alcohol and OD determined. Results were compared to miR-Neg transfected cells at 48 hs post transfection. One-way ANOVA $*p<0.05$, $**p<0.01$, $*p<0.005$

(3) However, when we examined the invasive potential of these miRNA transfected cells, we find that four miRNA (miR202-3p, miR-487a-3p, miR554-5p and miR-573-5p) decreased the invasive potential as determined using the Boyden chamber matrix assay (**Figure 4**).

Since miR202-3p, miR-573-5p and miR623-5p had effects on cell viability, we proceed with these 3 miRNA

(4) **Next, we treated cells with miR-Neg, miR623, miR554 and miR-510 (we had not tested miR510 before) and determined the cell viability in presence and absence of Abi (10 µM). We found that miR-510 was the only miRNA which consistently imparted sensitivity to abiraterone.**

Hence, we will use this miRNA-510, in addition to miR623-5p. These two miRNA gave the most consistent results for cell viability assay.

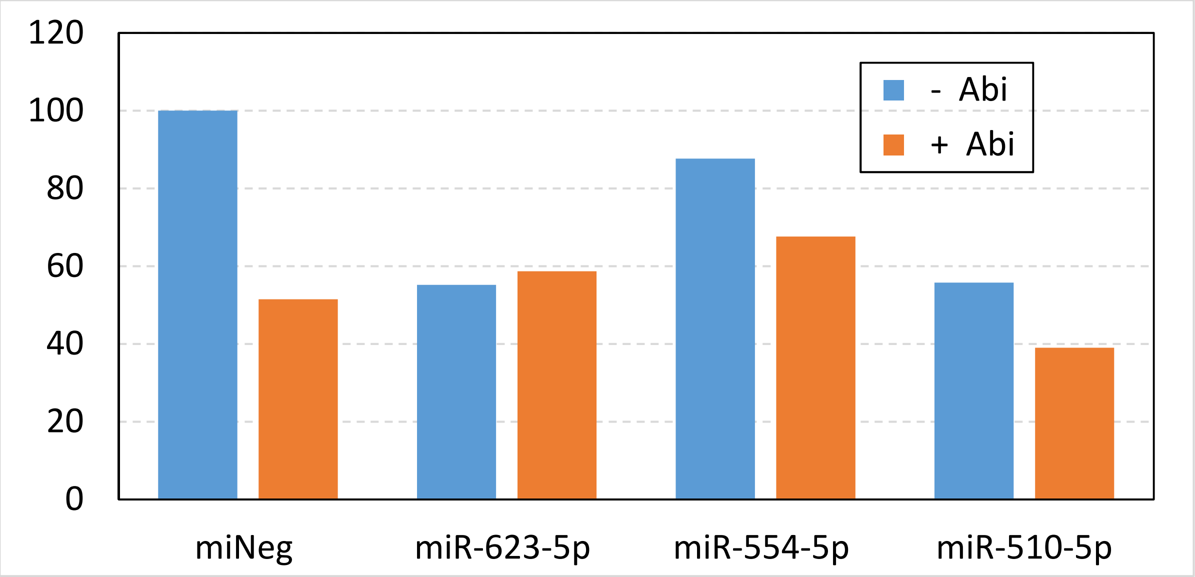

Figure 5. Transfection of miR-623 and miR-510 into C4-2 cells inhibited cell viability or numbers in absence of Abi (blue bars), however, only miR-510 imparted sensitivity to abiraterone (compare orange bar miR-neg and miR-510). miRNA mimics were transfected into C4-2. Cell viability was determined using the MTS assay (Promega) after 5 days.

Task 3. To identify function of miRNA: Determining the network of miRNA-regulated genes (Months 1-12)

This part is currently ongoing. We have transfected C4-2 and PC-3 cells with Ago expressing construct. However, our transfection was not very successful. We are now making stable clones, so that we can express enough Ago protein to proceed. At the same time we are re-cloning to change the vector of the pCMV-Ago construct for puromycin selection, and to a lower expressing promoter, to determine whether the expression was too toxic to cells.

Task 4. Design functionally active aptamer-pre-miRNA and aptamer-mature-miRNA chimeric molecules (aptPSMA-iRNA) (Months 7-12).

(1) We have designed and made the T7 transcribed aptPSMA-24mer, aptScrab-24mer, miR-623-5p-24merReverse and miR-510-5p-24merReverse. They have been annealed to give PSMA-Aptamer-iMT (aptPSMA-iRNA, **Figure 6**). Further testing for correct processing, resistance to degradation, and cleavage activity will be done.

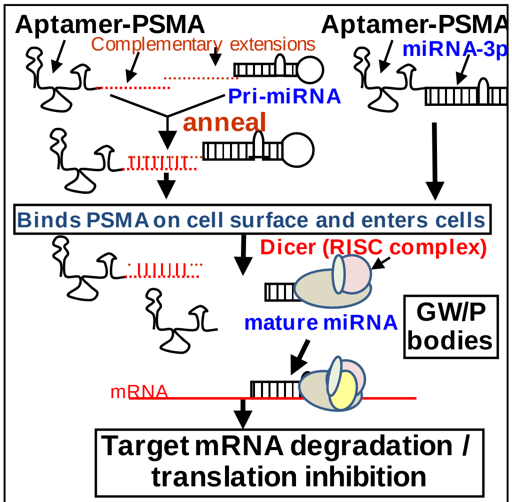

Figure 6. Schematic representation of the miRNA and aptPMSA annealing.

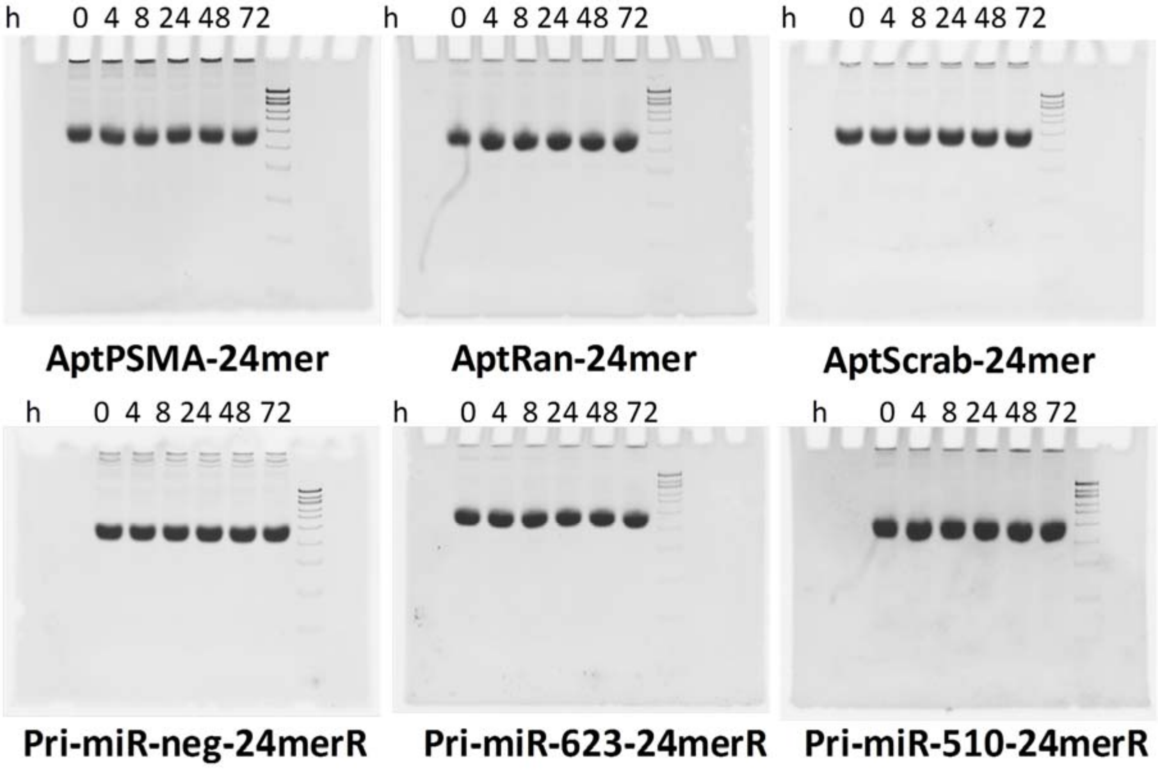

Figure 7. The aptamer-24 mer and miRNA-24merReverse **(R)** were tested for resistance to degradation in presence of Rat serum for indicated times.

(2) Test the aptamer-24 mer and miRNA-24merReverse **(R)** for correct processing for resistance to degradation. The individual chimeras above were incubated with rat serum

for 4h, 8h, 24h, 48h and 72hs. The constructs were analysed on a non-denaturing gel and sizes compared. See **Figure 7.** The aptamers and miRNA are stable for 72 hs.

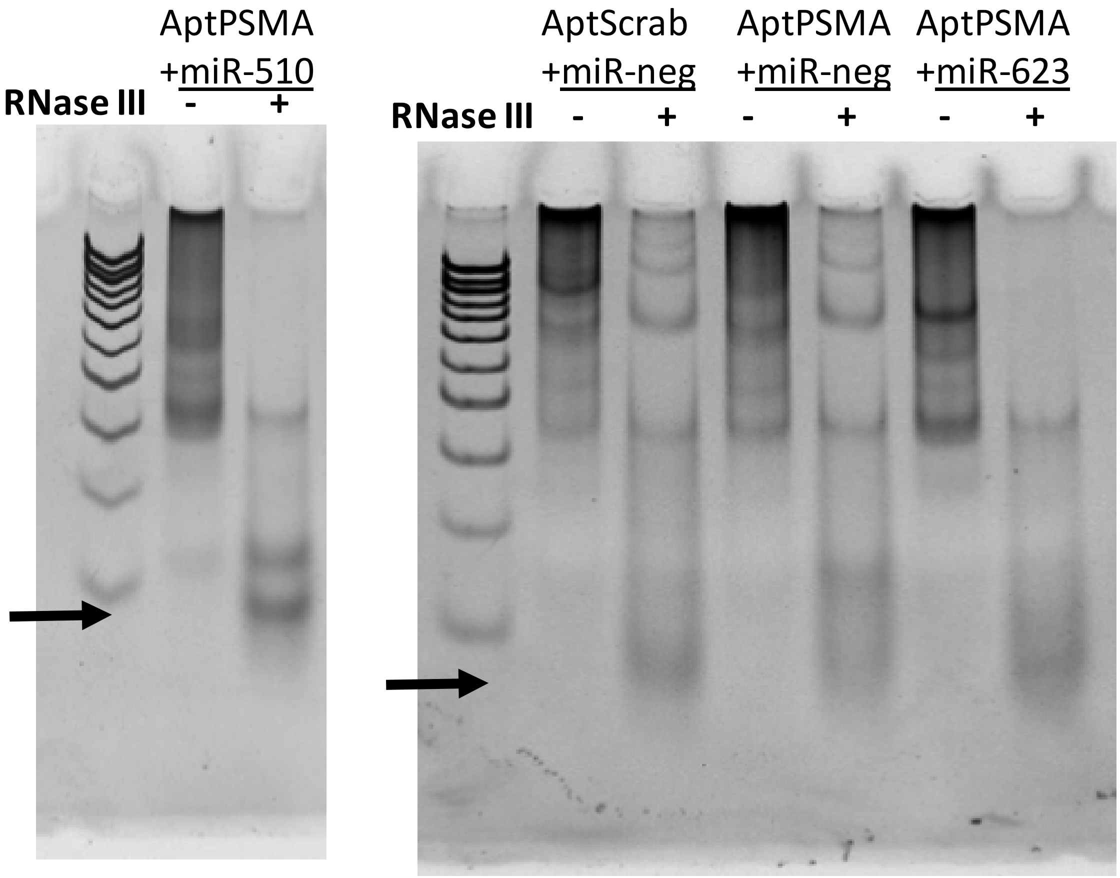

Figure 8. The annealed chimeras aptPSMA-miRNA as labeled were tested for correct RN'ase III processing. The processing with dicer gave the correct sized fragments (arrow).

(3) Test the aptamer-miRNA for correct dicer processing.

The miRNA-24merReverse and Aptamer-24 mer were annealed as indicated. The annealed aptamer-miRNA were digested with dicer (RN'ase III). See **figure 8**. The processing with dicer gave the correct sized fragments (arrow).

(4) The 2.5 pmole of aptamer-miRNA were tested for their ability to inhibit cell viability of C4-2 cells on treatments. aptPSMA-miR-623 significantly inhibited cell growth. While the results are promising, we need to repeat this experiment with increases in the concentration of chimeras, and perform this experiment in presence and absence of Abi (**Figure 9**).

We are getting ready, so that in the next year we will further test these chimeras in vitro and in vivo using the xenograft model. We are making enough aptamer-24mer and miRNA-24merR constructs so we can perform these studies in animals.

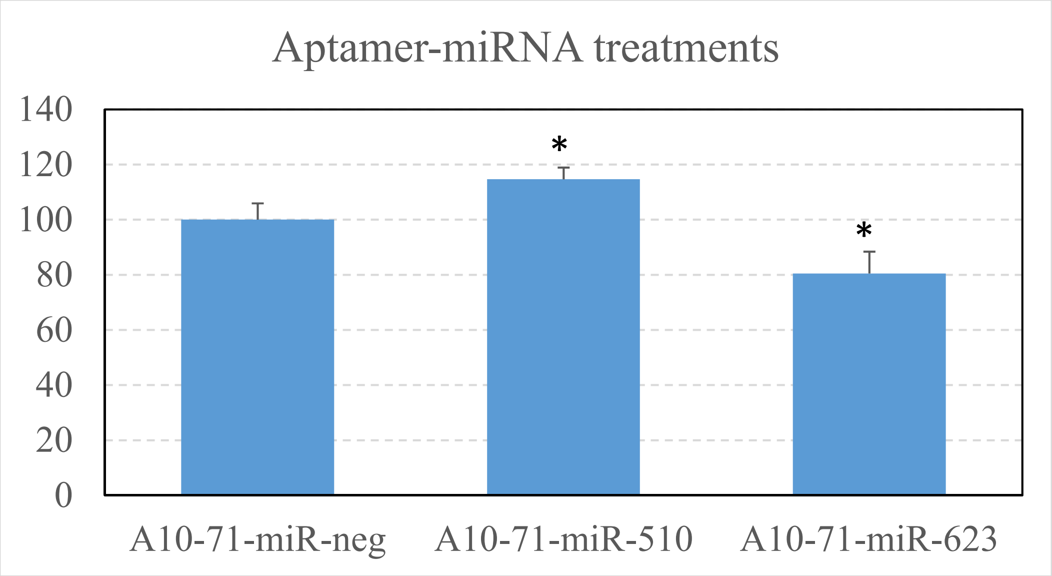

Figure 9. The C4-2 cells were treated with annealed chimeras aptamer-miRNA as indicated for 5 days and tested by MTT assay. AptPSMA (A10-71)-miRNA623 significantly decreased viability of C4-2 cells compared to AptPSMA (A10-71)-miR-neg

Specific Aim 2: To generate Abi-resistant (Abi-R) PCa Patient-derived xenograft (PCa-PDX) mice and identify the differentially expressed miRNA.

Task 2. Order and castrate the 32 patient derived engrafted (32 PCa-PDX mice - Jackson labs) male mice

The PDX-mice was ordered and the PDX tumor allowed to grow. We are now expanding the original tumor (**Figure 10**), in order to perform the castration and Abiraterone treatment study (**Figure 10**). We have the protocol in place at the DoD. Due to distribution of work resulting in personnel issues, we had a delay in initiating this part, but are now on track and proceeding with this part of the specific aim.

Plan for the next reporting period: We have high hope that we will be able to catch up with all our studies as outlined in the SOW and attain our goals in the near future. During the next reporting period, we will test out our aptamer-miRNA constructs in 22rv1 cells in presence and absence of Abi for cell viability, motility and invasiveness. We will then use the xenograft tumor model to perform in vivo studies in mice. We will also start with the second aim where we expand the PDX-PCa to required number of mice, and generate castration resistant/abiraterone resistant tumors in PDX-PCa mice.

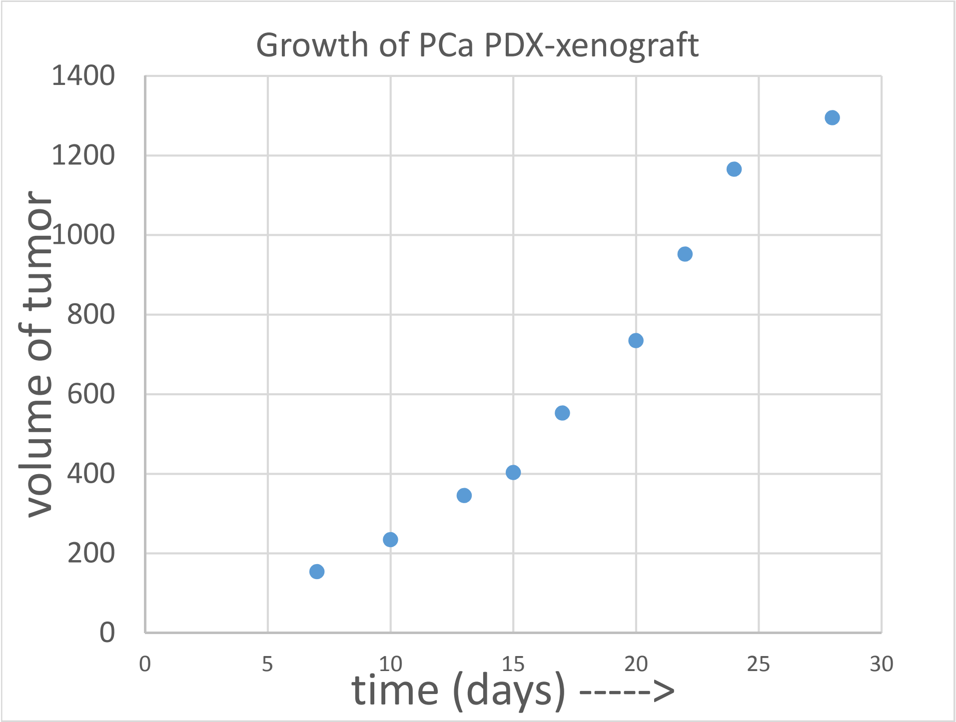

Figure 10. Growth curve for the F2 PCa-PDX tumor being used for our study.

4. IMPACT

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

6. PRODUCTS:

5 papers were published during this reporting period. However, they are not specifically related to this study.

1. **Tarapore P****, Hennessy M, Ouyang B, Leung YK, Janakiram V, Song D, Rao R, and **Ho SM****. High butter-fat diet and bisphenol A additively impair male rat spermatogenesis. (**corresponding authors) Reprod.Toxicol. 2017; 68:191-199. dx.doi.org/10.1016/j.reprotox.2016.09.008. PM:27658648. Impact Factor: 3.4.
2. Zhou Z, Kennell C, Lee JY, Leung YK, and **Tarapore P**. Calcium Phosphate-Polymer Hybrid Nanoparticles for Enhanced Triple Negative Breast Cancer Treatment via Co-Delivery of Paclitaxel and miR-221/222 Inhibitors. Nanomedicine. 2017; 13(2):403-410. PM:27520723. DOI: 10.1016/j.nano.2016.07.016. Impact Factor: 5.7.

3. Smithrud D, Powers L, Lunn J, Abernathy S, Peschka M, Ho SM, **Tarapore, P**. Ca2+ selective host Rotaxane is highly toxic against prostate cancer cells. ACS Medicinal Chemistry Letters. 2017; 8(2): 163-167. PM: 28197305. dx.doi.org/10.1021/acsmedchemlett.6b00347.
4. Ho SM, Rao RC, To S, Schoch E, **Tarapore P**. Bisphenol A and its analogues disrupt centrosome cycle and microtubule dynamics in prostate cancer. Endocr Relat Cancer 2017; 24(2): 83-96. doi:10.1530/ERC-16-0175; PM: 27998958. Impact Factor: 4.8.
5. **Tarapore P****, Hennessy M, Ouyang B, Leung YK, Janakiram V, Song D, Rao R, and **Ho SM****. Data on spermatogenesis in rat males gestationally exposed to bisphenol A and high fat diets. (**corresponding authors) Data Brief. 2016; 9:812-817. PMC5107686. PM:27872877.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Pheruza Tarapore
Project Role: PI
Researcher Identifier:
Nearest person month worked: 12
Contribution to project: Worked on designing the cell culture experiments, designed primers and templates for making the aptamer-miRNA constructs, did experiments related to these constructs.
Funding Support: This award and NIH grants

Name: Shuk-mei Ho
Project Role: co-I
Researcher Identifier:
Nearest person month worked: 1
Contribution to project: Helped in overall direction and overview of grant, generating reports, some proceedures.
Funding Support: This award and NIH grants

Name: Sarah To
Project Role: Post-doctoral researcher
Researcher Identifier:
Nearest person month worked: 1
Contribution to project: Performed the cell culture based experiments with microRNA mimetics and inhibitors.
Funding Support: National Health and Medical Research Council (GNT1070112 (ST)), Australia.

Name: Dan Song
Project Role: Technician
Researcher Identifier:
Nearest person month worked: 4
Contribution to project: Assisted in writing the animal protocol for DoD application. Has been getting materials ready for doing the PDX model
Funding Support: This award and NIH grants

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES:

Bibliography and References Cited

1. Cooperberg MR, Lubeck DP, Meng MV, Mehta SS, Carroll PR. The changing face of low-risk prostate cancer: trends in clinical presentation and primary management. J Clin Oncol. 2004; 22(11): 2141-9. PMC2997214. http://www.ncbi.nlm.nih.gov/pubmed/15169800.

2. Etzioni R, Berry KM, Legler JM, Shaw P. Prostate-specific antigen testing in black and white men: an analysis of medicare claims from 1991-1998. Urology. 2002; 59(2): 251-5. http://www.ncbi.nlm.nih.gov/pubmed/11834397.

3. American Cancer Society. Cancer Facts & Figures. Atlanta: American Cancer Society 2013; http://www.cancer.org/research/cancerfactsstatistics/allcancerfactsfigures/index

4. Pendleton J, Pisters LL, Nakamura K, Anai S, Rosser CJ. Neoadjuvant therapy before radical prostatectomy: where have we been? Where are we going? Urol.Oncol. 2007; 25(1): 11-8. http://www.ncbi.nlm.nih.gov/pubmed/17208133.

5. Hadaschik BA, Sowery RD, Gleave ME. Novel targets and approaches in advanced prostate cancer. Curr.Opin.Urol. 2007; 17(3): 182-7. http://www.ncbi.nlm.nih.gov/pubmed/17414516.

6. Hadaschik BA, Gleave ME. Therapeutic options for hormone-refractory prostate cancer in 2007. Urol.Oncol. 2007; 25(5): 413-9. http://www.ncbi.nlm.nih.gov/pubmed/17826663.

7. Kosaka T, Miyajima A, Yasumizu Y, Miyazaki Y, Kikuchi E, Oya M. Limited in vitro efficacy of CYP17A1 inhibition on human castration resistant prostate cancer. Steroids 2014; http://www.ncbi.nlm.nih.gov/pubmed/25150014. S0039-128X(14)00208-6;10.1016/j.steroids.2014.07.017

8. Bruno RD, Vasaitis TS, Gediya LK, Purushottamachar P, Godbole AM, Ates-Alagoz Z, Brodie AM, Njar VC. Synthesis and biological evaluations of putative metabolically stable analogs of VN/124-1 (TOK-001): head to head anti-tumor efficacy evaluation of VN/124-1 (TOK-001) and abiraterone in LAPC-4 human prostate cancer xenograft model. Steroids 2011; 76(12): 1268-79. PMC3171567. http://www.ncbi.nlm.nih.gov/pubmed/21729712. S0039-128X(11)00196-6;10.1016/j.steroids.2011.06.002

9. Mostaghel EA, Marck BT, Plymate SR, Vessella RL, Balk S, Matsumoto AM, Nelson PS, Montgomery RB. Resistance to CYP17A1 inhibition with abiraterone in castration-resistant prostate cancer: induction of steroidogenesis and androgen receptor splice variants. Clin.Cancer Res. 2011; 17(18): 5913-25. PMC3184252.

http://www.ncbi.nlm.nih.gov/pubmed/21807635. 1078-0432.CCR-11-0728;10.1158/1078-0432.CCR-11-0728

10. Mostaghel EA. Abiraterone in the treatment of metastatic castration-resistant prostate cancer. Cancer Manag.Res. 2014; 6: 39-51. PMC3912049. http://www.ncbi.nlm.nih.gov/pubmed/24501545. 10.2147/CMAR.S39318;cmar-6-039