AWARD NUMBER: W81XWH-20-1-0338

TITLE: Role of AR-derived Circular RNA in Prostate Cancer

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CONTRACTING ORGANIZATION: Johns Hopkins University, Baltimore, MD

REPORT DATE: June 2021

TYPE OF REPORT: Annual Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2021	Annual	15May2020-14May2021

4. TITLE AND SUBTITLE
Role of AR-derived circular RNA in prostate cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-20-1-0338

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development 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

The androgen receptor is a key therapeutic target in prostate cancer. Multiple androgen receptor alterations are known to affect prostate cancer progression and treatment efficacy. In this proposal, we will focus on a novel form of non-coding circular RNA originated from the androgen receptor gene. We will test the hypothesis that AR-derived, non-coding circular RNAs (circARs) can act as competitive endogenous RNAs through sponging micro RNA (miRNA), or RNA-binding proteins to regulate prostate cancer progression. To this end, we proposed three Specific Aims. Aim 1 will identify and validate circARs in castration resistance prostate cancer (CRPC). Aim 2 will define the functional roles of circARs in CRPC. Aim 3 will determine the regulatory factors involved in circAR generation. During Year 1 of the funding period, we have successfully initiated the study in spite of limitations and challenges posed by the pandemic. All regulatory documents are now in compliance with the latest regulations. We established and validated the methodology to enrich AR transcripts for identifying circular ARs by RNA-seq in prostate cancer cell lines. Although we have not generated definitive results, with expansion of the established method to prostate cancer patient tissues, we expect to report main in the next two years of the project period.

15. SUBJECT TERMS
Prostate cancer, androgen receptor, circular RNA, circular AR

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	9	

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1. **INTRODUCTION:**

In this project, we will test the hypothesis that AR-derived, non-coding circular RNAs (circARs) can act as competitive endogenous RNAs through sponging micro RNA (miRNA), or RNA-binding proteins to regulate prostate cancer progression. Deregulation of AR signaling by different mechanisms contributes to the development of castration resistance prostate cancer (CRPC), a lethal disease for which effective therapeutic approaches and biomarkers are urgently needed. Given the critical role of AR, the project will focus on a novel layer of *AR* gene regulation that may lead to new targets for the development of novel treatments and biomarkers. Circular RNA (circRNA) is a novel type of non-coding RNA implicated in prostate cancer. However, there is a gap of knowledge in relation to the potential role of circRNA. To test our central hypothesis, we will conduct an exploratory study to profile circARs and define their functions in prostate cancer. First, we will identify circARs in prostate cancer cell lines and clinical specimens. We will then conduct functional studies to determine the roles of circARs in CRPC. Finally, we will determine the regulatory factors involved in circAR generation.

2. **KEYWORDS:**

Prostate cancer, castration resistant prostate cancer, androgen receptor, circular RNA, circular AR

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Major Task 1: Establish AR-targeted RNA-seq pipeline in profiling circARs and identify major circARs in PCa

Subtask 1: To conduct essential project planning activities including obtaining Animal Care and Use Review Office (ACURO) and Human Research Protection Office (HRPO) approvals (months 1-4). Completed.

Subtask 2: To profile circARs in PCa cell lines and tumor tissues by AR-targeted RNA-seq: To design AR probe panel and optimize capture-based targeted RNA-seq in LNCaP and LNCaP95 cell lines (5-12 mths). Ongoing (70%).

Subtask 3: To confirm and to determine circAR expression in different PCa tissues (5-12 mths). Ongoing (50%).

Major Task 2: Define the role of circARs in PCa cell survival and identify interacting miRNAs

Subtask 1: To investigate the role of circARs in promoting cancer cell proliferation, migration, invasion, and their effect on current therapeutic responses (13-18 mths). Ongoing (5%).

Subtask 2: To identify non-coding RNAs interacting with circARs (13-18 mths). Yet to start.

Major Task 3: Identify genes and microRNAs regulated by circARs in PCa cells

Subtask 1: To explore the effect of circARs on whole transcriptome, especially the AR signaling (18-24 mths). Yet to start.

Major Task 4: Determine the circAR decay time vs linear AR transcripts and identify regulatory factors including cis-elements and RNA binding protein involved in circAR formation.

Subtask 1: To explore the correlation of linear AR transcripts and circARs (25-30 mths). Yet to start.
Subtask 2: To identify cis-element in modulating circular AR formation by gene editing (25-30 mths). Yet to start.
Subtask 3: To explore the RNA binding protein (RBP) in assisting circAR generation in PCa (31-36 mths). Yet to start.

- **What was accomplished under these goals?**

1) Major activities: during Year 1 of the project period, major activities included essential study planning and organization activities including IRB/HRPO approval, ACURO approval, ordering of reagents, equipment readiness, protocol review, SOP review. We have initiated cell line studies on circular AR identification by next generation sequencing and proceeded to patient prostate cancer tissues.
2) Specific objective: we have two specific objectives for this period. First objective was to establish protocol for AR-targeted RNA-seq to profile circARs in PCa cell line. Second objective was to profile circARs in variable PCa cell lines and tumor tissues using the established RNA-seq method.
3) Significant results or key outcomes:
   (A) All regulatory documents were filed and approved by IRB/HRPO and ACURO.
   (B) Although reportable results are not available for the reasons stated, we have made essential preparations and conducted studies necessary for the specific tasks outlined for Year 1 of the project period. To test cell lines for circAR detection, total RNA from 5 PCa cell lines and one normal prostate epithelial cell line were prepared. Among these cell lines, LNCaP (LN), LNCaP95 (LN95), and CWR22Rv1 expresses higher levels of AR wild type (Fig. 1A) and PSA (Fig. 1B) compared to normal prostate epithelial cell RPWE-1, while the expressions of AR and PSA in DU145 and PC3 cells were similar to those measured in RPWE-1 cells.

Figure 1. Prostate cancer (PCa) cell lines used for detecting circARs. PCa cell lines included in circAR search were: androgen sensitive cells: LNCaP, androgen-resistant cells: LN95 and CWR22Rv1, PCa cells with low or no AR expression: DU145 and PC3, and normal prostate epithelial cell line: RPWE-1. By qRT-PCR, expression levels of AR-FL and PSA were measured, and the relative expression level of each cell lines was normalized to RPL13A gene expression and then compared to RPWE-1 cell.

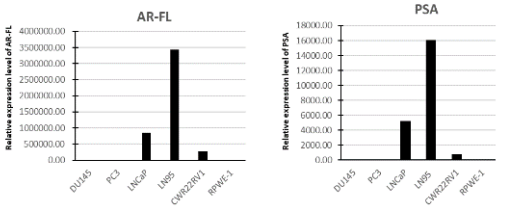
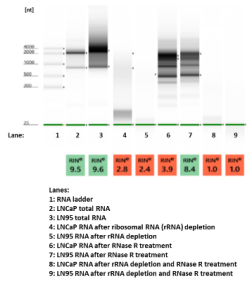
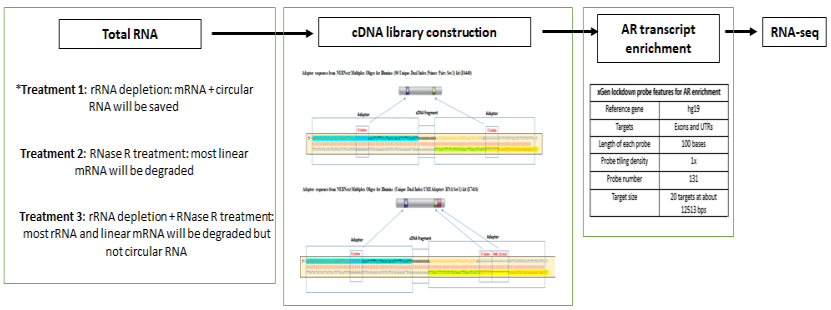


Figure 2. Quality analysis of RNA after different treatments by Agilent TapeStation system



(C) During this period, we optimized the construction of AR transcript-enriched cDNA library for RNA-seq. We tested two approaches of circAR enrichment. In the first method, we used RNase R to degrade linear RNAs, such as mRNA, lncRNA, and ribosomal RNA (rRNA). In the second method, we removed rRNA first and then usedAR gene-specific probes to enrich AR transcripts. In addition, we tested three RNA treatment methods: rRNA depletion, RNase R treatment, and rRNA depletion + RNase R treatment (Figure 2 & 3). We conducted quality analysis on Agilent Tapestation system, and found that with RNase R treatment alone (lanes 6 & 7, Fig. 2), the rRNA was not removed efficiently from total RNA, while higher efficiency was achieved in samples that underwent rRNA depletion step (lanes 4 & 5, 8 & 9, Fig. 2). We subsequently designed a DNA probe panel targeting AR exons and UTR (Fig. 3). AR transcripts including circAR were successfully enriched in cDNA library.

Figure 3. Different conditions tested in cDNA library prep for RNA-seq in circAR detection. LNCaP and LN95 total RNA were undergone different treatment followed by Illumina cDNA library prep with dual index/UMI adapters and AR probe enrichment. (* This treatment was finally chosen.)



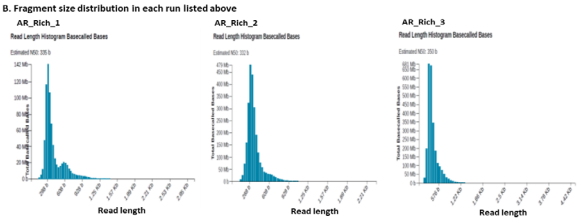
(D) Identification of circARs in PCa cell lines with AR transcript-enriched cDNA library by NGS: for cDNA library preparation, dual index adapters (with or without UMI) were used followed by AR probe enrichment before proceeding to RNA-seq (Figure 3). We have identified several circARs from LN95 and LNCaP cells (Figure 4), though additional validation is necessary.

Figure 4. Sequencing summary of cDNA library with different process for circAR detection

A. Representative runs and their cDNA library features

Nanopore sequencing run	Read Generated	Bases Generated	Cell type and RNA treatments
AR_Rich_1	3.42 M	1.29 Gb	Pooled samples: including LN95/LNCaP with rRNA depletion +/- RNase R treatment; dual i7-i5 indexed; AR gene panel enriched
AR_Rich_2	10.95 M	3.9 Gb	Pooled samples: including LN95 with rRNA depletion; UMI-i7-i5 indexed; AR gene panel enriched
AR_Rich_3	10.74 M	3.6 G	Pooled samples: including LN with rRNA depletion; UMI-i7-i5 indexed; AR gene panel enriched

B. Fragment size distribution in each run listed above



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

Nothing to report

- **How were the results disseminated to communities of interest?**

Nothing to report

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

We will continue our efforts on the proposed goals and timelines according to SOW and expect to accelerate our studies during Year 2 and Year 3.

4. **IMPACT:**

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

Nothing to report

- **What was the impact on other disciplines?**

Nothing to report

- **What was the impact on technology transfer?**

Nothing to report

- **What was the impact on society beyond science and technology?**

Nothing to report

5. **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**

We experienced some delay in achieving our objectives for Year 1 mainly due to the pandemic. We expect to be able to accelerate the pace of relevant activities during Year 2 and Year 3 of the project period.

- **Changes that had a significant impact on expenditures**

Nothing to report

Nothing to report

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

- **Significant changes in use or care of human subjects**

Nothing to report

- **Significant changes in use or care of vertebrate animals.**

Nothing to report

- **Significant changes in use of biohazards and/or select agents**

Nothing to report

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  - **Journal publications.**

    Nothing to report

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

    Nothing to report

  - **Other publications, conference papers, and presentations.**

    Nothing to report

- **Website(s) or other Internet site(s)**

  Nothing to report

- **Technologies or techniques**

  Nothing to report

- **Inventions, patent applications, and/or licenses**

  Nothing to report

- **Other Products**

  Nothing to report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name	**Project Role, contribution, and (ORCID ID)**	**Person Month**
Lu, Changxue	Principle Investigator, overall management and experiment performance (0000-0001-7565-8796)	9
Isaacs, William B.	Co-Investigator, oversight the project, and provide clinical specimens (0000-0001-6599-6775)	1.2

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

Nothing to report

- **What other organizations were involved as partners?**

Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**
- **QUAD CHARTS:**

N/A

9. APPENDICES:

Nothing to report