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14. ABSTRACT
Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related death in men in the United States. Androgen deprivation therapy (ADT) is the front-line treatment, but it eventually fails and disease almost always progresses as castration-resistant prostate cancer (CRPC). Nearly all prostate cancer deaths are due to CRPC. It has been demonstrated that intratumoral androgen synthesis is required to drive CRPC progression. The enzyme 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1), which catalyzes the initial rate-limiting step in the conversion of the adrenal-derived steroid dehydroepiandrosterone to dihydrotestosterone (the most potent natural stimulus of the androgen receptor), is likely a critical enzymatic gatekeeper that confers on tumors the ability to harness adrenal androgens. 3βHSD1 has been mechanistically linked to CRPC and is associated with prostate cancer resistance to ADT. Recently, we reported that a *HSD3B1* germline variant regulates dueling abiraterone metabolite effects and affects prognosis. Protein phosphorylation is a reversible post-translational modification mediated by kinases that modifies protein function. 3βHSD1 is critical to androgen synthesis and prostate cancer growth, but no functional phosphorylation sites have been identified. My preliminary studies show that in the LNCaP and C4-2 PCa cell lines, Compound C, an inhibitor of AMP-activated protein kinase (AMPK), inhibits 3βHSD1 activity without affecting its expression but decreases its phosphorylation, suggesting that phosphorylation of 3βHSD1 is important to its activity and that the regulatory kinase may be a potential treatment target. Therefore, I hypothesize that phosphorylation of 3βHSD1 modifies its function and the functional phosphorylation or regulatory kinase promotes CRPC. If so, targeting the functional phosphorylation or kinase could be new strategy for CRPC treatment.

15. SUBJECT TERMS
3βHSD1, phosphorylation, kinase, DHEA metabolism, CRPC

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Table of Contents

	Page
1. Introduction……	2
2. Keywords……	2
3. Accomplishments……..……...…….	2-6
4. Impact……...…….	7
5. Changes/Problems...……	7
6. Products…….……..…..…….	7-8
7. Participants & Other Collaborating Organizations…….	8
8. Special Reporting Requirements…….	8
9. Appendices…….	8

INTRODUCTION:

PCa is the most common malignancy in men and the second leading cause of cancer-related death in men in the United States. Prostate cancer progression depends on continued androgen receptor (AR) activity. Testosterone and its more potent metabolite, 5α-dihydrotestosterone (DHT), are the endogenous ligands that activate the AR in health, and in PCa they are the ligands fueling the growth of malignant prostate cells. Androgen deprivation therapy (ADT), either by medical or surgical castration, is the front-line treatment, but it almost always eventually progresses as castration-resistant prostate cancer (CRPC). The Sharifi laboratory has demonstrated that intratumoral androgen synthesis is required for driving CRPC progression. Intratumoral synthesis of DHT from precursors that are secreted from the adrenal gland occurs through a pathway that circumvents testosterone. This synthesis requires three enzymes: 3β-hydroxysteroid dehydrogenase (3βHSD; encoded by HSD3B), steroid-5α-reductase (SRD5A), and 17β-hydroxy steroid dehydrogenase (17βHSD) isoenzymes. 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1), which catalyzes the initial rate-limiting step in the conversion of the adrenal-derived steroid dehydroepiandrosterone (DHEA) to DHT, is likely a critical enzymatic gatekeeper that confers on tumors the ability to harness adrenal androgens. *HSD3B1*(1245A>C) variant has been mechanistically linked to CRPC by encoding an altered enzyme that augments dihydrotestosterone synthesis. Inheritance of the *HSD3B1* allele that enhances dihydrotestosterone synthesis is associated with prostate cancer resistance to ADT. And it has been reported that *HSD3B1* variant regulates dueling abiraterone metabolite effects in prostate cancer. Recently we identified and genotyped 475 Caucasian men enrolled in the CHAARTED trial, which evaluated the ability of early chemotherapy to improve overall survival of patients starting ADT for metastatic prostate cancer, and found that inheritance of the *HSD3B1*(1245C) allele that augments DHT synthesis from adrenal precursors may be associated with more rapid development of CRPC and lower overall survival in Caucasian men with low-volume metastatic prostate cancer treated with ADT with or without docetaxel.

Protein phosphorylation is a reversible post-translational modification of proteins in which an amino acid residue is phosphorylated by protein kinase by the addition of a covalently bound phosphate group. Phosphorylation alters the structural conformation of a protein, causing it to become activated, deactivated, or otherwise modifying its function. 3βHSD1 is critical to androgen synthesis and prostate cancer growth, but no functional phosphorylation sites have been reported. AMP-activated protein kinase (AMPK), which is activated when intracellular ATP levels lower, is one of the central regulators of cellular and organismal metabolism in eukaryotes. It is widely known that AMPK has play an essential in cancer cell survival. My preliminary studies show that in the LNCaP and C4-2 PCa cell lines, Compound C, an inhibitor of AMPK, inhibits 3βHSD1 activity without affecting its expression but decreases its phosphorylation, suggesting that phosphorylation of 3βHSD1 is important to its activity and that the regulatory kinase may be a potential treatment target. Here, I propose to identify the functional phosphorylation sites and their clinical significance in prostate cancer therapy.

KEYWORDS:

3βHSD1, phosphorylation, kinase, DHEA metabolism, CRPC

ACCOMPLISHMENTS:

What were the major goals of the project?

Training-Specific Tasks:

Milestone(s) Achieved: Attend monthly Prostate Cancer Working Group & Seminar Series. (12/24 months).

Milestone(s) Achieved: Attend and present research at weekly lab meetings and journal clubs. (12/24 months).

Research-Specific Tasks:

Specific Aim 1: Confirm that phosphorylation of 3βHSD1 indeed modifies its function and plays a role in prostate cancer cell growth.

Major Task 1: Identify the functional phosphorylation sites on 3βHSD1(6 months).

Milestone(s) Achieved: identification of the functional phosphorylation sites on 3βHSD1. (6 months)

Major Task 2: Determine whether phosphorylation of 3βHSD1 affects prostate cancer cell growth (11 months).

Milestone(s) Achieved: determination that phosphorylation of 3βHSD1 at functional site is required by prostate cancer cell growth (11 months).

Major Task 3: Determine whether phosphorylation of 3βHSD1 is increased or decreased in patients with prostate cancer (16 months).

Milestone(s) Achieved: Not yet.

Specific Aim 2: Determine the effect of AMPK on androgen synthesis and prostate cancer cell growth, and identify the mechanism.

Major Task 4: Determine the mechanism by which AMPK regulates 3βHSD1 activity (18 months).

Milestone(s) Achieved: Not yet.

Major Task 5: Determine whether sh-AMPK or the AMPK inhibitor, Compound C, affects prostate cancer cell growth (21 months).

Milestone(s) Achieved: Not yet.

Major Task 6: Analyze AMPK expression in tumor tissue from prostate cancer patients and the correlation of AMPK with patient survival (24 months).

Milestone(s) Achieved: Not yet.

What was accomplished under these goals?

Accomplished tasks:

Specific Aim 1: Confirm that phosphorylation of 3βHSD1 indeed modifies its function and plays a role in prostate cancer cell growth.

Major Task 1: Identify the functional phosphorylation sites on 3βHSD1 (6 months).

Milestone(s) Achieved: identification of the functional phosphorylation sites on 3βHSD1. (6 months).

Results:

In order to find the phosphorylation site(s), we purified 3βHSD1 and performed mass spectrometry. As shown in **Fig 1A**, several phosphorylation sites on 3βHSD1 were identified through mass spectrometry. We confirmed the phosphorylation of Y255 and Y344 by mutating these sites to phenylalanine and then performing western blots (**Fig 1B**). Further, we found that phosphorylation of Y344 is required for 3βHSD1 enzyme activity because the metabolism of DHEA was significantly reduced after mutated to phenylalanine (**Fig 1C**).

A.

Phosphorylated HSD3B1 peptides identified in LC-MS/MS analysis.

[M+H]	m/z	z	Site	Sequence	PA(phospho)/PA(unmod) (%)
1712.853	571.6225	3	-	DLAYKPLYSWEEAK	
1792.8193	598.278	3	Y340/Y344	DLAYKPLYSWEEAK + PO3	0.045
2869.2951	957.1032	3	-	GQFYYISDDTPHQSYDNLNYTLSK	
2949.2615	983.7587	3	Y255	GQFYYISDDTPHQSYDNLNYTLSK + PO3	0.017

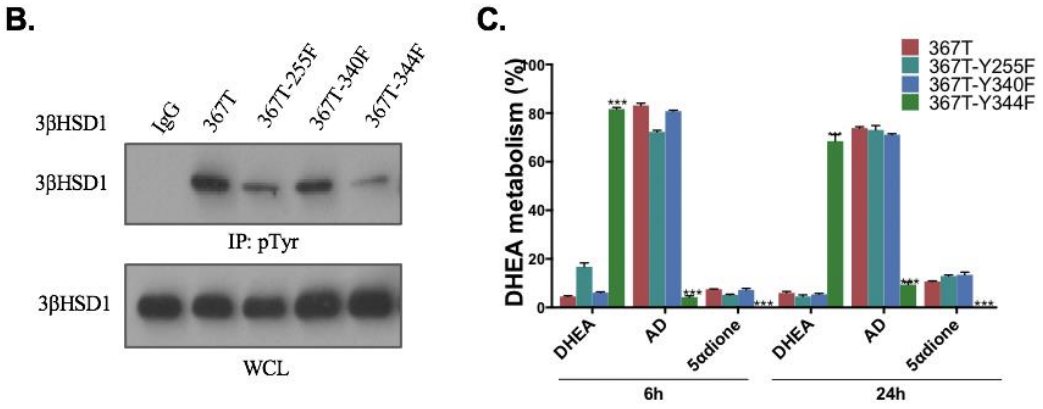

Figure 1. 3β-hydroxysteroid dehydrogenase (3βHSD1) pY344 is required for DHEA metabolism. **A.** 3βHSD1-GST was transfected and cells were treated with DHEA for 1 hour. GST-pull down complexes were immunoblotted and the indicated phosphopeptides on human 3βHSD1 were identified by LC-MS/MS. **B.** Cells were transfected with 3βHSD1 mutants and pan-phospho-tyrosine (pTyr) was detected by immunoprecipitation and western blot. WCL = whole-cell lysate. **C.** Cells were transfected with 3βHSD1 mutants and subsequently treated with [3H]-DHEA for the indicated times, followed by steroid extraction from media, steroid separation and quantitation with HPLC.

Major Task 2: Determine whether phosphorylation of 3βHSD1 affects prostate cancer cell growth (11 months).

Milestone(s) Achieved: determination that phosphorylation of 3βHSD1 at functional site is required by prostate cancer cell growth (11 months).

Results:

To determine whether phosphorylation of 3βHSD1 affects prostate cancer cell growth, we generate C42 cell lines that stably expressed 3βHSD1 (WT) or 3βHSD1-Y344F. As shown in **Fig 2A**, the metabolism of DHEA was slower in the 3βHSD1-Y344F cell line. We also tested cell viability and found that Y344F mutation of 3βHSD1 inhibits DHEA-increased cell viability (**Fig 2B**). We further analyzed the expression of AR downstream genes -- as shown in **Fig 2C**, Y344F mutation of 3βHSD1 inhibits AR transcriptional activity.

Additionally, our xenograft study revealed that Y344F mutation of 3βHSD1 inhibited DHEA-induced tumor growth and prolonged progression-free survival in C42 xenograft models of CRPC (**Figure 3A–B**). By assessing steroids in xenograft tissues by mass spectrometry (**Figure 3C**), we demonstrated that the inhibition of tumor growth by Y344F mutation was mediated by loss of intratumoral androgen production. The reduction of AR transcriptional activity also was detected in xenograft tumors carrying mutated Y344F (**Figure 3D**). In contrast, Y344F mutation had no significant effect on untreated tumors (**Figure 4A–B**).

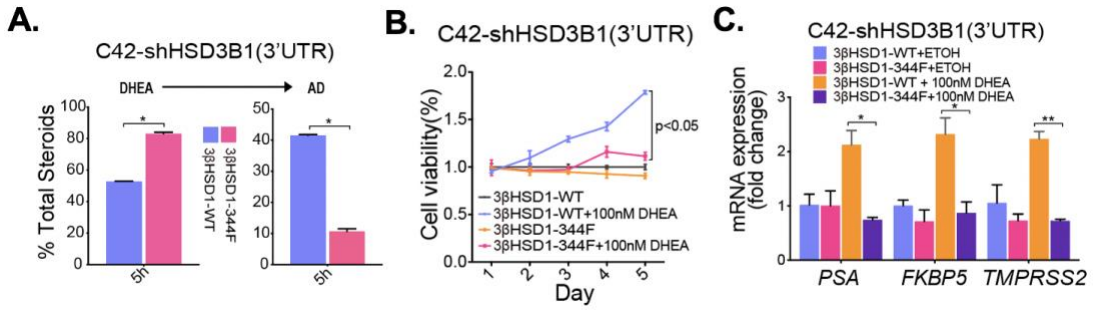

Figure 2. Y344F mutation of 3βHSD1 inhibits prostate cancer cell growth. **A.** C42 cells with stable shRNA-mediated knockdown of 3βHSD1 were stably infected with the lentivirus expressing 3βHSD1 (WT), or 3βHSD1-Y344F and subsequently treated with [3H]-DHEA for the indicated times, followed by steroid extraction from media and steroid separation and quantitation with HPLC. **B.** As in (A), but cells were deprived of serum overnight and followed by treatment with DHEA for the indicated times; a cell viability assay was performed. **C.** As in (A), but cells were deprived of serum overnight and treated with DHEA for 48 hours, followed by RNA extraction and qPCR. Expression is normalized to control and RPLP0 expression. Data are presented as the mean ± SEM of biological quadruplicates and are representative of at least three independent experiments.

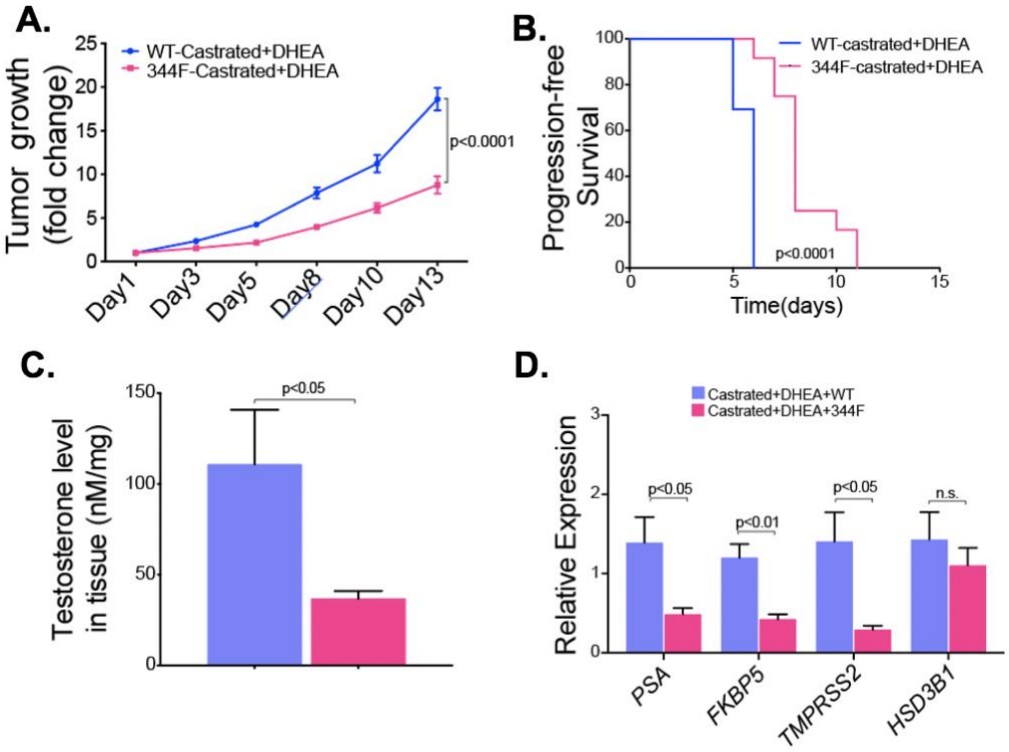

Figure 3. Y344F mutation of 3βHSD1 inhibits prostate cancer cell growth *in vivo*. **A.** C42 cells with stable shRNA-mediated knockdown of 3βHSD1 were stably infected with a lentivirus expressing 3βHSD1 (WT) or 3βHSD1-Y344F, and then 10 million cells were injected subcutaneously in 9- to 11-week-old mice. Castration and DHEA treatment were performed after tumors reached 200 mm^3. Tumor growth was analyzed. Data were plotted as mean ± SEM. **B.** As in (A), but progression-free survival was analyzed. **C.** As in (A), but tumor steroid levels were detected by mass spectrometry. **D.** As in (A), but RNA was extracted from tumor tissue, and AR target gene expression was analyzed.

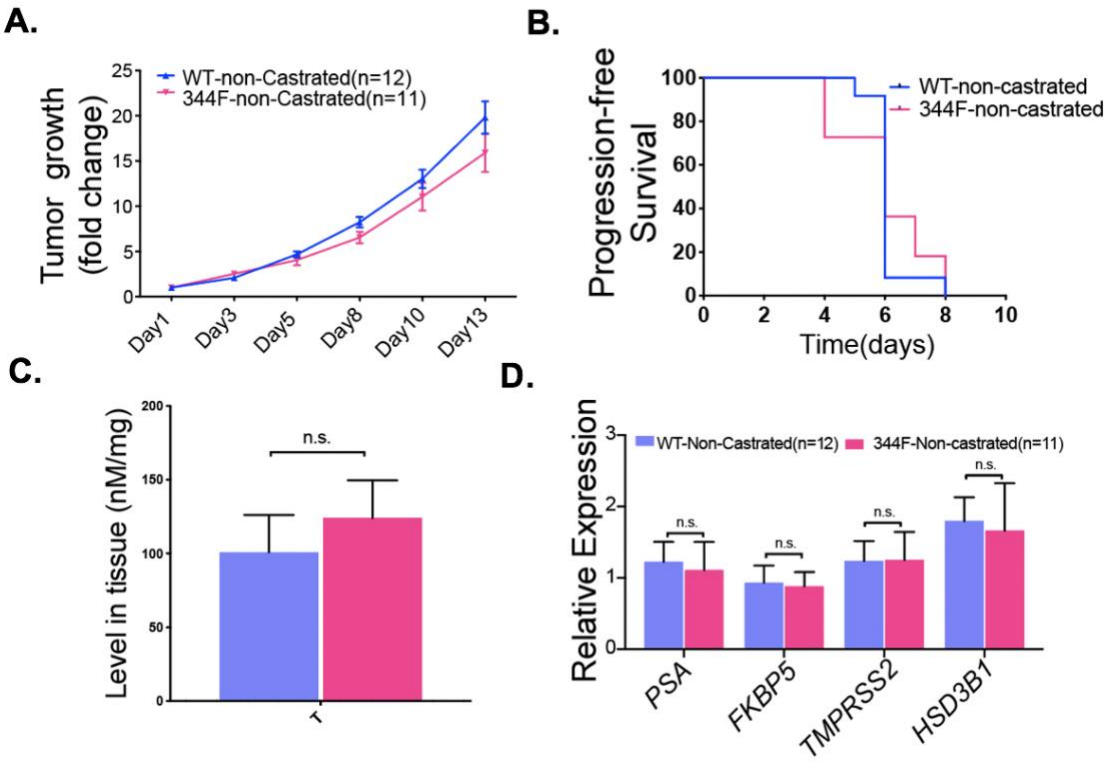

Figure4. Y344F mutation had no significant effect on untreated tumors. **A.** C42 cells with stable shRNA-mediated knockdown of 3βHSD1 were stably infected with a lentivirus expressing 3βHSD1 (WT) or 3βHSD1-Y344F and then 10 million cells were injected subcutaneously in 9- to 11-week-old mice. Tumor growth was analyzed. Data were plotted as mean ± SEM. **B.** As in (A), but progression-free survival was analyzed. **C.** As in (A), but tumor steroid levels were detected by mass spectrometry. T, Testosterone. **D.** As in (A), but RNA was extracted from tumor tissue, and AR target gene expression was analyzed.

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

1. Attended Conduct of Research and Human Subjects training.
2. Attended monthly Prostate Cancer Working Group and Seminar Series
3. Attended and presented research at the weekly lab meetings and journal clubs
4. Attended and presented work at the weekly CCF Department of Cancer Biology seminars
5. Attend workshop “Biomedical and Scientific Writing” held by Kenyon Institute, Ohio.

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?

During this reporting period, I have identified the functional phosphorylation sites on 3βHSD1 (Specific Aim 1, Major Task 1). I also determined that phosphorylation of 3βHSD1 at the functional site is required for prostate cancer cell growth (Aim 1, Major Task 2). The next step is to detect phosphorylation of 3βHSD1 in patients with prostate cancer during the next reporting period. I also plan to identify the regulatory kinase of p-Y344-3βHSD1, and then determine whether the kinase could be a target for prostate cancer treatment.

Then we will publish 1-2 peer reviewed papers.

IMPACT:

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

If there is nothing significant to report during this reporting period, state "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

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

Nothing to Report

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

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

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

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Xiuxiu Li*
Project Role:	*Principle investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-4106-318*
Nearest person month worked:	*12*
Contribution to Project:	*Xiuxiu Li is responsible for designing, performing and interpreting the experiments.*
Funding Support:	

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 Change.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report.

QUAD CHARTS:

Nothing to Report.

APPENDICES:

Nothing to Report.