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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our preliminary study demonstrates that advanced prostate cancer is addicted to glutamine, and a glutaminase isoform switch contributes to the development of therapy resistance and disease progression. The major goal of the project was to study the molecular mechanisms of GLS1 isoform switch and explore the possibility of targeting glutamine metabolism as a novel therapeutic approach. In year 1, we have made the following Key Research Accomplishments:

1. We have verified a general phenomenon that ADT decreases the metabolic rate to suppress tumor growth. That is for the first time demonstrating how hormonal therapy works initially from a metabolic basis.
2. With powerful evidence delivered from accomplished tasks, we have also raised a high possibility for the therapeutic failure which could be that in addition to reduction of the majority of metabolites, hormonal therapy accumulates glutamine at the same time. This restored glutamine replaces androgen to become the crucial energy and nutrient source.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction:

Prostate Cancer (PCa) leads to ~30,000 deaths yearly in the US[1]. Although androgen receptor (AR)-targeted therapies, including conventional androgen deprivation therapy (ADT) and the second generation of antiandrogen drugs (e.g abiraterone and enzalutamide), remain the standard management for metastatic PCa, the therapeutic failure and tumor recurrence are inevitable[2]. This is because tumor cells develop various resistance mechanisms to overcome the consequences of AR inhibition[3, 4]. Therefore, instead of targeting AR which regulates numerous signaling pathways and plays critical physiologic roles, identifying AR's independent effectors that are directly responsible for tumor biology, can yield better therapeutic targets to achieve more durable therapeutic responses and reduce side effects associated with AR targeting.

The rewired metabolism is one of the most significant cancer hallmarks, and targeting metabolism has become an important therapeutic strategy for many tumors[5, 6]. In PCa, our preliminary studies have revealed that androgen withdrawal causes a significant accumulation of intracellular glutamine by blocking glutamine being utilized. More interestingly, after cancer recurs, glutamine becomes the major nutrient instead of glucose to support growth and proliferation of recurrent tumor cells. We hypothesize that the resumed glutamine metabolic function is achieved by AR-governed isoform switch of the rate-limited enzyme glutaminase (GLS1) because of the differential expression of the two variants (KGA and GAC) in primary and advanced stage of PCa, respectively. Specifically, in primary tumor where KGA dominates, hormonal therapy inhibits AR activity to

suppress KGA expression and glutamine utilization, leading to tumor suppression. However, tumor cells eventually switch to express the other isoform GAC, which is dominant in therapy-resistant cancer type and independent of androgen, leading to more efficient glutamine usage and development of castration resistance. We are hoping to provide metabolic mechanisms to explain why hormonal therapy works initially but fails eventually and to explore novel therapeutic strategy with more better efficacy and less adverse effects for patients who have developed resistance to AR-targeted therapies.

This research proposal has following specific aims: 1. To study functions of glutamine metabolism and GLS1 in PCa and hormonal therapy; 2. To mechanistically study GLS1 isoform switching during therapy failure and disease progression of PCa.

2. **Key Words: Prostate cancer, Glutamine, Glutaminase, KGA, GAC**

3. **Research accomplishments associated with Task 1: In this task, we will demonstrate that ADT-induced inability to utilize glutamine mediates the inhibition effects of hormonal therapy on PCa.**

Subtask 1: Test if ADT-induced inability of glutamine utilization is general for all androgen sensitive PCa cell lines (Time frame: Months 1-12).

AR function is critical for the survival of PCa cells[7]. However, exactly how PCa stops proliferation after hormonal therapy is unclear despite decades of studies on AR signaling pathways. To metabolically address this question, we previously used charcoal-stripped FBS to treat hormone-sensitive LNCaP cells which mimics clinical androgen deprivation therapy. One of the main findings was that ADT resulted in a global inhibition of the majority of metabolites while dramatically increasing intracellular glutamine level. To confirm that this observation is a general phenomenon in primary PCa, we selected another hormone-sensitive, AR-positive PCa cell line, LAPC4, to conduct metabolite profiling

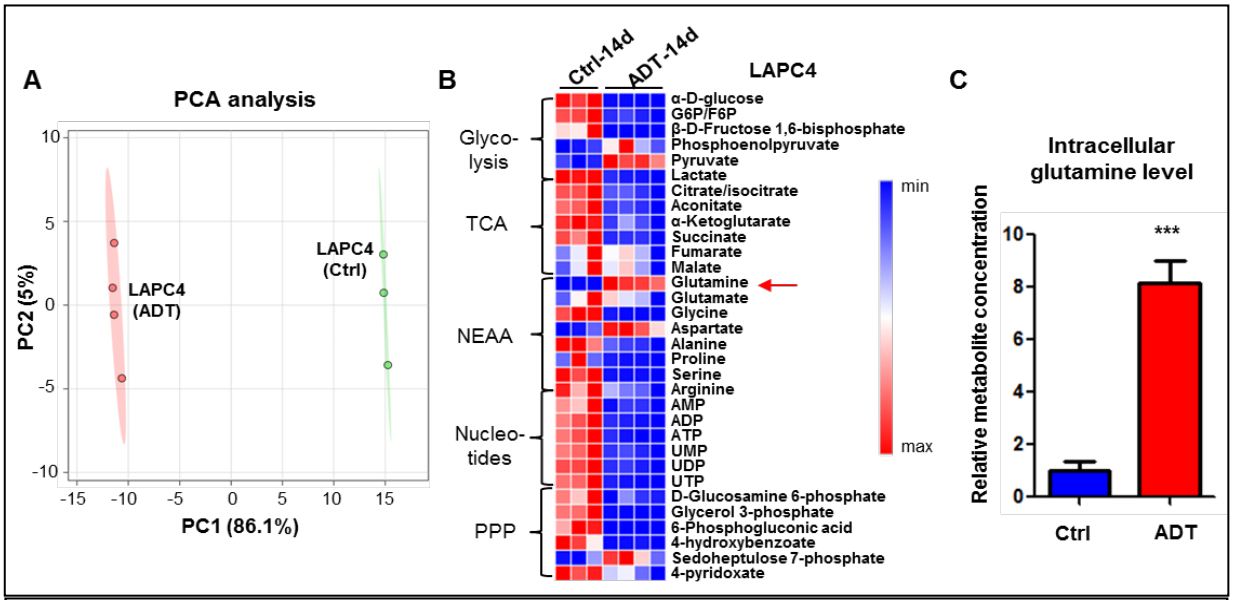

Fig. 1 ADT decreases most metabolites while increases intracellular glutamine level in hormone-sensitive PCa cells. **A and B.** Principal component analysis and heat map showing metabolite distribution and concentration after ADT treatment. **C.** Detailed intracellular glutamine level.

experiments. Similarly, cells clustered nicely based on whether they were cultured with (Ctrl) or with androgen (ADT) (**Fig. 1A**). Specifically, after treatment for two weeks, ADT decreased the levels of majority of the metabolites involved in important energy metabolic pathways such as glycolysis, TCA cycle, non-essential amino acids (NEAA), nucleotides, pentose phosphate pathway (PPP) and glutaminolysis (**Fig. 1B**). Similar to what was observed in LNCaP cells, we found a near 8-fold increase of glutamine level (**Fig. 1B-C**), indicating that ADT treatment, while inhibiting metabolite levels in general, significantly raises intracellular glutamine accumulation.

Subtask 2: Test if GLS1 (GAC) overexpression rescues the cell proliferation under the presence of ADT (Time frame: Months 12-24).

To be performed.

Subtask 3: Examine how AR regulates GLS1 expression (Time frame: Months 1-24).

Our hypothesis is that AR is involved in the regulation of the two isoforms of GLS1. To address this point, we need to demonstrate that *GLS1* is an AR-regulated gene. In addition to the previous findings from AR ChIP sequencing datatsets[8, 9] revealing a strong AR binding peak located in the 3' untranslated region (UTR) of *GLS1* after dihydrotestosterone (DHT) stimulation, we designed specific primers flanking the androgen response elements (AREs) regions to validate whether AR is bound to those putative sites. GLS1-ChIP-Forward: TGGTCTCAAATCCCAAGATTTAAAT; GLS1-ChIP-Reverse: GACAGGATAAAATGTATGTGTAACTCC. After AR was cross-linked to its DNA binding sites and pulled down by an anti-AR antibody, qPCR showed a dramatically increased activity upon DHT stimulation, which confirmed the binding of AR to this region (**Fig. 2A**).

Next, we cloned 3'UTR of GLS1 containing the two AREs into a CMV-driven Renilla luciferase reporter construct (pRL) and measured the luciferase activity in the presence or absence of DHT (**Fig. 2B**). DHT upregulated luciferase activity of the construct containing the GLS1 wide-type-3'UTR in comparison to the vector control (**Fig. 2B**). We then used site-directed mutagenesis kit (Agilent) to mutate all the AREs. Three mutant constructs containing the GLS1-3'UTR with deletions of each ARE individually or in combination were generated and luciferase reporter assay showed that each ARE mutation diminished the luciferase activity and the mutation combination had the strongest effect (**Fig. 2B**), further suggesting that AR binds to the AREs of *GLS1* and upregulates *GLS1* expression.

Since AR directly promotes GLS1 expression, we will continue to pursue whether AR differentially mediates production of the two splice isoforms of *GLS1*. We will knockdown AR in AR-positive cells as well as overexpress ectopic AR in AR-null cells to detect the alteration of KGA/GAC ratio. The expected results would be that with the presence of full length AR, *GLS1* tends to generate *KGA*. However, in the late stage where AR

is mutant or absent (such as neuroendocrine PCa)[10], GAC predominates at the transcript level of GLS1 due to the absence of AR regulation.

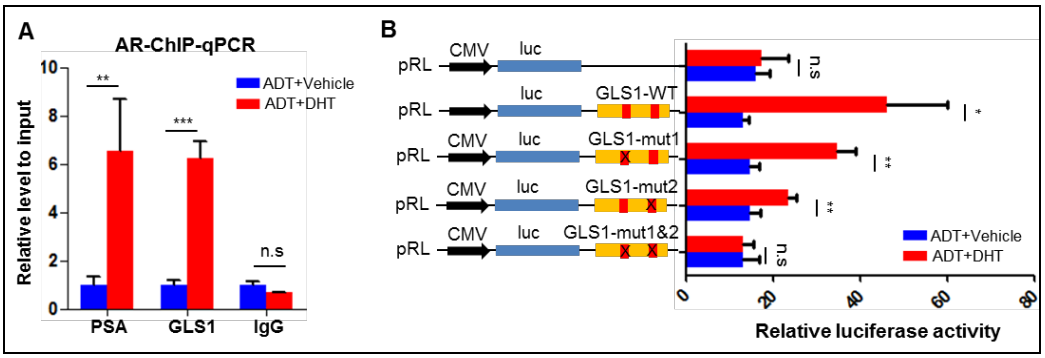

Fig. 2 *GLS1* is an AR-regulated gene. **A.** ChIP-qPCR showing that the activity of AR-bound GLS1 regions is upregulated upon DHT treatment. **B.** Luciferase activity of pRL and modified constructs containing wild type (wt) GLS1-3'UTR or mutant (mut) GLS1-3'UTR.

Research accomplishments associated with Task 2: In this task, we will demonstrate that the GLS1 isoform switch from KGA to GAC drives castration resistance.

Subtask 1: Test if glutamine utilization is efficient initially in the therapy-sensitive stage, inhibited after treatment and efficient again after development of therapy resistance (Time frame: Months 24-36).

To be performed.

Subtask 2-3: Generate *GLS1* knockout cell lines by CRISPR-Cas9 system and test if the switch from KGA to GAC mediates castration resistance (Time frame: Months 1-36).

Since GAC emerges at the late stage of PCa, and the emergence of GAC is likely the main reason causing therapeutic resistance, it is critical to investigate the biological functions of these two isoforms. We specifically knocked down KGA and GAC, respectively, using shRNAs in LNCaP and PC3 cells (**Fig. 3A, C**). LNCaP cells were more sensitive to the reduction of KGA in terms of cell proliferation and glutamine utilization (**Fig. 3A, B**), consistent with our observation that KGA is the dominant isoform of GLS1 in androgen-dependent cells including LNCaP cells. In PC3 cells, in contrast, loss of GAC caused a significant decrease of glutamine utilization and a dramatic cell growth arrest while knocking down KGA had little effect (**Fig. 3C, D**). These results indicate that the stronger enzymatic activity of GAC confers the rapid proliferation of advanced PCa through enhanced glutamine consumption. To demonstrate that GAC is the more potent isoform, we next intended to establish a more specific genetic model with exclusive expression of either KGA or GAC. To achieve this goal, we planned to knock out *GLS1* gene and introduce ectopic KGA and GAC back to observe their resultant biological phenotypes in both LNCaP and PC3 cells. CRISPR sgRNA plasmids were purchased from GeneCopoeia (HCP258798-CG12-3-B). Cells were transfected by sgRNAs via Lipofectamine LTX (Invitrogen) followed by geneticin selection. Protein knockout was confirmed by western blotting **(Fig. 3E)**.

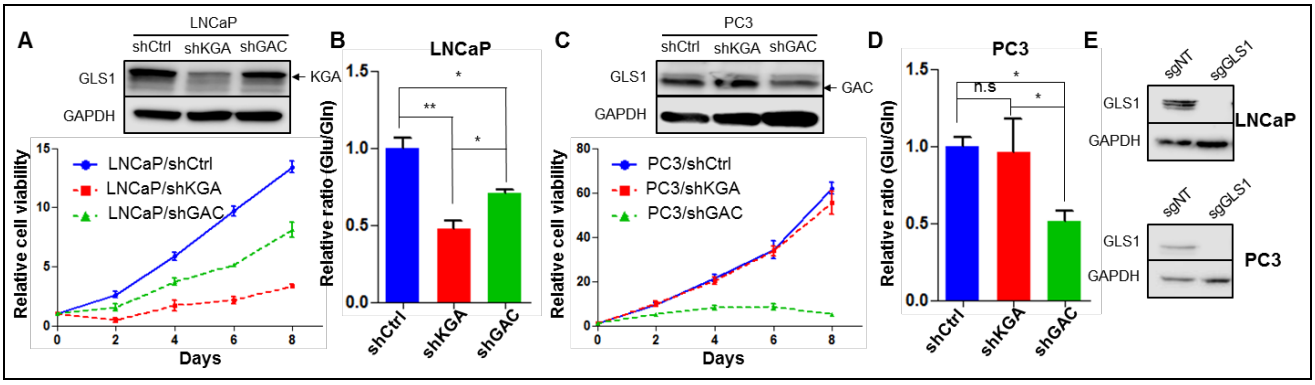

Fig. 3 Biological functions of GLS1 two isoforms. **A and C.** Western blot showing KGA and GAC expression of LNCaP and PC3 cells transduced with KGA-specific, GAC-specific or control shRNAs, and the corresponding cell proliferation rate for each cell line. **B and D.** Glutamine utilization indicated by the ratio of intracellular glutamate/glutamine of cells generated in (A and C). **E.** Western blot showing knockout of GLS1 mediated by CRISPR/Cas9.

Next, we will introduce either KGA or GAC in the GLS1-deficient cell models. We will observe several crucial aspects, including cell viability, sensitivity to glutamine deprivation, efficiency of glutamine utilization, etc. Based on the proposed assays, we expect that the GAC isoform is much more potent in promoting glutamine utilization and tumor proliferation than the KGA isoform and its function is independent of AR status (both AR-positive and AR-null cell lines will be incorporated). This will elucidate that GLS1 switches its variant from KGA to GAC leading to castration resistance most likely due to the powerful enzymatic activity of GAC independently of AR status.

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 finish the remaining work proposed to be done within the first 24 months.

4. Impact:

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

1. We have verified a general phenomenon that ADT decreases the metabolic rate to suppress tumor growth.

That is for the first time demonstrating how hormonal therapy works initially from a metabolic basis.

2. With powerful evidence delivered from accomplished tasks, we have also raised a high possibility for the therapeutic failure which could be that in addition to reduction of the majority of metabolites, hormonal therapy accumulates glutamine at the same time. This restored glutamine replaces androgen to become the crucial energy and nutrient source when tumor cells develop into recurrent or castration-resistant stage.
3. We have mechanistically demonstrated that the key enzyme of glutaminolysis, GLS1, is regulated by AR, which is an important foundation for our overarching aim to explore AR downstream target with more specificity and less side effects.
4. In terms of the biological functions of the two isoforms of GLS1, we have preliminarily elucidated that GAC is the enzymatically more potent one. Rebuilding the genetic construct of GLS1 by exclusively expressing either KGA or GAC will further demonstrate whether the isoform switch from KGA to GAC drives therapy resistance and disease progression of PCa.

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:

- **Nothing to Report**

6. Products

- **Nothing to report**

7. Participants & Other Collaborating Organizations

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Contribution to Project:	*Oversight of the entire project, Hypothesis, design, results interpretation*
Funding Support:	

Name:	*Qing Yang*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Experimental design, power calculation, statistics*
Funding Support:	

Name:	*Hailiang Hu*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Hypothesis, design, results generation, results interpretation*
Funding Support:	

Name:	*William Butler*
Project Role:	*PhD Research Assistant*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Performance of experiments, results interpretation*
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?

- **Nothing to Report."**

What other organizations were involved as partners?

- **Nothing to Report.**

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8. Special Reporting Requirements

Nothing to Report.

9. **Appendices**

None