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Title**: Investigating the Expression, Role, and Targeting of Collagen Modifying Prolyl 4-Hydroxylase P4HA1 in Prostate Cancer Progression and Metastasis**

Principal Investigator: **Sooryanarayana Varambally**

Contracting Organization: **University of Alabama at Birmingham**

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Abstract

Prostate cancer (PCa) is the most common malignancy and the second most common cause of cancer mortality of men in the United States. Complex molecular and signaling events converge leading to PCa initiation, unregulated growth, invasion, and metastasis. There is a growing concern that PSA screening leads to over-diagnosis resulting in excessive treatment of indolent PCa without significant clinical benefit. Thus identification and validation of novel diagnostic and prognostic molecular biomarkers of PCa as well as the oncogenic therapeutic targets are of critical importance for the early detection, management, and cure of PCa. Our recent studies utilizing gene expression profiling and next generation sequencing of prostate cancer tissues identified prolyl hydroxylase P4HA1, a key enzyme in collagen modification resulting in extracellular matrix modification in cancer as upregulated in primary PCa and castration resistant PCa. Being an enzyme, P4HA1 is a viable therapeutic target amenable to small molecule inhibition.

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INTRODUCTION

Prostate cancer (PCa) is the most common malignancy and the second most common cause of cancer mortality of men in the United States. Complex molecular and signaling events converge leading to PCa initiation, unregulated growth, invasion, and metastasis. There is a growing concern that PSA screening leads to over-diagnosis resulting in excessive treatment of indolent PCa without significant clinical benefit. Thus identification and validation of novel diagnostic and prognostic molecular biomarkers of PCa as well as the oncogenic therapeutic targets are of critical importance for the early detection, management, and cure of PCa. Our recent studies utilizing gene expression profiling and next generation sequencing of prostate cancer tissues identified prolyl hydroxylase P4HA1, a key enzyme in collagen modification resulting in extracellular matrix modification in cancer as upregulated in primary PCa and castration resistant PCa. Being an enzyme, P4HA1 is a viable therapeutic target amenable to small molecule inhibition.

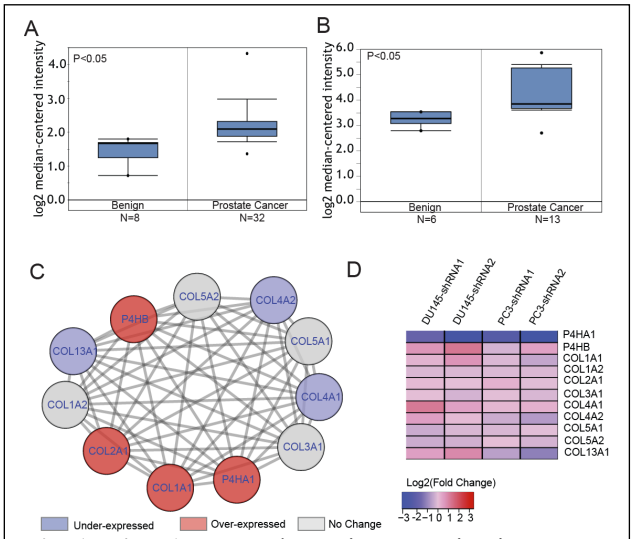

Fig. 1. P4HA1 gene and protein expression in prostate cancer. **A and B.** P4HA1 Gene expression analysis of normal prostate cells and primary prostate cancer using Oncomine cancer data analysis tool (www.oncomine.org) **C.** Investigation of P4HA1 interacting partners and their expression. Red indicated overexpression, blue downregulation and grey, no change. The data from TCGA. **D.** The gene expression changes in the P4HA1 interacting genes upon P4HA1 knockdown in DU145 and PC3 prostate cancer cells.

SPECIFIC AIMS:

Aim1. Evaluate the significance of P4HA1 expression in PCa.
Aim2. Investigate the functional role of P4HA1 in PCa metastasis.
Aim3. Targeting P4HA1 in PCa using specific small molecule inhibitor**.**

BODY

In order to establish the significance of P4HA1 expression in cohorts of prostate cancer patients, we have analyzed the P4HA1 expression in multiple prostate cancer datasets, using Oncomine cancer data analysis platform (www.oncomie.org) and identified a direct correlation with tumor grade and P4HA1 expression. Results of this analysis indicated that P4HA1 expression is increased in prostate cancer samples compared to benign prostate cells (**Fig. 1A** and **B**). Our analysis of P4HA1 interacting proteins also indicated multiple collagen genes and P4HB as interacting partners in this cascade (**Fig. 1C**). Furthermore, our microarray analysis of P4HA1 knockdown RNA from DU145 and PC3 prostate cancer cells (**Fig. 1D)** changes in the expression of these P4HA1 interacting partners, suggesting a dynamic relationship between these interacting proteins. Furthermore analysis of gene expression data after knockdown showed many genes either up or downregulated upon P4HA1 knockdown (**Fig. 2A**). These genes are involved in performing various cellular function as see in **Fig. 2B**. Further analysis of the interactions between these up and downregulated genes upon P4HA1 knockdown showed multiple potentially interesting genes alterations (**Fig. 2C**).

To further elucidate molecular mechanisms of P4HA1 functions in prostate cancer, we have generated 2 aggressive prostate cancer cell lines PC3-Luciferace and DU145-Luciferase in which we have performed P4HA1 knockdown by using shRNA. These clonal derivatives will be used for *in vitro* and *in vivo* experiments including tumor growth and metastasis study.

We have also generated P4HA1 adenovirus for overexpression studies in PrEC normal prostate cells. We have shown previously that the treatment of prostate cancer cells DU145 and PC3 with P4HA1 inhibitor PythiDC resulted in reduced cell proliferation and colony formation. Hence, are now using *in vivo* system to evaluate the tumor growth and metastasis in mice with PythiDC treatment.

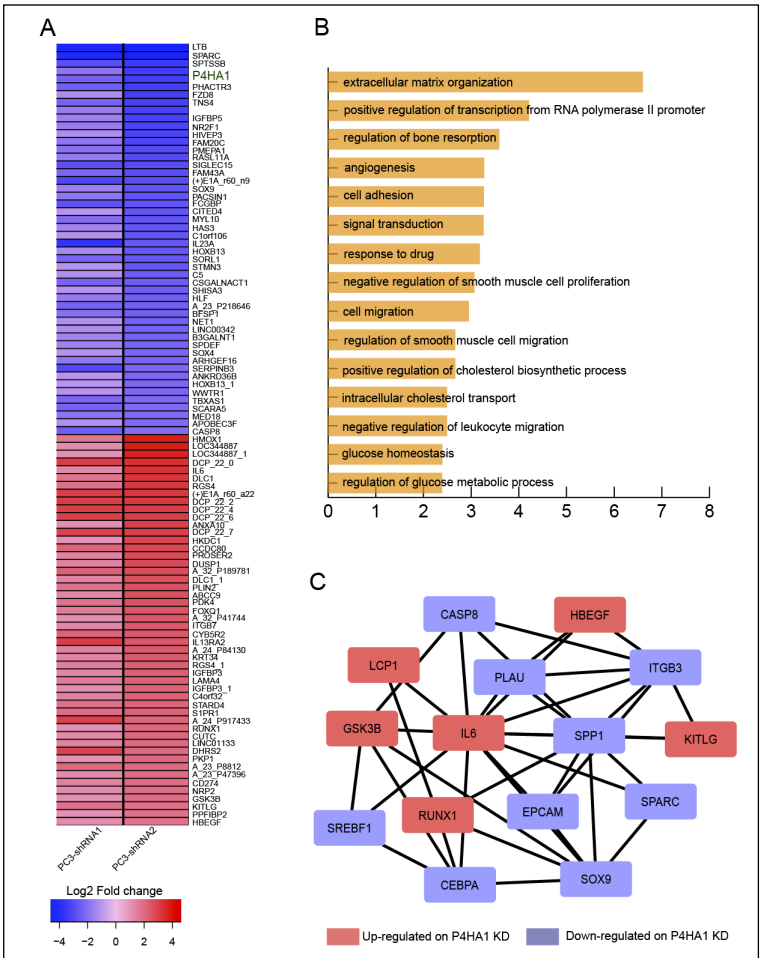

Figure. 2. Transcription profiling of Prostate cancer cells on P4HA1 knockdown. A. Heatmap showing top 100 up-/down-regulated genes on P4HA1 knockdown in PC3 cells. **B.** Top 15 biological processes enriched in genes altered on P4HA1 knockdown in PC3 cells. **C.** Differentially expressed hub genes on P4HA1 knockdown identified from protein-protein interaction network analysis using cytohubba application in Cytoscape.

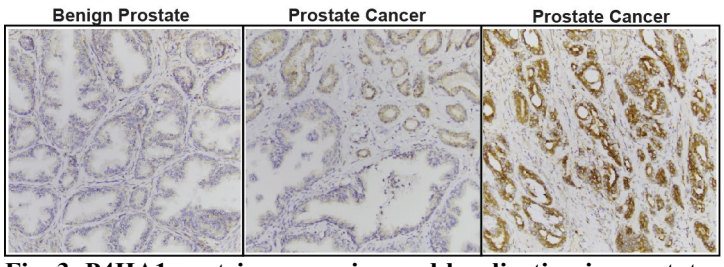

Fig. 3. P4HA1 protein expression and localization in prostate cancer. Immuno-histochemical staining was performed on prostate cancer tissues using specific antibody against P4HA1 protein. Varying degree of staining intensity was observed. On the left shows a benign prostate tissue showing no staining for P4HA1 protein. Middle and right hand panels show prostate cancer tissues with positive staining for P4HA1 protein.

In parallel, and towards relating the molecular characteristics of P4HA1 in actual patient-tumors, we have performed immunohistochemical analysis to detect and quantify P4HA1 protein expression in prostate cancer tissues. We used prostate cancer tissue microarray (TMA) for this. The staining showed increased P4HA1 protein in prostate cancer tissues (**Fig. 3**) in both Caucasian and African American prostate cancer tissues. The staining intensity has been quantified and is being analyzed to understand the pattern of P4HA1 protein expression with disease progression parameters such as Gleason grade and disease recurrence.

Key Words: PCa: Prostate cancer, TMA: Tissue microarray, P4HA1: Prolyl 4-Hydroxylase Subunit Alpha 1,

KEY RESEARCH ACCOMPLISHMENTS:

- Analyzed multiple prostate cancer gene and protein expression datasets to evaluate the expression of P4HA1 and other enzymes related to collagen metabolism.
- Constructed the tissue microarray containing PCa patient samples for immune-histochemical staining.
- Performed IHC staining and quantification of TMA. The TMA contained both Caucasian and African American Prostate cancer patient samples.
- Generated stable prostate cancer cell line PC3-Luc and DU145-Luc in which P4HA1 is modulated.
- Obtained IACUC approval for animal experiments and will be initiating the *in vivo* studies and initiated in vivo studies to evaluate the role of P4HA1 in prostate cancer metastasis and its inhibition with PythiDC.

Impact:
The current study developed a valuable prostate cancer tissue microarray containing various grades of prostate cancer. Furthermore, this TMA contains various race groups and will serve as a valuable tool to identify additional PCa biomarkers.

Changes/Problems: The beginning of COVID19 pandemic did effect to an extent some of the proposed experimental work and the timeline of the performance. Despite the problems faced and COVID related restrictions at the work place, we were successful in accomplishing some of the tasks and in line to finish majority of the tasks in the coming year. We will continue and finish the remaining part of the project in the coming years and will kindly request no cost extension if need arises.

REPORTABLE OUTCOMES

Generated PCa TMA's for evaluation of prostate cancer biomarkers.
Performed immune-histochemical staining of P4HA1 using prostate cancer TMAs.
Generated P4HA1 shRNA lenti-virus for stable knockdown of P4HA1.
Generated P4HA1 adenovirus for overexpression of P4HA1 in normal prostate cells.

Publication:

1) Marie-Lisa Eich , Mohammad Athar , James E Ferguson 3rd , Sooryanarayana Varambally. EZH2-Targeted Therapies in Cancer: Hype or a Reality. ***Cancer Research.*** 2020 Dec 15;80(24):5449-5458. doi: 10.1158/0008-5472.CAN-20-2147. PMID: 32978169

2) Robinson AD, Chakravarthi BVSK, Agarwal S, Chandrashekar DS, Davenport ML, Chen G, Manne U, Beer DG, Edmonds MD, **Varambally S**. Collagen modifying enzyme P4HA1 is overexpressed and plays a role in lung adenocarcinoma. ***Transl Oncol.*** 2021 Aug; 14(8): 101128. PMCID: PMC8170159

CONCLUSIONS

We have initiated the proposed studies and finished analyzing the genomic and transcriptomic data showing the overexpression of P4HA1 in PCa. We are now performing various bioinformatics analysis of gene expression using P4HA1 knockdown prostate cancer cell RNA. We have also constructed PCa TMA. The TMA contains multiple prostate cancer tissues and collected from diverse demographics including African American prostate cancer patients. This TMA contains wide ranging PCa tissues with different Gleason scores. During this funding period, we have optimized immuno-histochemical staining of prostate cancer tissues and TMA's using P4HA1 specific antibodies. The staining was evaluated by expert pathologist and scored for P4HA1 staining intensity. Currently we are performing statistical analysis to find the correlation between P4HA1 staining intensity and prostate cancer progression. We have also obtained PC3 and DU145 cells with Luciferase tag and performing the knockdown of P4HA1 to utilize in mice models to study the role of P4HA1 in prostate cancer metastasis. Currently, we have initiated the *in vivo* studies to evaluate the role of P4HA1 in prostate cancer metastasis and also the effect of targeting P4HA1 using PythiDC in reducing the prostate tumor growth.

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