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14. ABSTRACT

We hypothesize that GR mediates resistance to enzalutamide in advanced prostate cancer through transactivation of SGK1 and activation of a non-luminal gene expression program. In this proposal, we aim to further explore the relationship between GR, SGK1, and enzalutamide resistance through experimental manipulation of GR and SGK1 in our previously reported laboratory models of enzalutamide resistance. In this research period, we engineered a CRISPR-mediated SGK1 deletion model of enzalutamide-resistant prostate cancer and observed modest reduction in tumor growth in response to enzalutamide. Our observation that SGK1 correlates with loss of AR signaling and EMT directly led us to the discovery that the immunomodulatory Wnt regulated cytokine, DKK1, is upregulated in AR-independent prostate cancer. We are currently validating whether DKK1 can be a useful biomarker and therapeutic target of this disease subtype.

15. SUBJECT TERMS

Androgen Receptor, AR; Glucocorticoid Receptor, GR; Serum and Glucocorticoid-Regulated Protein Kinase 1, SGK1; Epithelial-Mesenchymal Transition, EMT; Castration-Resistant Prostate Cancer, CRPC; Dickkopf-1, DKK1

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INTRODUCTION

Castration-resistant prostate cancer (CRPC) inevitably develops resistance to the 2nd generation androgen receptor (AR) antagonist, enzalutamide, despite continued enzalutamide-mediated suppression of AR activity. A recent study from the Sawyers laboratory found that the nuclear hormone receptor transcription factor, glucocorticoid receptor (GR), can activate an overlapping set of AR target genes and can mediate resistance to enzalutamide in a mouse xenograft model. This study and others have led to considerable efforts to develop GR antagonists for the treatment of GR-driven prostate cancer. However, pharmacologic inhibition of GR-dependent physiology is of unknown clinical feasibility and we proposed to test whether serum and glucocorticoid-regulated kinase 1 (SGK1), a target gene of the GR transcriptional program, might be more suitable for targeted inhibition. GR and serum and glucocorticoid-regulated kinase 1 (SGK1) were among the most upregulated genes in an in vivo model of enzalutamide resistance. We hypothesize that GR mediates resistance to enzalutamide through transactivation of SGK1. In this proposal, we aim to further explore the relationship between GR, SGK1, and enzalutamide resistance through experimental manipulation of GR and SGK1 in our previously reported laboratory models of enzalutamide resistance. This project has the potential to identify a critical and druggable component of the GR program to enable a safer and more effective strategy for treating AR-independent enzalutamide-resistant prostate cancers.

KEYWORDS

Androgen Receptor, AR
Glucocorticoid Receptor, GR
Serum and Glucocorticoid-Regulated Protein Kinase 1, SGK1
Epithelial-Mesenchymal Transition, EMT
Prostate Cancer
Castration-Resistant Prostate Cancer, CRPC
Dickkopf-1, DKK1

PROJECT GOALS
1. Validate SGK1 as a driver of GR-dependent resistance to enzalutamide
2. Explore the role of SGK1-mediated epithelial-mesenchymal transition in CRPC

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Phase 1 Milestones (proposed completion September 2016)

1. Modulate SGK1 expression in a previously established GR-driven prostate cancer cell line model and determine the effects on resistance to enzalutamide and AR activity
2. Determine the importance of SGK1-dependent cellular reprogramming in a 3D organoid human prostate cancer culture system.

Task	% Completed	Status
Clone SGK1 overexpression construct	100%	Complete

Generate SGK1 overexpressing cell models	75%	Ongoing
Clone SGK1 CRISPR	100%	Complete
Generate SGK1-deficient cell models	100%	Ongoing
Test SGK1 inhibitor	100%	Complete
Treating SGK1-deficient cells with enzalutamide	50%	Ongoing

Phase 2 Milestones (proposed completion September 2017)
Evaluate the importance of EMT-related cellular reprogramming in SGK1-dependent enzalutamide resistance

Task	**% Completed**	**Status**
Develop flow cytometry-based EMT assay	75%	Ongoing
Generate loss of function EMT models	75%	Ongoing
Generate gain of function EMT models	75%	Ongoing

ACCOMPLISHMENTS

In our preliminary data, we reported that SGK1 overexpression could promote the growth of prostate cancer resistant to enzalutamide in a xenograft model. Our previous data supported the importance of the translational isoforms of GR – GR-A, GR-C, GR-D - in mediating resistance to enzalutamide. Because of this, we tested whether that these GR isoforms could upregulate SGK1 in response to the presence of corticosteroid ligand. A previously described GR-expressing human prostate cancer cell line known as LREX[1] was plated as single cell clones. A single cell clone, expressing low levels of GR, was then expanded (LREX-GR^{low}). LREX-GR^{low} cells transduced with lentivirus expressing either GR-A, GR-C, or GR-D were then treated with DMSO alone or with increasing concentrations of hydrocortisone and RNA was collected at the time points specified. Our results demonstrate that all isoforms of GR can promote SGK1 transcription – albeit the ability of GR-D to promote SGK1 transcription appears is weaker compared to GR-A and GR-C (Figure 1).

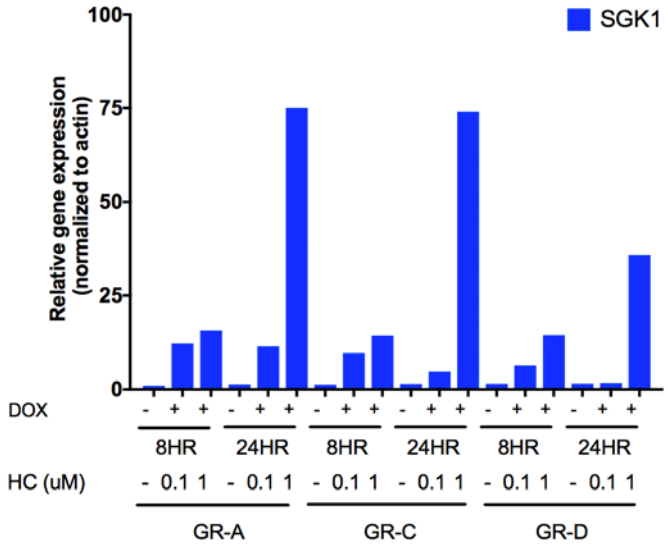

Figure 1. Glucocorticoid treatment leads to upregulation of SGK1 mRNA. SGK1 was quantitated using RT-qPCR from RNA collected from human prostate cancer cells expressing GR-A, C, or GR-D treated with increasing doses of hydrocortisone in vitro.

We next asked whether SGK1 was required for enzalutamide resistance. To address this question, LREX cells transduced with lentiviral CRISPR/Cas9 with guide RNAs targeting SGK1 or non-targeting (GFP) control were injected into castrated NOD/SCID mice and treated with enzalutamide from the day of injection. At 28 days post-implantation, sgSGK1 cells were growth suppressed compared to sgGFP control, but this difference did not reach statistical significance (586 mm^3 +/- 102 vs. 733 mm^3 +/- 59 vs., p = 0.27, Wilcoxon signed rank test). Based on our prior data that SGK1 expressing tumors demonstrated loss of AR signaling and an enrichment in genes associated with epithelial-mesenchymal transition, we hypothesized that loss of luminal features and an increase in mesenchymal associated gene expression might be an important new paradigm in understanding resistance to AR-directed therapies in mCRPC.

To investigate the clinical relevance of this finding, we turned our attention to the human prostate cancer genomics dataset and the cases that had lost expression of AR and PSA. To study prostate cancer with low AR activity, we classified metastatic prostate cancer specimens with publicly available RNA-seq data by AR and PSA expression. Of the recently published SU2C/PCF cohort of 150 patients[2], 11 (7%) show remarkably low PSA and AR expression ($AR^{low}PSA^{low}$) based on RNA sequencing analysis but no evidence of histological neuroendocrine features (small cell or large cell differentiation). Histological tumor content of $AR^{low}PSA^{low}$ and $AR^{high}PSA^{high}$ cancers was no different as assessed by histopathologic review. As was recently reported regarding the low prevalence of AR amplifications in NEPC, the $AR^{low}PSA^{low}$ mCRPC cases showed no AR amplifications or point mutations compared to 80% of the $AR^{high}PSA^{high}$ cases which either had a point mutation or amplification ($p < 0.001$). Of the patients whose tumors were biopsied prior to initiation of 2nd generation AR-directed therapy, patients with $AR^{low}PSA^{low}$ disease demonstrated a significantly shorter median time on treatment (2.5mo vs. 8.6mo, $p< 0.005$) and a higher prevalence of loss of Rb1 (50% vs. 4%, $p<0.0001$, Fisher's exact test) and p53 (80% vs. 51%, $p = 0.1$, Fisher's exact test) (Figure 2).

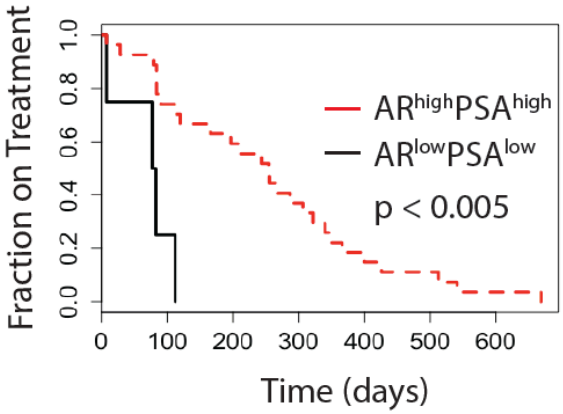

Figure 2. Patients with mCRPC with low AR and PSA expression respond poorly to AR-directed therapies.

Consistent with the relationship between GR, AR, and SGK1 that we have observed in our laboratory models, we found an inverse relationship between SGK1 mRNA and AR mRNA levels across all cases of castration-resistant prostate cancer (Pearson coefficient = -0.2) whereas we found a positive correlation between GR and SGK1 (Pearson coefficient = 0.4). To determine the set of genes enriched in $AR^{low}PSA^{low}$ we carried out a differential expression analysis

comparing 10 $AR^{low}PSA^{low}$ cases with 10 $AR^{high}PSA^{high}$ tumors selected to best match the organ biopsy site and the institution at which the biopsy was processed. Gene Set Enrichment Analysis (GSEA) of the set of differentially expressed genes demonstrated enrichment of non-luminal lineage programs including basal-type (NES 2.0, FDR q = 0.002), neuroendocrine (NES 2.1, FDR q = 0.005), and mesenchymal enrichment (NES 2.2, FDR q = 0.002) in the $AR^{low}PSA^{low}$ compared to the $AR^{high}PSA^{high}$ cases (Figure 3). Notably, of the neuroendocrine-associated genes, N-Myc was significant upregulated (4.4 vs 0.3 RPKM, Mann-Whitney test $p < 0.005$), Aurora Kinase A was not significantly upregulated (9.1 vs. 8.6 RPKM, Mann-Whitney test $p = 0.91$), and DLL3 was of borderline significance (0.6 vs. 0.23, $p = 0.08$, Mann-Whitney test) in $AR^{low}PSA^{low}$ compared to $AR^{high}PSA^{high}$ tumors. Collectively, this work supports the hypothesis that non-luminal programs including the EMT transcriptional program are enriched in a subset of mCRPC that has developed resistance to enzalutamide.

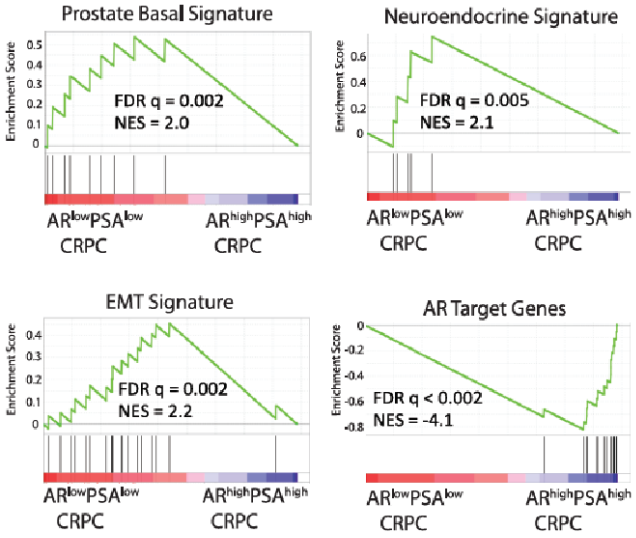

Figure 3. $AR^{low}PSA^{low}$ is enriched in non-luminal gene expression programs. GSEA analysis of the set of genes differentially expressed between $AR^{low}PSA^{low}$ and $AR^{high}PSA^{high}$ using NE (Neuroendocrine), Basal, and EMT (Epithelial-Mesenchymal Transition) and AR-signaling gene sets.

Given the absence of AR alterations and the high prevalence of tumor suppressor loss and the clinically aggressive phenotype of these tumors, we set out to identify genes expressed by this subtype that are known to be secreted to facilitate the clinical detection of this subtype through liquid biopsy. Among the list of differentially expressed genes, we focused on one of the most upregulated genes in the $AR^{low}PSA^{low}$ subset, Dickkopf-1 (DKK1) (11.2 RPKM vs. 0.28 RPKM, $q < 0.05$) (Figure 4A), because it is a mesenchymal oncogene, it has recently been implicated in tumor immune evasion[3,4], and it is secreted in sufficient quantities so as to be detectable in circulating blood specimens[5]. Upregulation of DKK1 in non-neuroendocrine $AR^{low}PSA^{low}$ mCRPC (9.2 FPKM vs. 0.99 FPKM, $p < 0.0001$) was confirmed in a DNPC validation cohort from Fred Hutchinson Cancer Center consisting of 77 metastatic biopsies annotated by AR and neuroendocrine gene expression status[6] (Figure 4B). Our finding that DKK1 is not elevated in neuroendocrine prostate cancer is consistent with a recent report and our analysis of this publicly available data in which DKK1 expression is upregulated in adenocarcinomas compared to NEPC defined by pathological/histological criteria (p = 0.005) (Figure 4C)[7].

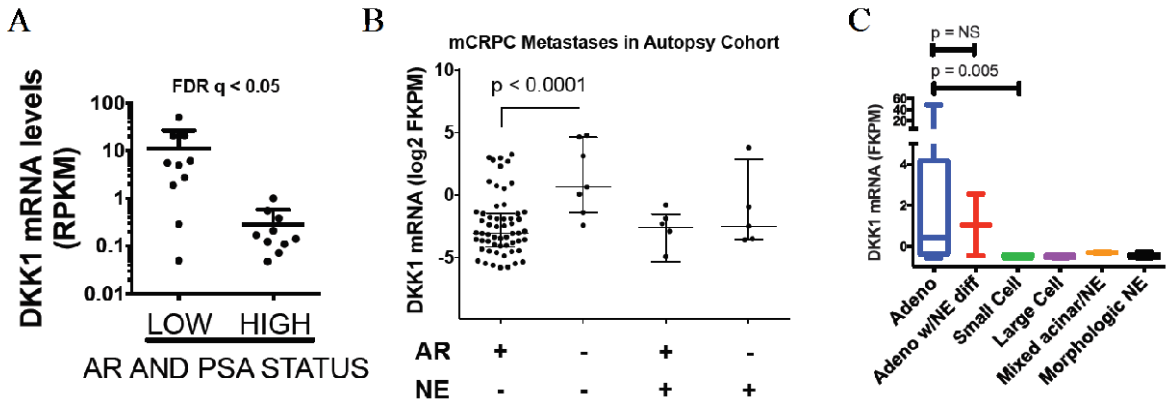

Figure 4. A. DKK1 mRNA levels derived from RNA-seq of $AR^{low}PSA^{low}$ (n=10) and $AR^{high}PSA^{high}$ (n=10). B. DKK1 mRNA levels derived from RNA-seq of mCRPC subdivided by AR and Neuroendocrine gene signatures from Fred Hutchinson Cohort. C. DKK1 mRNA levels derived from publicly available RNA-seq from Weill-Cornell Medical College mCRPC cohort.

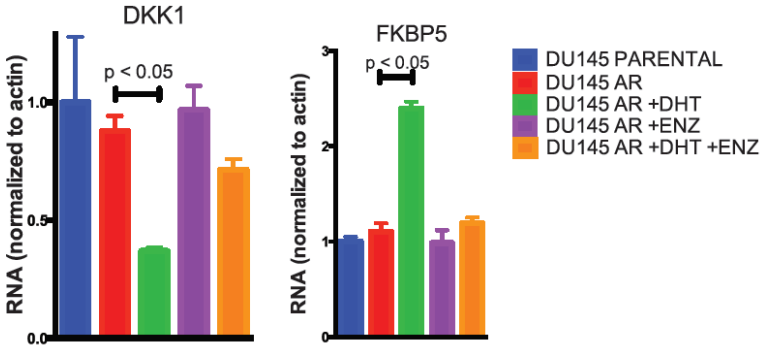

Figure 5. DKK1 and FKBP5 (AR target genes) RNA collected from DU145 cells expressing AR cDNA treated DHT, ENZ, or the combination

The finding that DKK1 was expressed in tumors with low levels of AR signaling prompted us to test whether AR signaling negatively regulated DKK1. To determine the contribution of AR signaling to the regulation of DKK1 levels, DU145 cells were transduced with a retroviral construct overexpressing AR. Treatment of these cells with 100nM DHT led to a reproducible reduction in DKK1 levels ($p < 0.05$) that could be reversed by the addition of the AR antagonists, enzalutamide (Figure 2A). We were not able to confirm any direct DNA binding by AR at the DKK1 locus with ChIP-seq of LNCAP and DU145 cells (data not shown).

Our interest in identifying a circulating biomarker for non-neuroendocrine PSA low prostate cancer led us to study the ability to detect the secretion of DKK1. Because DKK1 is a known secreted protein, we quantitated DKK1 protein in the cell culture medium of an AR negative non-NEPC cancer cell line (DU145) and we were able to detect DKK1 protein at a concentration of 4ng/mL/million cells, which could be selectively reduced through CRISPR/Cas9 targeting of the DKK1 locus (data not shown).

We next optimized an assay to detect DKK1 in the blood of prostate cancer patients. Serum DKK1 levels have been reported to be surprisingly higher than plasma DKK1 levels in healthy controls, likely due to the release of DKK1 from platelet-activation-mediated release of DKK1 into the serum fraction of whole blood[8 9]. Consistent with this report, we found serum DKK1 to be dramatically higher than plasma DKK1 in healthy controls (4613 vs. 665 pg/ml, $p < 0.0001$) (Supplementary Figure 1D). Whereas serum DKK1 levels in mCRPC patients were no different than levels measured in male healthy controls (3405 vs. 3529, pg/ml, $p = 0.4$) (Supplementary Figure 1E), plasma DKK1 levels were significantly higher in mCRPC patients compared to controls (1880 vs. 665 pg/ml, $p < 0.0001$) (Figure 2C). Of the mCRPC patients analyzed, 10/23 (43%) had plasma DKK1 levels higher than the maximum level measured in the 50 healthy controls analyzed. Next-generation DNA sequencing of tumors from 15 of these 23 patients demonstrated an increased frequency of alterations in TP53, PTEN, and RB1 in patients with DKK1 levels above the upper limit of normal compared to patients with normal DKK1 levels (data not shown).

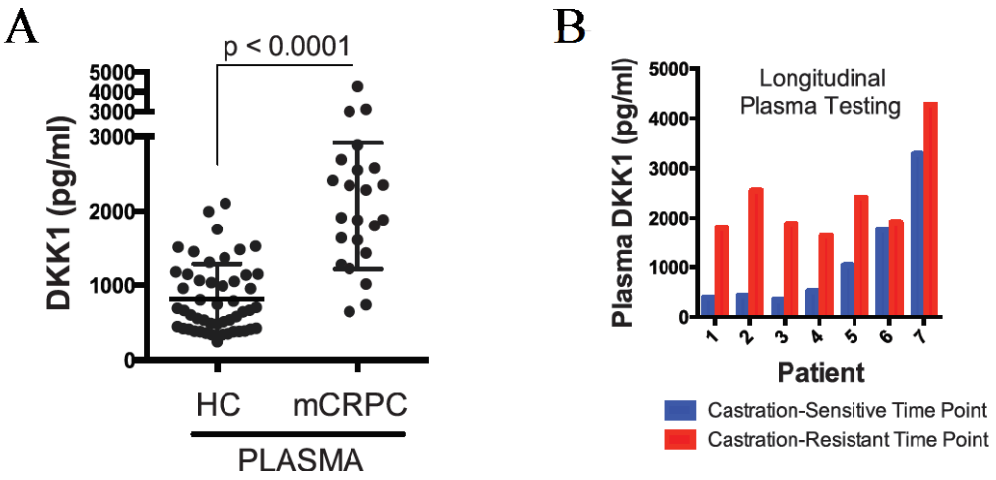

Figure 6. A. Plasma DKK1 protein quantitated in healthy controls and mCRPC patients. B. Plasma DKK1 protein quantitated in treatment naïve metastatic non-castrate patients (mCSPC) and matched samples from onset of mCRPC.

Based on our observation that AR activation by DHT could lower DKK1 levels, we hypothesized that the degree of FDHT uptake in a tumor should inversely correlate with the secreted serum DKK1 levels detected in patient peripheral blood-derived serum. To test this hypothesis, serum DKK1 and PSA levels were measured in a cohort of patients who had undergone PET-FDHT imaging[10]. Consistent with PSA as a direct readout of activation of AR, serum PSA and the average tumoral FDHT uptake were strongly positively correlated (R^2 = 0.7, p = 4e-11) (Figure 7A). In contrast, serum DKK1 and average FDHT uptake were inversely correlated (R^2 = -0.2, p = 0.1) and patients with higher tumoral FDHT uptake tended to have lower serum DKK1 levels compared to patients with lower tumoral FDHT uptake (p < 0.05) (Figure 7B).

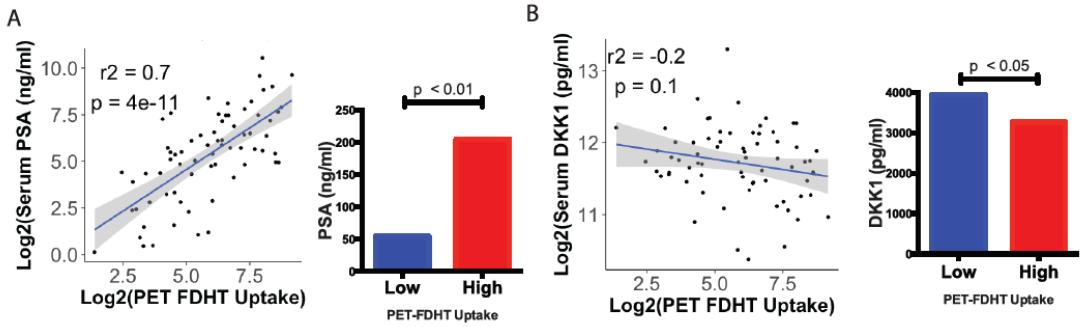

Figure 7. Serum DKK1 levels correlate with lower FDHT uptake in advanced prostate cancer A. Serum PSA levels and total F^{18}-DHT uptake in a cohort of patients with mCRPC B. Serum DKK1 levels and total F^{18}-DHT uptake in a cohort of patients with mCRPC.

Collectively, our data point to $AR^{low}PSA^{low}$ mCRPC as a subset of advanced prostate cancer with a poor prognosis and a distinct non-luminal biological profile. Given the known role of SGK1 in EMT, it is not surprising that $AR^{low}PSA^{low}$ mCRPC is enriched in the EMT gene expression program and that a known mesenchymal oncogene, DKK1, is overexpressed in this mCRPC subtype. We are now exploring whether DKK1, a secreted protein with known immunosuppressive activity, may be contributing to the immune exclusion phenotype seen in advanced prostate cancers.

PENDING GOALS: The major goals of this project have been completed.

TRAINING OPPORTUNITIES: This year, I had the opportunity to present the work resulting from this project as an abstract at ASCO 2017 entitled “The immunomodulatory protein Dickkopf-1 (DKK1) defines a non-neuroendocrine subtype of metastatic castration-resistant prostate cancer (mCRPC) with low AR and low PSA expression. J Clin Oncol 35, 2017 (suppl; abstr 5054). This abstract was awarded the ASCO merit award. To acquire additional data science skills that were critical for this project, I participated in a massively open online course on R for biomedical data science.

FUTURE PLANS FOR THIS PROJECT: This project has directly led to several ongoing projects:

1) **Validation of DKK1 as a biomarker of AR-independent prostate cancer.** Our observation that DKK1 is overexpressed in a subset of prostate cancer led us to develop a CLIA assay to detect DKK1 in patient plasma. We have also developed an RNA in-situ assay for DKK1 in tissue. We are now validating the concordance of these assays in retrospective and prospective cohorts and testing the extent to which DKK1 expression predicts loss of AR signaling in prostate cancer tissue.

2) **Exploring the contribution of DKK1 to immune evasion in prostate cancer.** We have developed a patient-derived 3D culture system to test the impact of DKK1 silencing on the activity of tumor resident immune cells.

3) **A Phase 1b/2 clinical trial targeting DKK1 for patients with treatment-refractory prostate cancer.** DKN-01, a DKK1 neutralizing antibody, has been found to be very well tolerated in phase 1 studies[11]. I am leading an early phase clinical trial that will test the activity of DKN-01 in mCRPC that has progressed on prior AR-directed therapies. The clinical trial will include extensive correlative molecular studies to determine the in vivo biological effect of DKK1 blockade.

IMPACT: This project has the potential to impact our understanding and the clinical management of mCRPC in the following ways:

1. By leading to the discovery of a potential biomarker of AR-independent disease, this project could facilitate earlier detection and intervention for this clinically aggressive subtype of prostate cancer.

2. The discovery that DKK1, a known immunosuppressive cytokine, is upregulated in AR-independent prostate cancer, might provide a mechanism for the observed immunologically "cold" phenotype of prostate cancer. Because DKK1 is also "druggable," this project also could provide a new therapeutic target to reactive the immune system as a strategy to treat prostate cancer. We are actively testing this hypothesis.

OTHER DISCIPLINES:

There is an increasing realization of the importance of molecular heterogeneity in leading to the difficulty in treating advanced prostate cancer. The use of non-invasive approaches to monitor this heterogeneity will be critical to advancing approaches to overcome it. In this project, we used a novel molecular imaging approach using 18F-DHT as a non-invasive indicator of AR activity. Our finding that lower DHT uptake correlates with higher serum DKK1 levels suggests that these results could be used as orthogonal approaches to detect this aggressive disease subtype.

TECHNOLOGY TRANSFER: nothing to report.

SOCIETY: nothing to report.

CHANGES/PROBLEMS: nothing to report.

PROJECTS: nothing to report.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name: David Wise
Project Role: Project PI
Researcher Identifier: N/A
Nearest person month worked: 7
Contribution to Project: David Wise planned all aspects of this project and contributed to the experimental procedures.

Name: Sarala Kal
Project Role: Laboratory Technician
Researcher Identifier: N/A
Nearest person month worked: 12
Contribution to Project: Sarala Kal performed tissue culture, cell line engineering, and biochemical techniques, including immunoblotting and qRT-PCR.
Funding Support: 5R01CA193837-02 (PI: Charles Sawyers)

SPECIAL REPORTING REQUIREMENTS: None

APPENDICES: None

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