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14. ABSTRACT The overarching goal of this grant award is to characterize differential splicing of oncogenes and tumor suppressor genes in prostate cancer disparities between African American (AA) and Caucasian American (CA) prostate cancer (PCa). In year 1 of this award, we have focused our efforts on two oncogenes, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (<i>PIK3CD</i>) and fibroblast growth factor receptor 3 (<i>FGFR3</i>), undergoing population-dependent differential splicing where the AA-specific variants are engendered with a more aggressive oncogenic phenotype <i>in vitro</i> and <i>in vivo</i> . Full-length cloning of the AA and CA variants of both <i>PIK3CD</i> and <i>FGFR3</i> have been accomplished. PCa cell lines genetically manipulated to predominantly express the AA-variant of <i>PIK3CD</i> or <i>FGFR3</i> exhibit greater proliferative and invasive capacity. Detailed analysis of PCa cell lines over-expressing the AA-variant of <i>PIK3CD</i> revealed enhanced activation of the PI3K/AKT pathway compared to the same lines over-expressing the CA-variant. Moreover, proliferative capacity of the CA-variant lines was sensitive to inhibition by CAL-101, a small molecule inhibitor designed specifically against PIK3CD. In contrast, proliferative capacity of the AA-variant lines was resistant to CAL-101 inhibition. And these findings (CA variants sensitive and AA variants insensitive to CAL-101) were recapitulated in a xenograft mouse model of proliferation. We are currently testing a xenograft mouse model of metastasis. Year 2 will focus on <i>in vitro</i> and <i>in vivo</i> characterization of the AA and CA variants of <i>FGFR3</i> .					
15. SUBJECT TERMS prostate cancer, cancer health disparities, alternative splicing, African American, Caucasian American, oncogenes, tumor suppressor genes					
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1. INTRODUCTION

There are striking population (race) disparities in prostate cancer risk and survival outcome borne out of current health statistics data. This is particularly evident between African Americans (AA) and their Caucasian American (CA) counterparts. Epidemiologic studies have shown that higher mortality and recurrence rates for prostate cancer are still evident in AA men even after adjustment for socioeconomic status, environmental factors and health care access. Thus, it is likely that intrinsic biological differences account for some of the cancer disparities. Our overarching hypothesis is that the biological component of prostate cancer health disparities is due, in part, to population-dependent differential splicing of oncogenes and tumor suppressor genes in cancer specimens. The application of genomic approaches has identified splice variants in AA specimens, but absent in CA specimens, encoding more aggressive oncogenic proteins, thereby producing a more cancerous phenotype.

2. KEYWORDS

Prostate cancer, cancer health disparities, alternative RNA splicing, African American, Caucasian American, oncogenes, tumor suppressor genes, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta, fibroblast growth factor receptor 3

3. ACCOMPLISHMENTS

Year 1 goals as stated in SOW:

Specific Aim 1. To define splice variant pairs (AA-specific variant versus CA-counterpart variant) associated with differential oncogenic behavior *in vitro*, and to delineate the mechanism of action.

Task 1. Full-length cloning and *in vitro* validation of splice variant pairs. Subtasks will be run concurrently and are as follows:

- 1a. Full-length cloning of splice variant pairs and ectopic over-expression into PCa cell lines.
- 1b. *In vitro* validation of differential oncogenic behavior by full-length splice variant pairs. Splice variant pairs (e.g. AA-specific versus CA-counterpart variant of PIK3CD and FGFR3) will be individually over-expressed in the same PCa cell line background, and screened for differential oncogenic behavior.
- 1c. *In vitro* validation of differential protein/enzyme activity by full-length splice variant pairs. Splice variant pairs will be individually over-expressed into appropriate cell line for enzyme activity assays and/or assessment of downstream activation of cell signaling components. Activation of downstream signaling components by splice variants will be assessed, for example, by measuring phosphorylation of downstream signaling components with phospho-specific antibodies (e.g. phospho-Akt, phospho-ERK, etc.).

Task 2. *In vitro* screening and full-length cloning of additional splice variant pairs. Subtasks will be run concurrently and are as follows:

- 2a. Exon-targeting and splice junction-targeting siRNAs will be used in appropriate PCa cell lines to identify splice variant pairs exhibiting differential oncogenic behavior following knockdown.

- 2b. From subtask 2a, we will select 5-10 splice variant pairs that exhibited differential oncogenic behavior for full-length cloning and ectopic over-expression in appropriate cell lines.
- 2c. Cell lines over-expressing individual full-length variant pairs (e.g. AA-specific variant versus CA-counterpart variant) will be validated *in vitro* for differential oncogenic behavior using *in vitro* screens described in subtask 1b. We will also test for differential sensitivity of splice variant pairs to small molecule inhibitors, if available.
- 2d. Cell lines over-expressing individual variant pairs (e.g. AA-specific variant versus CA-counterpart variant) will be screened *in vitro* for differential protein/enzyme activity and cell signaling as described in subtask 1c. We will also test for differential sensitivity of splice variant pairs to small molecule inhibitors, if available.

Specific Aim 2. To characterize oncogenic differences of splice variant pairs *in vivo* using xenograft animal models.

Task 1. Validate differential oncogenic behavior of the splice variant pair for PIK3CD *in vivo*. Stably expressed S (AA-specific) or L variants (CA-counterpart) of *PIK3CD* in appropriate cell line(s) will be transplanted (1×10^6 to 10^7 cells) into male SCID-NOD immuno-deficient mice for proliferation and metastasis assays.

Task 2. Validate differential oncogenic behavior of additional splice variant pairings *in vivo*. We will test *in vivo* an additional 4-9 splice variant pairings defined in Aim 1, Task 1, Subtasks 1b-1c (e.g. one variant pairing could be the AA-specific and CA-counterpart variants for *FGFR3*), or defined in Aim 1, Task 2, Subtasks 2c-2d.

Year 1 major accomplishments include the following:

i. Full-length cDNA cloning of AA and CA variants of *PIK3CD* and *FGFR3*

We have cloned the full-length cDNA sequences of the AA and CA splice variants for the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (*PIK3CD*) and fibroblast growth factor receptor 3 (*FGFR3*) genes (**Figure 1**). Cloning was accomplished by 5'- and 3'-RACE in AA and CA PCa cell lines, and an epitope tag (6×His tag) was attached to the 3'-end of each variant prior to subcloning into the plasmid vector pGEM using standard approaches in our lab. The AA variants of both genes have not been described in the literature nor the UCSC (genome.ucsc.edu) or Ensembl Genome Browsers (www.ensembl.org). The AA *PIK3CD-short* variant is missing exon 20 (encoding a 54 amino acid long segment within the kinase catalytic domain) while the AA *FGFR3-short* variant is missing exon 14 (encoding a 41 amino acid long segment within the tyrosine kinase domain) (**Figure 2**). The CA *PIK3CD* and *FGFR3* versions are long variants, suggesting that kinase activity of the AA variant proteins may differ from the CA variants. To date, the AA and CA cDNA variants of *PIK3CD* and *FGFR3* have been subcloned into the mammalian expression vector pcDNA3, and the *PIK3CD* cDNA variants have been sequence validated and ectopically and stably expressed into PCa cell lines for *in vitro* and *in vivo* investigations completed in Year 1 (see description below). We plan to likewise stably express the *FGFR3* long and short cDNA variants in PCa cell lines for Year 2 studies.

FIGURE 1. Full-length cDNA sequences of the AA short and CA long variants for *PIK3CD* and *FGFR3*.

>PIK3CD-long CA variant. Sequence shown below is the full length coding sequence of CA variant 3,135 nt, 1,044 aa. PIK3CD-short AA variant is missing exon 20 (which is highlighted in yellow in the CA variant sequence below).

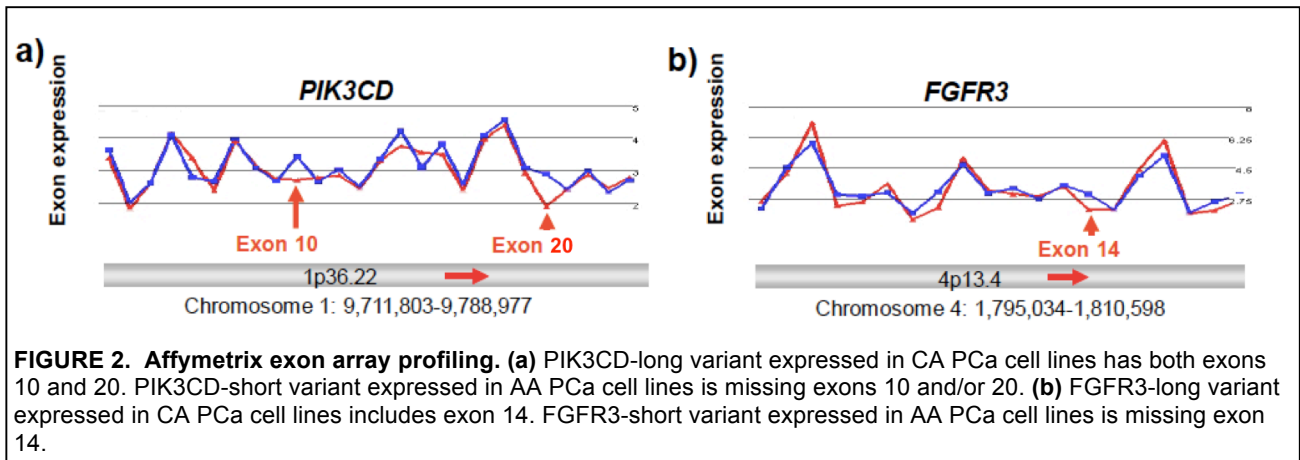
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>FGFR3-long CA variant. Sequence shown below is the full length coding sequence of CA variant 2,427 nt, 809 aa. FGFR3-short AA variant is missing exon 14 (which is highlighted in yellow in the CA variant sequence below).

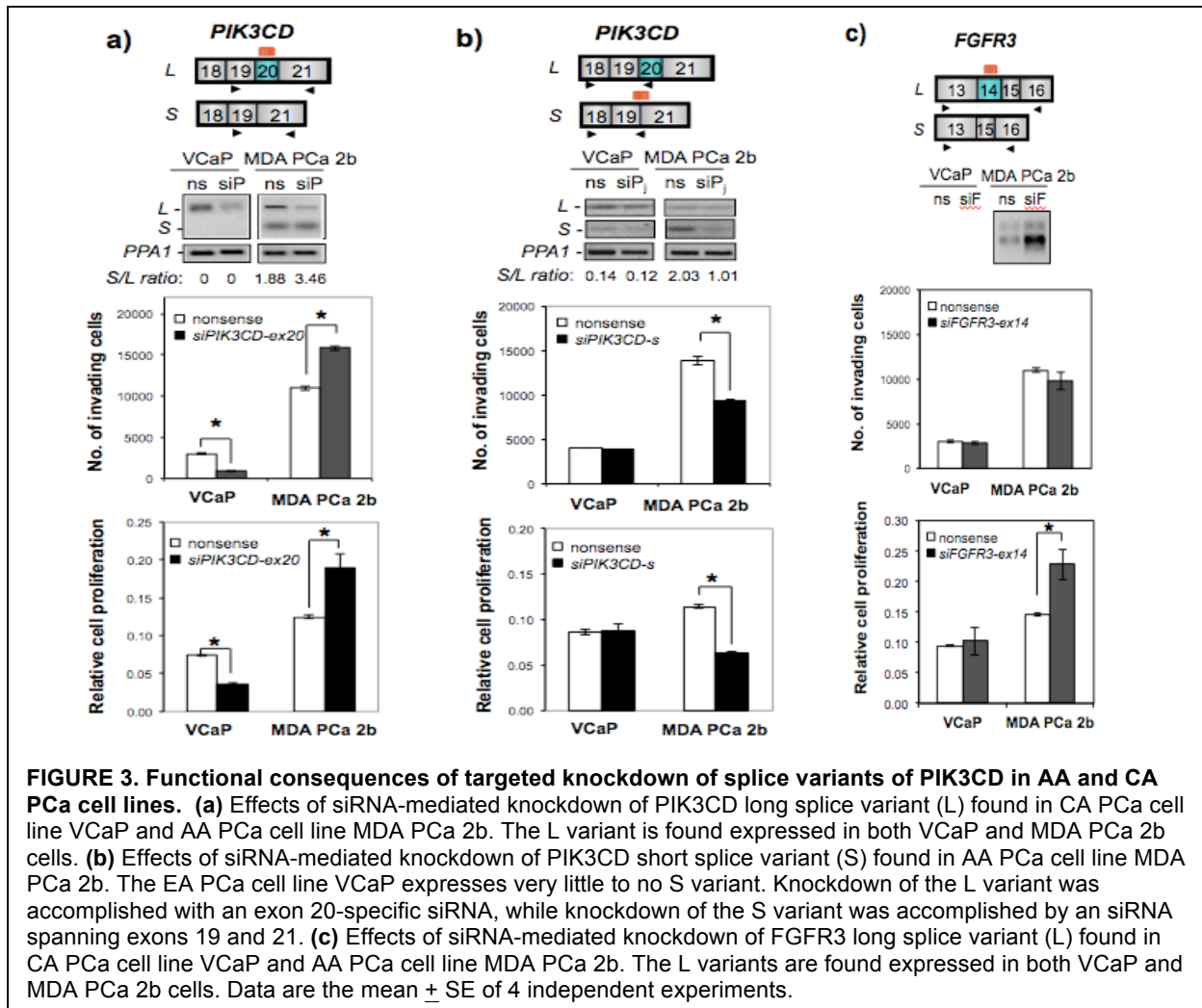
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- ii. **Validation that the AA short variant of *PIK3CD* is phenotypically more aggressive than the CA long variant based on *in vitro* assays.** The assays include genetic manipulation of AA and CA variant expression in PCa cell lines, proliferation assays, invasion assay in Matrigel

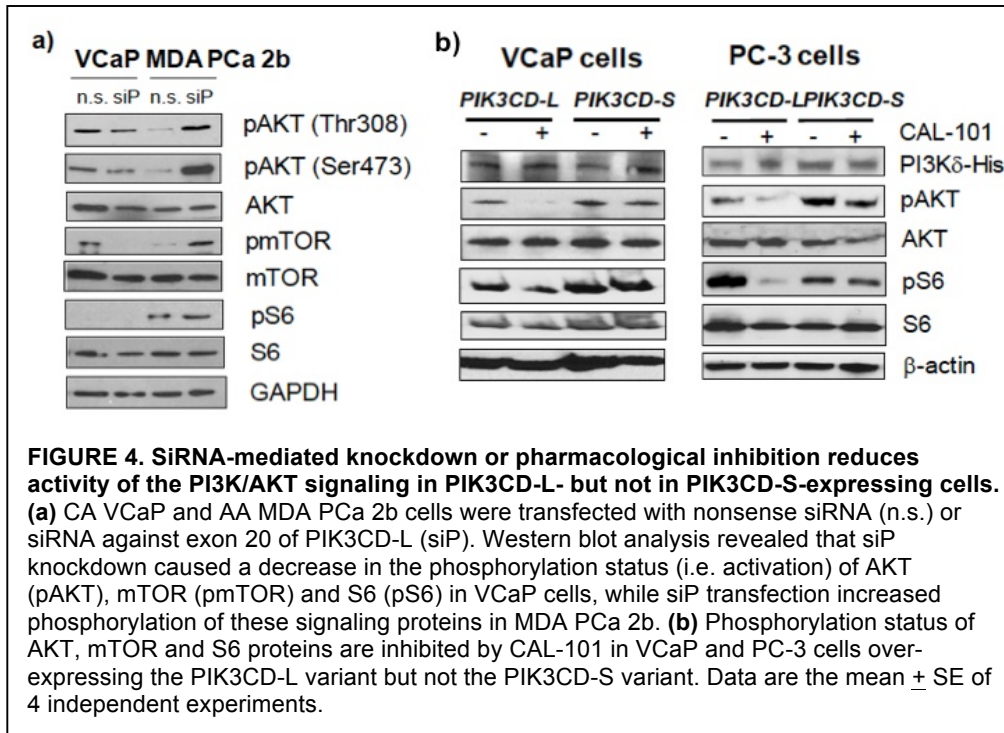
We demonstrate that the AA short splice variant (S variant) for *PIK3CD* encodes a more



aggressive version of the gene (i.e. leading to greater proliferation and invasion of cancer cells) compared to the CA long (L) variant counterpart. SiRNA-mediated knockdown of the L variant in CA PCa cell line VCaP (**Figure 3A**, middle panel qRT-PCR, siP lane) leads to a decrease in Matrigel invasion and a decrease in proliferation as assessed by BrdU incorporation (**Figure 3A**, bottom panels). By comparison, the AA PCa cell line MDA PCa 2b expresses both L and S variants, and knockdown of the L variant leads to predominant expression of the S variant and a corresponding increase in Matrigel invasion and increase in proliferation (**Figure 3A**, middle and bottom panels). Next, we investigated S variant knockdown. VCaP cells express little to no S variant; hence, targeted siRNA-mediated knockdown of this variant led to no change in Matrigel invasion and proliferation (**Figure 3B**). In contrast, targeted knockdown of the S variant in MDA PCa 2b cells leads to decreased Matrigel invasion and decreased proliferation (knockdown of S variant leads to predominant expression of L variant) (**Figure 3B**). These data indicate that the overall S to L ratio in MDA PCa 2b cells dictates the oncogenic profile of this AA PCa cell line. Namely, knocking down the L variant in MDA PCa 2b cells increases the S/L ratio, leading to a higher proportion of the aggressive S variant and consequently increased invasiveness and proliferation of the cell line. In contrast, knocking down the S variant in MDA PCa 2b cells decreases the S/L ratio, leading to a higher proportion of the less aggressive L variant and

consequently decreased invasiveness and proliferation of the cell line. An analogous increased proliferative behavior was obtained when the AA-‘specific’ variant (found exclusively or nearly exclusively in AA cell lines) to CA-counterpart variant (found in both EA and AA cell lines) ratio was increased for FGFR3 (**Figure 3C**).

The more aggressive invasive behavior observed in the AA PCa cell line MDA PCa 2b upon increasing the S/L variant ratio was associated with an augmented activation of the PI3K/AKT pathway. This was evidenced by the increased phosphorylation of AKT at amino acids Thr308 and Ser473, mTOR and ribosomal protein S6 (S6) (**Figure 4A**).

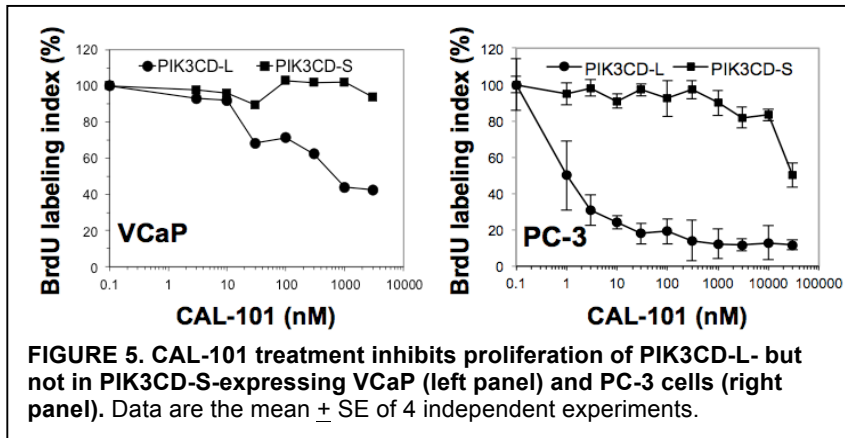


iii. **Demonstration in our *in vitro* assays that the AA variant of PIK3CD is resistant to inhibition by CAL-101, a small molecule inhibitor designed specifically against PIK3CD.** In contrast, the CA variant of PIK3CD is sensitive to inhibition by CAL-101

We subsequently stably over-expressed the AA PIK3CD-short (PIK3CD-S) and CA PIK3CD-long variants (PIK3CD-L) variants individually in the CA PCa cell lines PC-3 and VCaP. These stably transfected cell lines were tested *in vitro* for sensitivity to CAL-101 treatment (**Figure 4B**). CAL-101 is a PIK3CD inhibitor in clinical trials for various cancers. PCa cell lines over-expressing the CA PIK3CD-L variant exhibited a decrease in the activity of the PI3K/AKT pathway following CAL-101 treatment, as seen by a loss of AKT, mTOR and S6 phosphorylation. Remarkably, the same CA PCa cell lines stably over-expressing equivalent levels of the AA PIK3CD-S variant were completely resistant to CAL-101. In other words, there was no significant change in AKT, mTOR and S6 phosphorylation levels before and after CAL-101 treatment.

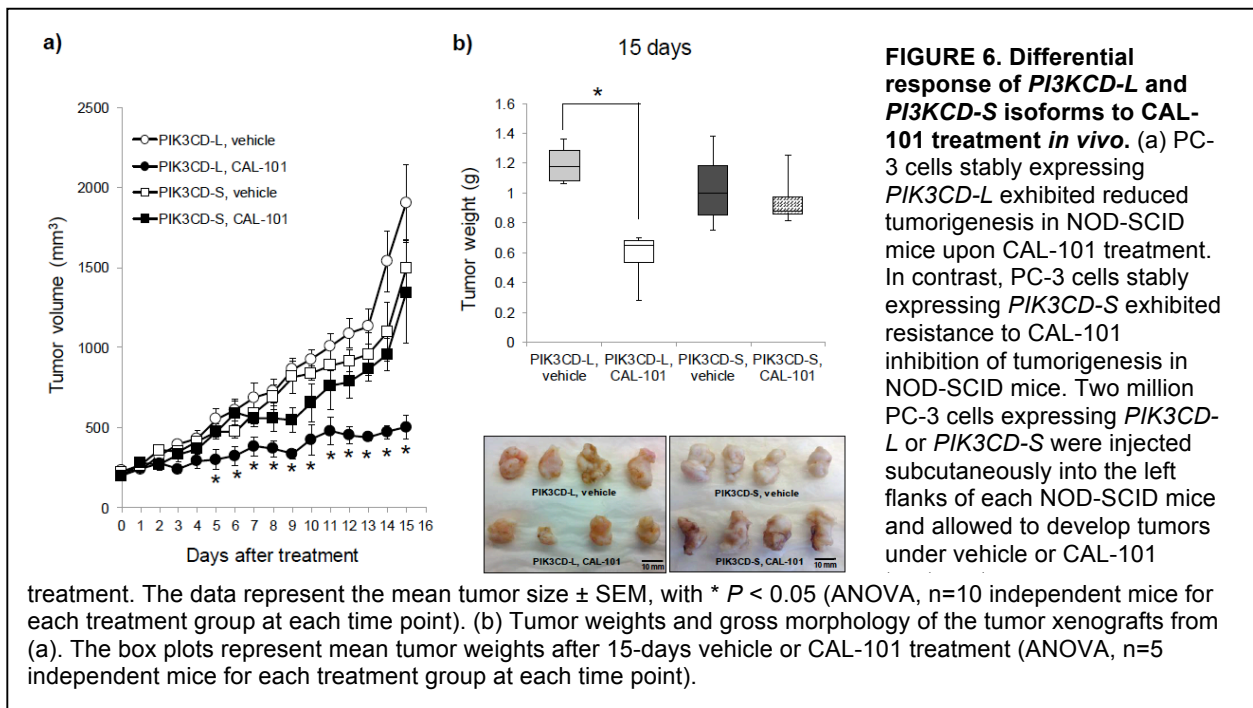
In cell proliferation assays, we demonstrate that BrdU labeling in VCaP and PC-3 cells over-expressing the CA PIK3CD-L variant was inhibited by CAL-101 in a dose-dependent manner,

while proliferation of VCaP and PC-3 cells over-expressing the AA PIK3CD-L variant were resistant to CAL-101 (Figure 5).



- iv. **Demonstration that subcutaneous injection of PCa cell line PC-3 over-expressing the AA PIK3CD-short variant into the hindflank of NOD-SCID mice (xenograft model for proliferation) is resistant to the anti-proliferative effects of CAL-101.** In contrast, PC-3 cells over-expressing the CA PIK3CD-long variant are sensitive to CAL-101 in the same xenograft model. Experiments are now underway to investigate a xenograft model for metastasis using the PC-3 cells over-expressing PIK3CD-long or PIK3CD-short.

Lastly, we demonstrate in a xenograft mouse model that growth of PC-3 cells over-expressing the AA PIK3CD-short variant were resistant to the inhibitory effects of CAL-101. By comparison, PC-3 cells over-expressing the CA PIK3CD-long variant were particularly sensitive to CAL-101 treatment (Figure 6).



Year 1 opportunities for training and professional development:

Year 1 of this proposal provided hands-on training for PhD graduate student Jacqueline Olender. The PI has served as Ms. Olender's mentor and she has participated in both the *in vitro* and *in vivo* work described herein. We are finalizing our xenograft mouse model experiments to investigate the metastatic potential of the PIK3CD-long and short variants over-expressed in the same PCa cell line described in **Figure 6**. Upon completion of these experiments, we plan to submit a manuscript for publication.

Dissemination of results and outreach to communities of interest:

During Year 1 of this grant, we had several freshman and sophomore students from local high schools (e.g. Thomas Jefferson High School for Science and Technology) rotate into our laboratory (1-3 weeks; 3-4 days per week). Students shadowed investigators/researchers in the PI's laboratory to observe and gain an appreciation of cancer health disparity research. Shadowing included didactic lectures to the students.

Year 2 goals:

- i. Submit manuscript by the end of 2014, related to work on CA PIK3CD-long and AA PIK3CD-short splice variants in prostate cancer health disparities
- ii. Fully characterize the AA FGFR3-short and CA FGFR3-long splice variants using the same *in vitro* and *in vivo* approaches outlined in our results section for PIK3CD.
- iii. Begin molecular cloning of 3rd set of splice variant cDNAs involved in prostate cancer disparities. Splice variant clones will be derived from AA and CA PCa cell lines, as was the case for PIK3CD and FGFR3.

4. IMPACT

Impact on the development of the principal discipline(s) of the project:

Principal discipline -- Understanding prostate cancer biology and disparities. Taken together, our *in vitro* and *in vivo* findings with the CA PIK3CD-long and AA PIK3CD-short variants provide evidence that differential splicing may play a role in PCa health disparities (our original hypothesis). Our future goal is to identify additional population-specific oncogene variants (i.e. FGFR3 which we are currently working on) that exhibit differential oncogenic behavior and/or sensitivity to small molecule inhibitors, thereby further supporting our hypothesis.

Impact on other disciplines:

Other disciplines -- Cancer chemoresistance. Our results in Year 1 demonstrate that the AA PIK3CD-short variant protein, but not the CA PIK3CD-long variant protein, is resistant to CAL-101, a small molecule inhibitor that has been specifically designed to inhibit PIK3CD and this inhibitor is undergoing clinical trials for treatment of hematological cancers. These findings have potential important clinical implications as it relates to population-specific differential splicing of oncogenes and primary chemoresistance.

Impact on technology transfer:

Our findings that the AA PIK3CD-short variant protein is resistant to CAL-101 has sparked interests in companies that are investigating small molecule inhibitors of kinases involved in cancer progression. These companies are gaining an appreciation that alternative splicing in kinases can affect the sensitivity these signaling proteins to cancer therapeutic agents. Our findings raise the issue related to prescreening patients for their variant protein in order to prognosticate whether a particular therapeutic agent will be efficacious in treating the cancer. We are in discussions with companies such as Celdara Medical (Lebanon, CT; <http://www.celdaramedical.com/>) concerning the leveraging of our findings.

Impact on society beyond science and technology:

Nothing to report

5. CHANGES/PROBLEMS

Changes in approach:

None

Actual or anticipated problems or delays:

None

Changes that had significant impact on expenditures:

None

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

None

6. PRODUCTS

Publications, conference papers, and presentations:

Invited speaking presentations

- i. American Association for Cancer Research, Sixth AACR Conference on The Science of Cancer Health Disparities, Atlanta Georgia, December 6-9, 2013. Presentation title: "Splice variants associated with African American prostate cancers promote oncogenesis and chemoresistance."
- ii. 4th Global Cancer Genomics Consortium Symposium, Kyoto University, Japan, November 14-15, 2014. Symposium on New Era of Molecular Medicine and Epigenetic Cancer Medicine: Cross section of Genomics and Epigenetics. Presentation title: "Methylation pattern, splicing factor expression and splice variants associated with African American prostate cancers promote oncogenesis and chemoresistance."

Website(s) or other Internet site(s):

None

Technologies or techniques:

None

Inventions, patent applications, and/or licenses:

- i. A provisional patent application has been filed. Application number: 61/948,218. Filing date: 3/5/2014. Application title: Companion Diagnostics for Cancer and Screening Methods to Identify Companion Diagnostics for Cancer Based on Splicing Variants

Other Products:

None

7. PARTICIPANTS & OTHER COLLABORATORS

Individuals working on this project:

Name:	Norman H Lee, PhD
Project Role:	PI
Nearest person month worked:	3
Contribution to project:	Direct and oversee entire project. Involved in experimental design and statistical analysis.

Name:	Bi-Dar Wang, PhD
Project Role:	Co-Investigator
Nearest person month worked:	3
Contribution to project:	Contributed to the cloning of variant cDNAs, in vitro and in vivo assays
Funding support:	Partially supported by GWU bridge support

Name:	Jacqueline Olender
Project Role:	PhD graduate student
Nearest person month worked:	12
Contribution to project:	Contributed to the cloning of variant cDNAs, in vitro and in vivo assays

Name:	Patricia Latham, MD
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Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to project:	Animal necropsy and immunohistochemistry of xenografts

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

None

Other organizations were involved as partners:

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

Nothing to report