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14. ABSTRACT

Recent studies demonstrate that for men with clinically localized, non-metastatic high-risk prostate cancer (PCa) receiving long-term androgen deprivation therapy (ADT) and dose-escalated radiotherapy (RT), a pre-RT PSA value greater than 0.5 ng/ml after ADT predicts for decreased time to distant metastases. African-American (AA) men were significantly associated with failure to achieve a pre-RT PSA value less than 0.5 ng/ml. These elevated PSA levels are a direct result of sustained androgen receptor signaling despite ADT. AA men would benefit greatly from more potent anti-androgenic therapies in combination with radiation. Several independent studies have shown that the aryl hydrocarbon receptor (AhR) can regulate androgen receptor signaling. Evidence is emerging that AhR may have intrinsic functions that promote prostate cancer progression. Published results from our laboratory recently revealed that AhR is constitutively active in advanced prostate cancer cell lines and no longer requires ligand activation for activity. Chemical and shRNA mediated ablation of AhR signaling decreases expression of androgen receptor. The ability of AhR to regulate androgen receptor signaling in advanced prostate cancer cells identifies it as a prime target to ablate androgen receptor signaling in AA men.

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1. INTRODUCTION:
   The objective of this proposal is to compare the level and effect of AhR activity in African-American (AA) and Caucasian-American (CA) prostate cancer cells and tissues. We hypothesize that constitutive AhR signaling is responsible for the sustained androgen receptor signaling seen in castration resistant prostate cancer (CRPC) and that AA men have elevated AhR activity compared to CA men. We have undertaken experiments to support previous data that knockdown of AhR expression ablates androgen receptor signaling. The current experiments show that over-expression of AhR in androgen sensitive AA cell lines enhances androgen receptor signaling. We have also added to the number of AA prostate cancer tissue samples in comparison to CA samples. Identification of this unknown mechanism of androgen receptor activation will provide a novel therapeutic target to ablate androgen receptor signaling that is not achieved by current therapies and could directly address the health disparity associated with prostate cancer. Previous studies have not considered the existence of constitutive AhR signaling in prostate cancer or its ability to promote prostate cancer progression.

2. KEYWORDS
   Aryl Hydrocarbon Receptor, Androgen receptor, Prostate cancer, Castration resistant prostate cancer, African-American, Prostate cancer health disparity.

3. ACCOMPLISHMENTS

   Major Task 1: Compare basal AhR activity in AA E006AA and E006HT cells to CA LNCaP and C4-2 prostate cancer cells.

   Major Task 3: Compare AhR activity in prostate cancer and matched normal tissue from AA and CA men with low, moderate and high Gleason scores as well as varying clinical PSA levels using AhR antibodies.

   Milestones Achieved: Identification of AhR as a regulator of androgen receptor signaling in AA prostate cancer cell models. Correlate AhR expression and nuclear localization to PSA progression in AA and CA prostate cancer tissue samples.

   Previous results have shown that African-American prostate cancer cell lines have increased AhR protein expression compared to their Caucasian-American (CA) counterparts. African-American Androgen insensitive cell line, E006HT, has a higher AhR expression than the androgen-sensitive cell line, E006AA. These set of experiments will validate the effect of AhR expression on androgen receptor signaling.

   Methods: E006AA cells were transfected with an AhR expression vector and cloned by serial dilution. Following expansion, protein samples were isolated using a commercially available cell lysis buffer (cell signaling) for total protein. Protein samples were resolved by SDS-PAGE and transferred to a PVDF membrane. Immunoblotting was carried out with AhR antibody (1:500). Blots were washed three times (15 minutes each) with TBST. The blots were then incubated in 1:2500 dilution in secondary antibody and washed three times (15 min each) with TBST, three times (10 min each) with TBS and once with ddH20 (10 mins). Bands were visualized with enhanced chemiluminescence kit as specified by manufacturer.

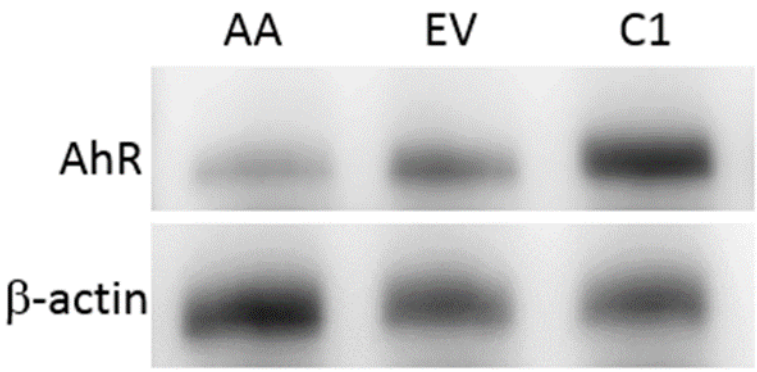

Figure 1: Expression of AhR protein in E006AA parental cell line (AA), E006AA empty vector control (EV) and E006AA Clone 1 (C1). Relative AhR expression is normalized with β-actin.

Results: Despite the anti-androgenic activity of many AhR ligands, studies concerned with the intrinsic functions of AhR have found that overexpression of the receptor may promote carcinogenesis. We have already shown that C4-2 cells endogenously overexpress AhR and that the increased expression is accompanied by nuclear localization and constitutive activity. In addition, our previous data revealed that androgen-insensitive African-American cell line, E006HT has enhanced AhR activity compared to C4-2 cells. Our previous data confirms overexpression of AhR protein in the African-American pair compared to the CA cell lines. The results below, show that ectopic over-expression of AhR in androgen sensitive African-American prostate cancer cell line E006AA enhances both AhR and AR expression as well as the expression of AhR responsive gene CYP1B1.

Methods: qRT-PCR was used to determine expression of AhR AR, CYP1B1 and KLK3 in E006AA parental cells, E006AA empty vector control (EV) and E006AA AhR over-expressing clone (C1). We isolated total RNA from cell monolayers using RNeasy Mini Kit (Qiagen) and 2 µg of the total RNA was reverse-transcribed using the Superscript II kit (Invitrogen), according to the manufacturer's recommendations. The cDNA served as a template in a 25 µl reaction mixture and was processed using the following protocol: an initial denaturation at 95˚C for 3 min, followed by 39 amplification cycles (95˚C for 10s and 55–65˚C for 30s), 95˚C for 10s, 65˚C for 5s and 95˚C for 50s. The 25 µl qPCR reaction mixture was mixed with GoTaq qPCR Master Mix (Promega). Melt curve analyses performed after each run was used to ensure a single product. Relative gene expression was determined using the ΔΔCq calculation method. The primer sequence and specificity of primer sets were validated in previously published work.

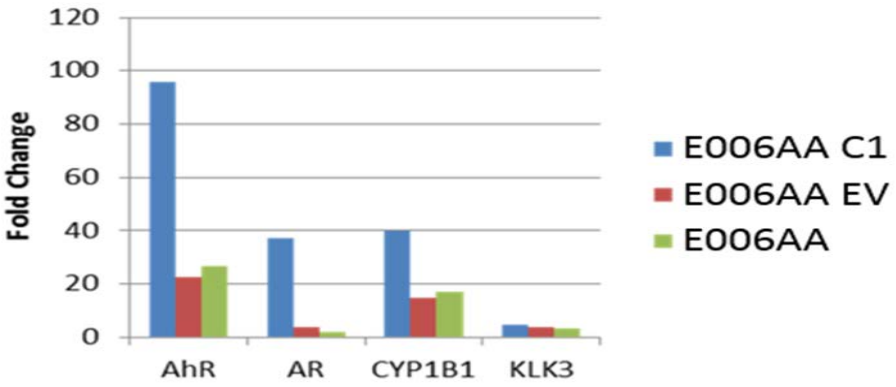

Figure 2: Quantitative real-time PCR was performed to determine AhR, AR, CYP1B1 and KLK3 gene expression in E006AA parental, control(EV) and over-expressing clone (C1).

AhR protein and mRNA expression is associated with phases of rapid proliferation and differentiation in certain tissues. AhR-defective cell lines demonstrate a reduced proliferation rate. Several studies suggest AhR promotes proliferation in the absence of exogenous ligands, whereas treatment with exogenous ligands inhibits cellular proliferation. Functional interaction between AhR and cyclin D1 has been suggested as a mechanism for cell cycle regulation. Cyclin D1 regulates cell cycle progression from G1 to S by regulating phosphorylation of retinoblastoma (Rb). Changes in AhR expression has been suggested to alter the phosphorylation of Rb by cyclin D1 thus regulating cell progression from G1 to S. Our results show that increasing AhR expression in E006AA cells increased G1 to S-phase cell cycle transition compared to the empty vector control and E006AA parental cell line.

Methods (Cell Cycle Analysis): E006AA parental cells, clones overexpressing AhR (C1) and E006AA empty vector (EV) human prostate cancer cell lines were grown in DMEM complete media. Cells were collected as a single cell in wash buffer and fixed overnight in cold 100% ethanol. Fixed cells were washed with PBS+1% FBS, resuspended in PBS containing propidium iodide (40µg/ml) and 50µl of RNase A solution, Then incubated for 3 hours at 4°C and immediately analyzed by flow cytometry to obtain cell cycle profiles.

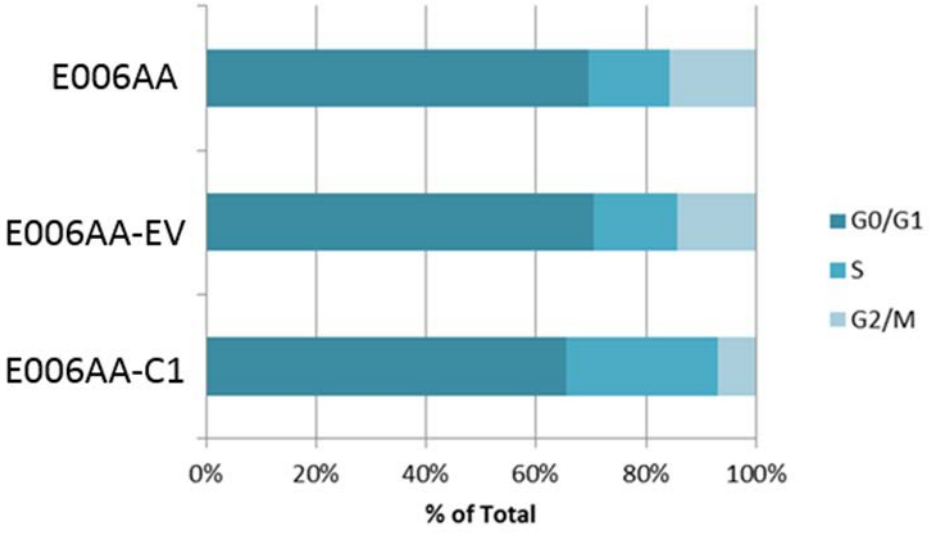

Figure 3: Cell cycle analysis of E006AA parental, control(EV) and over-expressing clone (C1) by flow cytometry. % of cells in G0/G1 phase, S-phase, and G2/M phase was determine by flow cytometry.

Methods (Proliferation Assay): E006AA overexpressing AhR (C1) and E006AA empty vector (EV) cells along with the parental E006AA cells were resuspended to a final concentration of 1.0×10^{5}/mL in DMEM. 100 µl of the cell suspension (10,000 cells) were added to each well of the 96-well plate. The micro plates were incubated at 37°C for 24 hours in a humidified, 5% CO2 atmosphere. Per manufacturer's instructions, following incubation, BrdU working stock was prepared by diluting the BrdU Label 1:2000 into fresh tissue culture media. 20 µl of this working stock was added to each well. BrdU was incubated with cells for 24 hours in the tissue culture incubator. Absorbances were read at 490 nm using the Synergy H1m multimode

micro plate reader.After removing contents of wells, 200 µl of the Fixative/Denaturing Solution was added to each well and they incubated for 30 min at room temperature. 1:100 Anti-BrdU Antibody diluted in the Antibody Dilution Buffer. 100 µl of this solution was added to each well and incubated for 1 hour at room temperature. Wells were washed 3 times with automatic plate washer with 1X Wash Buffer. 100 µl of Peroxidase Goat Anti-Mouse IgG HRP conjugate was added into each well and incubated for 30 min at room temperature. After each well was washed 3 times with 1X Wash Buffer, 100 µl of Substrate Solution was added to each well and incubated at room temperature for 15 min. 100 µl of Stop Solution was added to each well in the same order as the previously added Substrate Solution. Absorbance was measured in each well using a spectrophotometric plate reader at dual wavelengths of 450-540 nm.

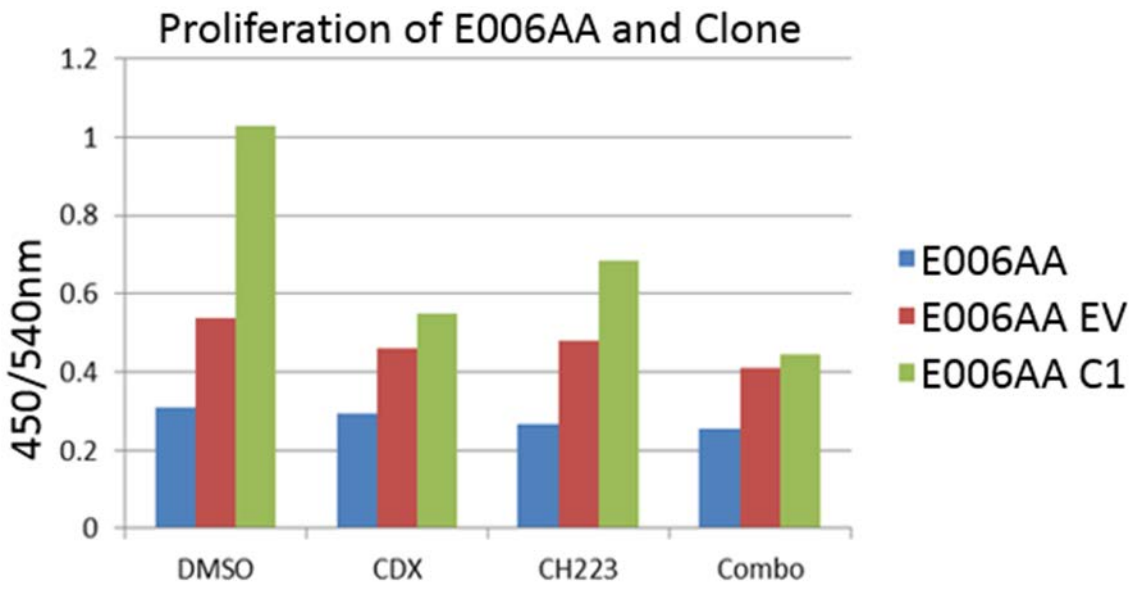

Figure 4: Growth of E006AA parental, control(EV) and over-expressing clone (C1) in the presence and absence of AR (casodex/CDX) and AhR inhibitor (CH223) was determined using an BrdU incorporation assay.

Results: Prostate cancer tissue samples were stained with AhR antibody. An Weight Index (WI)-score for AhR cytoplasmic and AhR nuclear intensity was determined by screening with digital pathology using Leica Biosystems imaging and individual sample WI-scores were also verified by an independent pathologist. We evaluated the correlation between WI of total, AA and CA samples. We were unable to gain statistical significance due to the limited number of samples. However, the trends indicate a comparable correlation of total WI for AA and CA samples. Noteworthy, there was a large difference in the correlation of nuclear WI for AA samples with an R^2 of 0.7409 and the nuclear WI of CA which revealed an R^2 of just 0.1317. Surprisingly, there appears to be a negative correlation emerging for the cytoplasmic WI of AA samples and PSA. Higher reported PSA showed lower cytoplasmic AhR reactivity.

Methods: Briefly, tissue slides were dewaxed with xylene, rehydrated in graded concentrations of alcohol and treated with hydrogen peroxide prior to blocking with normal goat serum. The slide were incubated with AhR antibody. The slides were incubated with 1 mg/ ml rabbit anti-AhR polyclonal antibody (1:100 dilution) overnight at 4^{o}C. Antibody binding was detected using a labeled streptavidinbiotin kit with 3'3-diaminobenzidine as the chromogen (DAKO). Hematoxylin was used as a counterstain. Results of staining was be scored by digital pathology and independent observers by rating staining intensity from 0 for below the level of detection to 3 for strongest expression multiplied by percent positive cells within sample.

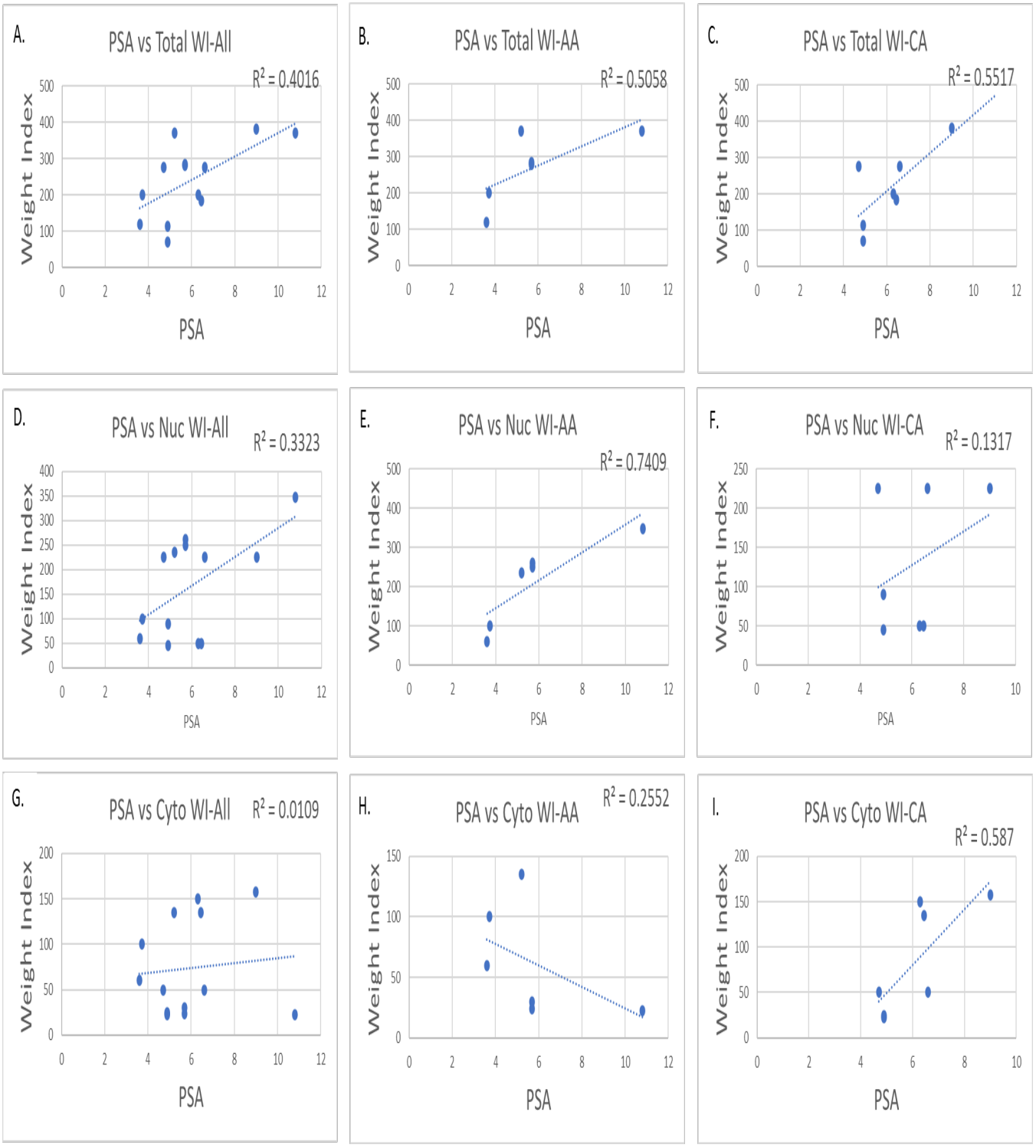

Figure 5: Weight Index (WI) was calculated for prostate cancer tissue samples. WI is the product of the staining intensity (0-3) of the sample and the percent positive cells. Patient PSA at time of diagnosis was plotted vs (A) total WI of all samples, (B) total WI of African-American/AA samples, (C) total WI of Caucasian-American/CA, (D) nuclear WI of all samples, (E) nuclear WI of AA samples, (F) nuclear WI of CA samples, (G) cytoplasmic WI of all samples, (F) cytoplasmic WI of AA samples and (I) cytoplasmic WI of CA samples.

4. IMPACT
   Nothing to report.

5. CHANGES/PROBLEMS
   Our lab experienced a significant and wide-spread mycoplasma contamination in September 2019. The cell culture incubator and biosafety cabinet was sterilized and recertified. Cultures of E006AA and E006HT as well as resulting clones were successfully decontaminated with a combination of two antibiotics, piperacillin and ciprofloxacin, at a concentration of 10 µg/ml each.

   Due to the Covid-19 pandemic. All University offices and labs closed in March 2020. We will regain access to the lab in August 2020. However, the University will continue to offer all courses online and the staff remains on telework status through January 2021. While we are able to continue working in the lab, new Covid-19 guidelines provide added restrictions such as limits on the number of people who can be present in labs at the same time and limited use of core labs.

6. PRODUCTS
   Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
   Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS
   Nothing to report.

9. APPENDICES
   Nothing to report.