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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.

15. SUBJECT TERMS
None listed.

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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:

What were the major goals of the project?

What was accomplished under these goals?

Major Activities: Compare the effect of AhR activity in African-American (AA) and Caucasian-American (CA) prostate cancer cell lines.

Specific Objective 1: Measured AhR activity in E006AA, E006HT, LNCaP and C4-2 prostate cancer cells by assessing protein levels, cellular localization, DNA interaction and expression of AhR responsive genes.

Results: 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 the highest AhR expression. The AhR expression in African-American androgen-sensitive cell line, E006AA is also slightly increased compared to the androgen independent Caucasian cell line, C4-2. In comparison, the androgen sensitive LNCaP cells have lowest AhR expression.

Methods: 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.

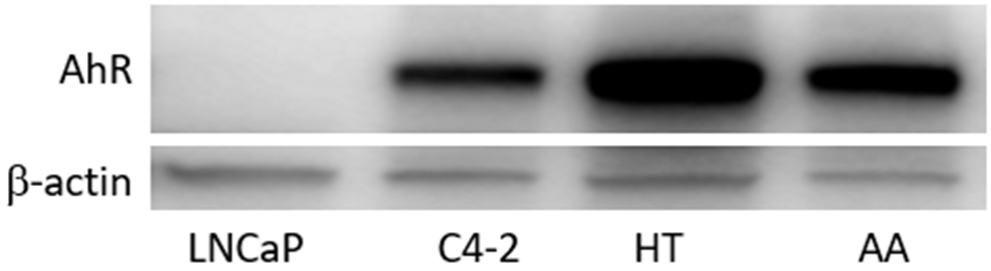


Figure 1: Expression of AhR protein in African-American (HT and AA) and Caucasian-American (LNCaP and C4-2) prostate cancer cell lines. C4-2 and HT are model castration resistant cell lines while LNCaP and AA are models of androgen sensitive prostate cancer cells.

Results: All cell lines contain cytoplasmic AhR protein. Despite AhR being identified as a ligand activated transcription factor, androgen-insensitive prostate cancer cell lines (C4-2 and E006HT) have nuclear AhR expression in the absence of exogenous ligands. African-American androgen sensitive prostate cancer cell line E006AA also has nuclear AhR protein expression.
Methods: To determine AhR cellular localization, cytoplasmic and nuclear protein samples were isolated using the Thermo Scientific NE-PER Extraction kit (Cell Signaling). Immunoblotting was carried out with 1 μg/ml mouse AhR monoclonal antibody (Santa Cruz) at 1:1000 dilution. Following TBST washes, the blots were incubated in 1:2500 dilution of b-tubulin/TOPO3 and washed extensively. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer.

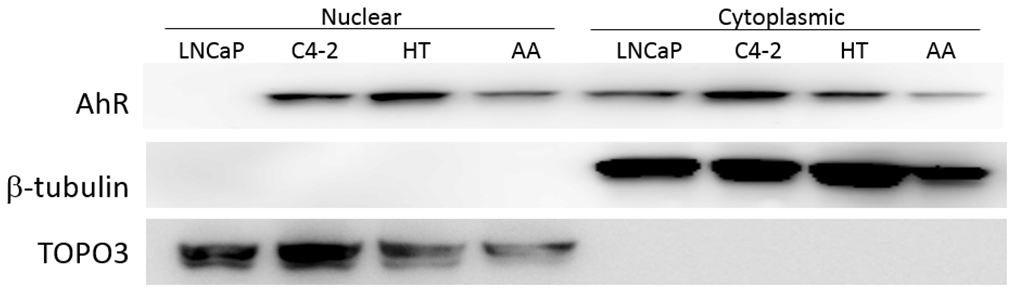


Figure 2: Subcellular localization of AhR in prostate cancer cell lines by nuclear and cytoplasmic staining. The nuclear and cytoplasmic extracts were analyzed by western blotting for AhR protein expression. The relative level of AhR was normalized by the respective B-tubulin or topoisomerase (TOPO3) levels.

Results: Subcellular localization studies show that AhR is localized in the nucleus of C4-2, HT and AA prostate cancer cells but not androgen sensitive LNCaP cells. The promoter binding studies evaluate if nuclear AhR interacts with DNA promoter regions specific to AhR, known as xenobiotic responsive elements (XRE). The results show that LNCaP has modest binding to XREs under normal culturing conditions. However, androgen sensitive African-American prostate cancer cells (AA), have increased binding to XREs compared to LNCaP. Also, HT and C4-2 both have significant promoter binding to XREs. Notably, the African-American cells lines have reduced binding to AREs compared to the Caucasian-American cell lines. These result may indicate that African-American cell lines have increased AhR activity.

Methods: Promoter binding assays were performed to assess constitutive AhR and androgen receptor (AR) binding to xenobiotic responsive elements (XREs) and androgen responsive elements (AREs) respectively. 4x104 cells were plated in a 96-well plate. Cells were transfected with the XRE or ARE reporter construct, as well as with positive and negative control reporter plasmids using attractene. After 18 hours of transfection, media was changed to standard assay media. A dual luciferase assay was performed, and promoter activity values are expressed as arbitrary florescence units (AFU).

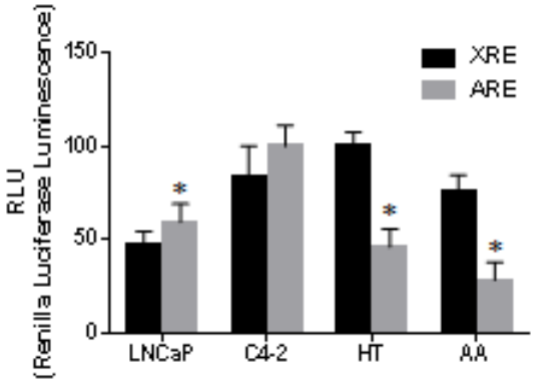


Figure 3: Promoter binding of AhR and AR in prostate cancer cell lines. AhR binding to XRE reporter construct under normal culturing conditions (dark bars). Androgen receptor binding to ARE reporter constructs under normal culturing conditions (light bars). Bar graphs represent mean ± SD of three separate experiments.

Results: AhR is a described as a ligand activated transcription factor. Therefore, AhR is commonly studied for its role as a xenobiotic receptor. However, recent studies indicate that AhR may possess differing intrinsic functions that require no ligand activation. We have already shown that C4-2 cells endogenously overexpress AhR and that the increased expression is accompanied by nuclear localization and constitutive activity. Here, comparison of AhR activity in the AA E006AA/E006HT isogenic pair to the LNCaP/C4-2 cells reveal that African-American cells have enhanced AhR activity over their Caucasian counterparts. Our preliminary data confirms overexpression of AhR protein in the African-American pair compared to the CA cell lines. The results below, in combination with figures 1-3, show that African-American prostate cancer cell lines E00HT and AA have enhanced AhR activity. Both the AhR and CYP1B1 genes are AhR responsive genes.

Methods: qRT-PCR was used to determine expression of AhR responsive genes (AhR and CYP1B1) in both isogenic pairs. 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 wasvprocessed 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.

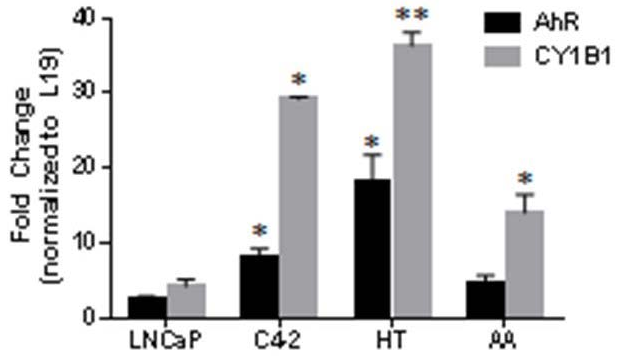


Figure 4: Quantitative real-time PCR was performed to quantify the expression of AhR and CYP1B1. The relative concentration of each PCR product 2aws determined using the ΔΔCq calculation method. L-19 was used as an internal control. Data are expressed as mean +/- SEM (n=3).

Specific Objective 2: Compare androgen receptor activity in E006HT clones following shRNA mediated depletion of AhR to androgen receptor activity in C4-2 clones with reduced AhR protein by measuring androgen receptor expression, phosphorylation, localization, DNA binding and expression of androgen responsive genes.

Results: We have already shown that depletion of AhR expression results in a decrease of androgen responsive genes KLK2 and KLK3 in C4-2 cells. Furthermore, we have shown that shRNA mediated depletion of AhR decreases AR expression and nuclear localization. This also

results in decreased expression of androgen related genes and growth. However, our previous studies did not include AA cell lines, which could have enhanced AhR signaling beyond the level seen in CA cell lines. We created and validated a E006HT cell line with shRNA mediated reduction of AhR protein. The clones will be used to assess the ability of AhR to revert a highly metastatic African-American prostate cancer cell line, which may be responsible for the disparity seen with PCa, to an androgen sensitive phenotype. It is possible that changes in AhR activity can confer a castration resistant phenotype independent of changes to the androgen receptor signaling pathway.

Methods: E006HT cells were incubated with 1 μg of pSuper expression plasmid lentiviral particles containing AhR- shRNA and an empty pSuper vector as a control. At 48 h post-transfection, puromycin was be added to the medium (0.5 mg/mL), and resistant colonies were selected and expanded by limited dilution. We used qRT-PCR (as previously described) to assess for expression AhR responsive genes (AhR and CYP1B1) to select clones for further characterization. Three clones were selected, expanded and assessed for AhR protein expression. The clones that were expanded tested positive for mycoplasma contamination. Therefore, we will repeat these methods to obtain sterile cultures before moving forward with characterization of AhR and AR signaling.

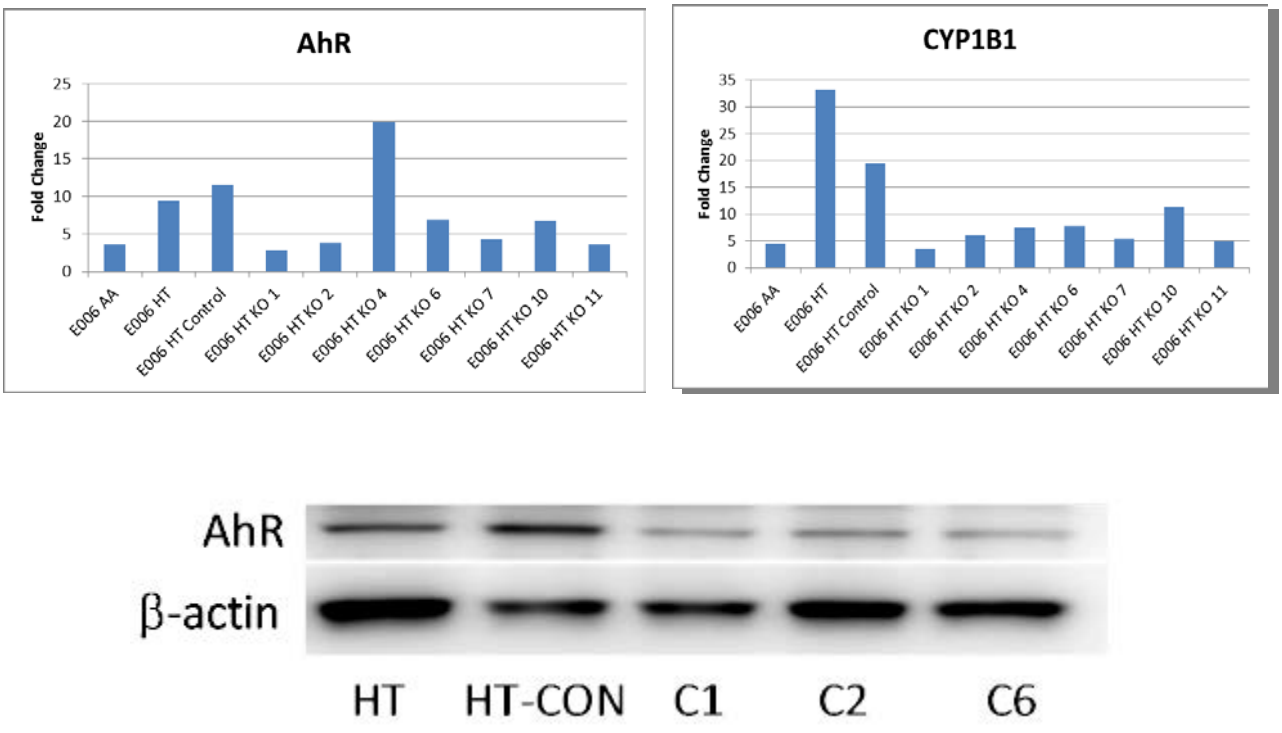


Figure 5: (A-B) Quantitative real-time PCR was performed to determine AhR and CYP1B1 expression in E006HT clones following shRNA mediated knock-down of AhR. The relative concentration of seven clones were assessed by the ΔΔCq calculation method. E006HT control and E006HT parental cells were used as control for basal AhR and CYP1B1 levels. L-19 was used as internal control for normalization of fold change. (C) AhR protein expression in HT parental, HT control and three knock-down clones.

Results: We also assessed expression of AR genes within the E006HT control and Clone 1. Relative to AhR, E006HT cells show modest AR and KLK3 expression. However, preliminary data shows a modest decrease in AR and KLK3. These results will be confirmed in new clones generated following shRNA mediated depletion of AhR.

Methods: Total RNA was isolated from cell monolayers of E006HT control and AhR knock-down 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. qRT-PCR was used to determine expression of AhR responsive genes (AhR and CYP1B1) and AR responsive genes (AR and KLK3). Due to mycoplasma contamination, methods will be repeated on new clones.

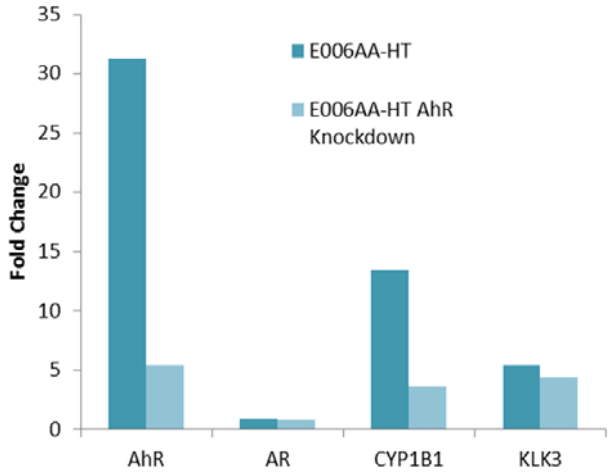


Figure 6: Quantitative real-time PCR was performed to determine AhR, CYP1B1, AR and KLK3 gene expression in E006HT control and knock-down clone 1 following shRNA mediated knock-down of AhR.

Results: We evaluated the growth rate of E006HT control and Clone 1 with decreased AhR expression to determine the effect of constitutive AhR signaling on the growth rate. Decreased AhR expression reduced the growth rate by 20%. However, E006HT (AhR knockdown) cells have a 50% decrease in proliferation in the presence of AhR inhibitor casodex (CDX). The HT control cells do not respond to casodex. Furthermore, AhR inhibitor CH223191 (CH223) decreased the growth rate of both the control and AhR knockdown.

Methods: Growth of E006HT CON and Clone 1 (AhR Knock-down) cells were assayed using the Promega CellTiter 96 Cell Proliferation Assay. Briefly, 50 µl of the 1.0 x 105/mL cell suspension (5,000 cells) were added to each well of the 96-well plate containing 50 µl of media resulting in a total volume of 100 µl. The plates were incubated at 37°C for 72 hours in a humidified, 5% CO2 atmosphere in the presence and absence of 20 µM casodex (CDX) and 50 µM AhR inhibitor CH223191 (CH223). Following incubation, 15 µl of dye solution was added to each well and incubated for 4 hours. Then, 100 µl of stop solution was added to each well and incubated for 1 hour. Absorbances were read at 570 nm using the Synergy H1m multi-mode microplate reader. Due to mycoplasma contamination, methods will be repeated on new clones.

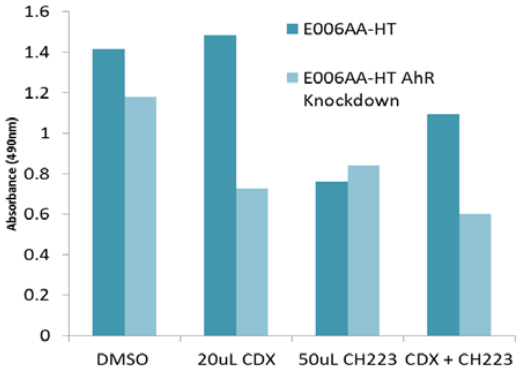


Figure 7: Growth of E006HT control and Clone 1 (AhR Knock-down) in the presence and absence of AR (casodex) and AhR inhibitor (CH223).

Specific Objective 3: Subtask 3: Measure proliferation, migration, invasion and anchorage independent growth of E006HT and C4-2 cells in the presence and absence of AhR inhibitor (CH223191) and androgen receptor inhibitor (casodex).

Results: Inhibition of AhR signaling with specific inhibitor CH223 reduced the growth of both castration resistant prostate cancer cell models (C4-2 and HT). Due to the enhanced AhR signaling in African-American cell line E006AA shown in figures 1-4, CH223 also decreased growth of these androgen sensitive cells. CH223 had no effect on LNCaP cells which showed the lowest level of AhR activity. Androgen receptor inhibitor (CDX) reduced growth of the two androgen sensitive cell lines (LNCaP and AA) but not the androgen-insensitive cell lines (C4-2 and HT).

Methods: Growth of E006HT and C4-2 cells were compared using the Promega CellTiter 96 Cell Proliferation Assay. 50 µl of the 1.0 x 105/mL cell suspension (5,000 cells) were added to each well of the 96-well plate containing 50 µl of media resulting in a total volume of 100 µl. The plates were incubated at 37°C for 72 hours in a humidified, 5% CO2 atmosphere in the presence and absence of 20 µM casodex (CDX) and 50 µM AhR inhibitor CH223191 (CH223). Following incubation, 15 µl of dye solution was added to each well and incubated for 4 hours. Then, 100 µl of stop solution was added to each well and incubated for 1 hour. Absorbances were read at 570 nm using the Synergy H1m multi-mode microplate reader.

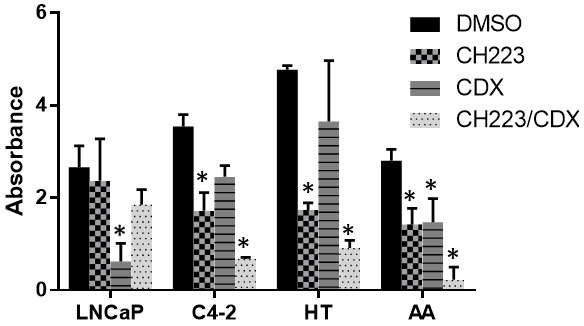
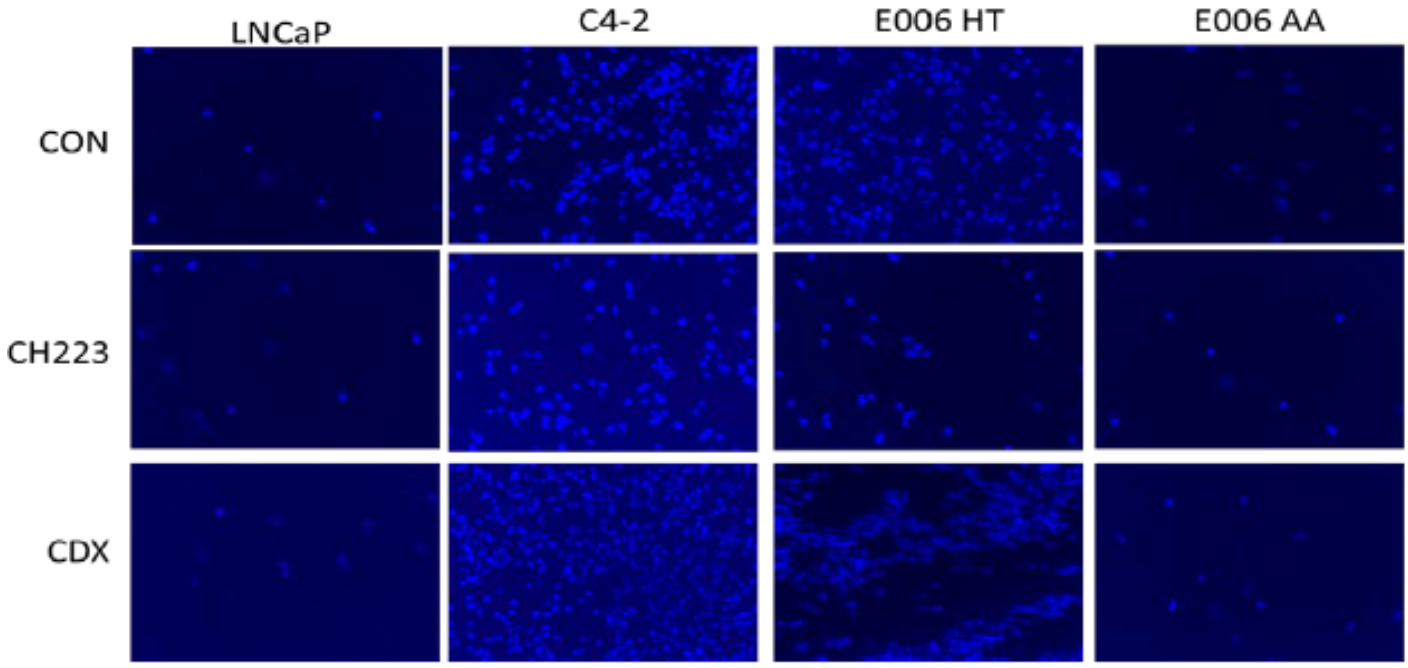


Figure 8: Growth of androgen sensitive (LNCaP and AA) and androgen insensitive (C4-2 and HT) in the presence and absence of AR (casodex) and AhR inhibitor (CH223). LNCaP and C4-2 were derived from Caucasian-American male and AA and HT cells were derived from an African-American male.

Results: Androgen-insensitive cell models C4-2 and E006HT have enhanced constitutive AhR signaling that regulates both cell migration and invasion. Androgen receptor inhibition by casodox (CDX) does not reduce the migration or invasion of either cell line. However, inhibition of AhR signaling by CH223 reduced migration of C4-2 cells by 50% and E006HT cells by over 60%. The level of inhibition correlates to the level of AhR activity revealed in figures 1-4 with E006HT cells having the highest level of AhR expression, nuclear localization, and XRE binding. Although E006AA (AA) does not respond to either CDX or CH223, there is a modest increase (but insignificant) increase compared to LNCaP cells.

Methods: The migratory and invasive potential of E006HT and C4-2 cells in the presence and absence of casodex and AhR antagonist CH223191 will be measured using a fluorescence-based tumor cell invasion assay (FluoroBlok invasion assay kits, BD Biosciences, Franklin Lakes, NJ). The assay has coupled a multi-well insert device containing a micro-porous membrane with a BD Matrigel coating process. The BD Matrigel coat functions as a barrier to the passage of non-invasive cells analogous to the in vivo extracellular basement membrane. Migration studies will be performed in chambers not coated with BD Matrigel. Cell suspensions were prepared by trypsinizing cell monolayers and resuspending the cells in serum-free medium at 5x104 cells/ml with and without bicalutamide. Media (750 µl) containing 5% fetal calf serum will be added to the bottom of each well as a chemo-attractant. A 500 µl aliquot of the cell suspension (2.5x104 cells) was added to the top chamber. The cells were incubated at 37ºC for 24 hours. Following incubation, the medium from the top chamber was carefully removed by aspiration and the insert transferred to a second plate containing 0.5 ml/well of 4 µg/ml DAPI (Molecular Probes, Eugene, OR). The plates were incubated for 1 hour at 37ºC and DAPI fluorescence of the invaded cells read from the bottom at excitation and emission wavelengths of 485 and 530 nm, respectively.



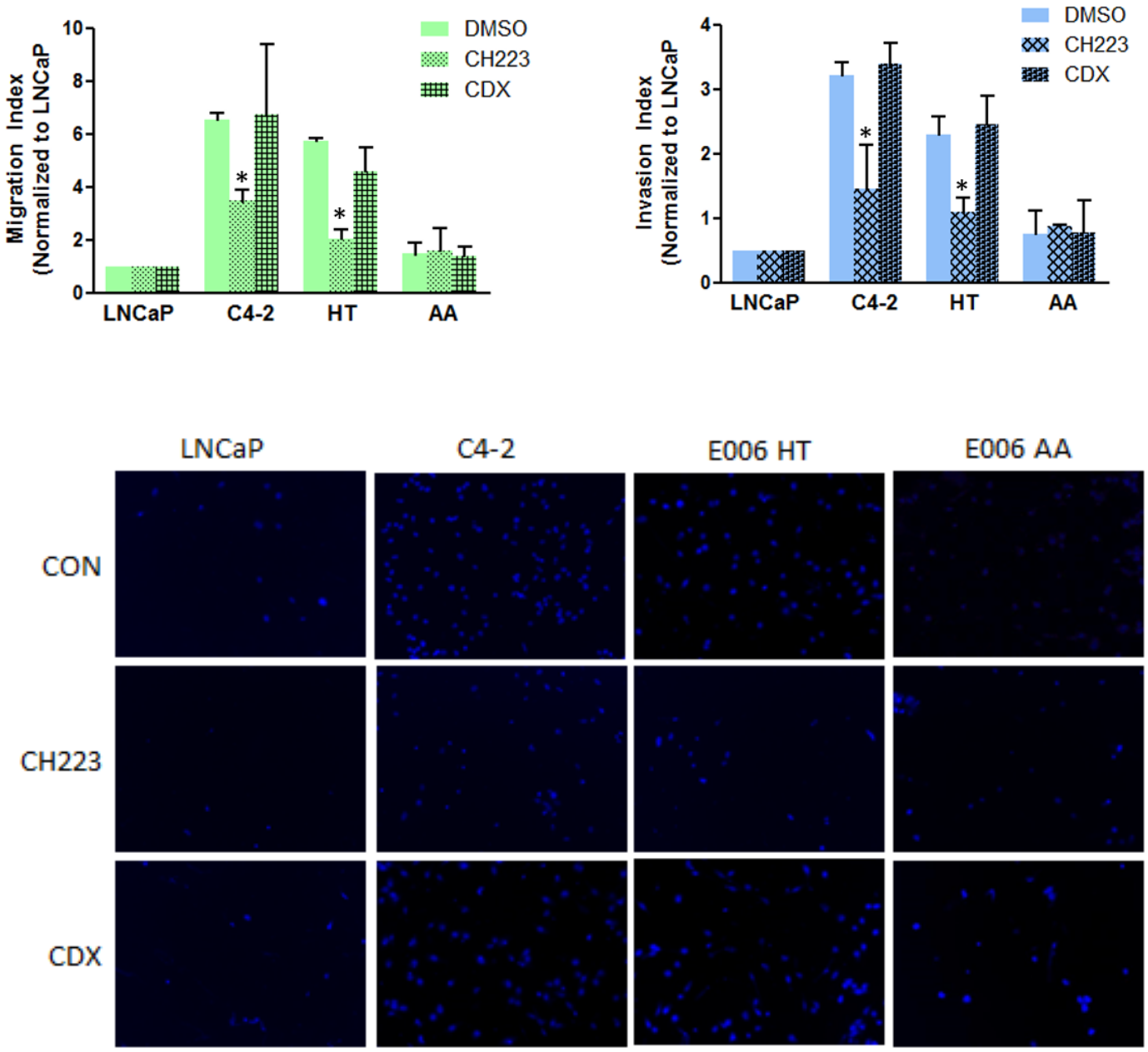


Figure 9: (A) Migration of prostate cancer cell lines in the presence and absence of AhR inhibitor, CH223191 (CH223) and androgen receptor inhibitor, casodex (CDX). Immunofluorescence expressions exhibited a blue-stained nucleus after 24 hours incubation. Bar graphs represent mean ± SD of three separate experiments.

Specific Objective 4: Compare AhR activity in prostate cancer and matched normal tissue from AAs and CAs to determine if AA cancer tissue has a higher AhR expression.

Results: 200 prostate tissue samples were stained with AhR antibody. An H-score for AhR cytoplasmic and AhR nuclear intensity was determined by screening with digital pathology using

Leica Biosystems imaging and individual sample H-scores were also verified by an independent pathologist. There was a measurable increase in cytoplasmic AhR staining in AA tumor compared to matched normal. However, there was a decrease in nuclear AhR staining in the AA tumor samples. This decrease could be a consequence of active AhR signaling which results in proteomic degradation of the AhR protein. Overall, there was not a significant increase in AhR expression, cytoplasmic or nuclear, in AA samples compared to CA tissue samples.

Methods: Analyzed 50 tumor and 50 matched normal prostate cancer tissue slides from African-American (AA) men as well as 50 tumor and 50 matched normal prostate cancer tissue slides from Caucasian-American (CA) men by immunohistochemistry (IHC) for expression of AhR. Briefly, tissue arrays will be dewaxed with xylene, rehydrated in graded concentrations of alcohol and treated with hydrogen peroxide prior to blocking with normal goat serum. The slide will be incubated with AhR antibody. The antibody titer, concentration and incubation time will be determined using corresponding optimization slides. Antibody binding will be detected using a labeled streptavidin-biotin kit with 3'3-diaminobenzidine as the chromogen (DAKO). Hematoxylin will be used as a counterstain. Results of staining will be scored by two independent observers by rating staining intensity from 0 for below the level of detection to 3 for strongest expression.

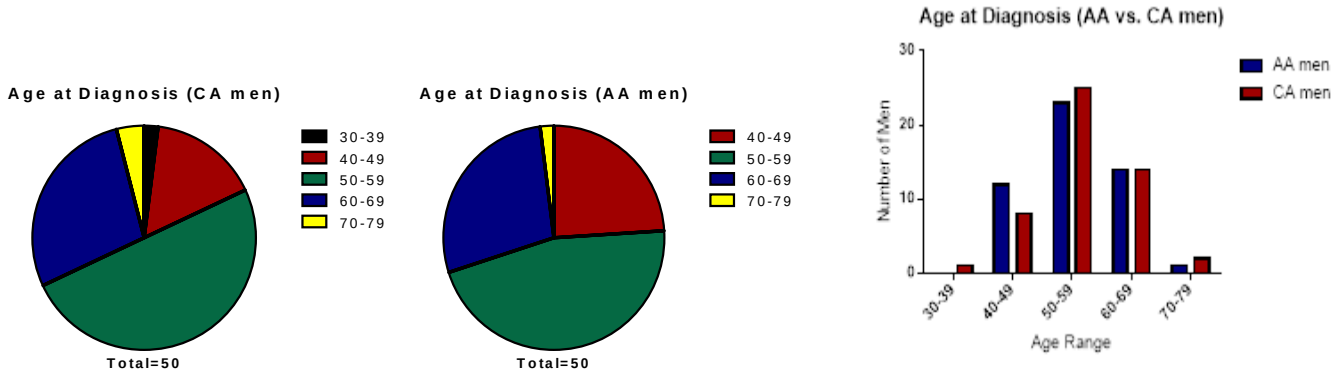


Figure 10: The AA samples ranged from age 43 to 72 with a median age of diagnosis of 55. The CA samples range from 39 to 70 with a median age of diagnosis of 56. The majority of AA (23) and CA (25) samples were from men who were diagnosed between age 50 and 59.

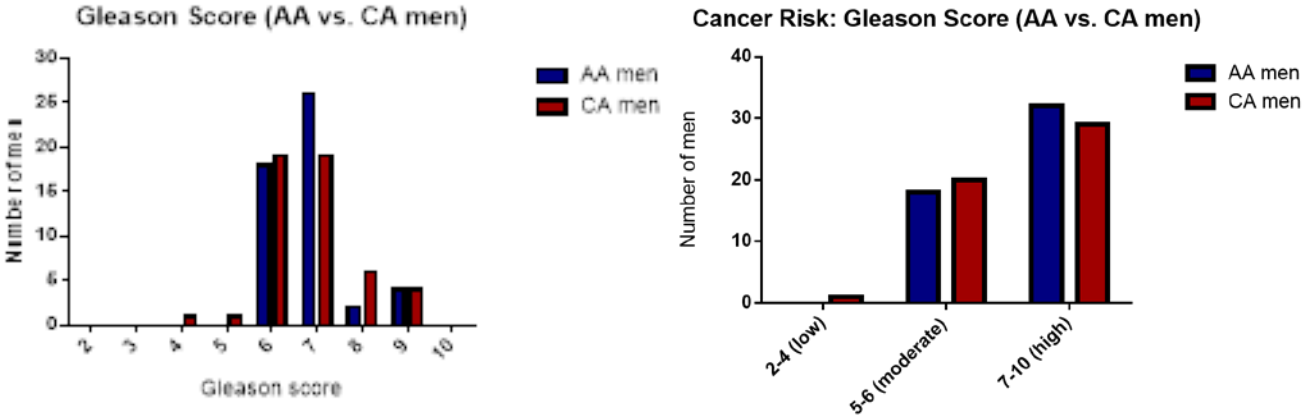


Figure 11: CA samples range from Gleason score 4 to 9. AA samples range from Gleason score of 6 to 9. The average Gleason score for both groups is 6.8 with a median of 7. 58% of CA samples are in the high Gleason range compared to 64% of AA samples.

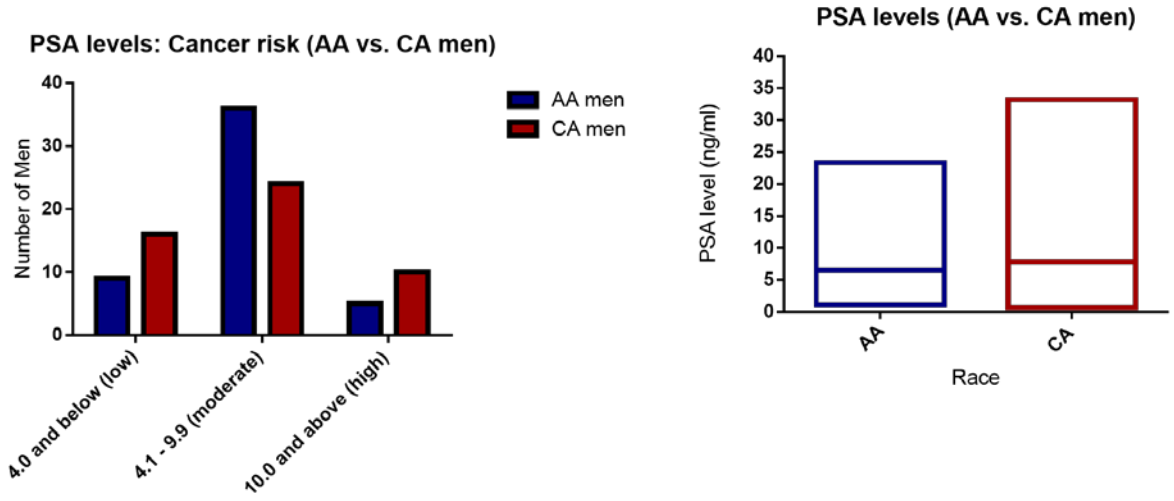


Figure 12: 82% of the AA tissue samples have a PSA of 4.0 or higher compared to 68% of the CA samples. The average PSA of AA samples is xx. The average PSA for CA samples is 7.8 vs 6.5 for AA samples.

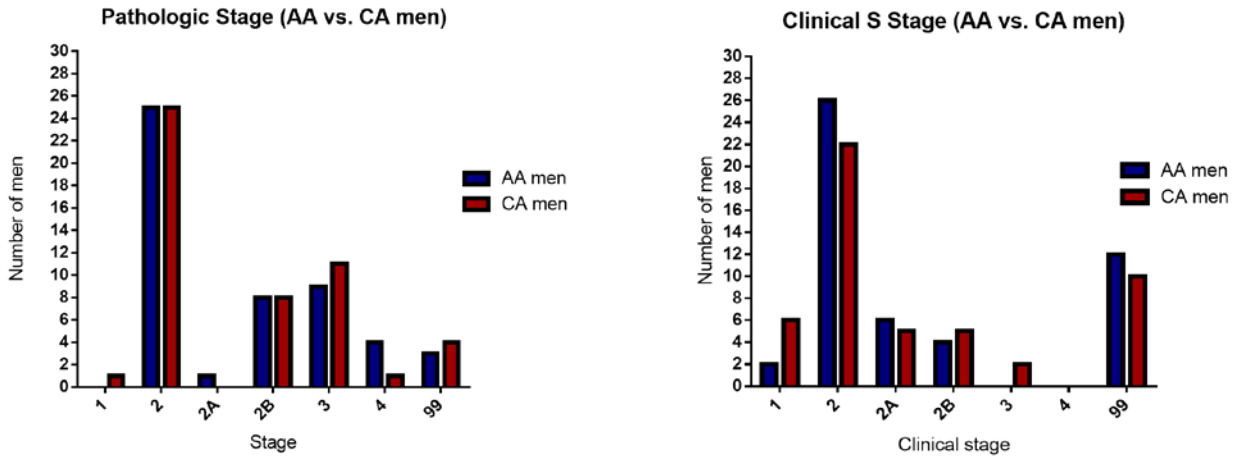


Figure 13: The majority, approximately 50% of the tissue samples were classified in clinical and pathological stage 2. A clinical classification was not assigned to 20% of the samples.

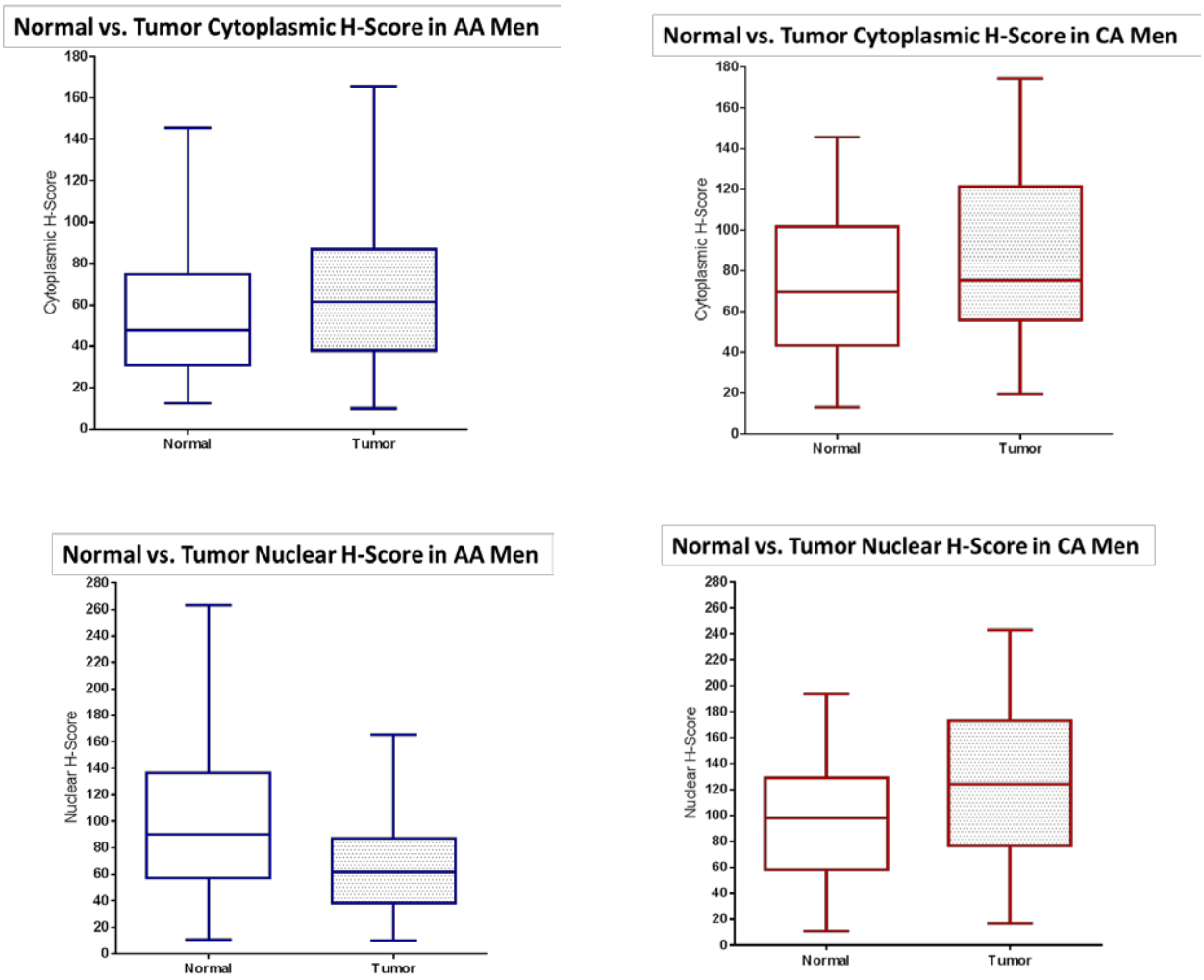
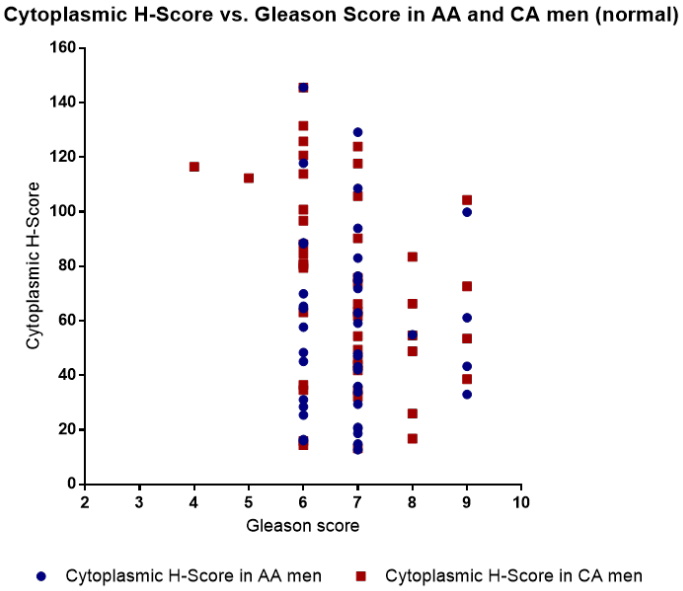
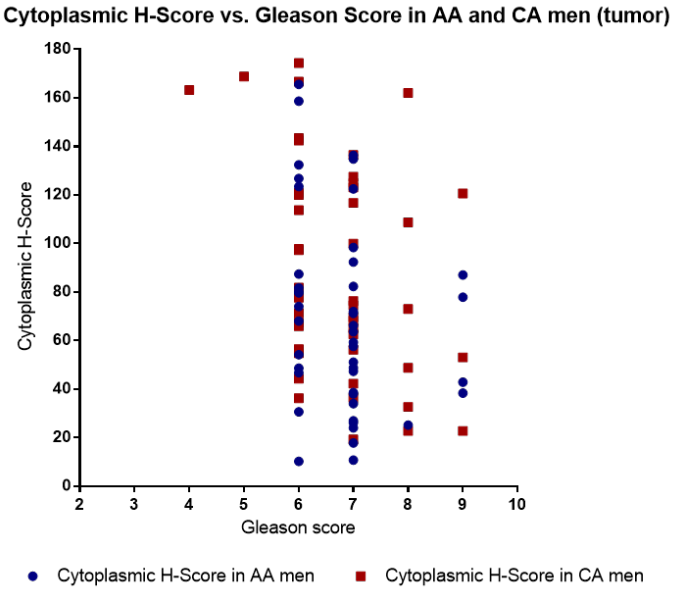
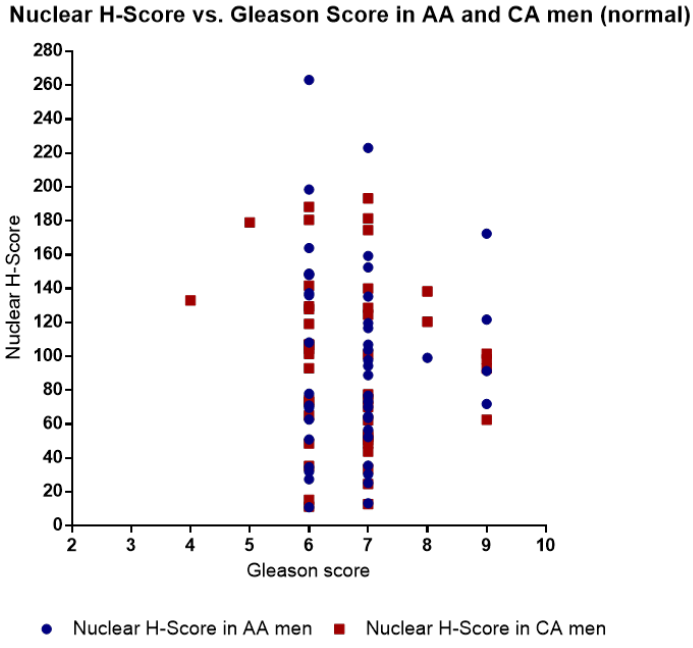


Figure 14: The cytoplasmic H-score for AhR staining increased in tumor (AA68/CA72) compared to matched normal samples (AA56/CA88). However, the nuclear H-score in AA normal (97) exceed the tumor H-score of 68).

Subtask: Compare AhR activity in low, moderate and high Gleason score prostate cancer tissue samples







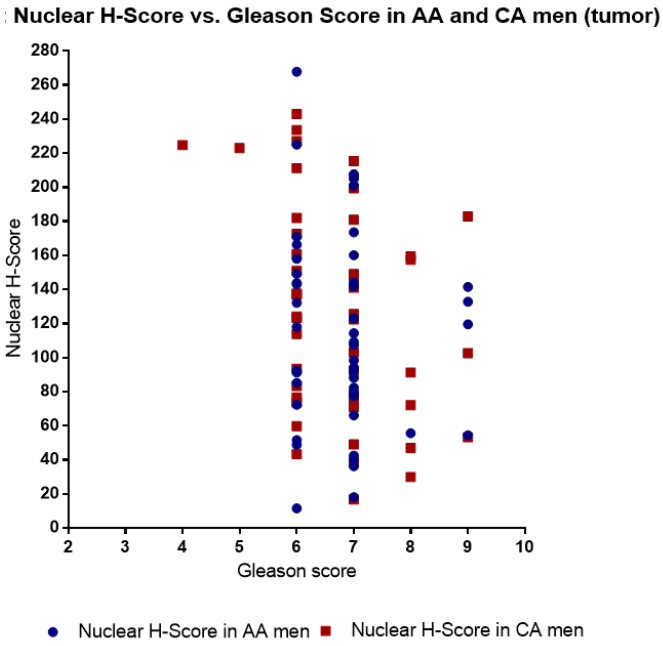


Figure 15: There was no observed correlation between the cytoplasmic or nuclear H-score and the Gleason Score in AA or CA tissues.

Subtask: Correlate AhR activity in clinical prostate cancer tissue to clinical indicators of progression (PSA).

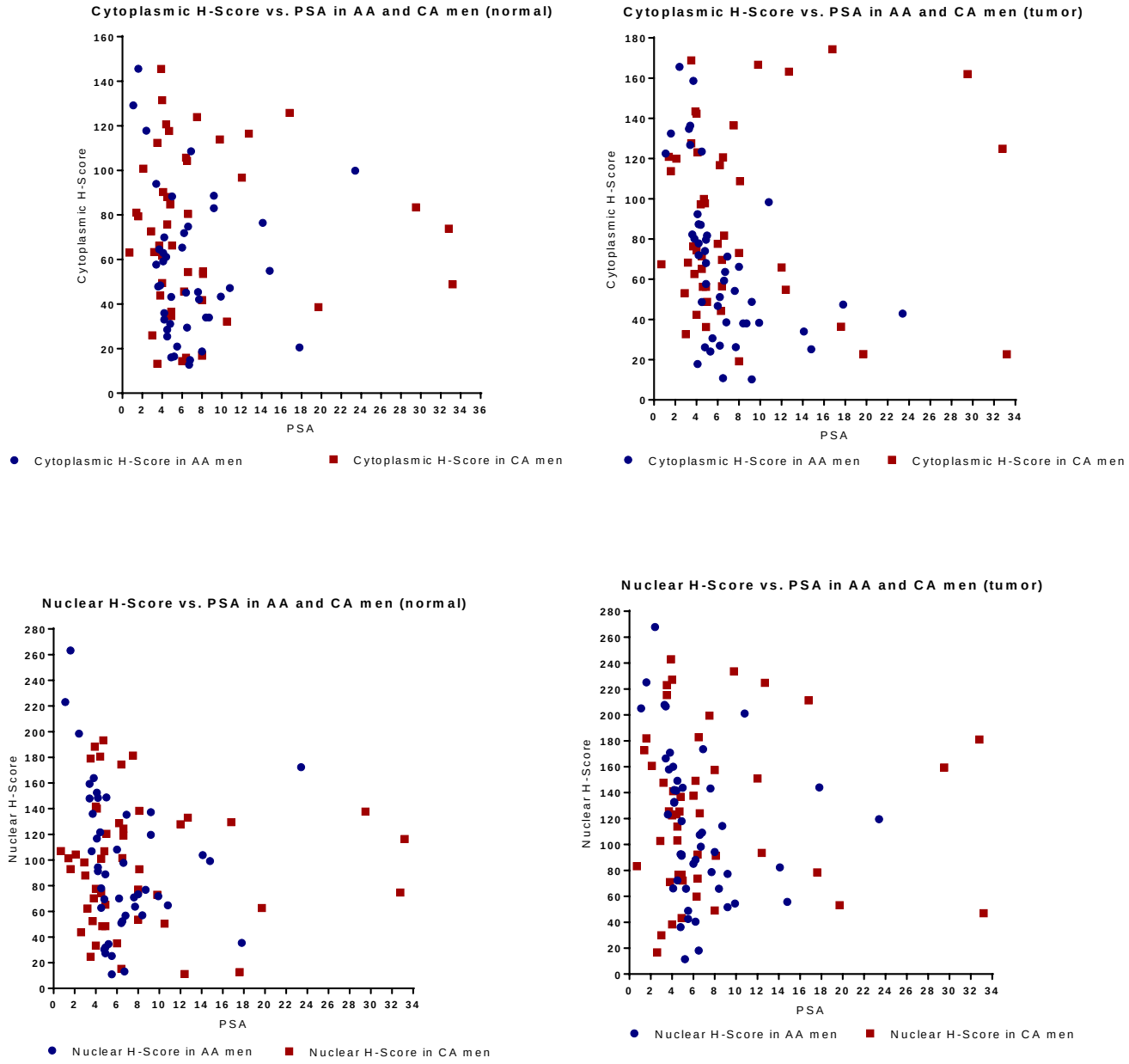


Figure 16: There was no observed correlation between the cytoplasmic or nuclear H-score and the PSA in AA or CA tissues.

Specific Objective 5: 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.

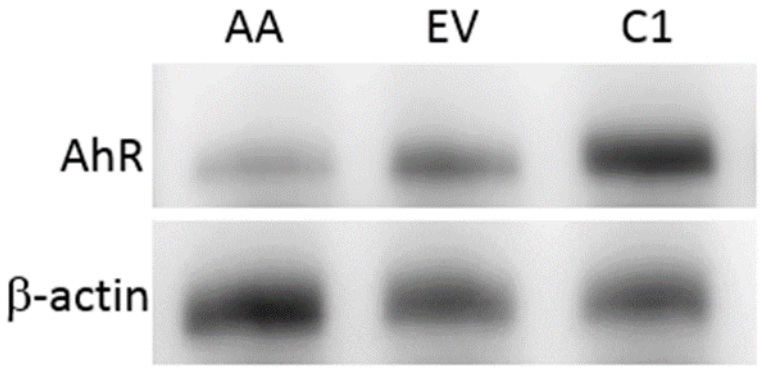


Figure 17: Expression of AhR protein in E006AA parental cell line (AA), E006AA empty vector control (EV) and E006AA Clone 1 (C1). Relative AhR expression is normalized with b-actin.

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.

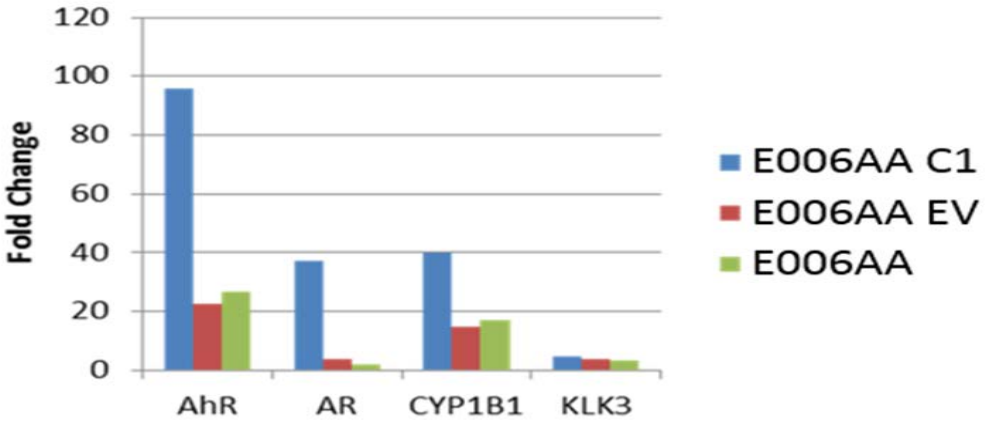


Figure 18: Quantitative real-time PCR was performed to determine AhR, AR, CYP1B1 and KLK3 gene expression in E006AA parental, control(EV) and over-expressing clone (C1).

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.

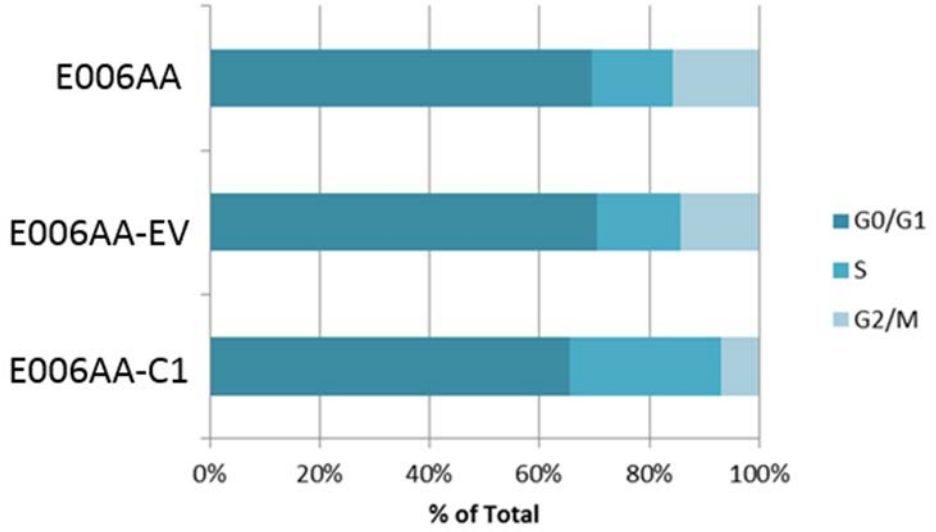


Figure 19: Cell cycle analysis of E006AA parental, control(EV) and over-expressing clone (C1) by flow cytometry. % of cells in G0/G1 phase, S-phase, and G2/M phase was determine by flow cytometry.

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.

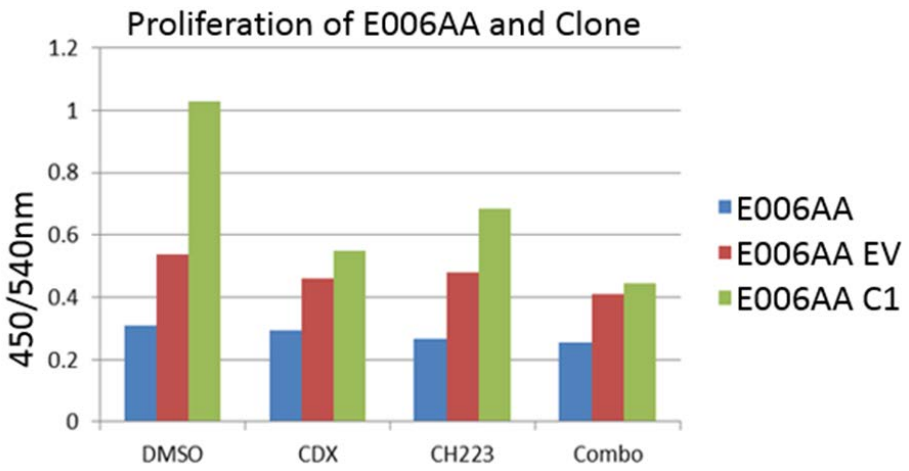


Figure 20: Growth of E006AA parental, control(EV) and over-expressing clone (C1) in the presence and absence of AR (casodex/CDX) and AhR inhibitor (CH223) was determined using an BrdU incorporation assay.

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

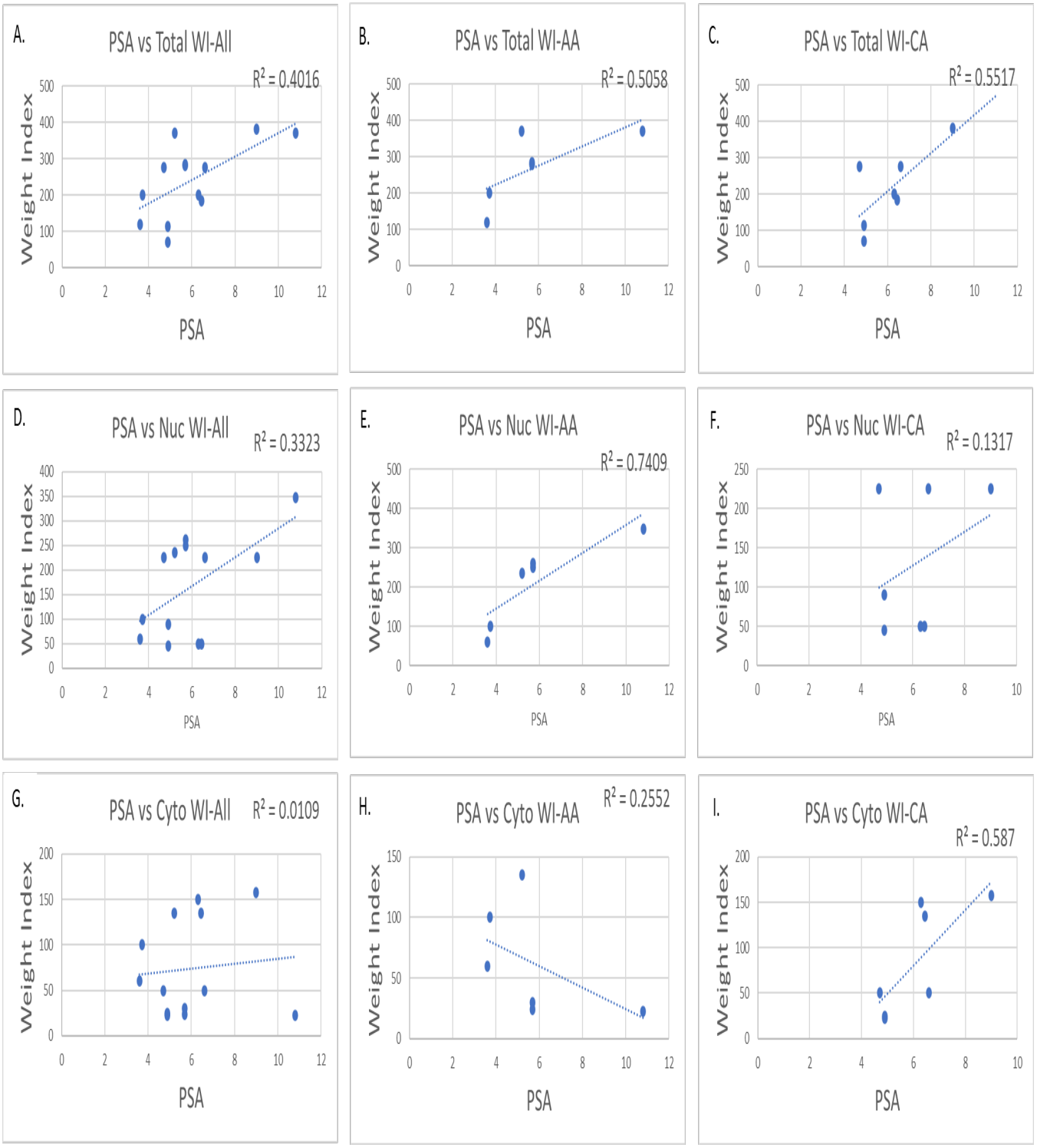


Figure 21: Weight Index (WI) was calculated for prostate cancer tissue samples. WI is the product of the staining intensity (0-3) of the sample and the percent positive cells. Patient PSA at time of diagnosis was plotted vs (A) total WI of all samples, (B) total WI of African-American/AA samples, (C) 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.

Specific Objective 6: Compare basal AhR activity in African-American MDA PCa cells to Caucasian American LNCaP and C4-2 prostate cancer cells.

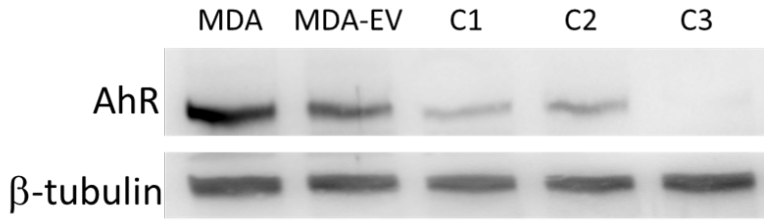


Figure 22: Expression of AhR protein in MDA parental cell line (MDA), MDA empty vector control (EV) and Clone 1 (C1), Clone 2 (C2), and Clone 3 (C3). Relative AhR expression is normalized with b-tubulin.

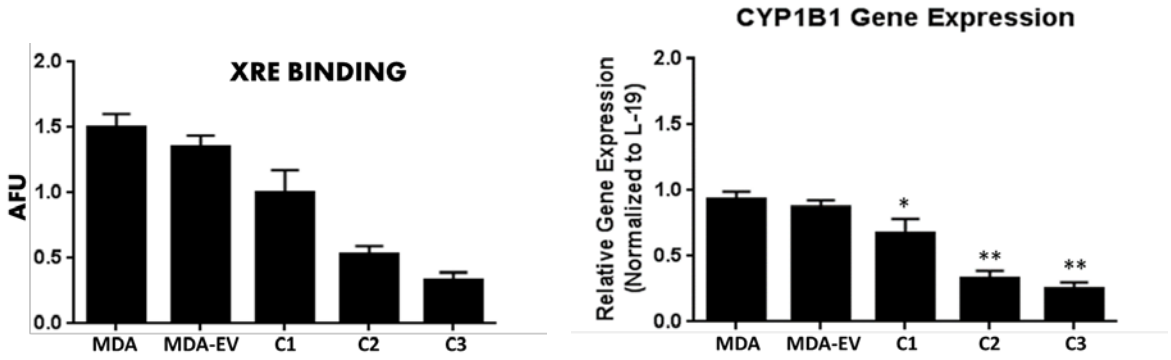


Figure 23: AhR binding to XRE reporter construct in MDA parental cell line (MDA), MDA empty vector control (EV) and Clone 1 (C1), Clone 2 (C2), and Clone 3 (C3) under normal culturing conditions. Quantitative real-time PCR was performed to determine CYP1B1 expression in parental cells, control and clones.

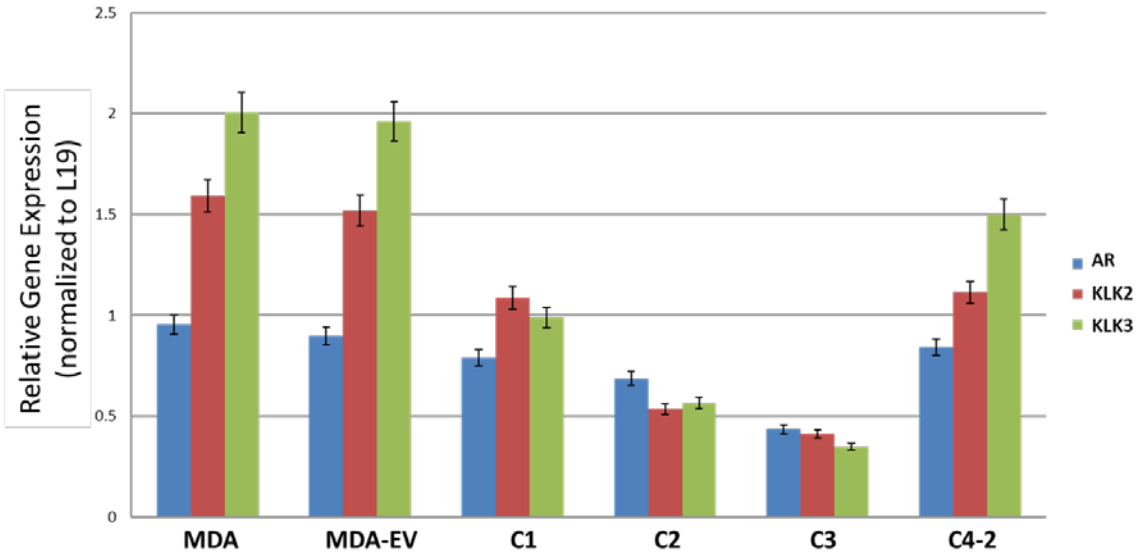


Figure 24: Quantitative real-time PCR was performed to determine AR, KLK2 and KLK3 gene expression in MDA-EV (empty vector) control and knock-down clones 1, 2 and 3 following shRNA mediated knock-down of AhR. MDA and C4-2 are included as positive controls for gene expression.

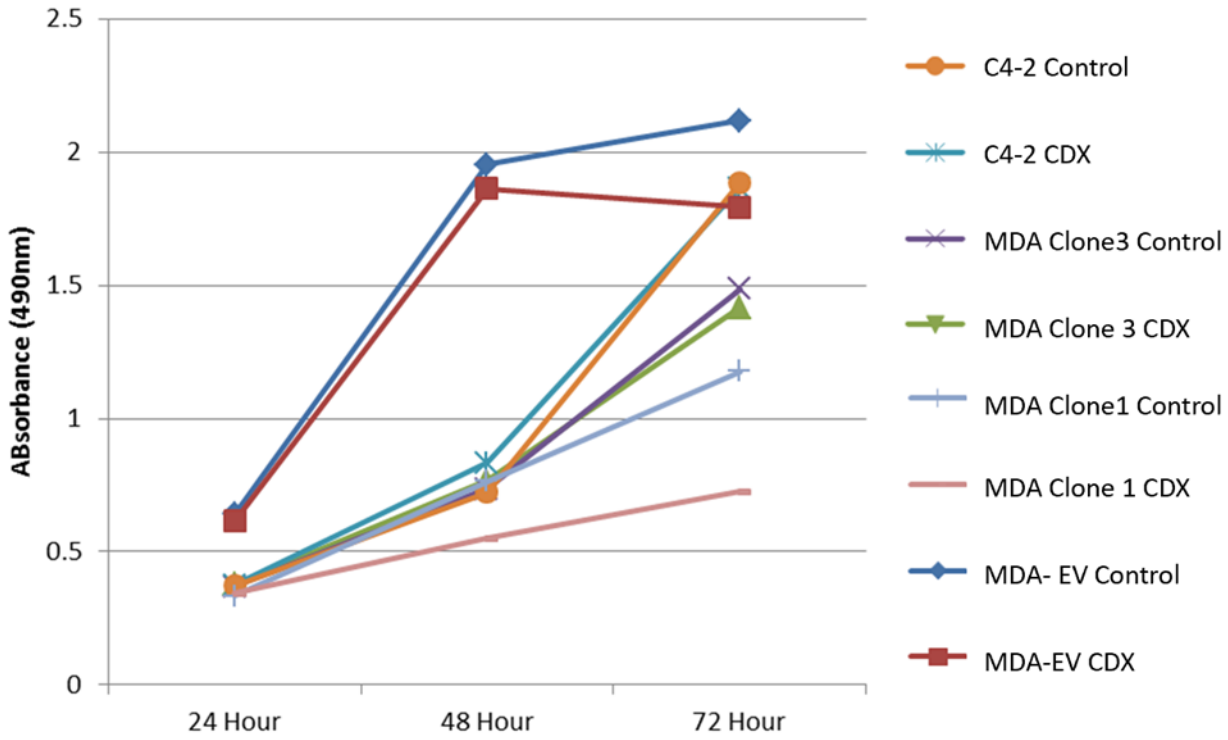


Figure 25: Growth of MDA parental cell line (MDA), MDA empty vector control (EV) and Clone 1 (C1) and Clone 3 (C3) in the presence and absence of AR (casodex/CDX) inhibitor was determined using an BrdU incorporation assay for 24- 72 hours.

What opportunities for training and professional development has the project provided?

Attendance/Presentations at the following conferences/symposia:
Maryam Ghotbaddini, Sakura McLaughlin and Joann B. Powell. Constitutive AhR signaling enhances androgen receptor signaling and growth in prostate cancer cells. AACR 2019 Annual Meeting. April 2019.
Kailen Turner, Sakura McLaughlin and Joann B. Powell. AhR Gene Expression During Prostate Cancer Progression. CAU Research Day. March 2019.
Maryam Chaudhary, Vivian Moultire and Joann B. Powell. Health Disparities and Aryl Hydrocarbon Receptor (AhR) Expression in Prostate Cancer. Annual Biomedical Research Conference for Minority Students (ABRCMS). November 2018.
Maryam Chaudhary, Vivian Moultire and Joann B. Powell. Health Disparities and Aryl Hydrocarbon Receptor (AhR) Expression in Prostate Cancer. 12th National Symposium on Prostate Cancer. September 2018.
Maryam Chaudhary, Vivian Moultire and Joann B. Powell. Aryl Hydrocarbon Receptor (AhR) Expression in Prostate Cancer. CAU Research Day. March 2018.

Describe how the results were disseminated to communities of interest.

Results were presented during the following oral presentations:
Powell, J. Role of Biomedical Researchers in Cancer Health Disparities Research. 33rd BEYA STEM Global Competitiveness Conference. February 2019. Oral Presentation.

Powell, J. Ectopic Over-Expression of AhR Induces Androgen Independent Signaling In LNCaP Prostate Cancer Cells. AhR in Paris Meeting. August 2018. Oral Presentation.

Powell, J. Role of Aryl Hydrocarbon Receptor in Prostate Cancer Progression. 14th International Symposium on Recent Advances in Environmental Health Research. September 2017. Oral Presentation.

Powell, J. Targeting of AhR Signaling in Advanced Prostate Cancer. Men's Minority Health Initiative Spring Retreat. February 2018. Oral Presentation

what you plan to do during the next reporting period to accomplish the goals and objectives.

This is the final reporting period.

4. IMPACT:

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

Nothing to report. While completing the requirements for IACUC approval, the article (Koochekpour et al. The Prostate 2019) was discovered. The article issues a correction for the classification of E006AA as a prostate tumor line which we've characterized for use during in vivo studies. Evidence now suggest that the E006AA may be a subclone of a renal adenocarcinoma cell line from a white male. Because the originators now believe that E006AA was contaminated with the renal adenocarcinoma cell line, there is no justification for use in animals. The uncertainty of the cell line also means thein vitro data obtained from E006AA is unreliable and can no longer be submitted for publication.

What was the impact on other disciplines?

Nothing to report due to the African-American cell lines used in the study being recalled.

What was the impact on technology transfer?

Nothing to report due to the African-American cell lines used in the study being recalled.

What was the impact on society beyond science and technology?

Nothing to report due to the African-American cell lines used in the study being recalled.

5. CHANGES/PROBLEMS:

While completing the requirements for IACUC approval, the article (Koochekpour et al. The Prostate 2019) was discovered. The article issues a correction for the classification of E006AA as a prostate tumor line which we've characterized for use during in vivo studies. Evidence now suggest that the E006AA may be a subclone of a renal adenocarcinoma cell line from a white male. Because the originators now believe that E006AA was contaminated with the renal adenocarcinoma cell line, there is no justification for use in animals. The uncertainty of the cell line also means thein vitro data obtained from E006AA is unreliable and can no longer be submitted for publication.

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.

Actual or anticipated problems or delays and actions or plans to resolve them

An updated statement of work was submitted. The statement detailed the new aim compare the effect of AhR activity in African-American (AA) and Caucasian-American (CA) prostate cancer cell lines. The major task was to compare basal AhR activity in AA MDA PCa cells to CA LNCaP and C4-2 prostate cancer cells.

Changes that had a significant impact on expenditures

Nothing to report.

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

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Because the originators now believe that E006AA was contaminated with the renal adenocarcinoma cell line, there is no justification for use in animals. The uncertainty of the cell line also means thein vitro data obtained from E006AA is unreliable and can no longer be submitted for publication.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Evidence now suggest that the E006AA may be a subclone of a renal adenocarcinoma cell line from a white male. Because the originators now believe that E006AA was contaminated with the renal adenocarcinoma cell line, there is no justification for use in animals. The uncertainty of the cell line also means the in vitro data obtained from E006AA is unreliable and can no longer be submitted for publication.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Evidence now suggest that the E006AA may be a subclone of a renal adenocarcinoma cell line from a white male. Because the originators now believe that E006AA was contaminated with the renal adenocarcinoma cell line, there is no justification for use in animals. The uncertainty of the cell line also means the in vitro data obtained from E006AA is unreliable and can no longer be submitted for publication.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Sakura McLaughlin
Project Role: Research Associate
Nearest Person month worked: 3 months (July 2020- September 2020)
Contribution to the project: Ms. McLaughlin provided technical assistance to the project. Specifically, she assisted on experiments in molecular biology, protein biochemistry and cell biology. She also assisted with the design of experimental protocols and maintenance of laboratory

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?

- *Other*.

None to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: