Award Number: W81XWH-12-1-0046

TITLE: Unique Genomic Alterations in Prostate Cancers in African American Men

PRINCIPAL INVESTIGATOR: Michael Ittmann MD PhD

CONTRACTING ORGANIZATION: BAYLOR COLLEGE OF MEDICINE
HOUSTON, TEXAS 77030

REPORT DATE: December 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2015	Final	1Apr2012 - 30Sep2015

4. TITLE AND SUBTITLE

Unique Genomic Alterations in Prostate Cancers in African American Men

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0046

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Michael Ittmann MD PhD

E-Mail: mittmann@bcm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

BAYLOR COLLEGE OF MEDICINE
HOUSTON, TEXAS 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Based on our published data we propose that specific patterns of genomic copy number alterations (CNAs) occur in prostate cancer in African American (AA) men which are associated with different levels of disease aggressiveness. Second, specific patterns of gene expression are associated with disease aggressiveness in AA prostate cancer and these reflect, in part, the specific CNAs at the relevant gene loci. Finally, we propose that 4p16.3, which is lost in 30% of AA prostate cancers, contains one or more tumor suppressor genes that impact prostate cancer initiation and progression in AA men. This proposal will test these hypotheses by carrying out high throughput copy number and expression microarray analysis of DNAs and RNAs from cancer and benign tissues from African American men with prostate followed by an in depth analysis of the 4p16.3 region.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

African American (AA) men have both a higher incidence and significantly higher mortality rates from prostate cancer (PCa) than Caucasian (CAU) men[1]. To what extent racial differences observed in PCa incidence and mortality are due to socioeconomic or biological factors remains controversial. Several groups have found that AA patients exhibited greater tumor volumes in comparison to similarly staged CAU patients [2, 3]. A major factor that has inhibited understanding the unique biology of PCa in AA men is the lack of clinical and pathological resources focused specifically on this problem. The vast majority of PCa molecular genetic and biological studies do not take differences in race into account when analyzing the results. This reflects the general under-representation of AA men in such studies, which substantially weakens the statistical power of any sub-group analysis. This is exacerbated by the generally lower percentage of AA patients in most tertiary referral centers where most such studies are performed. While some of the difference in mortality due to PCa can be attributed to socioeconomic factors, a number of studies have shown that there is a still a higher mortality rate from prostate cancer in AA men even after adjustment for socioeconomic factors [4]. Thus, as concluded by Freedland and Isaacs[4], that in addition to socioeconomic and cultural factors, biological differences account for some of the disparity in incidence and mortality for prostate cancer in AA men in comparison to CAU men. **The central problem addressed in this project is to understand the biological basis for the more aggressive clinical behavior of PCa in AA men and to develop predictive tools to help manage PCa in AA men.**

We have analyzed 20 PCas from AA men with high density single nucleotide polymorphism arrays [5] to detect genomic copy number alterations (CNAs). Comparison of our primary tumors with tumors from CAU patients from a previously published cohort with similar pathological characteristics showed higher frequency of loss of at numerous loci, all of which had higher frequencies in metastatic lesions in this previously published cohort. **This difference may in part explain the more aggressive clinical behavior of prostate cancer in AA men and indicates that AA men will need specific prognostic tools based on the biology of their PCa.** Furthermore, when we performed cluster analysis of CNAs with both AA and CAU patients, almost all of the AA patients fell into two clusters, one associated with less aggressive organ confined disease and a second associated with more aggressive, invasive disease. **This is an exciting finding indicating that analysis of CNAs and patterns of CNAs may have prognostic value in AA men with PCa.** Finally, we indentified a novel region on chromosome 4p16.3 that is lost in 30% of AA PCas which has not been previously shown to be lost in PCa. This region has previously shown to be lost in breast, colon and bladder cancer and harbors several potential tumor suppressor genes.

The overall goal of this proposal is to identify novel oncogenes and tumor suppressor genes that are altered at higher rates in AA PCa that may explain the increased clinical aggressiveness of AA PCa. The long range goal is to develop new markers that can be used to enhance treatment planning and identify new therapeutic targets in AA PCa.

BODY

Task 1: High resolution analysis of genomic alterations in African American prostate cancers.

Subtask 1: Sixty pairs of samples will be obtained from the Baylor Prostate Cancer Tissue Bank. Samples will be from African American (AA) men undergoing radical prostatectomy for treatment of prostate cancer and were collected with informed consent. Prostate cancer (PCa) samples will have 80% tumor and will have a matched benign tissue available from the same patient. DNA and RNA will be extracted by standard methodologies. Assess DNA and RNA integrity by standard techniques. **(Months 1-2)**

Progress: We have extracted DNA and RNA from 69 pairs of prostate cancer and benign tissues. Quality was assessed and is high as shown in Figure 1.

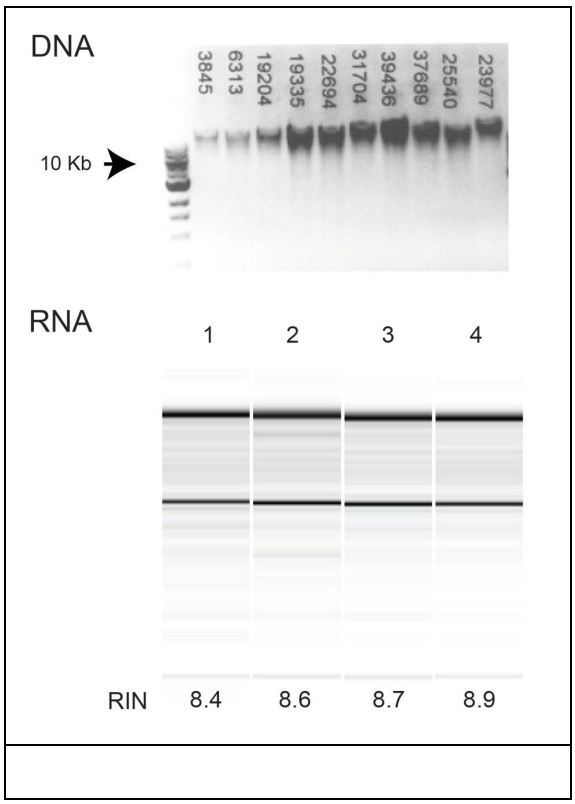

Figure 1. DNA and RNA from African American prostate cancer and prostate tissues.

Top: agarose gel of DNA showing undegraded high molecular weight DNA' The 10 Kb marker is shown on the left. Numbers are sample IDs.

Bottom: Agilent Bioanalyzer analysis of RNA. RNA integrity numbers (RIN) are shown at the bottom of each lane. A RIN of > 7 is required for optimal analysis by expression microarrays.

Subtask 2: Copy number analysis of DNAs from PCa and benign tissues from AA prostate cancers will be carried out as we have described previously except using Affymetrix 6.0 SNP arrays. **(Months 3-18)**

Progress: A total of 98 Affymetrix 6.0 Arrays have been completed (49 cancer/benign pairs).

Subtask 3: Continuous quality control of data **(Months 3-18)**

Progress: We have carried out continuous quality assurance during data acquisition and analysis.

Subtask 4: Data analysis for copy number alterations in PCas from AA. Hierarchical clustering (complete linkage method) of copy gain/loss profiles of prostate tumors from: AA PCa (new dataset); all Baylor AA PCa (new and old dataset); all AA PCa (our datasets and published AA datasets). We will also compare our AA cases (new and old) and the published CAU datasets. **(Months 18-36)**

Progress: We have carried out extensive data analysis of the copy number changes in the new data set and comparing it to our previous dataset as well as published datasets. We are using both the LaPointe dataset[6] and the Taylor dataset[7]. The data is shown in Figure 2 with our new data labeled Ittmann. Results of cluster analysis are shown in Figure 3. As in our previous analysis with a more limited data set, the majority of the AA cases are clustering together (right cluster). To better understand the basis for this clustering we have identified the statistically significant differences in gain or loss of specific cytobands between AA and CAU cases. This analysis is shown is summarized in Figure 4. It should be noted that are the loss of 4p16.3 is confirmed in this new analysis. In addition, it appears that the AA cases cluster into 4 major groups indicated b yred lines. From left to right there is a group without changes in the differentially lost loci (A); a group with widespread gains in these loci (B); a group with widespread losses (C) and a group on the far right with more focal gains (D). Of note, the middle two groups cluster adjacent to CAU cases with metastasis. Overall the data indicates that AA and CAU PCa have differences in CNAs and these differences are associated with aggressive disease.

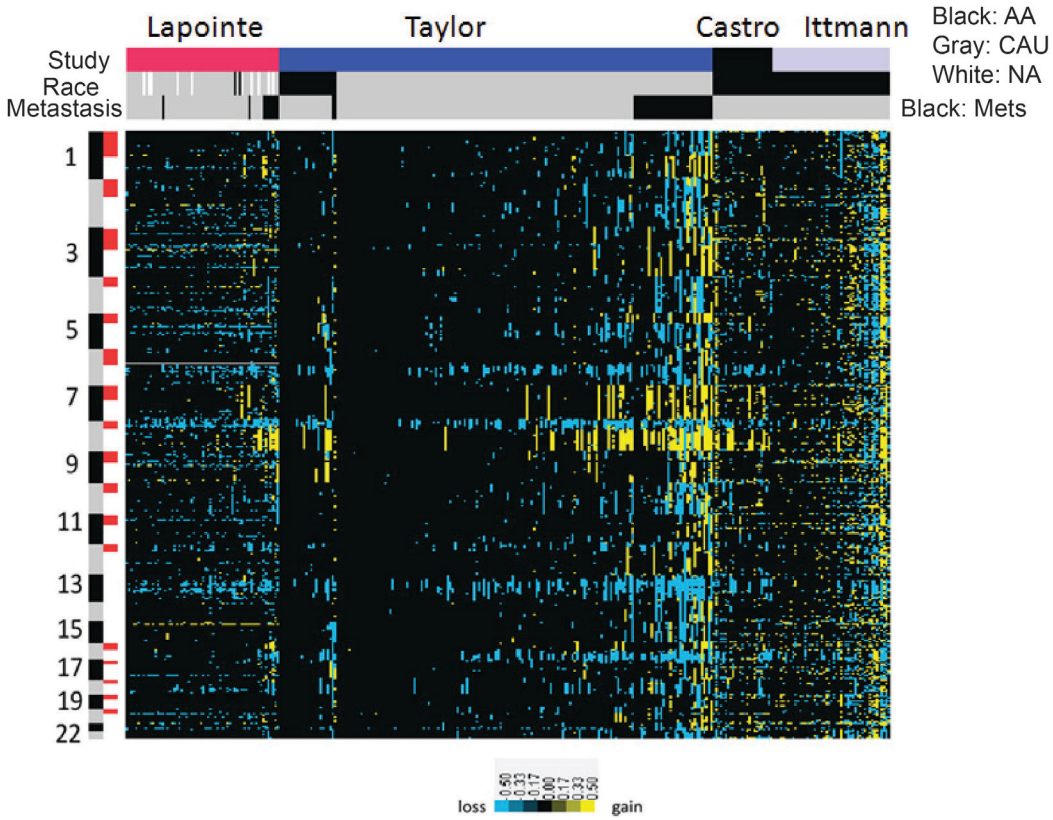

Figure 2. Copy number analysis of new data (Ittmann), our previous data (Castro) and two large publicly available data sets for with race data available (Taylor and LaPointe).

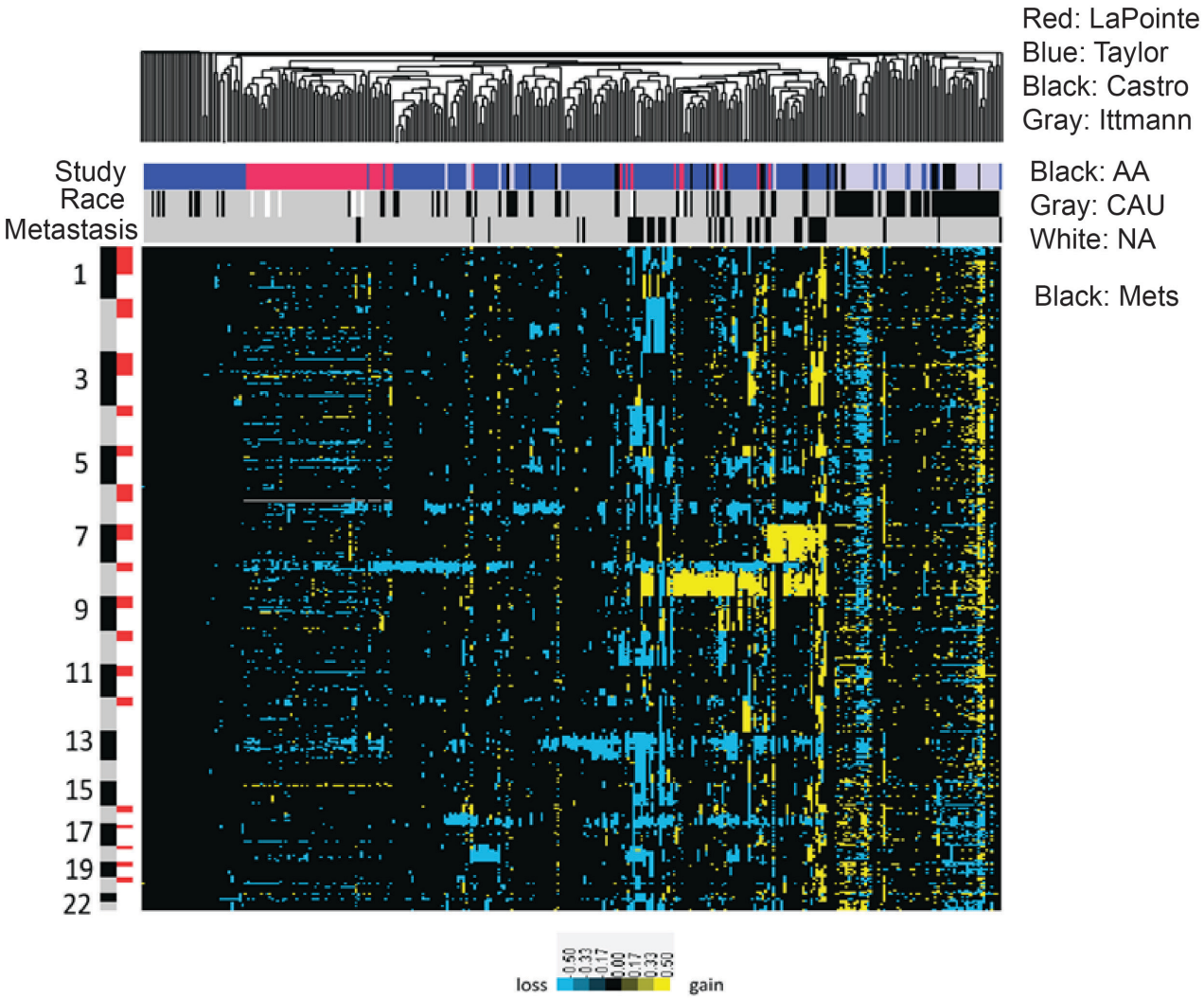

Figure 3. Cluster analysis of CAN data. Note that the majority of the AA cases cluster together in a major cluster to the right of the heat map.

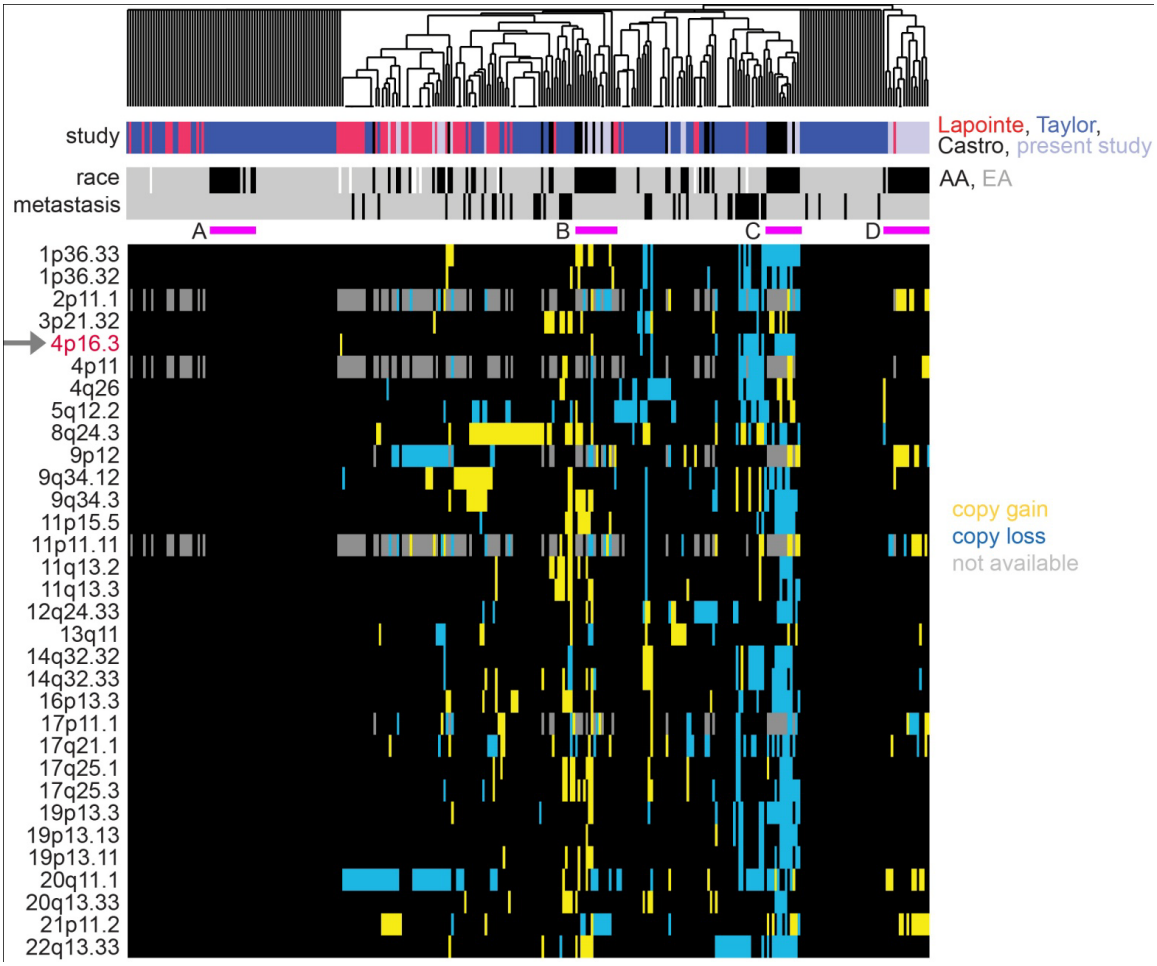

Figure 4. Cytobands lost or gained at significantly higher rates in AA Vs CAU (European American or EA) prostate cancers (p<.001)

Subtask 5: We will determine the extent to which any CNA or pattern of CNAs is associated with PSA recurrence using both the new dataset and the combined dataset (new plus prior study) using Cox proportional hazard regression modeling of biochemical recurrence to develop multivariate survival models with specific CNA cluster groups, specific CNAs and/or groups of CNAs. **(Months 18-36)**

We carried out analysis of the impact of CNA alterations on PSA recurrence. Unfortunately the total number of PSA recurrences was too small to develop robust models based on CNA alterations that would be predictive of PSA recurrence.

Task 2: Whole genome expression array analysis in African American prostate cancers.

Subtask 1. Expression array analysis of prostate cancers from AA men using RNAs extracted from PCa tissues containing 70% or more tumor from AA men in Task 1 above and matching benign tissues. We will use human whole genome arrays from Agilent for expression microarray

analysis as described previously. Each of these arrays contains 60-mer oligos that can detect 41,000 transcripts corresponding to the known human transcriptome. **(3-18 months)**

Progress: RNA expression analysis has been carried out on 96 RNAs (48 matched pairs)

Subtask 2. Continuous quality control of data **(3-18 months)**

Progress: We have carried continuous quality assurance.

Subtask 3. Initial data analysis using unsupervised cluster analysis of the AA expression dataset. Compare our data to existing publically available datasets available on the Web for both AA and CAU men. **(18-24 months).**

We have carried out extensive data analysis of the expression array data. This analysis revealed a total of 4341 probes altered in cancer Vs benign (at p<.01, t-test) in AA PCa corresponding to 1803 genes. Of these, 469 genes were upregulated >1.5 fold and 1146 were decreased 0.7 fold or more. Inspection of the data revealed multiple genes that are well known to be upregulated (AMACR[8], Hepsin [9], TFF3[10] and TDRD1[11]) and downregulated (GSTP1[12]) in PCa (Fig 5), confirming the overall quality of the data.

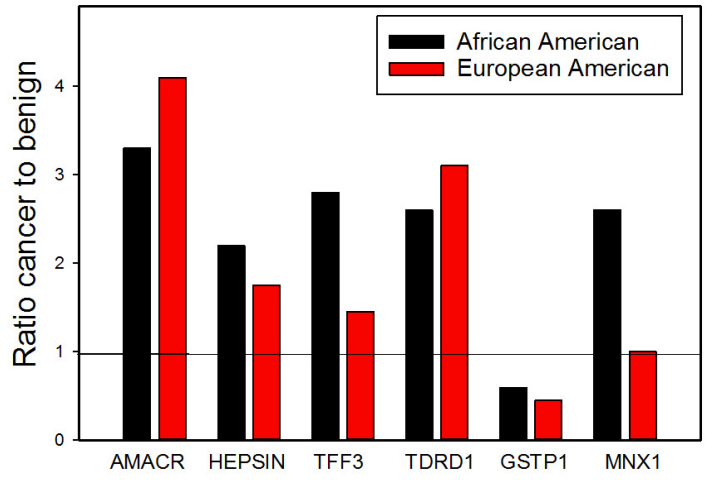

Figure 5. Expression array analysis of genes known to be altered in PCa and MNX1. Fold change (cancer versus benign) of specific gene expression in PCa tissues from African-American men and for same genes in the Taylor dataset for European American (Caucasian) men. The horizontal line shows one-fold for reference. All differences are statistically significant except MNX1 in European Americans.

To identify genes that were specifically upregulated (>1.5-fold) or down regulated (at least 0.7-fold) in PCa from AA PCa we compared expression of all such genes (all significant at p<.01, t-test) with the same genes in CAU patients (Taylor dataset [7]). We are using the Taylor data[7] set as our main comparator since it is the largest high quality dataset available until the recent publication of the TCGA dataset in November 2015. As expected, the majority of significant genes were significant in both groups. We identified 24 protein coding genes that were specifically increased in AA PCa. The most highly upregulated protein coding gene was the homeobox gene MNX1/HLXB9. This gene plays an important role in pancreas development [13] and is a causative oncogene in infantile AML [14]. However it has not been shown to be an oncogene in an epithelial cancer. As shown in Figure 5, MNX1 is increased 2.6 fold by array analysis, similar to AMACR and Hepsin; (p<.001, t-test) in AA PCa but not significantly altered in CAU PCa (1.03 fold, p=.16; Taylor dataset). Upregulation of MNX1 has been confirmed by

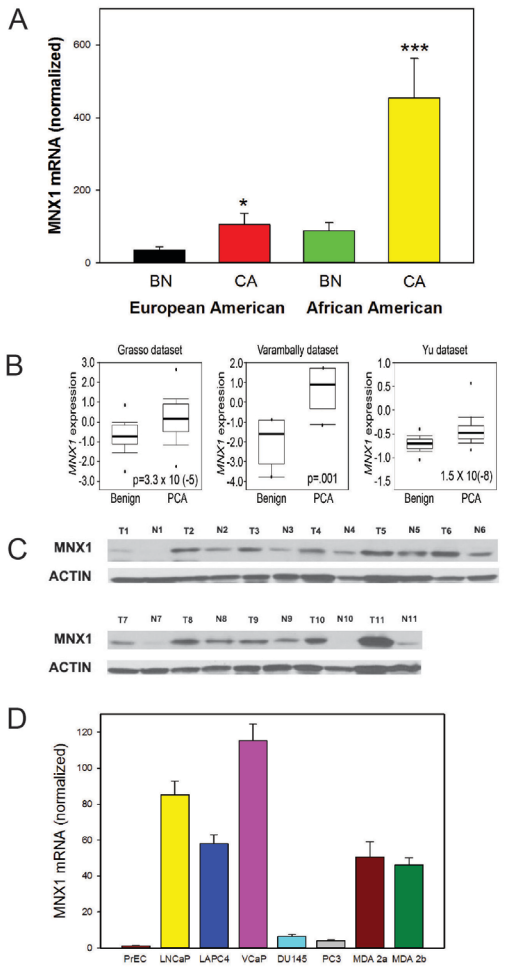

Figure 6. Expression of MNX1 in PCa.
A. MNX1 mRNA levels in EA and AA PCa and benign tissues by Q-RT-PCR. Asterisks indicate statistically significant differences between cancer and benign tissues. Mean +/- SEM; * p<.05; *** p<.001 by t-test
B. Examples of Oncomine datasets with increased expression of MNX1 relative to benign tissues; p value shown in bar and whisker plot.
C. Western blot on MNX1 from PCa and matched benign tissues from AA men. β-actin is a loading control.
D. Expression of MNX1 mRNA in primary prostatic epithelial cells (PrEC) and PCa cell lines.

Q-RT-PCR of AA RNAs (5.1 fold; p<.002, t-test, Figure 6A). Direct analysis of RNAs from CAU PCa and matched benign tissues shows a smaller but still significantly increased expression of MNX1 mRNA in PCa. Consistent with this finding, 3 of 13 Oncomine PCa tissue datasets (predominantly CAU) showed statistically significant increases in MNX1 mRNA (p≤.001), although the Taylor dataset was not among those with MNX1 upregulation (Figure 6B). As a comparison, ERG was upregulated in 10 of 19 datasets at p≤.001. We then carried out Western blots using protein extracts from matched PCa and benign tissues from AA men (Fig 6C). While variable expression was seen in both cancer and benign tissues, the mean increase in MNX1 protein in cancer compared to matched benign tissues was 12.4-fold (range 0.83 to 81.1-fold) by densitometry. RNA in situ hybridization showed expression of MNX1 in PCa cells and to a much lesser extent benign epithelial cells but no expression in benign or tumor associated stroma (Figure 7). Expression of MNX1 mRNA was almost undetectable in benign prostate epithelial cells (PrECs) but was markedly elevated in the androgen receptor (AR) expressing LNCaP, LAPC4, VCaP, MDA PCa 2a and MDA PCa 2b PCa cells but not in AR negative DU145 and PC3 (Fig 6D). MDA PCa 2a and 2b are cell lines from an AA patient. Thus MNX1 is expressed at increased levels in PCa, with more marked increases in cancers from AA men.

We have also identified genes that are significantly associated with aggressive pathological features (high Gleason score, seminal vesicle invasion and extracapsular extension).

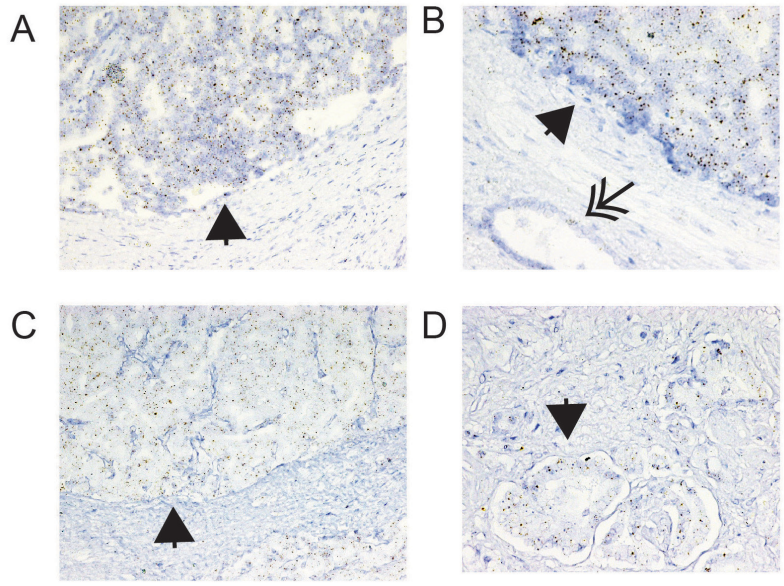

Figure 7. Localization of MNX1 mRNA in prostate cancer tissues. MNX1 mRNA was detected using RNAScope detection technology with brown dots represent mRNA signals.
A, B. Strong expression of MNX1 mRNA in prostate cancer cells (arrowheads). Minimal expression was seen in benign epithelium (double arrow). No expression was seen in stromal tissues.
C, D. Weak expression of MNX1 mRNA in prostate cancer cells (arrowheads).

Subtask 4: Correlate expression and CNA analysis **(18-36 months)**

We have correlated copy number and gene expression in our datasets. A total of 317 genes showed correlation of gene expression and copy number (based on cytobands) at $P<0.01$ in AA PCa. As expected, PTEN was among these genes given that losses in PTEN alleles are well known to be associated with gene expression in PCa. Of note, of the 32 cytobands that were more commonly altered in AA PCa than CAU PCa (Fig 4), 11 showed correlation with gene expression with one or more genes in that cytoband. This finding indicates that the specific genomic alterations observed in AA PCa are functional in driving gene expression in many instances.

Subtask 5: Carry out pathway analysis of expression data **(18-36 months)**

To discover specific pathways regulated by MNX1 we overexpressed MNX1 in DU145 cells and knocked down its expression in LNCaP cells and carried expression microarray analysis. Analysis of this data indicated that MNX1 plays a role in driving lipid synthesis. As shown in Fig 8A, DU145 cells express lower levels of MNX1, fatty acid synthetase (FASN) and sterol regulatory element binding transcription factor 1 (SREBP1) mRNAs than LNCaP cells (which have higher MNX1). SREBP1 is a transcription factor which is known to drive expression of FASN. DU145 cells with overexpression of MNX1 show increased expression of both FASN and SREBP1, while LNCaP cells with MNX1 knockdown show a 60-70% decrease in FASN and SREBP1. Of note, MNX1 was knocked down to a similar extent as FASN and SREBP1 in LNCaP cells, indicating that MNX1 plays a significant role in driving these two genes in PCa and thus in driving lipid synthesis.

To confirm the connection between MNX1 and lipid synthesis we analyzed LNCaP MNX1 knockdown tumors and controls for palmitic acid, a major product of FASN. As can be seen Fig 8B, tumors showed a marked decrease in the content of palmitic acid ($p<.001$, t-test). To confirm that MNX1 drives SREBP1 and FASN in human PCa we analyzed FASN in RNAs from benign

and PCa tissues from AA men. As show n in Fig 8C, both SREBP1 and FASN are significantly increased in AA PCa compared to benign tissues (p<.01, Mann-Whitney). We then examined the correlation between MNX1 and FASN in the cancer samples. The correlation was extremely high (r=.548, p<.001, Pearson's) in AA samples (Fig 8D). There was a similar significant, but somewhat lower correlation, of MNX1 with SREBP1 mRNA levels (r=.335, p=.02, Pearson's). As a positive control we examined the correlation of SREBP1 and FASN, since FASN is a known direct target of SREBP1. As predicted the correlation was quite high (r=.63, p<.001). Overall this data strongly supports the idea that one important pathway by which MNX1 promotes oncogenesis is by increasing expression of SREBP1 and its target FASN in AA PCa.

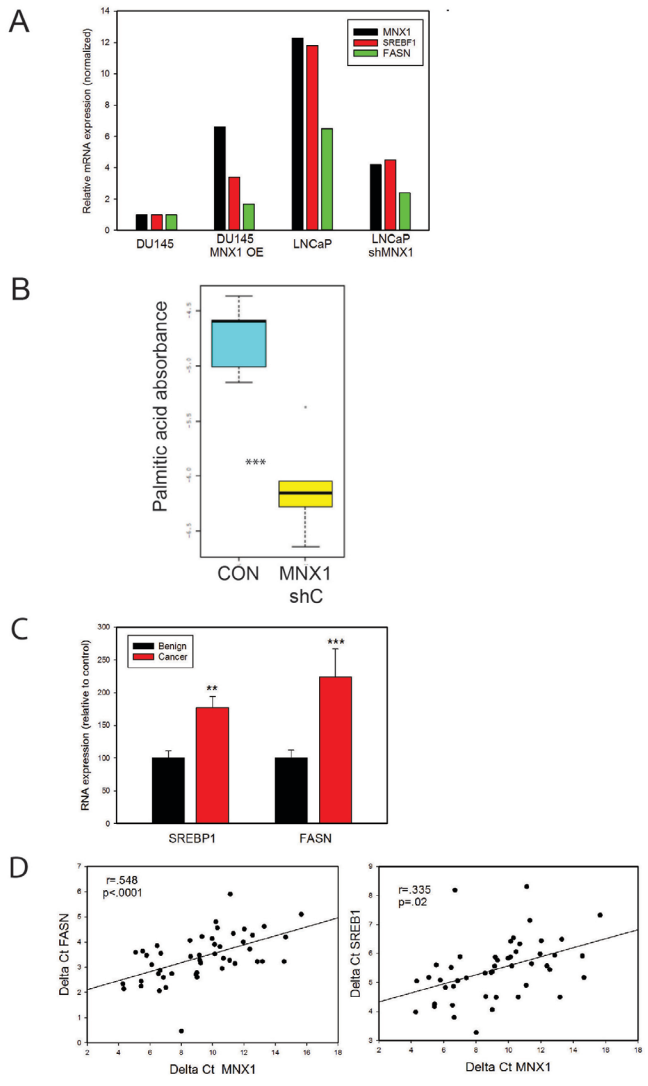

Figure 8. MNX1 regulates lipid synthesis.
A. Expression of MNX1, SREBF1, and FASN in DU145 overexpressing MNX1 or LNCaP with MNX1 knockdown versus controls. Data is expressed as percent of vector control for DU145 for each gene. Means of triplicate determinations are shown.
B. Palmitic acid content of LNCaP xenograft tumors versus scrambled controls (see Fig 3D); *** p<.001 by t-test.
C. SREBP1 and FASN mRNA expression in AA benign prostate and PCa tissues. Mean +/- SEM is shown. ** p<.01; *** p<.001; Mann Whitney.
D. Correlation between SREBP1 or FASN with MNX1 mRNA in AA PCa by Pearson Product Moment. Correlation coefficient and p-value are shown.

Subtask 6: Validation of key gene expression changes in PCa identified during data analysis using quantitative RT-PCR **(18-36 months).**

We have multiple genes with increased or decreased expression in AA PCa identified using the expression array analysis including MNX1 (Fig 6 above), RGS12 (see below), cartilage oliomeric protein (COMP), FOXL2 and CXCL14 (data not shown). Overall the expression arrays have been highly accurate in showing altered expression.

Task 3: Identification of potential tumor suppressor gene(s) on 4p16.3 in AA PCa.

Subtask 1: Identify minimal deleted region on 4p16.3 by analysis of CNA data from Task 1. Identify any homozygous deletions. **(Months 18-21).**

Subtask 2: Further define minimal deleted region by analysis of gene expression data from Task 2 **(Months 18-21).**

Progress on Subtasks 1 and 2:

Gene	Het Loss	Homo Loss	r value	p value
HTT	1	0	-0.0455	0.86714
C4orf44	13	3	0.35822	0.17308
RGS12	4	0	0.59163	0.01578

Table 1. Genes in region of interest at 4p16.2/16.3 boundary

We have analyzed the 4p16 region in detail for the presence of heterozygous and homozygous deletions, loss of expression in AA PCa and correlation of gene copy number changes with gene expression. We have identified a single region of interest and the genes in this region are shown in Table 1. This region encompasses approximately 400 Kbp and is located at 4p16.3,

To identify the potential an AA PCa specific tumor suppressor on 4p16.3 we systematically examined expression of genes on 4p16.3 in AA and CAU (Taylor dataset) PCa. We found that two genes that are adjacent on 4p16.3 (HTT and RGS12) and which both show downregulation of mRNA in AA PCas (but not in CAU PCa). **Detailed examination of deletions showed that losses are more concentrated in RGS12 and CNA changes correlate with expression levels for RGS12 (r=.59, p=.016) but not HTT (Table 1).**

C4orf44 is a gene of unknown function adjacent to RGS12 and shows high levels of LOH as well as homozygous deletions but copy number is not correlated with expression and its expression is not decreased in AA PCa. We hypothesize that deletion in C4orf44 may impact expression of RGS12 since it is only 50 Kbp upstream of RGS12. Alternatively, C4orf44 might encode a novel tumor suppressor. HTT does not appear to be target in this region.

Subtask 3: Exome capture of minimal deleted region defined in Sub-tasks 1 and 2 above and perform next generation sequencing to identify mutations **(Months 21-26)**

Progress: The approach above is now obsolete given the availability of high quality next generation sequencing data in public databases. Analysis of public databases (SU2C and University of Michigan) reveals that approximately 3% of advanced metastatic prostate cancers have a mutation of RGS12. For comparison, approximately 10% of advanced prostate cancers have mutations in PTEN, which has been repeatedly validated as a PCa tumor suppressor gene. A single point mutation was also identified in a clinically localized cancer. This finding is consistent with RGS12 being a tumor suppressor gene in PCa,

Subtask 4: In vitro functional studies of potential tumor suppressor genes identified based on homozygous deletion, mutation or decreased expression with relevant known or predicted biological functions. **(26-36 months)**

RGS12 is a negative regulator of G-protein coupled signaling and also regulates RAS/Raf/ERK signaling[15, 16]. Its role in cancer has not been studied in detail but it might potentially function as a tumor suppressor. As shown in Figure 9A, Q-RT-PCR analysis confirmed decreased RGS12 expression in AA PCa ($p=.024$, paired t-test). There was a trend for decreased expression of RGS12 in EA PCa by Q-RT-PCR that was not statistically significant ($p=.19$). Examination of Oncomine datasets revealed that 9 of 14 Oncomine PCa tissue datasets (all mainly EA patients) for which RGS12 data was available showed decreased expression of RGS12 ($p\leq.01$). Examples are shown in Figure 9B. For comparison, 10 of 16 datasets showed PTEN loss at $p\leq.01$. Overall the CNA and expression analysis data indicate that there is loss of RGS12 alleles and/or gene expression that is more pronounced in AA PCa but also occurs in EA PCa. We then examined expression in prostate and PCa cells in vitro. All seven PCa cell lines, including the related AA PCa cell lines MDA PCa 2a and MDA PCa 2b, showed decreased expression relative to normal prostate epithelial cells (PrEC) in culture (Fig 9C). We then knocked down RGS12 in LNCaP cells using stable expression of four different shRNAs and observed significantly increased growth in all cases, indicative of tumor suppressive activity by RGS12 (Fig 9D). Knockdown of RGS12 in LNCaP resulted in increased tumor growth of xenograft tumors in SCID mice compared to scrambled controls (Fig 10). Taken together, our data shows that RGS12 is a tumor suppressor gene that is decreased in AA PCa and, to a lesser extent, in CAU PCa as well.

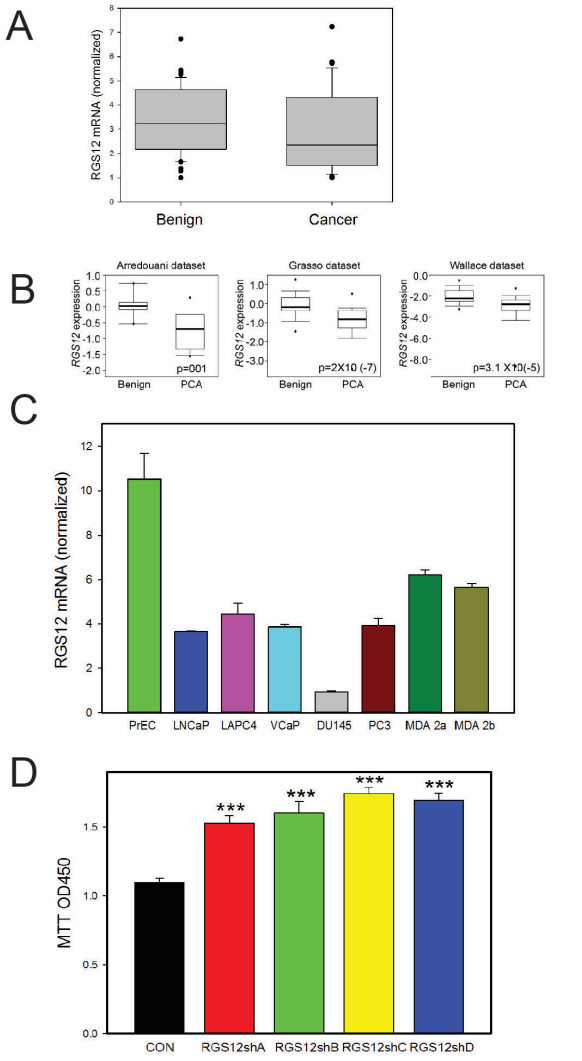

Figure 9. RGS12 in prostate cancer.
A. Expression of RGS12 in AA PCa and matched benign tissues by Q-RT-PCR (p=.024, paired t-test).
B. Examples of Oncomine datasets with significant loss of RGS12; p value shown in bar and whisker plot.
C. Expression of RGS12 mRNA in primary prostatic epithelial cells (PrEC) and PCa cell lines. Mean +/- standard error of the mean (SEM) for triplicates are shown.
D. Proliferation of LNCaP cells with knockdown of RGS12 using four different shRNAs. Mean +/- SEM for triplicate determinations; *** p<.001 by t-test

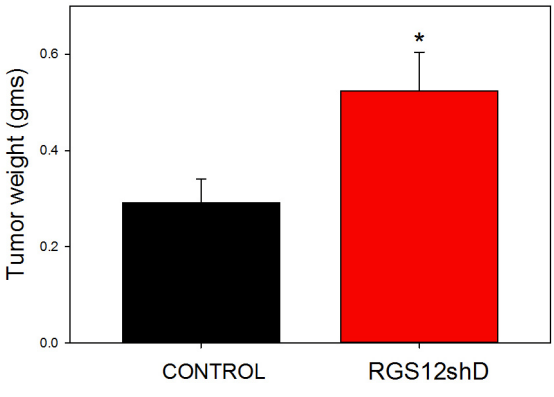

Figure 10. RGS12 is a tumor suppressor in PCa
Tumor size of LNCaP subcutaneous xenografts with knockdown of RGS12 or scrambled vector controls. * p<.05

KEY RESEARCH ACCOMPLISHMENTS

- We have identified and extracted high quality DNAs and RNAs from matched cancer and benign tissues from 69 radical prostatectomies.
- Copy number and expression array analysis has been performed and generated large amounts of data relevant to the biology and clinical behavior of AA PCa.
- We have identified RGS12 as a novel tumor suppressor gene on 4p16.3 that is decreased in AA PCa.
- We have identified MNX1 as a novel oncogene that is increased in AA PCa and increases lipid synthesis
- Analysis of other genes and pathways of interest is ongoing

REPORTABLE OUTCOMES

- A manuscript reporting our findings with MNX1 Has been submitted to *Cancer Research*
- A manuscript reporting our findings with RGS12 is in preparation

CONCLUSION

We have carried out large scale CNA and expression array analysis of AA PCa to provide a comprehensive picture of the genomic landscape in African American prostate cancer. This data will yield important insights into the biology of AA PCa as well as potential biomarkers of disease aggressiveness. The first two genes RGS12 and MNX1 discovered using this approach are a novel tumor suppressor and oncogene, respectively. Further mining and of this data by our laboratory and other groups will almost certainly yield additional novel insights into AA PCa

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