Prostate Carcinomas in African Americans Have Distinct miRNA Expression and Biological Markers for Poor Prognosis

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African American (AA) men have been disproportionately affected by prostate cancer (PCa), with almost twice the incidence and mortality as compared to non-Hispanic Whites. Controlling for socioeconomic and environmental factors, a biological basis for this disparity persists and its identification is crucial in providing early diagnosis and focused treatment. Much of the study of cancer biology has been focused on non-Hispanic Whites, leaving AA out of research and clinical trials. This has left a gap in medical literature, resulting in worse healthcare outcomes for this population. In this study, we have characterized demographic and clinico-pathological features and molecular markers of PCa in AA men to determine the biological basis of disparities in this minority population. Formalin-fixed paraffin-embedded tissues from 91 proctectomies for primary PCa and 23 biopsies for metastatic PCa with their clinico-pathological data and survival information were retrieved from the records of a tertiary-care medical center. Tissue microarrays were constructed and the expression of Programmed Cell Death 1 (PD-1), Programmed Death Ligand 1 (PD-L1) and Mismatch Repair (MMR) (MLH1, MSH2, MSH6 and PMS2) proteins was assessed by immunohistochemistry. miRNA expression of 24 cases of primary PCa and adjacent normal prostate tissue from proctectomies was identified by microarray in situ hybridization. Association between these variables were analyzed by Spearman's rank-correlation and p values were calculated for all pairs, $p < .05$ were considered significant. TargetScan and DIANA analysis software were used to identify modulation in miRNA expression. A distinct pattern of miRNA expression was identified in our patient population which could distinguish tumor from normal tissue. Specific miRNA associated with defined pathways of carcinogenesis were identified. miR-182 and miR-375 showed significant up-regulation; these have been linked to more aggressive PCa. PD-L1 expression was low in primary PCa, making these patients poor candidates for immunotherapy. The expression of PD-L1 in metastatic PCa was higher than primary PCa. PD-1 expression was high in almost half of primary and metastatic PCa patients and MMR protein expression was largely intact in both primary and metastatic PCa cases; both of these protein markers have been associated with biochemical recurrence and low survival. Smoking correlated with pathological features of aggressive primary PCa. High Body Mass Index (BMI) was linked to high PD-1 and PD-L1 expression in metastatic PCa, biomarkers of aggressive disease. The biological characteristics (low expression of PD-L1, high expression of PD-1, intact MMR and distinct miRNA signature) identified in our cohort of exclusively AA men are indicators of aggressive PCa. These results explain the biological basis of widely documented poor prognosis of PCa in AA men. This characterization of PCa can help fill the gap in knowledge of pathogenesis in PCa in AA men thus empower physicians to advise minority patients of risk factors and recommend individualized treatment plans. The miRNA panel identified in our study can be a promising tool for future studies on early diagnosis and predicting prognosis of PCa in AAs.

Health Disparities | Prostate Carcinoma | African American | miRNA | PD-1 | PD-L1 | MLH1 | MSH2 | PMS2 | MSH6 | MMR |

Prostate cancer (PCa) is the most common form of cancer in American men, and ranks second most common cause of cancer death after lung and bronchus (1). African Americans (AA) with PCa have 1.7 times higher incidence (158.3/100,000 AA vs 90.2/100,000 non-Hispanic Whites (NHW)) and twice the mortality rate as compared to NHW (39.9/100,000 AA vs 18.2/100,000 NHW) (2). AA men are diagnosed with PCa at a younger age, at a more advanced stage, have higher Prostate Specific Antigen (PSA) levels, and a greater rate of biochemical recurrence after treatment than their NHW counterparts (3). These disparities are a cause for concern amongst the healthcare community, and have been linked to cultural, socioeconomic and genetic factors (4). Even studies which adjusted for socioeconomic factors, clinical tumor stage, Gleason grade for prostate carcinoma grading and PSA have corroborated that PCa mortality remained statistically higher in AA men (5). Further, these studies suggest a biological basis for difference between NHW and AA men that is responsible for the differences in outcomes (5). Therefore, it is imperative that studies focusing on biological differences between AA and NHW men should be attempted to determine the molecular mechanisms responsible for this disparity.

Significance Statement

High incidence and mortality of prostate cancer in African-Americans has been a challenge for communities of color. As much of the study of cancer biology has focused on whites, a gap remains in medical literature, resulting in misdiagnosis and undertreatment for African-Americans. We designed this study to bridge this gap by characterizing the unique biological basis of disease in African-American patients through molecular analysis. These findings can empower physicians to advise minority patients of risk factors and design individualized treatment plans. This knowledge will also help in the development of new screening tools for early diagnosis and improved survival for this at-risk group.

Prostate Specific Antigen. No national consensus exists for the value of PSA screening since the United States Preventative Services Task Force (USPSTF) recommended against PSA screening for early detection of PCa in 2012 (6). Recognizing the increased risk many AA men face to develop PCa, USPSTF released an updated guideline in 2017 recommending PSA-based screening for AA men, and emphasized educating AA patients about increased risk of PCa and mortality in their population (7). This new report highlights the recognition within the healthcare community about the biological differences that exist in AA men as compared to the general population. The lack of reliable biological markers to guide therapy and prognosis results in overtreatment or under-treatment resulting in avoidable side effects and disease advancement (8). Furthermore, if the patient develops metastatic or hormonally unresponsive disease, few effective treatment options exist. Therefore, there is a need to better understand the biology of disease in AA and identify reliable biological markers for early diagnosis and focused treatment. Thus, in this study we have attempted to identify clinical and biological characteristics of PCa in AA men that can be used to better define their tumor biology, identify early disease, provide accurate prognosis and enhance treatment options.

DNA Mismatch Repair Pathway. The DNA Mismatch Repair Pathway (MMR) is a highly conserved mechanism which is required for genomic stability through multiple replication cycles. DNA damage occurs over time due to exposure to physical and chemical mutagens, causing base-base mismatches during replication. This damage, if unrepaired, can lead to potentially lethal mutations in somatic and germ-line cells. To prevent these defects and maintain genome integrity, cells operate the DNA Mismatch Repair Pathway. It resolves DNA mismatches, preventing the buildup of mutations from becoming permanent and harming the cell (9, 10). MMR is responsible for reducing errors in DNA replication through cell cycle arrest and/or apoptosis; defects in this pathway lead to abnormally high mutation rates and the failure of MMR has been linked to hereditary and somatic cancer (9–14). Microsatellite Instability (MSI) results from MMR inability to correct DNA replication errors and it has been associated with many tumors, including gastrointestinal and gynecologic carcinomas (15). MMR deficient tumors also exhibit acquired resistance to chemotherapy drugs which target apoptotic pathways (16). Identification of MSI in a tumor is of particular prognostic relevance because of 2014 Federal Drug Administration (FDA) approval of a new class of immunotherapy drug, pembrolizumab (Keytruda®), to treat patients with metastatic tumors with MSI. Few studies have characterized MMR status of primary and metastatic PCa; MMR deficiency has been correlated with high grade prostate tumors (17). In this study, we investigated MMR protein loss in both primary and metastatic PCa, calculated its incidence in AA patients, and correlated it with clinico-pathological features.

Programmed Cell Death Protein 1 (PD-1) and Programmed Death Ligand 1 (PD-L1). PD-1 is a T-cell immune checkpoint that reduces self-recognition in T-cell activation, conferring immune tolerance to cells expressing PD-L1 (18). PD-L1 is the principal ligand of PD-1 and is a co-inhibitory receptor that is expressed in normal myeloid, lymphoid and epithelial cells and in cancer (19). The interaction between these two proteins is important in the normal development of immune tolerance, thereby preventing autoimmune disease (Fig.1) (20). Various malignancies have utilized the expression of PD-L1 to evade immune recognition through expression of PD-L1 on cancer cells giving them survival advantage and poor prognosis (21, 22). The expression of PD-L1 on tumors has been utilized to predict the response to anti-PD-1/PD-L1 therapies (23). The development of new immunotherapies which inhibit the interaction between PD-L1 (present on the surface of the tumor), and PD-1 (present on the surface of activated lymphocytes) have generated promising patient outcomes. There have been few studies focusing on expression of PD-1/PD-L1 expression in PCa of AA men. Our study explored the presence of PD-1 and PD-L1 in primary and metastatic PCa in AA men, which may offer a new class of treatment options for this group.

microRNA. micro RNA are small, noncoding RNA nucleotides which regulate post-transcriptional gene expression (24). Complimentary binding of miRNA to mRNA can inhibit translation or induce the hydrolysis of mRNA in the cytoplasm (25). miRNA plays an important role as tumor suppressive genes that suppress cellular growth and proliferation and contribute to apoptotic pathways (26). miRNA have been found to be widely dysregulated in many tumors, including PCa (26). Polymerase Chain Reaction has recently been used to identify miRNA as key biomarkers for the diagnosis and prognosis of PCa (27, 28). To better define miRNAs that could be used for early diagnosis and accurate prognosis of PCa, we examined primary and metastatic PCa tissues from AA patients and correlated them with respect to clinico-pathologic features and disease outcomes.

In this study, we have attempted to comprehensively characterize clinico-pathologic features of primary and metastatic PCa in AA patients. We have compiled demographic, clinical and pathologic features from a tertiary-care medical center and analyzed expression of MMR, PD-L1 and PD-1 proteins and miRNA from tumor tissue of primary and metastatic PCa patients. We have attempted to characterize these variables and explore common biological pathways in PCa for AA.

Materials and Methods

Patients and Tissue Samples. 100 prostatectomies for primary PCa and 24 biopsies for metastatic PCa were identified from the electronic medical records of an urban tertiary-care medical facility from 2003. Clinico-pathological data and survival information were retrieved until 2018 (Table S1 & Table S2). Death records of all patients were reviewed from hospital files and Ancestry.com. Inclusion criteria for primary prostatectomy included all patients with diagnosis of PCa who have had a prostatectomy without pre-operative chemotherapy. Of the 100 patients with prostatectomies 91 AA patients were included in the study. 5 Asian and 4 NHW patients were excluded (Fig.2). Metastatic carcinoma cases included in the study did not have a prior prostatectomy and were first diagnosed from biopsy of the metastatic site. All AA patients (23 of 24) were included in the study; one Asian patient was excluded (Fig.2). The slides of all the cases included in the study were retrieved and reviewed for confirmation of the diagnosis. In prostatectomy cases, two representative slides with a large focus of PCa and one slide with benign prostate tissue were identified. In patients with metastatic carcinoma, only one

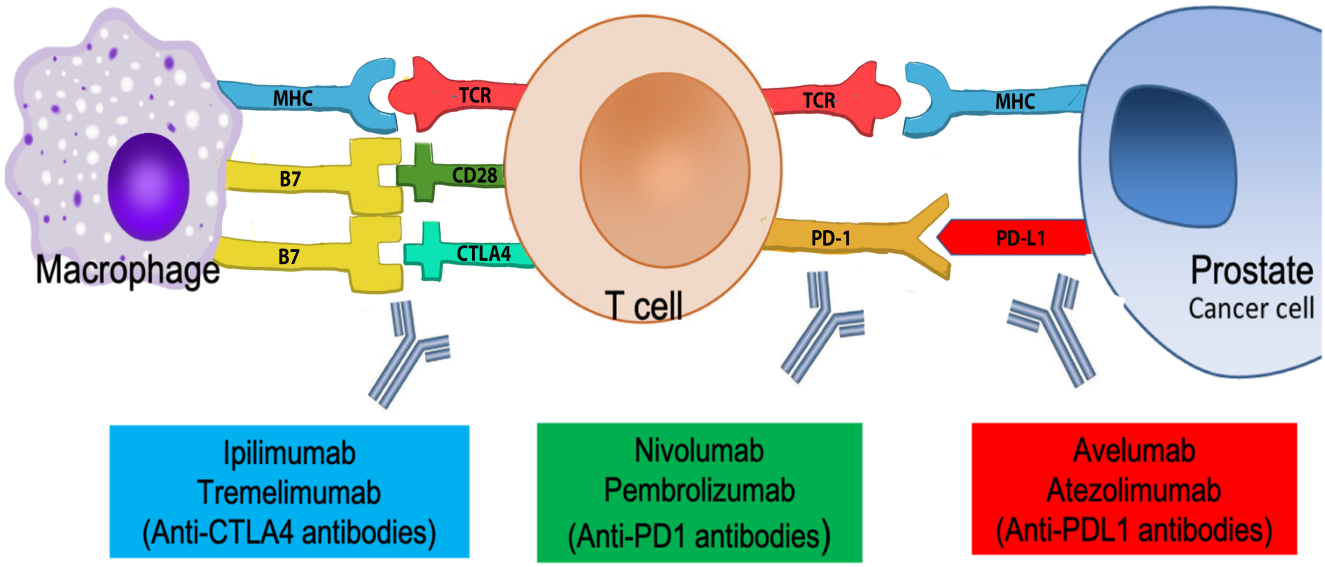

Fig. 1. Immune Checkpoint receptors on immune cells and prostate cancer cell, namily CTLA4, CD28, PD-1, and PD-L1 with their pharmacological inhibitors. MHC: major histocompatibility complex; TCR: T-cell receptor; CTLA-4: cytotoxic T lymphocyte antigen-4; PD-1: programmed cell death protein 1; PD-L1: programmed cell death protein ligand 1.

representative slide of diagnostic biopsy was identified. The respective blocks for these slides were retrieved. The prostatectomy tumor slides and blocks were marked for tumor outline and prepared for tissue microarray (TMA) construction. The biopsies of metastatic cancer were used as full surface sections because of their small size (Fig.4).

Tissue Microarray. The tumors in paraffin blocks of prostatectomies were traced with an outline after matching them with the corresponding slide. Two cores of paraffin, 3 mm in diameter, were punched from tumor in the paraffin block. These cores were inserted into the recipient block using a tissue arrayer (Quick-Ray, Tissue Microarrayer, IHC World LLC, USA). Each tissue microarray block accommodated approximately 38 cores (19 cases). The block was warmed in the incubator at 55ºC for 1 hour to fix the inserted cores in the paraffin block. The block was cooled on ice and 4 µm sections from each block were cut and affixed to glass slides for staining (Fig.4 & Fig.3).

Survival. Recurrence/survival were calculated as Biochemical Recurrence (BR) and, overall survival (OS). BR was characterized as any increase in PSA >0.2ng/ml any time after prostatectomy. OS was defined as total survival from the date of prostatectomy and/or date of diagnosis of metastasis to the last follow-up date (in increments of 2, 5 and 10 yr.) or date of death. Lost to follow up was defined as patients who did not come back for clinical follow up after prostatectomy.

Immunohistochemistry. Immunohistochemistry was performed on a Ventana ultra autostainer by using primary antibodies: PD-L1 (rabbit monoclonal clone SP263), PD-1 (mouse monoclonal antibody NAT105) and MMR [VENTANA MMR IHC Panel: anti-MLH1 (M1), anti PMS2 (A16-4), anti-MSH2 (G219-1129), anti-MSH6 (SP93)], Ventana Medical Systems, Inc. The paraffin sections were deparaffinized, and heat-induced antigen retrieval was performed with a CC1 buffer at 36º C (Ventana Medical Systems, Inc) for PD-L1 (64 min), PD-1 (40 min), MLH1 & MSH2 (24 min), MSH6 (32 min) and PMS2 (56 min). It was followed by incubation with PD-L1 antibody (16 min), PD-1 (16 min), MLH1 & MSH2 (16 min) and MSH6 & PMS2 (32 min). Antigen-antibody reactions were visualized using Optiview DAB Detection Kit (Ventana Medical Systems, Inc). Staining was performed using the standard Ventana protocol (BenchMark Ultra; Ventana Medical Systems, Inc.) on 4µm, thick, formalin-fixed, paraffin-embedded tissue sections from tissue microarrays of prostatectomies and surface sections of metastatic prostate tumors.

After chromogenic detection, all slides were counterstained with hematoxylin. Human placenta was used for optimization and as a positive control for PD-L1, which shows a strong cytoplasmic and membranous staining. The positive control utilized for PD-1 was tonsillar tissue, which showed strong cytoplasmic staining in lymphocytes. Positive control for all MMR antibodies was colonic tissue which highlighted strong diffuse nuclear staining of mucosal nuclei, stromal cells and lymphocytes.

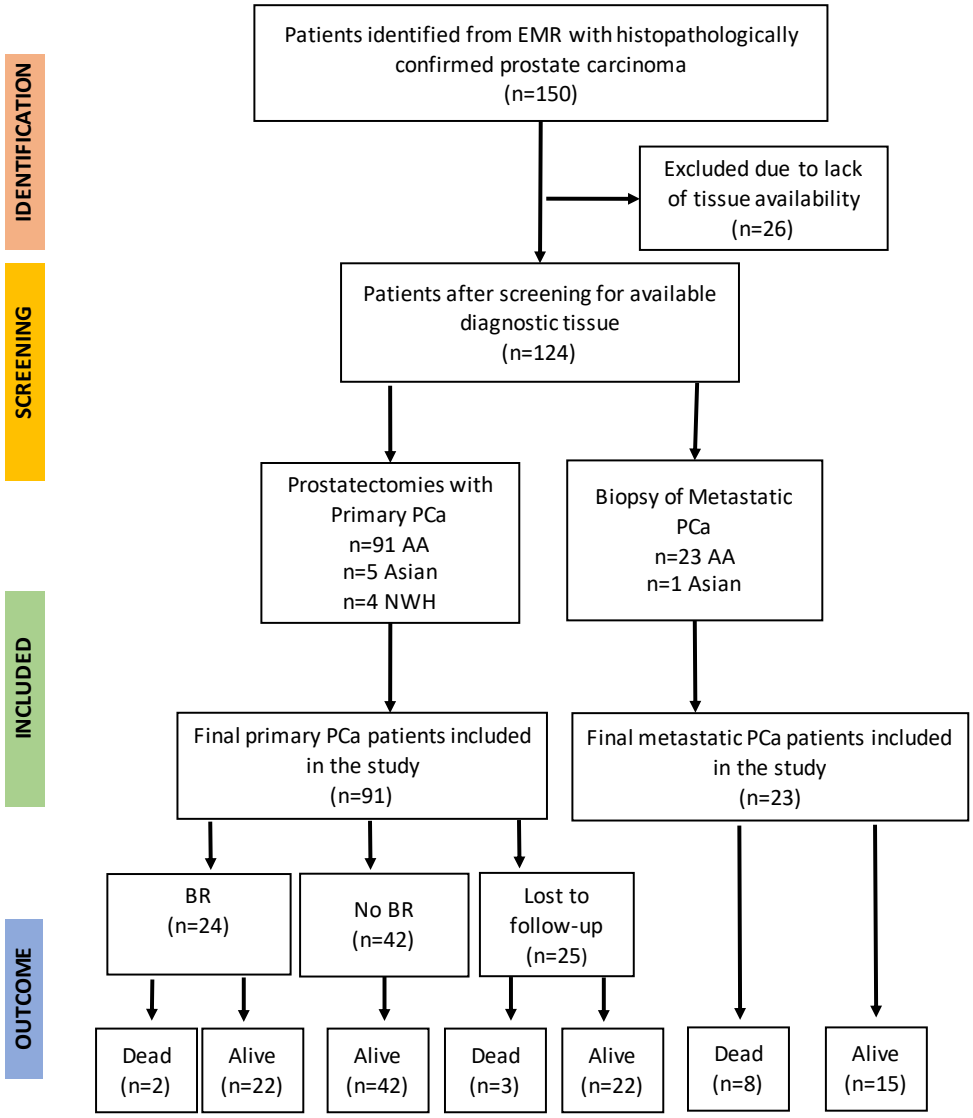

Fig. 2. Flowchart illustrating the selection of study population for primary and metastatic prostate cancer and their outcome. EMR: electronic medical record; PCa: prostate cancer; BR: biochemical recurrence.

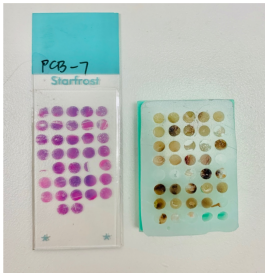

Fig. 3. Prostate carcinoma tissue microarray paraffin block and slide stained with Hematoxylin and Eosin.

miRNA. 48 FFPE blocks (24 tumor and 24 adjacent normal tissue from the same patient) were faced off and trimmed on a Microm HM315 microtome (Walldorf, Germany). 10 sections of 10 µm were shaved. They were collected into separate sterile test tubes at room temperature.

Extraction and purification of nucleic acids. Standardized approaches and kits were used for nucleic acid extraction. Genomic DNA was extracted from FFPE sections according to manufacturer's instructions using the QIAamp® DNA FFPE Tissue kit (Qiagen, Valencia, CA) and eluted into a total volume of 50 microliters of ATE (supplied) buffer. A single 10 µm FFPE section was used to extract both miRNA using the miRNeasy FFPE kit (Qiagen, Valencia, CA) and RNA using the RNeasy® FFPE kit (Qiagen, Valencia, CA) according to manufacturer instructions and both derivatives were individually eluted to 16 microliters of RNase-free water.

Analysis of nucleic acids and protein extracts. The quality and the concentrations of total RNA were assessed using the NanoDrop (Thermo Fisher Scientific) and Agilent Bioanalyzer (Agilent Technologies). Briefly, four microliters of each sample were placed on the NanoDrop Microplate and 0.5 mm path length cover slide. Absorbance measurements were taken at 230 nm, 260 nm, and 280 nm for all samples and Spectrum wavescans stored (220 nm-350 nm with a step size of 4 nm). Absorbance ratios were determined via SoftMax Pro v6.4.1 software and concentration was determined using default software formulas with the following concentration factors: RNA (40)Total RNA was processed and hybridized to Agilent Human miRNA microarrays using standard protocols. Total RNA (100 ng) was dephosphorylated with calf intestinal phosphatase and end-labeled with Cy3-pCp by T4 RNA ligase prior to an overnight hybridization at 55 °C onto Agilent Human miRNA v3 (Sanger release 12.0) microarrays which contains probes sets for 112 human miRNAs. The arrays were washed and scanned on a high resolution GC2565CA Agilent Scanner using the manufacturer's recommended settings. The raw data was extracted using Agilent Feature Extraction software v10.1.1 and imported into GeneSpring GX11.5 for further analysis.

Statistical analysis of miRNA gene targets. The pathway analysis was done based on the changes in miRNA expression between normal and tumor in each sample. After import into GeneSpring X11.5, the 112 miRNA array data was log2 transformed and normalized to the 75th percentile. TargetScan and DIANA analysis software were used to identify targets of the miRNA that were significantly under or overexpressed (Table 1). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was then used to identify the Gene Ontologies that are most affected by the changes in the miRNA, which were overlaied over known pathways involved in prostate carcinoma (Table S1). Entities with fold changes greater than 1.5 were considered significant ($p < 0.05$).

Statistical Analysis for clinico-pathological data. All statistical analyses were performed using the statistical package IBM SPSS, version 24 (SPSS Inc. Chicago, IL). Spearman's rank-correlation was used to examine the association between different variables in primary PCa and metastatic PCa. p value was calculated for all pairs and $p < 0.05$ was considered significant. All survival analyses were carried out with respect to biochemical recurrence and outcome.

Scoring of Immunohistochemical Reaction.

PD-L1. All tissue samples were scored as high or low for protein expression, while blinded to pathologic, or survival data. Number of of PD-L1 positive tumor cells and staining patterns were noted based on Ventana Urothelial Cancer SP263 PD-L1 Scoring Guidelines (29). PD-L1 expression was determined by the percentage of tumor cells with any membrane staining above background or by the percentage of tumor-associated immune cells with staining (IC+) at any intensity above background. The percent of tumor area occupied by any tumor-associated immune cells (Immune Cells Present, ICP) was used to determine IC+, which is the percent area of ICP exhibiting PD-L1 positive immune cell staining. PD-L1 status was considered High if any of the following were met: • ≥25% of tumor cells exhibit membrane staining; or, • ICP > 1% and IC+ ≥25%; or, • ICP = 1% and IC+ = 100%.

PD-1. PD-1 was evaluated in lymphocytes around the tumor cells. 100 lymphocytes were counted and the number of lymphocytes with cytoplasmic staining with PD-1 were recorded. Median of the stained lymphocytes was derived and cases with less than the median were recorded as low and higher than the median as high.

MMR. MMR status was assigned based on the evaluation of the presence or absence of specific staining with the four MMR IHC assays in the VENTANA MMR IHC Panel. Protein expression was considered intact on visualization of unequivocal nuclear staining in viable tumor cells, and in the presence of acceptable internal positive controls (nuclear staining in lymphocytes, fibroblasts or normal epithelium near the tumor). Loss of protein was assigned to cases with unequivocal loss of nuclear staining or focal weak equivocal nuclear staining in the viable tumor cells in the presence of internal positive controls (30). The Institutional Review Board approved the study [IRB: 1093234].

Results

Demographics. 91 AA primary PCa patients with prostatectomy and 23 AA metastatic PCa patients were selected for the study. Of the former, a majority of the cases were in the 60-69 yr (49%) age group followed by 50-59 yr (36%). 85% patients had pathological stage 2 and 15% had stage 3 PCa. Group grade 1 (low grade) was most common in 45% of the patients followed closely by grade 2 in 40% with the remaining

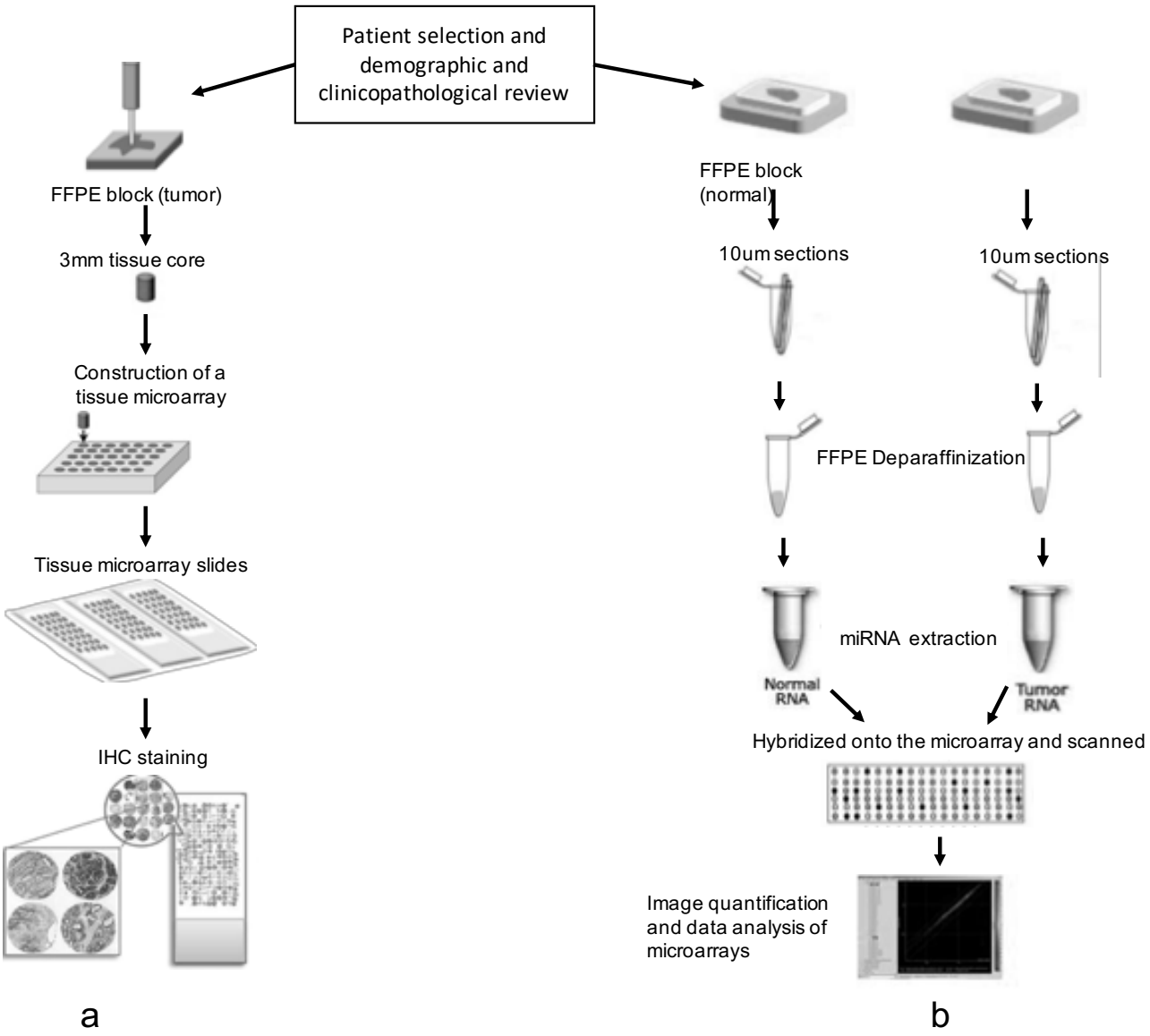

Fig. 4. Workflow for (a) tissue microarray construction and (b) miRNA analysis. FFPE: formalin fixed paraffin embedded; IHC: Immunohistochemistry

	PD1	PDL1	MLH1	Size	Stage	Smoking	PI	CI	BMI
PD-1		0.015	0.049	0.004			0.026		
PD-L1	0.015					0.008			
MLH1	0.049								
Size	0.004				0.001		0.014	0.001	
Stage				0.001		0.045	0.005	0.001	
Smoking		0.008			0.045		0.048	0.003	
PI	0.026			0.014	0.005	0.048		0.002	
CI				0.001	0.001	0.003	0.002		
BMI*	0.01	0.018							
PD-1*									0.01
PD-L1*									0.018

Table 1. Significant correlation between demographics, protein expression and clinicopathological variables in primary and metastatic PCa. p values <.05 are listed. * Represents data from metastatic carcinoma. PD-1: programmed cell death protein; PD-L1: programmed death-ligand 1; PI: Perineural invasion; CI: Capsular invasion.

sequential grades in 8%, 2% and 4% of the patients respectively. Pathological staging correlated significantly with the size of tumor (p = .001), perineural (p =.005) and capsular (p =.001) invasion and smoking (p =.045)(Table 1). Perineural invasion of the tumor was seen in 54%, while capsular invasion was seen in only 5% of patients (Table S1).

Smoking was infrequent in this cohort: 66% were non-smokers and of the remaining 34% only 14% were current smokers at the time of diagnosis. Smoking showed significant correlation with the pathological stage of tumor (p = .045), perineural invasion (p = .048), capsular invasion (p =.003) and PD-L1 (p =.008) (Table 1 & Table S2). Majority (47%) of the patients were overweight or obese (BMI>25), followed by 40% with a normal BMI (18.5-24.9) BMI did not correlate with the grade, stage of tumor or survival. At the last follow up in October 2018, 35% of the prostatectomy patients had experienced biochemical failure: 27% within 2 years, 36% within 2-5 years and 36% in >5 years. Overall survival was available in 66 primary PCa patients; 59 at 2-years, 36 at 5-year and 19 at 10-year. At the time of final analysis, 5 were dead and 25 were lost to follow up.

23 AA metastatic PCa patients were included in the study. Age group for this cohort was older with 34% at 60-69 yr followed by 70-79 yr (30%), 80-89 yr (13%) and 4% in >90 yr. Sites of metastasis included lymph nodes, bone marrow and other unusual sites: mediastinal, periaortic, lung and chest wall masses. BMI at the time of metastasis was lower than in primary PCa, with majority of the patients (54%) with normal and lower BMI than overweight and obese. High BMI did not correlate with any demographic variable or survival, but correlated significantly with high PD-1 and PD-L1 (p = .010, .018) (Table 1). Similar to the results of primary PCa, majority of the patients (58%) were never-smokers (Table S2). At the time of writing the manuscript, of the 23 patients with metastatic disease 8 patients had died of disease and 15 were still alive.

PD-1. In primary PCa PD-1 staining in intra/peritumoral lymphocytes was scored in 91/91 cases. Median of stained lymphocytes are calculated as 5. High PD-1 expression was seen in 42/91 (46%) cases. High PD-1 correlated with high PD-L1 (p = .015) and MLH-1 (p = .049) expression, larger volume of the prostate involvement with tumor (p = .004) and perineural invasion (p = .026) (Table 1, Table 2 & Fig.5).

	PD-L1	PD-1
Primary PCa		
Total patients (n)	87	91
High patients	14	42
High (%)	16	46
Metastatic PCa		
Total Patients (n)	23	23
High patients	8	12
High (%)	35	52

PCa: prostate carcinoma
PD-L1: Programmed death ligand 1
PD-1: Programmed cell death protein

Table 2. High protein expression of PD-L1 and PD-1 in primary and metastatic PCa.

In metastatic PCa PD-1 staining in intra/peritumoral lymphocytes was scored in 23 cases. High PD-1 was seen in 12/22 (50%) cases. High PD-1 showed significant correlation with high BMI (p = .010) and high PD-L1 of the patients (p = .015). It did not show any other correlation with other variables (Table 1, Table 2 & Fig.5).

PD-L1. In primary PCa, PD-L1 staining was scored in 87/91 (96%) tumors. High PD-L1 was seen in 14/87 (16%) patients. High PD-L1 showed significant correlation with high PD-1, and smoking (p = .008) (Table 1, Table 2 & Fig.5).

In metastatic PCa PD-L1 staining was scored in 23 tumors. High PD-L1 was seen in 8/23 (35%) patients. High PD-L1

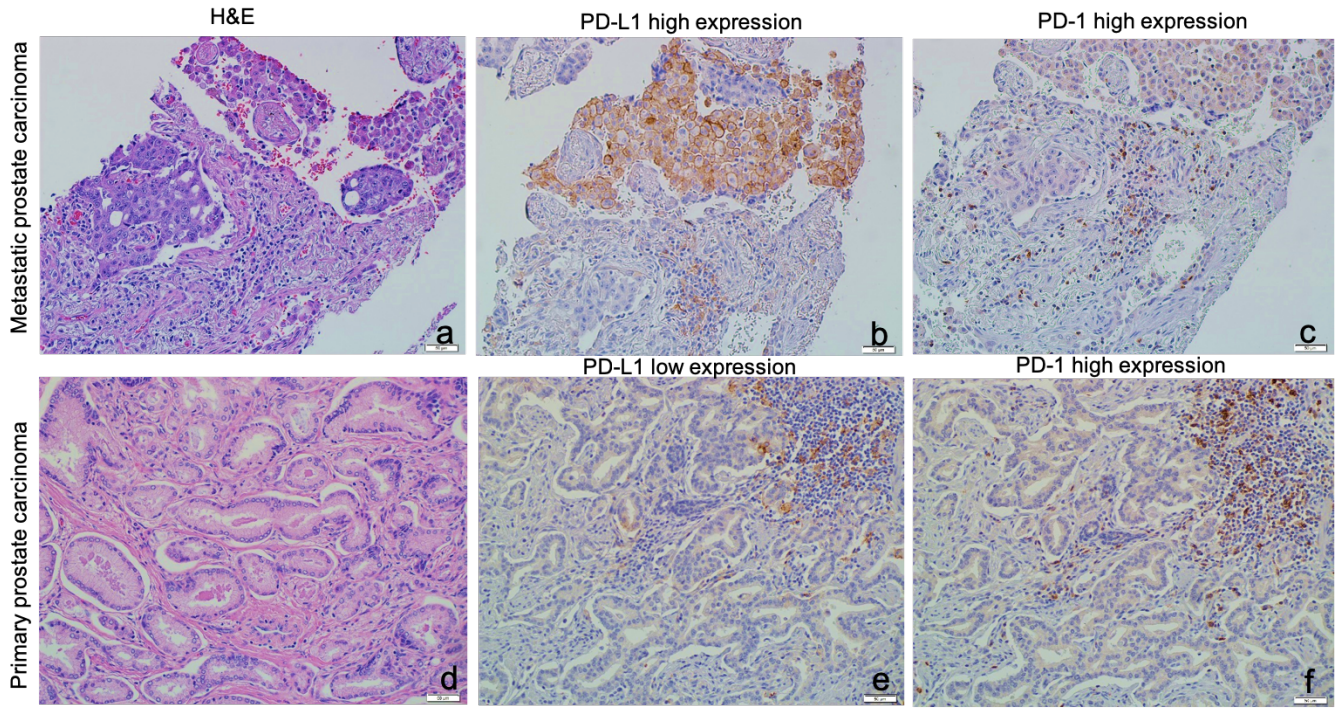

Fig. 5. Expression of PD-L1 and PD-1 in primary and metastatic PCa. Metastatic PCa (a-c) a. Hematoxylin and eosin stain showing metastatic PCa in lung. b. PD-L1 high: IHC showing membranous and cytoplasmic PD-L1 expression on immune cells, and negative tumor cells. c. PD-1 high: IHC for PD-1 showing >5% lymphocytes of peritumoral lymphocytes with positive cytoplasmic staining. Primary PCa (d-f) . d. Hematoxylin and eosin stain showing primary Pca with Gleason score 6. e. PD-L1 low: IHC for PD-L1 showing no expression in tumor cells and low expression in lymphocytes. f. PD-1 high: IHC for PD-1 showing >5% peritumoral lymphocytes with cytoplasmic staining.

expression in metastatic PCa showed significant correlation with high BMI ($p = .018$) and PD-1 ($p = .008$). It did not have any correlation with demographic/ clincopathaological variables (age, tobacco use, obesity, group grade, stage or survival) (Table 1, Table 2 & Fig.5).

MMR. In primary PCa, MLH1 (4%), PMS2 (2%) and MSH2 (1%) did not show significant loss. Loss of staining was most frequent in MSH6 (40%). Since MLH1/PMS2 and MSH6/MSH2 heterodimerize together in a functional pair, we determined the absence of the pairs MLH1/PMS2 in 2/91 (2%) and MSH6/MSH2 2/90 (2%) together. MLH1 and PMS2 stain stronger than MSH2 and MSH6. Loss of staining either individually or in pairs was not associated with biochemical or clinical recurrence. Loss of MLH1 showed correlation with high PD-1 ($p = .049$) (Table 3 & Fig.6).

In metastatic PCa, there was no loss of staining in any of the MMR antibodies (Table 3 & Fig.6). There was no correlation between MMR status any biological, clinico-pathological or survival variable.

miRNA. In a supervised heat map, overall the downregulation of microRNA was more than upregulation in tumor tissue. Volcano plot analysis revealed that 23 microRNA showed modulation ($p < .05$). 11 miRNA were upregulated in the prostate carcinoma tissue (miR - 203, - 3651, -202, - 375, - 182, - 1301, - 548ai, - 3940-5p, - 4776-5p, - 4787-5p, - 345) and 12 were downregulated (miR - 342-5p, - 4688, - 4763-3p, - 155-5p, - 320e, -378i, - 125a-3p, - 7c, - 125b-2, - 32, -125b, - 501-3p). miRNA associated with defined pathways of carcinogenesis - including MAPK (miR - 4763-3p, - 125b,), Jak-STAT (miR - 4688, - 202) and PI3-Akt (miR - 320e, - let7c, - 32, - 4787-5p) were identified. The most significant upregulation was seen with miR – 345 ($p = 9.4E-2$) (Table 4, Fig.7, Fig.8 & Fig.9).

Discussion

Prostate cancer (PCa) remains one of the most commonly diagnosed cancers among men worldwide with a remarkably high mortality in African Americans (AA) as compared to Non-Hispanic Whites (NHW). Further, AA men are more likely to be diagnosed with an advanced stage and high-grade PCa as compared to other races/ethnicities (31). Although some studies have been attempted to explore the underlying biological basis for this disparity, specific mechanisms of disease remain unexplained. New research suggests that several environmental factors, such as socioeconomic status, access to healthcare, engagement in physical activity and diet play a role; however, biological factors at the genetic and epigenetic level could be more influential in predicting racial disparities in the incidence and outcomes of PCa (32). In this study, we have characterized the demographic and clinico-pathological features, PD-1, PD-L1 and Mismatch Repair (MMR) protein expression and miRNA pattern of primary and metastatic PCa to understand the biology of PCa in AA patients.

In our study, we found that expression of PD-L1 on primary PCa was present in only a few patients. This is a significant finding for PCa of AA as it decreases their options for immunotherapy. PD-1 expression was high in almost half of the patients, which could provide a potential target for therapy in these patients. On the other hand, metastatic PCa showed biological evolution with one third of patients express-

	MLH1	MSH2	MSH6	PMS2	MSH2/MSH6	MLH1/PMS2
Primary PCa						
Total patients (n)	91	89	91	90	90	91
Negative patients (n)	4	1	31	2	2	2
Negative (%)	4	1	40	2	2	2
Metastatic PCa						
Total patients (n)	21	22	23	23	22	21
Negative patients (n)	0	2	0	0	0	0
Negative (%)	0	9	0	0	0	0

IHC, Immunohistochemistry; PCa: prostate carcinoma; MMR: mismatch repair

Table 3. Loss of MMR protein expression by IHC in primary and metastatic PCa. The absence of MMR proteins was more pronounced in primary PCa.

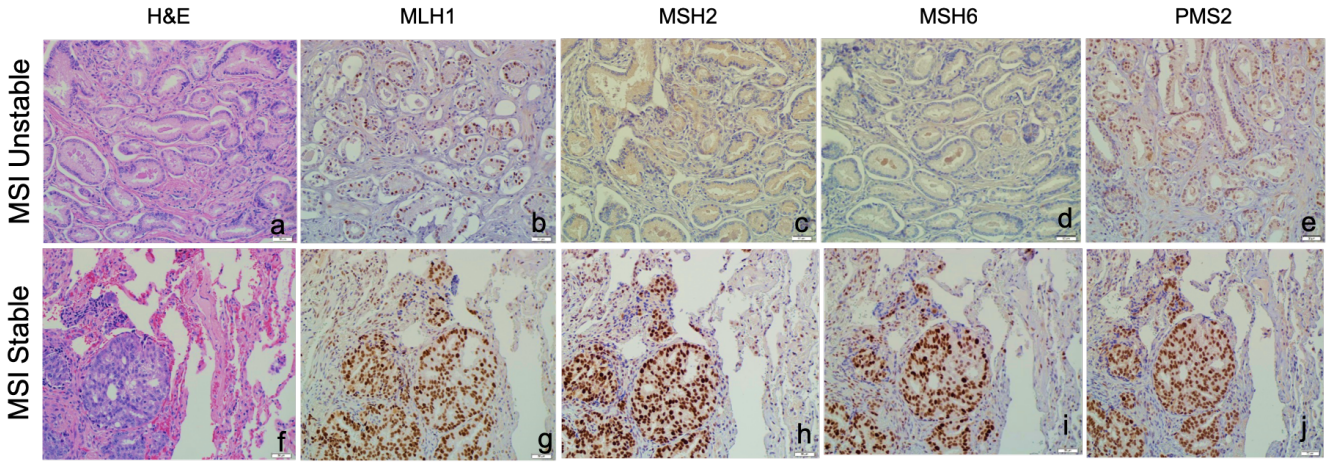

Fig. 6. Expression of MMR proteins in primary and metastatic PCa. Primary prostate carcinoma (a-e). (a)Hematoxylin and eosin stained section showing Gleason 6 PCa (b) and (d) IHC showing nuclear expression of MLH1 and PMS2. (c) and (d) IHC showing loss of nuclear expression of MSH2 and MSH6. Metastatic PCa (f-j). (f)Hematoxylin and eosin stained section showing metastatic Pca. (g-j) IHC showing strong nuclear expression of MLH1, MSH2, MSH6 and PMS2. All images magnification x200.

miRNA Entity	Fold Change	*P*-Value	Direction of Regulation	GO Term/Pathway
hsa-miR-4763-3p	0.19	0.035	Down	MAPK signaling pathway
hsa-miR-4688	0.435	0.717	Down	Jak-STAT signaling pathway
hsa-miR-342-5p	0.493	0.049	Down	Negative regulation of endothelial cell proliferation/ Notch signaling pathway
hsa-miR-155-5p	0.504	0.266	Down	Negative regulation of endothelial cell proliferation; positive regulation of apoptosis/ MAPK signaling pathway
hsa-miR-320e	0.523	0.203	Down	PI3K-Akt signaling pathway
hsa-miR-378i	0.53	0.411	Down	RNA Transport
hsa-miR-125a-3p	0.542	0.157	Down	
hsa-let-7c	0.56	0.122	Down	PI3K-Akt signaling pathway
hsa-miR-125b-2*	0.601	0.046	Down	Cell Cycle
hsa-miR-32	0.637	0.467	Down	PI3K-Akt signaling pathway
hsa-miR-125b	0.646	3.16E-01	Down	Negative regulation of angiogenesis; MAPK signaling pathway
hsa-miR-501-3p	0.655	0.64	Down	PI3K-Akt signaling pathway
hsa-miR-203	1.516	0.268	Up	MAPK signaling pathway
hsa-miR-3651	1.52	0.031	Up	Metabolic pathway
hsa-miR-202	1.764	0.61	Up	Jak-STAT signaling pathway
hsa-miR-375	2.253	0.011	Up	Positive regulation of endothelial cell apoptotic process; MAPK signaling pathway
hsa-miR-182	2.994	0.055	Up	PI3K-Akt signaling pathway
hsa-miR-4776-5p	3.062	0.362	Up	RNA transport
hsa-miR-1301	3.12	0.063	Up	mRNA surveillance pathway
hsa-miR-548ai	3.194	1.01E-01	Up	PI3K-Akt signaling pathway
hsa-miR-3940-5p	3.292	0.233	Up	RNA transport
hsa-miR-4787-5p	4.845	0.264	Up	PI3K-Akt signaling pathway
hsa-miR-345	5.795	9.40E-02	Up	Mismatch repair; DNA replication

Table 4. Modulation of miRNA expression ($p < 0.05$) between normal and tumor tissue.

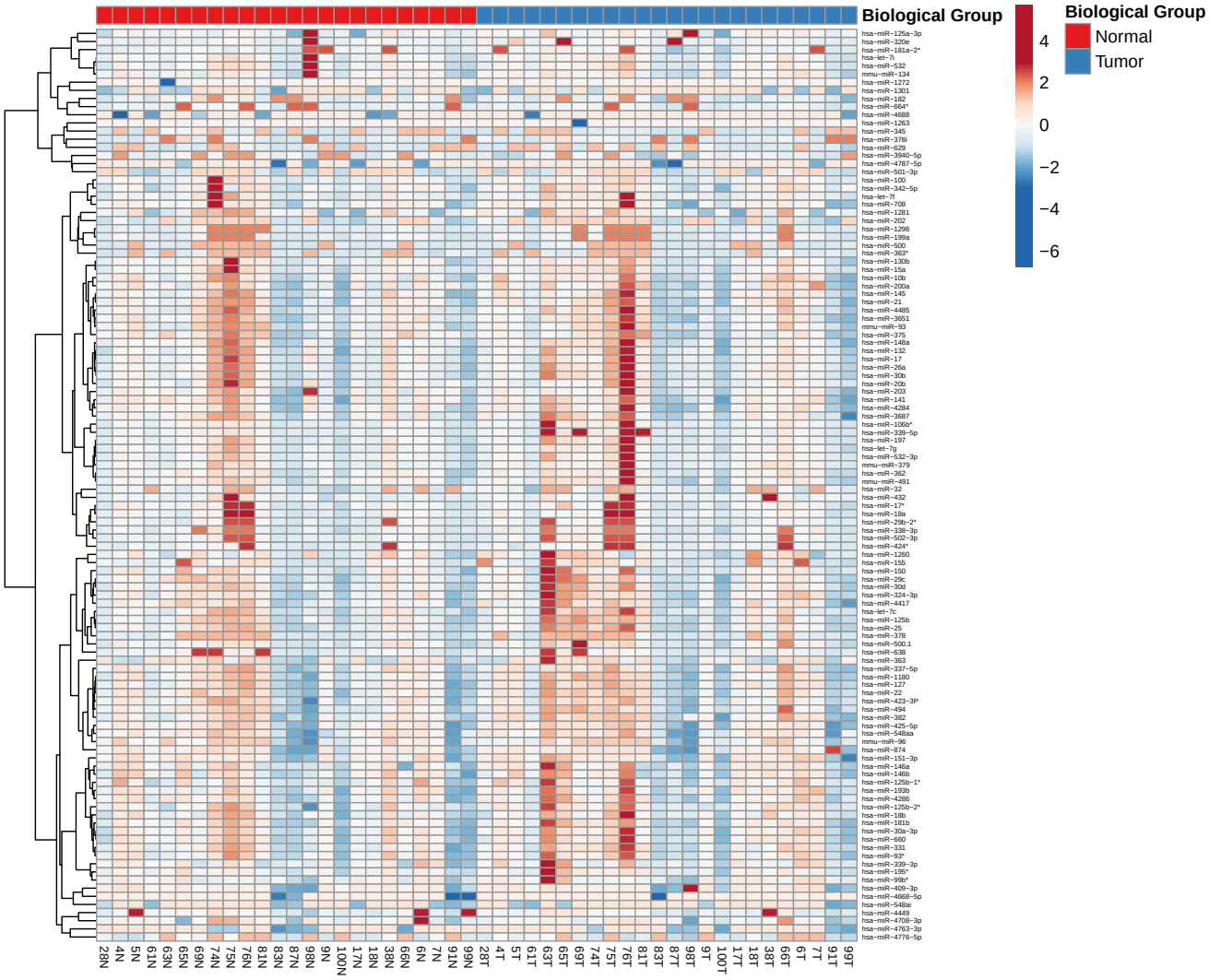

Fig. 7. A supervised heat map separating tumor and neighboring normal tissue. The tissue samples are arrayed vertically and the miRNAs that are differentially expressed are arrayed horizontally. Red and blue indicate relative upregulation and downregulation of miRNA expression.

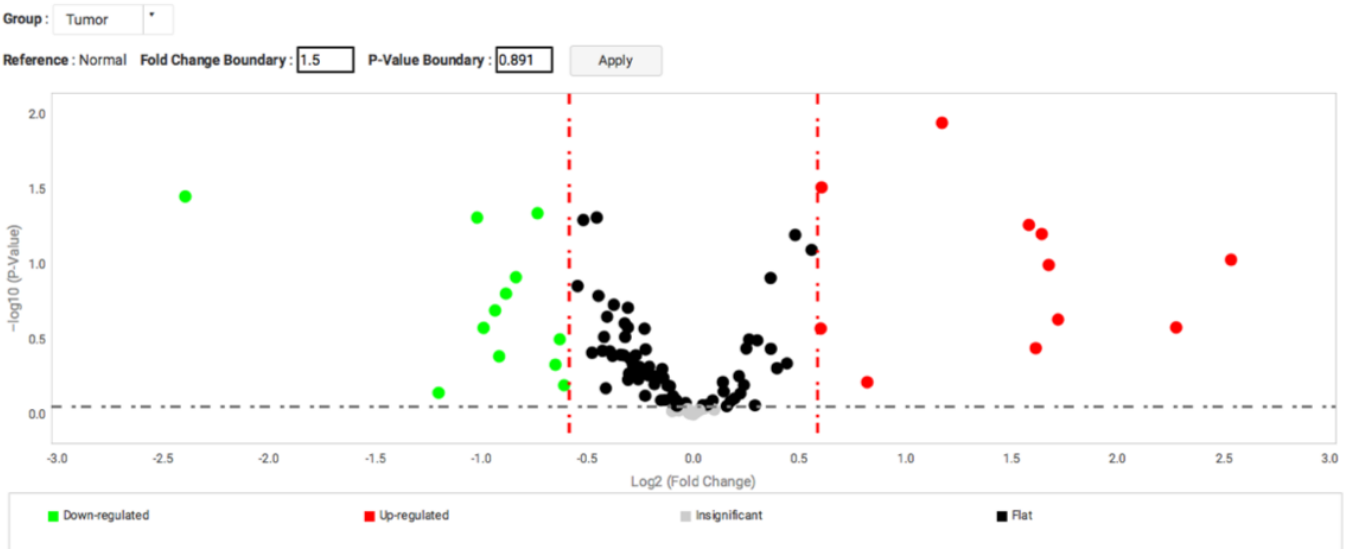

Fig. 8. Volcano plot showing the changes in the expression of individual miRNA sequences at the log fold difference of 1.5 (the mean of expression of each sequence in the tumor samples compared to the expression in the normal neighboring tissue.) Upregulated miRNA are in red, downregulated miRNA are in green and the miRNA with no change are black.

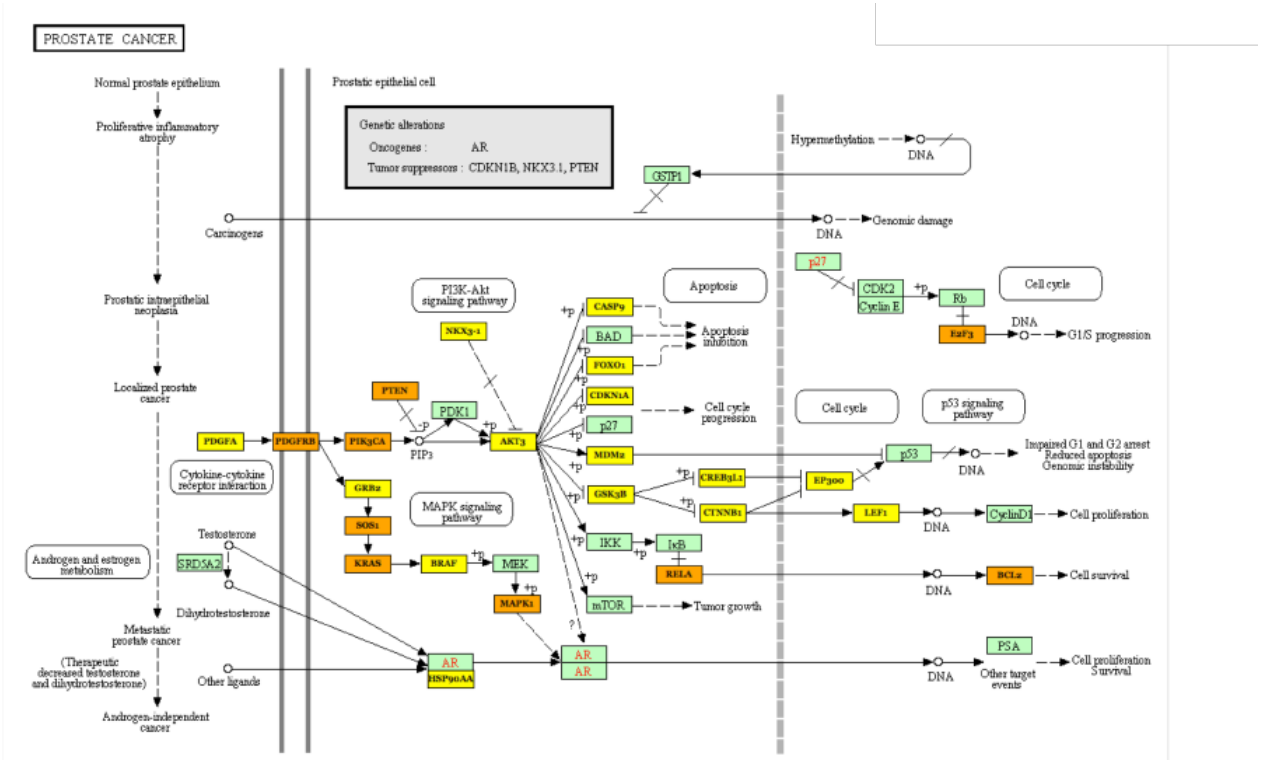

Fig. 9. Diagrammatic representation of known pathways for carcinogenesis in prostate carcinoma. The 37 genes whose expression is modulated miRNA expression are highlighted.

ing high PD-L1 and about half with high PD-1 expression, making them good candidates for immunotherapy. MMR proteins were mostly intact in both primary and metastatic PCa, pointing to a different biological carcinoma pathway than Microsatellite Instability. A panel of miRNA helped to distinguish benign from tumor tissue, suggesting that they may be promising tools for early detection of PCa. Specific sets of miRNA involved in carcinogenetic pathways of PCa were identified. Two oncogenic miRNA associated with aggressive disease were identified in AA patients, presenting a possible biological basis of high grade PCa in this cohort. Pathological features of the tumor corresponding to an aggressive disease (large tumor volume, perineural and capsular invasion) showed significant correlation with smoking, emphasizing that smoking in AA men is linked to a more aggressive disease. Demographic features (age, Body Mass Index (BMI)) in AA men did not correspond to recurrence or overall outcome in both primary and metastatic carcinoma.

In our study, 34% of patients were smokers and smoking showed a significant correlation with worse prognostic indicators in primary PCa, including higher stage, perineural and capsular invasion. This strong association of smoking in AA has been correlated with increased odds of overall cancer diagnosis and aggressiveness as compared to NHW in literature (33). BMI was associated with high PD-1 and PD-L1 expression in metastatic PCa. A similar association of overexpression of PD-1 and PD-L1 has been seen in obese mice. This finding links the role of immune-evasive tumor environment up-regulated by these receptors to the development of PCa and poor outcomes (26).

Higher density of PD-1+ lymphocytes was seen in both primary and metastatic PCa. In primary PCa its expression independently correlated with large size of the tumor and perineural invasion: both being indicators of a more aggressive disease. Similar correlation with high PD-1 expression, unfavorable prognosis and high risk of treatment failure has been shown in PCa patients (34). Presence of activation of PD-1 pathway and its association with worse prognosis indicates tumor evasion from immune cells, a mechanism for escaping detection by tumor specific CD8+ T cells (35). Another mechanism of host immune evasion has been up-regulation of regulatory T cells (FOXP3+, CD25+, CD4+) which have been associated with multiple high grade/stage cancers, including PCa (36, 37). In the environment of debated accuracy of PD-L1 as a predictor of treatment response, due to variations in cutoff definitions and lack of working standard across tumors exploring PD-1 pathway for new treatment options could prove effective (18). A recent trial with PD-1 inhibitor pembrolizumab on metastatic, hormonally unresponsive PCa showed measurable disease response (38).

PD-L1 expression in primary and metastatic PCa differed, indicating evolution of PCa pathways in advanced disease. High expression of PD-L1 was seen in only a few cases of primary tumor but it was seen in 35% of metastatic cases. PD-L1 antibody did not show any significant expression in tumor epithelial cells but most of the expression was limited to stromal lymphocytes and macrophages. PD-L1 expression did not correlate with any other patient characteristic, or clinical or prognostic data. High PD-L1 expression only correlated significantly with smoking in primary PCa and a high BMI in metastatic PCa. Expression of PD-L1 in prostate tumor epithelial cells has been conflicting in the literature, ranging from negative (39–41) to focal (42) to cases with high expression (43). Similarly, inconsistent reports of PD-L1 expression in PCa cells and its negative prognostic value are present in the literature (44). We were not able to reproduce these results with any clinico-pathologic or survival indicators. These results are similar to those of recent studies which did not to reach statistical significance with any survival data (31).

The lack of concordance between these studies may be due to the different antibodies used by different groups. We have used Ventana antibody, which has received FDA approval to predict treatment response with pembrolizumab (Keytruda®). Another explanation may be the lack of standardization in interpreting PD-L1. Ventana has released guidelines for non-small cell lung cancer and urinary bladder cancer, and in our study, due to lack of manufacturer released prostate specific guidelines, we utilized the latter guidelines, which may not be uniformly used by all investigators. The lack of induction of PD-L1 in PCa is under investigation and the two likely mechanisms, adaptive and innate immunity, have not been demonstrated to be active in this oncogenic pathway (34). Use of anti-PD-1/PD-L1 in primary or metastatic PCa has not yet been FDA-approved. There has been preliamniary evidence that they may represent promising targets, especially in metastatic PCa (45). Further exploration of checkpoint inhibitors, such as PD-1/PD-L1, in PCa may find a subpopulation that will respond to immunotherapy.

MMR deficiency is associated with loss of mismatch repair enzymes: MLH1, MSH2, MSH6 and PMS2 (46). Studies have reported MMR defects in PCa ranging from <1% to 14% (47, 48). These include both primary and metastatic PCa across different populations. In this study we have found mostly intact expression of MMR proteins in primary and metastatic PCa except singular loss of MSH6, limited to primary PCa cases. There is no correlation with biochemical recurrence or survival with loss of one or more MMR proteins. This result is consistent with recent studies that have not found any association between loss of MMR protein expression in PCa and biochemical recurrence (49). Some studies, however, have found decreased biochemical recurrence and a survival advantage in patients with low MSH2 expression in PCa (50, 51). In metastatic PCa we did not find any loss of expression in all four MMR proteins. MMR deficit has been reported in up to 12% of metastatic PCa, with association of lower MSH2/MSH6 expression and decreased patient survival; findings not confirmed in our study (47, 52, 53). These differences may be due to different study populations, ours being exclusively African American. As MMR deficient tumors have been shown to have decreased risk of recurrence, intact MMR status in AA patients in our study parallels the high mortality seen in this population.

Tissue microarray (TMA) was used to assess PD-L1, PD-1 and MMR protein expression in PCa specimens. Although TMA has limited tissue for evaluation as compared to a full section, it is a reasonable choice when evaluating large number of specimens with multiple antibodies. TMAs have been effectively used to evaluate MMR expression in other studies examining cancers like ovarian, endometrial, colon and prostate. (49, 50, 54, 55) Results of TMA compare well with whole slides, as demonstrated by Hendricks et al. in the assessment of MMR proteins in 129 patients, showing a good

concordance (56).

miRNA have an important role in regulating cell processes, including division, differentiation, survival and programmed death (57). Individual miRNA have been identified as either tumor suppressors or oncogenes depending on downstream targets, thus making them useful biomarkers for cancer diagnosis, treatment and prognosis (58). miRNA have recently become promising candidates for urologic diseases because of their easy extraction from a variety of biological samples, including body fluids like urine, and their stability over long periods of time (59–61). Their detection in body fluids eliminates the need for invasive biopsies for early detection of PCa. Of the studies investigating miRNA signature in prostate carcinoma, few have been done on AA patients. In our study of 24 specimens of PCa from exclusively AA patients, we observed global downregulation of miRNA in cancer tissue. This trend of low miRNA expression may be significant in this population as recent studies in a broader population reported global upregulation. (62, 63).

Identification of a panel of miRNA which can diagnose prostate cancer in AA patients can be further explored for early detection of PCa through non-invasive techniques. The diagnostic panel of the miRNA in our study consisted of eleven oncogenes and twelve tumor-suppressor genes. These miRNA have been reported to influence multiple carcinogenesis pathways - including MAPK (miR - 4763-3p, - 125b,), Jak-STAT (miR - 4688, - 202) and PI3-Akt (miR - 320e, - let7c, - 32, - 4787-5p). Of the significant 23 modulated miRNAs identified in our study, miR-182 and -375 have been reported in association with PCa by other investigators; Schaeffer et al. included these two miRNA in his six miRNA signature diagnostic panel for the diagnosis of PCa (miR-96, -149, -181b, -182, -285 and -375) with statistical significance (64) (65). In a study comparing AA and NHW PCa specimens, miRNA-182 was found to be upregulated in the former, also seen in our findings (66). Another significant miRNA upregulated in our panel was miR-375, which is known to be involved in DNA repair and DNA replication; its upregulation has been documented to be linked to metastatic PCa and biochemical recurrence. Therefore, upregulation of these two miRNA in our study population highlights the cellular basis for a more aggressive PCa in AA men and provides a biological explanation for racial health disparities (67, 68). A majority of miRNA profiling in the literature has utilized NHW populations, therefore our study in AA patients is unique in identifying race-specific miRNA that may play a role in early diagnosis and prognosis of PCa in African Americans.

Limitations of this study include use of tissue microarray cores. Although two cores from each tumor were used for immunohistochemical analysis, the limited tissue surface area hampers observation. Our sample size of 91 primary and 23 metastatic PCa was limited by number for biomarker expression which has a low incidence, like PD-L1. It is important to recognize that upregulation of the miRNA most likely results in an increase in the levels of the cognate RNA and the functional protein; however since individual proteins can activate or repress a particular pathway, these analyses cannot directly establish the biological consequences of dysregulation of miRNA to tumor biology. Further in vitro studies will be needed to determine the significance of these correlations.

Conclusion

In this study, we have characterized demographic and clinicopathological features and molecular markers of PCa in AA men to determine the biological basis of disparities in incidence and mortality amongst this minority population. A distinct pattern of miRNA expression was identified in our patient population, which has been linked to more aggressive disease. Specific miRNA linked to common carcinogenetic pathways were identified, which can be further explored to delineate mechanisms of pathogenesis in this group. Metastatic PCa had a higher expression of PD-L1 as compared to primary PCa, making the latter poor candidates for immunotherapy. PD-1 expression was high in almost half of primary and metastatic PCa patients and MMR protein expression was largely intact in both primary and metastatic PCa cases; both of these protein markers have been associated with biochemical recurrence and low survival. Smoking in AA correlated with pathological features of aggressive PCa. The biological characteristics (low expression of PD-L1, high expression of PD-1, intact MMR and distinct miRNA signature) identified in our cohort of exclusively AA men are indicators of aggressive PCa. These results explain the biological basis of widely documented poor prognosis of PCa in AA men. This characterization of PCa in AA men can help in appropriate management, preventing overdiagnosis and undertreatment, in this at-risk population. The unique miRNA panel identified in our study can be a promising tool for future studies to understand the biological pathways of PCa and improve the outcomes in AA patients.

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Supplementary Material

Parameter		n (%)	Total
Age (years)	Median	68	91
	Range	40-79	
Race	AA	91 (100%)	91
Tobacco	Current	10(14%)	71
	Ex-smoker	14(20%)	
	Never smoker	47(66%)	
BMI	Underweight <18.5	2(2%)	82
	Normal: 18.5 to 24.9	33(40%)	
	Overweight: 25 to 29.9	28(34%)	
	Obese: >30	19(23%)	
Biochemical Recurrence	Yes	22(35%)	63
	No	41(65%)	
Path Stage	2 (a, b, c)	77(85%)	90
	3 (a, b, c)	13(15%)	
Tumor Grade	1	41(45%)	
	2	37(40%)	
	3	7(8%)	
	4	2(2%)	
	5	4(4%)	
Perineural Invasion	Positive	51(56%)	91
	Negative	40(44%)	
Capsular Invasion	Positive	40(43%)	91
	Negatie	51(56%)	

Table S1. Patients characteristics, clinical pathological variables, and outcomes in primary PCa Patients.

Parameter		n(%)	Total
Age (years)	Median	68	23
	Range	40-99	
Race	AA	23(100%)	23
Tobacco	Current	0	13
	Ex-smoker	5 (39%)	
	Never smoker	8 (61%)	
BMI	Underweight: <18.5	3 (15%)	19
	Normal: 18.5 to 24.9	8 (40%)	
	Overweight: 25 to 29.9	7 (35%)	
	Obese: >30	1 (5%)	

Table S2. Patients characteristics, clinical pathological variables, and outcomes in primary PCa Patients.