Identification and Functional Validation of Reciprocal microRNA–mRNA Pairings in African American Prostate Cancer Disparities

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Abstract

Purpose: African Americans (AA) exhibit higher rates of prostate cancer incidence and mortality compared with European American (EA) men. In addition to socioeconomic influences, biologic factors are believed to play a critical role in prostate cancer disparities. We investigated whether population-specific and -enriched miRNA–mRNA interactions might contribute to prostate cancer disparities.

Experimental Design: Integrative genomics was used, combining miRNA and mRNA profiling, miRNA target prediction, pathway analysis, and functional validation, to map miRNA–mRNA interactions associated with prostate cancer disparities.

Results: We identified 22 AA-specific and 18 EA-specific miRNAs in prostate cancer versus patient-matched normal prostate, and 10 'AA-enriched/-depleted' miRNAs in AA prostate cancer versus EA prostate cancer comparisons. Many of these population-specific/-enriched miRNAs could be paired with target mRNAs that exhibited an inverse pattern of differential expression. Pathway analysis revealed EGFR (or ERBB) signaling as a critical pathway significantly regulated by AA-specific/-enriched miRNAs and miRNA–mRNA pairings. Novel miRNA–mRNA pairings were validated by qRT-PCR, Western blot, and/or IHC analyses in prostate cancer specimens. Loss/gain of function assays performed in population-specific prostate cancer cell lines confirmed miR-133a/MCL1, miR-513c/STAT1, miR-96/FOXO3A, miR-145/PPAR2, and miR-34a/PPP2R2A as critical miRNA–mRNA pairings driving oncogenesis. Manipulating the balance of these pairings resulted in decreased proliferation and invasion, and enhanced sensitization to docetaxel-induced cytotoxicity in AA prostate cancer cells.

Conclusions: Our data suggest that AA-specific/-enriched miRNA–mRNA pairings may play a critical role in the activation of oncogenic pathways in AA prostate cancer. Our findings also suggest that miR-133a/MCL1, miR-513c/STAT1, and miR-96/FOXO3A may have clinical significance in the development of novel strategies for treating aggressive prostate cancer. Clin Cancer Res; 21(21); 4970–84. ©2015 AACR.

Introduction

MiRNAs (miRNAs) are small regulatory RNAs of approximately 21 to 25 nucleotides in length that complementarily target mRNAs to inhibit translation and/or promote mRNA degradation. Recently, several reports have suggested that miRNA aberrations may be an important factor in cancer development (1, 2). The potential connection between miRNA regulation and cancer has been made at several levels, suggesting that miRNAs play critical roles in cellular growth and differentiation, which are two cellular processes commonly defective in tumor cells (3). Additional evidence for the involvement of miRNAs in human cancer comes from observations that approximately 50% of these small regulatory RNAs are transcribed from genomic regions associated with a loss of heterozygosity, minimal amplicons, or breakpoint cluster regions (4). Cancer-related miRNAs have been identified in various cancers (5). In general, oncogenic miRNAs upregulated in tumors act as oncogenes (repressing tumor-suppressor and apoptosis-associated genes), whereas tumor-suppressor miRNAs are downregulated (leading to derepression of oncogenes and proliferation-related genes; ref. 6). Although many miRNAs are differentially expressed in various cancers, the identity of the miRNAs specifically targeted by these miRNAs, functional
mRNA–mRNA Pairs Drive Prostate Cancer Disparities

Translational Relevance
Prostate cancer tends to be more aggressive and lethal in African Americans (AA) compared with European Americans (EA). An understanding of the molecular mechanisms associated with prostate cancer disparities can aid in the development of innovative and improved therapeutic options for the AA population. Integrative functional genomics analysis of patient specimens and prostate cancer cell lines has identified novel AA-specific and -enriched mRNA–mRNA pairs, including miR-133a/MCL1, miR-513c/STAT1, miR-96/FOXO3A, miR-145/ITPR2, and miR-34a/PPP2R2A, that reside in key oncogenic signaling pathways. The presence of these mRNA–mRNA pairs is computationally predicted to augment activation of EGFR–PI3K–AKT signaling in AA compared with EA cancers. Specific manipulation of these pairs reduced cell proliferation/invasion and enhanced docetaxel-induced cytotoxicity in AA prostate cancer cell lines. Converse manipulation resulted in a more aggressive phenotype in EA cell lines. Thus, targeting these novel mRNA–mRNA pairs may provide a potential clinical strategy for reducing AA prostate cancer burden.

consequences of mRNA–mRNA pairings and their contributions to cancer pathogenesis remain to be elucidated.

Prostate cancer is now the most frequently diagnosed cancer and the second most common cause of cancer-related deaths in men residing in the United States (7). AAs have among the highest incidence of prostate cancer and mortality attributable to this disease, being 1.6 times more likely to develop prostate cancer, and 2.4 times more likely to die from prostate cancer compared with their EA counterparts (8). Multiple socioeconomic and environmental factors have been postulated to explain the observed prostate cancer health disparities, such as access to care, attitudes toward health care, socioeconomic differences, diet, and differences in the type and aggressiveness of treatment (8). However, adjustment for these factors does not preclude the higher mortality and recurrence rate in AA men and suggests that intrinsic biologic differences exist (9). The application of epidemiology and genomics has revealed biologic factors implicated in prostate cancer health disparities between AA and EA, such as differences in the hormonal milieu of the tumor (10), oncogenic activation (11), and tumor immunobiology (12). More recently, our genomic analysis identified multiple signaling pathways converging on the androgen receptor (AR) to activate transcription of AR-target genes promoting prostate cancer progression and aggressiveness in AA patients (13).

Given the importance of miRNAs in cancer, studies have been forthcoming on the association of miRNAs in prostate cancer pathogenesis. Volinia and colleagues (1) performed large-scale analysis of miRNA expression profiles in 540 samples derived from six types of solid tumors, and demonstrated that 46 miRNAs were differentially expressed when comparing prostate cancer with patient-matched normal prostate (NP), including upregulated let-7d–miR-17-5p, and miR-21, and downregulated miR-24, miR-29, and miR-128a. An miRNA profiling study by Ozen and colleagues (14) revealed that 76 of the 85 differentially expressed miRNAs were downregulated (such as let-7c, miR-145, and miR-125b) in the prostate cancer clinical samples compared with normal tissues. More recently, Wang and colleagues (15) identified a set of deregulated miRNAs associated with cell-cycle regulation in aggressive prostate cancer by combining mRNA–miRNA expression profiling and coexpression network analysis. Although these profiling studies have begun to shed light on the involvement of miRNAs in prostate cancer development, questions on the role of miRNAs in prostate cancer disparities still remain. A recent study evaluated the impact of miRNAs contained in the region of 8q24, a genetic risk locus conferring prostate cancer in AAs. However, no empirical evidence of miRNA transcription was found within the 8q24 prostate cancer risk locus (16). In the present study, we applied a systems biology approach, by combining genome-wide miRNA and mRNA expression profiling in prostate cancer patient specimens, miRNA target predictions, and mRNA–miRNA pairing and pathway analyses, to identify the oncogenic signaling pathway most significantly regulated by AA-specific/-enriched miRNAs and mRNA–mRNA pairings. The AA-specific/-enriched miRNA and mRNA elements were also evaluated in AA and EA prostate cancer cell lines for their functional relevance in cell proliferation, invasion, and chemosensitivity to cytotoxic agents.

Materials and Methods

Acquisition and characteristics of prostate cancer clinical specimens
Tissues were procured from the George Washington University Medical Faculty Associates adhering to IRB approved protocols (IRB#020867), as detailed in Supplementary Materials and Methods. High-quality prostate cancer and patient-matched NP biopsy cores from each of 20 AA and 15 EA patients were collected and processed for the microarray analyses. Prostate cancer cores were determined by pathologist to have Gleason score of 6 to 7 (17 AA and 13 EA) or 8 to 9 (3 AA and 2 EA), whereas NP cores were negative for cancer. There was no significant difference between the two racial groups with respect to age (average age for AAs was 62.3 ± 8.2, average age for EAs was 63.3 ± 9.2) and Gleason score (Supplementary Table S1A).

Prostate cancer cell lines
Prostate cancer cell lines were purchased from the ATCC and passaged less than 6 months after receipt/resuscitation. Cell lines were tested and authenticated at the ATCC by short tandem repeat profiling of multiple unique genetic loci (D3S1818, D13S317, D7S820, D16S539, vWA, TH01, Amelogenin, TPOX, and CSF1PO).

Gene-expression microarrays
Total RNA was isolated from prostate cancer and patient-matched NP biopsy cores. For mRNA profiling, total RNA (1 µg) from each biopsy core was purified using the RNeasy Micro Kit (Qiagen) and interrogated with the Affymetrix Human Exon 1.0 ST GeneChip. For miRNA profiling, 250 ng of RNA from each biopsy core was isolated using the miRNeasy Kit (Qiagen,) and interrogated with the Agilent Human miRNA microarray V3 (Agilent Technologies). High-quality RNA samples were confirmed on the Agilent 2100 Bioanalyzer (Agilent Technologies). Affymetrix exon array data were normalized by quantile normalization with GC-RMA background correction, and data visualization and statistical analysis were performed by Partek Genomics.
Suite 6.6 software (Partek) as previously described (13). Raw data from Agilent miRNA microarray analysis were quantile normalized and analyzed in GeneSpring GX program version 12.5 (Agilent Technologies). Identification of statistically significant, differentially expressed-regulated mRNAs and miRNAs was based on ANOVA or the paired \( t \) test with a 10% FDR criterion to correct for multiple testing (13). Microarray data can be assessed at GEO using accession numbers GSE64331 and GSE64318 for Affymetrix exon and Agilent miRNA arrays, respectively.

Principal component analysis (PCA) plots and hierarchical clustering of mRNA and miRNA data were performed using the Partek Genomics Suite 6.6. Two-dimensional (2D) hierarchical clustering analysis used average linkage and a Euclidean distance metric.

miRNA–mRNA pairings and pathway analysis

TargetScanHuman 6.2 was used to identify mRNAs predicted to be targets of the ANOVA-defined differentially expressed miRNAs. The list of predicted target mRNAs was intersected with the ANOVA-defined differentially expressed mRNAs to generate a catalog of experimental miRNA–mRNA pairings. Pairings were categorized as having reciprocal (e.g., miRNA up and mRNA down, or mRNA up and miRNA up), positive (i.e., miRNA up and mRNA up), or negative correlations (i.e., miRNA down and mRNA down) in AA prostate cancer vs. NP or AA prostate cancer vs. EA prostate cancer comparisons. The differentially expressed mRNAs not belonging to any pairings are herein referred to as unpaired mRNAs.

Global test (17) [and Gene Set Enrichment Analysis (GSEA: ref. 18) as a secondary confirmatory approach] was implemented to identify statistically significant canonical signaling pathways containing differentially regulated gene sets that may be associated with AA prostate cancer aggressiveness, based on AA prostate cancer versus AA NP, AA prostate cancer versus EA prostate cancer, and EA prostate cancer versus EA NP comparisons (detailed description in Supplementary Materials and Methods). Note that significant genes identified by the Global test and ANOVA may be mutually exclusive. Representative genes in different pathways identified by the Global test were chosen for validation if these genes were also identified by ANOVA and TargetScan prediction analyses as unpaired mRNAs or mRNAs belonging to miRNA–mRNA pairings. The underlying assumption was that genes fulfilling the above criteria would have a greater likelihood of validation success. Validation of differential gene expression was accomplished by quantitative RT-PCR (qRT-PCR) and immunohistochemistry (IHC) in cohorts of patient specimens separate from those used in microarray analysis (Supplementary Table S1B and S1C). Western analysis and functional assays in prostate cancer cell lines were performed to validate predicted reciprocal miRNA–mRNA pairings.

qRT-PCR validation of mRNAs and miRNAs

qRT-PCR validation was performed as previously described (19, 20). qRT-PCR determinations of mRNAs and miRNAs were performed in duplicate and normalized to levels of housekeeping genes EIF1AX and miR-103, respectively. EIF1AX and miR-103 are constitutively expressed and resistant to expression changes (19, 20). qRT-PCR primer pair sequences for mRNA and miRNA determinations are provided in Supplementary Tables S2 and S3, respectively. Sequences to entire mature miRNA are reported in miRBase database (21).

Tissue processing, IHC, and Western blot analysis

Serial sections of formalin-fixed, paraffin-embedded (FFPE) prostate cancer specimens from AA and EA patients with Gleason score 6 to 8 were immunolabeled. Western blot analysis, as previously described (13), was performed on AA and EA prostate cancer cell lines MDA PCa 2b, RC777/E, VCaP, LNCaP, and PC-3. Details for tissue processing, IHC, image capturing/quantification, and cell line information can be found in Supplementary Materials and Methods.

Antibodies

Antibodies used in IHC assays and Western blotting analysis were rabbit monoclonal antibodies for STAT1 and pFOXO3A (Cell Signaling Technology), FOXO3A (Millipore), and AMACR (Dako), rabbit polyclonal antibody for MCL-1 (Santa Cruz Biotechnology), mouse monoclonal antibodies for p63 (Biocare Medical), and β-actin (Santa Cruz Biotechnology).

Functional analysis of prostate cancer cell lines following miRNA mimic or inhibitor transfections

Prostate cancer cells were transfected with either miRNA mimics or antagonirs using DharmaFECT4 transfection reagent (Dharmacon), according to the manufacturer's protocol. MiR-133a mimic, miR-513c mimic, miR-96 mimic, miR-34a mimic, miR-145 mimic, miR-133a antagonist, miR-513c antagonist, miR-96 antagonist, and nonsense miRNA mimic and antagonist controls were purchased from Life Technologies.

In vitro functional assays, including cell proliferation, apoptosis, and invasion assays were conducted following miRNA mimic/antagonir transfections. Cell proliferation and apoptosis assays were performed using the BrdUrd Cell Proliferation Assay Kit (Calbiochem) and the Apo-ONE Caspase-3/7 Assay Kit (Promega) as described by the manufacturers. Detailed experimental design and protocols can be found in Supplementary Materials and Methods. Matrigel invasion assays were performed as previously described (19, 20).

Results

Microarray analysis reveals differentially expressed mRNAs and miRNAs in AA and EA prostate cancer patient specimens

In an earlier study (13), a total of 70 prostate biopsy cores (20 cancerous and 20 patient-matched NP from AA patients; 15 cancerous and 15 patient-matched NP from EA patients) were subjected to mRNA profiling, and a three-way comparison identified 2,908 significant (ANOVA, 10% FDR multiple test correction) differentially expressed mRNAs. In the present study, we have classified these mRNAs as follows, 433 mRNAs are "AA-enriched" (significantly overexpressed in AA) and 755 mRNAs are "AA-depleted" (significantly underexpressed in AA) based on the AA prostate cancer versus EA prostate cancer comparison (Supplementary Table S4). Another 980 mRNAs (up or down) are defined as "AA-specific" based on the AA prostate cancer versus AA NP comparison (and not significant in EA prostate cancer vs. EA NP), whereas 740 mRNAs are "EA-specific" based on EA prostate cancer versus EA NP (and not significant in AA prostate cancer vs. AA NP, Supplementary Table S4). PCA and 2D hierarchical clustering demonstrated clear separation and consistency of gene-expression profiles in the three separate comparisons (Fig. 1A).
Figure 1. mRNA and miRNA expression profiling of prostate cancer (PCa) specimens and patient-matched normal tissues derived from AA and EA patients. A, prostate cancer plots and hierarchical 2D clustering of mRNA expression in AA prostate cancer versus EA prostate cancer, and prostate cancer versus patient-matched normal tissue. B, prostate cancer plots and hierarchical clustergrams of miRNA expression in AA prostate cancer versus EA prostate cancer, and prostate cancer versus patient-matched normal tissue. For both A and B, samples are in rows, and mRNAs or miRNAs are in columns. Plots demonstrated clear separation and consistency of mRNA and miRNA expression profiles in group comparisons. For mRNA profiling, n = 20, 20, 15, and 15 for AA prostate cancer, AA-matched normal, EA prostate cancer, and EA-matched normal, respectively. For miRNA profiling, n = 14, 14, 13, and 13 for AA prostate cancer, AA-matched normal, EA prostate cancer, and EA-matched normal, respectively.
We also sought to investigate the relationship between miRNA and mRNA profiles in the same cohort of patients. Of the original 70 biopsy cores used for mRNA expression analysis, 54 provided sufficient material for miRNA expression profiling (14 cancers and 14 patient-matched NP from AA patients; 13 cancers and 13 patient-matched NP from EA patients). MiRNA profiling revealed 10, 33, and 29 miRNAs that were differentially expressed (ANOVA or paired t test, 10% FDR, fold change ≥1.5) between AA prostate cancer versus EA prostate cancer, AA prostate cancer versus AA NP and EA prostate cancer versus EA NP, respectively. Eleven of these miRNAs represent race-independent noncoding RNAs (miRNAs found significant in both AA prostate cancer vs. AA NP and EA prostate cancer vs. EA NP comparisons), along with 2 AA-enriched, 8 AA-depleted, 22 AA-specific and 18 EA-specific miRNAs (Supplementary Table S5). Prostate cancer and 2D hierarchical clustering demonstrated clear separation of miRNA profiles (Fig. 1B). In summary, we postulate that AA-enriched, AA-depleted, and race-specific miRNAs and mRNAs (but not race-independent miRNAs and mRNAs) may be associated with the biologic component of prostate cancer disparities.

### Novel reciprocal miRNA–mRNA pairings and dysregulated-unpaired mRNAs in oncogenic signaling pathways promoting prostate cancer disparities

AA-enriched/-depleted, AA-specific and EA-specific miRNAs were analyzed by TargetScanHuman 6.2 (implemented in IPA miRNA Target Filter), resulting in the identification of 3,153, 5,244, and 3,812 predicted target mRNAs, respectively. We focused attention on those miRNA–mRNA pairings with the following criteria: (i) the predicted target mRNA was also differentially expressed in our microarray analysis (13), and (ii) the miRNA exhibited a reciprocal expression relationship with its target mRNA (‘up–down’ or ‘down–up’). Using these criteria, we have compiled 150 reciprocal miRNA–mRNA pairings in AA prostate cancer versus EA prostate cancer, 103 pairings in AA prostate cancer versus AA-matched NP and 137 pairings in EA prostate cancer versus EA-matched NP (Supplementary Table S6).

In a separate analysis to identify biologic pathways most significantly associated with AA prostate cancer aggressiveness, we applied the Global test to our gene-expression data from the original 70 biopsy cores. The Global test is a permutation-based approach, coupled with a penalized logistic regression model, to identify gene sets in pathways most significantly associated to clinical phenotypes/outcomes (17). Using this approach, we identified 124, 106, and 137 significant KEGG-annotated signaling pathways (FDR < 0.05) in AA prostate cancer versus EA prostate cancer, AA prostate cancer versus AA NP, and EA prostate cancer versus EA NP comparisons, respectively (Supplementary Table S7).

Among the significant KEGG oncogenic pathways associated with AA prostate cancer were ERBB, MTOR, WNT, JAK-STAT, TGFβ, P53, and VEGF. Noteworthy was the ERBB pathway in AA prostate cancer, where a great majority of pathway genes (miRNAs) identified as significant by the Global test were upregulated in AA prostate cancer versus EA prostate cancer and AA prostate cancer versus AA NP comparisons (Fig. 2A; Supplementary Table S7). Conversely, the vast majority of significant genes in the ERBB pathway of EA prostate cancer were downregulated according to Global testing of EA prostate cancer versus AA prostate cancer and EA prostate cancer versus EA NP comparisons (Fig. 2B; Supplementary Table S7). Similar findings were obtained when analyzing our gene-expression data by the GSEA approach (Supplementary Table S8; ref. 18). Collectively, our pathway analysis suggests that differential gene regulation of ERBB signaling components in AA versus EA prostate cancer may play a critical role toward promoting prostate cancer disparities. A finding that may be particularly relevant given the well-developed targeted therapies for this critical oncogenic pathway (22, 23).

Next, we mapped the population-associated miRNAs and miRNA–mRNA pairings (Supplementary Tables S4, S5, and S6) onto the ERBB signaling pathway (Fig. 2). Altogether, 17 AA-specific miRNAs (miR-15b, miR-20a, miR-25, miR-148a, miR-203, miR-129−, miR-659, miR-125-3p, miR-513c, miR-671-3p, miR-887, miR-145, miR-130b, miR-634, miR-767-3p, miR-1225-3p, and miR-197-3p), 2 AA-enriched miRNAs (miR-96 and miR-130b) and 4 AA-depleted miRNAs (miR-133a, miR-758, miR-34a, and miR-99b) were predicted to target 104 signaling genes of the ERBB pathway in AA prostate cancer (Fig. 2A; Supplementary Table S6), leading to a projected overall activation of oncogenic signaling based on GO-Elite analysis (24). Of the reciprocal miRNA–mRNA pairings in the ERBB pathway of AA prostate cancer (Fig. 2A), 14 were novel (i.e., predicted miRNA targeting of mRNA not validated in literature), namely miR-133a/MCL1 (down–up), miR-96/PPP2R3A (up–down), miR-133a/PPP2R2D (down–up), miR-767-3p/MTOR (down–up), miR-1225-3p/MTOR (down–up), miR-1297/MTOR (down–up), miR-1297/PIK3API1 (down–up), miR-96/COL5A1 (up–down), miR-34a/IKBKE (down–up), miR-1297/IKBKB (down–up), miR-933/IKBKB (down–up), miR-145/MKK4 (down–up), miR-634/MKK4 (down–up), and miR-1297/MKK4 (down–up; Supplementary Table S6).

In contrast with the projected activation of ERBB signaling in AA prostate cancer, EA prostate cancer was comprised mostly of downregulated oncogenes and upregulated EA-specific/enriched miRNAs (predicted to target oncogenes) that were projected by GO-Elite to restrain ERBB pathway activity (Fig. 2B). Note that AA- and EA-specific miRNAs do not overlap by definition. Hence, the inverse expression pattern of AA- and EA-specific/enriched/miRNAs.

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**Figure 2.** The ERBB signaling pathway is highly activated in AA prostate cancer (PCa) specimens. Differentially expressed mRNAs (identified by Global test or Global test plus ANOVA (indicated by asterisk)) and miRNAs (identified by ANOVA or paired t test) populating the ERBB signaling pathway in AA prostate cancer (A) and EA prostate cancer (B). Upregulated (red) and downregulated (green) mRNAs with underline representing population-specific miRNAs, whereas miRNAs not underlined represent population-enriched (red) or -depleted (green) miRNAs. The same coloring and underlining scheme is used for differentially expressed miRNAs. The ERBB pathway in AA prostate cancer (A) is more highly activated compared with EA prostate cancer (B) as determined by GO-Elite. Eight novel reciprocal miRNA–mRNA pairings are highlighted, including miR-133a/MCL1, miR-96/FXO13A, miR-513c/STAT1, miR-34a/PPP2R2A, miR-145/ITPR2, miR-145/MKK4, miR-634/MKK4, and miR-1297/MKK4. MiRNAs listed in boxes represent the population-specific (underlined) or -enriched/-depleted miRNAs predicted to target genes in the ERBB signaling pathway belonging to positively or negatively correlated pairings or nondifferentially expressed targets (see Supplementary Table S6).
depleted miRNAs targeting different components of the ERBB signaling pathway likely plays a critical role in the differential aggressiveness of prostate cancer progression in the two racial populations.

**Figure 3.** qRT-PCR validation of population-enriched/-depleted and -specific mRNAs and miRNAs in AA and EA prostate cancer (PCa). A, qRT-PCR validation of differentially expressed mRNAs in AA prostate cancer versus EA prostate cancer. B, qRT-PCR validation of differentially expressed miRNAs in AA prostate cancer versus EA prostate cancer. C, qRT-PCR validation of population-specific mRNAs. D, qRT-PCR validation of population-specific miRNAs. The expression levels of mRNA or miRNAs from AA and EA patients are presented as Box-and-Whiskers plots (in A-D). Box: top quartile, median and bottom quartile. Whiskers: top extreme (90 percentile of the dataset) and bottom extreme (10 percentile of the dataset). Dot plots represent the relative expression levels of mRNA or miRNA from individual patient samples; *, P < 0.05 using the Student's t test (n = 6–9 independent experiments in A and B), or a paired Student's t test (n = 5–8 independent experiments in C and D).
We specifically reassessed a combination of 30 differentially expressed miRNAs and mRNAs (identified as significant by both Global test and ANOVA; the exception being BCL2L11 that was identified as significant by ANOVA only) residing in the ERBB signaling pathway, as well as four additional signaling pathways [i.e., non–small cell lung cancer (NSCLC) signaling, the JAK–STAT pathway, tight junction signaling, phosphatidylinositol signaling]. A comparison of the microarray and qRT-PCR results revealed high concordance (28 of 30) in our expression measurements. Successful validations included AA-enriched and -depleted mRNAs (Fig. 3A), AA-enriched and -depleted miRNAs (Fig. 3B), population-specific mRNAs (Fig. 3C) and population-specific miRNAs (Fig. 3D). Encompassed within the validations were the novel reciprocal miRNA–mRNA pairings miR-133a/MCL1 (down–up; target mRNA significant in the ERBB pathway by the Global test), miR-96/FOXO3A (up–down; NSCLC signaling), miR-513c/STAT1 (down–up; JAK–STAT pathway), miR-34a/PPP2R2A (down–up; tight junction signaling), miR-145/IPTPR2 (down–up; phosphatidylinositol signaling) and miR-145/MKK4 (down–up; ERBB pathway; Figs. 2A and 3).

Interestingly, four of the target mRNAs (FOXO3A, STAT1, PPP2R2A, and ITPR2) in these pairings are also known to participate downstream of ERBB signaling, and hence included in Fig. 2A for illustration (25–29). qRT-PCR assessment of population-specific prostate cancer cell lines

We also assessed the expression of AA-enriched and -depleted miRNAs and mRNAs (depicted in Fig. 3) in a panel of prostate...
cancer cell lines derived from AA (MDA PCa 2b, RC77T/E) and EA patients (VCAp, LNCaP, and PC-3; see Supplementary Materials and Methods). There was strong overall agreement between the microarray data from patient specimens and qRT-PCR results of prostate cancer cell lines. Specifically, AA-depleted miRNAs (FOXO3A and BCL2L11) tended to be upregulated in AA versus EA prostate cancer cell lines, and AA-enriched miRNAs (PIK3CB, PPP2R2A, MCL1, 14-3-3e, ITGB5, and STAT1) tended to be overexpressed in AA versus EA prostate cancer cell lines (Fig. 4A). An analogous consistency was observed for the miRNAs (Fig. 4B). Again, contained within these validations were the novel reciprocal miRNA–mRNA pairings miR-133a/MCL1 (down–up), miR-96/FOXO3A (up–down), and miR-513c/STAT1 (down–up; Fig. 4C). As a final consistency check, miRNA–mRNA pairings were found to be consistent with Western blot analysis where FOXO3A was underexpressed, whereas MCL-1 and STAT1 were overexpressed in AA versus EA prostate cancer cell lines (Fig. 4D).

**Immunohistochemical assessment of MCL-1, STAT1, and FOXO3A in AA and EA prostate cancer specimens**

Next, we examined protein expression of MCL-1, STAT1, and FOXO3A by immunohistochemical examination of archived FFPE prostate cancer specimens from AA and EA patients, representing a third cohort with associated Gleason scores ranging from 6 to 9 (Fig. 5A and B; Supplementary Table S1C and Fig. S1). To ensure that MCL-1, STAT1, and FOXO3A protein expression was indeed present in cancerous cells, another series of IHC was performed where our proteins of interest were examined along the basis of congruent qRT-PCR, Western and immunohistochemical findings (Figs. 4 and 5). Transfection of a miR-133a mimic into AA and EA lines led to a downregulation of MCL-1 protein compared with cells transfected with nonsense control RNA (Fig. 6A, left). Conversely, miR-133a antagonim transfection into AA and EA lines led to an upregulation of MCL-1 protein compared with nonsense control (Fig. 6A, right). This antagonism-mediated upregulation in prostate cancer cells was anticipated given the “converse” mimetic-downregulated in prostate cancer cells. Analogous confirmatory findings were also demonstrated for AA-enriched miR-96 (predicted target FOXO3A) and downregulated AA-specific miR-513c (predicted target STAT1; Fig. 6A). Taken together, our in vitro mimic/antagonim manipulation of population-specific prostate cancer cell lines was consistent with observations in patient specimens (see Figs. 3 and 5), providing strong evidence of a causal link between our reciprocal miRNA–mRNA pairings.

The oncogenic consequences of disrupting steady-state expression of our prototype reciprocal pairings were assessed in AA lines RC77T/E and MDA PCa 2b, and EA lines LNCaP and PC-3. In the first set of functional assays, prostate cancer lines were transfected with a series of mimics, antagonimirs, or nonsense control RNA and tested for proliferative activity using a bromodeoxyuridine (BrdUrd)-labeling assay. In each case, the miR-133a mimic, miR-513c mimic, and miR-96 antagonim significantly suppressed proliferation of the AA and EA prostate cancer cell lines compared with nonsense control (Fig. 6B, top). Conversely, the majority of “converse” antagonim/mimic treatments (miR-133a antagonim, miR-513c mimic, and miR-96 mimic) significantly enhanced proliferation in both AA lines and EA line LNCaP, as anticipated (Fig. 6B, bottom). Interestingly, EA line PC-3 was completely resistant to the proliferating—inducing effects of all three “converse” antagonist/mimic treatments (Fig. 6B).

Next, apoptotic sensitivity in the absence and presence of 11 nmol/L docetaxel, a cytotoxic agent used in prostate cancer chemotherapy (31), was assessed in prostate cancer cell lines by caspase-3/7 activity assay. In the absence of any antagonist or mimic treatment, AA lines RC77T/E and MDA PCa 2b were chemoresistant to docetaxel-induced apoptosis (see nonsense control transfected cells in Fig. 6C, top). In contrast, docetaxel treatment alone significantly induced apoptosis in EA lines LNCaP and PC-3 (see nonsense control transfected cells in Fig. 6C, top). In the absence of docetaxel treatment, transfection of AA and EA cell lines with the miR-133a mimic, miR-513c mimic, or miR-96 antagonim precipitated a generalized (exception being miR-513c mimic-transfected RC77T/E and LNCaP cells) and significant increase in apoptosis compared with nonsense control transfected cells (Fig. 6C, top). Strikingly in AA prostate cancer cells (but not in EA cells), the combination of a mimic or antagonim treatment with docetaxel
treatment resulted in apoptotic activity that was greater than either treatment alone, suggesting that disruption of key miRNAs sensitized cells to docetaxel (Fig. 6C, top). Interestingly, the "converse" antagomir/mimic treatments (miR-513c antagomir, and miR-96 mimic) in the absence of docetaxel had the effect of rendering AA lines, but not EA lines, more resistant to apoptosis (Fig. 6C, bottom). On the basis of the proliferative and apoptotic findings, EA prostate cancer lines compared with AA lines appear to be less susceptible to the oncogenic-promoting effects of the reciprocal pairs mir-133a/miRCL1, miR-96/FOXO3A, and miR-513c/STAT1.

Finally, the consequences of disrupting steady-state expression of our prototype reciprocal pairings on the invasive activity of prostate cancer cell lines were assessed by Matrigel assay. Both miR-513c mimic and miR-96 antagonist treatments in AA lines RC77/T/E and MDA PCa 2b, and EA lines LNCaP and PC-3 resulted in a significant decrease in invasive activity (Fig. 6D), though we cannot discount the possibility that this decrease may be due in part to decreased proliferative activity (Fig. 6B, top). In an attempt to identify reciprocal pairings that modulate invasion without affecting proliferation, we tested two additional down-up pairings (mir-145/ITPR2 and miR-34a/PPP2R2A in the EGF–PI3K–AKT pathway) in the AA prostate cancer lines. Western blot analysis confirmed a causal link for these two reciprocal pairings, as transfection with either the miR-145 mimic or miR-34a mimic in AA lines resulted in a reduction of ITPR2 or PPP2R2A protein levels, respectively (Supplementary Fig. S2A). As shown in Supplementary Fig. S2B, the miR-145 mimic affected both proliferation and invasion, whereas the miR-34a mimic was associated with a significant decrease in invasion and had no effect on proliferation in both AA prostate cancer cell lines. Taken together, these findings support the notion that depletion of miR-133a (leading to upregulation of MCL1), miR-513c (upregulation of STAT1), miR145 (upregulation of ITPR2), and miR-34a (upregulation of PPP2R2A), coupled with enrichment of miR-96 (downregulation of FOXO3A) collectively drives proliferation, chemoresistance and/or invasion in AA prostate cancer cells.

Discussion

In this study, we performed an integrated analysis of differential miRNA and mRNA expression profiles in prostate cancer and NP specimens derived from AA and EA patients. Our goal was to identify significant oncogenic signaling pathways that are populated with AA-specific/enriched reciprocal miRNA–mRNA pairs. Emphasis was placed on cataloging novel reciprocal pairs (i.e., predicted miRNA targeting of the mRNA has yet to be experimentally validated). The underlying hypothesis being that these novel reciprocal pairs may play a mechanistic role in prostate cancer disparities (i.e., more aggressive nature of AA prostate cancer), which could be assessed by systematically disrupting reciprocal pairings with mimic/antagomir treatment of population-specific prostate cancer cell lines and testing for a loss (or gain) of oncogenic function. To date, the integrated analysis of miRNA–mRNA pairs has been limited to a handful of prostate cancer studies (32, 33) and none have been related to prostate cancer disparities.

There are a number of available miRNA-target mRNA prediction algorithms (34). However, it is estimated that up to 40% of all miRNA-target mRNA predictions are false positives (35), representing a major obstacle in the identification of true miRNA–mRNA interactions with functional consequences in cancer. An approach exploited by this study was to incorporate both a sequence-based algorithm for miRNA target predictions and focusing on miRNA–mRNA predictions exhibiting reciprocal differential expression profiles (up-down and down-up). Such a strategy has been demonstrated to provide more accurate predictions (35). A total of 390 reciprocal pairings were identified in prostate cancer and NP specimens from AA and EA patients. These pairs (along with unpaired differentially expressed miRNAs and mRNAs) were found populated in 19 and 18 significant cancer signaling pathways from the perspective of AA and EA prostate cancer, respectively.

ERBB signaling pathway in prostate cancer disparities

The ERBB signaling pathway is regarded as a critical oncogenic signaling pathway in cancer, as mutations and/or overexpression of the EGFR and mutations in multiple PI3K isoforms are frequently detected in various types of cancers, including prostate, head and neck, renal, lung, breast, colon, ovarian, glioma, pancreas, and bladder cancers (22, 23). In terms of prostate cancer disparities, EGFR overexpression has been shown to be significantly associated with AA patients (11). Our findings suggest that 18 reciprocal miRNA–mRNA pairs populating the EGFR–PI3K–AKT signaling pathway in AA prostate cancer, and likely working in concert with overexpressed EGFR (11), drive AA prostate cancer.

miR-513c/STAT1 (down–up) represented a novel predicted pairing, and miR-513c has previously been shown to be downregulated in neuroendocrine lung tumors (36). However, the role of miR-513c in cancer and the identification of its target mRNA(s) have remained undetermined. Our results demonstrate for the first time that STAT1 serves as a target of miR-513c. The STAT1
protein is a transcription factor and its overexpression in prostate cancer cells has been associated with docetaxel resistance (37). Interestingly, the AA prostate cancer cell lines investigated in this study were resistant to docetaxel-induced apoptosis, but became sensitized upon treatment with a miR-513c mimic that downregulated STAT1. Additional functions of the miR-513c/STAT1 pair in AA prostate cancer cells include proliferation and invasion, as disruption of this pairing with a miR-513c mimic resulted in a loss of proliferative and invasive activities. The role of miR-513c/STAT1 in driving AA prostate cancer was further supported by experiments using a “converse” targeting approach (i.e., miR-513c antagonist) in EA prostate cancer cell lines, resulting in STAT1 upregulation and a more aggressive phenotype reminiscent of the AA prostate cancer lines (i.e., increased proliferation and chemoresistance).

Downregulation of miR-133a has been observed in various cancers (38), acting as a tumor suppressor by targeting multiple oncoproteins, such as FSCN1, MMP14, LASP1, EGFR, IGF1R, and GSTP1 (39). In our study, MCL1 was identified as a novel target of miR-133a, and overexpression of a miR-133a mimic in prostate cancer cell lines led to a downregulation of MCL-1 protein and a corresponding decrease in proliferative activity, as well as loss of chemoresistance to docetaxel. MCL-1 has been demonstrated to be overexpressed in prostate cancer and is linked to higher chemoresistance to docetaxel. MCL-1 has been demonstrated to be overexpressed in prostate cancer and is linked to higher chemoresistance to docetaxel.

Our analysis identified many miR-513c and miR-133a tumor suppressor-mRNA pairings, it is important to stress that the miRNAs in these pairings would be expected to coordinate target other miRNAs (i.e., MCL1, FSCN1, MMP14, LASP1, EGFR, IGF1R and GSTP1 by miR-133a, and FOXO3A, FOXO1 and RECK by miR-96), presumably leading to the aggressive phenotypic features found in AA prostate cancer. Finally, our findings suggest that these deregulated miRNA–mRNA pairs, uniquely found in AA prostate cancer, appear to target the EGFR mRNA, in an anticipated up- and downregulation of the EGFR protein, respectively. This finding would be consistent with the observed racial disparity of EGFR overexpression in AA prostate cancer (11).

Although our analysis has focused on five reciprocal miRNA–mRNA pairings, it is important to stress that the miRNAs in these pairings would be expected to coordinate target other miRNAs (i.e., MCL1, FSCN1, MMP14, LASP1, EGFR, IGF1R and GSTP1 by miR-133a, and FOXO3A, FOXO1 and RECK by miR-96), presumably leading to the aggressive phenotypic features found in AA prostate cancer. Finally, our findings suggest that these deregulated miRNA–mRNA pairs, uniquely found in AA prostate cancer, appear to target the EGFR mRNA, in an anticipated up- and downregulation of the EGFR protein, respectively. This finding would be consistent with the observed racial disparity of EGFR overexpression in AA prostate cancer (11).

Additionally, our analysis focused on five reciprocal miRNA–mRNA pairings, it is important to stress that the miRNAs in these pairings would be expected to coordinate target other miRNAs (i.e., MCL1, FSCN1, MMP14, LASP1, EGFR, IGF1R and GSTP1 by miR-133a, and FOXO3A, FOXO1 and RECK by miR-96), presumably leading to the aggressive phenotypic features found in AA prostate cancer. Finally, our findings suggest that these deregulated miRNA–mRNA pairs, uniquely found in AA prostate cancer, appear to target the EGFR mRNA, in an anticipated up- and downregulation of the EGFR protein, respectively. This finding would be consistent with the observed racial disparity of EGFR overexpression in AA prostate cancer (11).

It should also be noted that AA prostate cancer was associated with a large number of upregulated oncoproteins (such as ITGA5, PIK3CB, PIK3AP, ITPR2, STAT1, CSN2, MK4, 14-3-3ε, MIF, and MCL1) as well as dysregulated unpaired miRNAs that are unique to AA prostate cancer (e.g., AA-specific/deleted miRNAs) and computationally predicted to target EGFR–PI3K–AKT signaling components (such as EGRF, AKT3, GSK3, JAK1, JUN, and SRC) and leading to pathway activation. Conversely, our analysis identified an equally large number of dysregulated oncoproteins plus unpaired miRNAs that were specific to EA prostate cancer and computationally predicted to target a different set of EGFR–PI3K–AKT signaling components leading to pathway suppression. Also noteworthy, unpaired AA-specific miR-767-3p (downregulated in AA prostate cancer vs. AA NP) and unpaired EA-specific miR-195 (upregulated in EA prostate cancer vs. EA NP) were both predicted to target the EGRF mRNA, resulting in an anticipated up- and downregulation of the EGFR protein, respectively. This finding would be consistent with the observed racial disparity of EGFR overexpression in AA prostate cancer (11).

Although our analysis has focused on five reciprocal miRNA–mRNA pairings, it is important to stress that the miRNAs in these pairings would be expected to coordinate target other miRNAs (i.e., MCL1, FSCN1, MMP14, LASP1, EGFR, IGF1R and GSTP1 by miR-133a, and FOXO3A, FOXO1 and RECK by miR-96), presumably leading to the aggressive phenotypic features found in AA prostate cancer. Finally, our findings suggest that these deregulated miRNA–mRNA pairs, uniquely found in AA prostate cancer, appear to target the EGFR mRNA, in an anticipated up- and downregulation of the EGFR protein, respectively. This finding would be consistent with the observed racial disparity of EGFR overexpression in AA prostate cancer (11).

Understanding the origins and etiology of cancer disparities is a complex endeavor and it is imperative that such disparities be addressed at all levels of intervention, both social and biologic. Evidence exists, indicating that one component of the disparity may be related to biologic differences in the molecular etiology of the disease resulting in tumor aggressiveness. We have used a population-based comparative approach in an attempt to discern potential drivers of prostate cancer aggressiveness and have identified novel pathway alterations in miRNA–mRNA pairs that may contribute to prostate cancer disparities. Given the projected use of miRNA mimics and antagonins as potential cancer therapeutics (50), our study serves as the first pass catalog of dysregulated miRNA–mRNA pairs residing in key oncogenic signaling pathways in AA prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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References

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