ORIGINAL ARTICLE

Role of a novel race-related tumor suppressor microRNA located in frequently deleted chromosomal locus 8p21 in prostate cancer progression

Divya Bhagirath, Thao Ly Yang, Z.Laura Tabatabai, Varahram Shahryari, Shahana Majid, Rajvir Dahiya, Yuichiro Tanaka and Sharanjot Saini*

Department of Urology, Veterans Affairs Medical Center, San Francisco and University of California, 4150 Clement Street, San Francisco, CA 94121, USA

*To whom correspondence should be addressed. Tel: +1 415 221 4810 ext. 23548; Fax: +1 415 750 6639; Email: sharanjot.saini@ucsf.edu

Abstract

The prostate cancer (PCa) genome is characterized by deletions of chromosome 8p21–22 region that increase significantly with tumor grade and are associated with poor prognosis. We proposed and validated a novel, paradigm-shifting hypothesis that this region is associated with a set of microRNA genes—miR-3622, miR-3622b, miR-383—that are lost in PCa and play important mechanistic roles in PCa progression and metastasis. Extending our hypothesis, in this study, we evaluated the role of a microRNA gene located in chromosome 8p—miR-4288—by employing clinical samples and cell lines. Our data suggests that (i) miR-4288 is widely downregulated in primary prostate tumors and cell lines; (ii) miR-4288 expression is lost in metastatic castration-resistant PCa; (iii) miR-4288 downregulation is race-related PCa alteration that is prevalent in Caucasian patients and not in African Americans; (iv) in Caucasians, miR-4288 was found to be associated with increasing tumor grade and high serum prostate-specific antigen, suggesting that miR-4288 downregulation/loss may be associated with tumor progression specifically in Caucasians; (v) miR-4288 possess significant potential as a molecular biomarker to predict aggressiveness/metastasis; and (vi) miR-4288 is anti-proliferative, is anti-invasive and inhibits epithelial-to-mesenchymal transition; and (vii) miR-4288 directly represses expression of metastasis/invasion-associated genes *MMP16* and *ROCK1*. Thus, the present study demonstrates a tumor suppressor role for a novel miRNA located with a frequently lost region in PCa, strengthening our hypothesis that this locus is causally related to PCa disease progression via loss of microRNA genes. Our study suggests that miR-4288 may be a novel biomarker and therapeutic target, particularly in Caucasians.

Introduction

Prostate cancer (PCa) is a leading cause of cancer incidence and mortality among males in the United States, with 164 690 new cases and 29 430 deaths estimated in 2018 (1). It is a disease characterized by remarkable heterogeneity (2) with the disease ranging from indolent to very aggressive forms. A major challenge in the PCa field is the inadequacy of current diagnostic tests such as serum prostate-specific antigen (PSA) testing and other risk stratification tools to distinguish and predict tumor aggressiveness (3). In the absence of effective biomarkers, aggressive PCa is associated with significant

morbidity and mortality (4), mostly resulting from metastasis that occurs locally or distantly in other organs. In 2017, metastatic disease accounted for ~16.5% of deaths from PCa (5), and studies point to an upward trend in metastasis incidence, particularly in white men when compared with other races (6). Furthermore, these rates are expected to increase at 0.38% per year, accounting for almost 42% of metastatic PCa cases by 2025 (7). An improved understanding of the molecular mechanisms underlying aggressive PCa is key to finding effective molecular biomarkers for better diagnosis, for prognosis and for developing

Received: September 19, 2018; Revised: February 7, 2019; Accepted: March 12, 2019

© The Author(s) 2019. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com.

Abbreviations

3'-UTR	3'-untranslated regions
AA	African Americans
AUC	area under the ROC curve
BPH1	benign prostate hyperplasia 1
chr8p	chromosome 8p
CI	confidence interval
CRPC	castration-resistant PCa
EMT	epithelial-to-mesenchymal transition
FFPE	formalin-fixed, paraffin-embedded
LOH	loss of heterozygosity
miRNAs	microRNAs
MMPs	matrix metalloproteinases
PCa	prostate cancer
PSA	prostate-specific antigen
ROCK1	Rho-associated, coiled-coil containing protein kinase 1

better therapeutic modalities against the disease. Furthermore, epidemiological studies show that compared with Caucasian men, African American (AA) men have a higher PCa incidence and mortality rate (8–10). In AA men, PCa presents itself at a younger age and often advanced stage compared with men of other ethnicities (8,9). Though the basis of this racial disparity in PCa incidence and outcome has been attributed to a combination of socioeconomic differences and different tumor biology (11), the molecular basis contributing to racial disparities in PCa is not fully understood.

Genomic studies have previously reported that loss of chromosome 8p (chr8p) region, particularly that of chr8p21–22 subregion, is a frequent event in PCa (12–15). This region harbors prostate-specific NKX3.1 gene (16) among other tumor suppressor genes and have been associated with PCa initiation. However, a significantly higher deletion frequency has been reported in advanced PCa (17,18), indicating that this region may play a significant role in PCa progression. A focused objective of our research is to understand the mechanistic role of this frequently deleted genomic region in PCa progression and metastasis. Toward this, we have proposed and validated a novel hypothesis that this region is associated with a set of microRNA genes—miR-3622a, miR-3622b, miR-383—that play important causal roles in PCa progression, recurrence and metastasis (19–21). MicroRNAs (miRNAs) are small, endogenous RNAs that suppress gene expression post transcriptionally via sequence-specific interactions with the 3'-untranslated regions (3'-UTRs) of cognate mRNA targets (22). miRNAs have been reported to influence key processes involved in progression and metastasis of various cancers (23). Our studies on chr8p miRNAs show a role for these miRs in PCa progression and metastasis, providing a paradigm shift in understanding the mechanistic involvement of this region in PCa (19–21). Within chr8p21 region, miR-3622a/b form a miRNA cluster. We demonstrated that miR-3622a is an important inhibitor of PCa epithelial-to-mesenchymal transition (EMT) (19). EMT is a process by which tumors have decreased expression of epithelial genes (such as E-cadherin) and increased expression of mesenchymal genes (such as vimentin), which promotes invasion and metastasis (24–26). Also, we reported a tumor suppressive role for miR-3622b in PCa via its regulation of epidermal growth factor receptor (20). In another study, we identified that miR-383 at chr8p22 is involved in PCa metastasis via direct regulation of PCa stem cell marker CD44 (21). Extending our hypothesis, in this study we evaluated the role of another miRNA gene located within this region—miR-4288—in PCa. miR-4288 is located on chr8p21 (chr8: 28505116–28505182 [–]) within

the Frizzled 3 (FZD3) gene. FZD3 is a member of the frizzled gene family, a family characterized by seven-transmembrane domain proteins that act as receptors for the wntless-type signaling proteins. Hao *et al.* reported that miR-4288 is upregulated during osteogenic differentiation of human periodontal ligament stem cells (27). The role of miR-4288 has never been investigated in PCa. In this study, we examined the role of miR-4288 in PCa employing clinical samples and cell lines. Our data suggest that (i) miR-4288 is widely downregulated in PCa clinical samples and cell lines; (ii) miR-4288 downregulation is a race-related PCa alteration that is prevalent in Caucasian patients and not in AA; (iii) in Caucasians, miR-4288 was found to be associated with disease progression; and (iv) miR-4288 plays a causal role in PCa invasion and metastasis. Thus, the present study demonstrates a tumor suppressor role for a novel miRNA located within a frequently lost genomic region in PCa, strengthening our hypothesis that this locus is causally related to PCa disease progression via loss of miRNA genes within this region. To our knowledge, this is the first study to ascribe a role to miR-4288 in PCa.

Materials and methods

Cell lines and cell culture

Primary prostate epithelial cells and prostate carcinoma cell lines (LNCaP, Du145, PC3, MDA-PCa-2b, E006) were obtained from the American Type Culture Collection (ATCC) and cultured under recommended conditions. Primary prostate epithelial cells were cultured in prostate epithelial cell basal media supplemented with prostate epithelial cell growth kit (ATCC). LNCaP and PC3 cell lines were maintained in RPMI 1640 media (UCSF cell culture facility), and Du145 cells were cultured in Minimum Essential Media media, each supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin (UCSF cell culture facility). MDA-PCa-2b was cultured in HPC1 media with 20% fetal bovine serum in poly-L-lysine (Sigma-Aldrich) coated culture dishes. E006 cell line was cultured in Dulbecco's modified Eagle's media. Immortalized non-transformed prostate epithelial cell line [benign prostate hyperplasia 1 (BPH1)] (28) was maintained in RPMI 1640 media supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Prostate cell lines were authenticated by DNA short-tandem repeat analysis. The experiments with cell lines were performed within 6 months of their procurement/resuscitation.

miRNA transfections

Cells were plated in growth medium without antibiotics ~24 h before transfections. Transient transfections were carried out by miRNA precursors (Ambion) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Control miRNA (miR-CON; AM17110)/miR-4288 precursor (AM17100) were used for miRNA transfections at a final concentration of 50 nM followed by functional assays. All miRNA transfections were for 72 h.

Generation of stable cell lines

PC3 cells were plated in growth medium without antibiotics ~24 h before transfections. Cells were transfected with the following lentiviral constructs (Applied Biological Materials): miR-4288 expression construct (catalog no. mh10667) or control construct (catalog no. m003) as per the manufacturer's protocol. Seventy-two hour post-transfection, the stably transfected cells were selected by culturing in the presence of puromycin (1 µg/ml).

Tissue samples

Formalin-fixed, paraffin-embedded (FFPE) PCa samples were obtained from the San Francisco Veterans Affairs Medical Center (SFVAMC) or Prostate Cancer Biorepository Network (PCBN). Written informed consent was obtained from all patients, and the study was approved by the UCSF

Committee on Human Research (IRB number 14-12956). The study was performed in accordance with the Declaration of Helsinki. All slides were reviewed by a board-certified pathologist for the identification of PCa foci as well as adjacent normal glandular epithelium. Tissues were microdissected as described in ref. (29) using the AutoPix System (Arcturus). Briefly, 8 μ m sections were placed on glass slides, deparaffinized, stained with hematoxylin, dehydrated and microdissected with an AutoPix instrument using manufacturer's instructions. Areas of interest were captured with infrared laser pulses onto CapSure Macro LCM Caps.

RNA extraction from FFPE tissues and cultured cells

RNA was extracted from microdissected FFPE tissues and cultured cells using a miRNeasy FFPE Kit (Qiagen) and an miRNeasy Mini Kit (Qiagen), respectively, following the manufacturer's instructions.

Quantitative real-time PCR

Mature miRNA miR-4288 (assay ID 242342_mat) was assayed using the TaqMan MicroRNA Assay, in accordance with the manufacturer's instructions (Applied Biosystems). miRNA expression was normalized to RNU48 control (assay ID 001006) (Applied Biosystems). The comparative Ct method was used to calculate the relative changes in gene expression with the 7500 Fast Real Time PCR System.

Cell viability and bromodeoxyuridine incorporation assays

Cell viability was determined at 24, 48 and 72 h after miR-CON/miR-4288 transient transfections by using the CellTiter 96 AqueousOne Solution Cell Proliferation Assay Kit (Promega), according to the manufacturer's protocol. For bromodeoxyuridine (BrdU) incorporation assay, 48 h post-transfection, BrdU reagent was added to cells for 24 h followed by fixation and detection of incorporated BrdU employing a BrdU Cell Proliferation Assay Kit (Millipore) according to the manufacturer's protocol.

Cell cycle analyses

Fluorescence-activated cell sorting for analyzing cell cycle was done 72 h post-transfection. Cells were harvested, washed with cold phosphate-buffered saline and fixed with 70% ethanol overnight at 4°C. Following two phosphate-buffered saline washes, cells were resuspended in propidium iodide/RNase staining buffer (catalog no. 554656, BD Biosciences) and incubated at room temperature for 15 min as per the manufacturer's protocol. Stained cells were immediately analyzed on FACSVerse (BD Biosciences).

Fluorescein isothiocyanate-phalloidin staining

Cells were transfected with miR-CON/miR-4288 precursor. After 72 h, cells were fixed with 4% paraformaldehyde for 15 min and stained with FITC-labeled phalloidin (Sigma) as per the manufacturer's instructions. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Cells were visualized and photographed on a Nikon Eclipse Ti-S fluorescence microscope equipped with NIS-Elements-D software. Photomicrographs were processed with Adobe Photoshop.

Invasion assays

Matrigel inserts (BD Biosciences) were used for performing *in vitro* transwell invasion assays as per the manufacturer's protocol. Briefly, 48 h post-transfection, cells were counted and 100 000 cells in a volume of 300- μ l serum-free medium were placed on Matrigel inserts. Cells were allowed to migrate for 24 h at 37°C. Cells were then removed from the top of the inserts, and cells that invaded through the polycarbonate/basement membrane were fixed, stained and counted under light microscope.

Western blotting

Whole cell extracts were prepared in RIPA buffer [50 mmol/l Tris (pH 8.0), 150 mmol/l NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate and 1.0% NP-40] containing protease inhibitor cocktail (Roche). Total protein was electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and western blotting was carried out according to standard protocols. The following antibodies were used for western

blotting: E-cadherin (3195, Cell Signaling), vimentin (5741, Cell Signaling), SLUG (9585, Cell Signaling), matrix metalloproteinases (MMP16) (Cell Signaling), Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) (611136, BD Biosciences) and GAPDH (sc-32233, Santa Cruz Biotechnology).

Luciferase assays

For ROCK1 and MMP16, the 3'-UTR region containing target sequences complementary to the miR-4288 seed sequence was cloned downstream of the luciferase gene in the pmiRGLO luciferase vector (Promega). The primers used for clonings were synthesized from Invitrogen and are listed in [Supplementary Table S4](#), available at *Carcinogenesis* Online. SLUG 3'-UTR reporter construct (catalog no. HmiT017502-MT01), along with control construct (CmiT000001-MT01) were obtained from Genecopoeia. These target clones are in SV-40 promoter-based vector pEZx-MT01 (Genecopoeia).

Statistics

All quantified data represent an average of triplicate samples or as indicated. Data are represented as mean $\pm$ SEM or as indicated. Two-tailed Student's *t*-test was used for comparisons between groups. The Wilcoxon signed rank and Mann-Whitney tests were used to assess the difference between miR-4288 expression in matched tumor/normal clinical tissues and independent tumor/normal serum samples, respectively. Correlations between miR-4288 expression and clinicopathological parameters were assessed using chi-square test. Receiver-operating characteristic (ROC) curves were generated based on dCt values of miR-4288 in test/control samples. Statistical analyses were performed using MedCalc version 10.3.2. Results were considered statistically significant at $P \leq 0.05$.

Results

MicroRNA-4288 located in frequently deleted chr8p21 region is underexpressed in PCa

We performed miR-4288 expression profiling in a PCa patient cohort seen at the SFVAMC ([Figure 1A](#)). miR-4288 expression was analyzed in microdissected PCa tissues ($n = 74$) and matched adjacent normal regions by real-time PCR and was found to be significantly downregulated in 48/74 (65%) of PCa cases ($P < 0.0001$, Wilcoxon signed rank test) ([Figure 1A](#), upper panel). 13.5% of PCa cases showed no change and ~21.5% of cases exhibited high miR-4288 expression when compared with normal adjacent tissue. Average miR-4288 expression in prostate tumor tissues was ~8.1-fold lower than that of normal tissues ($P < 0.0001$) ([Figure 1A](#), lower panel). Patients' demographics and clinicopathological characteristics are summarized in [Supplementary Table S1](#), available at *Carcinogenesis* Online. Furthermore, analyses of miR-4288 expression in prostate cell lines showed that its expression is specifically attenuated in prostate carcinoma cell lines (LNCaP, DU145, PC3) compared with normal primary prostate epithelial cells and BPH1 cell line ([Figure 1B](#)). Collectively, these data confirm the widespread attenuation of expression of miR-4288 in PCa.

Diagnostic potential of miR-4288 expression in PCa

In view of the observed attenuated miR-4288 expression in PCa clinical specimens, we asked if miR-4288 expression can be used as a diagnostic parameter for PCa. We performed ROC curve analyses based on dCt values of tumor and adjacent normal samples ([Figure 1C](#)). Our analyses showed that miR-4288 expression can discriminate between normal and tumor tissues with an area under the ROC curve (AUC) of 0.701 [95% confidence interval (CI): 0.620–0.773, $P < 0.0001$], ~82% sensitivity (95% CI: 71.8–90.3%) and ~49% specificity (95% CI: 36.9–60.6%) ([Figure 1C](#)). To validate the diagnostic potential of miR-4288, we profiled miR-4288 expression in a set of donors with BPH

Figure 1. MicroRNA-4288 located in frequently deleted chr8p21 region is underexpressed in PCa. (A) Relative miR-4288 expression levels in microdissected prostate tumor tissues ($n = 74$) and matched adjacent normal tissues as assessed by real-time PCR. Lower panel: Average miR-4288 expression in normal and tumor tissues. (B) Relative miR-4288 expression levels in prostate cell lines as assessed by RT-PCR. Data were normalized to RNU48 control. Data are represented as mean $\pm$ SEM (* $P < 0.05$). (C) ROC curve analysis showing the ability of miR-4288 expression to discriminate between tumor and normal samples.

($n = 23$) and performed ROC curve analyses based on dCt values in BPH versus PCa cases (Supplementary Figure S1, available at Carcinogenesis Online). Our analyses validated the potential of miR-4288 to distinguish between cancer and benign conditions with an AUC of 0.620 (95% CI: 0.516–0.717) (Supplementary Figure S1, available at Carcinogenesis Online).

miR-4288 expression is correlated with race in PCa

We next analyzed if low miR-4288 expression is associated with clinicopathological parameters such as age, race, serum PSA, Gleason score, pathological stage and biochemical recurrence of PCa (Figure 2A). Although there was no significant correlation with age, decreased miR-4288 expression was observed in 57% of cases with low Gleason score (4–6) versus 73% of cases with Gleason score of ≥ 7 (79% of Gleason 7 and 58% of Gleason 8–10). Furthermore, decreased miR-4288 expression was observed in 40% of cases of pathological stage pT2a, 75% of cases of pT2b, 65% of pT2c, 75% of pT3 and 100% of pT4 cases. Low miR-4288 expression was observed in 69% of cases with biochemical recurrence versus 54% cases without recurrence. However, the correlations between miR-4288 expression and Gleason score, pathological stage and biochemical recurrence failed to achieve statistical significance within the tested cases (Figure 2A). Furthermore, we stratified our clinical cohort based on median PSA to examine the correlation between miR-4288 expression

and serum PSA. We found that miR-4288 expression was not statistically significantly correlated with serum PSA levels ($P = 0.5018$). Interestingly, we observed a significant correlation between miR-4288 expression and race ($P = 0.0393^*$). In AA, low miR-4288 expression was observed in 54% of cases, whereas 18 and 28% of cases showed no significant change or high miR-4288 expression, respectively. Significantly, among Caucasians, 81% of cases showed low miR-4288 expression in tumors, whereas a much lower proportion of cases showed unaltered/high (5 and 14%, respectively) miR-4288 expression compared with adjacent normals. These data suggest that miR-4288 expression is a race-related molecular alteration in PCa. We further performed Kaplan–Meier survival analyses for PCa patients, stratified based on miR-4288 levels (low expression and high/no change groups). Our analyses showed that patients with low miR-4288 expression trend toward reduced overall survival when compared with those with higher levels (Figure 2B).

miR-4288 downregulation is a race-related PCa alteration associated with increasing tumor grade and high serum PSA in Caucasians

In view of the observed significant association between low miR-4288 expression and race, we also examined miR-4288 expression in AA cell lines MDA-PCa-2b and E006 versus BPH1 and found that miR-4288 expression is higher in these cell lines

Figure 2. miR-4288 downregulation is a race-related PCa alteration associated with increasing tumor grade and high serum PSA in Caucasians. (A) Correlation of miR-4288 expression with clinicopathological characteristics of PCa patients. (B) Kaplan-Meier survival analyses of PCa patients stratified based on miR-4288 expression levels. (C) Relative miR-4288 expression levels in prostate cell lines as assessed by RT-PCR. (D) Correlation of miR-4288 expression with clinicopathological characteristics in Caucasians versus AA PCa patients. P-values are based on chi-square test.

when compared with BPH1 cells (Figure 2C). This expression pattern in AA cell lines was opposite to that observed in Caucasian-derived cell lines LNCaP, PC3 and Du145 (Figure 1B). We further examined if attenuation of miR-4288 expression in Caucasians is associated with the clinicopathological parameters of the disease and compared this association with that of AA/Blacks (Figure 2D). For this analysis, we stratified our clinical cohort into two subgroups—Caucasians versus AA—and examined the association of relative miR-4288 expression with race-related age, Gleason score, pathological stage and biochemical recurrence (Figure 2D). Although no statistically significant associations were observed between age, Gleason score and biochemical recurrence, we found that low miR-4288 expression was significantly associated with increasing pathological stage in Caucasians ($P = 0.0151^*$) and not in AA ($P = 0.7997$). Furthermore, we examined the association of miR-4288 expression with serum PSA in the two races. We used race-specific, age-defined normal PSA ranges as defined earlier (30,31) (also listed in Supplementary Table S2, available at Carcinogenesis Online) to stratify the Caucasian and AA populations into low ($\leq$ age-defined range) or high ($>$ age-defined range) PSA groups and used age-adjusted PSA ranges to examine the correlation between miR-4288 expression and serum PSA. We found that miR-4288 expression was statistically significantly correlated with age-adjusted PSA levels in Caucasians ($P = 0.0327^*$) and not in AA ($P = 0.4257$). In Caucasians, low miR-4288 expression

was observed in 33% of cases with low age-adjusted PSA levels and in 88% of cases with PSA levels higher than the age-defined range. These findings suggest that miR-4288 downregulation is a race-related PCa alteration associated with increasing tumor grade and high serum PSA in Caucasians.

MicroRNA-4288 expression is highly downregulated in metastatic castration-resistant PCa tissues

In view of the observed association of low miR-4288 expression with high pathological stage in Caucasian patients within the SFVAMC cohort, we examined miR-4288 alterations in an independent cohort of metastatic PCa (Figure 3). This cohort included metastatic castration-resistant PCa (CRPC) cases ($n = 33$, Supplementary Table S3, available at Carcinogenesis Online). Interestingly, we found that 100% of metastatic tissues showed downregulated expression of miR-4288 (Figure 3A) when compared with normals ($n = 19$). ROC curve analyses (Figure 3B) showed that miR-4288 expression can be a single significant parameter to discriminate between normal and metastatic tissues with an AUC of 0.982 ($P < 0.0001$), 100% specificity and 90.91% sensitivity. We validated these findings by performing ROC curve analyses based on dCt values in CRPC ($n = 33$) and BPH cases ($n = 23$) (Supplementary Figure S2, available at Carcinogenesis Online). Our analyses showed that miR-4288 can distinguish between metastatic PCa and BPH conditions with an AUC of 0.926 (95% CI: 0.824–0.979; $P < 0.0001$) (Supplementary

Figure 3. MicroRNA-4288 is expression is highly downregulated in metastatic CRPC tissues. (A) Relative miR-4288 expression in microdissected metastatic CRPC tumor tissues ($n = 33$) when compared with normals ($n = 19$) as assessed by real-time PCR. (B) ROC curve analysis showing the ability of miR-4288 expression to discriminate between metastatic tumor and normal samples.

Figure S1, available at *Carcinogenesis* Online). These data suggest that miR-4288 possess significant potential as a molecular biomarker to predict PCa aggressiveness/metastasis.

miR-4288 overexpression suppresses proliferation of PCa cell lines

In view of the preceding data showing downregulated expression of miR-4288 in PCa clinical tissues and cell lines, we examined the functional significance of this miRNA by modulating its levels in PCa cell lines PC3 and LNCaP followed by functional assays (Figure 4). We overexpressed miR-4288/control miRNA (miR-CON) in PCa cell lines (LNCaP, PC3) by transient transfections (Figure 4A). miR-4288 overexpression led to a significant decrease in cellular viability in both cell lines when compared with controls (Figure 4B). Cell cycle analyses (Figure 4C) showed that miR-4288 expression causes G0–G1 arrest as we observed an increase in the fraction of cells in the G0–G1 cell cycle phase upon miR-4288 transfection when compared with miR-CON transfected LNCaP and PC3 cells. To confirm this effect of miR-4288, we performed BrdU cell proliferation assay upon control/miR-4288 expression (Figure 4D). We observed decreased BrdU incorporation upon miR-4288 expression. Further, we analyzed the expression of cell cycle-related proteins upon control/miR-4288 expression (Figure 4E) and found that cyclin D1 levels decrease upon miR-4288 overexpression while CDK inhibitors p21 and p15 were not altered significantly. Collectively, these data suggest that miR-4288 plays a tumor suppressor role in PCa by suppressing cellular proliferation.

miR-4288 expression reduces invasiveness of PCa cell lines and induces mesenchymal to epithelial transition

We also examined the potential role of miR-4288 in other cellular processes such as apoptosis, and invasion upon its overexpression in LNCaP and PC3 cells. While no significant effects were observed on apoptosis, we observed that miR-4288 overexpression led to reduced invasiveness in PCa cell lines (Figure 5A). This suggests that miR-4288 plays an anti-invasive role in PCa. Also, miR-4288 overexpression led to morphological changes in these cell lines consistent with mesenchymal to epithelial transition. We assessed these morphological

alterations by examining actin cytoskeletal organization (Figure 5B). Fluorescein isothiocyanate-labeled phalloidin staining of miR-CON/miR-4288 overexpressing LNCaP and PC3 cells suggest that miR-4288 induces actin cytoskeletal reorganization with miR-4288 expressing cells presenting a prominent cortical actin distribution when compared with control (Figure 5B). We further stably overexpressed miR-CON/miR-4288 in PC3 cells (Figure 5C) and examined the expression of epithelial and mesenchymal genes by real-time PCR (Figure 5D, upper panel) and Western blotting (Figure 5D, lower panel). When compared with control, epithelial marker *CDH1*/E-cadherin was found to be upregulated in miR-4288 overexpressing PC3 cells with a concomitant decrease in expression of mesenchymal markers *CDH2* and *VIM*/vimentin.

miR-4288 directly regulates metastasis-associated genes

Ongoing studies in our laboratory on microRNA genes located in the frequently deleted chr8p region suggest that these genes are important players in PCa EMT, stemness and PCa progression and metastasis (19–21). *In silico* analyses using miRANDA (32) or TargetScan (33) algorithm identified that miR-4288 potentially targets an array of metastasis and stemness-related genes including *CD44*, *KLF4*, *SRC*, *SLUG*, *MMP16* and *ROCK1*. To validate the potential targets, we performed immunoblot analyses of miR-CON/miR-4288 overexpressing LNCaP and PC3 cells. Our data shows that miR-4288 expression represses *MMP16*, *ROCK1* and *SLUG* protein levels (Figure 6A) suggesting that miR-4288 may directly target these metastasis-associated genes. The 3'-UTR regions of *ROCK1*, *MMP16* and *SLUG* each possess one potential miR-4288 binding site (Figure 6B). To examine if these genes as direct miR-4288 targets, we cloned their respective 3'-UTR regions into luciferase reporter vector and performed reporter assays in miR-CON/miR-4288 transfected PC3 cells (Figure 6C). Reporter assays with *ROCK1* and *MMP16* 3'-UTR constructs led to repression of their luciferase reporter activity with miR-4288 cotransfection when compared with corresponding control 3'-UTR constructs while non-significant changes were observed with *SLUG* 3'-UTR (not shown). This data suggests that *ROCK1* and *MMP16* are direct miR-4288 targets while *SLUG* is not a direct target of this miRNA.

Figure 4. miR-4288 overexpression suppresses proliferation of PCa cell lines. Control miRNA (miR-CON)/miR-4288 precursor was transfected in LNCaP/PC3 cell lines at a final concentration of 50nM followed by functional assays (performed 72 h post-transfection). (A) Relative miR-4288 expression after miRNA transfections as assessed by RT-PCR. Data were normalized to RNU48 control. ($P < 0.05$). (B) Relative cellular viabilities at the indicated time points after transient transfections as assessed by MTS cellular viability assay. (C) Cell cycle analyses in LNCaP (upper panels)/PC3 cells (lower panels) after miR-CON (left panels) or miR-4288 (right panels) transfections. (D) BrdU cell proliferation assay upon control/miR-4288 expression in LNCaP and PC3 cells. (E) Western blot analyses for indicated proteins after miR-CON/miR-4288 transfection in PC3 cells. GAPDH was used as a loading control.

Figure 5. miR-4288 expression reduces invasiveness of PCa cell lines and induces mesenchymal to epithelial transition. (A) Trans-well invasion assay in miR-CON/miR-4288-transfected LNCaP and PC3 cells. (B) Morphological alterations upon miR-CON/miR-4288 transfections as assessed by fluorescein isothiocyanate-labeled phalloidin staining in LNCaP and PC3 cells 72 h post-transfection. (C) Relative miR-4288 expression in PC3 cells stably transfected with control miR/miR-4288 overexpression construct as assessed by RT-PCR. Data were normalized to GAPDH. (D) Upper panel: Real-time PCR analyses of relative transcript levels of CDH1, CDH2 and VIM in PC3 cells stably transfected with miR-CON/miR-4288 overexpression construct. Data were normalized to GAPDH. Lower panel: Immunoblots of endogenous E-cadherin and vimentin in PC3 cells stably transfected with miR-CON/miR-4288.

Figure 6. miR-4288 directly regulates metastasis-associated genes. (A) Immunoblots for indicated proteins in PC3 and LNCaP cells treated with miR-CON/miR-4288. GAPDH was used as a loading control. (B) Schematic representation of SLUG, ROCK1 and MMP16 3'-UTRs showing putative miR-4288 target sites. (C) Luciferase reporter assays with the indicated wt and mutated 3'-UTR constructs or control luciferase construct co-transfected with miR-CON/miR-4288 in PC3 (left panels) and LNCaP cells (right panels). Firefly luciferase values were normalized to Renilla luciferase activity and plotted as relative luciferase activity (* $P < 0.05$ when compared with miR-CON).

Discussion

Here, we report for the first time that a novel microRNA gene—miR-4288—is downregulated in PCa and plays a tumor suppressive role. Interestingly, our data suggest that miR-4288 downregulation is a race-related PCa alteration that was found to be associated with increasing tumor grade and high serum PSA in Caucasians and not in AA. It has been reported previously that there is a lower incidence of loss of heterozygosity (LOH) at chr8p12–23 in AA when compared with Caucasians (34). Although the overall incidence of LOH at 8p12–23 was 53%, and 16% showed homozygous deletions, the incidence of LOH in Caucasians was 68% compared with 35% in AA. This study showed that Caucasian race was a significant predictor for chr8p LOH, whereas other clinicopathologic parameters did not have any significant effect on LOH incidence (34). In agreement with this study, we found that miR-4288 expression was lost more frequently in Caucasian patients than in AA patients. Furthermore, we found that low miR-4288 expression was associated with increasing tumor grade and high serum PSA in Caucasians and not in AA. This suggests that miR-4288 downregulation/loss may be associated specifically with tumor progression in Caucasians and that this molecular alteration is not frequent in AA. Similar observations have been reported for NKX3.1 (located on chr8p region) in PCa. Yamoah *et al.* examined the association of 20 biomarkers associated with aggressive PCa in AA versus European Americans and reported that the loss of NKX3.1 expression was associated with an increased risk of pT3 disease in European Americans, whereas it was associated with a decreased risk of pT3 among AA men (35). These studies support the concept that PCa may arise from distinct molecular pathways/alterations in AAs than Caucasians. When considering the more aggressive form of disease in AAs and association of chr8p loss with aggressive PCa (18,36), these findings seems

paradoxical. Similarly, Lindquist *et al.* examined aggressive (Gleason 7, stage T2b) prostate tumors from 24 AA patients and reported that TMPRSS2-ERG gene fusions (chr21) and PTEN losses (chr10q23) are far less common in AA patients than in patients of European ancestry (11). It has been suggested and reported that the underlying tumor genetics in AA versus other ethnicities differ and that disease in AAs may arise from different tumor progenitors (35). In light of this, we suggest that loss of chr8p miRNA—miR-4288—may affect underlying oncogenic pathways and thereby, specifically influence aggressiveness in Caucasians. MicroRNAs have been implicated in racial disparity in AAs when compared with Caucasians (9,37–39). In this direction, Wang *et al.* reported that AA-specific/-enriched miRNA-mRNA pairings such as miR-133a/MCL1, miR-513c/STAT1, miR-96/FOXO3A, miR-145/ITPR2 and miR-34a/PPP2R2A may play a critical role in the activation of oncogenic pathways in AA PCa cells (39).

Furthermore, functional studies with PCa cell lines derived from Caucasian patients—LNCaP and PC3 cells—showed that miR-4288 is anti-proliferative as it causes G0–G1 arrest. Our functional studies also suggest that it plays an anti-invasive role in PCa. Also, miR-4288 overexpression led to morphological and molecular alterations in PCa cell lines consistent with mesenchymal to epithelial transition. This data suggest that miR-4288 promotes epithelial characteristics and may inhibit PCa EMT. We have previously shown that miR-3622a located within chr8p region is a novel regulator of PCa EMT by direct targeting of EMT effectors ZEB1 and SLUG (19). A role for miRNAs that affects EMT, cancer progression and metastasis is being increasingly realized (23). Prominent examples are the miRNAs from miR-200 family, miR-205 (40–42) and miR-203 (43), which as a result regulate PCa invasion and metastasis. We found that miR-4288 represses the expression of SLUG. However, SLUG was not found to be a direct miR-4288 target.

Furthermore, we found that miR-4288 directly represses metastasis/invasion-associated MMP16 and ROCK1. MMPs are a family of proteolytic enzymes that play a crucial role in cancer metastasis by degrading the extracellular matrix, along with a variety of cell surface receptors and signaling molecules (44). Jiang et al. reported that high expression of MMP16 is associated with advanced prostate tumor stage and PCa metastasis (45). Knockdown of MMP16 in PC3 cells led to decreased migratory and invasive properties of PC3 cells (45). ROCK1 is an effector kinase of the small GTPase RhoA (46). Rho GTPases as well as Rho effector proteins ROCK1 and ROCK2 play a role in the generation of actomyosin contractile forces underlying cellular behaviors including motility, survival and proliferation. Elevated expression of ROCK1 is commonly observed in various human cancers and has been associated with promoting tumor cell dissemination and angiogenesis, thereby promoting more invasive and metastatic phenotypes (47,48). In view of our present results, we propose that miR-4288-mediated repression of MMP16 and ROCK1 inhibits PCa invasion and metastasis. Loss of miR-4288 expression upregulates the expression of ROCK1 and MMP16, promoting PCa progression and metastasis. We observed an increased loss of miR-4288 in metastatic tumors and high pathological grade primary tumors, supporting a role for this miR in PCa metastasis and progression. Chr8p losses have been reported to occur in 90.5% cases of advanced PCa than in 55.7% cases of localized PCa (49). These deletions have been reported to increase significantly with tumor grade (18), linking this region with tumor progression and poor prognosis (36). In addition, our data suggest that miR-4288 possess significant potential as a molecular biomarker for PCa diagnosis and as a predictor of aggressiveness/metastasis.

In conclusion, our study suggests that a novel microRNA gene is lost in chr8p deletions associated with PCa, thereby promoting PCa progression and metastasis by upregulation of MMP16 and ROCK1. In conjunction with our earlier studies on miR-3622a (19), miR-3622b (20) and miR-383 (21) in this region, we have identified an additional important miRNA-mediated regulatory loop that associates the frequently lost chr8p region with PCa progression and metastasis. Our findings lend credence to the hypothesis that copy number alterations frequently include multiple genes that cooperatively produce aggressive disease (50). Our study suggests that miR-4288 may be a novel target for developing effective therapies against advanced PCa, particularly in Caucasians.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

Funding

National Cancer Institute at the National Institutes of Health (RO1CA177984, U01CA184966); Department of Defense Prostate Cancer Research Program (Award Nos W81XWH1810303, W81XWH-14-2-0182, W81XWH-14-2-0183, W81XWH-14-2-0185, W81XWH-14-2-0186 and W81XWH-15-2-0062 Prostate Cancer Biorepository Network (PCBN)).

Acknowledgements

We thank Dr Roger Erickson for his support and assistance with preparation of the manuscript.

Author contributions

D.B., T.L.Y., S.S. and Z.L.T. acquired data; S.S., R.D. and D.B. were involved in conception and design; D.B., S.S., R.D., S.M. and

T.L.Y. developed methodology; D.B., T.L.Y. and S.S. analyzed and interpreted data; D.B., T.Y., Z.L.T., V.S., S.M., R.D., Y.T. and S.S. provided administrative, technical or material support; S.S. supervised the study and wrote the manuscript.

Conflict of Interest Statement: None declared.

References

1. Siegel, R.L. et al. (2018) Cancer statistics, 2018. *CA. Cancer J. Clin.*, 68, 7–30.
2. Boyd, L.K. et al. (2012) The complexity of prostate cancer: genomic alterations and heterogeneity. *Nat. Rev. Urol.*, 9, 652–664.
3. Saini, S. (2016) PSA and beyond: alternative prostate cancer biomarkers. *Cell. Oncol. (Dordr.)*, 39, 97–106.
4. Loberg, R.D. et al. (2005) Pathogenesis and treatment of prostate cancer bone metastases: targeting the lethal phenotype. *J. Clin. Oncol.*, 23, 8232–8241.
5. Siegel, R.L. et al. (2017) Cancer statistics, 2017. *CA. Cancer J. Clin.*, 67, 7–30.
6. Dalela, D. et al. (2019) Contemporary trends in the incidence of metastatic prostate cancer among US men: results from nationwide analyses. *Eur. Urol. Focus*, 5, 77–80.
7. Kelly, S.P. et al. (2018) Past, current, and future incidence rates and burden of metastatic prostate cancer in the United States. *Eur. Urol. Focus*, 4, 121–127.
8. Hoffman, R.M. et al. (2001) Racial and ethnic differences in advanced-stage prostate cancer: the prostate cancer outcomes study. *J. Natl. Cancer Inst.*, 93, 388–395.
9. Karakas, C. et al. (2017) Molecular mechanisms involving prostate cancer racial disparity. *Am. J. Clin. Exp. Urol.*, 5, 34–48.
10. Platz, E.A. et al. (2000) Racial variation in prostate cancer incidence and in hormonal system markers among male health professionals. *J. Natl. Cancer Inst.*, 92, 2009–2017.
11. Lindquist, K.J. et al. (2016) Mutational landscape of aggressive prostate tumors in African American men. *Cancer Res.*, 76, 1860–1868.
12. Kim, J.H. et al. (2007) Integrative analysis of genomic aberrations associated with prostate cancer progression. *Cancer Res.*, 67, 8229–8239.
13. Lapointe, J. et al. (2007) Genomic profiling reveals alternative genetic pathways of prostate tumorigenesis. *Cancer Res.*, 67, 8504–8510.
14. Lapointe, J. et al. (2004) Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc. Natl. Acad. Sci. USA*, 101, 811–816.
15. Taylor, B.S. et al. (2010) Integrative genomic profiling of human prostate cancer. *Cancer Cell*, 18, 11–22.
16. He, W.W. et al. (1997) A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. *Genomics*, 43, 69–77.
17. Oba, K. et al. (2001) Two putative tumor suppressor genes on chromosome arm 8p may play different roles in prostate cancer. *Cancer Genet. Cytogenet.*, 124, 20–26.
18. Matsuyama, H. et al. (2003) Clinical significance of chromosome 8p, 10q, and 16q deletions in prostate cancer. *Prostate*, 54, 103–111.
19. Bucay, N. et al. (2017) A novel microRNA regulator of prostate cancer epithelial-mesenchymal transition. *Cell Death Differ.*, 24, 1263–1274.
20. Bucay, N. et al. (2016) Novel tumor suppressor microRNA at frequently deleted chromosomal region 8p21 regulates epidermal growth factor receptor in prostate cancer. *Oncotarget*, 7, 70388–70403.
21. Bucay, N. et al. (2017) MicroRNA-383 located in frequently deleted chromosomal locus 8p22 regulates CD44 in prostate cancer. *Oncogene*, 36, 2667–2679.
22. Bartel, D.P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell*, 136, 215–233.
23. Sekhon, K. et al. (2016) MicroRNAs and epithelial-mesenchymal transition in prostate cancer. *Oncotarget*, 7, 67597–67611.
24. Kalluri, R. et al. (2009) The basics of epithelial-mesenchymal transition. *J. Clin. Invest.*, 119, 1420–1428.
25. Thiery, J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer*, 2, 442–454.
26. Zhang, J. et al. (2012) MicroRNA control of epithelial-mesenchymal transition and metastasis. *Cancer Metastasis Rev.*, 31, 653–662.

27. Hao, Y. et al. (2017) Identification of microRNAs by microarray analysis and prediction of target genes involved in osteogenic differentiation of human periodontal ligament stem cells. *J. Periodontol.*, 88, 1105–1113.
28. Hayward, S.W. et al. (1995) Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1. *In Vitro Cell. Dev. Biol. Anim.*, 31, 14–24.
29. Bucay, N. et al. (2015) miRNA expression analyses in prostate cancer clinical tissues. *J. Vis. Exp.* doi:10.3791/53123
30. Morgan, T.O. et al. (1996) Age-specific reference ranges for serum prostate-specific antigen in black men. *N. Engl. J. Med.*, 335, 304–310.
31. Oesterling, J.E. (1995) Age-specific reference ranges for serum prostate-specific antigen. *Can. J. Urol.*, 2 (suppl. 1), 23–29.
32. Betel, D. et al. (2008) The microRNA.org resource: targets and expression. *Nucleic Acids Res.*, 36, D149–D153.
33. Agarwal, V. et al. (2015) Predicting effective microRNA target sites in mammalian mRNAs. *Elife*, 4. doi:10.7554/eLife.05005
34. Kalapurakal, J.A. et al. (1999) Racial differences in prostate cancer related to loss of heterozygosity on chromosome 8p12–23. *Int. J. Radiat. Oncol. Biol. Phys.*, 45, 835–840.
35. Yamoah, K. et al. (2015) Novel biomarker signature that may predict aggressive disease in African American men with prostate cancer. *J. Clin. Oncol.*, 33, 2789–2796.
36. El Gammal, A.T. et al. (2010) Chromosome 8p deletions and 8q gains are associated with tumor progression and poor prognosis in prostate cancer. *Clin. Cancer Res.*, 16, 56–64.
37. Hashimoto, Y. et al. (2017) The role of miR-24 as a race related genetic factor in prostate cancer. *Oncotarget*, 8, 16581–16593.
38. Shiina, M. et al. (2017) Differential expression of miR-34b and androgen receptor pathway regulate prostate cancer aggressiveness between African-Americans and Caucasians. *Oncotarget*, 8, 8356–8368.
39. Wang, B.D. et al. (2015) Identification and functional validation of reciprocal microRNA-mRNA pairings in African American prostate cancer disparities. *Clin. Cancer Res.*, 21, 4970–4984.
40. Gandellini, P. et al. (2009) miR-205 Exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. *Cancer Res.*, 69, 2287–2295.
41. Gregory, P.A. et al. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.*, 10, 593–601.
42. Korpai, M. et al. (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J. Biol. Chem.*, 283, 14910–14914.
43. Saini, S. et al. (2011) Regulatory role of mir-203 in prostate cancer progression and metastasis. *Clin. Cancer Res.*, 17, 5287–5298.
44. Pei, D. (2005) Matrix metalloproteinases target protease-activated receptors on the tumor cell surface. *Cancer Cell*, 7, 207–208.
45. Jiang, C. et al. (2017) Membranous type matrix metalloproteinase 16 induces human prostate cancer metastasis. *Oncol. Lett.*, 14, 3096–3102.
46. Olson, M.F. et al. (2009) The actin cytoskeleton in cancer cell motility. *Clin Exp Metastasis*, 26, 273–287.
47. Croft, D.R. et al. (2004) Conditional ROCK activation in vivo induces tumor cell dissemination and angiogenesis. *Cancer Res.*, 64, 8994–9001.
48. Lochhead, P.A. et al. (2010) Activating ROCK1 somatic mutations in human cancer. *Oncogene*, 29, 2591–2598.
49. Williams, J.L. et al. (2014) Recurrent copy number alterations in prostate cancer: an in silico meta-analysis of publicly available genomic data. *Cancer Genet.*, 207, 474–488.
50. Xue, W. et al. (2012) A cluster of cooperating tumor-suppressor gene candidates in chromosomal deletions. *Proc. Natl Acad. Sci. USA*, 109, 8212–8217.