miRNA-29b Suppresses Prostate Cancer Metastasis by Regulating Epithelial–Mesenchymal Transition Signaling

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Abstract

Prostate cancer remains the second leading cause of cancer deaths among American men. Early diagnosis increases survival rate in patients; however, treatments for advanced disease are limited to hormone ablation techniques and palliative care. Thus, new methods of treatment are necessary for inhibiting prostate cancer disease progression. Here, we have shown that miRNA-29b (miR-29b) expression was lower in prostate cancer cells (PC3 and LNCaP) as compared with immortalized prostate epithelial cells. Between these two prostate cancer cell lines, metastatic prostate cancer PC3 cells displayed lower expression of miR-29b. We also observed a significant downregulation of miR-29b expression in human prostate cancer tissues as compared with patient-matched nontumor tissues. PC3 cells ectopically expressing miR-29b inhibited wound healing, invasiveness, and failed to colonize in the lungs and liver of severe combined immunodeficient mice after intravenous injection, while PC3 cells expressing a control miRNA displayed metastasis. Epithelial cell marker E-cadherin expression was enhanced miR-29b transfected in prostate cancer cells as compared with cells expressing control miRNA. On the other hand, N-cadherin, Twist, and Snail expression was downregulated in PC3 cells expressing miR-29b. Together these results suggested that miR-29b acts as an antimetastatic miRNA for prostate cancer cells at multiple steps in a metastatic cascade. Therefore, miR-29b could be a potentially new attractive target for therapeutic intervention in prostate cancer. Mol Cancer Ther; 11(5): 1166–73. ©2012 AACR.

Introduction

Prostate cancer remains the second leading cause of cancer deaths among American men (1). Early diagnosis increases survival rate in patients. However, improvement in survival has remained relatively small over the past several years. Therefore, new approaches are needed to further reduce the incidence/mortality of this disease. Metastasis is the predominant cause of prostate cancer death (1). Primary tumor cells are released by the production of proteases, which allows tumor cells to cross small blood vessels in the adjacent normal tissue and enter the circulation. Many mechanisms are associated with cancer progression, in which activation of epithelial–mesenchymal transition (EMT) plays a pivotal role in the initiation of metastasis, a process in which epithelial cells lose adhesion and cytoskeletal components concomitant with a gain of mesenchymal components and the initiation of a migratory phenotype (2–4).

miRNAs are a class of small noncoding RNAs that are processed from precursors with a characteristic hairpin secondary structure (5). Hundreds of evolutionarily conserved miRNAs have been identified in plants, animals, and viruses (6, 7). miRNAs are transcribed genes processed to single-stranded regulatory RNA of approximately 22 nucleotides and regulate target mRNAs (8, 9). In mammalian cells, miRNAs effect gene silencing via both translational inhibition and mRNA degradation. An individual miRNA is capable of regulating several distinct mRNAs, and together more than 1,000 human miRNAs are believed to modulate more than one-third of the mRNA species encoded in the genome (10). Dysregulation of miRNA expression has been identified in various cancers, and accumulating data suggest that miRNAs function as classical oncogenes or tumor suppressor genes (11). In addition, several miRNAs have been shown to impact critical steps in the metastatic cascade, such as EMT, apoptosis, and angiogenesis by acting on multiple signaling pathways and targeting various proteins that are major players in this process (9). Furthermore, several clinical studies have identified correlations between miRNA expression and cancer recurrence, development of metastasis, and/or survival (12–14).

Matrix metalloproteinases (MMP) as promoters and mediators of developmental and pathogenic EMT processes can degrade and modify the extracellular matrix (ECM) as well as cell–ECM and cell–cell contacts, facilitating detachment of epithelial cells from the surrounding tissue (15). We have previously reported that miR-29b inhibits MMP-2 expression and may be involved in the
modulation of prostate cancer metastasis (16). In this article, we observed a significant downregulation of miR-29b expression in human prostate cancer tissues as compared with patient-matched nontumor tissues. We have shown that reintroduction of miR-29b into metastatic prostate cancer cells inhibits colonization in the lungs by these cells and modulates the expression of EMT marker proteins. Therefore, miR-29b was identified as a new metastatic suppressor and has potential as a new therapeutic target in metastatic prostate cancer.

Materials and Methods

Cell lines and cell culture

Immortalized prostate epithelial cell line (PZ-HPV-7) and prostate cancer cell lines (PC3 and LNCaP) were procured from American Type Culture Collection and maintained in Dulbecco’s Modified Eagle’s Medium or RPMI medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified CO2 incubator. PC-3M-luc, a luciferase-expressing cell line, is derived from PC3 with high metastatic ability and was obtained from Caliper Life Sciences. Cell lines have not been independently authenticated. PC-3M-miR-29b stable cell line was generated by overexpressing miR-29b into PC-3M-luc cells following selection of G418.

Antibodies and miRNAs

Rabbit antibodies to E-cadherin, N-cadherin, Twist, and Snail were purchased from Cell Signaling Technology. Goat anti-actin-horseradish peroxidase antibody was purchased from Santa Cruz.

miRNA control (Cont miR) and miR-29b were purchased from Invitrogen. Cytomegalovirus promoter driven mammalian expression vector expressing miR-29b or control (Origene) was used for generation of stable transfectants after selection with G418.

In situ hybridization

For in situ hybridization (ISH), paraffin-embedded tissue (6 cancers and 6 nontumors) slides were deparaffinized, transferred into diethyl pyrocarboxylate-treated water, treated with protease K at 37°C for 30 minutes using a protocol adapted from Valastyan and colleagues (17). The sections were incubated in hybridization buffer (50% formamide, 5× SSC, 0.1× Tween 20, 9.2 mmol/L citric acid for adjustment to pH 6, 50 μg/mL heparin, 300 μg/mL yeast RNA) at 50°C (based on the Tm) for 4 hours.

Digoxigenin-labeled, LNA-modified miR-29b or scrambled miRNA probe was incubated at 42°C for overnight. Sections were washed in hybridization buffer (0.1× SSC, 2× SSC, and PBS Tween 20 (PBST). Slides were treated with blocking solution (2% goat serum, 2 mg/mL bovine serum albumin) at room temperature for 1 hour. The anti-digoxigenin-fluorescein isothiocyanate antibody (Roche) was added at room temperature for 1 hour. Tissue sections were washed 3 times with PBST and counterstained with hematoxylin.

Mouse strain and animal care

All animals used in the studies were male severe combined immunodeficient (SCID)/beige mice, 4 to 5 weeks old. Strict animal care procedures set forth by the Institutional Animal Care and Use Committee based on guidelines from the NIH guide for the Care and Use of Laboratory Animals were followed for all experiments. Ten mice were randomly divided into 2 groups. Control group received 6 x 10^6 PC-3M-luc cells expressing control miRNA, and the experimental group received 6 x 10^6 PC-3M cells expressing miR-29b through tail vein injection. After 5 weeks of implantation of cells, mice were injected 100 μL D-luciferin (30 μg/μL) per mouse for bioluminescent imaging (BLI) once a week for 3 weeks.

BLI

In vivo BLI was carried out with an IVIS Spectrum Imaging System (Caliper) comprised of a highly sensitive, cooled CCD camera mounted in a light tight specimen box. Images and measurements of bioluminescent signals were acquired and analyzed with Living Image software (Caliper). Animals received the substrate D-luciferin (Caliper) in DPBS by intraperitoneal injection 15 minutes before in vivo imaging and were anesthetized using 1% to 3% isoflurane (Abbott Laboratories). Animals were placed onto the warmed stage inside the camera box and received continuous exposure to 1% to 2% isoflurane to sustain sedation during imaging. Imaging times ranged from 1 second to 1 minute, depending on the bioluminescence of the tumors or metastatic lesions and 3 to 4 mice were imaged at a time. The light emitted from the bioluminescent tumors or cells was detected in vivo by the IVIS Spectrum Imaging System, was digitized and electronically displayed as a pseudocolor overlay onto a gray scale animal image. Regions of interest (ROI) from displayed images were drawn around the tumor sites and quantified as photons per second with the Living Image software (Xenogen). Background in vivo bioluminescence was measured as approximately 1 x 10^5 to 2 x 10^5 photons/s/cm^2/sr for similarly sized ROIs at nontumor sites of mice. Bioluminescence was quantified and the mean photons/s/cm^2/sr was calculated for the control and experimental groups.

Western blot analysis

Cell lysates were subjected to SDS-PAGE. Specific antibodies were used for Western blot analysis followed by enhanced chemiluminescence (Pierce). Protein load was normalized to actin, using an anti-actin antibody.

Quantitative real-time reverse transcriptase PCR

miRNA expression was determined by isolating total RNA using TRIzol reagent (Invitrogen). Total RNA (10 ng) was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) with miRNA-specific primers. To quantify the miRNA levels, the 7500 Real-Time System (Applied Biosystems) was
used in conjunction with gene-specific TaqMan assays for miR-29b or U6 RNA as an endogenous control. Relative miR-29b expression and SE were calculated by the 7500 Real-Time System software.

Wound healing and invasion assays
Cells were grown to confluency and then scratched using a pipette tip. Two wounds were made for each sample. Migration distance of cells was photographed and measured at 0 time and after 24 hours. For invasion assays, $8 \times 10^4$ cells were plated in the top chamber with Matrigel-coated membrane (24-well insert; pore size, 8 μm; BD Biosciences). Cells were plated in medium without serum or growth factors, and medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated for 22 hours and cells that did not invade through the pores were removed by a cotton swab. Invading cells on the lower surface of the membrane were stained with hematoxylin and counted. After staining, the total number of invading cells was counted from 5 distinct fields for each well at $\times20$ magnification. The relative percentage of invading cells were calculated by dividing the total number of invaded cells in PC-3M-miR-29b by the total number of invaded cells in control prostate cancer cells. The results shown are averages from 3 independent experiments. The Student t test was used to determine statistical significance.

Luciferase assay
Luciferase reporter constructs containing the putative miR-29b binding sites from Snail 3' untranslated region (UTR) was used. Plasmid DNAs were cotransfected into cells cultured in a 12-well plate using Lipofectamine 2000. Briefly, 10 nmol/L miRNA mimic or control miRNA was transfected with 50 ng of the pMIR Snail 3'UTR clone. Luciferase activity was measured 48 hours posttransfection as described previously (16).

Results
Expression of miR-29b is suppressed in prostate cancer
We examined the expression level of miR-29b in prostate cancer cell lines with different metastatic ability and compared with the immortalized human prostate PZ-HPV-7 cells (immortalized, nontransformed human prostate epithelial cell line). Results from real-time PCR displayed significantly higher miR-29b expression in PZ-HPV-7 cells, as compared with prostate cancer LNCaP or PC3 cells (Fig. 1A). We next examined clinical prostate cancer specimens to assess miR-29b expression. Coded clinical
specimens were obtained after Institutional Review Board approval. Expression of miR-29b was examined by real-time PCR in prostate cancer tissues and compared with patient-matched noncancerous prostate tissue (Fig. 1B). miR-29b expression was significantly lower in cancer tissues (9 of 10) than in patient-matched noncancerous tissues ($P < 0.0001$, paired 2-tailed $t$ test). To further validate the expression of miR-29b, we used ISH assay. Paraffin-embedded tissues (6 cancers and 6 noncancers) were hybridized with digoxigenin-labeled, LNA-modified miR-29b, or scrambled probe, and counterstained with hematoxylin. miR-29b expression was undetectable in 4 prostate cancer tissues by ISH as compared with control samples. Two other cancer tissues displayed a low level of miR-29b expression. We did not detect fluorescence from scrambled probe. A representative of control (a) and prostate cancer tissue (b) probed with miR-29b and miR-scrambled are shown (Fig. 1C). A correlation of the miR-29b expression with the grade of disease was not determined due to availability of a limited number of specimens.

Ectopic expression of miR-29b inhibits prostate cancer metastasis in vivo

Because miR-29b expression was significantly inhibited in the highly metastatic PC3 cells, we speculated that miR-29b may be involved in the metastatic process. To examine the antimetastatic activity, we ectopically expressed miR-29b in metastatic human prostate carcinoma PC-3M-luc cell line. The expression level of miR-29b in PC-3M-miR-29b cells was similar to PZ-HPV-7 cells (Fig. 2A). PC-3M-luc cells were stably transfected with control miR and used as a control in parallel. As an experimental metastasis model, stable transfectants were injected into the tail vein of male SCID/beige mice. Luciferase expression (in vitro) was similar in PC-3M-control and PC-3M-miR-29b cell lines. Bioluminescence was quantified (after 5, 6, and 7 weeks of tail vein injection of cells), and the mean of total photon flux was calculated for the control (PC-3M-luc cells expressing control miR) and experimental (PC-3M-luc cells expressing miR-29b) groups. Representative bioluminescence images of 2 mice from each group, taken at week 7, indicated that miR-29b expression in PC-3M cells decreases the metastatic lesions (Fig. 2B). Quantification of the bioluminescence from the whole body surface on week 5, 6, and 7 showed that miR-29b reexpression had a negative effect on the development of metastases (Fig. 2C). The mean photon flux (i.e., luciferase expression) of control group was 10- to 1,000-fold higher (between week 5 and 7) than that in the experimental group, suggesting that miR-29b can inhibit the metastasis of prostate cancer in vivo.

Introduction of miR-29b in metastatic prostate cancer cells inhibits metastasis-relevant traits in vitro

We next examined the antimetastatic potential of miR-29b in vitro. PC-3M cells stably expressing miR-29b exhibited marked morphologic changes compared with the parental cell line, and a pronounced shift was observed from an elongated, spindle-shape fibroblast-like appearance to a more epithelial-like phenotype (Fig. 3A). To address whether these morphologic changes resulted in altered functionality, wound healing and invasion assays were conducted. PC-3 cells expressing miR-29b were less proficient in closing an artificial wound compared with control miR (Fig. 3B). Similarly, Transwell invasion assay (Fig. 3C and D) showed...
that restoration of miR-29b significantly reduced the invaded cells numbers as compared with control PC-3M cells ($P < 0.01$, $n = 3$).

EMT is an important cellular process during tumor development by which epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility (18). The characteristics of EMT are the loss of epithelial cell markers (e.g., E-cadherin) and gain of mesenchymal cell markers (e.g., N-cadherin; ref. 19). Because we have observed phenotypic change of miR-29b stable transfectants, including change in morphology, reduced migration/invasion abilities, we asked whether miR-29b introduction could influence the EMT marker expression. Western blot analysis revealed that PC-3M cells overexpressing miR-29b resulted in upregulation of E-cadherin and concomitant decrease of N-cadherin, Twist, and Snail (Fig. 4A and B). To further confirm the role of miR-29b in EMT signaling, we knocked down miR-29b in nonmetastatic LNCaP cells. An increase expression of Snail was noted (Fig. 4C). Basal level E-cadherin protein level was high in LNCaP cells, and we were unable to detect the differences of this protein in miR-29b knockdown LNCaP cells.

**miR-29b directly targets Snail**

To identify the underlying mechanisms how overexpression of miR-29b inhibits metastasis, potential miR-29b target genes (other than MMP-2) were searched using TargetScan and miRanda computer prediction methods. We found a putative miR-29b target site in the Snail 3’UTR (Fig. 5). Snail is a key marker for mesenchymal phenotype and negative regulator of E-cadherin (20). We examined the interaction between miR-29b and 3’UTR of Snail by a luciferase reporter assay. The results showed that miR-29b repressed wild-type Snail 3’UTR directly as evident from decreased the luciferase activity.

**Discussion**

In this article, we have shown that miR-29b represses the expression of multiple prometastatic proteins and
thereby inhibit several steps of the invasion metastasis cascade. Moreover, we found that miR-29b expression is decreased in a number of human prostate cancer tissues and prostate cancer cells. miRNAs can modulate a wide variety of biological processes. To assess the biological significance of decreased miR-29b expression, we introduced miR-29b into metastatic prostate cancer cell line followed by in vitro and in vivo functional assays. Our results suggested that exogenous expression of miR-29b significantly attenuated the development of metastasis in an experimental prostate cancer metastatic mouse model.

A handful of miRNAs with pro-(miR-10b, miR-21, miR-125b, miR-220/-221, miR-141, and miR-373/520c) or antimetastatic (miR-34b/c, miR-126, miR-148a, miR-206, miR-101, miR-200 family, miR-330, miR-146a, miR-203, and miR-335) functions have been identified (13, 14, 21, 22). A single miRNA can regulate expression of several or multiple principal targets in a specific microenvironment. miR-29b expression was reported to be suppressed in different cancers including cholangiocarcinoma, acute myeloid leukemia, hepatocellular carcinoma, and myeloma (23–26). miR-143 and miR-145 inhibit the migration and invasion of prostate cancer cells and regulate the EMT by inducing E-cadherin (27). miR-34a, a p53 target, can inhibit prostate cancer stem cells and metastasis by targeting CD44 (28). miR-203 expression is specifically attenuated in bone metastatic prostate cancer cell lines and inhibits prostate cancer metastasis by targeting a class of prometastatic genes and decreasing N-cadherin (22, 29). The same miRNA may exhibit diverse functions, depending on the repertoire and stoichiometry of its direct mRNA targets (30).

Metastasis reflects the acquisition of multiple molecular traits by tumor cells, including the ability to counteract anoikis, migrate, invade, survive, and proliferate in unrelated microenvironments (31). We have previously shown that exogenous expression of miR-29b in prostate cancer cells inhibits Mcl-1 and MMP-2 protein expression (16). miR-29b exerts pleiotropic effects by affecting multiple steps of the metastatic cascade including invasion, motility, cellular survival, and proliferation (16, and this report) that contribute to the metastatic dissemination of prostate cancer cells. As loss of miR-29b expression is noted in prostate cancer, we believe that its effects on cellular proliferation and apoptosis may be relevant to the development of distant metastases. In the normal prostate gland, the basal cells inside the lumen attach to the basement membrane and form a cell layer. Normal epithelial cells above the basal cell layer are restricted to move by the adhesion to each other as well as to ECM. Cell to cell adhesion in normal epithelium is maintained by many different junctions, such as adherens
References

10. In summary, the findings of this study suggest that the loss of miR-29b can facilitate the completion of multiple distinct steps of the invasion metastasis cascade in prostate cancer (Fig 6). miR-29b was known as a tumor suppressor in different cancers, but we first investigated the regulation of miR-29b in prostate cancer metastasis. The study of the pleiotropic functions of miR-29b may help in further understanding prostate cancer metastasis. Distant metastases are responsible for patient mortality in the vast majority of human carcinomas. Therefore, including miR-29b seems to be promising for therapeutic modalities against its specific functions in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Writing, review, and/or revision of the manuscript: P. Ru, K. Toth, R.B. Ray
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