miR-200b suppresses cell proliferation, migration and enhances chemosensitivity in prostate cancer by regulating Bmi-1

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Abstract. microRNAs (miRNAs) are a class of small non-coding RNAs that can post-transcriptionally regulate gene expression and play critical roles in many important biological processes. The role of miRNAs in prostate cancer (PCa) development and pathogenesis remains largely unknown. In the present study, we showed that miR-200b was downregulated in clinical prostatic tumors when compared to normal prostate tissue and in advanced PCa cell lines when compared to normal epithelial prostatic cells. Enforced miR-200b expression suppressed PCa cell proliferation and migration and enhanced chemosensitivity to docetaxel by targeting B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1). Bmi-1 was detected at higher levels in PCa, and knockdown of Bmi-1 showed similar effects as miR-200b overexpression in PCa cells. Moreover, we confirmed that these effects were correlated with increased levels of E-cadherin and P16 and a reduction in vimentin expression and expression of stem cell markers (CD44 and OCT4). These findings suggest that miR-200b plays vital roles as a tumor-suppressor by targeting Bmi-1 and may be a promising therapeutic target for PCa treatment.

Introduction

Prostate cancer (PCa) is one of the most common lethal malignant diseases in males in the United States (1). Disease confined to the prostate is treatable, while metastatic PCa eventually leads to accelerated disease progression and mortality (2). Thus, to further improve patient survival, it is essential to further understand the molecular and cellular mechanisms of PCa development and to establish novel therapeutic strategies to target PCa progression and metastasis.
Moreover, we demonstrated that silencing of Bmi-1 revealed its function as an oncogene in PCa.

Materials and methods

Tissue specimens. Human PCa specimens were obtained from 30 patients who underwent radical prostatectomies at the Department of Urology, Shanghai First People's Hospital, School of Medicine, Shanghai Jiaotong University between March 2011 and December 2012. Fifteen benign prostatic hyperplasia (BPH) tissue samples used as the control were obtained by transurethral resection of the prostate (TURP). All of the samples were confirmed by pathological examination and stored in liquid nitrogen for miRNA analysis. Formalin-fixed, paraffin-embedded samples for immunohistochemistry from 60 PCa tissues and 30 BPH tissues obtained by radical prostatectomy and TURP, respectively, were from the Tissue Paraffin Block Bank of Pathology in our hospital. The Institutional Review Board of Shanghai First People's Hospital approved all experimental procedures, and patient consent was obtained before tissue collection.

Cell lines and cell culture. Human PCa cell lines LNCaP, PC3 and DU145 were obtained from the Shanghai Cell Bank, the Chinese Academy of Sciences, and the benign hyperplastic epithelial cell line BPH-1 was developed in our laboratory. All cell lines were maintained in RPMI-1640 medium (Gibco-BRL, Rockville, MD, USA) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal bovine serum (Gibco-BRL) in a humidified atmosphere at 37˚C in 5% CO2.

Real-time quantitative RT-PCR. Total RNA from PCa cell lines was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Real-time quantitative RT-PCR was carried out using the PrimeScript Reverse Transcription System and SYBR Premix Ex Taq™ II kit (Takara, Dalian, China) according to the manufacturer's instructions. The primer sequences used were as follows: Bmi-1 (forward primer, 5'-TGGACTGACAAATGCTGGAG-3' and reverse primer, 5'-GGCAAGACAGAAGGTGGAA-3'); internal control GAPDH (forward primer, 5'-TGGACAGTCA GCCGCATCTTTT-3' and reverse primer, 5'-ACCAATCG TTGACTCGAGGCT-3'). PCR conditions included denaturation at 94˚C for 2 min, followed by 40 cycles of 5 sec at 94˚C, 30 sec at 60˚C and 30 sec at 72˚C.

Expression of mature miR-200b was assayed using stem-loop RT followed by real-time PCR analysis. Bulge-Loop™ miRNA qRT-PCR Primer Set and miRNA qRT-PCR Control Primer Set (RiboBio, Guangdong, China) were used for quantitative real-time PCR analysis of miR-200b and U6 small nuclear RNA, respectively. PCR conditions included denaturation at 95˚C for 20 sec, followed by 40 cycles of 10 sec at 95˚C, 20 sec at 60˚C and 10 sec at 70˚C. U6 expression was used as an internal control for miR-200b expression. The relative expression fold change of miRNAs and miRNAs was calculated using the 2-ΔΔCt method (15). All experiments were performed in triplicates.

Transient transfections. PC3 and DU145 cells seeded at 1.5x10^5 cells/well in 6-well plates were transfected with miR-200b mimic and a scramble control (RiboBio) performed at a concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Transfection efficiency was confirmed by qRT-PCR. For the silencing experiments, PC3 and DU145 cells were transfected with small interfering RNAs (siRNAs) targeting Bmi-1 (siRNA-Bmi-1) and their negative controls (siRNA-NC) (RiboBio) using Lipofectamine 2000 reagent according to the manufacturer's instruction. The sequences of the siRNA were identical to those described in a previous study (16). Forty-eight hours after transfection, cell proliferation and migration were assayed.

Cell Counting Kit-8 (CCK-8) and EdU analysis. Cell proliferation was assessed using the CCK-8 assay (Dojindo, Kumamoto, Japan). The transfected PC3 and DU145 cells were plated with 100 µl culture medium in 96-well plates at a density of 3,000 cells/well. After 24, 48 and 72 h, 10 µl of CCK-8 reagent (5 mg/ml) was added to each well, and incubation was carried out at 37˚C for 2 h. Viable cells were evaluated by absorbance measurements at 450 nm. Each assay was performed in 6 replicates for 3 independent experiments. The impact of miR-200b on PC3 and DU145 cell proliferation was also assessed by the EdU cell proliferation assay kit, according to the manufacturer's instructions. The assays were performed as recommended by the manufacturer of the EdU detection kit Apollo 488 (RiboBio).

Migration assay. The migratory capacity of the PCa cells following miR-200b and siRNA-Bmi-1 or NC transfection was assessed using a Corning Transwell assay according to the manufacturer's protocol. A total of 0.5x10^5 PCa cells in 200 µl of serum-free medium were seeded into the upper chamber of the system. Lower chambers were filled with 0.75 ml of complete medium. After 24 h of incubation, the cells in the upper chamber were removed with a cotton swab, and the transmigrated cells were fixed in methanol and stained with crystal violet. Stained cells were counted by photographing 5 fields/membrane.

Chemosensitivity assay. Rates of sensitivity to drugs were determined by the cell proliferation reagent CCK-8. Forty-eight hours after transfection, cells were digested and plated in 96-well plates at a density of 3,000 cells/well. After an overnight incubation, the cells were treated with docetaxel (10 nM). After 72 h, 10 µl of CCK-8 reagent was added to each well and the plate was incubated at 37˚C for 2 h. Viable cells were evaluated by absorbance measurements at 450 nm. Each assay was performed in 6 replicates in 3 independent experiments.

Immunofluorescence staining. BPH-1, LNCaP, PC3 and DU145 cells were grown on glass coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and then blocked with 5% bovine serum albumin in phosphate-buffered saline (PBS). The coverslips were then exposed to the primary antibody, anti-Bmi-1, overnight at 4˚C, followed by secondary antibodies. Images were captured using a laser scanning confocal fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany).
Western blot analysis. Cells were washed twice with cold PBS and homogenized in ice-cold RIPA buffer (Beyotime, Jiangsu, China) containing phosphatase and protease inhibitors. Total protein was separated by denaturing SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto PVDF membranes. Membranes were incubated with the primary antibodies for rabbit anti-Bmi-1, rabbit anti-E-cadherin, vimentin, P16 and rabbit anti-GAPDH (Cell Signaling Technology, Inc., Beverly, MA, USA) at 4˚C overnight and subsequently with horseradish peroxidase-conjugated secondary antibody. Signals were visualized with an ECL chemiluminescence kit (Boster, Wuhan, China) and exposed to X-ray film.

Immunohistochemical staining of tissues. Immunohistochemistry was conducted on archived paraffin-embedded formalin-fixed tissues of the study patients. Bmi-1 immunohistochemistry was performed according to the avidin-biotin-peroxidase complex method, with primary antibodies specific against human Bmi-1 (Cell Signaling Technology, Inc.) used at a 1:400 dilution. The secondary antibody (Long Island Biotech, Shanghai, China) was applied at a dilution of 1:400. Bmi-1 immunoreactivity was assessed by the intensity of the positive reaction (no staining, 0; low staining, 1; medium staining, 2; strong staining, 3). Immunohistochemical evaluation was performed in a blinded manner by two independent pathologists.

Statistical analyses. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as means ± SD from at least 3 independent experiments. The two-tailed t-test and χ² tests were used to assess statistically significant differences. A P-value <0.05 was considered to indicate a statistically significant result.

Results

miR-200b is reduced in PCa when compared with normal prostate cells and tissues. We determined the expression levels of miR-200b in human prostate cell lines, which included prostate carcinoma cell lines (LNCaP, PC3 and DU145) and the benign hyperplastic epithelial cell line BPH-1 by real-time qPCR analysis. We found that miR-200b was expressed at significantly lower levels in the PC3 and DU145 cells when compared with that in the non-malignant BPH-1 cells, while there was no difference between the BPH-1 and LNCaP cells (Fig. 1A). We next investigated the clinical relevance of miR-200b. Its expression levels were analyzed by real-time qPCR in PCa tissue and BPH tissue samples. miR-200b expression was significantly downregulated in the cancer tissues when compared with that in the BPH tissue samples (P<0.05) (Fig. 1B).

miR-200b suppresses cell proliferation, migration and improves chemosensitivity to docetaxel. We performed gain-of-function studies using miR-200b mimics, and miRNA transfection efficiency was determined by real-time qRT-PCR. After PC3 and DU145 cells were transfection with 50 nM miR-200b mimics and negative control, the expression of miR-200b was significantly upregulated (Fig. 2A). The effects of ectopic expression of miR-200b on cell proliferation in PCa cell lines were examined by CCK-8 assay. Transfection with miR-200b mimics resulted in a significant decrease in cell growth of the PCa cell lines (P<0.05) (Fig. 2B). We further investigated the effect of miR-200b on cell proliferation using an EdU incorporation assay. Fewer EdU-positive cells were observed in the miR-200b mimic-transfected cells when compared to the NC-transfected cells (Fig. 2C). These data indicate that miR-200b has a vital role in reducing the growth of PCa cells.

A Transwell assay was introduced to investigate whether miR-200b regulates tumor migration. PC3 and DU145 cells were transfected with either miR-200b mimics or the negative control. PCa cells transfected with miR-200b exhibited a decrease in cell motility (Fig. 2D). Our results are consistent with a previous study that miR-200b inhibits EMT and cancer cell migration (17). Moreover, miR-200b-treated PC3 and DU145 cells showed higher chemosensitivity to 10 nM docetaxel.
docetaxel than the NC-treated cells (Fig. 2B), demonstrating that upregulation of miR-200b improved PC3 and DU145 cell chemosensitivity to docetaxel.

miR-200b inhibits Bmi-1 and increases E-cadherin expression in PCa cells. Real-time qRT-PCR demonstrated that Bmi-1 mRNA expression was decreased in PC3 and DU145 cells after transfection with miR-200b mimics when compared with the negative control (Fig. 3A). Furthermore, the protein expression level of Bmi-1 was also markedly reduced in the PCa cell lines after miR-200b transfection (Fig. 3B). These results indicate that Bmi-1 may be a target of miR-200b in PC3 and DU145 cells. Furthermore, enforced expression of miR-200b in PC3 and DU145 cells resulted in increased expression of E-cadherin and reduced the expression of vimentin when compared with the control cells (Fig. 3C).

Bmi-1 is efficiently inhibited by siRNA. To explore the biological functions of Bmi-1 in PC3 and DU145 cells, we transfected these two cell lines with siRNA-Bmi-1. The
efficiency of siRNA-Bmi-1 inhibition was measured by qRT-PCR and western blotting. The Bmi-1 mRNA (Fig. 4A) and protein (Fig. 4D) levels were downregulated in the PC3 and DU145 cells when compared with the level in the control cells. GAPDH was used as an internal control. *P<0.05. (B) Western blotting showed that the Bmi-1 protein level was decreased in the miR-200b-transfected PC3 and DU145 cells. (C) E-cadherin was increased while the vimentin protein level was decreased in the PC3 and DU145 cells following transfection. The results were normalized to GAPDH expression. Bmi-1, B-cell-specific Moloney murine leukemia virus insertion site 1.

miRNAs have recently been described as important players in human cancer and their role as therapeutic targets has been proposed. The expression of miRNAs was found to be markedly deregulated in prostate cancer (PCa), strongly suggesting that miRNAs are involved in the initiation and progression of this disease (20). Recent reports indicate that miR-200b is highly correlated with epithelial-mesenchymal transition (EMT) and is identified as a critical regulator of tumor invasion, metastasis and chemosensitivity (6,21). The miR-200 family was found to directly target the mRNA of the E-cadherin transcriptional repressors ZEB1 and ZEB2. Ectopic expression of miR-200 reduced cancer cell motility by upregulation of E-cadherin protein levels. Conversely, inhibition of miR-200 reduced E-cadherin expression, increased expression of vimentin and induced EMT (22). Moreover, recent studies have discovered that miR-200b and miR-1 inhibit EMT via a Slug-dependent mechanism and tumorigenesis via a Slug-independent mechanism (23). In the present study, we confirmed that miR-200b expression levels

Figure 3. Effects of miR-200b on the expression of Bmi-1 in PC3 and DU145 PCa cells. (A) The expression of Bmi-1 mRNA was evaluated by real-time qPCR after transfection of miR-200b for 48 h. The Bmi-1 mRNA expression level was reduced in the miR-200b-transfected PCa cells when compared with the level in the control cells. GAPDH was used as an internal control. *P<0.05. (B) Western blotting showed that the Bmi-1 protein level was decreased in the miR-200b-transfected PC3 and DU145 cells. (C) E-cadherin was increased while the vimentin protein level was decreased in the PC3 and DU145 cells following transfection. The results were normalized to GAPDH expression. Bmi-1, B-cell-specific Moloney murine leukemia virus insertion site 1.
were underexpressed in advanced prostate cancer cell lines and in PCa clinical specimens. Consistent with our results, miR-200b has frequently been found to be downregulated in cancers, including gastric (24), lung cancer (25), renal cell carcinoma (26) and bladder cancer (27), as well as PCa (28).

The downregulation of miR-200b in PCa indicates that it may play a vital role in PCa tumor development and progression as a potential tumor-suppressor.

In PCa cells, miR-200b has an impact on pathways governing cell proliferation, drug sensitivity and cell migration. We found...
that overexpression of miR-200b in PCa cells suppressed cell growth and cell migration. Moreover, PC3 and DU145 cells showed a higher sensitivity to 10 nM docetaxel when cells were treated with miR-200b mimics, when compared to the negative control. These findings demonstrate that miR-200b may have significant future clinical implications.

Figure 5. Expression of Bmi-1 in prostate cancer cell lines and patient tissue samples. (A) The expression of Bmi-1 protein levels in BPH-1, LNCaP, PC3 and DU145 cell lines. GAPDH was used as an internal control. (B) Immunofluorescence staining in human prostate adenocarcinoma cell lines LNCaP, PC3 and DU145 and human prostate epithelium cell line BPH-1 showed that Bmi-1 was overexpressed in different degrees in the prostate cancer cell lines, when compared with the BPH-1 cell line. (C) Different expression levels of Bmi-1 in normal prostate and prostate cancer tissues. a, Nonmalignant epithelia showing low staining; b, malignant cells showing strong staining. c, Scoring of the staining intensities with the anti-Bmi-1 antibody indicates that Bmi-1 protein expression was significantly increased in PCa when compared with the BPH tissue specimens (original magnification, x400). P<0.05 (\( \chi^2 \) tests). Bmi-1, B-cell-specific Moloney murine leukemia virus insertion site 1; BPH, benign prostatic hyperplasia.
In addition, we further explored the mechanism by which miR-200b affects proliferation, migration and sensitivity to docetaxel in PCa cells. We used target prediction databases (miRanda, Targetscan and PicTar) to search for genes regulated by miR-200b and found that miR-200b may target Bmi-1. Recent research has revealed that miR-200b functions as a tumor-suppressor gene and that Bmi-1 may be the target of function in miR-200b in human tongue cancer (29).

Previous studies have suggested that Bmi-1 acts as an oncogene and plays a crucial role in cell proliferation, transformation, EMT, self-renewal of stem cells and cancer initiation and chemoresistance in various human tumors (19,30,31). We discovered that Bmi-1 was overexpressed in PCa tumor tissues when compared with that in the BPH normal tissues. Bmi-1 overexpression was also observed in PCa cell lines when compared with the BPH-1 normal prostate epithelial cells. Consistent with our findings, previous publications also reported that Bmi-1 was overexpressed in PCa (32,33), and its overexpression was associated with a high Gleason score and increased risk of recurrence after prostatectomy (34). In the present study, we showed that rescue of miR-200b expression led to downregulation of Bmi-1 mRNA and protein levels in PCa cells. These results suggest that miR-200b may directly target the 3’-UTR of Bmi-1 mRNA and act as a potential tumor-suppressor in PCa.

We demonstrated that knockdown of Bmi-1 in PC3 and DU145 cells significantly reduced cell proliferation and upregulated the p16 tumor-suppressor. There is an enormous body of evidence suggesting that Bmi-1 regulates cell proliferation and senescence through suppressing p16 (35). Previous studies have discovered that targeting Bmi-1 improves the outcome of docetaxel therapy in animal models bearing chemoresistant prostatic tumors (32). Crea et al (36) identified that Bmi-1 silencing impairs antioxidant defense and sensitizes PCa cells to docetaxel. The present study also found that inhibition of Bmi-1 enhanced the antitumor activity of docetaxel in PCa cells. These results indicate that Bmi-1 may be exploited as a potential molecular target for therapeutics to treat chemoresistant tumors. Recent studies have demonstrated that many metastatic cancers undergo EMT resulting in enhanced cell motility and invasion (37). The hallmark of EMT is the loss of adherens protein E-cadherin and acquisition of mesenchymal markers such as vimentin (38). A recent study showed that Bmi-1 is regulated by Twist1 in HNSCC and is essential in EMT (39). Yet, the role of Bmi-1 in PCa cell migration has not been thoroughly explored. The present study is consistent with the literature reported. Following inhibition of the expression of Bmi-1, the migration ability of PCa cells was reduced. Here we also provided evidence that Bmi-1 is correlated with E-cadherin and vimentin protein expression in PCa cells.

In summary, our data suggest that Bmi-1 is expressed at a high level in PCa. miR-200b plays a pivotal role in PCa at least in part via downregulation of the oncogene Bmi-1 and may serve as a therapeutic target by which to inhibit PCa cell proliferation and migration and enhance chemosensitivity.

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References


