MiR-1266 suppresses the growth and metastasis of prostate cancer via targeting PRMT5

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Abstract. – OBJECTIVE: To elucidate the correlation between microRNA-1266 (miR-1266) and prostate cancer (PCa) progression, and to investigate the possible underlying mechanism.

PATIENTS AND METHODS: The expression level of miR-1266 and protein arginine methyltransferase 5 (PRMT5) in PCa tissues and cell lines was first detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). After up-regulating or down-regulating miR-1266 expression in cells, cell proliferation, migration and invasion abilities were detected. Possible target genes of miR-1266 were predicted and validated by bioinformatics analysis and dual-luciferase reporter gene assay, respectively. Finally, abnormal expression of PRMT5 was ascertained after transfection.

RESULTS: MiR-1266 was lowly expressed in PCa tissues and cell lines, whereas PRMT5 exhibited the opposite results. Up-regulated expression of miR-1266 significantly inhibited the proliferation, migration and invasion abilities of PC-3 cells. However, the growth and migration of DU145 cells with low miR-1266 expression were significantly accelerated. Meanwhile, the number of invading cells was significantly increased. PRMT5 was verified as a potential target gene of miR-1266. Furthermore, results found that miR-1266 was negatively correlated with PRMT5. In addition, the expression of PRMT5 was remarkably decreased after miR-1266 overexpression, which could be restored after knockdown of miR-1266.

CONCLUSIONS: MiR-1266 inhibits the growth and metastasis of PCa by targeting PRMT5. We may provide a potential and prospective therapeutic target for PCa.

Key Words: MiR-1266, Proliferation, Metastasis, PRMT5, PCa.

Introduction

Prostate cancer (PCa) remains one of the most common malignant tumors in men, with high mortality and morbidity1,2. The incidence of PCa has increased year by year, which is already higher than that of bladder cancer3. The five-year survival rate of patients with focal PCa is close to 100%. Most patients with advanced or recurrent PCa have developed resistance to current multiple therapies, eventually leading to failure of routine treatment and high mortality4,5. Therefore, finding reliable early diagnosis is essential to reduce the recurrence rate and mortality of PCa patients6. MicroRNAs (miRNAs) are a type of small non-coding RNAs with about 21-25 nucleotides in length. MiRNAs partially bind to complementary sequences in the 3′-untranslated region of mRNA (3′-UTR), and negatively affect post-transcriptional regulation7. Currently, Tutar et al8 has indicated that miRNAs are involved in the proliferation, differentiation and signal transmission of cells in a variety of cancers, which are also closely related to the prognosis of cancer patients. Recent investigations have found that miR-375 inhibits the growth and metastasis of renal clear cell carcinoma. MiR-224 promotes the proliferation and migration of pancreatic cancer cells9,10. Meanwhile, miRNAs also act as biomarkers to guide the prognosis of PCa11. For example, miR-218 inhibits epithelial-mesenchymal transition of PCa stem cells by degrading glioma-associated oncogene homolog 1 (GLI1). MiR-126 inhibits the proliferation and metastasis of prostate cancer cells by modulating a disintegrin and metalloprotease 9 (ADAM9)12,13. Previous works14,15 have demonstrated that miR-1266 can suppress the progression of gastric cancer and papillary thyroid cancer by slowing the rate of tumor growth and metastasis. Furthermore, miR-1266 promotes resistance to GEM and induces chemo-resistance by regulating various negative regulators of signal transducers and activators of
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transcription 3 (STAT3) and nuclear factor-kappa B (NF-kB) pathways in pancreatic cancer. At the same time, highly expressed miR-1266 may be an important cause of recurrence, metastasis and chemical resistance in ER (+) breast cancer patients. Therefore, it is of great significance to explore the role of miR-1266 in PCa. Protein arginine methyltransferase 5 (PRMT5) is a member of the PMRT family. It participates in the regulation of gene transcription and cellular signal transmission. Previously Huang et al. have indicated that PRMT5 enhances the transcription of STC1 to accelerate the metastasis of breast cancer. Meanwhile, PRMT5 promotes lung cancer metastasis by enhancing miR-99 family/FGFR3 axis. It also accelerates the proliferation of hepatocellular carcinoma (HCC) cells by down-regulating BTG2/ERK pathway. However, the biological mechanism of PRMT5 in PCa remains unclear. In this study, we first discovered that miR-1266 was lowly expressed in PCa patients and cell lines. MiR-1266 promoted the proliferation and migration of cancer cells in vitro. Results also indicated that PRMT5 was negatively correlated with miR-1266. Bioinformatics prediction and dual luciferase reporter gene assay demonstrated that PRMT5 was a downstream target of miR-1266. Hence, miR-1266 could be used as a potential and prospective therapeutic target for PCa.

Patients and Methods

Tissues
This work was approved by the Ethics Committee of Tongren Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). Signed written informed consents were obtained from all participants before the study. 36 pairs of matched PCa and normal prostate tissues were obtained from patients who underwent radical prostatectomy at Tongren Hospital, Shanghai Jiao Tong University School of Medicine from February 2018. Meanwhile, patients who received radiation or chemotherapy prior to surgical resection were excluded. PCa patients were staged according to the US Seventh Joint Cancer Joint Committee (AJCC) (Chicago, IL, USA) classification system. All tissues were conserved in nitrogen for subsequent use.

Cell Lines
Human PCa cell line DU145, PC-3, and normal human prostate epithelial cell line NHPE were provided by Shanghai Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin in a 37°C, 5% CO₂ incubator.

Cell Transfection
MiR-1266 mimics (5'-CCUCAGGGCGUAGAAGCGAGCU-3'), inhibitor (5'-AGCCCUUGUUCUAACGAGCCUGAGG-3') and negative controls (5'-CAG UACUUUUGUGUAAGACAA-3') were synthesized by GenePharma (Shanghai, China). These nucleotides were transiently transfected into cells (2 × 10⁵ cells/well) according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 36 hours of transfection, cells were harvested for subsequent experiments.

Total RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)
Total RNA was extracted from transfected cells or PCa tissues in strict accordance with TRIzol reagent (TaKaRa, Otsu, Shiga, Japan). Extracted RNA was then subjected to polyadenylation and reverse transcription. qRT-PCR was performed with SYBR Green Master Mix II (TaKaRa, Otsu, Shiga, Japan) on an ABI 7500 Real-time PCR system (ABI, Foster City, CA, USA). Primers used in this study were as follows: PRMT5: F, 5'-CTGACACACTAGGGGCTGTG-3' and R, 5'-ACTAGTCTGCTTCTCCGT-3'; GAPDH: F, 5'-AAGGGAAGGTTGCTGGATAGG-3' and R, 5'-CACATCCACCTCCTCCACATC-3'. Relative expression of genes was calculated by the 2⁻ΔΔt method. This experiment was repeated for three times in each group.

Cell Proliferation Assays
Cell proliferation assay was performed by using cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan). Transfected cells were first seeded into 96-well plates at a density of 1000 cells/well, and cultured in 100 µL of medium. 10 µL CCK-8 reagent were added to each well, followed by incubation for 2 h in dark. The absorbance at the wavelength of 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA). On the other hand, 1×10³ cells were seeded into 60 mm dishes for colony formation assay. After in-
Cubation for 12 days, cells were stained with crystal violet staining solution (Beyotime, Shanghai, China) for 10 minutes. Colonies containing ≥ 50 cells were counted. Each experiment was performed for three times.

**Cell Migration and Invasion Assays**

Wound healing assay was first chosen to perform. Artificial wounds were generated from cells at 48 hours after transfection using a pipette tip. To observe wound healing, images were captured randomly after 24 hours. Simultaneously, the effect of miR-1266 on cell invasion was also examined. A total of 1 × 10^5 cells were seeded into the upper chamber of the insert. Cells were cultured in serum-free medium, and medium supplemented with 10% FBS was added to the lower chamber as a chemoattractant. After that, the cells were incubated for 36 hours. Cells migrating through the matrix to the other side of the insert were stained with crystal violet staining solution. 10 fields were randomly selected for each well (magnification, 100×).

**Bioinformatics Analysis**

MicroRNA.org was chosen to predict the potential target genes of miR-1266.

**Dual Luciferase Reporter Gene Assay**

The target sequence or mutant sequence was inserted into the psiCHECK-2 luciferase reporter vector (Promega Corporation, Madison, WI, USA) to construct psiCHECK-2-PRMT5-WT-3′-untranslated region (UTR) or psiCHECK-2-PRMT5-MUT-3′-UTR. Briefly, DU145 and PC-3 cells were seeded into 24-well plates and transfected with psiCHECK-2-PRMT5-WT-3′-UTR or psiCHECK-2-PRMT5-MUT-3′-UTR. Lipofectamine 2000 and miR-1266 mimics/negative controls were transfected into cells after 24 hours. Luciferase activities were determined by Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

**Western Blot**

Total protein was extracted from PCa tissues or cell lines using radio-immunoprecipitation assay (RIPA, Beyotime, Shanghai, China). Protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Beyotime, Shanghai, China). After blocking in 5% fat-free milk at room temperature for 2 hours, the membranes were incubated with primary antibodies of anti-PRMT5 (1:1,000; ab109451; Abcam, Cambridge, MA, USA) and anti-GAPDH (1:1,000; ab37168; Abcam, Cambridge, MA, USA) at 4°C overnight. The membrane was then incubated with horse reddish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000) at room temperature for 2 hours next day. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as an internal control. Bound secondary antibodies were detected by enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL, USA).

**Statistical Analysis**

GraphPad software 7.0 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 19.0 were used for all statistical analysis (IBM, Armonk, NY, USA). Student’s t-test, Spearman’s test, and one-way ANOVA followed by post-hoc test (Least Significant Difference) was used to compare the differences among groups. p<0.05 was considered statistically significant.

**Results**

**MiR-1266 Was Down-Regulated in PCa**

As shown in Figure 1A, we first detected miR-1266 expression in 36 pairs of PCa tissues and matched normal tissues. Results showed that the expression of miR-1266 in PCa tissues was significantly lower than that of matched normal tissues (Figure 1B). Expression of miR-1266 was also detected in PCa cell lines and NPHE. As indicated in Figure 1C, miR-1266 expression in DU145 and PC-3 cells was also significantly lower than that of NPHE cells. Subsequently, the correlation between miR-1266 expression and clinic-pathological features of PCa patients was analyzed. As shown in Table I, miR-1266 expression was closely related to tumor stage, higher Gleason score, and lymph node status. However, no statistical difference was found between miR-1266 expression and age, as well as prostate specific antigen (PSA) levels. These findings revealed that miR-1266 might play a vital role in PCa.

**MiR-1266 Inhibited the Proliferation of PCa in vitro**

To explore the role of miR-1266 in PCa, we transfected miR-1266 mimics, inhibitor and negative controls into PCa cells to modulate the expression level of miR-1266. As illustrated in Fig-
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Figure 1. MiR-1266 expression was downregulated in PCa cell lines and tissues. A, QRT-PCR analysis of miR-1266 expression in 36 pairs of PCa tissues and matched normal prostate tissues. The expression of miR-1266 was normalized to U6 small nuclear RNA. B, The expression of miR-1266 in PCa tissues was significantly lower than that of adjacent tissues. C, Expression levels of miR-1266 in DU145 and PC-3 PCa cell lines compared with NHPE cells. The data were shown as mean ± SD (*p<0.05; **p<0.01).

Table I. Distribution of miR-1266 expression status in human prostate cancer according to clinicopathological characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients, n</th>
<th>miR-1266 expression, n</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td>Low</td>
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<tr>
<td>≥60</td>
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<td>10</td>
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<td>Tumor stage</td>
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PC-3 cells. However, lowly expressed miR-1266 obviously increased the proliferation of DU145 cells. In addition, miR-1266 up-regulation significantly reduced the number of colonies in PC-3 cells. However, after knocking down the expression of miR-1266, the number of colonies in DU145 cells was significantly increased (Figure 2C). Taken all together, these results re-

![Figure 2](image)

**Figure 2.** Upregulation of miR-1266 inhibited cell proliferation. A, Expression of miR-1266 after transfection. B, CCK-8 proliferation assay revealed that overexpression of miR-1266 significantly inhibited the growth rate of PC-3 cells. Conversely, transfection with miR-1266 inhibitor significantly promoted the proliferation of DU145 cells. C, Colony formation assay showed that overexpression of miR-1266 significantly inhibited the growth rate of PC-3 cells. MiR-1266 inhibitor transfection significantly promoted the proliferation of DU145 cells. The data were shown as mean ± SD (*p<0.05; **p<0.01).
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Transwell assay showed the same results with wound healing assay (Figure 3B). The results above suggested that miR-1266 inhibited the metastasis of PCa.

**MiR-1266 Inhibited the Migration and Invasion of PCa in vitro**

To further explore the role of miR-1266 in cell migration and invasion, wound healing assay and transwell assay were performed. As indicated in Figure 3A, overexpression of miR-1266 significantly inhibited the wound healing ability of PC-9 cells, whereas decreased expression of miR-1266 in DU145 cells resulted in the opposite effect. Regarding to the changes in cell invasion, transwell assay showed the same results with wound healing assay (Figure 3B). The results above suggested that miR-1266 inhibited the metastasis of PCa.

**PRMT5 was a Potential Target of miR-1266**

Bioinformatics analysis revealed that the 3'-UTR of PRMT5 contained a conserved putative target site for miR-1266 (Figure 4A). The effect of miR-1266 on PRMT5 was verified by dual luciferase reporter gene assay (Figure 4B). As illustrated in Figure 4C and 4D, the re-
sults of qRT-PCR showed that the mRNA expression level of PMRT5 in PCa tumor tissues and cell lines was remarkably higher than that of matched normal prostate tissues and NHPE cells, respectively. More importantly, the level of PMRT5 was negatively correlated with miR-1266 in 36-paired PCa tissues, with a R²-value of 0.339 (Figure 4E). Besides, we also detected the protein expression levels of PMRT5 in transfected PC-3 and DU145 cells. Results indicated that the expression level of PMRT5 in PC-3 cells transfected with miR-1266 mimics was significantly lower than that of the NC group. However, the expression level of PMRT5 in DU145 cells transfected with miR-1266 inhibitor was significantly reversed (Figure 4F and 4G). The above findings indicated that PMRT5 might be a potential target gene for miR-1266.

**Discussion**

PCa remains the second leading cause of cancer-related mortality in men. Further researches on the molecular mechanism of PCa tumorigenesis and progression, as well as continued

Figure 4. PRMT5 was a potential target of miR-1266. A, A computational algorithm revealed that the putative miR-1266-binding sequence was in the 3’ UTR of PRMT5. B, Relative luciferase activity of the WT group and the Mut group. C, QRT-PCR analysis of mRNA expression of PRMT5 in PCa tissues and matched normal prostate tissues. D, QRT-PCR analysis of mRNA expression of PRMT5 in PCa cells and NHPE. E, Correlation between miR-1266 and PRMT5 expression in 36 PCa tissues. F, Expression of PRMT5 mRNA in PCa cells after transfection. G, Western blot analysis of PRMT5 protein expression in PCa cells after transfection. The data were shown as mean ± SD (*p<0.05; **p<0.01).
development of targeted therapy are the current tendency for PCa. MiRNAs have been found to exhibit important effects on the progression of cancer. Meanwhile, they are involved in various cellular processes, including proliferation, differentiation, apoptosis, metastasis, stem cell maintenance and metabolism. We first identified miR-1266 as an abnormal expressed miRNA in PCa. Results showed that the expression of miR-1266 in PCa tissues was significantly higher than that of matched normal tissues. Subsequently, this phenomenon was reconfirmed in PCA cell lines and NHPE cells. The effect of miR-1266 on the growth and metastasis of PCa were elucidated in vitro. Overexpression of miR-1266 significantly inhibited the proliferation, migration and invasion of PC-3 cells. After knocking down miR-1266, the proliferation and migration abilities of DU145 cells were significantly accelerated. Meanwhile, the number of invading cells was increased. It was originally hypothesized that miR-1266 could bind to the 3'-UTR of PRMT5 mRNA by using microRNA.org. Wang et al. have demonstrated that PRMT5 is involved in gene transcription, signal transduction, and DNA repair. AS1411 alters the subcellular localization of PRMT5-nucleolar complex cells, which also has an effect on cell proliferation in prostate cancer. Overexpression of PRMT5 is an important factor in tumorigenesis, and is occurred in a large number of cancers, including lung cancer27, glioblastoma28, melanoma and bladder cancer. In this study, we used a vector containing two alleles to assess luciferase activity. Results demonstrated that miR-1266 directly targeted 3'-UTR of PRMT5. Compared with the Mut group, luciferase activity of the WT group was significantly decreased. This indicated that miR-1266 inhibited the mRNA expression of PRMT5 by directly targeting its 3'-UTR. At the same time, PRMT5 was highly expressed in both PCa tissues and cell lines. Moreover, there was a negative correlation between the expression of PRMT5 and miR-1266. After overexpression of miR-1266, the mRNA and protein levels of PRMT5 were remarkably reduced. Thus, miR-1266 inhibited the protein expression of endogenous PRMT5 by modulating the stability of PRMT5 mRNA. Considering the results of this work, it was proposed that miR-1266 negatively inhibited cell proliferation, migration and invasion by regulating PRMT5. However, our findings did not rule out the possibility that other signaling pathways might also be affected by miR-1266. In addition to the PRMT5 pathway, there might be other mechanisms that regulated tumor growth and metastasis. Therefore, whether miR-1266 could predict the prognosis of PCa patients still needed further studies.

Conclusions
We showed that miR-1266 acts as a tumor suppressor in PCa. MiR-1266 inhibits the proliferation, migration and invasion of PCa cells by targeting PRMT5. Although no further studies have identified other direct targets downstream of miR-1266, this preliminary data suggests that the recovery of miR-1266 may be a promising therapeutic option for PCa.

Conflict of Interest
The Authors declare that they have no conflict of interest.

References


