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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 PCa 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 showed that miR-1266 was negatively correlated with PRMT5. In addition, the expression of PRMT5 was remarkably increased 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 morbidity[1,2]. The incidence of PCa has increased year by year, which is already higher than that of bladder cancer[3]. The five-year survival of patients with local PCa is close to 100%. Most patients with advanced or recurrent PCa have developed resistance to current multiple therapies, eventually leading to failure of radical treatment and high mortality[4,5]. Therefore, reliable early diagnosis is essential to reduce the recurrence rate and mortality of PCa patients[6]. 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 regulation[7]. Currently, Tutar et al[8] hasindicated 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 cells[9,10]. Meanwhile, miRNAs also act as biomarkers to guide the prognosis of PCa[11]. 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 works[14,15] 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-κB) pathways in pancreatic cancer[16]. At the same time, highly expressed miR-1266 may be an important cause of recurrence, metastasis and chemical resistance in ER (+) breast cancer patients[17]. 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[18]. Previously Huang et al[19] 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[20]. It also accelerates the proliferation of hepatocellular carcinoma (HCC) cells by down-regulating BTG2/ERK pathway[21]. 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 luciferase reporter gene assay demonstrated that PRMT5 was a downstream target of miR-1266. Hence, miR-1266 could be used as a potential 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 cancer and normal prostate tissues were obtained from patients who underwent radical prostatectomy at Tongren Hospital, Shanghai Jiao Tong University School of Medicine from February 2016 to February 2017, while patients who received radiation or chemotherapy prior to surgical resection were excluded. All patients were staged according to the US seventh Joint Cancer Joint Committee (AJCC) (Chicago, IL, USA) classification system. 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 at 37°C, 5% CO_2 incubator.

Cell Transfection

MiR-1266 mimics (5'-ACCUCCCUGUGGCUGUAGAACAGGGCU-3'), inhibitor (5'-AGCCCUGUUCUACAGCCACUGAGGGU) and negative controls (5'-UUCUACUCCUCUGUAGUACAA-3') were synthesized by GenePharma (Shanghai, China). These nucleotides were transiently transfected into cells (2 × 10^5 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 and 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'-ACTAGTCTGCCCTTCTCCGT-3'; GAPDH: F, 5'-AAGGGAAGGTTGCTGGATAGG-3' and R, 5'-CACATCCACCTCCTCCACATC-3'. Relative expression of genes was calculated by the $2^{-\Delta\Delta t}$ 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, 1×10^3 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 (http://www.micror[illegible]) was chosen to predict the potential target[illegible]es of miR-1266.

Dual Luciferase Reporter Ge[illegible] Assay

The target sequence or m[illegible]nce was inserted into the psiCHEC[illegible] lucifer[illegible] reporter vector (Promega Cor[illegible]on, M[illegible] WI, USA) to construct psiC[illegible]K-2[illegible] 3'-untranslated regi[illegible]UTR[illegible]iCHECK-2-PRMT5-MUT-3'-U[illegible] Briefly, [illegible]5 and PC-3 cells were seede[illegible] 24-well pla[illegible] transfected with ps[illegible]EC[illegible]PRMT5-WT-[illegible]-UTR or psiCHECK-2-[illegible]MT5-M[illegible]'-UTR. Lipofectamine 20[illegible]nd miR-1266 [illegible]s/negative controls w[illegible]ransfected into ce[illegible] after 24 hours. Luci[illegible]e acti[illegible]s were determined by Dual-[illegible]rase [illegible]orter [illegible]ssay System (Promega Corp[illegible]adison[illegible], USA).

[illegible]ern B[illegible]

T[illegible] protein[illegible]s extracted from PCa tissues or [illegible] using radio-immunoprecipitation as-s[illegible]otime, Shanghai, China). Protein [illegible]ples were separated by 10% sodium dodecyl [illegible]hate-polyacrylamide gel electrophoresis (S[illegible]AGE) and transferred onto polyvinylidene diflu[illegible]ide (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; Abca[illegible] bridge, MA, USA) and anti-GAPD[illegible] ab37168; Abcam, Cambridge, MA[illegible]A) at 4°C overnight. The membrane was[illegible] incubated with horse reddish peroxidase (H[illegible]njugated anti-rabbit IgG (1:2000) at r[illegible] tempe[illegible] for 2 hours next day. Glyceral[illegible]de 3-phosp[illegible] hydrogenase (GAPDH)[illegible]s taken[illegible]s an in[illegible] control. Bound secon[illegible] antib[illegible]s were detected by enhanced che[illegible]in[illegible]nce (E[illegible] system (Pierce Biot[illegible]olog[illegible]kford, [illegible]USA).

Statistical [illegible]sis

GraphP[illegible]o[illegible]7.0 (La Jolla, CA, USA) and Statistical [illegible]roduct [illegible]ervice Solutions (SPSS) 19.0 w[illegible]used for a[illegible]tical analysis (IBM, A[illegible]Y, USA). Stu[illegible]s *t*-test, Spearman's t[illegible]and one-way ANOVA followed by post-hoc [illegible](Least Sig[illegible]ant Difference) was used to [illegible]pare the dif[illegible]nces among groups. $p<0.05$ w[illegible]nsidered [illegible]stically significant.

Results

Mi[illegible]66 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 NHPE 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-

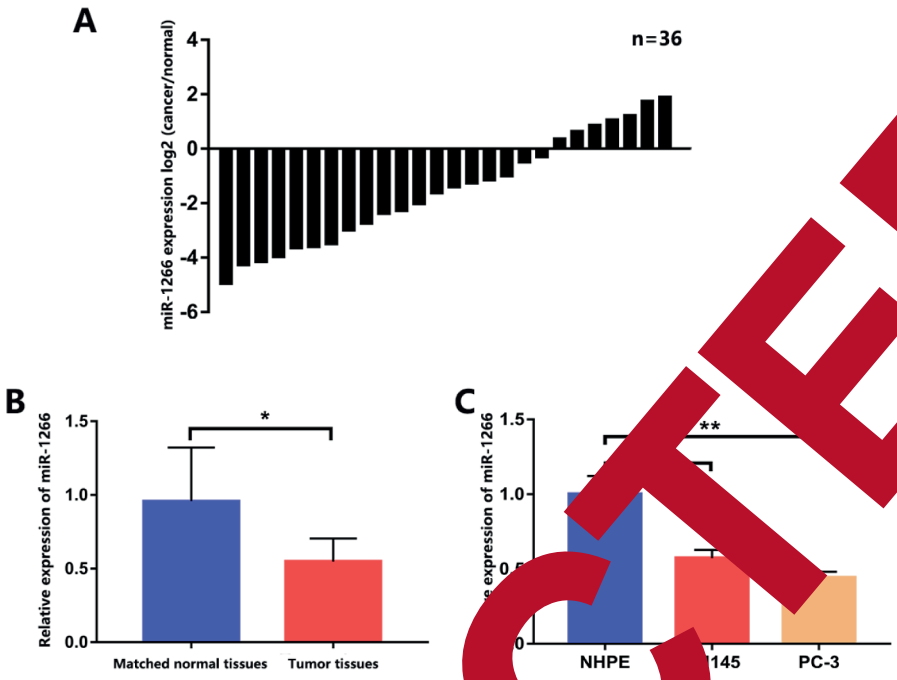

Figure 1. MiR-1266 expression was downregulated in PCa cell [illegible] and tissues. [illegible]RT-PCR analysis of miR-1266 expression in 36 pairs of PCa tissues and matched normal prostate tiss[illegible] of miR-1266 was normalized to U6 small nuclear RNA. ***B,*** The expression of miR-1266 in PCa tissues was [illegible] lower than that of adjacent tissues. ***C,*** Expression levels of miR-1266 in DU145 and PC-3 [illegible] lines compared with NHPE cells. The data were shown as mean ± SD (*p<0.05; **p<0.01).

ure 2A, the expression level of mi[illegible]266 in PC[illegible] 1266 inhibitor was significantly lower than that cells transfected with miR[illegible]ics was of the NC group. Subsequently, cell proliferation remarkably higher than th[illegible] transf[illegible]ed with rate was detected in transfected cell lines. As negative controls. In con[illegible] miR-1[illegible] observed in Figure 2B, overexpression of miR-sion level in DU145 c[illegible]lls [illegible]fec[illegible] 1266 significantly inhibited the proliferation of

Table I. Distributio[illegible]-1266 expressi[illegible]s in human prostate cancer according to clinicopathological characteristics.

Charact[illegible]cs	Pati[illegible]	miR-1266 expression, n		
		Low	High	*p*-value
Age				
≥[illegible]	22	10	12	NS
<[illegible]	14	7	7	
Tumor				
[illegible]2a-c	17	5	12	0.045
[illegible]4	19	13	6	
Gr[illegible] score				
≥[illegible]	15	9	6	0.030
	21	4	17	
[illegible]al st[illegible]				
[illegible]N0	20	14	6	0.047
[illegible]1	16	5	11	
[illegible]vels, ng/mL				
≥[illegible]	13	8	5	NS
<10	23	13	10	

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 i[illegible] (Figure 2C). Taken all together, thes[illegible]

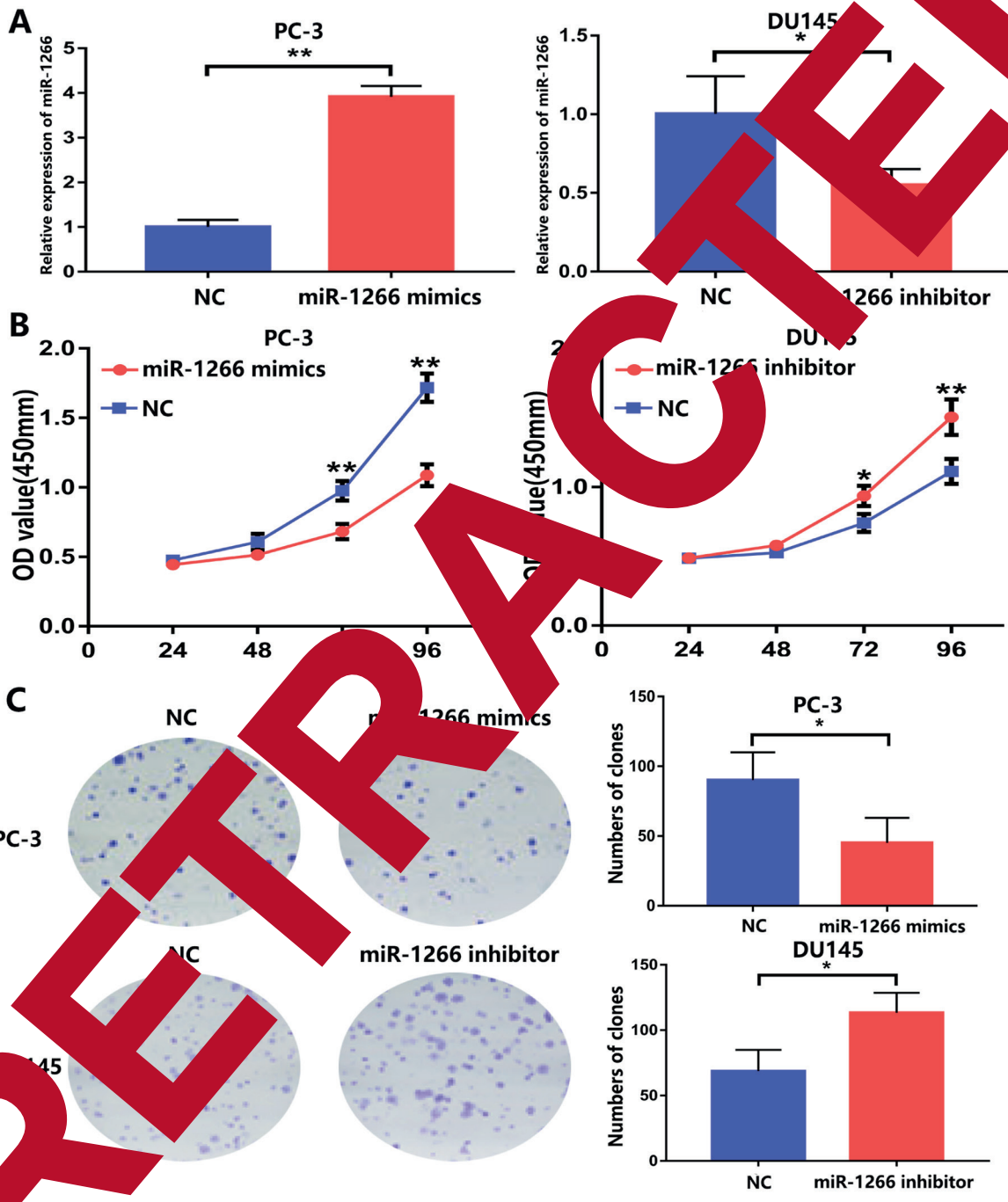

Figure 2. Upregulation of miR-1266 inhibited cell proliferation. ***A,*** Expression of miR-1266 after transfection. ***B,*** CCK-8 [illegible]ation assay revealed that overexpression of miR-1266 significantly inhibited the growth rate of PC-3 cells. Conversely, [illegible]ection with miR-1266 inhibitor 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).

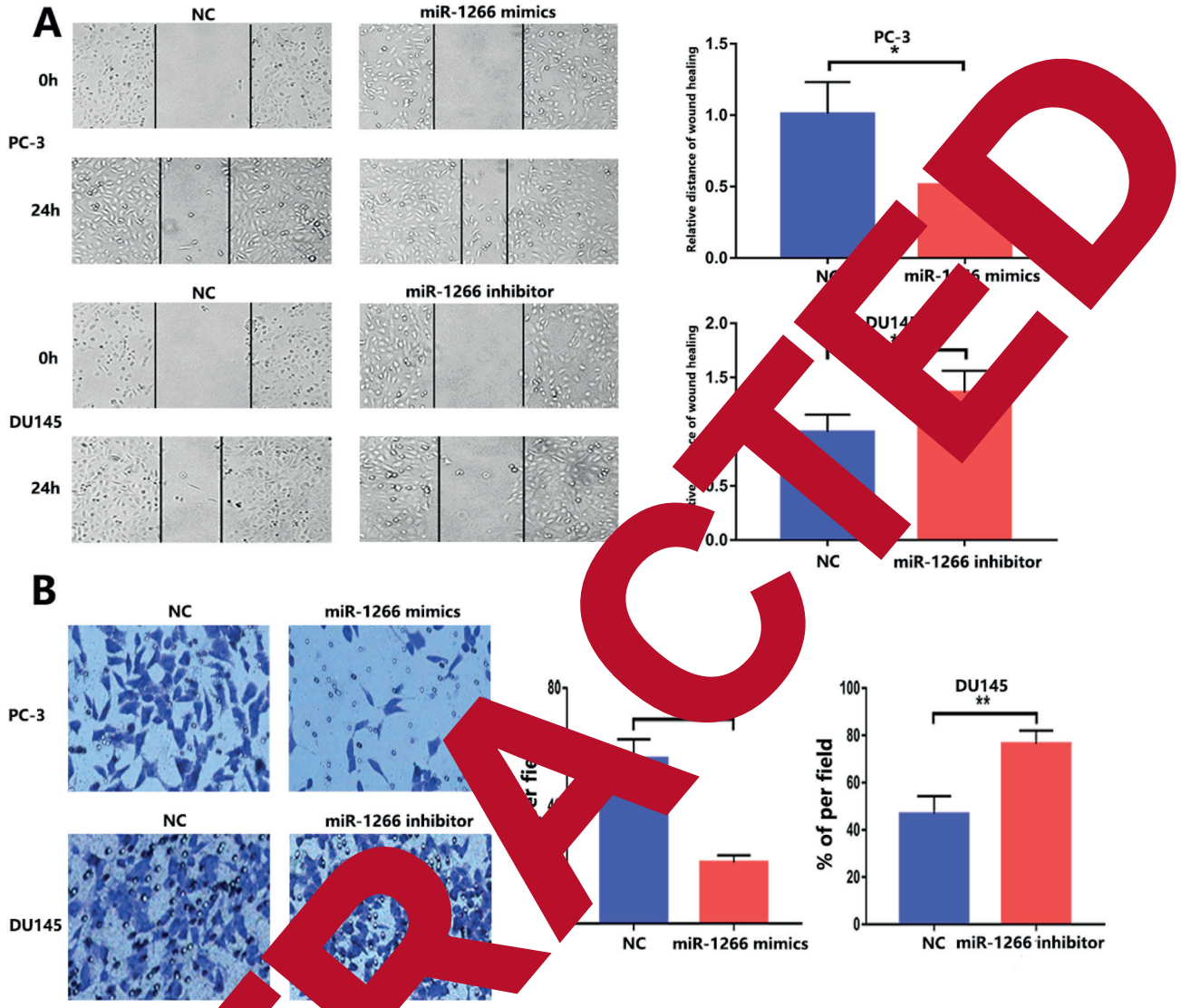

Figure 3. MiR-1266 i ted cell mig nd invasion. ***A,*** MiR-1266 promoted cell migration *in vitro*. ***B,*** MiR-1266 promoted cell invasion . All images w ired at 100× magnification. The data were shown as mean ± SD (**p*<0.05; ***p*<0.01).

veal at mi 6 inhibited the proliferation ab f PC s.

iR-12 hibit he Migration nvas a in vitro

T rther ex e the role of miR-1266 in cell mi and invasion, wound healing assay a assay were performed. As indi- ed in Figure 3A, overexpression of miR-1266 ficantly inhibited the wound healing ability o cells, whereas decreased expression of miR 266 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 PRMT5 was negatively correlated with miR-1266 in 36-paired PCa tissues, with a R^2-value of 0.339 (Figure 4E). Besides, we also detected the protein expression levels of PRMT5 in transfected PC-3 and DU145 cells. Results indicated that the expression level of PRMT5 in PC-3 cells transfected with miR-1266 mimics was significantly lower than that of the NC group. However, the expression level of PRMT5 in DU145 cells transfected with miR-1266 inhibitor was significantly reversed (Figure 4F and 4[illegible] above findings indicated that PRMT[illegible] a potential target gene for miR-12[illegible]

Discussi[illegible]

PCa remains the sec[illegible] leadin[illegible] cause of cer-related mortality [illegible]men[22]. [illegible]er research-es on the molecular [illegible]ni[illegible]of PCa [illegible]nori-genesis and pro[illegible]sio[illegible]ell as [illegible]tinued

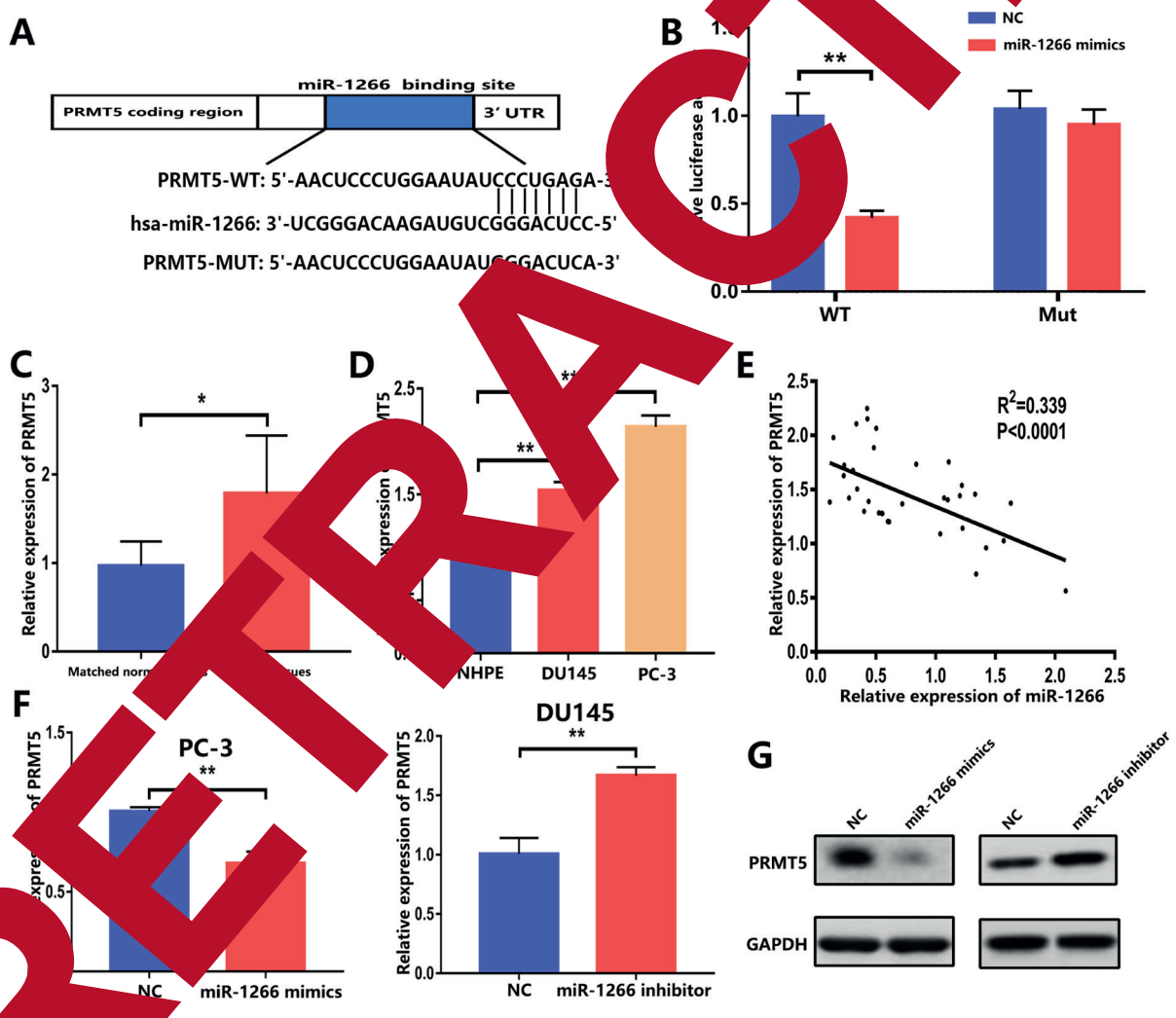

Figure 4. PRMT5 was a potential target of miR-1266. ***A,*** A computational algorithm revealed that the putative miR-1266-binding sequence was in the 3' UTR of PRMT5. ***B,*** Relative luciferase activity of the WT group and the Mut group. ***C,*** QRT-PCR analysis of mRNA expression of PRMT5 in PCa tissues and matched normal prostate tissues. ***D,*** QRT-PCR analysis of miR-1266 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[23,24]. 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[25] have demonstrated that PRMT5 is involved in gene transcription, signal transduction, and DNA repair. AS1411 ... the subcellular localization of t PRMT5 ... lar complex cells, which also has an effect ... ell proliferation in prostate cancer[26]. Overexpre... of PRMT5 is an important factor in tumorige... sis, and is occurred in a large nu... of cancer... including lung cancer[27], glio... melano-ma[29] and bladder cancer[30]. ... is stu... ve used a vector containing two ... s to as... ifer-ase activity. Results de... ed ... directly targeted 3'-... of ... Compared with the Mut grou... iferase ac... of the WT group was signi... decreased. ... indicated that miR-12... nh... the mRNA expression of PRMT5 by ... irectly ... ing its 3'-UTR. At the same ... e, PRMT5 wa... ly expressed in both P... ssues and cell line... Moreover, there was ... gative c... lation between the expression of ... T5 a... iR-12... After overexpression of mi... ne mR... and protein levels of PRMT5 ... rema... ly reduced. Thus, miR-... nhibit... otein expression of endog-eno... PRMT5 ... modulating the stability of PR... mRNA. Considering the results of this ... roposed that miR-1266 negatively ...hibited cell proliferation, migration and inva... by regulating PRMT5. However, our findings d... clude out the possibility that other signaling path...ays 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 pati... ll needed further studies.

Conclusions

We showed that miR... acts as ... suppressor in PCa. Mi...66 inh...its the p... eration, migration a... vasion ... PCa cells by targeting PRMT5. A... h... further ...udies have identified o... dire... ets dow... eam of miR-1266, thi... liminary ... s that the recovery of ...266 may be ... sing thera-peutic opt...

Co... Interest

T... uthors declare that they have no conflict of interest.

...eferences

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