

miR-486-5p suppresses prostate cancer metastasis by targeting Snail and regulating epithelial–mesenchymal transition

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Abstract: The most common cause of death from prostate cancer (PCa) is metastases. There is an increasing body of evidence that microRNAs play an important role in the development of PCa by regulating target genes involved in tumor metastasis. Here, we identified that expression of miR-486-5p was decreased in metastatic C4-2 cells compared to non-metastatic LNCaP cells. Further validation in clinical samples showed that miR-486-5p expression was significantly decreased in metastatic PCa tissues compared to localized PCa tissues. Functional studies demonstrated that increased miR-486-5p expression can suppress cell migration and the invasive ability of C4-2 cells. Moreover, Snail, a key regulator of the epithelial–mesenchymal transition, was verified as a target gene of miR-486-5p. In conclusion, these findings suggest that miR-486-5p plays a suppressive role in mediating the migration and invasion of PCa by directly suppressing the protein expression of Snail and may provide a potential therapeutic target for the disease.

Keywords: microRNA, prostate cancer, metastasis, EMT

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-related deaths among North American males.¹ Most deaths from this disease are related to metastases. However, the mechanisms underlying PCa metastasis are not fully understood. Further understanding of the machinery driving this process is urgently needed to improve PCa treatment and patient survival rates.

Metastasis is a multi-step process of interrelated events, which is thought to be driven by changes in the gene expression of cancer cells and the interaction between cancer cell and its microenvironment.² In view of this, it is likely that genes and their upstream regulators, found to be differentially expressed in closely related metastatic and non-metastatic PCa, may play a critical role in metastasis. Such genes and/or their regulators may provide new targets for the therapy of metastatic disease.

MicroRNAs (miRNAs) are a class of single-stranded noncoding RNAs, which act as posttranscriptional regulators of gene expression.³ Many studies have demonstrated that some miRNAs may play important roles in cancer cell invasion, migration, and metastasis by regulating the expression of their target genes.⁴ Reduced miR-486-5p expression has been observed in multiple types of tumors, such as gastric cancer, lung cancer, colorectal carcinoma, and glioblastoma.^{5–11} In these cancers, it may play a suppressive role in tumor progression by regulating tumor proliferation and metastasis. However, miR-486-5p was observed to be upregulated and can enhance tumor growth

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and survival of some malignancies, such as renal cancer and chronic myeloid leukemia, indicating a context-dependent role of it in cancer progression.^{12,13} To date, the function of miR-486-5p in PCa, especially its role in PCa metastasis, is not clear. In this study, we tested the expression of miR-486-5p in non-metastatic and metastatic PCa cell lines and clinical samples, investigated the roles of miR-486-5p in the regulation of PCa cell migration and invasion, and explored the underlying mechanisms regulated by miR-486-5p.

Materials and methods

Clinical samples

All patients provided written informed consent and a total of 28 fresh PCa specimens were collected from Tianjin Third Central Hospital and Tianjin Institute of Urology. In all, 17 primary PCa tissues were obtained from radical prostatectomy. Lymph node metastasis tissues were obtained from 11 patients with PCa. Following surgical resection, samples were immediately frozen in liquid nitrogen. This study was approved by the research ethics committee of Tianjin Third Central Hospital and Tianjin Institute of Urology.

Cell culture

LNCAp and C4-2 cells were cultured in a Roswell Park Memorial Institute-1640 medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) at 37°C under a humidified atmosphere containing 5% CO₂.

Cell transfection

C4-2 cells were transfected with miR-486-5p mimics or scramble (Sangon Biotech Co., Ltd., Shanghai, People's Republic of China) for 48 hours by Lipofectamine 2000 (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

RNA extraction and real-time PCR analysis

The total RNA from cultured cells and clinical PCa tissues were extracted using TRIzol reagent (Thermo Fisher Scientific). miRNA-specific complementary DNA was synthesized using a TaqMan MicroRNA Reverse Transcription Kit and miRNA-specific RT primers from a TaqMan MicroRNA Assay (Thermo Fisher Scientific). The miR-486-5p expression was measured by TaqMan miRNA Assays according to the manufacturer's instructions (Thermo Fisher Scientific). The expression of miR-486-5p was normalized to U6 small nuclear RNA.

Western blot

The total protein from the cultured cells was prepared using radioimmunoprecipitation assay lysis buffer. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). For immunoblotting, the following antibodies were used: anti-E-cadherin antibody (Abcam, Cambridge, UK), anti-N-cadherin antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-Snail antibody (Santa Cruz Biotechnology Inc.), and anti-actin (Sigma-Aldrich Co., St Louis, MO, USA). Signals were detected by Super Signal West Pico Chemiluminescent Substrate Kit (Pierce).

Cell migration assay

C4-2 cells were plated onto six-well plates. Once the cells reached confluence, the cells were rinsed with prostate-specific antigen (PSA). Straight scratches were created using P200 pipette tips. Cells were incubated in a 0.1% fetal bovine serum medium afterward. Images of wounds were taken immediately and after 24 hours of incubation. Wound closure was quantified by measuring the areas covered by cells.

Invasion assay

Modified Boyden chambers (BD Biosciences, San Jose, CA, USA) were used according to the manufacturer's manual for the tumor cell invasion assay. In brief, C4-2 cells were seeded to the upper part of the chambers. In all, 5% fetal bovine serum was added to the lower chambers. The cells were incubated at 37°C for 22 hours. The cells that were attached to the upper surface of the chamber were wiped off using a cotton swab. The cells on the lower surface were fixed with paraformaldehyde and stained with crystal violet. The cells were counted under a light microscope.

Luciferase reporter gene assay

The 3'-untranslated region (UTR) sequence of SNAI1 predicted to interact with miR-486-5p was synthesized and cloned downstream of the Renilla luciferase gene in psiCHECK-2 vector (Promega, Madison, WI, USA). Plasmid DNAs were co-transfected into C4-2 cells with miR-486-5p mimics or scramble sequence. Luciferase activities were measured 48 hours post-transfection using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Renilla luciferase activities were normalized to the internal control firefly luciferase activity.

Statistical analysis

Data were presented as mean ± SD. Statistical significance was established using the Student's *t*-test. Statistical analyses

were performed using SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at 0.05 to indicate statistical significance ($P < 0.05$).

Results

Expression of miR-486-5p was downregulated in the metastatic PCa cell line and clinical metastatic PCa tissues

At first, miR-486-5p expression was examined in a non-metastatic LNCaP cell line and its metastatic derivative cell line C4-2. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) results demonstrated that the metastatic C-4 cell line showed significantly decreased expression of miR-486-5p than the non-metastatic LNCaP cells ($P < 0.01$), which indicates an association of miR-486-5p expression with PCa metastasis (Figure 1A). To validate this association, the expression of miR-486-5p was further examined in 28 fresh PCa specimens, including 17 primary PCa cases and 11 metastatic PCa cases. The qRT-PCR results showed that the expression of miR-486-5p in metastatic PCa tissues was significantly lower than that in localized PCa tissues ($P < 0.01$), which is consistent with the findings in cell line models (Figure 1B).

miR-486-5p suppresses PCa cell migration and invasion

To study the function of miR-486-5p in PCa, the mimics of miR-486-5p and scramble sequence (negative control) were transfected into C4-2 cells, which were known to express relatively low levels of miR-486-5p. qRT-PCR confirmed the overexpression of miR-486-5p in C4-2 cells transfected with miR-486-5p mimics (Figure 2A). The wound healing assay

and Matrigel (Boyden chamber) invasive assay showed that increased miR-486-5p expression significantly suppressed C4-2 cell migration and invasion (Figure 2B and C).

miR-486-5p targets *SNAIL* and inhibits epithelial–mesenchymal transition

To study the mechanisms underlying miR-486-5p-regulated PCa metastasis, a number of potential target genes of miR-486-5p were identified using TargetScan Release 7.0 (Figure 3A). Further validation of the protein expression of these candidates in LNCaP and C4-2 using Western blot demonstrated that Snail, a protein encoded by the *SNAIL* gene, showed increased expression in C4-2 cells compared to LNCaP cells (Figure 3B). In C4-2 cells transfected with miR-486-5p mimics, the expression of Snail was decreased compared to that transfected with scramble sequence. This result suggested that *SNAIL* is likely a target gene of miR-486-5p in PCa. Moreover, the expression of the epithelial marker E-cadherin was increased and the expression of N-cadherin was decreased in the miR-486-5p-overexpressed C4-2 cells compared to the negative control cells (Figure 3C). The direct regulation of miR-486-5p was further validated using a luciferase reporter assay by cloning the *SNAIL* 3'-UTR sequence into a psiCHECK-2 vector. We observed that the miR-486-5p-overexpressed C4-2 cells showed an evidently suppressed luciferase activity compared to the negative control cells (Figure 3D). Furthermore, a significant negative correlation between miR-486-5p and Snail expression was observed in the clinical samples ($r = -0.56$ and $P < 0.01$). These results indicated that the overexpression of miR-486-5p may reverse epithelial–mesenchymal transition (EMT) by suppressing Snail expression.

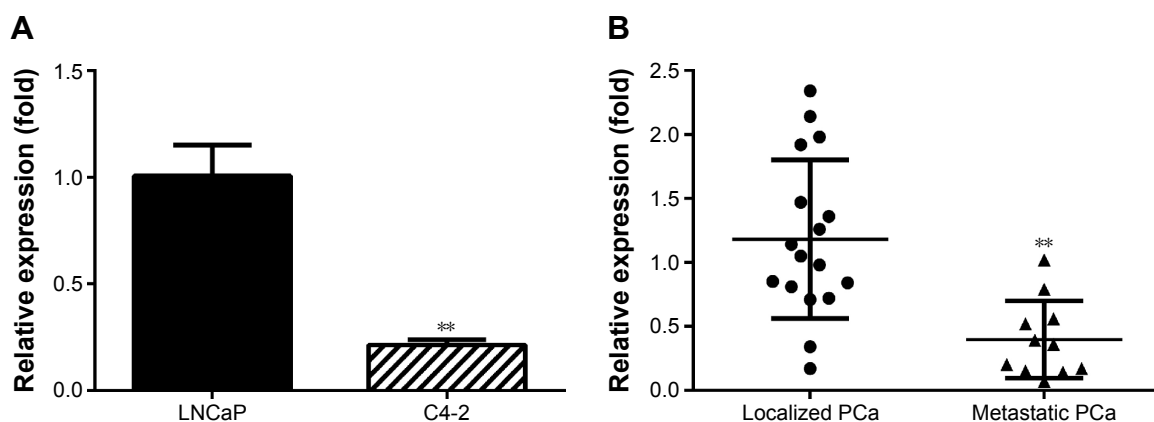


Figure 1 Expression of miR-486-5p in prostate cancer cell lines and clinical samples.

Notes: (A) The relative expression of miR-486-5p in the metastatic PCa cell line C4-2 is much lower than that in the non-metastatic LNCaP cell line. (B) The expression of miR-486-5p in clinical metastatic PCa specimens is significantly decreased compared to that in primary PCa tissues (** $P < 0.01$). Each symbol represents the relative expression of each localized (●) or metastatic prostate cancer sample (▲).

Abbreviation: PCa, prostate cancer.

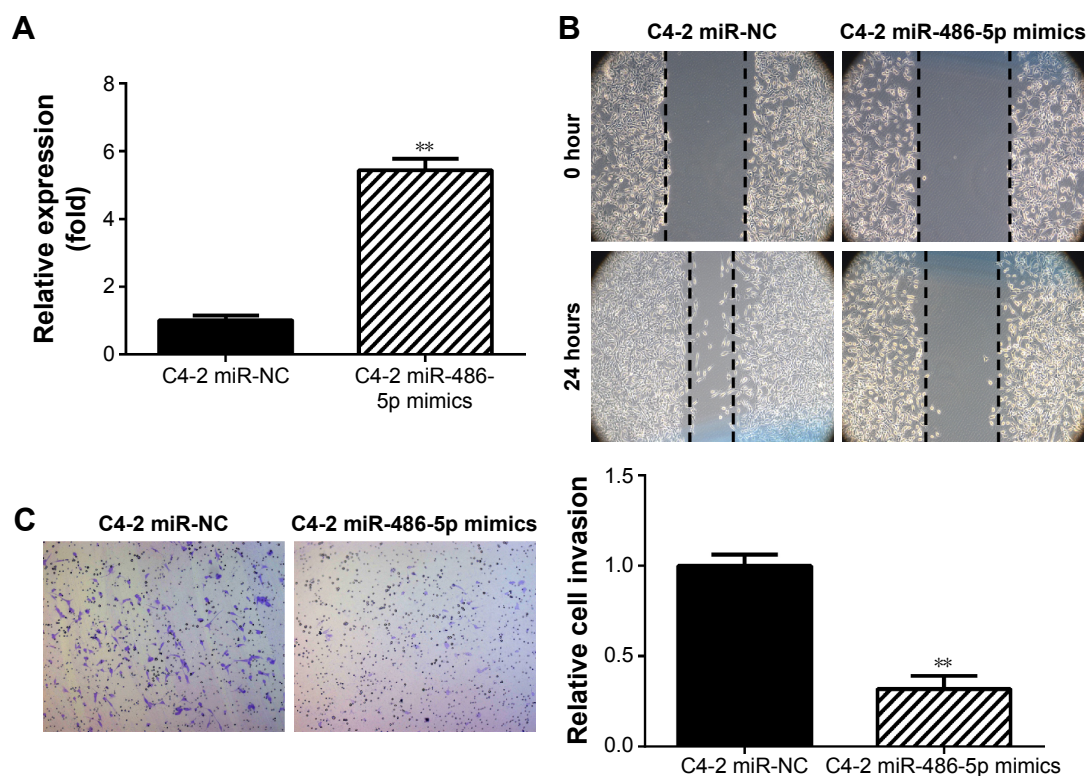


Figure 2 miR-486-5p suppresses migration and invasion of PCA cells.

Notes: (A) qRT-PCR confirmed the miR-486-5p expression in C4-2 cells transfected with miR-486-5p mimics or scramble miR (miR-NC). (B) Representative images of wound healing assay showed the decreased cell migration of C4-2 cells transfected with miR-486-5p mimics compared to cells transfected with miR-NC. (C) Matrigel (Boyden chamber) invasive assay showed that increased miR-486-5p significantly suppressed C4-2 cell invasion (** $P < 0.01$).

Abbreviations: PCA, prostate cancer; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

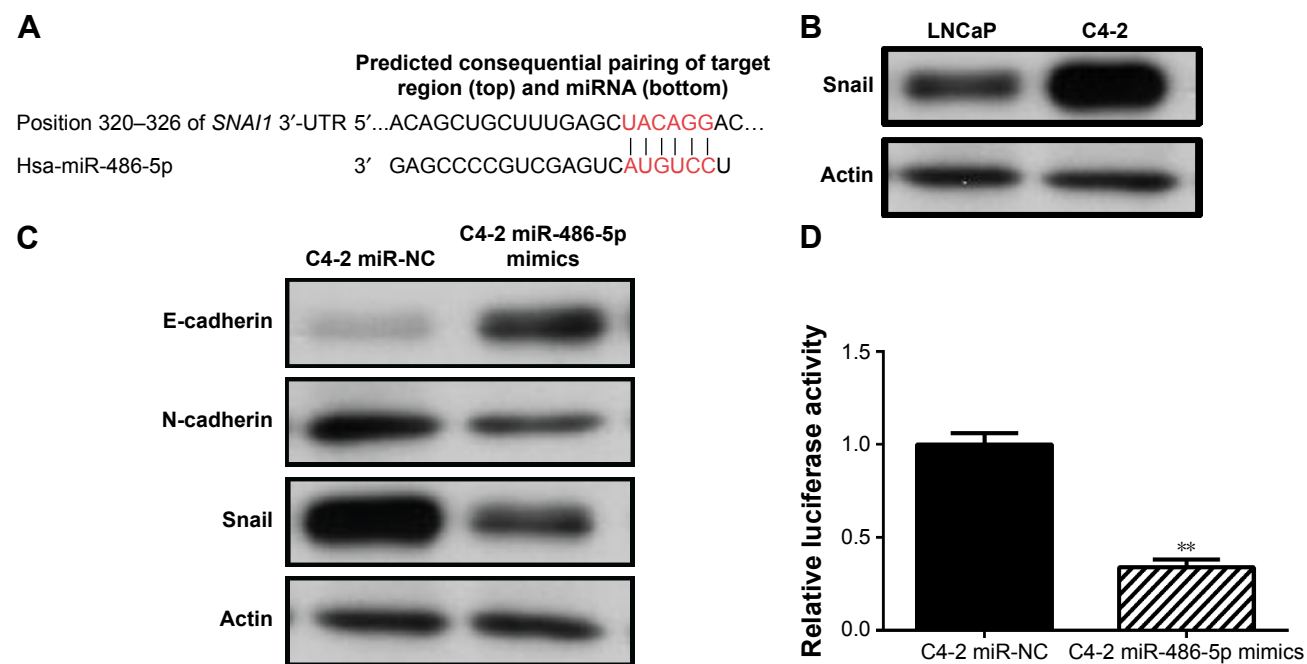


Figure 3 miR-486-5p directly targets *SNAIL*.

Notes: (A) TargetScan program predicts that *SNAIL* is a direct target of miR-486-5p. The seed sequence of miR-486-5p in the 3'-UTR of *SNAIL* is indicated in red. (B) Western blot results show the increased expression of Snail protein in metastatic C4-2 cells compared to the non-metastatic LNCaP, which is reversely correlated with miR-486-5p expression. (C) Western blot results show a decreased expression of Snail and N-cadherin and an increased expression of E-cadherin in the C4-2 cells transfected with miR-486-5p mimics compared to those transfected with scramble sequence. (D) The luciferase activity is significantly decreased in C4-2 cells transfected with miR-486-5p mimics (** $P < 0.01$).

Abbreviation: UTR, untranslated region.

Discussion

Most PCa deaths are due to metastases. However, the mechanisms underlying PCa metastasis remain largely unclear. There is an urgent need to identify reliable metastatic biomarkers and effective therapeutic targets, which may lead to improved management of the disease. A number of miRNAs have been reported to be deregulated and play an important role in PCa development and progression by regulating the expression of key target genes. It has been observed that miR-486-5p is downregulated in several types of tumors and may play a suppressive role in tumor progression. Recently, Song et al¹⁴ reported a significant downregulation of miR-486-5p in PCa tissues compared to benign tissues using high-throughput Illumina sequencing and qRT-PCR methods, which suggest that miR-486-5p may act as a tumor suppressor in PCa. However, previous studies of miR-486-5p in PCa mainly focused on primary disease and lacked functional assays.¹⁴ In this study, we demonstrated the downregulation of miR-486-5p in the metastatic PCa cell line compared to the non-metastatic cell line and further validated the finding in clinical PCa samples. We did not observe a significant correlation between miR-486-5p, androgen receptor, and PSA expression. In addition, functional analysis showed that an increased miR-486-5p can suppress PCa cell migration and invasion, suggesting a metastasis-suppressing role in PCa.

EMT is a crucial mechanism that guides proper cell movement during several phases of embryonic development¹⁵ and is also commonly observed in the process of cancer metastasis.¹⁶ Such transition from a non-invasive to an invasive phenotype is characterized by decreased expressions of epithelial cell adhesion molecules, such as E-cadherin, and increased expressions of mesenchymal markers, such as N-cadherin and Vimentin.¹⁶ Such changes in cell phenotype and behavior are mediated by a number of key transcription factors, including Snail.^{17,18}

It has been reported that Snail can repress the expression of epithelial genes like E-cadherin by binding to E-box DNA sequences through their carboxy-terminal zinc-finger domains followed by recruitment of the PRC2.^{19,20} As a key EMT transcription factor, Snail can also activate other transcription factors and markers that contribute to the mesenchymal phenotype, such as TWIST, ZEB1, ZEB2, and N-cadherin.²¹ In this study, we predicted that Snail may be directly regulated by miR-486-5p and validated the direct binding of miR-486-5p and 3'-UTR of *SNAIL1*. Western blot analyses confirmed that Snail expression was decreased in C4-2 PCa cells transfected with miR-486-5p mimics when compared with the cells transfected with scramble sequence. Furthermore, E-cadherin expression was significantly

increased and N-cadherin expression was decreased in miR-486-5p-overexpressed C4-2 cells. These data suggested that miR-486-5p may directly control Snail protein expression and further regulate EMT and cell invasion in PCa.

Conclusion

This study demonstrated that miR-486-5p is downregulated in metastatic PCa cell lines and clinical samples compared to non-metastatic ones. Functional studies showed that miR-486-5p suppresses PCa cell migration and invasion by directly regulating Snail protein expression. The findings suggest that miR-486-5p plays a metastasis-suppressor role in PCa and may provide a potential therapeutic target for the disease.

Acknowledgment

This work was supported by Tianjin Science and Technique Foundation (13RCGFSY19100).

Disclosure

The authors report no conflicts of interest in this work.

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