MicroRNA-106b functions as an oncogene and regulates tumor viability and metastasis by targeting LARP4B in prostate cancer

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Abstract. Prostate cancer (PCa) is the most common malignancy among males worldwide, and is one of the leading causes of cancer-related mortality. MicroRNAs (miRs) are a type of endogenous, noncoding RNA that serve a key role in pathological processes, and have been demonstrated to be involved in the formation and progression of PCa. Previous studies have reported that miR-106b acts as an oncogene; however, the specific effects of miR-106b on PCa have not been fully elucidated. The present study aimed to investigate the role and underlying molecular mechanisms of miR-106b in the initiation and progression of PCa. In this study, miR-106b was reported to be overexpressed and la-related protein 4B (LARP4B) was downregulated in PCa tissues compared with paracancerous tissues. In addition, LARP4B was identified as a target gene of miR-106b by bioinformatics prediction analysis and a dual luciferase reporter gene assay. Furthermore, MTT, wound healing and Transwell assays were performed to evaluate PCa cell viability, migration and invasive abilities. The data revealed that inhibition of miR-106b significantly suppressed the viability, migration and invasion of PCa cells. In addition, inhibition of miR-106b significantly suppressed the mRNA and protein expression of cancer-related genes, including matrix metalloproteinase-2, cluster of differentiation 44 and Ki-67, and increased that of the tumor suppressor, mothers against decapentaplegic homolog 2. Collectively, the findings of the present study indicated that miR-106b may target LARP4B to inhibit cancer cell viability, migration and invasion, and may be considered as a novel therapeutic target in PCa.

Introduction

Prostate cancer (PCa) is the most common malignancy among males in Europe and USA, with increasing incidence (1). Additionally, ~1/7 of Australian males are diagnosed with PCa annually, at a mean age of 75 years; PCa is considered to be the second most fatal malignancy among the European and American male populations (2). In China, with the improvement of the living standards, changes in diet composition and the progressive aging of the population, the incidence of malignant tumors has notably increased; PCa in particular is quickly becoming the leading cause of mortality among middle-aged and elderly men (3,4). At present, the main methods for diagnosing PCa are digital rectal examination (DRE), measurement of serum prostate-specific antigen (PSA) levels, magnetic resonance imaging and transrectal ultrasound-guided prostate biopsy (5-8). In addition, DRE combined with PSA is considered as the standard method for early screening of PCa (6); however, the findings on DRE may be subjective, and the value of DRE in the diagnosis of early PCa without notable nodules is limited. On the contrary, PSA detection is characterized by high sensitivity and relatively low specificity, and the detection rate of PCa is only ~25%, particularly when PSA is between 4-10 ng/ml (PSA diagnostic gray zone) (9). Furthermore, PSA detection often leads to inaccurate diagnoses and poor therapeutic strategies for the treatment of PCa (10-12). In addition, the exact molecular mechanism underlying the development and progression of PCa remains unclear. Therefore, elucidating this mechanism is crucial for the clinical diagnosis, treatment and follow-up monitoring of patients with PCa.

MicroRNAs (miRNAs/miRs) are noncoding RNAs comprising ~20-22 nucleotides in length and are highly conserved among species, which act as key factors in tumor inhibition or promotion via the regulation of oncogenes or tumor suppressors (13). The association between miRNAs and the development, diagnosis and treatment of PCa has attracted notable attention (14-16). For example, miR-206 was reported to exert antitumor effects on PCa through regulating Annexin A2 and C-X-C motif chemokine 11 (17,18); miR-33a acts as a tumor suppressor and was observed to be downregulated in PCa (19). Furthermore, miR-605 (20), miR-106a (21) and miR-483-5p (22) act as oncogenes in PCa. Conversely, miR-154 (23), miR-331-3p (24) and miR-625 (25) act as tumor suppressors in PCa. miR-106b is a member of the miR-106b-25 family that is highly expressed in laryngeal, gastric, breast and hepatic cancer, and has important functions in regulating tumor cell migration, invasion and proliferation (26-29). Furthermore, it was previously reported that miR-106b induces apoptosis and inhibits invasion of thyroid
cancer cells by downregulating the expression of chromosome 1 open reading frame 24, suppresses the ability of Smad7 to enhance epithelial-to-mesenchymal transition and promotes the metastasis of esophageal cancer cells; inhibition of miR-106b induces apoptosis, and suppresses proliferation and migration of renal cell carcinoma cells (30-32). However, the role and the underlying molecular mechanisms of miR-106b in PCa require further investigation.

The aim of the present study was to investigate the effects of miR-106b on PCA cell viability, migration and invasion and the underlying mechanism of action, in order to determine whether PCa may be a novel biomarker for the diagnosis and treatment of PCa.

Materials and methods

Human tissue samples. The present study was approved by the Ethics Committee of Ningbo First Hospital (Ningbo, China). A total of 40 patients with PCa from Ningbo First Hospital were investigated, none of whom had received radiotherapy, chemotherapy or immunotherapy prior to tumor resection. All the patients provided written informed consent for their tissues to be used for research purposes.

Cell culture and transfection. The human PCA cell line LNCaP was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and routinely cultured in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO₂.

miR-106 inhibitors, miR-negative control (NC) inhibitor, miR-106 mimics and miR-NC mimics were purchased from Ambion (Thermo Fisher Scientific, Inc.). Inhibitors and mimics were transfected at a concentration of 100 nM. LNCaP cells were seeded into 6-well plates and transfected with 100 nM of miR-106 inhibitor, miR-NC inhibitor, miR-106 mimics and miR-NC mimics using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocols for 48 h. The sequences of miR-106 inhibitor, miR-NC inhibitor, miR-106 mimics and miR-NC mimics were as follows: miR-106b mimics, 5'-Taa aGT GcT Gca cuu ua-3'; miR-nc inhibitor, 5' -caG uac uuu uGu Gca cuu ua-3'; mir-106b inhibitor, 5'-auc uGc uca Gca cuu ua-3'; mir-nc inhibitor, 5' -uuc ucc Gaa cGu Guc acG uTT-3'; mir-106b mimics, 5'-Taa aGT GcT Gca cuu ua-3'; miR-nc inhibitor, 5' -caG uac uuu uGu Gca cuu ua-3'; mir-106b inhibitor, 5'-auc uGc uca Gca cuu ua-3'; mir-nc inhibitor, 5' -uuc ucc Gaa cGu Guc acG uTT-3';

Target prediction and luciferase assay. The prediction of the 3’-untranslated regions (3’-UTRs) of lra-related protein 4B (LAR4B) as a binding target of miR-106b was checked by using TargetScan (version 7.1; www.targetscan.org/vert_71/). Subsequently, the 3’-UTR of LAR4B was mutated using a mutagenesis kit (Promega Corporation, Madison, WI, USA) according to the manufacturer’s protocol. After transfection, cells were maintained at 37°C in 5% CO₂ for 48 h. Wild-type (WT) and mutant (Mut) sequences of LAR4B were amplified and inserted into the pmirGLO vector (Shanghai GenePharma Co., Ltd., Shanghai, China) to construct luciferase reporter plasmids according to the manufacturer’s protocol (Promega Corporation). Cells were transfected with miR-106 mimics or miR-NC mimics and LAR4B 3’-UTR WT or LAR4B 3’-UTR Mut plasmids. Cells were maintained at 37°C in 5% CO₂ for 48 h after transfection. Luciferase activity was detected with a dual luciferase reporter kit (Promega Corporation). Renilla luciferase was used to normalize the luciferase activity.

Cell viability assay. Cells at a density of 5x10⁴ cells/well were seeded in 96-well plates and transfected. After incubation for 0, 12, 24 and 48 h, the transfected cells were treated with 0.5 mg/ml MTT solution and incubated in the dark at 37°C for 4 h. Subsequently, the supernatants were removed and dimethyl sulfoxide was added to dissolve the formazan crystals. Then, the optical density at 490 nm was recorded using a microplate spectrophotometer. The experiment was performed in triplicate.

Wound healing assay. Cells at a density of 5x10⁴ cells/well were seeded into a 6-well plate. After cells attained 90% confluence, the cell monolayer was scratched using a 10-µl pipette tip, and the cells were cultured in a serum-free DMEM (Thermo Fisher Scientific, Inc.) for cell recovery. Subsequently, the cells were imaged at 48 h with an inverted light microscope (Olympus, Tokyo, Japan; magnification, x200) and samples were observed in five randomly-selected fields of view.

Transwell invasion assay. Matrigel was diluted with serum-free medium (1:3) and then added to the upper chambers (50 µl per well) and allowed to form a gel for 30 min at 37°C with 5% CO₂. Then, transfected LNCaP cells (1x10⁵ cells/well) were seeded into the upper chamber with serum-free RPMI-1640 medium, whereas RPMI-1640 supplemented with 10% FBS was added to the lower chamber. After incubation for 48 h in 5% CO₂ at 37°C, the cells on the top of membranes were removed and the invading cells were fixed with 70% ethanol at room temperature for 30 min and stained with 0.5% crystal violet solution at room temperature for 30 min, and counted using an inverted light microscope (magnification, x200) and samples were observed in five randomly-selected fields of view.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from PCA tissues and cell lines was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and miRNA was extracted using the miRcute miRNA Isolation kit (Tiangen, Shanghái, China). TaqMan MicroRNA Reverse Transcription kit and TaqMan High-capacity cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to reverse transcribe miRNA and mRNA, respectively. The RT conditions were the following: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. The expression of miR-106b was determined by RT-qPCR using the TaqMan miR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.); the mRNA expression of LAR4B, matrix metalloproteinase-2 (MMP2), mothers against decapentaplegic homolog 2 (Smad2), cluster of differentiation (CD)44 and Ki-67 was measured using a TaqMan RT-qPCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were the following: Initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. U6 and GAPDH
were used as controls for miRNA and mRNA, respectively. Data were acquired by using a HT-7900 TaqMan instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mRNA relative expression levels were calculated using the 2^ΔΔCt method (33). The PCR primers were as follows: mir-106b, 5’-TTT TcG ccc TTa GcG TGa aGa-3’ (forward) and 5’ -GaG Gca GTc Gaa GcT cTc G-3’ (reverse); u6, 5’-cTc GcT TcG Gca Gca ca-3’ (forward) and 5’ -aac GcT Tca cGa aTT TGc GT-3’ (reverse); lar P4B, 5’-TGG Tcc TaT aTc Gca aac cac T-3’ (forward) and 5’-Gca cTa cTc GcT Tcc aaa TGT-3’ (reverse); MMP2, 5’ -GcT aTG Gac cTT GGG aGa a-3’ (forward) and 5’ -TGG aaG cGG aaT GGa aac-3’ (reverse); Smad2, 5’ -caT caG cca aTG Gca aGT Gaa-3’ (forward) and 5’-aGa aca GGG TcT Gca Tcc aTc aTa-3’ (reverse); cd44, 5’ -aca acT GGT GaT GGa Gac Tca Tcc-3’ (forward) and 5’ -caG aGT GGc TTa Tca TcT TGG-3’ (reverse); and Ki-67, 5’ -Gca GGa cTT cac TTG  cTT cc-3’ (forward) and 5’ -Tca TTT  GcG TTT GTT Tca cG-3’ (reverse); GAPDH, 5’ -aca acT TTG GTa TcG TGG aaG G-3’ (forward); and 5’ -Gcc aTc acG cca caG TTT c-3’ (reverse).

Western blot analysis. Total protein from PCa tissues and lnCaP cells was extracted with radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology, Beijing, China), and the protein concentration was measured using the Bca Protein assay kit (Vazyme, Piscataway, NJ, USA). Equal amounts of protein (10 µg) were separated via 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% non-fat milk for 1 h, followed by incubation at 4°C overnight with the following primary antibodies (1:1,000): LARP4B (cat. no. ab197085; Abcam, Cambridge, MA, USA), MMP2 (cat. no. 40994; Cell Signaling Technology, Inc.), Smad2 (cat. no. 8685; Cell Signaling Technology, Inc.), CD44 (cat. no. 37259; Cell Signaling Technology, Inc.), Ki-67 (cat. no. 4400; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174; Cell Signaling Technology, Inc.). Subsequently, the membranes were washed with tris-buffered saline with 0.1% Tween-20 three times (15 min each time) and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000) for a further 2 h at room temperature including anti-mouse (cat. no. 7076; Cell Signaling Technology, Inc.) and anti-rabbit (cat. no. 5127; Cell Signaling Technology, Inc.). Protein bands were examined via Quantity One software (version 4.5; Bio-Rad Laboratories, Inc., Hercules, CA, USA) with an Ecl kit (Abcam). GAPDH was used as the loading control.

Statistical analysis. Each experiment was performed in triplicate. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the experimental data. The data were presented as the mean ± standard deviation. Statistical differences between two groups were analyzed by a Student’s t-test. Statistical differences among multiple groups were analyzed by one-way analysis of variance followed by a Bonferroni post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological features of the patients. As presented in Table I, the expression of miR-106b was significantly associated with pT stage, histological grade and lymphatic metastasis, but had no significant association with gender and age.

miR-106b is upregulated in patients with PCa. To evaluate the expression and clinical value of miR-106b in PCa, 40 pairs of PCa tissues and matched adjacent normal tissues were collected and the expression of miR-106b was examined by RT-qPCR. The results demonstrated that the expression of miR-106b was significantly upregulated in PCa tissues relative to the respective adjacent normal tissues (Fig. 1). Therefore,

![Figure 1. miR-106b is upregulated in patients with PCa. The expression of miR-106b in PCa tissues was measured by reverse transcription-quantitative polymerase chain reaction assay. The results are expressed as the mean ± standard deviation of three independent experiments and each was performed in triplicate. **P<0.01 vs. adjacent normal tissues. miR, microRNA; PCa, prostate cancer.](image-url)
miR-106b may have the potential as a novel biomarker for the diagnosis and prognosis of PCa.

**Downregulation of miR-106b inhibits in PCa cell viability.** To further investigate the role of miR-106b in the progression and development of PCa, miR-NC inhibitor and miR-106b inhibitor were respectively transfected into LNCaP cells, and RT-qPCR was performed to evaluate the transfection efficiency after 48 h. The results demonstrated that the expression of miR-106b was significantly decreased relative to the negative control group in LNCaP cells (Fig. 2A). Subsequently, an MTT assay was conducted to evaluate the effects of miR-106b on the viability of LNCaP cells. As presented in Fig. 2B, inhibition of miR-106b exerted a significant inhibitory effect on the viability of LNCaP cells compared with the controls.

**Inhibition of miR-106b suppresses the migration and invasion of PCa cells.** In addition, to investigate the effects of miR-106b on the migration and invasion of PCa cells, wound healing and Transwell invasion assays, were respectively performed with LNCaP cells. The results revealed that knockdown of miR-106b significantly suppressed LNCaP cell migration after 48 h (Fig. 3A and B) compared with the control. Furthermore, the invasive ability of LNCaP cells was significantly decreased following transfection with miR-106b inhibitors for 48 h compared with the control (Fig. 3C and D). Collectively, these results indicate that the downregulation of miR-106b decreased the migration and invasive abilities of LNCaP cells.

**Expression of miR-106b.** As presented in Fig. 4, the expression of miR-106b was significantly increased following transfection with miR-106b mimics compared with the control; no significant difference between the control and miR-NC mimics groups was observed.

**LARP4B is a direct target of miR-106b.** To further characterize the possible downstream regulators of miR-106b affecting the development and progression of PCa, TargetScan was used to predict the target genes regulated by miR-106b. The results indicated that LARP4B is a potential target gene of miR-106b (Fig. 5A). Furthermore, a luciferase reporter assay was used to further confirm whether miR-106b directly targets LARP4B in LNCaP cells. As presented in Fig. 5B, miR-106b could directly bind to the 3'-UTR of LARP4B after co-transfection of miR-106b mimic or miR-NC mimic, and the luciferase reporter vector. Upregulation of miR-106b significantly suppressed the luciferase activity of wild-type LARP4B 3'-UTR compared with the miR-NC group, but not that of mutated LARP4B 3'-UTR.

In addition, to further confirm whether LARP4B is a direct target gene of miR-106b, RT-qPCR and western blot analyses were performed to evaluate the mRNA and protein levels of LARP4B in PCa tissues and the matched adjacent normal tissues. As presented in Fig. 5C and D, the expression of LARP4B was decreased at the mRNA and protein level in PCa tissues compared with in the matched adjacent normal tissues. Furthermore, the expression of LARP4B in LNCaP cells after transfection with miR-106b inhibitors was measured by RT-qPCR, respectively. The results revealed that miR-106b downregulation significantly increased the mRNA (Fig. 5E) and protein (Fig. 6E and F) expression of LARP4B compared with the control.

**Knockdown of miR-106b regulates the expression of MMP2, Smad2, CD44 and Ki-67.** To further confirm the role of miR-106b in the progression and development of PCa, the expression of proteins associated with the proliferation and metastasis of PCa, including MMP2, Smad2, CD44 and Ki-67, was measured by RT-qPCR analysis and western blotting. As presented in Fig. 6A-F, inhibition of miR-106b significantly suppressed the expression of MMP2, CD44 and Ki-67, whereas it markedly increased the expression of Smad2 compared with the control.

**Discussion**

miR-106b was reported to be aberrantly expressed in numerous types of cancers, such as non-small cell lung cancer (34), breast cancer (35) and renal cell cancer (36). Additionally, miR-106b was differentially expressed in colorectal cancer (37), esophageal squamous cell carcinoma (38) and
hepatocellular cancer (39). miR-106b was downregulated in thyroid cancer tissues, while overexpressed miR-106b inhibited the migration and invasion of thyroid cancer cells (30); however, miR-106b was upregulated in gastric cancer, and downregulated miR-106b reduced the migration and invasion of gastric cancer cells (27). Therefore, miR-106b may serve an oncogenic or anti-tumor role in cancer. However, the possible roles of miR-106b in prostate cancer require further investigation.

In the present study, miR-106b was overexpressed in PCA tissues. The expression of miR-106b in PCA cells was significantly decreased following treatment with miR-106b inhibitor. We proposed that miR-106b may act as oncogene in PCA, and was associated with the progression and prognosis of PCa, suggesting that miR-106b could serve as novel clinical markers of PCa (40,41). However, the underlying mechanisms remain unclear.

LARP4 is a class of RNA-binding proteins that are located at different subcellular sites and interact with RNA, serving an important role in cell transcription and translation (42,43). In recent years, LARP4 has been reported to serve important roles in the proliferation, differentiation, migration and angiogenesis of several malignant tumors (44). LARP4B, a member of the LARP family, acts as a tumor suppressor in glioma (45); however, its expression and regulatory mechanism in PCA have not been elucidated. In the present study, we determined that the expression of LARP4B was downregulated in PCA tissues. Furthermore, LARP4B was reported as a target gene of miR-106b. Additionally, downregulation of miR-106b increased the expression of LARP4B. These results support LARP4B as a critical downstream mediator of miR-106b, involved in the progression and development of PCa.

We further investigated the potential roles of miR-106b on the behavior of PCA cells. Downregulated miR-106b suppressed the cell viability, migration and invasion of PCA cells. In addition, knockdown of miR-106b downregulated the expression of MMP2, Ki67 and CD44, but increased that of Smad2. Upregulated MMP2 promoted the migration and invasion of glioblastoma multiforme cells (46). Mutations or deletions in Smad2 can interrupt transforming growth factor (TGF)-β signal transduction, and lead to reduced growth inhibition of induced by TGF-β, resulting in tumor development (47-49). CD44 is highly expressed in malignant tumor cells and its expression is closely associated with patient prognosis (50-52). miR-200b-3p regulated the proliferation and apoptosis of colorectal cancer cells, and inhibited Ki-67 signaling (53). MMP2, Smad2, Ki-67 and CD44 serve an
Figure 5. LARP4B is a direct target of miR-106b. (A) The prediction of the binding between miR-106b and LARP4B was performed using TargetScan. (B) Luciferase reporter assays were performed to verify the binding of miR-106b to the 3'-UTR of LARP4B in cells treated with miR-106b mimic or miR-NC mimic. (C) The mRNA and (D) protein expression levels of LARP4B in PCa tissues were examined by RT-qPCR and western blot assays. (E) The mRNA expression levels of LARP4B in LNCaP cells transfected with miR-106b inhibitor or miR-NC inhibitor after transfection with miR-106b was examined by RT-qPCR. The band intensity was quantified by ImageJ software. The results are expressed as the mean ± standard deviation of three independent experiments and each was performed in triplicate. *P<0.01 vs. control group. hsa, homo sapiens; LARP4B, la-related protein 4B; PCa, prostate cancer; miR, microRNA; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; UTR, untranslated region; WT, wild-type.

Figure 6. Knockdown of miR-106b regulates the expression of MMP2, Smad2, CD44 and Ki-67. (A-D) The mRNA and (E and F) protein expression levels of MMP2, Smad2, Ki-67 and CD44 in LNCaP cells after transfection with miR-106b, which were examined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The band intensity was quantified by ImageJ software. The results are expressed as the mean ± standard deviation of three independent experiments and each was performed in triplicate. *P<0.05, **P<0.01 vs. control group. cd, cluster of differentiation; MMP, matrix metalloproteinase; Smad2, mothers against decapentaplegic homolog 2.
important role in the proliferation, migration and invasion of tumor cells (54-56). The role of miR-106b in regulating the expression of MMP2, Smad2, Ki-67 and CD44 further suggested that miR-106b could suppress the viability, migration, and invasion of PCa cells.

In conclusion, the present study demonstrated that miR-106b targets LAR4B to suppress cancer cell viability, migration and invasion; thus; miR-106b may represent a novel target for the treatment of patients with PCa. However, there was a limitation in the present study, in which no evidence was provided to link LARP4B exclusively to miR-106b and its function in cancer, which will be further investigated in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WY drafted the manuscript. WY, JC and GW collected, analyzed and interpreted the data. DZ conceived and designed the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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