miR-130b supresses the invasion and migration of prostate cancer via inhibiting DLL1 and regulating the PI3K/Akt pathways

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Abstract. Prostate cancer occurs in the prostatic epithelium and poses a threat to the health of middle-aged and older males. The objective of the present study was to explore the roles of microRNA (miRNA/miR)-130b in prostate cancer and potential molecular mechanisms in order to control the migration and invasion of prostate cancer. For this purpose, reverse transcription-PCR was performed to evaluate the mRNA levels of DLL1, phosphoinositide-3 kinase (PI3K), protein kinase B (Akt) and matrix metalloproteinase (MMP)9, and western blot analysis was carried out to detect the protein expression levels of DLL1, phosphorylated (p)-PI3K, p-Akt and MMP9. A Transwell assay was conducted to examine the invasion rate of prostate cancer cells. Furthermore, a scratch wound assay was performed to examine the migration rate of prostate cancer cells. A luciferase assay was performed to examine the interaction between miRNA and its target mRNA. The results revealed that miR-130b had abnormal (low) expression in tumor tissues compared with that in the adjacent normal tissue. An miR-130b mimic suppressed the expression of DLL1. The expression of p-PI3K, p-Akt and MMP9 in prostate cancer cells transfected with the miR-130b mimic was decreased in comparison to the negative control and control groups. Furthermore, migration and invasion were significantly suppressed in the miR-130b mimic group. In conclusion, a novel pathway interlinking miR-130b and MMP9, p-Akt and p-PI3K, which regulates the migration and invasion of prostate cancer cells, was identified. These findings provide an intriguing biomarker and treatment strategy for patients with prostate cancer.

Introduction

Prostate cancer occurs in the prostatic epithelium and poses a threat to the health of middle-aged and older males (1). After lung cancer, prostate cancer is the second most common malignant tumor affecting men in numerous western countries (2). Although the prostate cancer mortality rate in China is lower than that in western countries, the incidence has gradually increased (3). It is therefore necessary to explore novel molecular mechanisms for prostate cancer treatment.

MicroRNAs (miRNAs/miR) are a large class of endogenous non-coding single chain small molecule RNAs (20-25 nt) (4,5). Bioinformatics shows that miRNAs, when specifically combined with target gene mRNAs, can control hundreds of transcriptional target genes, thus promoting degradation or inhibiting translation (6). miRNAs may affect multiple pathways, thus altering the expression levels of the target mRNA and regulating the signaling pathways of target genes during the occurrence and development of tumors (7). miRNA expression profiles which vary depending on tumor type, play a crucial role in early diagnosis and prognosis as well as being molecular markers in cancer treatment (8). miRNA expression is abnormal in various tumors (9). miRNAs play a role as oncogenes or tumor suppressor genes (4,10). miR-130b inhibits migration and invasion in breast cancer (11). Exploring the relationship between miRNA and prostate cancer may provide new therapies for its treatment.

Delta-like 1 (DLL1), as a canonical ligand of Notch signaling, participates in cell fate determination, cellular differentiation and pattern formation (12). Overexpressed DLL1 contributes to the progression of choriocarcinoma and hepatocellular carcinoma, while downregulated DLL1 promotes the apoptosis and suppresses the proliferation of multiple glioma cells (13-15). In prostate cancer, DLL1 was overexpressed in LNCaP, but decreased DLL1 promotes the lateral motility of PC-3 cells (16). The roles of DLL1 in prostate cancer is intriguing and the effects of DLL1 on the progression of prostate cancer has not been fully elucidated.

Phosphatidylinositol 3 kinase (PI3K)-protein kinase B (Akt)-mammalian target of rapamycin (mTOR) is one of three major signaling pathways in cancer. mTOR is a key kinase downstream of PI3K/Akt, which regulates tumor cell proliferation, growth, survival and angiogenesis (17-19). Cancer
cells may escape detection and normal biochemical systems regulating the balance between apoptosis (programmed cell death) and survival (20). PI3K-Akt-mTOR generally promotes survival via the inhibition of proapoptotic factors and activation of anti-apoptotic factors (21). Previous studies suggest that the PI3K-Akt-mTOR signaling pathway plays an important role in cancer recurrence and metastasis (22-24).

In the present study, the roles of miR-130b in prostate cancer were investigated. It was found that miR-130b regulated migration and invasion of prostate cancer cells via the PI3K-Akt-mTOR axis.

Materials and methods

Tissue sample collection. A total of 30 prostate cancer tissues and 30 para-carcinoma tissues were obtained from patients (56-87 years; average age, 71 years) diagnosed with prostate cancer at Yulin Traditional Chinese Medicine Hospital Yulin, China from September, 2015 to January, 2017. The tissue samples were preserved in liquid nitrogen at -80°C. Informed consent was obtained from all patients. The present study was approved by the Ethics Committee of Yulin Traditional Chinese Medicine Hospital.

Cell culture. The prostate cancer cell line C4-2B was obtained from the American Type Culture Collection. The cells were incubated at 37°C and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) including 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) as well as 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). The incubator was humidified with 5% CO₂. Cell propagation was conducted every other day. Cells in the logarithmic (log) phase were used for experimentation.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. miRNAs were extracted with a miRNeasy Mini kit (Qiagen, Inc.). Thereafter, a Primer‑Script™ One Step RT‑qPCR kit (Takara Biotechnology, Co., Ltd.) was used for cDNA synthesis at 50˚C. qPCR was performed to amplify the cDNA template using a SYBR® (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cDNA synthesis at 50˚C. qPCR was performed to detect miR-130b expression in each group. miR-130b mimics and miR-130b NC were synthesized by Ribobio and the sequences used were as follow: hsa-miR-130b mimics, 5'-CAGUGCAUGAGAA AGGGCAU-3'; hsa-miR-130b NC, 5'-AUCCCCUUCAU CAUUGCAUG-3'.

Scratch wound assay. Cell migration was measured using a scratch wound assay. When cells reached 90% confluency, cells were digested and suspended at 5x10⁵ cells/ml. Thereafter, cells were seeded into 6-well plates (5x10⁵ cells/well) for a monolayer culture. Cells were then scratched with a 10 µl pipette tip (Thermo Fisher Scientific, Inc.). Cells were then incubated with RPMI-1640 medium supplemented with 10 g/l bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc.) and 1% FBS for 24 h. Thereafter, the cell migration rate was measured using a light microscope (magnification, x200; BioTek Instruments).

Transwell assay. Cell invasion was measured via Transwell assay with the 8.0 µm pore size FluoroBlok membrane (Corning which was coated with BD Matrigel Matrix (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Transfected cells were seeded (3x10⁵ cells/well) in the upper chamber with serum-free medium. The lower chamber contained DMEM (Thermo Fisher Scientific, Inc.) with 10% FBS. After 48 h incubation, non-migrated cells were removed with a cotton swab. Invaded cells were fixed with 100% methanol at room temperature for 10 min. Cells were then counted under a light microscope (x100 magnification; BioTek Instruments) in five predetermined fields. Each experiment was performed in triplicate.

Western blot analysis. Western blot analysis was used to determine protein levels. Protein samples were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology). The supernatant was retained in order to detect its protein concentration. Total protein was extracted by using a Total Protein Extraction kit (Phygene Life Sciences). Thereafter, the cell migration rate was measured using a light microscope (magnification, x200; BioTek Instruments) for each conditioned media. Each experiment was performed in triplicate.

Transfection. The log-phase prostate cancer cells (1x10⁴ cells/well) were divided into groups, namely the control group, the negative control (NC) group and the miR-130b mimic group. Cells in the miR-130b mimic group were transfected with miR-130b mimic using Lipofectamine™ 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells in the NC group were transfected with NC mimic. The control group was left untreated. After 72 h transfection, RT-qPCR was used to detect miR-130b expression in each group. miR-130b mimics and miR-130b NC were synthesized by Ribobio and the sequences used were as follow: hsa-miR-130b mimics, 5'-CAGUGCAUGAGAA AGGGCAU-3'; hsa-miR-130b NC, 5'-AUCCCCUUCAU CAUUGCAUG-3'.

PI3K-Akt-mTOR axis. The role of PI3K-Akt-mTOR in cancer recurrence and metastasis (22-24). Previous studies suggest that the PI3K-Akt-mTOR signaling pathway plays an important role in cancer recurrence and metastasis (22-24).
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1:1,000; Cell Signaling Technology, Inc.). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) for 1 h at 37˚C. Protein bands were visualized using an enhanced chemiluminescence kit (ECL Prime Western Blotting Detection Reagent; GE Healthcare) and quantified using ImageJ software (Fiji version 1.8.0; National Institutes of Health).

Luciferase assay. The target of miR-130b was predicted via Online database TargetScan (http://www.targetscan.org/vert_72/). The 3’untranslated region (UTR) fragment of DLL1 containing mutant fragments was cloned into luciferase reporter vectors (Shanghai GeneChem Co., Ltd.). Prostate cancer cells were cultured in 96-well plates. miR-130b mimic (60 nM) or NC were co-transfected with 200 ng of the 3’UTR wild-type (WT) or mutant (Mut) plasmid DNA into cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.). Luciferase activity was measured and normalized to that of Renilla luciferase after 48 h using a Dual Luciferase Reporter Assay kit (Thermo Fisher Scientific, Inc.), according to manufacturer’s protocol. All transfection assays were performed independently in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software (IBM, Corp.). Data are presented as the mean ± standard deviation. A Student’s t-test (two-tailed) or a one-way analysis of variance followed by Dunnett’s post-hoc test was used to analyze data. P<0.05 was considered to indicate a statistically significant difference. All the experiments in the present study were conducted in triplicate.

Results

Table I. Relationship between miR-130 and clinico-pathological parameters of patients with prostate cancer.

| Clinical features     | Number | miR-130 high | miR-130 low | P-value |
|-----------------------|--------|--------------|-------------|---------|
| Age (years)           |        |              |             | 0.201   |
| <50                   | 9      | 3            | 6           |         |
| >50                   | 21     | 7            | 14          |         |
| Tumor size            |        |              |             | 0.004a  |
| <3 cm                 | 13     | 8            | 5           |         |
| >3 cm                 | 17     | 2            | 15          |         |
| Histological grade    |        |              |             | 0.018a  |
| Well-intermediately   | 12     | 7            | 5           |         |
| Poor                  | 18     | 3            | 15          |         |
| Metastasis            |        |              |             | 0.166   |
| No                    | 5      | 3            | 2           |         |
| Yes                   | 25     | 7            | 18          |         |

aP<0.05 and bP<0.01, miR-130 low group vs. miR-130 high group. miR, microRNA.

Figure 1. miR-130b is downregulated in prostate cancer. (A) miR-130b expression was significantly downregulated in tumor tissues in comparison to para-carcinoma tissue. (B) Transfection of prostate cancer cells with miR-130b mimics significantly increased miR-130b expression in comparison with the NC and control groups. Data are presented as the mean ± standard deviation. "P<0.01 vs. NC group. miR, microRNA; NC, negative control.

The expression of miR-130b and patients’ clinical features. As shown in Table I, the expression of miR130b was significantly associated with tumor grades and size; however, there was no significant correlation with the expression of miR-130 and patients’ age, gender, and tumor metastasis.

Decreased miR-130b expression in prostate cancer tissue. RT-qPCR was used to determine miR-130b expression. The
results showed abnormally low miR-130b expression in tumor tissues compared with adjacent tissues (P<0.01; Fig. 1A). Furthermore, in cells transfected with the miR-130b mimic, significantly increased miR-130b expression was observed compared with the NC and control groups (P<0.01; Fig. 1B).

Impact of miR-130b on cell migration and invasion. Scratch wound and Transwell assays were used to estimate the effect of miR-130b on the migration of prostate cancer cells. Wound healing was significantly augmented post-miR-130b mimic treatment, suggesting that miR-130b suppressed the migration of prostate cancer cells (P<0.01; Fig. 2A and B). Furthermore, the number of migrated cells treated with miR-130b mimic significantly decreased compared with the NC and control groups, thus proving that miR-130b significantly inhibited prostate cancer cell migration (P<0.01; Fig. 2C and D).

DLL1 expression in prostate cancer. Western blot analysis was used to examine the DLL1 protein level. The results...
showed that the DLL1 protein expression level in prostate cancer tissues was increased compared with in normal adjacent tissues (P<0.01; Fig. 3).

**miR-130b inhibits the protein level of DLL1.** The DLL1 protein level in cells treated with miR-130b mimics was significantly decreased compared with in the NC and control groups (P<0.01; Fig. 4).

**miR-130b targets DLL1.** Online software (TargetScan) suggested that DLL1 was a target gene of miR-130b (http://www.targetscan.org/vert_72/) (Fig. 5A). The results of the luciferase assay suggested that relative luciferase activity of WT DLL1 was significantly reduced after miR-130b mimic treatment (P<0.01), while the luciferase activity of Mut DLL1 in the NC and miR-130b mimic groups showed no significant difference (Fig. 5B), thus proving that DLL1 was the direct target of miR-130b.

**miR-130b suppresses the mRNA level of PI3K, AKT, MMP9 and DLL1 expression.** The RT-qPCR results showed that the mRNA expression of PI3K, AKT, MMP9 and DLL1 was significantly downregulated after miR-130b mimic treatment (P<0.01; Fig. 6).
miR-130b suppresses the mRNA level of p-PI3K, p-AKT, MMP9 and DLL1. Protein levels of p-PI3K, p-AKT, MMP9 and DLL1 were then checked. The results showed that miR-130b mimic or DLL1 siRNA significantly decreased p-PI3K, p-AKT, MMP9 and DLL1 protein levels relative to GAPDH while there were no significant differences in PI3K and AKT levels (P<0.01; Fig. 7).
Discussion

Paralogous miRNA sequences of the miR130 family, miR-130a and miR-130b are situated on chromosomes 11 and 22, respectively (26-28). miR-130b inhibits cell proliferation and induces apoptosis in gastric cancer cells by targeting CYLD (29). Moreover, miR-130b is upregulated in triple-negative breast cancer and participates in its progression (30). Furthermore, miR-130b inhibits the cell proliferation and invasion of pancreatic cancer by targeting signal transducer and activator of transcription 3 (31). By contrast, overexpression of miR-130b promotes cell proliferation in esophageal squamous cell carcinoma (32) and in hepatocellular carcinoma (33). The roles of miR-130b vary across cancer types. However, evidence for the function of miR-130b in prostate cancer is not clear. In the present study, it was found that the expression of miR-130b was suppressed in prostate cancer tissues. It was also suggested that miR-130b may act as an antitumor gene in prostate cancer. To fully understand the role of miR-130b in prostate cancer, scratch wound and Transwell assays were performed; the results of which suggested that miR-130b inhibited the migration and invasion rates of prostate cancer cells.

To understand the mechanisms underlying the regulatory effect of miR-130b on the migration and invasion of prostate cancer cells, the present study investigated the possible target genes of miR-130b. It was identified that miR-130b may directly target DLL1. DLL1 expression was suppressed in the miR-130b mimic group. Furthermore, downregulated expression of DLL1 promotes lateral motility of PC-3 cells (16). DLL1-activated Notch1 stimulates C4-2B differentiation (34). Therefore, it was concluded that miR-130b may affect prostate cancer cell migration and invasion by targeting DLL1.

To further understand the potential mechanisms of miR-130b in prostate cancer, the present study assessed related pathways. The PI3K/Akt/mTOR signaling pathway is one of the most abnormal pathways in human cancer. PI3K/Akt/mTOR plays a role in controlling the migration and invasion of glioblastoma cells (35). Key gene mutations, such as PI3K, PTEN, Akt, TSC, LKB and mTOR may lead to the abnormal activation of certain pathways including PI3K/Akt, thus resulting in tumorigenesis (36). Akt is the switching locus for the cancer phenotype and a key factor in the downstream regulation of PI3K (37). Previous studies have shown that the PI3K/Akt pathway plays a crucial role in the migration and invasion of prostate cancer cells (38-40). PI3K/Akt expression was increased post-DLL1 treatment (41). Moreover, previous studies revealed that miR-130b mediates the progression of breast cancer and epithelial ovarian cancer via regulating the PI3K/Akt and MMP9 signaling pathway (42,43). In the present study, it was shown that miR-130b downregulated PI3K/Akt expression. miR-130b mimic significantly inhibited PI3K/Akt expression, thus restricting migration and invasion abilities. Moreover, knockout of DLL1 inhibited the expression of p-PI3K/Akt. Therefore, the inhibition of the PI3K/Akt pathway may be important in tumor treatment.

MMPs are family members of extracellular proteinases that regulate cellular biological processes, including cell proliferation, invasion and migration. MMPs are best known for their role in the degradation and removal of extracellular matrix molecules (44). Therefore, MMP expression was shown to be related to the invasive ability of prostate cancer. DLL1 and MMP9 play an important role in breast cancer cell invasion (45). The present study showed that miR-130b and knockdown of DLL1 suppressed MMP9 activity. This finding was consistent with previous studies (46-49).

However, there are some limitations in this study. Firstly, an miRNA may have several targets and a gene may be targeted by several miRNAs. Secondly, the present study was limited to the number of participants enrolled and lack of in vivo study. Although PI3K, Akt and MMP9 were suppressed in the mimic group, the current study did not confirm these molecules were a target of miR-130b directly or indirectly. Moreover, the use of 1% FBS in wound healing assays is also a limitation of the study.

In conclusion, the present study identified a novel pathway, interlinking PI3K/Akt, miR-130b, DLL1 and MMP9, p-Akt and p-PI3K, which regulates the migration and invasion of prostate cancer cells. It was found that miR-130b expression in prostate cancer was decreased. Furthermore, miR-130b reduced the migration of prostate cancer cells by targeting DLL1 and inhibiting the PI3K/Akt and MMP9 pathways. These findings provide an intriguing biomarker and treatment strategy for patients with prostate cancer.

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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

LiJ was involved in the preparation of the manuscript and worked with BL and HG to perform the experiments and collect the data; and cooperated with LinJ, DL and JH to interpret the data. LinJ and BJ were responsible for design of this study. LiJ and BJ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all patients and the study was approved by Yulin Traditional Chinese Medicine Hospital Yulin.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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