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*Original Article*

# miR-10b represses the proliferation and invasion of prostate cancer by targeting LRH1

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**Abstract:** Liver receptor homolog 1 (LRH1) plays an important role in the onset and progression of many cancer types. However, the role of LRH1 in prostate cancer has not been well investigated. In this study, the critical role of LRH1 in prostate cancer cells was described. Quantitative polymerase chain reaction and Western blot analysis results revealed that LRH1 was highly overexpressed in prostate cancer cells. Bioinformatics analysis results showed that LRH1 was potential direct target of miR-10b, which was further confirmed by a dual-luciferase activity reporter assay. LRH1 knocked down by small interfering RNA (siRNA) significantly inhibited prostate cancer cell proliferation and invasion. miR-10b was overexpressed in prostate cancer cells through transfection of miR-10b mimics and overexpressed miR-10b significantly inhibited LRH1 expression, cell proliferation and invasion. In conclusion LRH1 is implicated in prostate cancer, and miR-10b-mediated suppression of LRH1 can be a novel treatment strategy of prostate cancer.

**Keywords:** Liver receptor homolog 1 (LRH1), miR-10b, prostate cancer, proliferation, invasion

## Introduction

Prostate cancer (PCa) is the most common malignancy among elderly men and the second leading cause of cancer death among males in western societies [1]. Despite improvements in treatment strategies, including surgical castration and chemotherapy, many prostate cancer patients eventually experience recurrence, leading to accelerated disease progression and death [2, 3]. Hence, it is urgent to reveal the molecular mechanism of prostate cancer development and find new treatment strategies.

Liver receptor homolog 1 (LRH1), also named nuclear receptor subfamily 5 group A member 2 (NR5A2), is an orphan member of the nuclear receptor superfamily that share substantial structural homology within their DNA and ligand binding domains, involving the regulation of metabolism, differentiation, and development [4-6]. Recently, accumulated studies demonstrated that LRH1 plays an important role in various human cancers, including liver, gastric, pancreatic cancers, breast cancer and colon [7-11]. For example, Bayrer et al. showed that silencing LRH1 in colon cancer cell lines impairs proliferation and alters gene expression programs [12]. In breast cancer, LRH1 is highly expressed and promotes cell proliferation by enhancing ERα transcription of growth-related target genes mediated by estrogen [7, 13]. LRH1 also reported to promote breast cancer motility and invasion by remodeling of the actin cytoskeleton and E-cadherin cleavage [11]. LRH1 overexpression is associated with the colony formation, cell proliferation, and tumor progression in pancreatic and hepatic cancer cells [10, 14]. However, the expression and function of LRH1 in prostate cancer remains poorly understood.

With the critical role of LRH1 in tumorigenesis and cancer progression, targeting LRH1 may be applied to develop novel cancer therapy. microRNAs (miRNAs) have been considered as a novel tool by binding to the 3'untranslated region (3'-UTR) of their target mRNAs and regulating oncogene/tumor suppressor gene expression [15-21]. Accumulated evidences proved that the deregulated miRNAs contribute to cancer progression as a result of changes in expre-

ssion of their target genes in various cancers including prostate cancer [22-24]. For instance, MicroRNA-940 suppresses prostate cancer migration and invasion by regulating MIEN1 [25]. microRNA-155 promotes the proliferation of prostate cancer cells by targeting annexin 7 [26]. Downregulation of miR-221, -30d, and -15a contributes to pathogenesis of prostate cancer by targeting Bmi-1 [27]. MicroRNA-19a regulates proliferation and apoptosis of castration-resistant prostate cancer cells by targeting BTG1 [28]. Therefore, miRNA-targeted therapy can be applied to treat prostate cancer. Recently, miR-10b was reported to be significantly downregulated in all the prostate cancer tissues in comparison with normal epithelium [29], however, the mechanism of miR-10b in prostate cancer remains unclear.

In this study, the potential role of LRH1 in prostate cancer was investigated. Our results indicated that LRH1 was significantly overexpressed in prostate cancer cells. Bioinformatics analysis results revealed that LRH1 contained putative binding sites of miR-10b, which was validated through a dual-luciferase activity reporter assay. miR-10b overexpression and LRH1 knocked down by small interfering RNA (siRNA) significantly inhibited LRH1 expression in prostate cancer cells and repressed cell proliferation and invasion. This study suggested that LRH1 plays an important role in regulating prostate cancer cell proliferation and invasion. Thus, targeting LRH1 by miR-10b can be applied to treat prostate cancer.

## Materials and methods

### *Cell culture and transfection*

Human prostate cancer cell lines PC3, Du145, and 22Rv1, the human prostate epithelial cell line RWPE and the human normal kidney cell line HEK293T were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA).

The miR-10b mimics and negative control molecules were purchased from GenePharma Co., Ltd. (Shanghai, China), added to culture media at a final concentration of 100 nM and transfected into cells using Lipofectamine™ 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. The siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) of LRH1 was transfected into cells according to standard transfection protocol. In transfection, siRNA was diluted in a transfection medium containing transfection reagent to obtain a concentration of 0.01 mg/ml and incubated for 30 min at room temperature.

### *Quantitative real-time PCR (qPCR)*

Total RNA was extracted from cells with the TRIzol reagent (Invitrogen, USA) and then reverse-transcribed into cDNA, following the thermal cycle program of 16°C for 30 min, 37°C for 60 min, and 85°C for 5 min, cDNA was stored at -20°C. The real-time quantitative PCR was performed by a fast real-time PCR system (7900HT, ABI, USA) using a TaqMan miRNA assay kit. the protocol was conducted for 35 cycles at 95°C for 3 minutes, 95°C for 12 seconds, and 58°C for 30 seconds. Finally, the relative expression level of miR-21 was normalized to that of internal control U6 by using $2^{-\Delta\Delta}$Ct cycle threshold method.

### *Western blot analysis*

The cells were harvested and lysed with radioimmunoprecipitation assay buffer. The concentration of protein was determined using a bicinchoninic acid assay kit. Following that, proteins of 20 μg/lane were loaded on a 10% SDS-PAGE to be separated, and then electrophoretically transferred to polyvinylidene fluoride membranes. Proteins on the membranes were then probed using primary antibodies, including anti-LRH1 and anti-GAPDH antibodies (Bioss, Beijing, China), according to the manufacturer's instructions. Following incubation with secondary antibodies, including rabbit anti-mouse secondary antibody, the results were visualized with horseradish peroxidase and an enhanced chemiluminescence system, and quantified by the Quantity One software (Bio-Rad, Hercules, CA, USA).

### *Cell proliferation assay*

Cell proliferation was assessed MTT assay. In brief, cells were seeded into a 96-well plate and transfected with siRNA or miRNAs. After the cells were incubated for 48 h, the old medium was replaced with an equal volume of fresh medium. MTT (0.5 mg/ml in PBS) was then added at 20 ml per well and incubated for

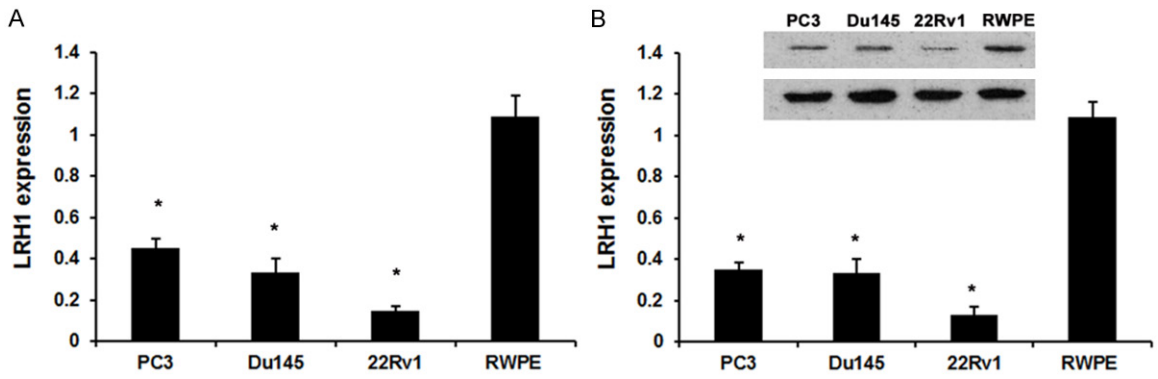


**Figure 1.** The expression of LRH1 in PCa cell lines. A. qRT-PCR analysis revealed the LRH1 expression in Human prostate cancer cell lines PC3, Du145, and 22Rv1, the human prostate epithelial cell line RWPE. B. Western blot analysis revealed the LRH1 expression in Human prostate cancer cell lines PC3, Du145, and 22Rv1, the human prostate epithelial cell line RWPE. Each bar represents the mean of three independent experiments. *P < 0.01 versus RWPE cell line.

another 4 h. Afterward, the medium was discarded and 150 ml of dimethyl sulfoxide was added to each well to dissolve formazan crystal. Absorbance was read at 490 nm by using a microplate reader (ThermoElectron Corporation, Vantaa, Finland).

*Cell invasion assay*

The capability of cell invasion was examined by transwell invasion assay. Cells were cultivated to 80% confluence on the 12-well plates. Then, we observed the procedures of cellular growth at 72 h. All the experiments were repeated in triplicate. The transwell migration chambers were used to evaluate cell invasion. Then invading cells across the membrane were counted under a light microscope.

*Dual-luciferase reporter assay*

The 3'-UTR of LRH1 containing the putative binding site of miR-10b was amplified and subcloned into pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The vector was co-transfected with miR-10b mimics into HEK293 cells for 48 h. The cells were harvested and relative luciferase activity was detected using a dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions.

*Wnt signaling activity assay*

The cells were introduced using a TCF-responsive reporter, a TOP Flash firefly luciferase reporter vector (Addgene, Cambridge, MA, USA), and Renilla luciferase vectors phRL-TK (Promega). After siRNA or miRNA transfection was performed for 24 h, the cells were harvested and luciferase activities were quantified using a dual-luciferase reporter assay kit (Promega).

*Statistical analysis*

Data are expressed as mean ± SEM. The significance of the results was analyzed using a student's t-test. The value of $P < 0.05$ was considered as a significant statistical difference.

## Results

*LRH1 is highly expressed in human prostate cancer cells*

To investigate whether LRH1 plays a key role in human prostate cancer, we initially detected the expression profile of LRH1 in human prostate cancer cell lines. The results showed that the mRNA expression level of LRH1 was significantly higher in human prostate cancer PC3, Du145, and 22Rv1 (**Figure 1A**) cells than in normal control RWPE cells. Western blot analysis results also revealed that the protein expression level of LRH1 was more abundant in PC3, Du145, and 22Rv1 (**Figure 1B**) cells than in normal control RWPE cells.

*LRH1 is a direct target gene of miR-10b*

mRNAs were reported to involve in tumorigenesis and metastasis by regulating oncogene/tumor suppressor gene expression. Here, we

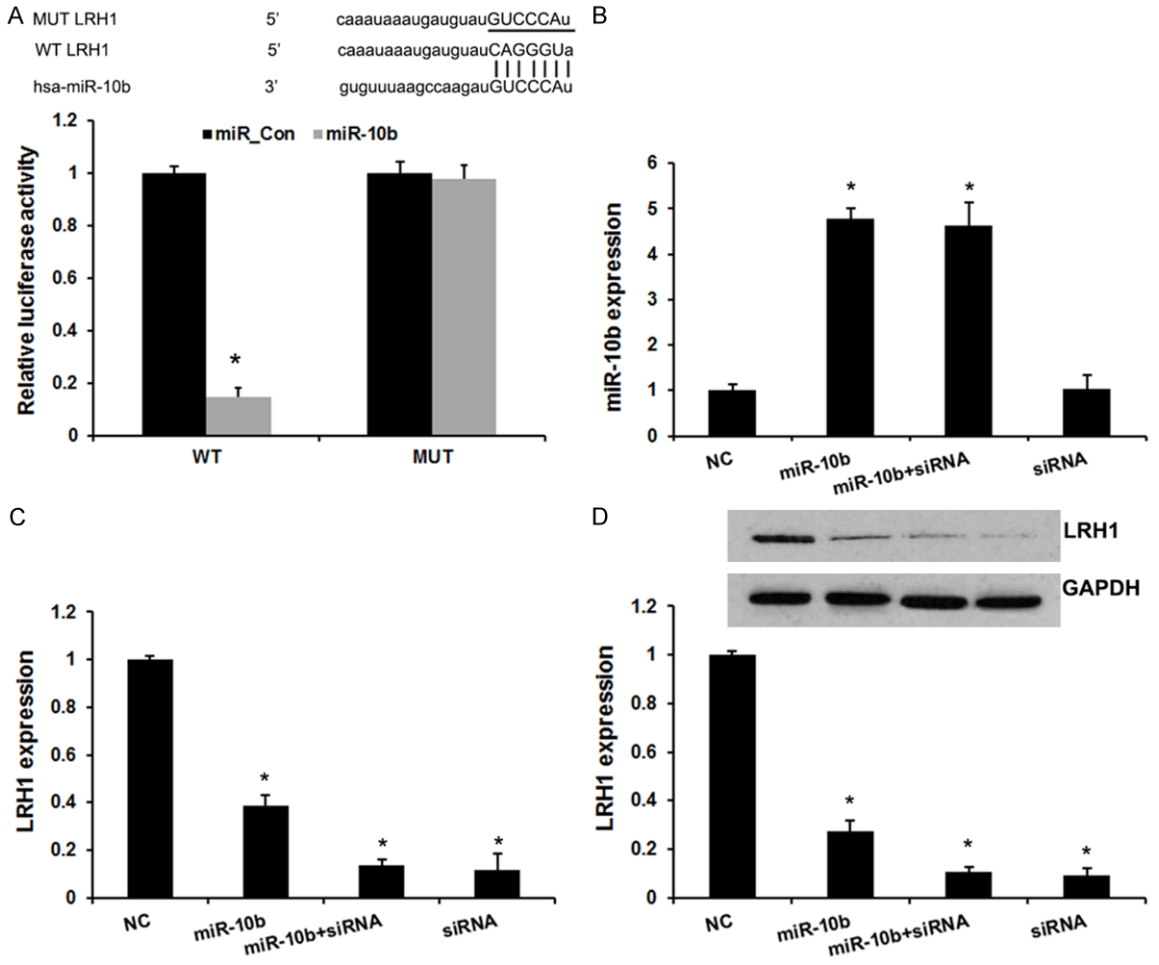


**Figure 2.** miR-10b directly targeted LRH1. A. Representative diagram of the predicted wild-type (WT) or mutant (Mut) binding site of miR-10b in the 3'-untranslated region (UTR) of LRH1 mRNA. The luciferase reporter plasmid containing the WT or Mut LRH1 3'-UTR was cotransfected into HEK293T cells with miR-10b mimics. Luciferase activity of the cells was assayed at 48 h after transfection, and the values were normalized to the normal control values. *P < 0.01 (compared with the control). B. qRT-PCR analysis revealed the effects of LRH1 siRNA and miR-10b mimics on the expression level of miR-10b. C. qRT-PCR analysis revealed the effects of LRH1 siRNA and miR-10b mimics on the expression level of LRH1. D. Western blot analysis revealed the effects of LRH1 siRNA and miR-10b mimics on the expression level of LRH1. Error bars represent ±S.E. and *P < 0.01 versus NC.

performed a bioinformatic analysis using mirco-RNA.org (http://www.microrna.org/microrna/home.do) to predict the possible miRNAs that target and regulate LRH1 (**Figure 2A**). To verify their relationship, we performed a dual-luciferase reporter assay. The results showed that miR-10b mimics remarkably decreased the luciferase activity in the 3'-UTR of wild-type (WT) LRH1 transfected cells (**Figure 2A**). By contrast, miR-10b mimics did not evidently affect the 3'-UTR of mutant-type (MT) LRH1 transfected cells. We further determined whether miR-10b regulates LRH1 expression in human prostate cancer cells. As shown in **Figure 2B**, miR-10b mimics markedly increased the expression of miR-10b, but silenced LRH1 did not affect miR-10b expression. Next, mRNA and protein expressions of LRH1 were detected in miR-10b mimic-transfected cells. The mRNA expression level of LRH1 was significantly decreased by miR-10b mimics and LRH1 siRNA (**Figure 2C**). Western blot analysis results showed that the protein expression level of LRH1 was also downregulated by miR-10b mimics and LRH1 siRNA (**Figure 2D**).

*miR-10b overexpression induces the inhibitory effect of the siRNA of LRH1 on cell proliferation and invasion*

To determine the role of LRH1 and miR-10b in the PCa cell growth and metastasis, cells were

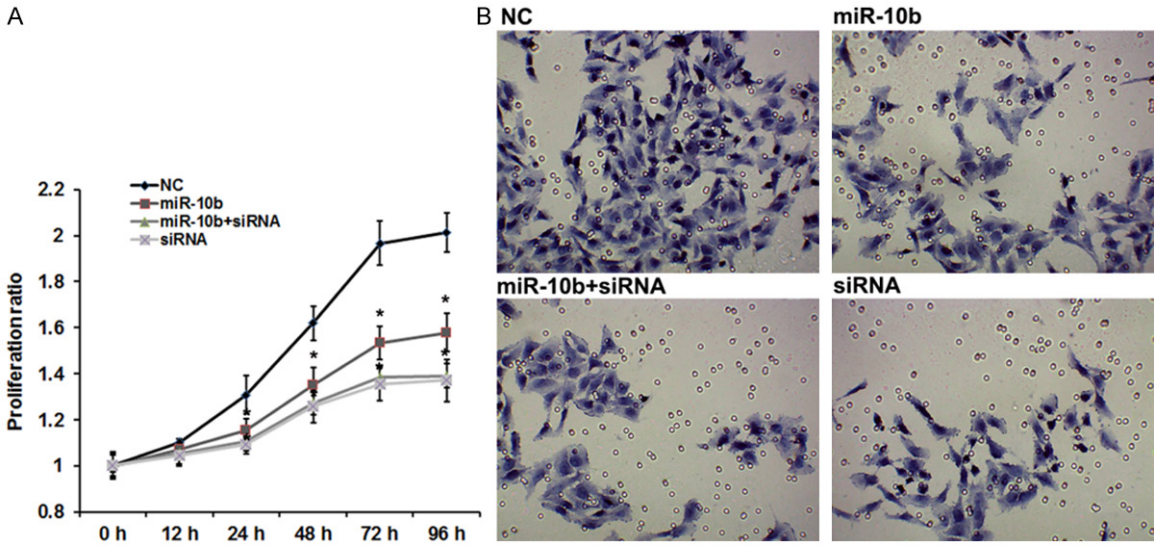


**Figure 3.** miR-10b regulated PCa cell proliferation and invasion by repressing LRH1 expression. A. The effects of miR-10b and LRH1 on cell proliferation. B. The effects of miR-10b and LRH1 on cell invasion. Error bars represent ±S.E. and *P < 0.01 versus NC.

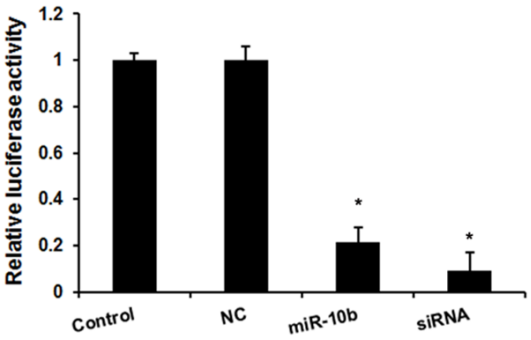


**Figure 4.** miR-10b suppresses PCa cell Wnt activity. 22Rv1 cells were transfected with miR-10b mimics, NC miRNA or siRNA. Effect of LRH1 siRNA or miR-10b mimics on Wnt signaling activity detected through TCF-dependent TOP Flash reporter activity assay. **P < 0.01 vs. Control or NC.

transfected with LRH1 siRNA (siRNA) and miR-10b mimic (miR-10b). Consistent with the effects induced by miR-10b overexpression, knockdown of LRH1 significantly suppressed the cell viability and invasion (**Figure 3A** and **3B**), whereas overexpression of miR-10b did not have further suppressive effects on cell growth and metastasis in LRH1-siRNA-transfected PCa cells.

*Loss of LRH1 in PCa cells impairs the activity of Wnt signaling*

LRH1 regulates cell proliferation by modulating Wnt/β-catenin [30]. To investigate the role of LRH1-mediated Wnt signaling in PCa cells, we detected Wnt signaling activity by performing a luciferase assay. The results showed that siRNA-silenced LRH1 significantly decreased Wnt activity compared with that of the control group (**Figure 4**). miR-10b-induced inhibition of LRH1 also impaired Wnt activity in prostate cancer cells. Therefore, siRNA-induced or miR-10b overexpression-induced inhibition of LRH1 impaired Wnt signaling activity.

## Discussion

Accumulated studies demonstrated that LRH1 plays an important role in various human cancers. This study is the first to demonstrate the critical role of LRH1 in PCa. We found that the mRNA and protein of LRH1 were significantly overexpressed in human PCa cell (PC3, Du145, and 22Rv-1) compared with the human prostate epithelial cell line RWPE. LRH1 was a direct target of miR-10b and miR-10b suppressed PCa cell proliferation and invasion by repressing LRH1-mediated Wnt signaling activity in PCa cells. Therefore, targeting LRH1 by miR-10b could be applied to repress PCa.

Accumulated studies showed that overexpressed LRH1 was observed in various tumor tissues, which promotes both uncontrolled proliferation and tumorigenesis. Overexpressed LRH1 has been detected in gastric cancer tissues and LRH1 overexpression promotes the

proliferation of gastric cancer cells [31]. LRH1 was also found to be highly expressed in breast carcinomas, notably in invasive ductal carcinoma and ductal carcinoma in situ [32, 33]. In breast cancer cells, knockdown of endogenous LRH1 inhibits tumor cell proliferation, migration and invasion [11, 34]. Likewise, Benod et al. reported that LRH1 is evidently overexpressed in pancreatic cancer tissues, and silenced LRH1 by specific siRNA significantly inhibits the proliferation of pancreatic cancer cells [10, 14]. Consistent with these findings, our data suggested that LRH1 function as an oncogene and overexpressed in PCa cells, implying that LRH1 may be used as a therapeutic target to treat PCa.

miRNAs have been considered as a novel tool to regulate oncogene/tumor suppressor gene expression. Recently, an increasing number of studies have demonstrated that the expression of miR-10b is deregulated in various cancers [35]. For example, upregulated miR-10b expression was observed in breast cancer [36], esophageal cancer [37], head and neck squamous cell carcinomas [38], bladder cancer [39] and colorectal cancer [40]. miR-10b expression in breast cancer tissues was significantly higher than that in adjacent tissues [35], and miR-10b modulates breast cancer metastasis by targeting E-cadherin [41]. miR-10b function as oncogenes in bladder cancer cells and targeting miR-10b mediated KLF4/E-cadherin and HOXD10/MMP14 axis may be helpful as a therapeutic approach to block bladder cancer cell metastasis [39]. Moreover, Li et al. demonstrated that decreased expression of miR-10b was observed in gastric cancer, miR-10b may function as a novel tumor suppressor and is partially silenced by DNA hypermethylation in gastric cancer [42]. Recently, miR-10b was reported to be significantly downregulated in all the prostate cancer tissues in comparison with normal epithelium [29], however, target genes regulated by miR-10b in prostate cancer cells have been rarely investigated. In this study, we demonstrated LRH1 as a predicted target gene of miR-10b. Therefore, high LRH1 expression in prostate cancer cells may be attributed to the decreased miR-10b expression. We further demonstrated that LRH1 expression was significantly inhibited by miR-10b mimics. miR-10b overexpression also significantly suppressed the proliferation and invasion of prostate cancer cells. The results implied that prostate cancer can be repressed by inhibiting LRH1 expression, as induced by miR-10b.

LRH1 is a coactivator of Wnt/β-catenin that synergistically promotes cell proliferation and invasion. Wnt/β-catenin pathway has been proposed as a critical signaling pathway involved in the initiation and progression of various cancers, including pancreatic cancer [10], osteosarcoma [43] and prostate cancer [44]. Our results showed that siRNA or miR-10b-induced inhibition of LRH1 significantly decreases the activity of Wnt/β-catenin signaling pathway in prostate cancer.

In this study, LRH-1, as an oncogene, was overexpressed in prostate cancer cells. miR-10b directly targeted and regulated LRH1 expression in prostate cancer cells; siRNA-induced and miR-10b-induced suppression of LRH1 inhibited cell proliferation and invasion. The loss of LRH1 by its siRNA or miR-10b mimics markedly impaired the activity of Wnt/β-catenin in prostate cancer cells. In conclusion, LRH1 plays an important role in prostate cancer; miR-10b-induced inhibition of LRH1 can be a novel therapy to treat this disease.

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## Disclosure of conflict of interest

None.

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