

## Original Research Article

# High miR-34a and miR-26b expressions inhibit prostate cancer cell OPCN-1 proliferation and enhances apoptosis

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### Abstract

**Purpose:** To investigate the effects of miR-34a and miR-26b on the targeted genes, LEF1 and EphA2, and proliferation and apoptosis of OPCN-1.

**Methods:** Sixty specimens of cancer tissue (CT) and equivalent tissue adjacent to tumors (TAT) were collected from prostate cancer patients. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine the mRNA expression levels of miR-34a, miR-26b, LEF1, and EphA2 in the above tissues, while protein expression levels of LEF1 and EphA2 were evaluated by Western blot.

**Results:** Compared with TAT, the expression levels of miR-26b and miR-34a in CT decreased significantly ( $p < 0.05$ ), whereas the mRNA and protein expression levels of EphA2 and LEF1 in CT significantly increased ( $p < 0.05$ ). TargetScanHuman7.2 assay data revealed that miR-26b targeted EphA2, while miR-34a targeted LEF1. MiR-26b MG showed decreased EphA2 mRNA and protein levels when compared with miR-26b-NC group after overexpression. The miR-34a MG exhibited decreased expression levels of LEF1 mRNA and protein compared with the miR-34a-NC group. Between 48 and 72 h, miR-26b MG grew more slowly than miR-26b-NC group; miR-34a MG also showed significantly slower growth than miR-34a-NC group. The miR-26b MG and miR-34a MG groups displayed higher apoptosis rate than miR-26b-NC and miR-34a-NC groups, respectively.

**Conclusion:** High expressions of miR-34a and miR-26b targeted the inhibition of LEF1 and EphA2, respectively, indicating that they inhibit the proliferation, and also control the increased apoptosis rate of OPCN-1 cells. Hence, miR-34a and miR-26b are probable molecular targets for the development of new prostate cancer drugs.

**Keywords:** Prostate, miR-34a, miR-26b, LEF1, EphA2

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## INTRODUCTION

Prostate cancer (PCa) is a common male cancer, and one of the leading causes of male death worldwide [1,2]. It is characterized by fast metastasis and high heritability [3,4]. All patients

who have died of PCa would have cancer cells that metastasized to the bones, lymph nodes, lungs, liver, etc. [5], which are important research topics in PCa treatment.

Treatment options of PCa mainly include active monitoring, surgery, radiotherapy, chemotherapy, and hormone therapy [6]. Recent studies have centered on molecular targets for PCa, such as microRNA (miRNA) [7]. miRNA detection has become a reliable diagnostic tool in PCa diagnosis and treatment. An miRNA is a small non-coding RNA molecule (containing about 22 nucleotides, which binds to the 3'-untranslated region (3'-UTR) of the target mRNA, in order to regulate mRNA and its expression [8,9]. Studies found that miR-34a and miR-26b are PCa-associated [10,11]. It was also found that miR-34a and miR-26b target the lymphoid enhancer-binding factor 1 (LEF1) and Eph receptor tyrosine kinase A2 (EphA2), respectively [10,12]. As a transcription factor, LEF1 contributes to cancer cell invasion and migration by regulating epithelial-mesenchymal transition, while EphA2 regulates blood vessel formation in tumors and promotes cell immigration, invasion, and metastasis [13,14]. This study is aimed at investigating the relationship between miR-34a and miR-26b, and the PCa cell OPCN-1 through LEF1 and EphA2, as well as the effects of overexpression of these two miRNAs on OPCN-1 proliferation and apoptosis.

## METHODS

### Cancer tissues

Cancer tissue (CT) and equivalent tissue adjacent to tumors (TAT) (60 specimens each) were collected from PCa patients undergoing surgery in Second Affiliated Hospital of Dalian Medical University, Dalian, China, from January 2017 to January 2019. The inclusion criteria were: patients diagnosed with PCa in the hospital, patients with complete medical records, patients aged  $\geq 18$  years, patients without accompanying cancer other than PCa; patients who signed informed consent. The exclusion criteria were: patients who previously received radiotherapy and patients who received hormone therapy. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Dalian Medical University. The study was conducted in compliance with the Declaration of Helsinki.

### Main reagents and equipment

#### Main reagents

Human prostate cancer cell line OPCN-1 (Beijing Crespo Biotechnology Co., Ltd., Beijing, China), Dulbecco's modified eagle medium (DMEM) medium (Hunan Fenghui Biotechnology Co., Ltd., Changsha, China), 10% fetal bovine serum

(FBS; Thermo Fisher Scientific, Waltham, MA, USA), cell cycle assay kit (Shanghai Enzyme Biotechnology Co., Ltd., Shanghai, China), cell counting kit-8 (CCK-8) kit (Shanghai Jingkang Biotechnology Co., Ltd., Shanghai, China), apoptosis detection kit and transfection reagent (Lipofectamine TM3000; Sigma-Aldrich (Shanghai) Trading Co., Ltd., Shanghai, China), TRIZOL reagent (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China), transcriptase (Shanghai Shifeng Biotechnology Co., Ltd., Shanghai, China), paraformaldehyde (Zibo Qixing Chemical Technology Co., Ltd., Zibo, China), crystal violet stain (Shenzhen Xin Yaosheng Industrial Co., Ltd., Shenzhen, China). miR-34a, miR-26b, LEF1, EphA2, and internal reference primers were synthesized by Shanghai Shenggong Biological Company (Table 1).

#### Main equipment

The following equipment were used in this study. UV spectrophotometer (Beijing Jiayuan Xingye Technology Co., Ltd., Beijing, China), Coulter CytoFLEX flow cytometer (Beckman Coulter, Franklin Lakes, NJ, USA), ABI7500 real-time PCR kit (Beijing Longyue Biotechnology Development Co., Ltd., Beijing, China), Transwell cell (Shanghai Shengbo Biomedical Technology Co., Ltd., Shanghai, China), and Microplate reader (Beijing Anmag Trading Co., Ltd., Beijing, China).

#### Evaluation of mRNA expression levels of miR-34a, miR-26b, LEF1, and EphA2

Total RNA was extracted as follows: Tissue sample (50 mg) was transferred into RNase-free centrifuge tube (1.5 mL). TRIZOL solution (0.5 mL) was added and the tissue was homogenized for 2 – 15 sec. Then, another 0.5 mL TRIZOL was added and allowed to stand for about 0.5 h. For every 1 mL of TRIZOL, 200  $\mu$ L chloroform was added and mixed by shaking for 30 sec, placed on ice for 5 min, followed by centrifugation at 1500 $\times$ g and 4 °C for 10 min.

A pipette was used to transfer 400 – 600  $\mu$ L supernatant to a new centrifuge tube, then 500  $\mu$ L isopropyl alcohol was added for every 1 mL TRIZOL and mixed well by repeatedly pipetting up and down for 10 min. After centrifugation at 1500 $\times$ g and 4 °C for 10 min, the supernatant was discarded and 1 mL of 75 % ethanol was added. The RNA was washed after centrifugation at 1500 $\times$ g and 4 °C for 10 min. The supernatant was discarded and the pellet air-dried for 5–10 min. DEPC water (20  $\mu$ L) was added to fully dissolve the total RNA obtained. The concentration and purity of total RNA was

measured by a UV spectrophotometer. RNA with an absorbance<sub>260</sub>/ absorbance<sub>280</sub> ratio of between 1.8 and 2.0 was used to synthesize cDNA with reverse transcriptase and oligonucleotides, according to the kit instructions.

PCR assay was performed as follows: pre-denatured at 95 °C for 5 min, then denatured at 95°C for 15 s, and annealed at 60 °C for 30 s over 40 cycles. The results were compared with those of the internal reference. The primers used in the PCR are shown in Table 1.

### Determination of protein expression levels of LEF1 and EphA2

The CT and TAT were ground with liquid chlorine and RIM cell lysate was added. Under low temperature, the homogenate was crushed and centrifuged at high speed, then the supernatant was stored at -80 °C. Western blot was used to detect the protein expression levels of LEF1 and EphA2 in CT and TAT. 30 µg of protein was added to each well and separated by 10 % polyacrylamide gel. The target proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) using SDS-PAGE (Bio-Rad, Hercules, CA, USA). The membrane was incubated with primary antibodies (1:1000) against LEF1 ([ab137872](#), Abcam), EphA2 ([ab217363](#), Abcam) and β-actin ([ab8226](#), Abcam) at 4 °C overnight. After blocking with skim milk, the membrane was incubated with a 1:5000 HRP-conjugated anti-rabbit secondary antibody (WLA024, Wanleibio, Shenyang, China) at room temperature. The membrane density was measured using a chemiluminescence digital image system (Bio-Rad). The ratios of LEF1/GAPDH and EphA2/GAPDH were considered to be relative expression levels.

### Dual luciferase reporter assay

3'-UTR of EphA2 and LEF1 DNA fragments containing potential binding sites of miR-26b and miR-34a, respectively, were merged, and the fragments were then subcloned into *XhoI* and *NotI* sites downstream of the Renilla luciferase

coding sequences of the psiCHECK-2 vector, and verified by Sangon Biotech sequencing. The dual luciferase reporter assay (Promega, Madison, WI, USA) was performed to evaluate the activity of firefly and Renilla luciferases after the cell culture and transfection.

### Cell culture and transfection

OPCN-1 cells were subcultured using a high-glucose DMEM medium containing 10 % FBS in a 37 °C and 5 % CO<sub>2</sub> incubator. Before transfection, cells were seeded into 96-well plates and then assigned to miR-26b-NC group, miR-26b MG, miR-26b-NC group and miR-34a MG. Lipofectamine TM3000 kit was used to transfect miR-26b mimic, miR-26b-NC, miR-34a mimic, and miR-34a-NC into OPCN-1 cells.

### Assessment of cell growth

The transfected OPCN-1 cells were inoculated into 96-well plates, and three replicates were set. MTS Cell Proliferation Colorimetric Assay Kit (CCK8) (20 µL) were added to each well 2 h before the end of culture, and was placed in a 37 °C, 5 % CO<sub>2</sub> incubator. After 2 h, the cell proliferation was observed by measuring the absorbance at 490 nm using an automatic microplate reader. The time points for assays were 24, 48, and 72 h.

### Cell invasion assay

Invasion assays were performed using the transwell method. Firstly, 10 µg/µL matrigel was dissolved at 4 °C to form a 0.25 µg/µL solution with DMEM, and then left in an icebox. A total of 100 µL solution per well was added in a 24-well transwell chamber and incubated for 1 h in a 37 °C, 5 % CO<sub>2</sub> incubator. The matrigel was allowed to solidify for about 30 min and the remaining unsolidified liquid was absorbed with a filter paper. Then, 100 µL DMEM containing 10 % FBS was added to the upper chamber while 600 µL DMEM containing 20 % FBS was added to the lower chamber. The cells were cultured in an incubator at 37 °C, 5 % CO<sub>2</sub> for 24 h and then counted.

**Table 1:** Primer sequences

Variable	Upstream primer	Downstream primer
miR-26b	5'-CCCAGTTC AAGTAATTCAGG-3'	5'-TTTGCCACTAGCACATT-3'
EphA2	5'-TGGCTCACACCCGTATG-3'	5'-GTCGCCAGACATCACGTTG-3'
MiR-34a	5'- ACACTCCAGCTGGGTGGCAGTGTCTTA GCTGGT-3'	5'-CTCAACTGGTGTCTGGA-3'
LEF1	5'-CTACCCATCCTCACTGTCTGAGTC-3'	5'-GGATGTTCCCTGTTTGACCTGAGG-3'
GAPDH	5'-ATGTTTCGTCATGGGTGTGAA-3'	5'-GGTGTCTAAGCAGTTGGTGGT-3'

## Apoptosis assay

The flow cytometer was used to detect cells that had been transfected for 48 h and stained with Annexin V and PI in a 6-well plate. The experiment was repeated thrice.

## Statistical analysis

Statistical analysis was performed using SPSS statistical analysis software (version 26.0). The count data was analyzed by  $\chi^2$ . Data were expressed as mean  $\pm$  SD. The variance between the two groups or more than two groups was analyzed using Student's t-test or ANOVA with post hoc Bonferroni test.  $P < 0.05$  indicated that the difference was significant.

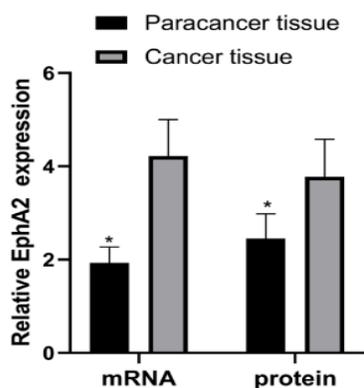
## RESULTS

### Relative expression levels of miR-26b and miR-34a in CT and TAT

Compared with CT, the relative expression levels of miR-26b and miR-34a in TAT significantly increased ( $p < 0.05$ , Table 2).

### Relative expression of EphA2 in CT and TAT

The relative expression levels of EphA2 mRNA and protein were significantly lower in TAT ( $1.93 \pm 0.34$  and  $2.45 \pm 0.53$ , respectively) than in CT ( $4.22 \pm 0.78$  and  $3.78 \pm 0.80$ , respectively) (Figure 1,  $p < 0.05$ ).



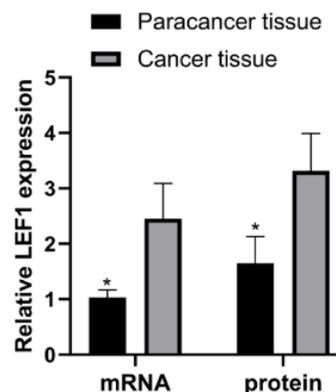
**Figure 1:** Relative expression of EphA2 in CT and TAT. The relative mRNA and protein levels of EphA2 in TAT were significantly lower than those in CT ( $p < 0.05$ ). **Note:** \*compared with CT

**Table 2:** Expressions of miR-26b, miR-34a in CT and TAT (n=60)

Factor	TAT	CA	t	P-value
miR-26b	2.32 $\pm$ 0.47	1.67 $\pm$ 0.23	9.62	<0.001
miR-34a	3.22 $\pm$ 0.52	2.01 $\pm$ 0.37	14.69	<0.001

### Relative expression of LEF1 in TAT and CT

The relative expression levels of LEF1 mRNA and protein were significantly lower in TAT ( $1.03 \pm 0.14$  and  $1.65 \pm 0.48$ , respectively) than in CT ( $2.45 \pm 0.64$  and  $3.32 \pm 0.67$ , respectively) (Figure 2,  $p < 0.05$ ).



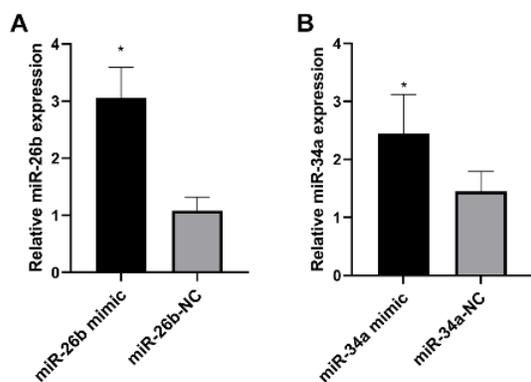
**Figure 2:** Relative expressions of LEF1 in TAT and CT. The relative mRNA and protein levels of LEF1 in TAT were significantly lower than those in CT ( $p < 0.05$ ). \*indicates comparison with CT

### Relative expression levels of miR-26b and miR-34a in each group after transfection

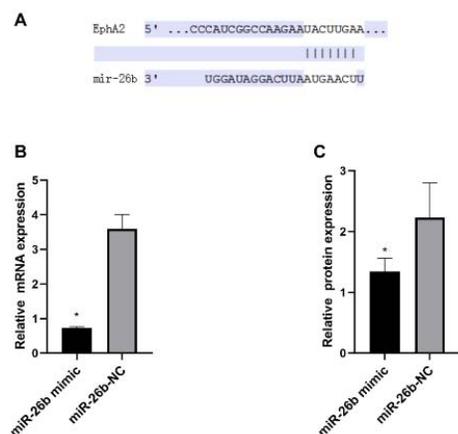
The miR-26b mimic group (miR-26b MG) ( $3.05 \pm 0.54$ ) showed significant increase in the relative expression level of miR-26b compared with miR-26b-NC group ( $1.08 \pm 0.24$ ) ( $p < 0.05$ ). Compared with miR-34a-NC group ( $1.45 \pm 0.34$ ), the relative expression level of miR-34a in the miR-34a mimic group (miR-34a MG) ( $2.45 \pm 0.67$ ) significantly increased (Figure 3,  $p < 0.05$ ).

### EphA2 expression decreased after miR-26b overexpression

TargetScanHuman7.2 predicted that miR-26b targets EphA2; our results showed that the miR-26b MG ( $0.73 \pm 0.03$  and  $1.34 \pm 0.22$ , respectively) showed significant decrease in the relative levels of EphA2 mRNA and protein compared with miR-26b-NC group ( $3.59 \pm 0.42$  and  $2.23 \pm 0.57$ , respectively) (Figure 4,  $p < 0.05$ ).



**Figure 3:** (A) Comparison of miR-26b level in miR-26b mimic and miR-26b-NC groups. miR-26b mimic > miR-26b-NC group ( $p < 0.05$ ). \*indicates comparison with miR-26b-NC group. (B) Comparison of miR-34a level in miR-34a mimic and miR-34a-NC groups. miR-34a mimic > miR-34a-NC group. **Note:** \* compared with miR-34a-NC group



**Figure 4:** EphA2 expression decreased after miR-26b overexpression. (A) There is a binding site between miR-26b and EphA2. (B) miR-26b mimic significantly reduced the expression of EphA2 mRNA ( $p < 0.05$ ). (C) miR-26b mimic significantly reduced the expression of EphA2 protein ( $p < 0.05$ ). Note: \*indicates comparison with miR-34a-NC group

### LEF1 expression decreased after miR-34a overexpression

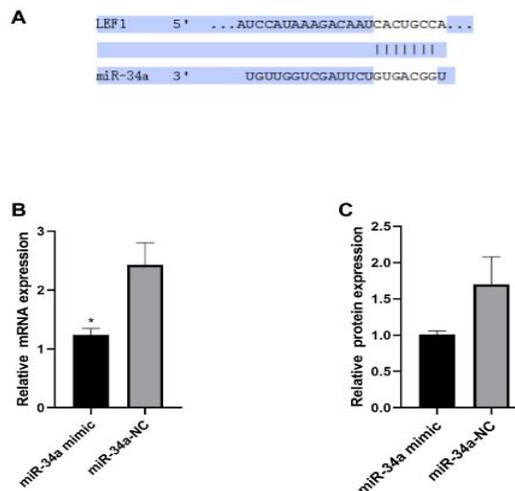
TargetScanHuman 7.2 found that miR-26b has a binding site with EphA2. It was found that the

**Table 3:** OPCN-1 cell growth at different time points after miR-26b overexpression (n = 60)

Time points (h)	miR-26b MG	miR-26b-NC group	t	P-value
24	1.44±0.12	1.43±0.15	0.40	0.688
48	3.04±0.23*	3.78±0.57*	9.33	<0.001
72	3.65±0.44*#	5.76±0.82*#	17.56	<0.001

Note: \* indicates comparison with that after 24 h, # indicates comparison with that after 48 h

miR-34a MG ( $1.24 \pm 0.11$  and  $1.01 \pm 0.05$ ) indicated significantly decreased relative expression levels of LEF1 mRNA and protein compared with miR-26b-NC group ( $2.43 \pm 0.37$  and  $1.70 \pm 0.38$ , respectively) (Figure 5,  $p < 0.05$ ).



**Figure 5:** (A) A binding site exists between miR-34a and LEF1. Comparison of LEF1 (B) mRNA and (C) protein relative expression levels in miR-34a mimic and miR-34a-NC groups. miR-34a mimic > miR-34a-NC group ( $p < 0.05$ ). \*compared with miR-34a-NC group

### Slow growth of OPCN-1 cells after miR-26b overexpression

From 48 to 72 h, the proliferation of the miR-26b MG was higher than that of the miR-26b-NC group ( $p < 0.05$ ). OPCN-1 proliferation in the three groups were significantly different between 24 and 72 h (Table 3,  $p < 0.05$ ).

### Slow growth of OPCN-1 cells after miR-34a overexpression

From 48 to 72 h, the miR-34a MG showed lower proliferation than miR-34a-NC group ( $p < 0.05$ ). No significant difference in growth rate of OPCN-1 was found in the three groups between 24 and 72 h (Table 4,  $p < 0.05$ ).

**Table 4:** OPCN-1 cell growth at different time points after miR-34a overexpression (n=60)

Time points (h)	miR-34a mimic	miR-34a-NC	t	P-value
24	0.44±0.02	0.43±0.04	1.73	0.09
48	1.38±0.19*	1.95±0.32*	11.86	<0.001
72	1.89±0.30*#	3.84±0.82*#	17.30	<0.001

Note: \*P<0.05, compared with 24 h group, # P<0.05, compared with 48 h group

**Table 5:** Apoptosis of OPCN-1 cells in miR-26 mimic and miR-26b-NC groups (n = 60)

Group	miR-26b mimic	miR-26b-NC	t	P-value
Apoptosis (%)	25.52±0.74	4.53±0.72	157.50	<0.001

**Table 6:** Apoptosis rate of OPCN-1 cells in miR-34 mimic and miR-34b-NC groups (n = 60)

Group	miR-26b MG	miR-26b-NC group	t	P-value
Apoptosis rate (%)	24.89±0.63	4.22±0.90	145.70	<0.001

### OPCN-1 apoptosis rate increased after miR-26b overexpression

Compared with miR-26b-NC group, the miR-26b MG showed higher apoptosis rate (Table 5,  $p < 0.05$ ).

### Apoptosis of OPCN-1 cells increased after miR-34a overexpression

Compared with that in miR-34a-NC group, the apoptosis rate in miR-34a MG increased significantly ( $p < 0.05$ , Table 6).

## DISCUSSION

MiRNA has been used as a biomarker, and its relationship with various cancers has been a crucial topic [15,16]. This study aimed to investigate the effects of two miRNAs, miR-26b and miR-34, on the proliferation and apoptosis of PCa cells.

Studies have found that miR-26b negatively correlated with EphA2 in lung injury of rat models [17]. In a clinical trial on bladder cancer, miR-34a was seen to target LEF1 [18]. PCa occurrence is related to both the increase of EphA2 levels and the  $\beta$ -catenin/LEF1 pathway activation [19]. Therefore, the expression levels of EphA2 and LEF1 mRNA and protein were also tested, and the results showed that they are significantly lower in TAT than in CT. Thus, in PCa, a negative correlation exists between miR-26b and EphA2, and miR-34a and LEF1.

Next, the relationship between miR-26b and EphA2 and between miR-34a and LEF1 was further explored. It was discovered that miR-26b and miR-34a have binding sites with EphA2 and LEF1, respectively. Then the over-expressed miR-26b and miR-34a transfected them into

OPCN-1 cells, and then tested the EphA2/LEF1 mRNA and protein level after miR-26b and miR-34a overexpression, respectively.

The research can therefore conclude that in PCa, miR-26b and miR-34a targeted the inhibition of EphA2 and LEF1, respectively. Wu *et al* has shown that miR-26b inhibits tumors by directly regulating EphA2 expression [20]. Gong *et al* has found that miR-34a directly targets LEF1, and miR-34a overexpression inhibits the activity of LEF1-related pathways [21].

Studies have found that in breast cancer, miR-34a and miR-26b inhibits cancer cell proliferation, while LEF1 and EphA2 have also been found to promote cancer cell proliferation in glioblastoma multiforme [22]. Many studies have also revealed that reducing EphA2 expression and inhibiting LEF1 regulated cell apoptosis [23]. However, it is not known whether these factors would show similar results in PCa. Besides, these studies do not explain the targeted inhibitory effect of miRNA, neither do they mention the underlying mechanism.

Studies have indicated that LEF1 activates the signal pathway inhibiting apoptosis by inhibiting the enzymes involved in cell mitosis, thereby promoting cell proliferation [24]. EphA2 inhibits cell apoptosis by regulating caspase-3, a key mediator of apoptosis. When EphA2 is silenced, caspase-3 activity is increased and apoptosis is promoted. Combining the above-mentioned results, it can be stated that miR-26b targets EphA2 inhibition. Hence, miR-26b overexpression inhibits EphA2-related promotion of cell proliferation, and slows signal pathway-related apoptosis inhibition, which in turn increases apoptosis and decreases proliferation. Similarly, since miR-34a targets LEF1 inhibition, miR-34a overexpression inhibits LEF1, thus

increasing the activity of enzymes related to apoptosis promotion, and thereby promoting cell apoptosis and slowing cell growth.

Due to the limitation of laboratory equipment, this study did not detect the expression levels of signaling pathway-related proteins and kinases regulating cell growth and apoptosis after the high expression of miR-34a and miR-26b. In future studies, improvements will be made to the study methods.

## CONCLUSION

The high expressions of miR-34a and miR-26b target LEF1 and EphA2, respectively, and hence inhibit OPCN-1 proliferation and increase apoptosis. Thus, miR-34a and miR-26b are potential molecular targets for the development of drugs for the management of PCa.

## DECLARATIONS

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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