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*Original Article*

# miR-486 inhibits Gli1 expression and attenuates epithelial mesenchymal transition and invasion of prostate cancer

Derong Zhou, Hao Lin, Xiaohong Chen, Junhong Zheng

*Department of Urology, The Second Affiliated Hospital of Shantou University Medical College, Shantou 515000, Guangdong, China*

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**Abstract:** Abnormal expression of glioma-associated oncogene protein 1 (Gli1) is associated with various tumors. Studies have found that decreased miR-486 expression is associated with prostate cancer. Bioinformatics analysis revealed that miR-486 has a complementary binding site in the 3'-UTR region of Gli1 mRNA. This study investigated whether miR-486 plays a role in regulating Gli1 expression and affects epithelial mesenchymal transition (EMT) and invasion of prostate cancer cells. The dual luciferase reporter gene assay validated the targeted regulation between miR-486 and Gli1. qRT-PCR was used to measure the expression of miR-486 and Gli1 mRNA. The Gli1 protein expression was detected by western blot. PC3M cells were cultured *in vitro* and divided into two groups: miR-NC group and miR-486 mimic group. The expressions of Gli1, E-cadherin and N-cadherin were detected, and the cell invasion ability was detected by Transwell assay. There was a targeted regulatory relationship between miR-486 and Gli1 mRNA. Compared with RWPE-1 cells, miR-486 expression was significantly decreased and Gli1 expression was significantly increased in PC3 and PC3M cells, and both of them were increased in high metastatic PC3M cells. Transfection of miR-486 mimic significantly down-regulated the expression of Gli1 and N-cadherin in PC3M cells, which increased E-cadherin expression, inhibited EMT and decreased the invasive ability. The decreased expression of miR-486 plays a role in up-regulating Gli1 expression, promotes EMT and invasion of prostate cancer cells. Increasing miR-486 expression can inhibit Gli1 expression and attenuate the EMT process and the invasion of prostate cancer cells.

**Keywords:** miR-486, Gli1, prostate cancer, EMT, invasion

## Introduction

Prostate cancer (PC) refers to epithelial malignant tumors that occur in the prostate. In recent years, the incidence of prostate cancer in China has been increasing year by year and has ranked sixth in the incidence of male malignant tumors, and third place in male urinary tumors [1, 2].

The Hedgehog signaling pathway is widely expressed in various tissues and cells and is involved in the regulation of various biological processes such as cell proliferation, apoptosis, migration and invasion [3-5]. Glioma-associated oncogene protein 1 (Gli1) is an important transregulatory factor in the Hedgehog signaling pathway, which can regulate the expression of a variety of genes, thereby affecting cell proliferation, apoptosis, migration, invasion, drug resistance and other biological processes [6-8]. The abnormal expression and function of Gli1 is associated with abnormal Hedgehog signaling and plays an important role in the occurrence and progression of various tumors [9-11]. Studies have found that elevated expression and functional activity of Gli1 is associated with prostate cancer [12]. MicroRNA is an endogenous non-coding single-stranded small molecule RNA with a length of about 22-25 nucleotides in eukaryotes. Through complementary binding to the 3'-untranslated region of the target gene mRNA (3'-untranslated region, 3'-UTR), microRNA regulates the expression of target genes by degrading mRNA or inhibiting mRNA translation, and thus participates in the regulation of biological processes such as cell prolif-

eration, differentiation, migration, etc., and plays a promoting role in the occurrence of tumors [13] or the role of the tumor suppressor genes [14]. In recent years, studies have found that abnormal expression of miR-486 is associated with prostate cancer [15]. Bioinformatics analysis showed that miR-486 has a target-complement relationship with Gli1's 3'-UTR. This study investigated whether miR-486 regulates Gli1 expression and affects the EMT process and invasion ability of prostate cancer cells.

## Materials and methods

### *Main reagents and materials*

Highly metastatic human prostate cancer cells PC3M, low metastatic prostate cancer cells PC3 were purchased from Nanjing Kezhen cell bank; HEK293T cells were purchased from Wuhan Punosi organism; human normal prostate epithelial RWPE-1 cells were purchased from Hunan Fenghui organism; DMEM culture Base, fetal bovine serum (FBS), Opti-MEM and penicillin were purchased from Gibco (USA); Lip 2000 was purchased from Invitrogen (USA); RNA extraction reagent EasyPure RNA Kit, fluorescent quantitative PCR reagent TransScript Green One-Step qRT-PCR SuperMix was purchased In Beijing, the whole-type golden biology; miR-NC, miR-486 mimic was designed and synthesized by Guangzhou Ruibo Bio; rabbit anti-E-cadherin, N-cadherin, Gli1, β-actin polyclonal antibody were purchased from American Abcam; HRP coupled two The anti-purchase was purchased from Shanghai Biotech; the Transwell cell was purchased from Corning (USA); the Matrigel gel was purchased from BD biosciences; the Dual-Luciferase Reporter Assay System, and the pGL3 plasmid was purchased from Promega (USA); the recombinant TGF-β1 protein was purchased from Peprotech (USA).

### *Cell culture and EMT induction*

PC3M, PC3 and RWPE-1 cells were cultured in DMEM medium containing 10% FBS in an incubator containing 5% $CO_2$ at 37°C, subcultured at a ratio of 1:4, and harvested in logarithmic phase for the experiment.

In the EMT induction experiment, PC3M cells were inoculated into 6-well plates at a density of $3 \times 10^4$. After adherence for 24 h, TGF-β1 at a final concentration of 10 ng/mL was added to the medium for 48 h to induce EMT. Conventional cultured cells induced without the addition of TGF-β1 were used as controls.

### *Dual luciferase gene reporter assay*

Using the PC3M cell genome as a template, the full-length 3'-UTR fragment of Gli1 gene was amplified, and the PCR product was digested. The amplified product was ligated into the luciferase reporter vector pGL3 and transformed into DH5α competent cells followed by screening positive clones by colony PCR to select the correct sequencing plasmid for transfection cells and subsequent experiments, which were named as pGL3-Gli1-WT, pGL3-Gli1-MUT.

pGL3-Gli1-WT (or pGL3-Gli1-MUT) was transfected into HEK293T cells with miR-486 mimic (or miR-NC) using Lipo2000. After 48 h of culture, dual luciferase assay was performed following the instructions of Dual-Glo Luciferase Assay System Kit.

### *Cell transfection and grouping*

PC3M cells were cultured *in vitro* and divided into two groups: miR-NC transfection group and miR-486 mimic transfection group. The general procedure for transfection was as follows: 10 μL of Lip 2000, 50 nmol miR-NC, 50 were diluted with 100 μL Opti-MEM. Nmol miR-486 mimic incubated for 5 min at room temperature, mixed Opti-MEM with Lip 2000, miR-NC or miR-486 mimic, incubated for 20 min at room temperature, added the transfectant mixture to the cell culture medium, continued to culture 72 hours followed by collection of cells for experiments.

### *qRT-PCR detection of gene expression*

The RNA extracted using the EasyPure RNA Kit was subjected to one-step qRT-PCR to detect the relative expression of the gene using TransScript Green One-Step qRT-PCR SuperMix in a 20 μL reaction system including: 1 μg of RNA template, 0.3 μM of pre-primer, 0.3 μM of post-primer, 10 μL of 2 × TransStart Tip Green qPCR SuperMix, 0.4 μL of RT Enzyme Mix, 0.4 μL of Dye II, and deionized water. qRT-PCR reaction conditions were: 45°C, 5 min, reverse transcription; 94°C, 30 s; (94°C, 5 s; 60°C, 30 s) ×

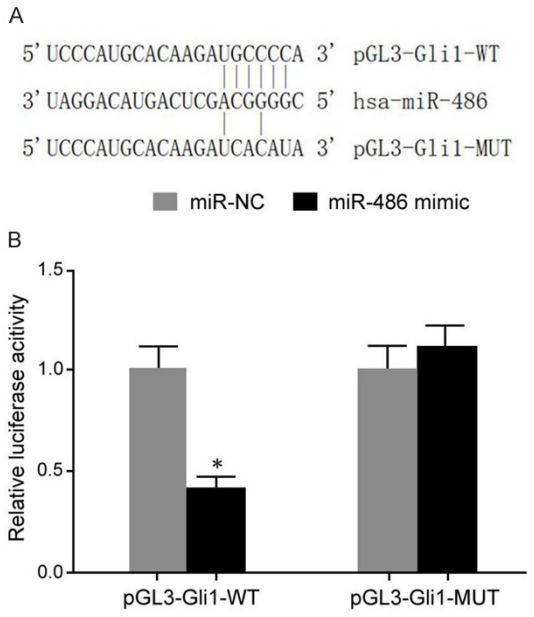


**Figure 1.** Dual luciferase reporter gene assay analysis of the targeted regulation relationship between miR-486 and Gli1. A. Schematic diagram of a targeted binding site between miR-486 and Gli1. B. Dual luciferase reporter gene assay. *P < 0.05 compared to miR-NC.

40 cycles, detection of gene expression on Bio-Rad CFX96 real-time PCR instrument. The primer sequence was: miR-486-Forward-5'-TGGGATCCATGAGGAAGGGACATGAAGA-3'; miR-486-Reverse-5'-ACCGAAGCTTAAAAAAGCTCGGTCCCAGAGTCAG-3'; U6-Forward-5'-CTCGCTTCGGCAGCACA-3'; U6-Reverse-5'-AACGCTTCACGAATTTGCGT-3'; GLI-1 (forward) 5'-CTCCCGAAGGACAGGTATGTAAC-3' and (reverse) 5'-CCCTACTCTTTAGGCACTAGAGTTG-3'; GAPDH (forward) 5'-CACCACCAACTGCTTAG-3' and (reverse) 5'-CTTCACCACCTTCTTGATG-3'; E-cadherin (Forward) 5'-CAGAAAGTTTTCCACCAAAG-3' and (reverse) 5'-ACTGAACCTGACCGTACAAAATGTGAGCAATTCTGCTT-3'.

*Western blot*

The total protein was extracted using RIPA lysis buffer, and the mass concentration was determined by BCA method according to the kit instructions. After adding 3 times the volume of 4 × protein loading buffer, it was boiled for 5 min, and 40 μg was loaded, and separated on 8-10% SDS-PAGE followed by being transferred to PVDF membrane (300 mA, 90 min), blocked with 5% skim milk powder for 60 min at room temperature, and incubated with the primary antibody at 4°C overnight (E-cadherin, N- The dilution ratios of cadherin, Gli1, and β-actin were 1:2000, 1:2000, 1:800, 1:5000, respectively). After that, the membrane was washed 3 times with PBST, and then HRP-conjugated secondary antibody (1:10000 ratio dilution) was added and incubated for 60 min at room temperature. After washing the membrane 3 times with PBST, added ECL luminescence solution for 2~3 min, exposure, developed, fixed in the dark room, scaned the film and saved the data.

*Transwell assay analysis of cell invasion*

100 μL of Matrigel gel was placed on the upper surface of the Transwell chamber filter and incubated in a 37°C cell incubator for 30 min to allow complete polymerization. We added 500 μL of complete medium containing 10% FBS to a 24-well plate, placed the Transwell chamber in a 24-well plate, and added 200 μL of PC3M cells resuspended in serum-free DMEM medium to the upper chamber to continue the culture. After 48 h, the medium in the upper chamber of Transwell was discarded, and the cells that failed to pass were wiped off with a sterile cotton swab. After fixation with methanol and crystal violet, the microscope was inverted (manufactured by Olympus, model: IX51) and counted. The number of perforated cells in each field of view was calculated and the average value was calculated.

*Statistical analysis*

Statistical analysis was performed using SPSS 18.0. The measurement data were expressed as mean ± standard deviation (SD). The comparison between the measurement data of the groups was analyzed by student t test. P < 0.05 was considered statistically significant.

## Results

*A targeted regulation relationship between miR-486 and Gli1*

Bioinformatics analysis revealed a complementary binding site between miR-486 and the 3'-UTR of Gli1 mRNA (**Figure 1A**). The dual luciferase gene reporter assay showed that transfection of miR-486 mimic significantly reduced the relative luciferase activity in pGL3-Gli1-WT-transfected HEK293T cells, while transfection of miR-NC or miR-486 mimic did not have a sig-

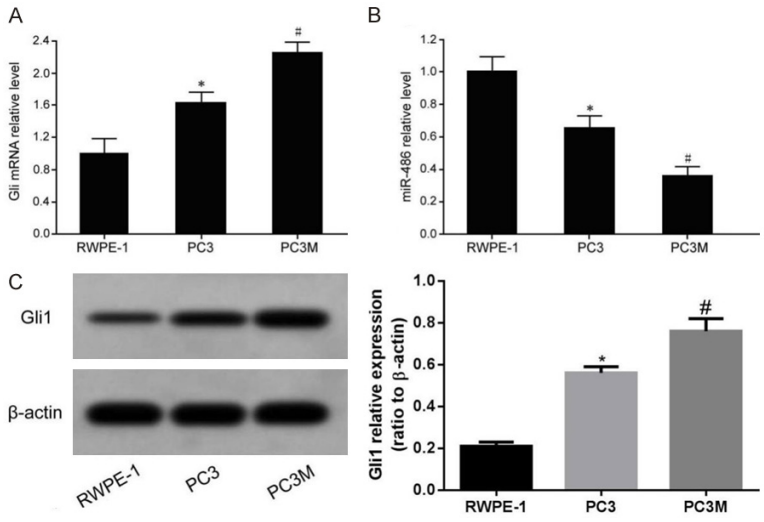


**Figure 2.** Abnormal expression of Gli1 and miR-486 in prostate cancer cells, and is related to cell transfer ability. A. qRT-PCR detection of intracellular Gli1 mRNA expression. B. qRT-PCR detection of intracellular miR-486 expression. C. Western blot analysis of intracellular Gli1 protein expression. *P < 0.05 compared with RWPE-1 cells; #P < 0.05 compared to PC3 cells.

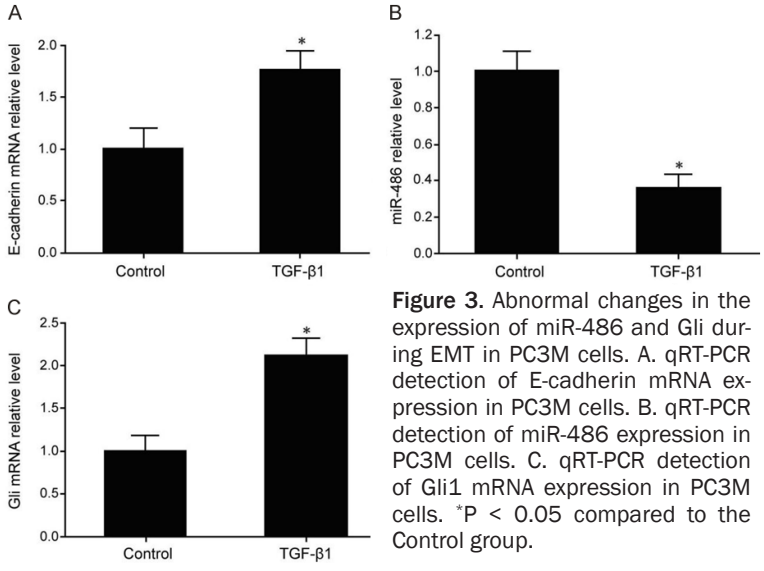


**Figure 3.** Abnormal changes in the expression of miR-486 and Gli during EMT in PC3M cells. A. qRT-PCR detection of E-cadherin mRNA expression in PC3M cells. B. qRT-PCR detection of miR-486 expression in PC3M cells. C. qRT-PCR detection of Gli1 mRNA expression in PC3M cells. *P < 0.05 compared to the Control group.

nificant effect on the relative luciferase activity in HEK293T cells transfected with pGL3-Gli1-MUT (**Figure 1B**), indicating a targeted regulatory relationship between miR-486 and Gli1 mRNA.

*Abnormal expression of Gli1 and miR-486 in prostate cancer cells*

The results of qRT-PCR showed that the expression of Gli1 mNRA in prostate cancer PC3 and PC3M cells was significantly higher than that in human normal prostate epithelial RWPE-1 cells (**Figure 2A**), while the expression of miR-486 was significantly decreased (**Figure 2B**). The expression of Gli1 mNRA in high metastatic PC3M cells was higher than that in low metastatic PC3 cells, while the expression of miR-486 was lower than that in low metastatic PC3 cells. Western blot analysis showed that the expression of Gli1 protein in human normal prostate epithelial RWPE-1 cells, prostate cancer PC3 and PC3M cells was significantly decreased, and the expression of Gli1 protein in PC3M cells was lower than that in PC3 cells (**Figure 2C**).

*Abnormal expression of miR-486 and Gli in EMT of PC3M cells*

The results of qRT-PCR showed that the expression of E-cadherin mRNA was significantly down-regulated during the EMT of PC-3M cells induced by TGF-β1 treatment, indicating that the epithelial properties were reduced (**Figure 3A**). Meanwhile, the expression of miR-486 was significantly decreased during the EMT of PC-3M cells induced by TGF-β1 treatment (**Figure 3B**), and the expression of Gli1 mRNA (**Figure 3C**) was significantly increased.

*Elevated miR-486 inhibits EMT process and invasion of PC3M cells*

The qRT-PCR results showed that the expression of miR-486 was significantly increased in PC3M cells in the miR-486 mimic group compared with the miR-NC group (**Figure 4A**), and the expression of Gli1 mRNA was significantly decreased (**Figure 4B**). The expression of E-cadherin mRNA was significantly increased (**Figure 4B**). Western blot analysis showed that compared with miR-NC, transfection of miR-486 mimic significantly decreased the expression of Gli1 and N-cadherin proteins in PC3M

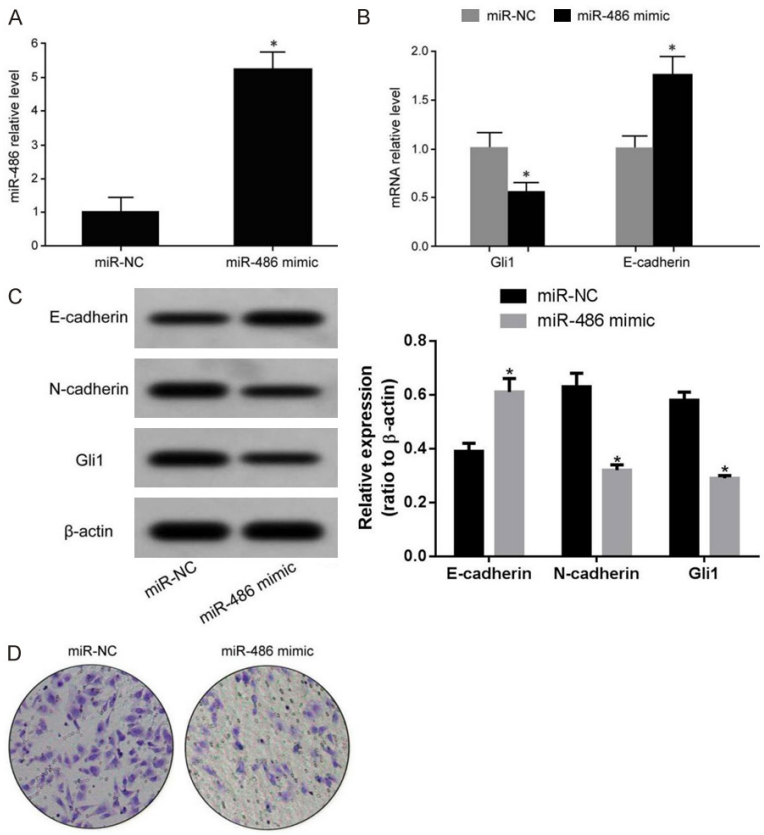


**Figure 4.** Elevated miR-486 reduces Gli1 expression and inhibits EMT process and invasion of PC3M cells. A. qRT-PCR detection of miR-486 expression in PC3M cells. B. qRT-PCR was used to detect the expression of Gli1 and E-cadherin mRNA in PC3M cells. C. Western blot detection of protein expression. D. Transwell assay to detect cell invasion ability. *P < 0.05 compared to their corresponding miR-NC.

cells, and significantly increased the expression of E-cadherin protein (**Figure 4C**). Transwell assays showed that the invasive ability of PC3M cells in the miR-486 mimic transfection group was significantly reduced compared to the miR-NC group (**Figure 4D**).

## Discussion

Prostate cancer has the characteristics of high malignancy, rapid progress and poor therapeutic effect, low survival rate and unsatisfactory prognosis. Therefore, studying the abnormal changes in the pathogenesis of prostate cancer is of great significance in improving the diagnosis, the therapeutic effect, the survival rate as well as the prognosis.

Under physiological conditions, the Hedgehog signaling pathway is usually in an inactive form or has low functional activity. When the Hedgehog signaling pathway is abnormally activated, it is associated with the occurrence, progression and metastasis of various tumors such as gastric cancer [16], bladder cancer [17], non-small cell lung cancer [18], and pancreatic cancer [19]. The Hedgehog signaling pathway includes the extracellular signal ligand HH, the transmembrane protein receptor Ptch, another transmembrane protein Smoothened (Smo), an intermediate delivery molecule, the transcription factor Gli and its downstream target genes. When the Hedgehog signaling pathway is inactivated, Ptch binds to Smo and inhibits its activity, causing phosphorylation of Gli and subsequent ubiquitination degradation, thereby failing to enter the nucleus in full-length form and regulating the transcription and expression of downstream target genes. When the Hedgehog signaling pathway is activated, HH ligand binds to the transmembrane protein receptor Ptch, which abolishes the inhibitory effect of Ptch on Smo, which in turn invalidates the degradation of Gli and enters the nucleus in full length to promote the transcription and expression of target genes, thereby promoting cell proliferation, migration, malignant transformation, leading to tumors [20]. The abnormal expression and function of Gli1 is associated with abnormal Hedgehog signaling pathway, and plays an important role in the occurrence and progression of several tumors such as breast cancer [9], cervical cancer [10], and lung cancer [11]. Studies have found that elevated expression and functional activity of Gli1 is associated with prostate cancer [12]. A number of studies have shown that the expression of miR-486 is decreased in multiple tumors such as non-small cell lung cancer [21], colorectal cancer [22], cervical cancer [23]. In recent years, studies have found that abnormal expression of miR-486 is associated with prostate cancer

[15]. This study investigated whether miR-486 plays a role in regulating Gli1 expression and affecting the EMT process and invasion ability of prostate cancer cells.

In this study, the dual luciferase gene reporter assay showed that transfection of miR-486 mimic significantly reduced the relative luciferase activity in pGL3-Gli1-WT-transfected HEK293T cells, but did not have significant effect on the relative luciferase activity in pGL3-Gli1-MUT-transfected HEK293T cells, confirming the targeted regulatory relationship between miR-486 and Gli1. The results of this study showed that compared with human normal prostate epithelial RWPE-1 cells, the expression of miR-486 in prostate cancer PC3 and PC3M cells was significantly decreased, while the expression of Gli1 was significantly increased. The abnormal expression of miR-486 and Gli1 was significantly higher in highly metastatic PC3M cells than that in low metastatic PC3 cells. The results showed that the decreased expression of miR-486 may play a role in up-regulating Gli1 expression and prostate pathogenesis, and this regulation is involved in the regulation of prostate cancer metastasis. Epithelial mesenchymal transition (EMT) is a biological process in which epithelial cells are transformed into mesenchymal cells, which mediate tight junctions between cell-cells and cells-extracellular matrix (E-cadherin). Decreased expression of cadherin is an important marker of EMT process. The EMT process of tumor cells is closely related to tumor progression, metastasis, recurrence and poor prognosis [24, 25]. In this study, TGF-β1 was administered to PC3M cells to induce EMT in PC3M cells, and the expression of miR-486 was significantly decreased in EMT, while the expression of Gli1 was significantly increased, further confirming the targeted regulation between miR-486 and Gli1 is involved in the regulation of EMT processes in prostate cancer PC3M cells. In the study of the relationship between miR-486 and prostate cancer, the sequencing results of Song et al [26] showed that the expression of miR-486 in prostate cancer patients was significantly lower than that of benign prostatic hyperplasia, and the results of qRT-PCR further confirmed the sequencing test results. The results of Zhang et al [15] showed that compared with non-metastatic prostate cancer LNCaP cells, the expression of miR-486 in metastatic prostate cancer C4-2 cells was significantly reduced. Compared with *in situ* prostate cancer tumor tissue, the expression of miR-486 in the metastatic prostate cancer tissue was significantly reduced. In this study, the decreased expression of miR-486 is associated with prostate cancer and high metastatic characteristics, consistent with the results of Song et al [26], and Zhang et al [15].

In this study, miR-486 mimic was further transfected into prostate cancer PC3M cells to observe changes in cell EMT and invasion ability. The results showed that transfection of miR-486 mimic in prostate cancer PC3M cells significantly decreased the expression of Gli, increased the expression of E-cadherin, decreased the expression of N-cadherin, and inhibited the cell EMT process. The ability of cells to invade was also significantly inhibited. Zhang et al [15] showed that the expression of miR-486 in high-metastasis prostate cancer C4-2 cells can inhibit the expression of target gene Snail and inhibit the EMT process of C4-2 cells. The ability to migrate and invade was significantly weakened. In this study, the effect of miR-486 expression in prostate cancer cells was attenuated by decreasing the expression of miR-486 in prostate cancer cells, consistent with the results of Zhang et al [15]. This study combines the targeted regulatory relationship between miR-486 and Gli1, revealing that decreased expression of miR-486 plays a role in up-regulating Gli1 expression and promoting EMT and invasion of prostate cancer cells, while increasing miR-486. The expression can inhibit the expression of Gli1 by targeting, and attenuate the EMT process and invasion ability of prostate cancer cells, which has not been reported in previous studies, and is the innovation of this study. Whether MiR-486 regulates Gli1 is related to prostate cancer invasion in human body is unclear and requires further investigations in the future.

## Conclusion

The decreased expression of miR-486 plays a role in up-regulating the expression of Gli1 and promoting EMT and invasion of prostate cancer cells. Increasing the expression of miR-486 can inhibit the expression of Gli1 and attenuate the EMT process and invasion of prostate cancer cells.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Derong Zhou, Department of Urology, The Second Affiliated Hospital of Shantou University Medical College, Dongxia North Road, Shantou 515000, Guangdong, China. Tel: +86-0754-88915666; Fax: +86-0754-88346-543; E-mail: bizhan389js@126.com

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