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miR-92a promotes proliferation and inhibits apoptosis of prostate cancer cells through the PTEN/Akt signaling pathway

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ABSTRACT

MicroRNAs (miRNAs) play an important role in the development of prostate cancer (PCa). Recent studies have shown that miR-92a expression is significantly increased in various cancers including PCa. However, its specific mechanism in PCa remains unknown. The goal of this study was to investigate the effect of miR-92a expression on the function and mechanism of PCa. PCa cell lines PC-3 and LNCap were transfected with miR-92a inhibitor to reduce the expression of miR-92a, respectively. The cell proliferation, cell viability, apoptosis, cell invasion and migration ability of PCa cells were examined by CCK8 assay, cell cloning, flow cytometry, Transwell assay and scratch assay, respectively. The effects of miR-92a on PTEN/Akt signaling pathway-related factors (PI3k, Akt, p-PI3k, p-Akt, PTEN) were also observed by RT-qPCR and Western blot. Compared with the control group and NC inhibitor group, the viability, cell migration and invasion ability of PC-3 and LNCap cells were decreased and apoptosis was significantly increased after interference with miR-92a expression. In addition, the mRNA and protein levels of PTEN in PC-3 and LNCap cells in the miR-92a inhibitor group were significantly increased, while the phosphorylation levels of PI3K and AKT were significantly decreased. MiR-92a might play a key role in regulating the proliferation, migration and invasion of PCa cells through the PTEN/Akt signaling pathway. Inhibition of miR-92a expression has practical value against PCa and provides ideas for further clinical treatment of patients with PCa.

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KEYWORDS
Mir-92a; prostate cancer; PTEN/Akt; cell proliferation and migration

1. Introduction

Prostate cancer (PCa) is a common malignancies of the male genitourinary system with the highest incidence in male tumors and the second most common cause of cancer-related death in Europe and the USA [1]. It is expected that there will be 174, 650 new PCa cases and 31, 620 deaths in the USA by 2019 [1]. Most patients with PCa present metastases at the time of initial diagnosis, which accounts for one of the reasons for poor treatment outcome and decreased survival rate [2]. Despite the progression in PCa treatment in recent years, the therapeutic efficacy remains unsatisfactory [3,4]. The mortality rate remains high, particularly for patients with metastases, with less than 33% of patients with metastatic tumors survive more than 5 years [5]. According to domestic statistics, the incidence and mortality of PCa is growing rapidly with an aging population, changing dietary patterns and the development of more sensitive diagnostic techniques. In recent years, many clinical epidemiological studies have shown that obesity and high Body mass index (BMI) are associated with the invasion and metastasis of PCa [6]. And obesity is one of the risk factors of PCa, and obese and overweight patients have an increased risk of developing PCa [7]. Therefore, there is an urgent need to discover biomarkers to further understand the regulatory mechanisms of PCa progression and develop new preventive and therapeutic approaches.

Although progress has been made in the study of oncogenes, tumor suppressor genes and related signal transduction pathways in recent years, oncogenesis mechanism is still not fully elucidated due to its complexity [8]. MicroRNAs (miRNAs) are found to be abnormally expressed in most tumors and play a role similar to tumor suppressor genes or oncogenes in tumor development, affecting cell proliferation through differential changes in upstream and downstream gene [9,10]. In addition, researches have confirmed that miRNAs adjust post-transcriptional regulations of various pathway genes including genes of cell cycle progression, cell death, epithelial–mesenchymal transition (EMT), angiogenesis, and tumor cell metabolism and metastasis [11]. For example, Bai et al. found that miR-32 was down-regulated in non-small cell lung cancer and was correlated with the progression and overall survival of patients [12]. Yang et al. showed that miR-506 was downregulated in clear cell renal cell carcinoma and inhibited the growth and metastasis of tumor cells by localizing FLOT1 [13]. Li et al. revealed

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that miR-205 inhibited the development of colorectal cancer by targeting the cAMP response regulatory tumor suppressor element-binding protein 1 (CREB1) [14]. While there is also a relevant literature showing that differential expression of miRNAs in PCa is an indicator in differentiating benign from malignant diseases, and further supporting the role of miRNAs as diagnostic and prognostic biomarkers in PCa [6,15]. However, the biological function and underlying mechanism of miRNAs in PCa await further exploration.

The deletion of phosphatase and tensin homologue gene (PTEN) on human chromosome 10 was first identified as a tumor suppressor in 1997 and later confirmed in following years [16]. PTEN, because of its demonstrated phosphatase activity, can hydrolyze PIP3 to generate PIP2, which intervenes the PI3K/Akt signaling pathway [17,18]. PI3K mediates the reversal of PIP2 to generate PIP3 and thereby activate the activity of a series of downstream signaling molecules, such as the phosphorylation of Akt. Enrinched p-Akt has been proven to promote cell survival, proliferation, growth, and differentiation, and to inhibit apoptosis and promote tumor tissue angiogenesis [19]. Many literature have reported that PTEN gene loses its cancer suppression ability due to genetic mutations in tumors, including PCa. Absence of PTEN fails to inhibit PI3K/Akt signaling pathway, resulting in its increased activity, thus exerting carcinogenic effect [20]. Recently, it has also been reported that miR-92a promotes colorectal cancer cell metastasis through PTEN-mediated PI3K/AKT pathway [21]; it enhances the metastatic potential of lung cancer cells by targeting PTEN-mediated AKT pathway [22]. Therefore, we hypothesize that miR-92a may also affect the biological function of PCa cells through the PTEN/Akt signaling pathway, and this study will investigate this hypothesis to provide new ideas for the treatment of PCa.

2. Materials and methods

2.1. Cell culture

Human PCa cell lines PC-3 and LNCap were purchased from Guangzhou Yeshan Biotechnology Co., Ltd. (Guangzhou, PRC), and cultured in RPMI-1640 medium (Sigma, USA) containing 10% fetal bovine serum (FBS; Sigma, USA) and 1% double antibody (penicillin 100 U/ml and streptomycin 100 μg/m1). And the cells were cultured in a constant temperature incubator at 37 □C and 5% CO_2. The medium was changed every 2 to 3 days. Cells were used for subsequent experiments when they grew to 80–90% density.

2.2. Cell transfection

When the cells density reached about 60%, miR-92a inhibitor (miR-92a group) or NC inhibitor (NC group) transfection was performed. The transfection process was performed in strict accordance with the instructions of Lipofectamine 2000 kit (Thermo Fisher Scientific, USA). Specific groupings are shown below: 1) miR-92a inhibitor group: PC-3/LNCap cells transfected with miR-92a inhibitor; 2) NC group: PC-3/LNCap cells transfected with NC inhibitor; 3) Blank group: PC-3/LNCap cells without transfection. After 6 h, the culture medium containing double antibody and FBS was used to replace the medium and continue the culture. Transfection efficiency was confirmed by real-time PCR (RT-qPCR).

2.3. CCK8 assay

The cells in logarithmic growth phase were digested to prepare single-cell suspension and seeded in 96-well culture plates (5×10^3 cells/well). The cells were subsequently transfected with miR-9a inhibitor and NC inhibitor. After overnight transfection, the 10 μl of CCK-8 solution (CCK-8, Beyotime, PRC) was added to 96-well plates and the plates were incubated for 1 h, followed by detection at 450 nm using a microplate reader (Bio-Rad Laboratory, USA). The experiment was repeated three times. Cell viability (%) = (OD interference group/OD control group 0 h) × 100 (%) [23].

2.4. Flow cytometry

The apoptotic rate was examined by flow cytometry combined with AnnexinV-FITC/PI. PC-3 and LNCap cells were seeded into cell plates at a density of 5×10^4 cells/well and transfected 24 h. After 48 h of transfection, 5 μl of AnnexinV-FITC and 10 μl PI were added after washing three times with PBS. The cells were mixed and reacted at room temperature in the dark for 10 min, and the apoptosis rate was detected on a flow cytometer (Becton Dickinson, USA).

2.5. Colony formation assay

Cells were counted and cultured into 12-well plates (1000 cells/well). Gently rotate the cells in dish by cross method to evenly disperse them, and incubate them at 37 □C and 5% CO_2 for 2–3 weeks. The culture was terminated when clones were visible to the naked eye and washed with PBS. Cells were fixed with 4% paraformaldehyde for 15 min and stained with Giemsa stain. Cell colony formation containing 50 cells or more was counted and recorded.

2.6. Transwell assay

100 μl of diluted Matrigel was vertically added to the center of the chamber bottom of Transwell and incubated at 37℃ to gelatinize. 1×10^5 cells containing serum-free medium were added to the upper chamber

of Transwell, and culture medium containing 10% FBS was added to the lower chamber. Samples were cultured in a constant temperature incubator at 5% CO2, 37°C for 24 h, washed twice with PBS, fixed with 10% formaldehyde for 15 min, stained with 0.1% crystal violet for 30 min, and counted under an inverted microscope.

2.7. Scratch assay

PC-3 and LNCap cells were seeded into 24-well plate at a density of 8×10^4 cells/well, and three duplicate wells were set for each group. The 24-well plate was vertically scratched with a 10 μl disinfection tip, and then washed with serum-free medium for 2 to 3 times. The culture medium was removed and cells without serum were incubated for another 24 h. Images were recorded on a microscope at 0 and 24 h, and the migration rate of the cells was measured by ImageJ software.

2.8. Quantitative reverse transcriptase-PCR (RT-qPCR)

Total RNA was extracted from PC-3 and LNCap cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. And the concentration and purity of RNA were measured with a spectrophotometer. RNA was reverse transcribed to cDNA, which was diluted 4x to prepare the reaction solution according to the instructions, and the qPCR reaction conditions were as following: 95°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. U6 and GAPDH were selected as internal references, and each set of samples was repeated three times. Quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were listed in Table 1.

2.9. Western blot

Total protein from cell lines was extracted with TriZol reagent (Sigma, USA) following the manufacturer's instructions. The total protein concentration was determined by BCA protein assay kit (Bio-rad). Electrophoretic total proteins were heated to 100°C and incubated for 5 minutes before electrophoresis with SDS-polyacrylamide gel (120 v, 100 min). The separated proteins were then transferred into the PVDF membranes. After blocking with 5% skimmed milk, the membranes were incubated with PI3k antibody, Akt antibody, p-PI3k antibody, p-Akt antibody, PTEN antibody, and GAPDH overnight at 4°C. On the second day, after incubation, the membranes were washed three times, and incubated with horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit IgG (catalog number ab6721) secondary antibody (1:10,000) at room temperature for 1 h. After washing with PBS three times, the membrane was visualized by enhanced chemiluminescence (ECL) reagents. Images were subsequently quantified with ImageJ (NIH, USA).

Table 1. Primer sequences of qRT-PCR.

Genes	Primers
miR-92a	5′-GCTGAGTATTGCACTTGTCCCG-3′& 5′-GTGTCGTGGAGTCGGCAA-3′
PTEN	5′-ATCAACAGCCAACAAATACC-3′&5′-TTCTTATCACCGTCACCCT-3′
Akt	5′-ATCAACAGCCAACAAATACC-3′&5′-TTCTTATCACCGTCACCCT-3′
PI3K	5′-ATCAACAGCCAACAAATACC-3′&5′-TTCTTATCACCGTCACCCT-3′
GAPDH	5′-AACGGATTTGGTCGTATTG-3′&5′-GGAAGATGGTGATGGGATT-3′

2.9 Western Blot

2.10. Statistical analysis

All measurements were repeated for at three times. All data were expressed as mean ± standard deviation (SD) and statistically analyzed with SPSS 18.0. ANOVA or Student's t-test was used to evaluate difference between more than two groups or two groups, respectively. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of miR-92a on PCa cells proliferation and activity

After transfection with NC inhibitor and miR-92a inhibitor, CCK8 and colony formation assay were used to examine the proliferation and activity of PC-3 and LNCap. The results showed that the growth rate and cell viability of PC-3 and LNCap cells were significantly decreased after interference with miR-92a expression compared with the NC group ($P < 0.05$), and there was no significant difference between NC group and Blank group (Figure 1A-B). These results confirm that interfering with miR-92a inhibits the proliferation of PCa cells.

3.2. Effect of miR-92a on PCa cells apoptosis

The apoptotic period of PCa cell lines PC-3 and LNCap was detected by flow cytometry using Annexin V-FITC/PI. The results revealed that the apoptotic rate of PC-3 and LNCap cells was significantly higher in the miR-92a inhibitor group compared with the NC group ($P < 0.05$), and there was no significant difference between the NC and Blank groups (Figure 2A-B). These results suggested that low expression of miR-92a could promote apoptosis in PCa cells.

3.3. Effect of miR-92a on PCa cell s migration and invasion

The invasion and migration abilities of PC-3 and LNCap cells after transfection with NC inhibitor and miR-92a

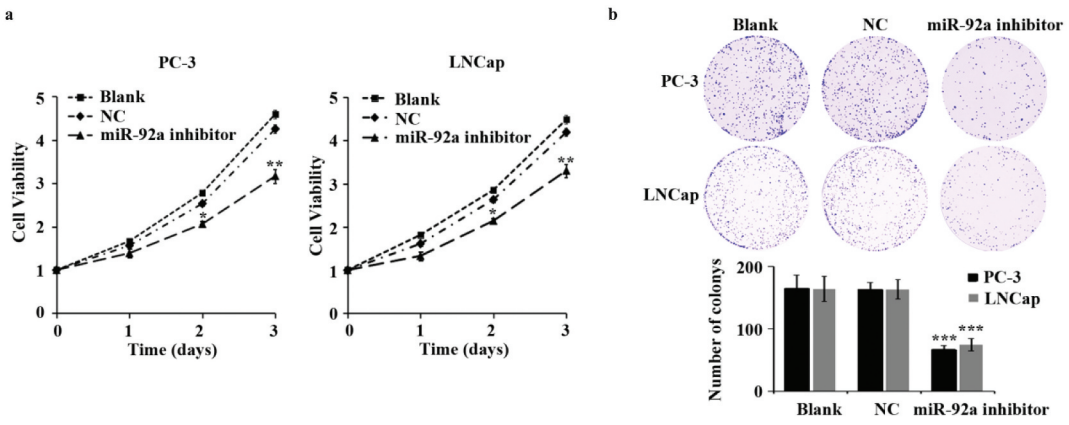

Figure 1. Effect of miR-92a on PCa cell line PC-3 and LNCap proliferation and activity.

(A) CCK-8 was used to detect the cell proliferation rate of PC-3 with LNCap after transfection with NC inhibitor and miR-92a inhibitor; (B) Colony formation assay was performed to detect the cell viability. Compared to NC group, $^{**}P < 0.01$, $^{***}P < 0.001$.

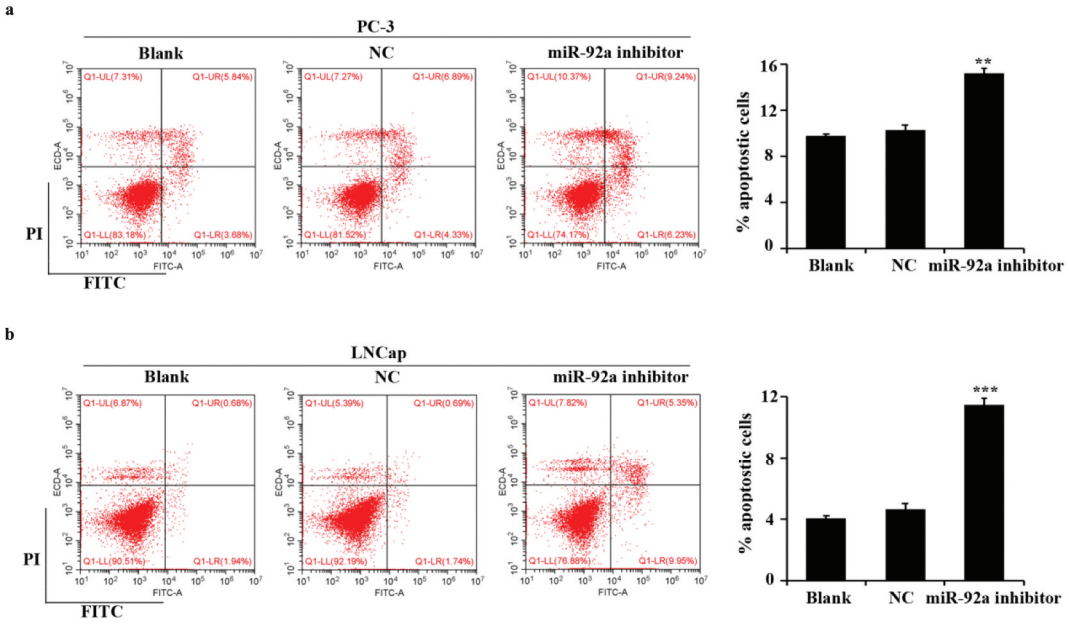

Figure 2. Effect of miR-92a on PCa cell line PC-3 and LNCap apoptosis.

PC-3 and LNCap cells were transfected with NC inhibitor and miR-92a inhibitor respectively after 48 h, Flow cytometry was applied to examine the apoptotic rate of PC-3 (A) and LNCap cells (B). Compared to NC group, $^{**}P < 0.01$, $^{***}P < 0.001$.

inhibitor, respectively, were detected by Transwell and cell scratch assay. The results of Figure 3 A-B showed that the invasion and migration abilities of PC-3 and LNCap cells in the miR-92a inhibitor group drastically elevated compared with those in the NC group ($P < 0.05$), and there was no significant difference between the NC and Blank groups. These results indicate that interference with miR-92a expression reduces the migration and invasive ability of PCa cells.

3.4. Effect of miR-92a on PTEN/Akt signaling pathway in PCa cells

Further we investigated the molecular mechanism of miR-92a inhibition of Pca function. Previous studies have reported that PTEN/Akt signaling pathway is abnormally activated in the development of tumors [24,25]. Therefore, the expression of the related genes of this signaling pathway were detected after transfection of NC inhibitor and miR-92a inhibitor in the PC-3 with LNCap cells. The results (Figure 4 A-D) showed that the mRNA expression levels of miR-92a were significantly decreased, while the mRNA and protein expression of PTEN were increased in the miR-92a inhibitor group compared with the NC group. In addition, the protein expression levels of p-Akt and p-PI3K significantly decreased after interfering with miR-92a expression, and the expression levels of Akt and PI3K were not significantly different. These results suggest that interference with miR-92a

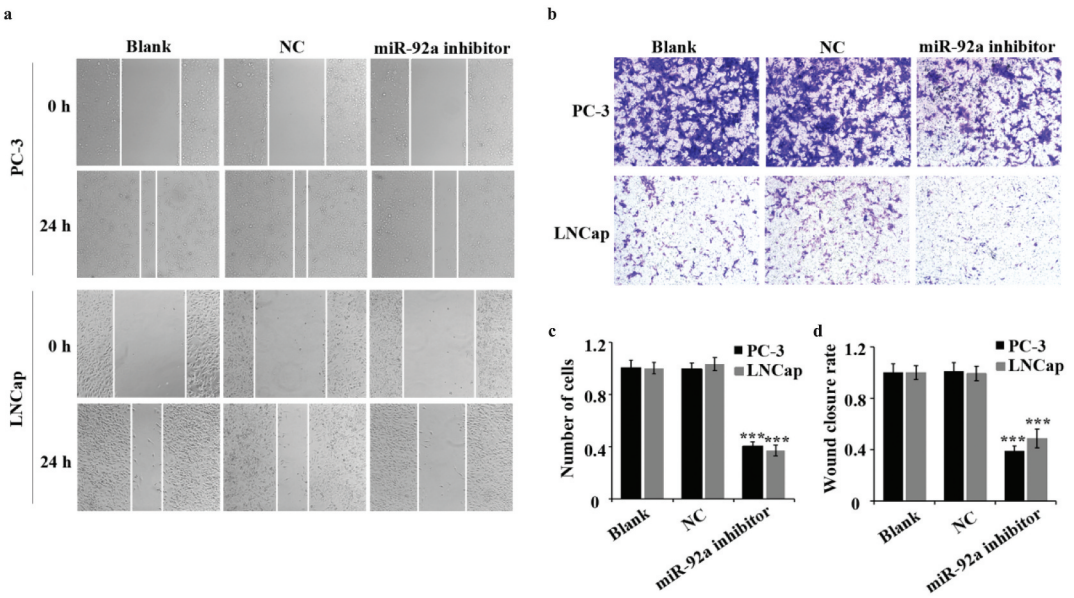

Figure 3. Effect of miR-92a on PCa cell line PC-3 and LNCap migration and invasion.

PC-3 and LNCap cells were transfected with NC inhibitor and miR-92a inhibitor respectively, (A) Transwell assay was applied to investigate the cell migration ability; (B) Scratch assay was used to measure the cell invasion ability; (C) Statistical analysis of transwell and scratch assay. Compared to NC group, ***$P < 0.001$

expression inhibits the activity of PI3K/Akt signaling pathway.

4. Discussion

Because of its high prevalence and mortality, PCa has gradually become the most common malignancy in male urinary system, exceeding lung cancer and gastric cancer in the USA, ranking first [26]. The proliferation, invasion and metastasis of PCa cells are important causes of death in the vast majority of patients [27]. The mechanism of invasion and metastasis of PCa remains unclear.

So far, multiple miRNAs have been demonstrated to be dysregulated in PCa, such as miR-15, miR-16, miR-21, and miR-137, all contributing to PCa development and progression [28,29]. As a member of the miRNA family, miR-92a also plays an important role in a variety of tumors [30–32]. MiRNA-92a is involved in the biological behavior of a variety of tumor cells. For example, miRNA-92a is highly expressed in lung cancer tissues, and the expression level correlates with the degree of malignancy [33]. Hu et al. found that the expression level of miRNA-92a in colorectal cancer was significantly higher than that in adjacent tissues, and was correlated with the pathological stage of cancer and prognosis [34]. MiRNA-92a is highly expressed in human cervical cancer cell tumors, and its invasion is closely related to FBXW7 expression [35]. Although miRNA-92a is well studied in other tumors, it is role in PCa remains under-studied. Therefore, in this study, we found that miRNA-92a was highly expressed in PCa cell lines, suggesting that miRNA-92a may be involved in the development of the disease. To investigate the role of miR-92a in PCa cells, in this study, PC-3 and LNCap cells were transfected with miR-92a inhibitor to reduce the expression. Subsequently, CCK8 assay and flow cytometry revealed that after inhibiting miR-92a expression, cell proliferation was inhibited and apoptosis was increased. While transwell and cell scratch assay results showed that cell invasion and migration were significantly attenuated, indicating that miR-92a may be involved in the pathogenesis of PCa. Similar to the results of this study, Lv et al. revealed that miR-92a can promote colorectal cancer cell proliferation and migration [36], and established miR-92a as a target for colorectal cancer prevention and treatment.

PTEN/Akt signaling pathway is involved in multiple biological processes such as tumor cell proliferation, apoptosis and malignancy transformation. Abnormal activation of this pathway has been confirmed to participate in the occurrence and development of a variety of malignant tumors [37]. PTEN is a tumor suppressor gene with phosphatase activity that negatively regulates P13 K/Akt cell signaling [38]. It was previously shown that PTEN could be a possible target of miR-92a by Targetscan [39]. The study by Xiao et al confirmed that miR-92a has a targeted regulatory effect on the PTEN/Akt signaling pathway [25]. To further investigate whether miR-92a exerts a regulatory effect on PCa cells through the PTEN/Akt signaling pathway, this study examined the expression level of the pathway-related proteins. PTEN protein expression elevated and the expression of p-Akt protein decreased in PC-3 and LNCap cells, confirming that miR-92a negative regulates PTEN/Akt signaling. This outcome verified that miR-92a has an

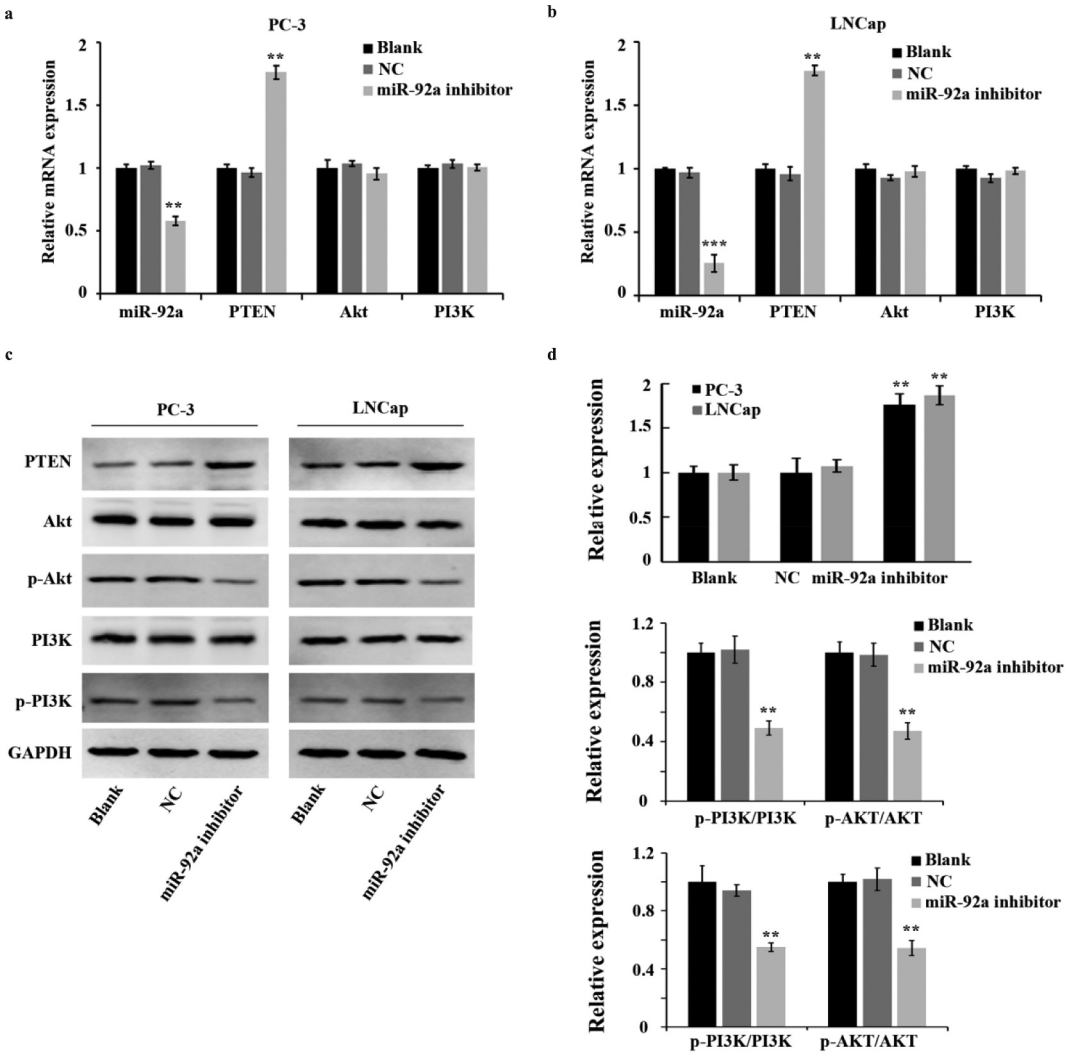

Figure 4. Effect of miR-92a on PTEN/Akt signaling pathway in PCa cell line PC-3 and LNCap.

PC-3 and LNCap cells were transfected with NC inhibitor and miR-92a inhibitor respectively after 48 h, (A-B) RT-qPCR was used to detect the mRNA expression of PTEN, p-PI3K/PI3K and p-Akt/Akt; (C)Western blot was conducted to determine the protein levels of PTEN, PI3K and Akt; (D) Western blot statistical analysis. Compared to NC group, ** $P < 0.01$.

oncogene-like role in PCa, and its abnormal overexpression leads to the effects of proliferation, invasion and migration of cancer cells, as well as the activation of PTEN/Akt signaling by inhibiting PTEN expression.

On the contrary, the present study also has shortcomings. On the one hand, we did not investigate the target genes of miR-92a, and the specific mechanism about miR-92a regulating PTEN/Akt signaling pathway is less clear. On the other hand, the effect of miR-92a on prostate cancer in vivo has not been investigated. All these need to be further explored by cellular and animal experiments.

In conclusion, our study found that miR-92a was highly expressed in PCa, and could promote the proliferation, invasion and migration of PCa cells, inhibit cell apoptosis, and activate the PTEN/Akt signaling pathway, thereby exacerbating the development of PCa. This discovery has important practical implications for miR-92a as a decisive marker and prospective therapeutic target for the diagnosis and treatment of PCa.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

ZYS, MHH and DZ: Participated in study design, helped in results and manuscript drafting; ZYS, YLF and WXL: Contributed in experimental manipulation, data analysis, and statistical analysis; ZYS and YLF: Participated in data collation and manuscript writing; MHH and DZ: Contributed in key revisions of the manuscript. All authors critically reviewed and agreed on the final version of the manuscript.

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