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Original Article

Unregulating miR-3074-3p enhanced cell proliferation ability in prostate cancer by suppressing Axin2

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Abstract: Activation of Wnt/β-catenin pathway promotes development of cancer, Axin2 is the center effector in this pathway. This study is to explore the role of miR-3074-3p in in prostate cancer. In our research, we examined that miR-3074-3p was upregulated in prostate cancer cell lines and tissues. Overexpressing miR-3074-3p promoted, while downregulating miR-3074-3p inhibited the proliferation capacity of prostate cancer cell lines *in vitro*. Moreover, we found that miR-3074-3p can directly targeted to Axin2 3'-UTR to activate the Wnt/β-catenin signaling pathway, and then further to influence the downstream genes of Wnt/β-catenin signaling pathway to enhance malignance of prostate cancer. In summary, our study inferred that miR-3074-3p took apivotal role on prostate cancer progression and may be acted as a novel therapeutic target of prostate cancer.

Keywords: MiR-3074-3p, prostate cancer, proliferation, Axin2, Wnt/β-catenin pathway

Introduction

The Wnt/β-catenin signaling pathway is related with development and morphogenesis of most organs by modulating embryonic induction, polarity of cell division, cell fate and proliferation, and the defects in components of pathway induce tumorigenesis and tumor progression [1]. The β-catenin is thecentral effector in this pathway, and its concentration is controlled by APC-Axin-GSK3, termed as destruction complex [2]. Specially, Axin acts as a scaffold protein and can directly interacted with many proteins of Wnt/β-catenin signaling pathway [3]. There are two homologue of Axin. Axin2, one of them, locates on chromosome 17q24 in human [4]. Resent researches have been demonstrated that Axin2 is mutant in many cancer, such as colorectal cancer, liver cancer and breast cancer and so on. Ishizaki et al. found the mutation of Axin2 changed the Wnt/β-catenin pathway to contribute the progression of hepatocellular carcinomas [5]. Chapman A. and his colleagues demonstrated that the Axin2 gene defects results in the β-catenin accumulation to activate Wnt signaling in adrenocortical carcinomas [6]. Hughes and Brady discovered that the expression of Axin2 mRNA is changed at levels of both total mRNA and relative proportions of alternative 5' untranslated regions in lung and colon cancer [7]. However, the report on specific mechanism of Axin2 in prostate canceris still rare.

Epigenetic regulation is characterized by the regulating gene on transcription and translation levels without the alteration of gene sequence [4]. Gene silence inducing by miRNAs is one of important pattern of epigenetic regulation. miRNA is a group of small non-coding RNA, and about 22 nucleotides in length [8]. miRNAs can directly target to the 3'-untranslated region of mRNA to suppress the expression of target genes [9]. miRNA deregulation can induce body disorder, including cancer. It has been reported that miR-205, miR-34a, miR-222 and miR-15a can bind to Axin2 to regulate cancer progress and development [10-12], but the related studies are limited to date.

In the present study, we detected the expression of miR-3074-3p was exceptional increased in prostate cancer cell lines and tissues. Subsequently, MTT, colony formation and anchorage-independent growth ability assays illustrated that overexpressing miR-3074-3p accelerated the proliferation of prostate cancer line. And then, we found that miR-3074-3p promoted prostate cancer line proliferation by directly binding to the 3'-UTR of the Axin2 mRNA, consequently induced the downstream molecules, including p21, Cyclin D1, c-myc, c-jun, changed. Our research informed that miR-3074-3p may take acritical role on the development and progression of prostate cancer.

Materials and methods

Cell lines and cell culture

Human prostate cancer cells (LNCaP1, PC3, DU145, 2B4, PC-3M IE8, PC-3M-2B4, LnCaP2) and immortalized normal prostate epithelial cells (RWPE-2) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were maintained under 37°C in a 5% CO_2 humidified incubator.

Patients and tissue specimens

8 tumor tissues (T) and adjacent tumor tissues (ANT) were collected at Peking University Shenzhen Hospital from 2014 to 2015. Before collecting, patient informed consent was obtained, and the study was approved by the Institutional Research Ethics Committee.

RNA extraction and quantitative PCR (qPCR)

Total miRNA of cells was extracted with the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction, and mRNA of prostate cancer tissues was extracted with TRIzol (Life Technologies). Then the cDNA was synthesized with 5 ng total miRNA or mRNA. The qPCR was performed using ABI 7500HT system (Applied Biosystems, Foster City, USA). U6 or GAPDH was as reference. And the relative expression was analyzed according to the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay

2×10^3 cells were seeded into 96-well plates. Subsequently, stained on the indicated time by 100 μl sterile MTT (Sigma-Aldrich, St Louis, MO) dye for 4 h under 37°C. Later, the culture mediums were removed, added 150 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich). And the absorbance was quantified under 570 nm wavelength, and 655 nm is as the reference wavelength.

Colony formation assay

0.2×10^3 cells were seeded into 6-well plates. 10 days later, fixed with 10% formaldehyde, and dyed by 1.0% crystal violet for 30 s.

Anchorage-independent growth ability assay

Firstly, put agar mixture (Sigma-Aldrich) containing 1% complete medium onto the bottom of the 6-well plates. Secondly, 3×10^3 cells were suspended with 2 ml complete medium adding 0.3% agar, and put the mixture onto the upper of the 6-well plates. Incubated for 10 days, the cell colonies were measured with an ocular micrometer. Eventually, we counted the colony whose diameter was larger than 0.1 mm.

Vector and transfection

The 3'-UTR of Axin2 contains one binding site of miR-3074-3p from 365 bp to 387 bp. The region of human Axin2 3'-UTR, from 324 bp to 454 bp, was amplified from genomic DNA and cloned into the pGL3-basic dual-luciferase report plasmid (Promega, Madison, WI, USA). The miR-3074-3p mimic's negative control, miR-3074-3p mimic, miR-3074-3p inhibitor negative control and miR-3074-3p inhibitor were purchased from RibiBio (RiboBio Co.Ltd, Guangzhou, China). Transfection was performed with the Lipofectamine 2000 (Invitrogen) according to the protocol.

Western blotting assay

20 μg protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred into PVF membranes (Millipore, Billerica, MA, USA). Probed with antibodies against p21, Cyclin D1, c-myc, c-jun and

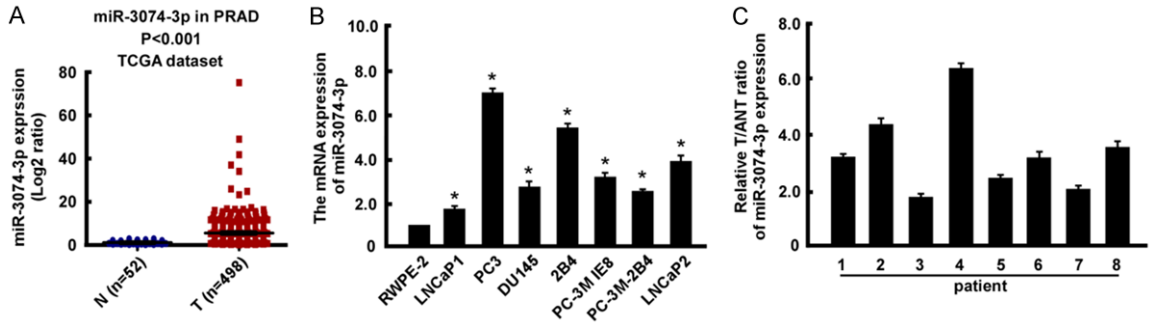

Figure 1. MiR-3074-3p was upregulated in prostate cancer cell lines and tissues. A. The analysis of miR-3074-3p expression of prostate cancer in TCGA dataset. B. The expression level of miR-3074-3p in immortalized normal prostate epithelial cell and prostate cancer cells via qPCR assay. C. The relative T/ANT ratio of miR-3074-3p expression in prostate cancer tissues. Each bar represents the mean ± standard deviation of three independent experiments. (The same below) *P<0.05.

GAPDH (Abcam, Cambridge, MA) overnight at 4°C and GAPDH as a loading control.

Luciferase assay

3.5×10^3 cells were cultured in 24-well plates for 24 h. When the cell confluence reached 60-80%, the cells were transfected with 100 ng pGL3-axin2-3'UTR plasmid, andmiR-3074-3p mimic negative control/miR-3074-3p mimic/miR-3074-3p-mut/miR-3074-3p inhibitor negative control/miR-3074-3p inhibitor, plus 10 ng pRL-TK ranilla plasmid (Promega, Madison, WI). 48 h later, the luciferase activity was measured with the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI).

Statistical analysis

All experiments were repeated three times and the data are presented as the mean ± SD. SPSS 18.0 software (SPSS, Chicago, IL, USA) was used to analyze the significant differences. Student's t-test was used to determine statistical differences. P<0.05 means significant.

Results

MiR-3074-3p expression promoted in prostate cancer cell lines and tissues

By analyzing the miRNA expression datasets in The Cancer Genome Atlas (TCGA), the analysis illustrated that miR-3074-3p was significantly promoted in prostate cancer tissues compared to the normal prostate tissues (Normal: n=52, Prostate cancer: n=498; P<0.001; **Figure 1A**). Then, we confirmed the results in the immortalized normal prostate epithelial cell line (RWPE-2) and prostate cancer cell lines (LNCaP1, PC3, DU145, 2B4, PC-3M IE8, PC-3M-2B4, LnCaP2), and the qPCR assay showed that miR-3074-3p was increased in prostate cancer cell lines compared with the normal prostate cell (**Figure 1B**). Next, to further investigate whether there is the same trend in the prostate tissues, we also performed the qPCR assay in 8 prostate cancer tissues, and the results showed that the miR-3074-3p expression in prostate cancer tissues was sharply enhanced compared to adjacent normal tissues (**Figure 1C**). Together, we examined that miR-3074-3p was upregulated in prostate cancer cell lines and tissues.

MiR-3074-3p overexpression enhanced the prostate cancer cell line proliferation

To further study the biological role of miR-3074-3p in the occurrence and development of prostate cancer, we next transfected the prostate cell line DU145, which expressed the moderate miR-3074-3p, with miR-3074-3p mimic negative control, miR-3074-3p mimic, miR-3074-3p inhibitor negative control and miR-3074-3p inhibitor, respectively. Subsequently, we assayed the Ki-67 positive cells by immunofluorescence; Ki-67 is characterized as a cell proliferative marker. The results showed that Ki-67 positive cells increased dramatically when miR-3074-3p overexpressed, while the Ki-67 positive cell decreased when miR-3074-3p was inhibited, suggesting that miR-3074-3p upregulation maybe promote the proliferation of prostate cell lines (**Figure 2A**). Then, we confirmed the effect of miR-3074-3p on prostate cell proliferation. By means of MTT and colony formation assays, we observed that miR-3074-3p

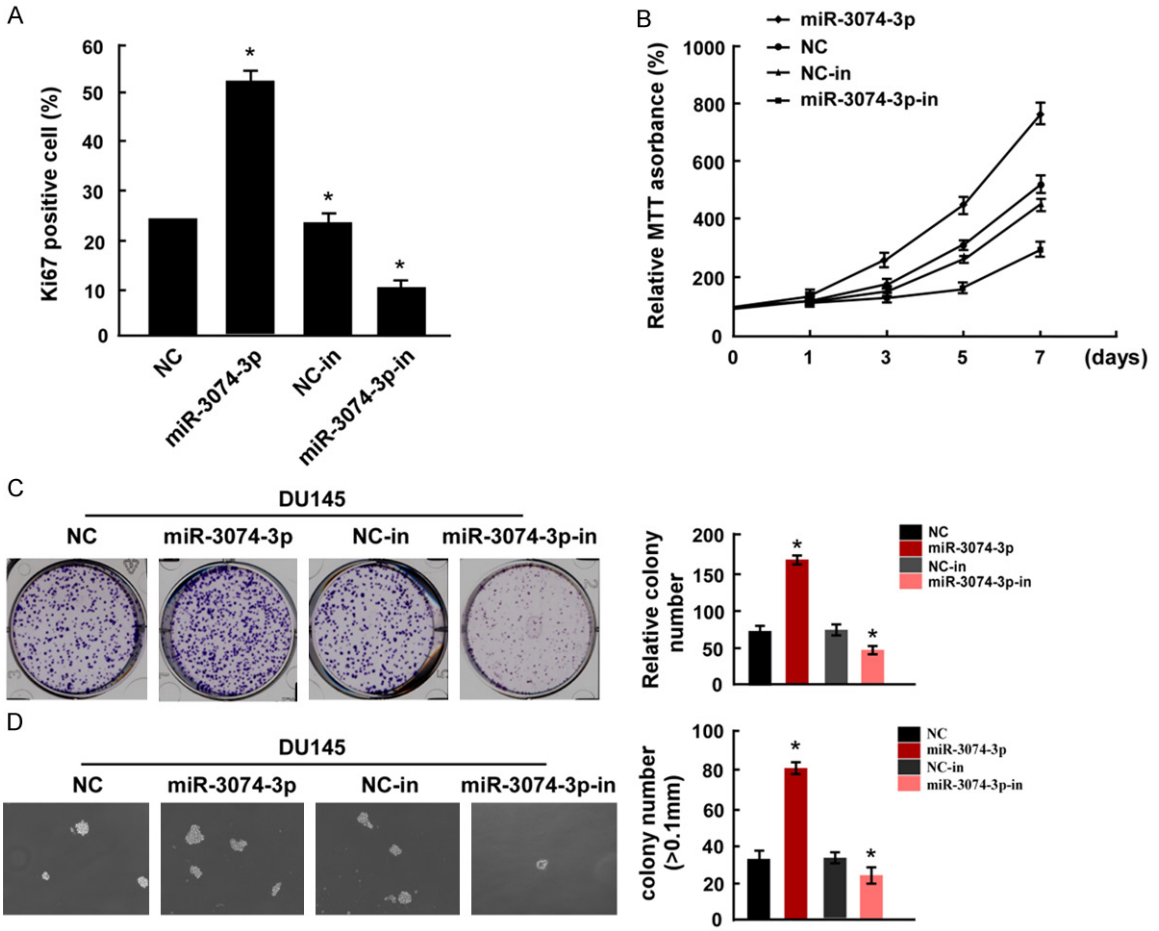

Figure 2. Overexpressing miR-3074-3p promoted, while inhibiting reduced the proliferation ability of DU145 *in vitro*. A. Overexpressing miR-3074-3p promoted, while inhibiting reduced the Ki67 positive cells of DU145 *in vitro*. B. The viability of indicated cells was measured by MTT. C. The representative images of colony formation (left panel) and corresponding colony numbers (right panel) of indicated cells. D. The representative images (left panel) and corresponding colony numbers (>0.1 mm) of anchorage-independent growth in indicated cells.

overexpression significantly enhanced cell proliferation ability and miR-3074-3p down-regulation dramatically reduced cell proliferation ability (**Figure 1B** and **1C**). Besides, ectopically upregulating miR-3074-3p severely promoted, but downregulating miR-3074-3p inhibited anchorage-independent growth capacity of DU145 cell lines (**Figure 1D**), such showed that miR-3074-3p could enhance tumorigenicity of prostate cancer cells *in vitro*. In conclusion, upregualting miR-3074-3p enhanced the proliferation ability and tumorigenicity of DU145 *in vitro*.

MiR-3074-3p directly targeted the 3'-UTR of Axin2 to hyperactivate the Wnt/β-catenin signaling pathway

Furthermore, we investigated the molecular mechanism by which miR-3074-3p enhanced development and progression of prostate cancer. Through publically available algorithms (Miranda, TargetScan) illustrated the Axin2 contains the binding sites of miR-3074-3p theoretically (**Figure 3A**). As predicted, upregulating the miR-3074-3p inhibited, while downregulating promoted the expression of Axin2 protein (**Figure 3B**). Since Axin2 is an important suppressor gene in Wnt/β-catenin, suggesting that the miR-3074-3p may activate the Wnt/β-catenin signaling pathway. Consistent with the predicted, Wnt/β-catenin signaling pathway was hyperactivated when miR-3074-3p overexpressed, but overexpression of miR-3074-3p mutation eliminate the effect, and the pathway was inhibited when the expression of miR-3074-3p was suppressed (**Figure 3C**). Next, we subcloned the Axin2 3'-UTR containing the miR-3074-3p binding sites into the dual luciferase system. Luciferase activity was decreased

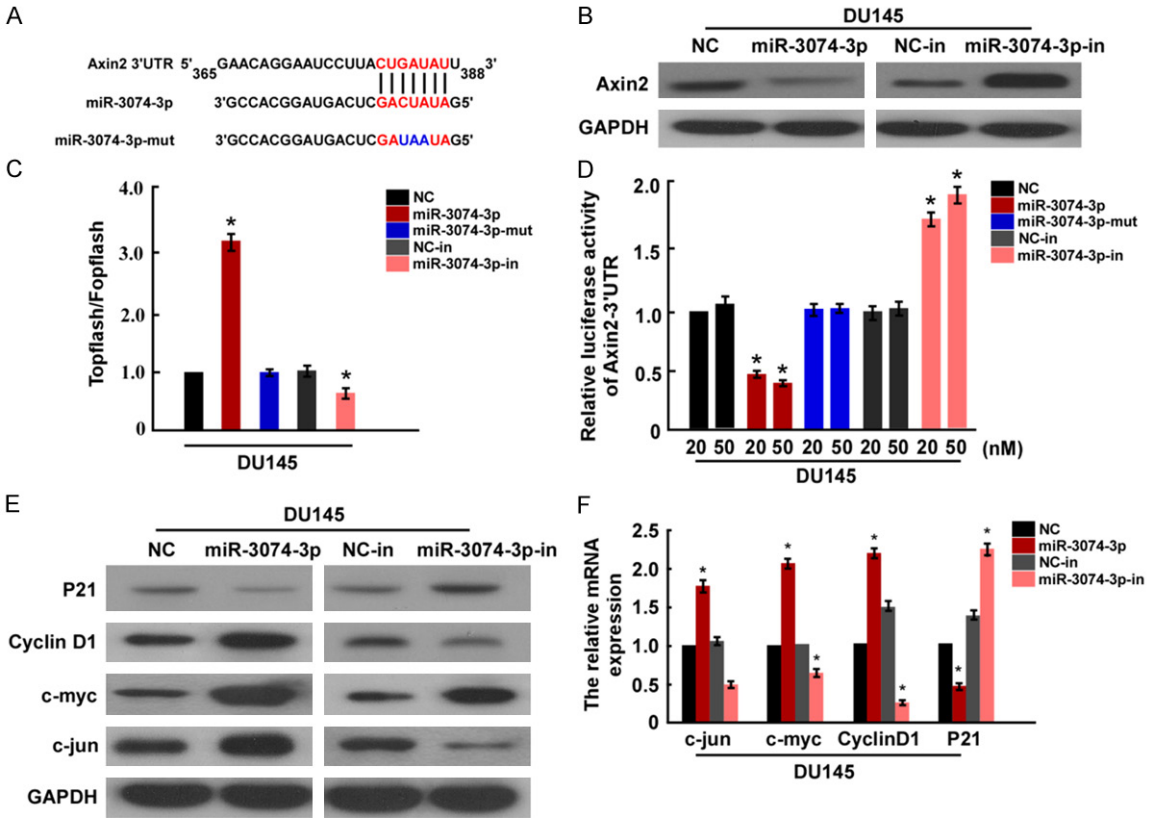

Figure 3. MiR-3074-3p can directly target to the Axin2 3'-UTR to activate Wnt/β-catenin signaling pathway. A. The Axin2 3'-UTR sequences interacting with miR-3074-3p. B. The protein expression in indicated cells by western blotting assay. C. The TOPflash/FOPflash reporter activity in the indicated cells. D. The relative dual luciferase activity in the indicated cells. E. The protein expression of P21, Cyclin D1, c-myc and c-jun, downstream genes of Wnt/β-catenin pathway. F. The expression of P21, Cyclin D1, c-myc and c-jun, downstream genes of Wnt/β-catenin pathway on mRNA level.

when DU145 cell transfected with miR-3074-3p mimic, miR-3074-3p mutation did not influence the luciferase activity, and miR-3074-3p inhibitor promoted the activity (**Figure 3D**). Moreover, we examined the expression of P21, Cyclin D1, c-myc and c-jun, downstream targets of Wnt/β-catenin signaling, on protein and mRNA levels. The results illustrated that the overexpression of miR-3074-3p inhibited P21, but enhanced Cyclin D1, c-myc and c-jun on transcription and translation levels. Whereas, there is the opposite effect when miR-3074-3p inhibited (**Figure 3E** and **3F**). Taken together, the above results inferred that miR-3074-3p overexpression hyperactivated the Wnt/β-catenin signaling pathway by targeting Axin2 in prostate cancer *in vitro*.

There was a negative relation between Axin2 and miR-3074-3p in prostate tissues

To investigate whether the above results were supported by human prostate cancer tissues, we examined the miR-3074-3p expression by qPCR and Axin2 protein expression profile by western blotting in 9 fresh prostate cancer tissues (**Figure 4A** and **4B**), and then we performed the correlation analysis. The correlation analysis demonstrated that the miR-3074-3p expression was negativelyrelated with the expression of Axin2 *in vivo* (**Figure 4C**). Such further strengthened the standpoint that miR-3074-3p mediated proliferation of prostate cancer via inhibiting the expression of Axin2.

Discussion

In the research, we provided evidence for a new notion between miR-3074-3p and Axin2 in prostate cancer. Firstly, we discovered that the miR-3074-3p was dramatically increased in prostate cancer lines and tissues. And then weverified that miR-3074-3p promoted proliferation of prostate cancer cell *in vitro*. Furthermore, we demonstrated that miR-3074-3p can bind to Axin2 3'-UTR to activate the

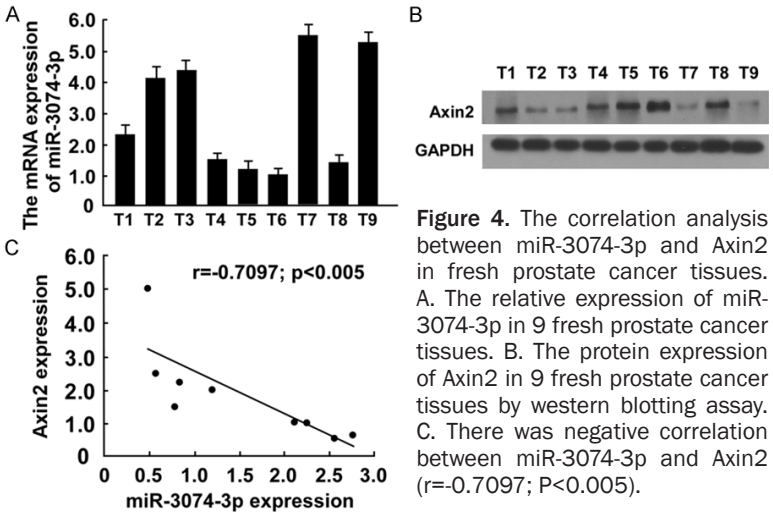

Figure 4. The correlation analysis between miR-3074-3p and Axin2 in fresh prostate cancer tissues. A. The relative expression of miR-3074-3p in 9 fresh prostate cancer tissues. B. The protein expression of Axin2 in 9 fresh prostate cancer tissues by western blotting assay. C. There was negative correlation between miR-3074-3p and Axin2 (r=-0.7097; P<0.005).

Wnt/β-catenin signaling pathway, and there was a negative correlation between miR-3074-3p and Axin2. Together, our research demonstrated that miR-3074-3p functioned as an oncogene in prostate cancer and may be a novel target for clinical therapy in prostate cancer.

Prostate cancer is the second most frequent occurrence cancer in men worldwide, but the molecular mechanism of prostate cancer remains largely unknown [13]. Axin2 is an important scaffold protein in Wnt/β-catenin signaling pathway. The study of Ma et al. showed that the variations of Axin2 in SNP (rs2240308, G/A) played a critical role in the progression of prostate cancer [14]. Hu et al. [15] demonstrated that the expression level of Axin2 closely influenced invasiveness, proliferation and tumor growth in prostate cancer. Wang et al. found that Axin2 upregulated in human prostate cancer cell lines, moreover, adding Dickkopf 1 (DKK1), a Wnt pathway inhibitor, into the culture medium dramatically suppressed the proliferation ability of prostate cancer cell [16]. Content with the previous study, we found that miR-3074-3p was targeted to Axin2 to inhibit its expression and further to activate the Wnt pathway in prostate cancer. Cyclin D1, c-myc, P21 and c-jun are the downstream genes of Wnt/β-catenin pathway [17, 18], and the qPCR and western blotting assays demonstrated that the expression of Cyclin D1, c-myc and c-jun were upregulated, while P21 was reduced when miR-3074-3p overexpressed, such suggested that miR-3074-3p promoted the development of prostate cancer via the Wnt pathway.

Prostate specific antigen (PSA) testing is a means of prostate cancer routine screening, but this means is debated due to high false positives and concerns about frequent overdiagnosis [13, 19]. miRNAs are frequently aberrantly expressed in human cancer, and stable in serum and allow for minimally invasive diagnostic separation of samples, so miRNAs can be acted asdetection markers. In our study, the miR-3074-3p was abnormally expressed in prostate cancer, and it may be as a novel detection marker of prostate cancer.

In summary, our study provided an important link between miR-3074-3p inducing cell proliferation and suppression of Axin2 in prostate cancer. Investigating the precise function of miR-3074-3p in prostate cancer will not only increase our theoretical knowledge but also provide a novel therapeutic strategy.

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Disclosure of conflict of interest

None.

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