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MicroRNA-95-3p promoted the development of prostatic cancer via regulating DKK3 and activating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Previous studies have shown that microRNA-95-3p (miR-95-3p) plays a crucial role in multiple human cancers except for prostatic cancer (PCa). Therefore, the function of miR-95-3p was investigated in PCa in the present work.

PATIENTS AND METHODS: The expression of miR-95-3p was measured by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) assay. Western blot assay was used to examine the protein expression of epithelial-mesenchymal transition (EMT) markers. In addition, the function of miR-95-3p was detected through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assays. Dual Luciferase assay was applied to confirm the relationship between miR-95-3p and dickkopf-3 (DKK3). The tumor growth was observed through xenograft tumor formation assay.

RESULTS: The upregulation of miR-95-3p was detected in PCa tissues and cell lines, which predicted poor prognosis of PCa patients. Moreover, miR-95-3p promoted cell proliferation, migration and invasion in PCa by targeting DKK3 and activating the Wnt/β-catenin pathway. MiR-95-3p also promoted the tumor growth of PCa in vivo. Besides that, downregulation of DKK3 was identified in PCa and low DKK3 expression predicted poor prognosis of PCa patients.

CONCLUSIONS: MiR-95-3p promoted the development of PCa via targeting DKK3 and activating the Wnt/β-catenin pathway.

Key Words:
MiR-95-3p, DKK3, Prostatic cancer, Wnt/β-catenin pathway.

Introduction

Prostate cancer (PCa) is the most common malignant tumor in male reproductive system[1]. PCa ranks sixth in male malignant tumors[2]. Moreover, the incidence of PCa increases with age, and the highest incidence is between 70 and 80 years old[3]. However, family-inherited PCa occurs at an earlier age, and 43% of patients with family-inherited PCa are under 55 years old[4]. The main treatments for PCa include surgery, radiotherapy and chemotherapy. Recently, proton therapy has become one of the most effective and safe treatments for PCa[5]. Despite this, the exact cause, process and mechanism of PCa are far from clear. Therefore, there are no effective concrete measures for the prevention of PCa.

MicroRNAs (miRNAs) act as negative regulators of gene expression by blocking mRNA translation or degradation of the target mRNAs. MiRNAs play an important role in various human cancers including PCa. For instance, miR-141 inhibited cell proliferation and migration and induced cell apoptosis *via* targeting RUNX1 in PCa[6]. Inversely, miR-194 promoted cell metastasis by inhibiting SOCS2 in PCa[7]. Moreover, miR-129 predicted prognosis and inhibited tumor growth in human PCa[8]. These studies suggested that the alternation of miRNAs expression might be involved in the tumorigenesis of PCa. At present, the specific effects of miR-95 on the pathogenesis of human cancers draw our attention. It was reported that the abnormal expression of miR-95 was associated with the radioresistance

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of lung cancer[9]. Moreover, miR-95 had been reported to be upregulated in pancreatic cancer by Real-Time PCR analysis[10]. Functionally, Zhao et al[11] proposed that the downregulation of miR-95 increased the apoptosis of colorectal cancer cells. In addition, the upregulation of miR-95 was implied to promote cell proliferation in human colorectal cancer[12]. Especially, Huang et al[13] demonstrated that miR-95 mediated the radioresistance of PC3 cells *via* targeting SGPP1. However, the specific function of miR-95 associated with the progression of PCa remains unclear and need to be investigated.

Dickkopf-3 (DKK3) is a member of the DKK gene family containing DKK1-4[14]. In addition, the Dickkopf family has been found to be useful as a Wnt modulator[15]. Moreover, DKK3 was downregulated in cervical cancer and acted as a negative regulator of β-catenin[16]. DKK3 was also found to inhibit the invasion and motility of osteosarcoma cells by modulating the Wnt/β-catenin pathway[17]. Besides that, several miRNAs have been identified to direct target DKK3 in different cancers. For example, a miR-92b inhibitor promoted apoptosis of glioma cells by targeting DKK3[18]. Haug et al[19] found that miRNA-92 could inhibit secretion of the tumor suppressor DKK3 in neuroblastoma. Furthermore, MYCN was found to be a novel oncogenic target in adult B-ALL that activated the Wnt/β-catenin pathway by inhibiting DKK3[20]. To the best of our knowledge, the abnormal expression and function of DKK3 are unclear and need to be elucidated in PCa.

In this study, changes in miR-95-3p expression were observed in PCa. In addition, the function of miR-95-3p and its target genes were systemically investigated in PCa. These findings will help develop and provide therapeutic targets and effective prognostic markers for PCa.

Patients and Methods

Clinical Tissues

After receiving the signed informed consent, 34 human PCa tissues and adjacent normal tissues were obtained from Wuhan Pu Ren Hospital. None of the patients with PCa received any treatment before surgery. These tissues were frozen in liquid nitrogen and then stored in the -80°C refrigerator for further experiment. This research was approved by the Ethics Committee of Wuhan Pu Ren Hospital, Wuhan, China.

Cell Lines Culture

The DU145, LNCaP, PC3 cells and the human normal prostate epithelium cell line PNT2 were used for this study. These cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) or Sigma-Aldrich (St. Louis, MO, USA). These cell lines were then inoculated into Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and cultured at 37°C with 5% CO_2.

Cell Transfection

The miR-95-3p mimic (5'-TGCTCAATAAATACCCGTTGAA-3'; NC, 5'-TTATCGCCATGTCCAATGAGGCT-3) or inhibitor (5'-UCAACAUCAGUCUGAUAAGCUA-3'; NC, 5'-TTATCGCCATGTCCAATGAGGCT-3), miR-95-3p plasmid and negative control (NC) were obtained from GeneChem (Shanghai, China). They were then transferred to DU145 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturers' protocols.

MTT Assay

MTT assay was performed to measure cell proliferation based on the manufacturer's instructions. 2×10^4 DU145 cells were then plated and incubated in 96-well plates for 0, 24, 48 and 72 h. They were put in an incubator with 5% CO_2 at 37°C. Next, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well for 4 hours (Dojindo, Kumamoto, Japan). Finally, they were detected using a microplate reader (Molecular Devices) at an absorbance of 450 nm.

Transwell Assays

Transwell chambers (8-μm pore size membranes) were used for cell migration and invasion assays. 10% FBS was added to the lower chamber and incubated at 37°C with 5% CO_2. The upper surface with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was then used for cell invasion, where cell migration assay was performed without Matrigel. 2×10^4 DU145 cells were cultured in the upper chamber with serum-free medium. After 24h, the migrated or invaded cells were fixed with methanol and stained with crystal violet. Finally, we counted the number of removed cells using a microscope.

Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the standard method. The PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan) was used for the synthesis of complementary deoxyribonucleic acid (cDNA) following the instructions of the manufacturer. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed by SYBR Green PCR Master Mix on the ABI 7300 system (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as a control for miR-95-3p and DKK3. Their expressions were calculated using the $2^{-\Delta\Delta ct}$ method. The forward and reverse primers of miR-95-3p and U6 are as follows: miR-95-3p F/R, 5'-TGCGGTTCAACGGGTATTTATTG-3'/5'-CCAGTGCAGGGTCCGAGGT-3'; U6 F/R, 5'-TGCGGGTGCTCGCTTCGGCAGC-3'/5'-CCAGTGCAGGGTCCGAGGT-3'. DKK3 F/R, 5'-AGGACACGCAGCACAAATTG-3'/5'-CCAGTCTGGTTGTTGGTTATCTT-3', GAPDH F/R,5'-GGAGCGAGATCCCTCCAAAAT-3'/5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western Blot Analysis

Protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein was then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and incubated with 5% non-fat milk in polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at room temperature. We then incubated the membranes with E-cadherin, N-cadherin, vimentin, and β-catenin, GAPDH primary antibodies (1:1000) (Abcam, Cambridge, MA, USA) overnight at 4°C. After washing, they were incubated with the corresponding secondary antibodies and the protein expression levels were measured by enanched chemiluminescence (ECL; Pierce, Rockford, IL, USA).

Dual Luciferase Assay

The 3'-UTR of wild or mutant type DKK3 was inserted into the pmirGLO Luciferase vector (Promega, Madison, WI, USA) for Luciferase reporter assays. Then, wild-type or mutant 3'-UTR of DKK3 and miR-95-3p mimic were transfected into DU145 cells. Subsequently, Dual Luciferase assay system (Promega, Madison, WI, USA) was used to analyze Luciferase activity.

Xenograft Tumor Formation Assay

We purchased nude mice (6 weeks) from the Shanghai SLAC Laboratory Animals (Shanghai, China). All animal experiments were approved by the Ethics Committee of Southern Medical University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health. 4×10^6 transfected cells with pre-miR-95-3p plasmid or negative control were injected into the oxter flank of nude mice. The tumor volume was observed every 4 days. After 4 weeks, the mice were sacrificed and the tumors were used for further studies.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) and GraphPad Prism 6 (La Jolla, CA, USA). Data were shown as mean ± SD. The relationship between miR-95-3p expression and clinicopathological features of PCa patients was analyzed by χ^2-test. One way ANOVA or Student's *t*-test was used to analyze differences between groups, followed by Post-Hoc Test (Least Significant Difference). Survival curves were plotted using Kaplan-Meier analysis, and log-rank test was used to compare survival differences. Significant difference was defined as $p < 0.05$.

Results

MiR-95-3p Was Upregulated in PCa Tissues

First, the expression of miR-95-3p was examined in PCa tissues by RT-qPCR. We found that miR-95-3p was significantly upregulated in PCa tissues compared to normal tissues (Figure 1A). In addition, the upregulation of miR-95-3p was found to be associated with Gleason score ($p = 0.002$), pathological stage ($p = 0.036$) and lymph node metastasis ($p = 0.031$) (Table I). Besides that, high miR-95-3p expression predicted a poor prognosis in patients with PCa ($p = 0.0274$) (Figure 1B). Therefore, miR-95-3p is thought to be involved in tumorigenesis of PCa.

MiR-95-3p Promoted Cell Proliferation, Migration and Invasion in PCa

Then, the expression of miR-95-3p was also observed in the PCa cell lines. Similarly, the upregulation of miR-95-3p was also identified in DU145, LNCaP and PC3 compared to PNT2 cells

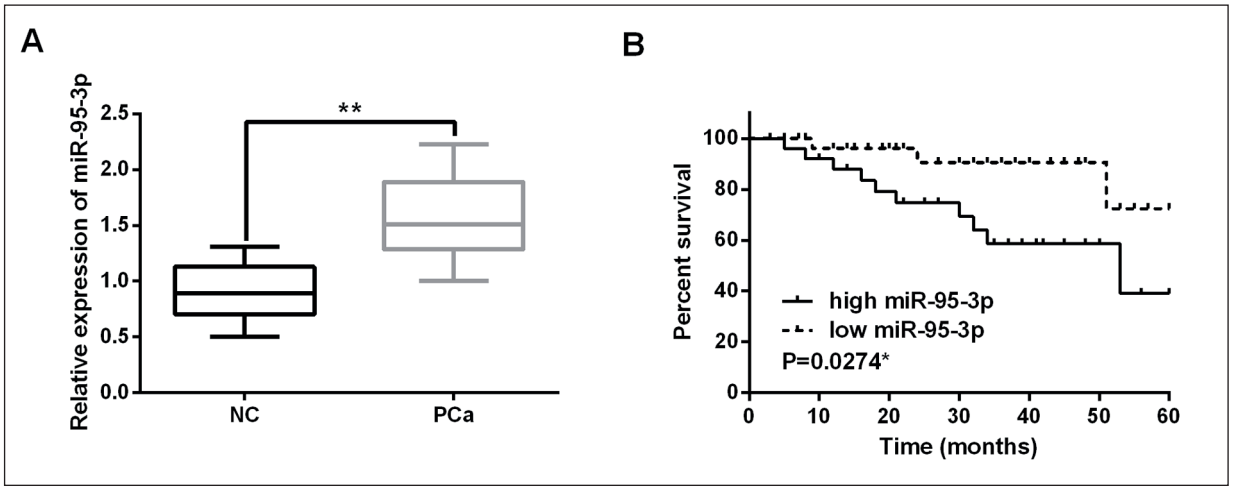

Figure 1. MiR-95-3p was upregulated in PCa tissues. ***A,*** The expressions of miR-95-3p in PCa tissues detected *via* RT-qPCR. ***B,*** High miR-95-3p expression was related to shorter overall survival in PCa patients. *$p < 0.05$, **$p < 0.01$.

(Figure 2A). Next, miR-95-3p mimics or inhibitor was transfected into DU145 cells to investigate its function in PCa. The results of RT-qPCR assay showed that the expression of miR-95-3p was promoted by miR-95-3p mimics and suppressed by the miR-95-3p inhibitor in DU145 cells (Figure 2B). After transfection, the overexpression of miR-95-3p indicated by CCK-8 assay promoted the proliferation of DU145 cells, while knockdown of miR-95-3p suppressed the proliferation (Figure 2C, 2D). In addition, transwell assay showed that the transfection of miR-95-3p mimics accelerated cell migration, when miR-95-3p inhibitor significantly blocked cell migration in DU145 cells (Figure 2E). Consistently, the same function of miR-95-3p was also identified for cell invasion in PCa (Figure 2F). Taken together, the miR-95-3p was found to promote cell proliferation, migration and invasion in PCa.

DKK3 Was a Direct Target of MiR-95-3p in PCa Cells

Next, it was predicted by TargetScan (http://www.targetscan.org/) that DKK3 is a target of

Table I. Relationship between miR-95-3p expression and their clinic-pathological characteristics of PCa patients.

Characteristics	Cases	miR-95-3p		*p*-value
		High	Low	
Age (years)				0.389
≥ 60	18	12	6	
< 60	16	9	7	
Gleason score				0.002*
< 7	20	13	7	
≥ 7	14	9	5	
Pathological stage				0.036*
I + II	24	16	8	
III + IV	10	7	3	
Preoperative PSA				0.261
< 10	15	9	6	
≥ 10	19	13	6	
Lymph node metastasis				0.031*
Negative	12	8	4	
Positive	22	14	8	

Statistical analyses were performed by the χ^2-test. *$p < 0.05$ was considered significant.

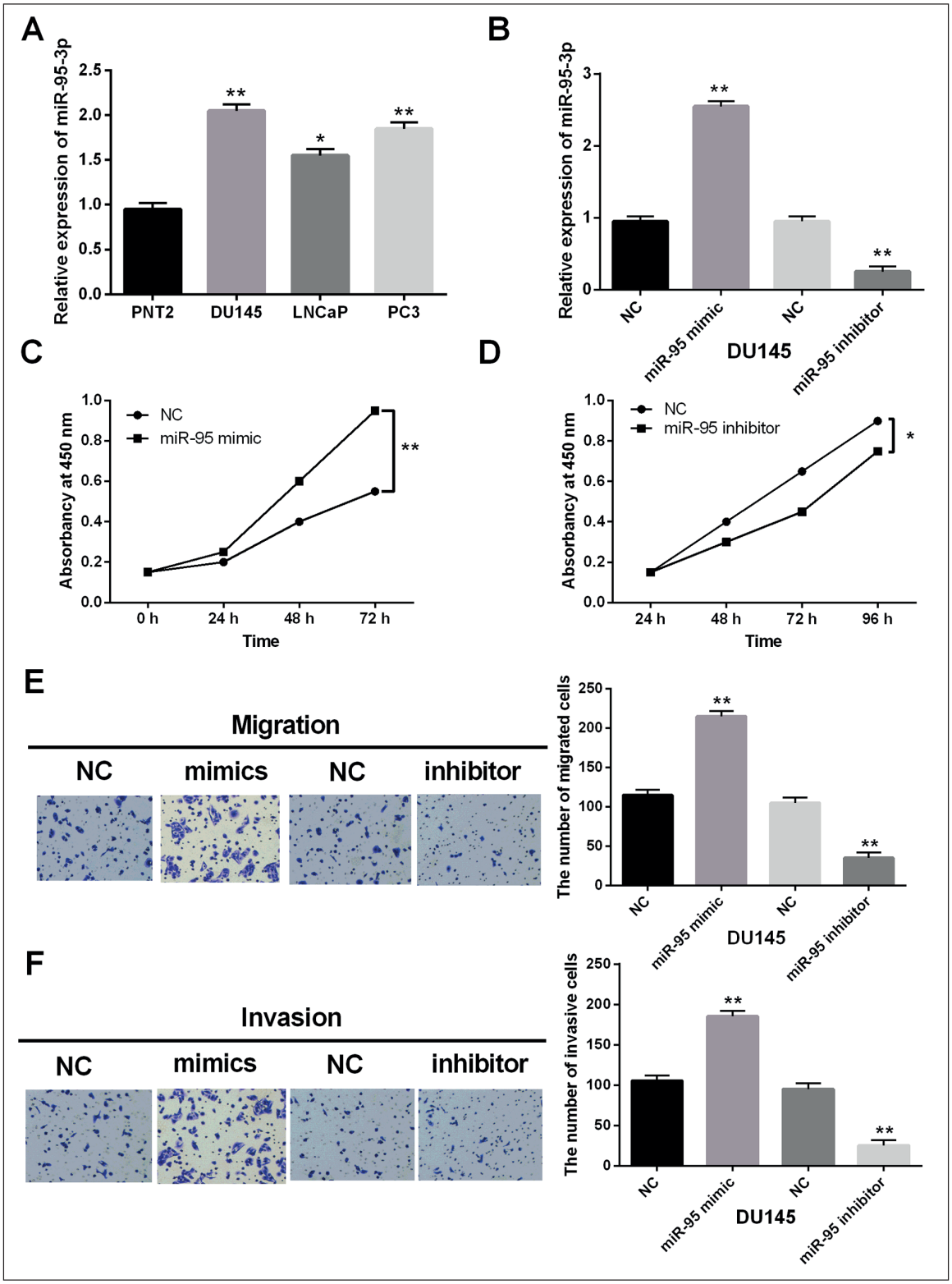

Figure 2. MiR-95-3p promoted cell proliferation, migration and invasion in PCa. ***A,*** MiR-95-3p expression in DU145, LNCaP, PC3, and PNT2 cell lines. ***B,*** The miR-95-3p expression was examined in DU145 cells with miR-935 mimics or inhibitor *via* qRT-PCR. ***C, D,*** The cell proliferation was measured in cells containing miR-95-3p mimics or inhibitor *via* MTT assay. ***E, F,*** Cell migration and invasion analysis in cells containing miR-95-3p mimics or inhibitor were detected by transwell assay. *$p < 0.05$, **$p < 0.01$.

miR-95-3p in PCa cells and that miR-95-3p has a binding site to DKK3, as shown in Figure 3A. Then, we performed a Luciferase reporter assay to verify the prediction. As we predicted, the reduction of Luciferase activity was examined in DU145 cells with miR-95-3p mimics and DKK3-Wt vector. However, the Luciferase activity of DKK3-Mut was not affected by miR-95-3p mimics (Figure 3B). Furthermore, DKK3 expression was negatively correlated with miR-95-3p in PCa tissues (p = 0.004, R^2 = 0.231) (Figure 3C). In addition, the expression of DKK3 was reduced by transfecting miR-95-3p mimics into DU145 cells (Figure 3D) and promoted by the transfection of miR-95-3p inhibitor (Figure 3E). Thus, miR-95-3p directly targeted DKK3 and negatively regulated its expression in PCa.

DKK3 Was Downregulated in PCa

Subsequently, an alternation of DKK3 expression was explored in PCa. We found that DKK3 was downregulated in PCa tissues (Figure 4A). The downregulation of DKK3 was also examined in the PCa cell lines (Figure 4B). Moreover,

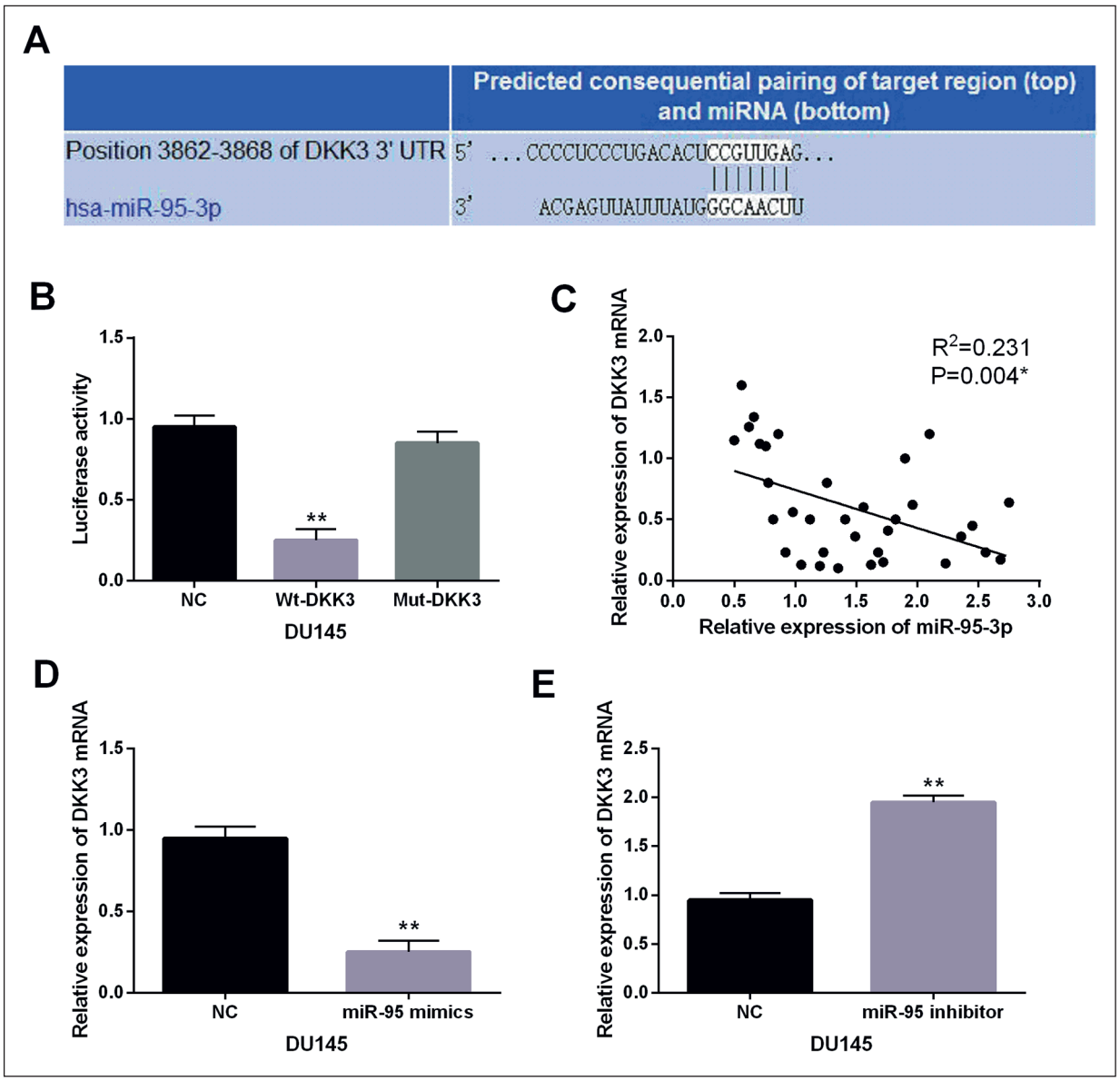

Figure 3. DKK3 was a direct target of miR-95-3p in PCa cells. ***A,*** The binding sites of miR-95-3p on the 3'-UTR of DKK3. ***B,*** Luciferase reporter assay. ***C,*** The correlation between miR-95-3p and DKK3. ***D, E,*** The expression of DKK3 was observed in DU145 cells containing miR-95-3p mimics or inhibitor $^{**}p < 0.01$.

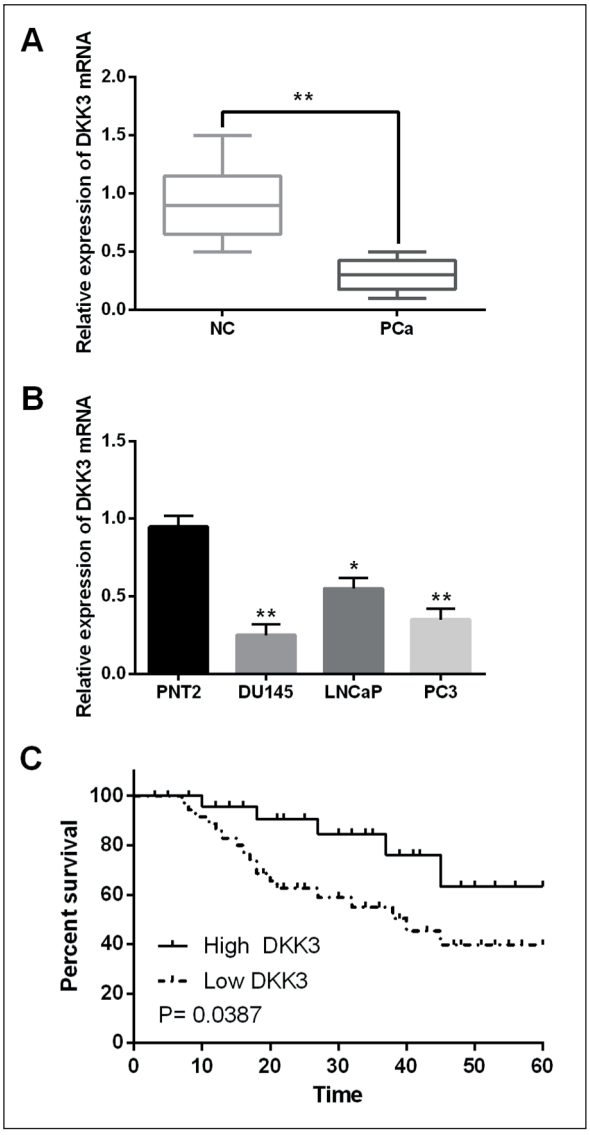

Figure 4. DKK3 was downregulated in PCa. ***A,*** The expressions of DKK3 in PCa tissues detected *via* RT-qPCR. ***B,*** The DKK3 expression in DU145, LNCaP, PC3 and PNT2 cell lines. ***C,*** High DKK3 expression was related to longer overall survival in PCa patients. *$p < 0.05$, **$p < 0.01$.

low DKK3 expression was found to predict poor prognosis in patients with PCa ($p = 0.0387$) (Figure 4C). These results suggest that DKK3 may also be involved in the pathogenesis of PCa.

MiR-95-3p Promoted EMT and Activated Wnt/β-Catenin Pathway in PCa

Furthermore, it is speculated that miR-95-3p regulates the EMT and Wnt/β-catenin pathway to affect the metastasis and proliferation of PCa cells. As we suspected, the overexpression of miR-95-3p suppressed E-cadherin expression and promoted N-cadherin and Vimentin expressions (Figure 5A). Silencing of miR-95-3p had the opposite results (Figure 5B). Thus, the overexpression of miR-95-3p promoted EMT to regulate cell metastasis. Next, the protein expression of β-catenin was detected in DU145 cells with miR-95-3p mimics or inhibitor. The protein expression of p-β-catenin was promoted by upregulation of miR-95-3p and inhibited by the knockout of miR-95-3p (Figure 5A). However, the protein expression of β-catenin was not affected by miR-95-3p. Therefore, miR-95-3p was considered to promote EMT and activate the Wnt/β-catenin pathway to regulate the development of PCa.

MiR-95-3p Promoted the Tumor Growth of PCa

Finally, we subcutaneously injected DU145 cells with miR-95-3p stable transfection plasmid or miR-NC into nude mice. Furthermore, the overexpression of miR-95-3p increased tumor volume compared with the control group (Figure 6A). Moreover, the growth of tumor with miR-95-3p stable transfection plasmid was faster than that of miR-NC (Figure 6B). Therefore, miR-95-3p promoted tumor growth of PCa *in vivo.*

Discussion

In the present study, there is much convincing evidence that miR-95-3p act as a carcinogenic miRNA in the development of PCa. First, the expression of miR-95-3p was increased in PCa, which was associated with shorter survival of PCa patients. Functionally, miR-95-3p was found to promote proliferation, migration and invasion of PCa cells. Of note, miR-95-3p also promoted EMT and activated the Wnt/β-catenin pathway in PCa. In addition, miR-95-3p promoted tumor growth in PCa.

Recently, the upregulation of miR-95-3p has been frequently identified in many human cancers. For example, the expression of miR-95 was increased and induced to proliferation in non-small cell lung cancer[21]. In addition, Huang et al[22] found that miR-95 promoted the proliferation of human colorectal cancer cells and targeted SNX1, which was consistent with our results. Besides that, downregulation of miR-95-3p was also found to inhibit the proliferation and invasion of glioma cells by targeting CELF2[23]. It was also reported that miR-95-3p was a diagnostic and prognostic marker for osteosarcoma. However,

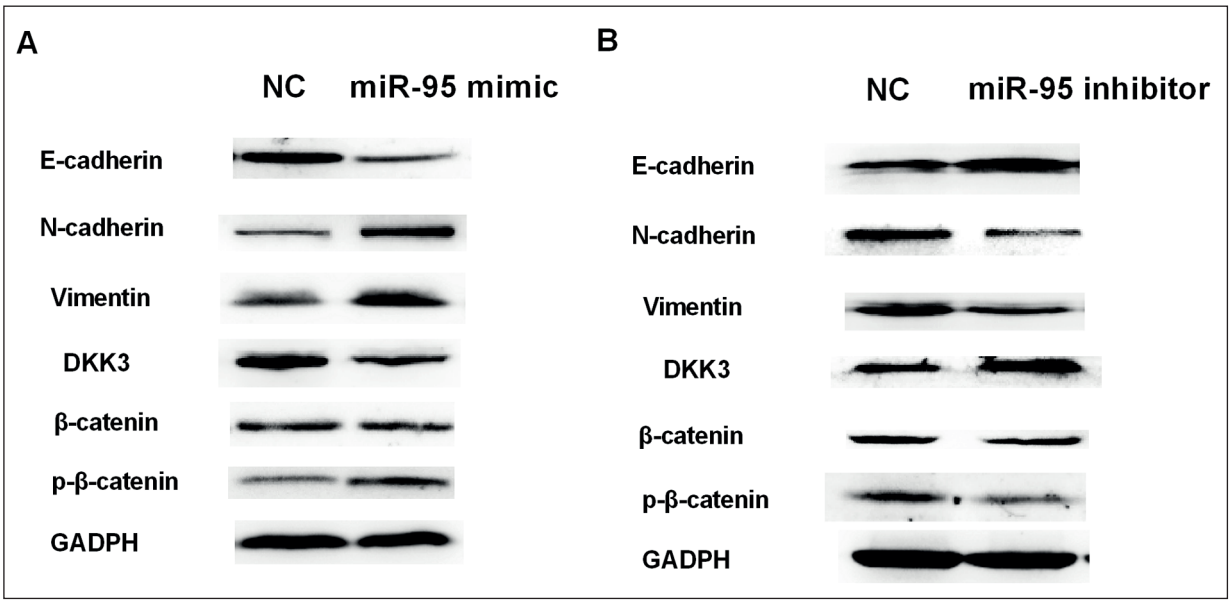

Figure 5. MiR-95-3p promoted EMT and activated the Wnt/β-catenin pathway in PCa. ***A, B,*** Western blot analysis of E-cadherin, N-cadherin, Vimentin, β-catenin, and the p-β-catenin in DU145 cells contained miR-95-3p mimics or inhibitor.

contrary to our results, low miR-95-3p expression predicted poor prognosis in patients with osteosarcoma[24]. Previous studies have shown that miR-95-3p has different roles in different cancers, but miR-95-3p was carcinogenic miRNA in PCa.

MiR-95-3p has been reported to regulate tumorigenesis in human cancers by targeting corresponding genes expression. For instance, the upregulation of miR-95-3p in hepatocellular carcinoma promoted tumorigenesis by targeting p21 expression[25]. In this work, we found that DKK3 is a direct target of miR-95-3p, which was downregulated in PCa. MiR-95-3p promoted the development of PCa *via* regulating DKK3. Moreover, DKK3 has been identified as a direct target gene for several miRNAs, such as miR-92a[26] and miR-92b[27]. MiR-183, proposed by Ueno et al[28], was an oncogene *via* targeting DKK-3 in PCa, which was similar to our findings. Besides that, upregulated miR-25 *via* the WNT/β-catenin pathway targets DKK3 to mediate migration of melanoma cells[29]. In this

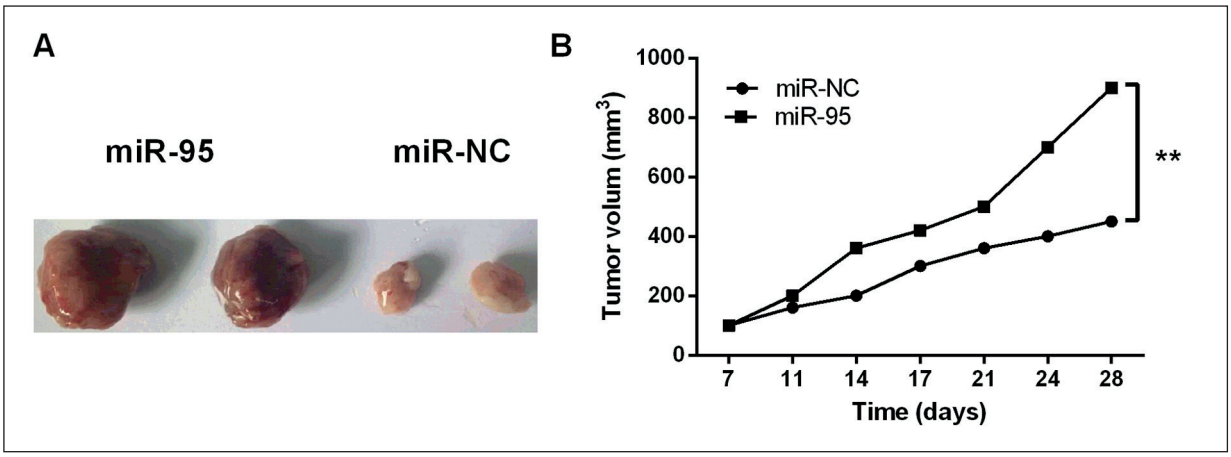

Figure 6. MiR-95-3p promoted the tumor growth of PCa. ***A,*** The tumors formed in nude mice by miR-95-3p stable transfection plasmid or negative control. ***B,*** The growth rate of tumors with miR-95-3p stable transfection plasmid was increased. $^{**}p < 0.01$.

study, miR-95-3p was also found to promote the development of PCa by regulating EMT and the Wnt/β-catenin pathway. Similarly, Guan et al[30] reported that miR-744 promoted PCa progression through aberrant activation of the Wnt/β-catenin signaling. In conclusion, miR-95-3p may be a potential new target for improving PCa diagnostic and therapeutic techniques.

Conclusions

We showed that miR-95-3p was up regulated in PCa tissues and cell lines and predicted a poor prognosis in patients with PCa. In addition, miR-95-3p promoted cell proliferation, migration and invasion *via* modulating DKK3 and activating the Wnt/β-catenin pathway in PCa. These findings will contribute to the diagnosis and treatment of PCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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