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MiR-20a acted as a ceRNA of lncRNA PTENPL and promoted bladder cancer cell proliferation and migration by regulating PDCD4

X.-L. ZHONG[1], L. WANG[1], X. YAN[1], X.-K. YANG[1], H. XIU[2], M. ZHAO[3], X.-N. WANG[4], J.-X. LIU[1]

[1]Urinary Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[2]Nursing and Hospitality Office, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[3]Qingdao University, Qingdao, Shandong, China
[4]Medical Record Management Center, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China

These authors contributed equally to this study and share first authorship

Abstract. – OBJECTIVE: Bladder cancer is the most frequent tumor of the urinary system. Despite variety of new treatment options, bladder cancer remains a main global medical problem. Our purpose was to explore the potential molecular and therapeutic targets of bladder cancer diagnosis.

PATIENTS AND METHODS: The qRT-PCR was used to assess the expression of miR-20a in tissues and cell lines. Counting Cell Kit-8 (CCK-8) assay was carried out to evaluate cell proliferation. Cell migration was calculated using the transwell assay.

RESULTS: The expression of miR-20a increased and PDCD4 decreased in bladder cancer tissues compared with normal tissues. Overexpression of miR-20a promoted T24 cell proliferation and migration, while miR-20a inhibitor suppressed cell proliferation and migration. MiR-20a targeted PDCD4 to regulate its expression in T24 cells. MiR-20a is inversely related to PDCD4 and PTENPL in bladder cancer tissues. Upregulation of PDCD4 suppressed T24 cell proliferation and migration.

CONCLUSIONS: The PTENP1/miR-20a/PTEN axis was involved in the progression of bladder cancer. Our study investigated the function of miR-20a in bladder cancer and provided new insights into the treatment of bladder cancer.

Key Words:
MiR-20a, PDCD4, Bladder cancer, CeRNA.

Introduction

Bladder cancer (BC) is one of the deadliest urothelial malignancies and the ninth most common cancer in the world[1,2]. The incidence and mortality of bladder cancer have gradually increased. Despite improvements in bladder cancer treatment, patients still have a poor prognosis[3,4]. Thus, there is an urgent need to explore new molecular targets for early diagnosis and treatment of bladder cancer.

MicroRNAs (miRNAs), endogenous single stranded non-coding RNAs with 19-22 nucleotides in length, could inhibit the translation of target genes via interacting with the 3'-UTR of mRNAs[5,6]. MiRNAs are carcinogenic or inhibitory in bladder cancers[7-10], such as miR-204, miR-4500, miR-132, and miR-3619. Previous study indicated that miR-20a suppressed tumor apoptosis and promoted tumor proliferation and tumorigenicity in multiple myeloma[11]. MiR-20a regulated cell viability, apoptosis, and autophagy of cervical cancer[12]. Moreover, miR-20a induced cellular radioresistance *via* activating PTEN/PI3K/AKT signaling in HCC[13]. However, the roles of miR-20a in bladder cancer remain unknown.

LncRNAs are a class of RNA transcripts that do not encode proteins greater than 200 nt[14,15]. Thin et al[16] have demonstrated that a series of lncRNAs were involved in multiple processes, including cell viability, metastasis, and carcinogenesis. A variety of lncRNAs have been expected to serve as independent biomarkers for the diagnosis and prognosis in bladder cancers[17-20], including BANCR, LINC00612, DANCR, and FOXD2-AS1. Of note, LncAR-

Corresponding Author: Junxiang Liu, MM; e-mail: beishijijie5725701@163.com

SR promoted cell proliferation, migration, invasion and EMT processes in bladder cancer cells[21]. LncRNA HCG22 suppressed the proliferation and metastasis of bladder cancer cells[22]. Phosphatase and tensin homolog pseudogene 1 (PTENP1) was a pseudogene of PTEN and promotes the binding between miRNAs and the mRNA of PTEN[23]. Hu et al[24] revealed that lncRNA PTENP1 suppressed cell proliferation, invasion, and migration in glioma. In our study, we discovered PTENP1 as a sponge of miR-20a to regulate the expression of PDCD4 in bladder cancer.

Patients and Methods

Patients and Specimens

A collection of paired fresh tumor tissues and adjacent non-tumor samples were obtained from 60 bladder cancer patients in the Affiliated Hospital of Qingdao University. The inclusion criteria of our collected cases were complete medical records, without chemotherapy, radiotherapy, endocrine therapy, and other anti-tumor treatments before surgery. The tissue samples were immediately frozen in liquid nitrogen after surgery and stored in -80°C until use. The study was approved by Ethics Committee of the Affiliated Hospital of Qingdao University and written informed consent has been obtained from all patients.

Cell Culture and Transfection

Human bladder cancer cell lines J82 and T24 and a normal bladder cell line SV-HUC-1 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and were incubated at 37°C in a humidified atmosphere of 5% CO_2.

The miR-20a mimic, miR-20a inhibitor, and NC were obtained from Ribobio (Guangzhou, China). PcDNA3.1-PDCD4, pEX-PTENPL, and sh-PTENPL together with control plasmids were purchased from Genepharma (Shanghai, China). The transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in T24 cells. After transfection, the transfected T24 cells were harvested 48 h.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total mRNAs were extracted from tissues or cell lines using the RNAiso-Plus (TaKaRa, Dalian, China). The first single-stranded cDNA was synthesized from 1 μg RNA using qRT-PCR cDNA synthesis kit (TaKaRa, Dalian, China). Finally, the iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA) was utilized to carry out the Real Time-PCR was on Applied Biosystems 7500 (Thermo Fisher Scientific, Waltham, MA, USA) Sequence Detection System. The primers sequences were listed as follows: miR-20a forward 5'-UAAAGUGCUUAUAGUGCAGGUAG-3' and reverse 5'-CUACCUGCACUAUAAGCACUUUA-3'; U6 forward 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse 5'-CGCTTCAGAATTTGCGTGTCAT-3'; PDCD4 forward 5'-GTTGGCAGTATCCTTAG-3' and reverse 5'-TCCACATCAGTTGTGCTCATTAC-3'; PTENP1 forward 5'-TCAGAACATGGCATACACCAA-3' and reverse 5-TGATGACGTCCGATTTTTCA-3'; GAPDH forward 5'-TGGCTTCATAGGTGACTTCCA-3' and reverse 5'-AAGGACCTGTCTAGGTTTGATGC-3'.

Cell Migration

Transwell assay was conducted to calculate the migratory ability using 8-μm pore size chamber (Corning, Corning, NY, USA) in T24 cells. In the lower 24-well transwell chamber, 600 μl medium containing 20% FBS was added and acted as the chemoattractant. Meanwhile, the upper chamber was appended with 100 μl cell suspension without FBS. Subsequently, the non-migratory cells were removed using the cotton swabs. The migrated cells were fixed with formaldehyde and stained 0.1% crystal violet. The migratory ability was evaluated using a microscope (Leica, Solms, Germany) on counting 5 random fields.

Cells Proliferation

Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) assay was conducted to calculate cell proliferation. T24 cells were seeded in 96-well plate and cultivated at 37°C overnight. Followed, CCK-8 solution was added in each well and the cells were further incubated for 4 h. Finally, the absorbance at 490 nm was assessed using a multifunctional microplate reader.

Luciferase reporter Assay

To investigate the connection between miR-20a, PDCD4, and PTENPL, the wild type or

mutant of PDCD4 or PTENPL and miR-20a mimic were co-transfected in T24 cells. The transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After cultivation for 48 h, the Luciferase activity was measured using Dual-Luciferase assay kit on Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical Analysis

All data analyzed as the mean ± SD are from at least three separate experiments. GraphPad Prism 7 (GraphPad, San Diego, CA, USA) was used for the statistical analyses. The comparison between two or more groups was carried out using Student's *t*-test or One-way analysis of variance (ANOVA) followed by post-hoc test. Spearman's correlation was conducted to analyze the correlation between PTENPL and miR-20a expression. $p<0.05$ was considered statistically significant.

Results

MiR-20a was Overexpressed in Bladder Cancer

To calculate the expression of miR-20a in bladder cancer, the expression of miR-20a was examined in 60 pairs of bladder cancer tissues and normal tissues. Not unfortunately, miR-20a was lowly expressed in tumor tissues compared to normal tissues ($p<0.05$) (Figure 1A). Similarly, downregulation of miR-20a was also detected in two bladder cancer cell lines J82 ($p<0.05$) and T24 ($p<0.05$) *vs.* a normal cell line SV-HUC-1 (Figure 1B).

Overexpression of MiR-20a Promoted Bladder Cancer Cell Proliferation and Migration

To investigate the functional significance of miR-20a in bladder cancer cells, the miR-20a mimic was used to overexpress miR-20a in T24 cells ($p<0.05$) (Figure 2A). Cell viability and migration were calculated using CCK-8 and transwell assays. As expected, the miR-20a mimic promoted the proliferation in T24 cells ($p<0.05$) (Figure 2B). In addition, high level of miR-20a enhanced the migratory capability of T24 cells compared with negative control ($p<0.05$) (Figure 2C).

Downregulation of MiR-20a Suppressed Cell Viability and Migration in Bladder Cancer

Moreover, to decipher the important roles of downregulation of miR-20a, the miR-20a inhibitor was used to knockdown miR-20a in T24 cells ($p<0.05$) (Figure 2A). Cell viability ($p<0.05$) and migration ($p<0.05$) were impeded after transfected with the miR-20a inhibitor (Figure 2B, C). All the results indicated that miR-20a promoted cell viability and migration of bladder cancer.

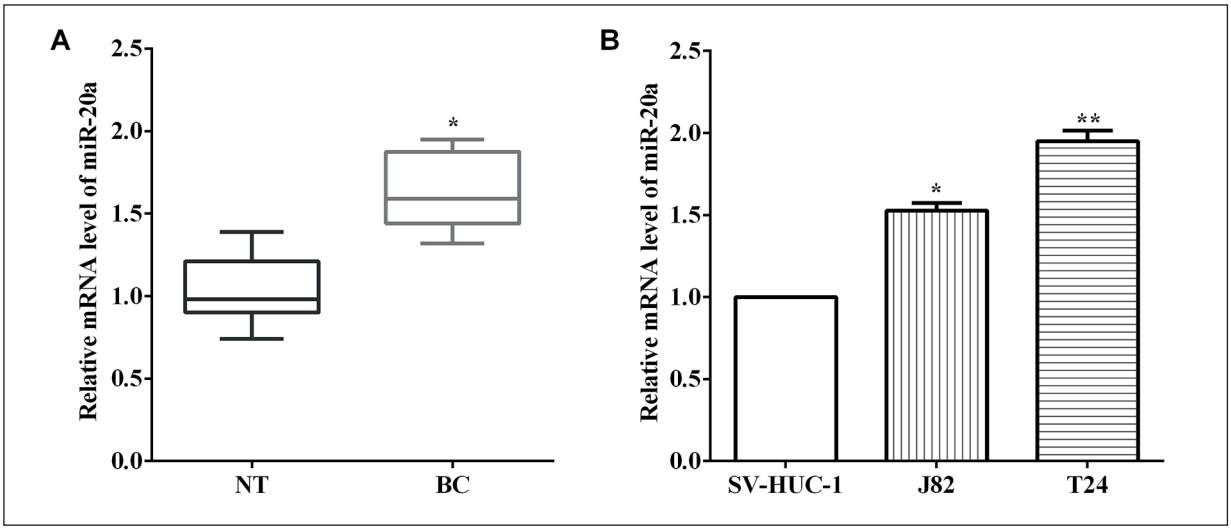

Figure 1. MiR-20a was overexpressed in bladder cancer. **A,** MiR-20a was low expressed in tumor tissues compared to the adjacent tissues. **B,** Downregulation of miR-20a was detected in bladder cancer cells J82 and T24 *vs.* normal cells SV-HUC-1

PDCD4 was verified to be a Direct Target of MiR-20a

TargetScan predicted the potential target genes for miR-20a, and we found that PDCD4 was selected as a candidate gene. To verify that miR-20a targeted PDCD4, mutations were made to the potential binding sequences, as shown in Figure 3A. Luciferase reporter assay analysis was performed to explore the interaction between miR-20a and PDCD4. The results demonstrated that miR-20a mimic reduced the wild type PDCD4 ($p<0.05$) and had no effect on mutant PDCD4 ($p>0.05$) (Figure 3B). Furthermore, the expression of PDCD4 was calculated after altering miR-20a in T24 cells. Not unfortunately, the miR-20a mimic attenuated the expression of PDCD4, whereas miR-20a inhibitor increased the expression of PDCD4 in T24 cells ($p<0.05$) (Figure 3C). The expression of PDCD4 was assessed in tissues and cell lines. As expected, PDCD4 was found to be lowly expressed in 60 bladder cancer tissues compared with the normal tissues ($p<0.05$) (Figure 3D). Thus, we wonder the connection between the expression of miR-20a and PDCD4 in bladder cancer tissues. We found that miR-20a was negatively correlated with PDCD4 in bladder cancer tissues ($p<0.05$) (Figure 3E). Also, PDCD4 was downregulated in bladder cancer cell lines J82 ($p<0.05$) and T24 ($p<0.05$) *vs*. normal cell line SV-HUC-1 (Figure 3F).

PDCD4 Inhibited the Viability and Migration in T24 Cells

To explore the functions of PDCD4 in bladder cancer, pcDNA3.1-PDCD4 was used to overexpress PDCD4 in T24 cells ($p<0.05$) (Figure 4A). Overexpression of PDCD4 attenuated cell proliferation by CCK-8 assay ($p<0.05$) (Figure 4B). On the other hand, transwell assay revealed that transfection of pcDNA3.1-PDCD4 impaired cell migratory ability ($p<0.05$) (Figure 4C). All the findings indicated that PDCD4 suppressed the viability and migration in T24 cells.

PTENP1 Sponging miR-20a and Regulated the Expression of PDCD4 in Bladder Cancer

StarBase was used to predict the ceRNAs of miR-20a, and we discovered that lncRNA PTENP1 sponged miR-20a. To verify that PTEBPL binds to miR-20a, we mutated the potential binding site from CAAUUU to GUGAAA (Figure 5A). Luciferase reporter assay demonstrated that miR-20a mimic reduced the Luciferase activity of wild type PTEBPL, and the Luciferase activity of mutant PTEBPL, revealing that miR-20a directly binds to PTEBPL ($p<0.05$) (Figure 5B). In addition, the expression of miR-20a was evaluated when PTENP1 was changed in T24 cells. As expected, the expression of miR-20a was inhibited by overexpressed PTENP1 ($p<0.05$), and miR-20a was increased after knockdown PTENP1 ($p<0.05$) (Figure 5C). Meanwhile, the expression of PDCD4 was assessed after alteration of PTENPL. Not unfortunately, PDCD4 was enhanced by transfecting with pEX-PTENPL. By contrast, when the knockdown of PTENPL in T24 cells verified, the expression of PDCD4 was also reduced ($p<0.05$) (Figure 5D). Spearman's correlation was conducted to detect the relationship between PTENP1, miR-20a, and PDCD4 in bladder cancer. As we expected, a negative connection was found between PTENP1 and miR-20a in bladder cancer tissues ($p<0.05$) (Figure 5E). The expression of PTENP1 was positively correlated with PDCD4 in bladder cancer tissues ($p<0.05$) (Figure 5F).

Discussion

In this study, it was found that miR-20a was upregulated, while PDCD4 was downregulated in bladder cancer. It was indicated that miR-20a mimic enhanced the viability and metastasis of T24 cells, whereas miR-20a inhibitor exerted an opposite effect. MiR-20a targeted PDCD4 to regulate the expression of PDCD4. MiR-20a has a negative connection with PDCD4 in bladder cancer tissues. PTENP1 targeted miR-20a to regulate PDCD4 expression in T24 cells.

MiRNAs, as post-transcriptional regulators of gene expression, are a variety of small noncoding RNAs with a length of 19-25 nucleotides[25]. MiRNAs are involved in tumor progression in multiple cancer[26,27]. As a new type of promising biomarker, miR-20a enhanced the invasiveness of glioma[28]. In this study, we found that miR-20a was overexpressed in bladder cancer tissues and cells. Liao et al[29] showed that miR-20a regulated cell proliferation, invasion, and apoptosis in glioma. Similarly, miR-20a enhanced the sensitivity of colorectal cancer to cisplatin by increasing the expression of ASK1[30]. Consistent with all the findings, we discovered that upregulation of miR-20a enhanced viability and migration. Conversely, silencing miR-20a suppressed cell

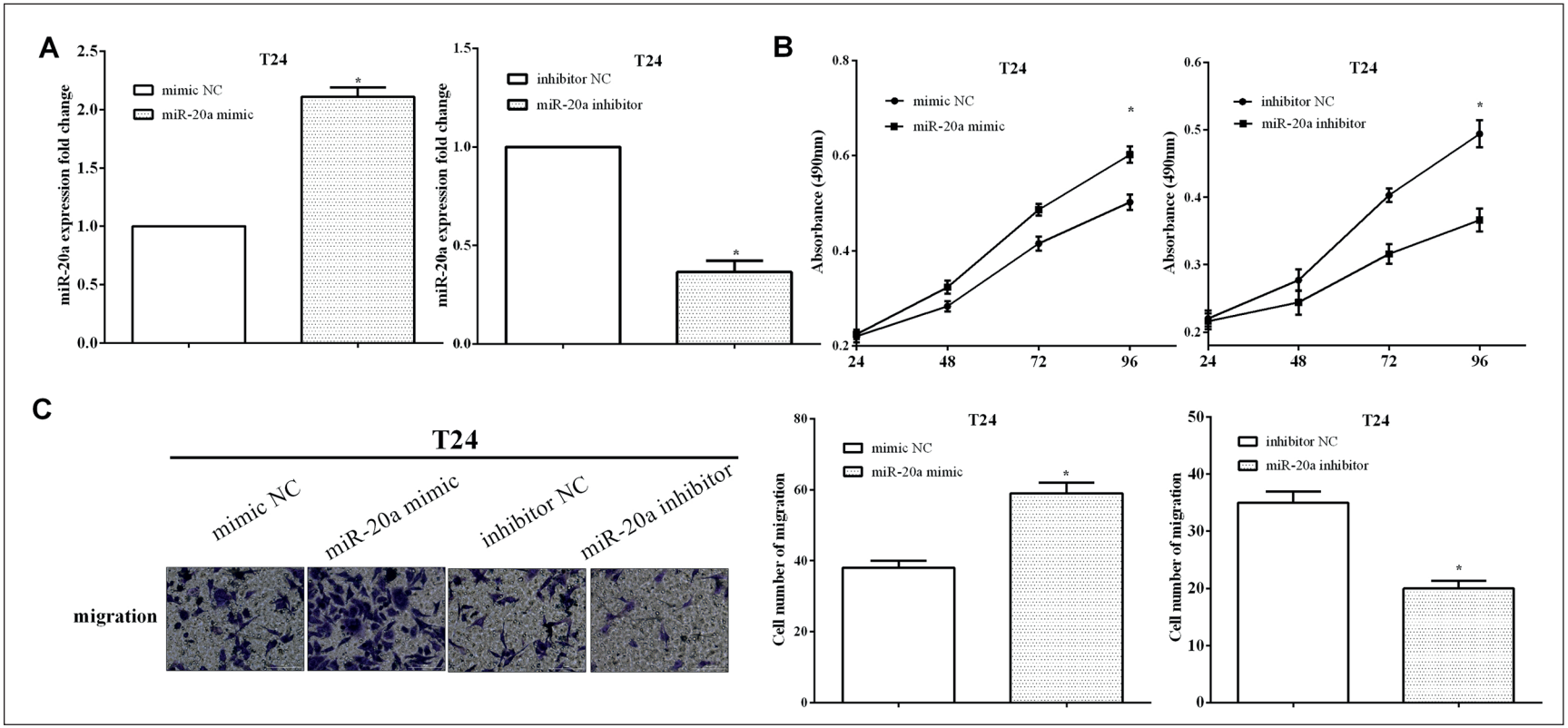

Figure 2. MiR-20a promoted cell proliferation and migration. **A,** The miR-20a mimic and inhibitor was used to overexpress or knockdown miR-20a in T24 cells. **B,** MiR-20a enhanced the proliferation in T24 cells. **C,** MiR-20a enhanced the migratory capability of T24 cells (×200).

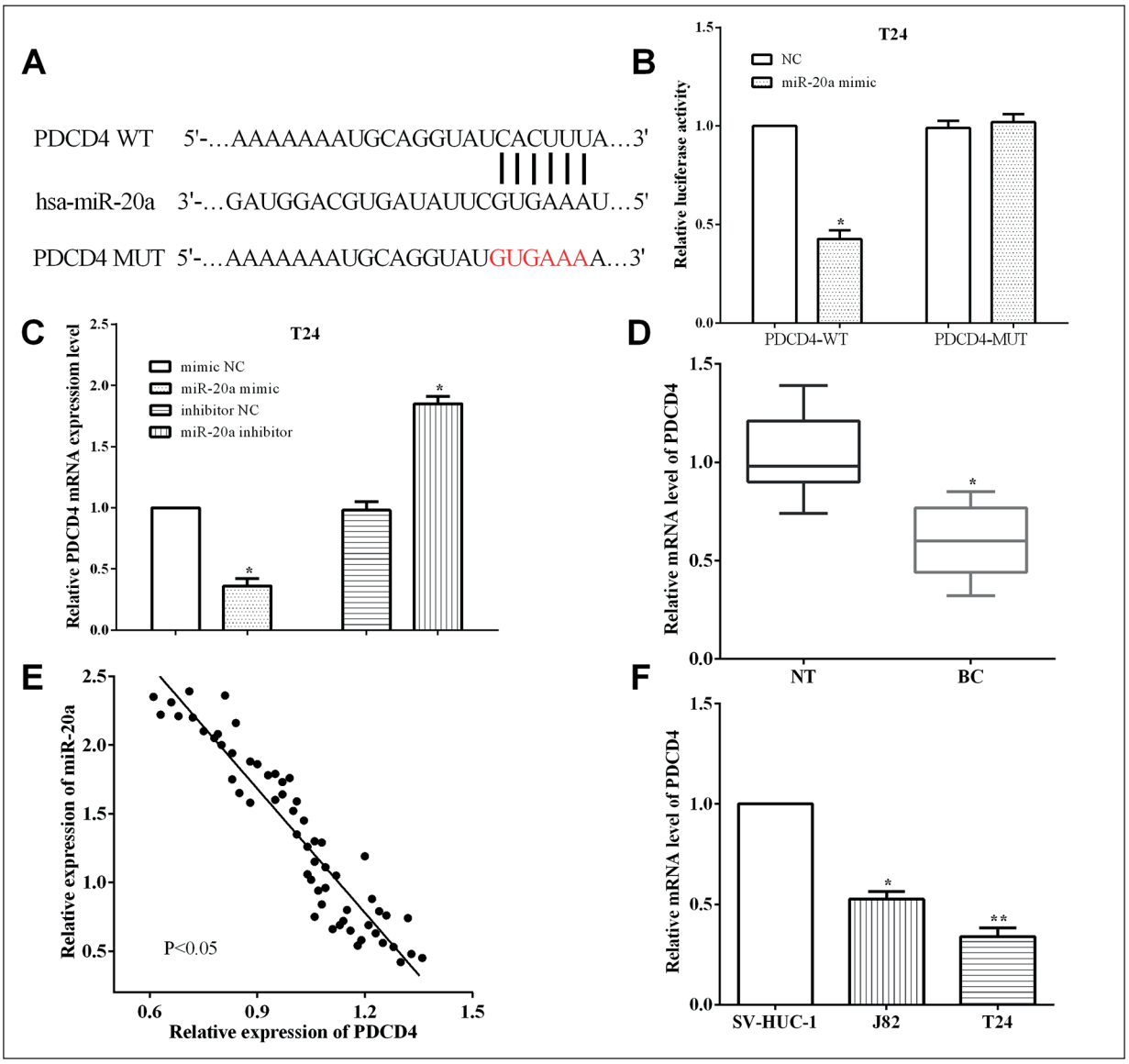

Figure 3. PDCD4 was verified to be a direct target of miR-20a. **A,** PDCD4 was predicted to be a candidate of miR-20a. **B,** MiR-20a mimic reduced wild type PDCD4, and has no effect on the mutated type PDCD4. **C,** MiR-20a mimic attenuated the expression of PDCD4, whereas miR-20a inhibitor increased PDCD4 expression in T24 cells. **D,** PDCD4 was found to be lowly expressed in 60 bladder cancer tissues compared with the normal tissues. **E,** MiR-20a has negative connection with PDCD4 in bladder cancer tissues. **F,** PDCD4 was downregulated in bladder cancer cells J82 and T24 versus normal cells SV-HUC-1.

viability and migration in T24 cells. We first explored the role of miR-20a in bladder cancer. Programmed cell death 4 (PDCD4) is a novel cancer suppressor gene that plays crucial functions in suppressing tumorigenesis[31,32]. In our study, PDCD4 was found to be downregulated in bladder cancer. PDCD4 was a target gene of miR-20a and miR-20a modulated the expression of PDCD4, which was consistent with the findings in colorectal cancer[33]. The expression of PDCD4 had a negative connection with miR-20a in bladder cancer tissues. Tumorigenesis suppressor PDCD4 was related to aromatase inhibitor resistance in estrogen receptor positive breast cancer[34]. Similarly, PDCD4 functioned as a tumor suppressive role and was associated with EMT in gastric cancer[35]. Our data agreed with all these findings, and we found that cell viability and migration were attenuated *via* overexpression of PDCD4. The expression of

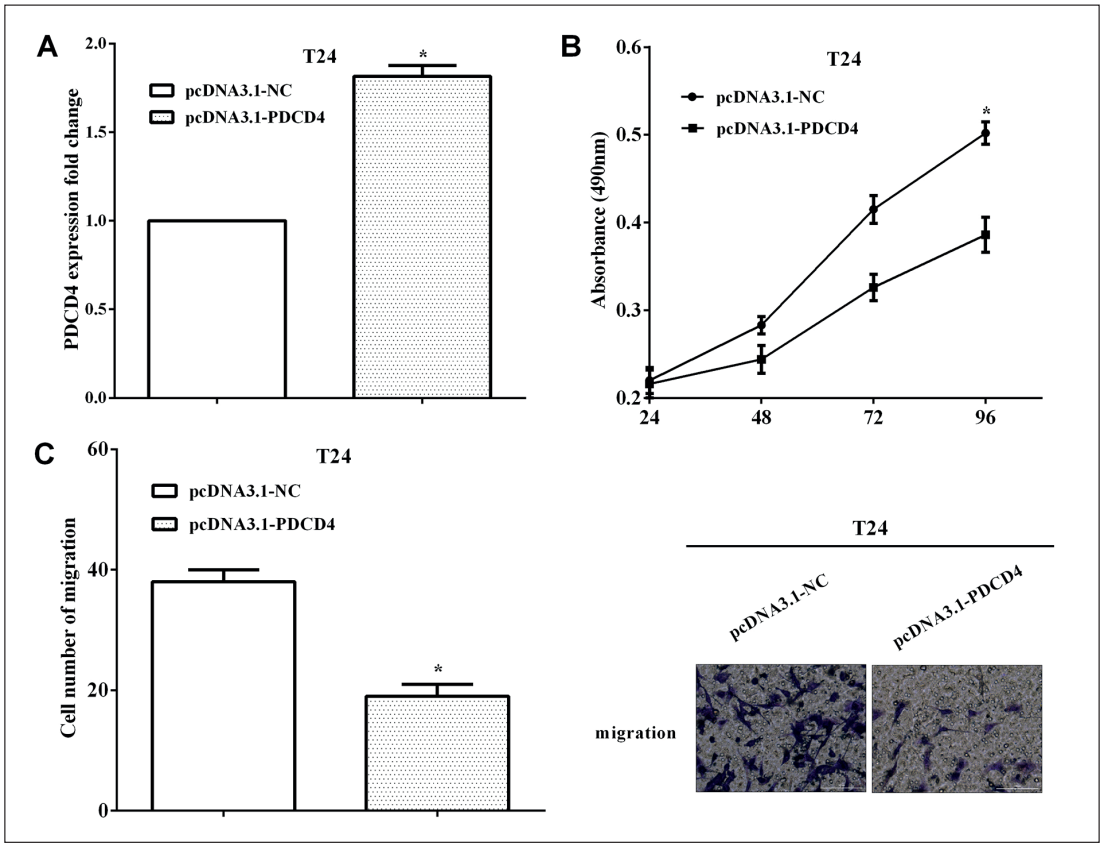

Figure 4. PDCD4 inhibited the viability and migration in T24 cells. **A,** pcDNA3.1-PDCD4 was applied to overexpress PDCD4 in T24 cells. **B,** Overexpression of PDCD4 attenuated cell proliferation. **C,** Transfection of pcDNA3.1-PDCD4 impaired the migratory ability in T24 cells (×200).

PDCD4 was inhibited by miR-20a mimic, while it was enhanced after transfecting miR-20a inhibitor. It had a negative connection between PDCD4 and miR-20a in bladder cancer tissues, which was the first time for us to propose the connection between PDCD4 and miR-20a in bladder cancer. However, due to the limitation of experimental conditions, we cannot detect the effect of PDCD4 on bladder cancer apoptosis, which is a deficiency of this article.

LncRNAs regulated the occurrence and development of cancer through gene silencing and histone modification at post-transcription level, therefore mediating cancer tumorigenesis[36]. LncRNAs modulate gene expression in bladder cancer through the interaction of miRNA and protein[37]. Fan et al[38] indicated that PTENP1 accelerated cell proliferation and slowed down cell apoptosis in cervical cancer. In our study, we discovered that PTENP1 was a ceRNA of miR-20a and was determined by Luciferase reporter assay. The expressions of miR-20a and PDCD4 were regulated by upregulating or downregulating PTENP1. Consistent with the findings in breast cancer, we discovered that PTENP1 sponged miR-20a to regulate PDCD4 expression in bladder cancer[39].

Conclusions

Altogether, these results showed that miR-20a significantly suppressed the viability of bladder cancer cells and reduced cell migration capacity. MiR-20a, serving as a ceRNA of PTENP1, modulated the biological behaviors of bladder cancer cells by mediating the expression of PDCD4. We indicated that miR-20a played a vital function in the development of bladder cancer and may help develop new diagnostic and treatment methods.

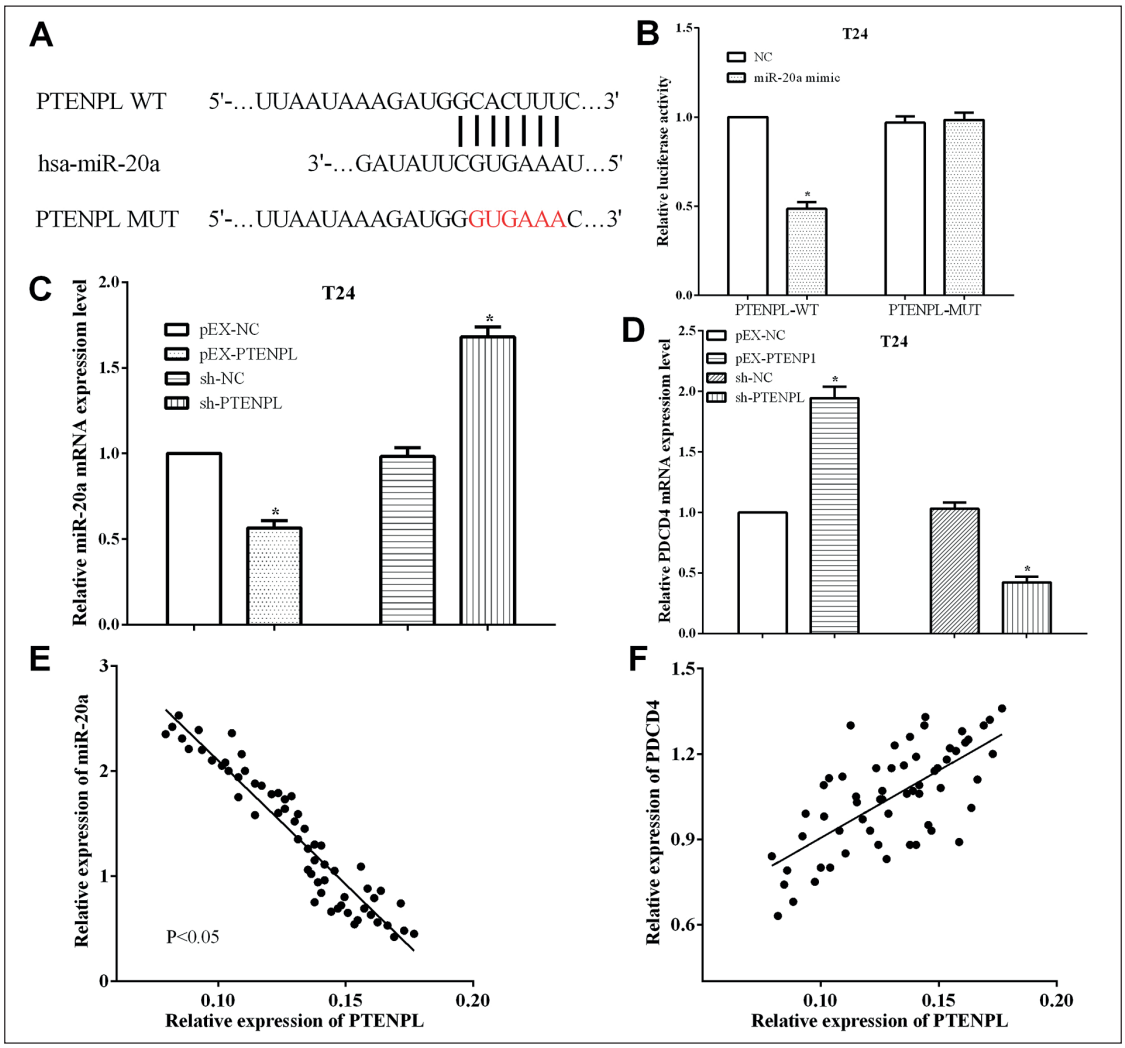

Figure 5. PTENP1 sponged miR-20a and regulated the expression of PDCD4 in bladder cancer. **A,** LncRNA PTENP1 was predicted to be a ceRNAs of miR-20a by StarBase. **B,** Luciferase reporter assay revealed that miR-20a binding to PTEBPL. **C,** The expression of MiR-20a was evaluated when changed PTENP1 in T24 cells. **D,** The expression of PDCD4 was assessed after alteration of PTENPL. **E,** Spearman's correlation detected the relationships among PTENP1 and miR-20a in bladder cancer. **F,** The expression of PTENP1 had a positive relationship with PDCD4 in bladder cancer tissues.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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