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MiR-335 suppresses cell proliferation and migration by upregulating CRKL in bladder cancer

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Abstract. – OBJECTIVE: microRNAs (miRNAs) abnormal expression was proved to regulate the bladder cancer (BC) development. Here, we aimed to investigate the role of miR-335 played in BC.

MATERIALS AND METHODS: Real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot were used to examine the miR-335 and CRKL (CT10 regulator of kinase-like protein) expression level in BC. Methyl thiazolyl tetrazolium (MTT) and RT-qPCR were used to examine cell viability of BC cells. Cell transwell assay was used to assess the migratory ability of BC cells. The direct target of miR-335 in BC was verified by luciferase reporter assay.

RESULTS: The results showed that the expression of miR-335 and CRKL in normal and adjacent tissues showed no significant differences. Whereas, miR-335 expression in BC was significantly lower and CRKL expression was observably higher than normal. CRKL was verified as a specific target of miR-335 in BC cells and the relationship between CRKL and miR-335 expression was negatively correlated in BC tissues. Furthermore, CRKL siRNA group in BC cells remarkably inhibited cell proliferation and migration. MiR-335 mimic in BC cells remarkably curbed cell proliferation and migration and CRKL could reverse the proliferative and migratory ability of BC cells regulated by miR-335.

CONCLUSIONS: miR-335 could suppress BC cell proliferation and migration by upregulating of CRKL.

Key Words:
miR-335, Proliferation, Migration, Bladder cancer (BC), CRKL.

Introduction

Bladder cancer (BC) is the most common malignant tumor in the urinary system and it is one of the ten most common tumors in the body[1-3]. In the national tumor registries in 2012, the mortalities of BC were 165,000, the ninth highest incidence of malignancy[4]. The causes of bladder cancer are complex, and two of the most significant risk factors are poisoning and smoking. Although there are several treatments currently available, including radiotherapy, surgery and chemotherapy, the annual incidence and mortality rate of BC disease has been increasing[5]. Therefore, it is necessary to explore the molecular mechanism of tumor development and progression of BC. MicroRNAs (miRNAs), a kind of endogenous 19 to 22-nucleotide non-coding RNA, were found in eukaryotic organisms and regulate protein expression by degrading or weakening the target mRNAs translation. Previous studies demonstrated that the abnormal expression of miRNA was closely correlated with the tumor development, including cell proliferation, migration, invasion or apoptosis[6] in lung cancer[7], gastric cancer[8], esophageal cancer[9], osteosarcoma[10], also bladder cancer[11] and so on. In these cancers, the abnormal expression of miRNA affected the malignant characteristics of tumor cells profoundly. Specifically, miR-335 has been proved to participate various tumors development and progression by regulating of the target mRNAs[12,13]. For instance, miR-335 was reduced in gastric cancer and had a close relationship with tumor infiltration depth, lymph node metastasis and patient survival by targeting of associated genes[14]. Dong et al[15] provided an evidence that miR-335 inhibited the motility and proliferation of breast cancer partly through downregulating of EphA4. We showed that miR-335 expression was reduced in bladder cancer and regulating the cell development by targeting ROCK1 or MAPK1[16,17]. Thus, determi-

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ning the target genes of miR-335 is very important for understanding their role in BC development and progression. CRKL (CT10 regulator of kinase like protein) is a member of the human CRK adapter protein, which was proved to play an important role in multiple cancers progression, including cell proliferation and invasion in pancreatic cancer[18], cell invasion in lung cancer[19] and the proliferation, migration and invasion of hepatocarcinoma[20]. In addition, CRKL was a target of miR-429 on the inhibition of cervical cancer[21]. Wang et al[22] showed that CRKL acted as an oncogene in gastric cancer and promoted cell proliferation regulated by miR-126. It has been stated[23] that the expression of CRKL was upregulated in bladder cancer and could regulate the growth and invasion of BC cells. However, the biological mechanism of CRKL in BC cell regulated by miR-335 has not been reported. In our study, we showed that miR-335 expression was increased while CRKL was decreased in BC tissues and cell lines. In addition, the relationship between miR-335 and CRKL mRNA expression was negatively correlated. We also explored the role of miR-335 played in the proliferation and migration of BC cells and found that miR-335 had the inhibition effect. However, CRKL overexpression promoted the proliferation and migration of BC cells, and CRKL could partially reverse the inhibition effect of miR-335 on the proliferation and migration. All these results suggested that CRKL was a specific target of miR-335 in regulating of the development of BC.

Patients and Methods

Tissue Samples

The human bladder cancer specimens were collected from BC patients during operations including 21 paired BC tissues and adjacent tissues, which were approved by the Ethics Committee of Tangdu Hospital of Air Force Military Medical University. Moreover, seven cases of normal tissues were collected too. We divided the 21 BC tissues into three phase cases on average (T1, T2a and T2b) on the basis of the tumor, node, metastasis of BC, 7 tissues in each group. The use of pathological classification criteria is in consistent with the urothelial cancer standard developed by the WHO. All patients signed informed consent before sample collection and were untreated before surgery. All specimens were stored at -80°C refrigerator.

Cell Culture

All BC cell lines (T24, 5637, BIU87, HT1376) and normal uroepithelial cell lines SV-HUV-1 were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cancer cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Solarbio, Beijing, China), which was incubated at 37°C under 5% CO_2 atmosphere.

Cell Transfection

The Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect miR-335 mimic or inhibitor and control mimic or inhibitor into BC cells. All of the transfection procedures were performed following the manufacturer's instructions. The transfected cells were divided into several groups: control mimic, control inhibitor, miR-335 mimic, miR-335 inhibitor and control mimic + control vector, miR-335 mimic + control vector, control mimic + CRKL vector, miR-335 mimic + CRKL vector.

Western Blot Assay

Radioimmunoprecipitation assay (RIPA) lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) and proteinase inhibitors (Beyotime, Shanghai, China) were used to extract total protein from the tissues or cells. The protein concentration measured using bicinchoninic acid (BCA) reagent kit (Beyotime, Shanghai, China). The total proteins (50 μg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to NC membrane (Millipore Corporation, Billerica, MA, USA), then, skim milk (5%-10%) was used to block the membranes at room temperature for 2 h and the primary antibodies (CRKL, 1:1000, Abcam, Cambridge, MA, USA; glyceraldehyde 3-phosphate dehydrogenase (GADPH), 1:2000, Cell Signaling Technology, Danvers, MA, USA), subsequently the secondary antibodies were added in to incubate the specimens at 4°C overnight or 2 h at room temperature, respectively. Finally, the enhanced chemiluminescence kit (ECL, Millipore, Billerica, MA, USA) was used to detect the signals. GADPH served as a loading control.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs exacted from tissues or cells used TRIzol reagent (Invitrogen, Carlsbad,

CA, USA). TaqMan PCR kit was used to perform RT-qPCR (TaKaRa, Otsu, Shiga, Japan). All reactions were performed three times. The sequences of the primers were as follows: miR-335-F: TCAAGAGCAATAACGAAAAATGT; miR-335-R: GCTGTCAACGATACGCTACGT. CRKL-F: CATTCCCGGGCGGCTCTCTC; CRKL-R: CACGCCTTAGCCCGGCAGAC, PCNA-F: GGTGTTG GAGGCACTCAAGG, PCNA-R: CAGGGTGAGCTGCACCAAAG; U6-F: CTCGC TTCGGCAGCACA; U6-R: AACGCTTCACGAATTTGCGT. GAPDH-F: TGGTATC GTGGAAGGACTC; GAPDH-R: AGTAGAGGCA GGGATGATG. GAPDH and U6 were used as an internal control. The $2^{\text{™}\Delta\Delta Ct}$ method was used to detect the expression of miR-335, CRKL and PCNA.

Cell Proliferation Assay

Cell proliferation was detected by Methyl thiazolyl tetrazolium (MTT) (R&D Systems, Minneapolis, MN, USA). The cells in each group were added into 96-well plates and cultured. After that, we added MTT medium (20 μL) to each group. After incubation at 37°C for 4 h, the MTT medium was sucked out and 100 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was then added in for additional 10 min, the plates were then read at a wavelength of 490 nm at 0, 24, 48, 72, 96 h to measure the absorbance of each well.

Transwell Assay

Cell migratory ability was performed using transwell assay. First, a transwell chamber with 8 μm pore size polycarbonic membrane (Costar, Corning, NY, USA) was placed into the 24-well plates to separate the top and the lower chambers. Then, we added BC cells (1×10^5) into the top chamber, and seeded Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 20% fetal bovine serum (FBS) to the lower chambers as an attractant. After incubation for 24 h at 37°C, the cells in upper chambers migrated into the lower chamber were stained with 0.1% crystal violet for another 30 min. Images of the migration cells were photographed under a microscope.

Dual Luciferase Reporter Assay

The recombinant pMIR-reportor luciferase vector was used for luciferase assays. The wild-type and mut-type miR-335 putative targets on CRKL 3'UTR were constructed into the pMIR-reporter luciferase vector. We used Lipofectamine 2000 to transfect T24 cells with control mimic, control inhibitor or miR-335 mimic, miR-335 inhibitor. Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was then used to measure the luciferase activity.

Statistical Analysis

All experiments were repeated in triplicate, we use Statistical Product and Service Solutions (SPSS) v.19.0 software (IBM, Armonk, NY, USA) to perform statistical analyses and GraphPad Prism 5.02 Software (La Jolla, CA, USA) to complete graph presentations. Results are represented as the mean ± SD, and the data was evaluated using Student's *t*-test or Tukey's post-hoc test after ANOVA in SPSS, with statistically significant difference considered as $p<0.05$.

Results

Lower Expression of miR-335 and Higher Expression of CRKL in BC Tissues and Cells

We used RT-PCR and Western blot to detect the miR-335 and CRKL expression in BC tissues and cell lines. The expression of miR-335 and CRKL in normal and adjacent tissues showed no significant differences (Figure 1). However, miR-335 mRNA expression in BC tissues expressed significantly lower than normal tissues and both CRKL mRNA and protein level were observably higher than normal tissues (Figure 1A-1C). We also found that miR-335 mRNA expression in both BC cell lines expressed significantly lower than normal cells and both CRKL mRNA and protein level were observably higher than normal cells (Figure 1D-1F).

MiR-335 Targeted CRKL in BC Cells

The binding site of CRKL 3'UTR and miR-335 were predicted based on the TargetScan calculation (Figure 2A). We used luciferase reporter assay to verify this targeting relationship in T24 cell line. The luciferase intensity in miR-335 mimic group was significantly reduced compared with the control group in wild-type as expected, while there have no significant difference in mut-type (Figure 2B). The miR-335 expression level of RT-qPCR analysis in T24 cells after overexpression or knockdown of miR-335 was shown in Figure 2C. The RT-qPCR and Western blot showed that the CRKL mRNA and protein expression was markedly decreased in miR-335 mimic group,

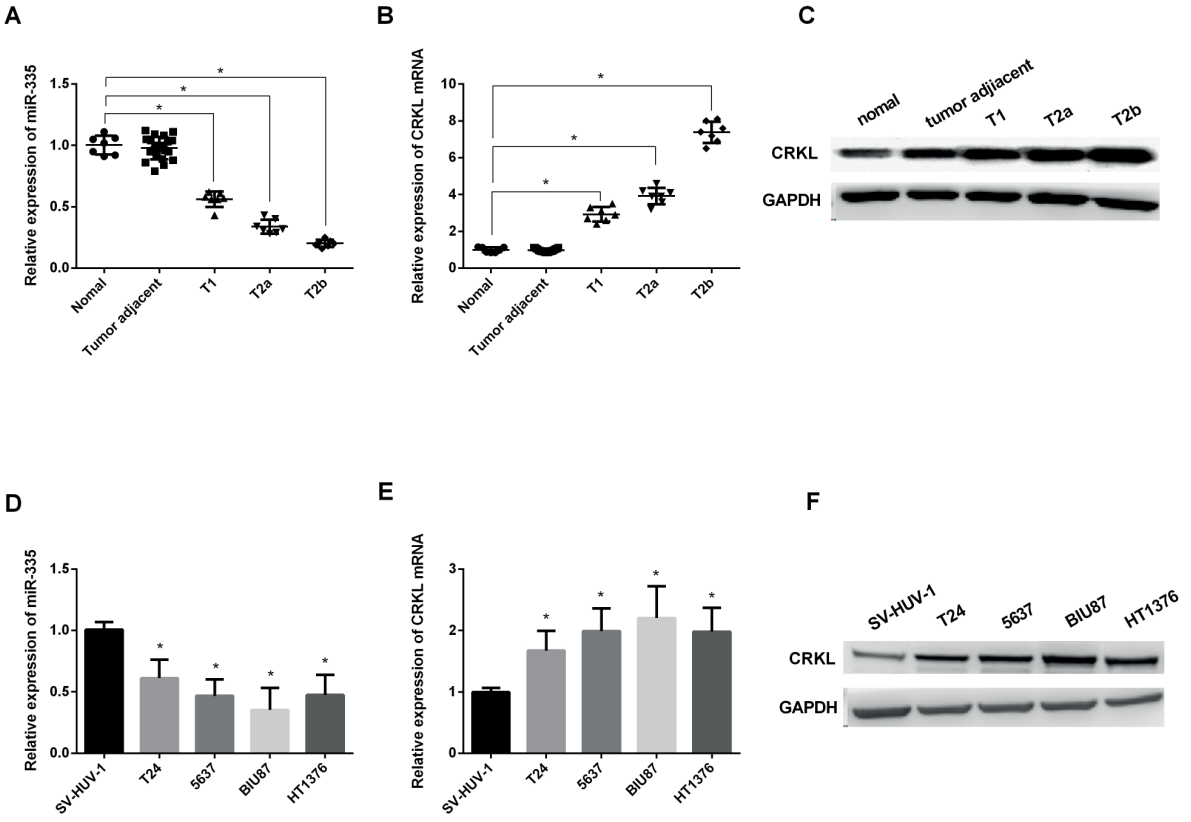

Figure 1. Downregulation of miR-335 and upregulation of CRKL in BC tissues and cells. ***A-B***, miR-335 and CRKL expression was examined by RT-qPCR in BC tissues of different phases. ***C,*** CRKL protein level was examined by Western blot in BC tissues of different phases. ***D-E***, miR-335 and CRKL expression was examined by RT-qPCR in all BC cell lines. ***F,*** CRKL protein level was examined by Western blot in all BC cell lines. *$p<0.05$

and miR-335 inhibitor could significantly upregulate the CRKL mRNA and protein expression (Figure 2D-2E). We also detected the relationship between miR-335 and CRKL and found that the fold change of miR-335 mRNA and CRKL was negatively correlated (Figure 2F).

Knockdown of CRKL Expression Inhibited BC Cell Proliferation and Migration

The T24 cells were transfected with CRKL siRNA to confirm the influence of CRKL on BC cell proliferation and migration. The transfection efficiency of CRKL siRNA was shown in Figure 3A-3B. Transwell assay was used to detect the effect of CRKL in regulating of BC cell proliferation and migration. Results showed that knockdown of CRKL decreased the proliferative and migratory ability of T24 cells (Figure 3C-3D).

MiR-335 Suppressed Cell Proliferation by Upregulating of CRKL in BC

We first explored the role of miR-335 played on BC cells proliferation. The cells with different transfection were divided into four groups: control mimic, miR-335 mimic, control inhibitor and miR-335 inhibitor. MTT assays the cell viability in the four groups at 0, 24, 48, 72, 96 h, respectively. The results showed that miR-335 mimic remarkably inhibited cell proliferation and silencing of miR-335 enhanced the cell proliferation (Figure 4A). We also examined the proliferating cell nuclear antigen (PCNA) mRNA expression in T24 cells. The results demonstrated that miR-335 mimic could inhibit the PCNA expression while miR-335 inhibitor promoted the PCNA expression (Figure 4B). Secondly, we investigated the effect of CRKL on the development of BC regulating by miR-335. MTT assay results stated that re-expression of CRKL could promote the cell viability of T24 cells, while overexpression of miR-335 suppressed the cell viability. However,

co-transfected with miR-335 and CRKL partially reversed the inhibitory effect of miR-335 in BC cells (Figure 4C). QRT-PCR analysis showed that PCNA expression was observably higher in the group of overexpression of CRKL than control group, while PCNA expression was observably lower in the group of overexpression of miR-335 than control group. Nevertheless, co-transfected with miR-335 and CRKL could promote cell proliferation compared with transfected with miR-335 alone in T24 cells (Figure 4D).

MiR-335 Suppressed Cell Migration by Upregulating of CRKL in BC

Firstly, we explored the role of miR-335 played on the migration of BC cells. The cells with different transfection were divided into four groups too: control mimic, miR-335 mimic, control inhibitor and miR-335 inhibitor. Transwell assays the cell migratory ability in the four groups, respectively. The results showed that miR-335 mimic remarkably inhibited cell migration and inhibiting miR-335 enhanced the cell migration (Figure 5A and 5C). Secondly, we investigated the effect of CRKL on the migration of BC regulated by miR-335. The results stated that re-expression of CRKL could promote the cell migratory ability of T24 cells, while miR-335 mimic curbed the cell migration. However, co-transfected with miR-335 and CRKL partially reversed miR-335 inhibitory effect in BC cell migration (Figure 5B and 5D).

Discussion

It is well known that among all of the urinary system tumors, BC is one of the most common cancers[24] and its incidence is rising every year[25].

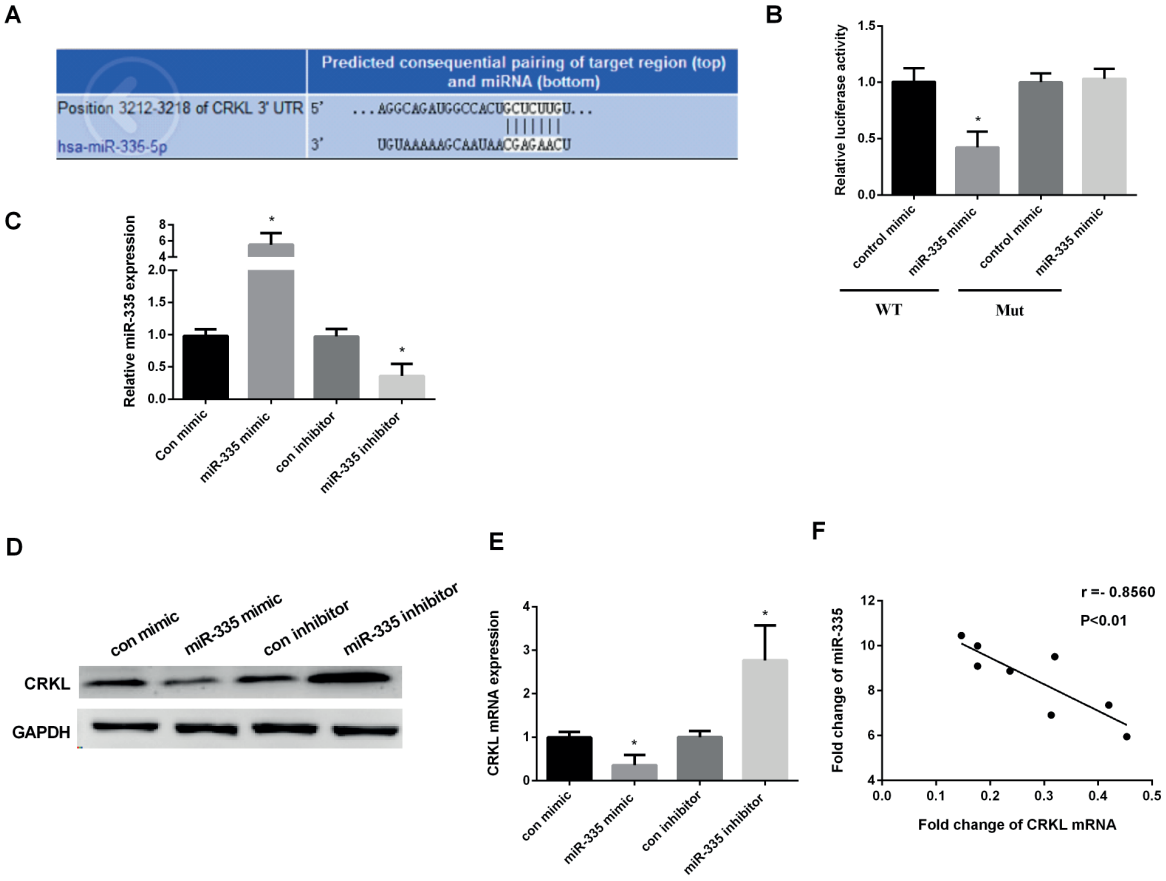

Figure 2. miR-335 directly targets CRKL by binding to its 3' UTR. ***A,*** Predicted consequential pairing of target region (top) and miR-335 (bottom). ***B,*** The relative luciferase activity was examined in T24 cells transfected with miR-335 mimic in wild-type and mut-type group. ***C,*** The relative miR-335 expression was examined in T24 cells after overexpression or knockdown of miR-335 by RT-qPCR. ***D-E***, The CRKL mRNA and protein expression were detected in T24 cells after overexpression or knockdown of miR-335 by RT-qPCR and Western blot. ***F,*** Regression analysis showed that the fold change of miR-335 and CRKL mRNA were inversely correlated (r = -0.8560, $p<0.01$). *$p<0.05$.

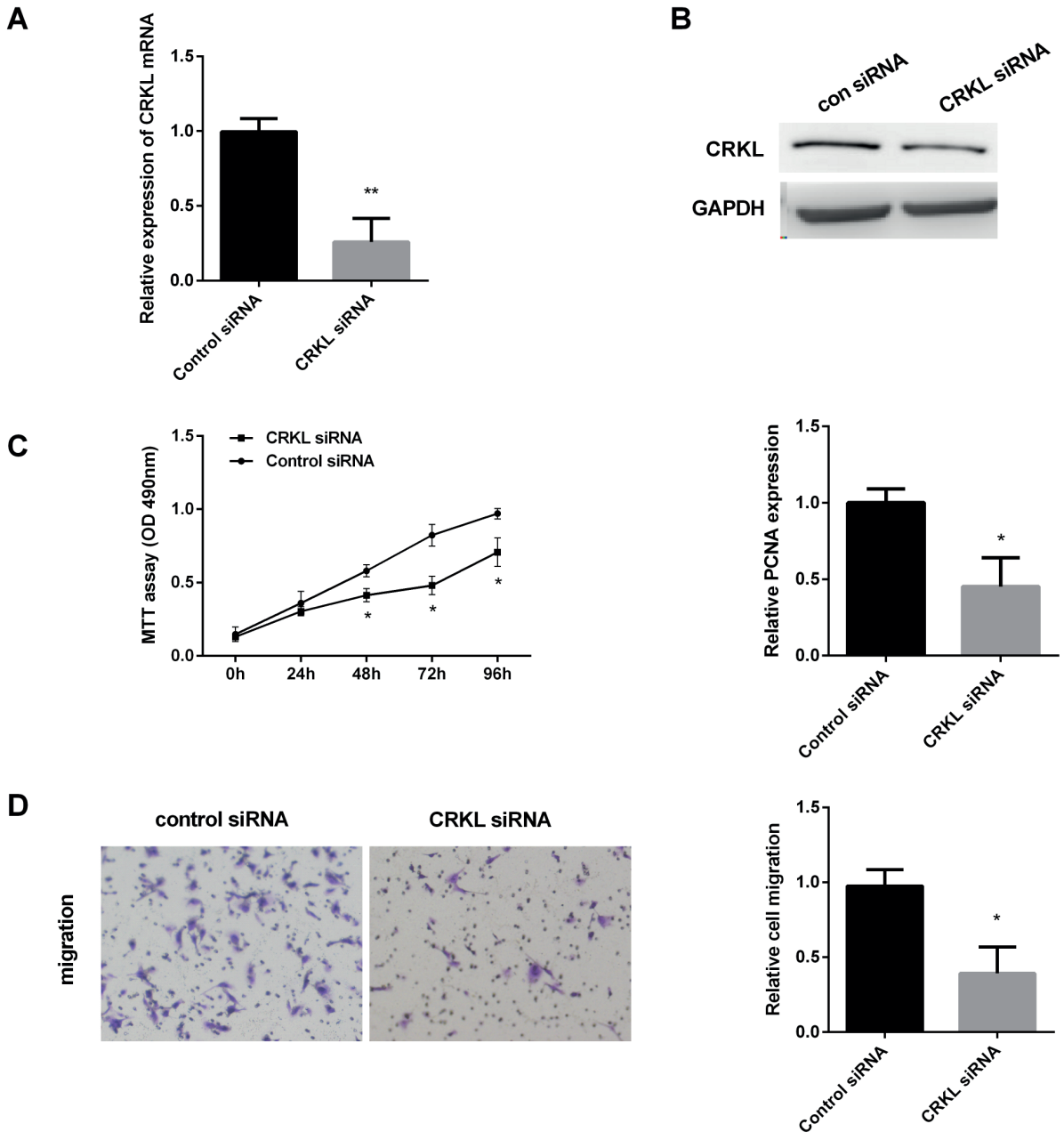

Figure 3. The inhibitory effect of siRNA CRKL in T24 cells. ***A,*** CRKL mRNA expression was examined in T24 cells transfected with siRNA CRKL by RT-Qpcr. ***B,*** CRKL protein expression was detected in T24 cells after silencing CRKL by Western blot. ***C,*** Relative cell viability of T24 cells examined by MTT assay and PCNA mRNA examined by RT-PCR in T24 cells after knockdown of CRKL. D, Relative migration of T24 cells examined by transwell assay after knockdown of CRKL. *p<0.05, **p<0.01.

Therefore, determining its potential mechanism may provide a research idea for the treatment of BC. In our study, we stated that miR-335 expression was decreased in BC cells and it could inhibit BC cell viabilities and migration *via* upregulating CRKL gene. Our work may provide an important treatment strategy for BC. MiR-335 functioned as either tumor promoters or suppressors in various human cancers. Previous researches have proved that miR-335 had lower expression level in hepatocellular carcinoma[26], renal cell carcinoma[27] and cervical cancer[28]. In our study, we clarified that miR-335 expression was lower in BC tissues and acted as a tumor suppressor in regula-

ting of the progression of BC, which in lines with the previous investigations that showed miR-335 was reduced in BC cells[16,17]. We also found that re-expression of miR-335 might inhibit the cell activity and migration while inhibiting miR-335 enhanced the progression of BC. In addition, we first identified CRKL as a target of miR-335 in BC. CRKL overexpressed has been proved to participate in various human cancers, including gastric cancer, endometrial carcinoma and cervical carcinoma[29-31], upregulating of the cell viability, migration and invasion. In our study, we stated that CRKL was up-regulated in BC tissues and cells. Additionally, CRKL could enhance BC cell proliferation and migration, consistently with the previous research showing that the CRKL expression was upregulated in bladder cancer and could regulate the growth and invasion of BC cells. However, it is the first time that we found CRKL could reverse partially miR-335 suppression effect and acted as a specific target of miR-335 in regulating of the development of BC.

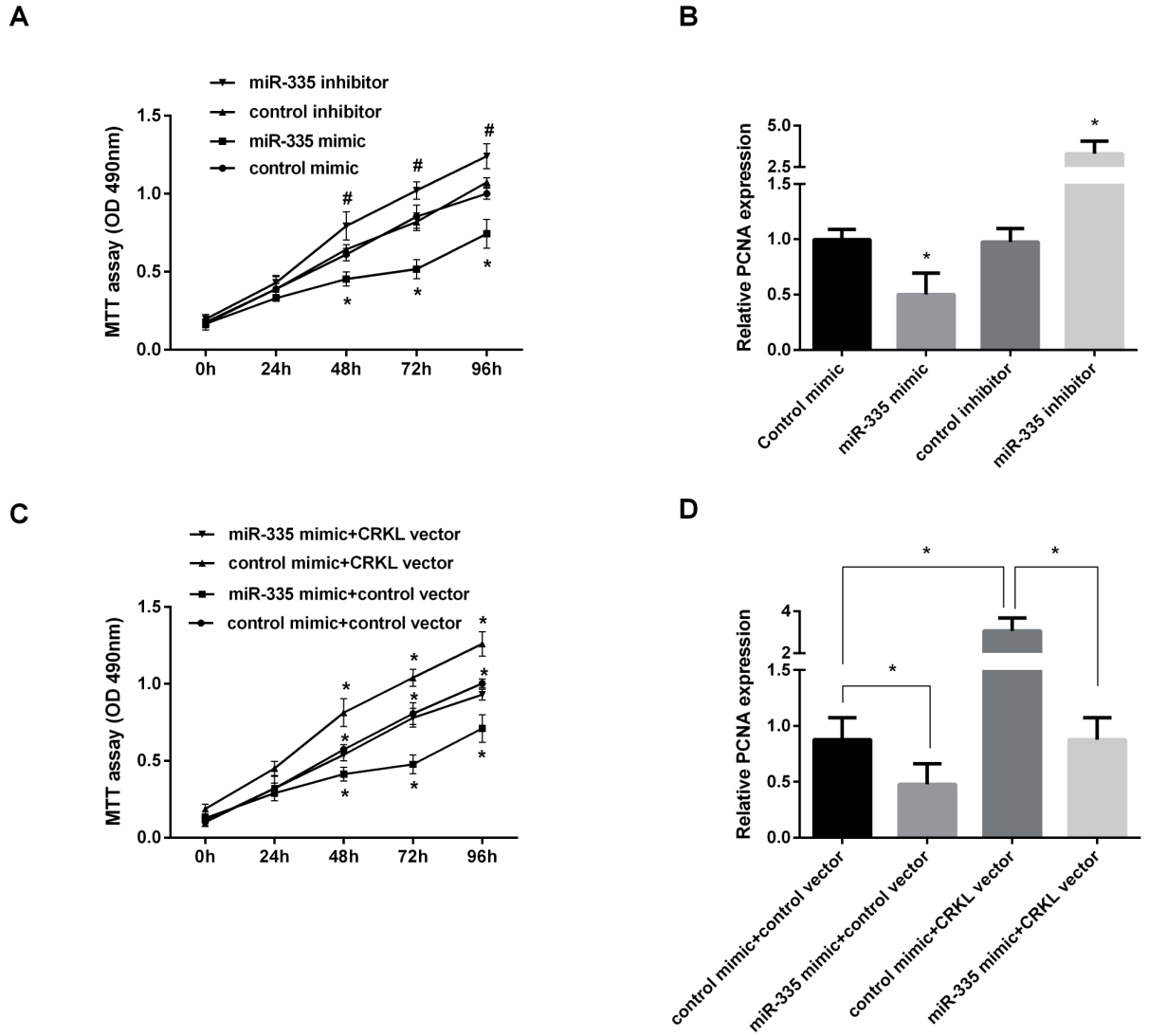

Figure 4. The suppression effect of miR-335 on BC cell proliferation by targeting CRKL. ***A,*** Cell viability of T24 cells was examined by MTT assay after promoting or inhibiting miR-335. ***B,*** The PCNA mRNA was examined in T24 cells by RT-PCR after promoting or inhibiting miR-335. ***C,*** MTT assay examined the cell viability of T24 cells at 0, 24, 48, 72 and 96 h in four groups (control mimic + control vector, miR-335 mimic + CRKL vector, control mimic + CRKL vector, miR-335 mimic + CRKL vector). D, RT-qPCR examined PCNA expression in the same four groups of T24 cells. *p<0.05, #p<0.05.

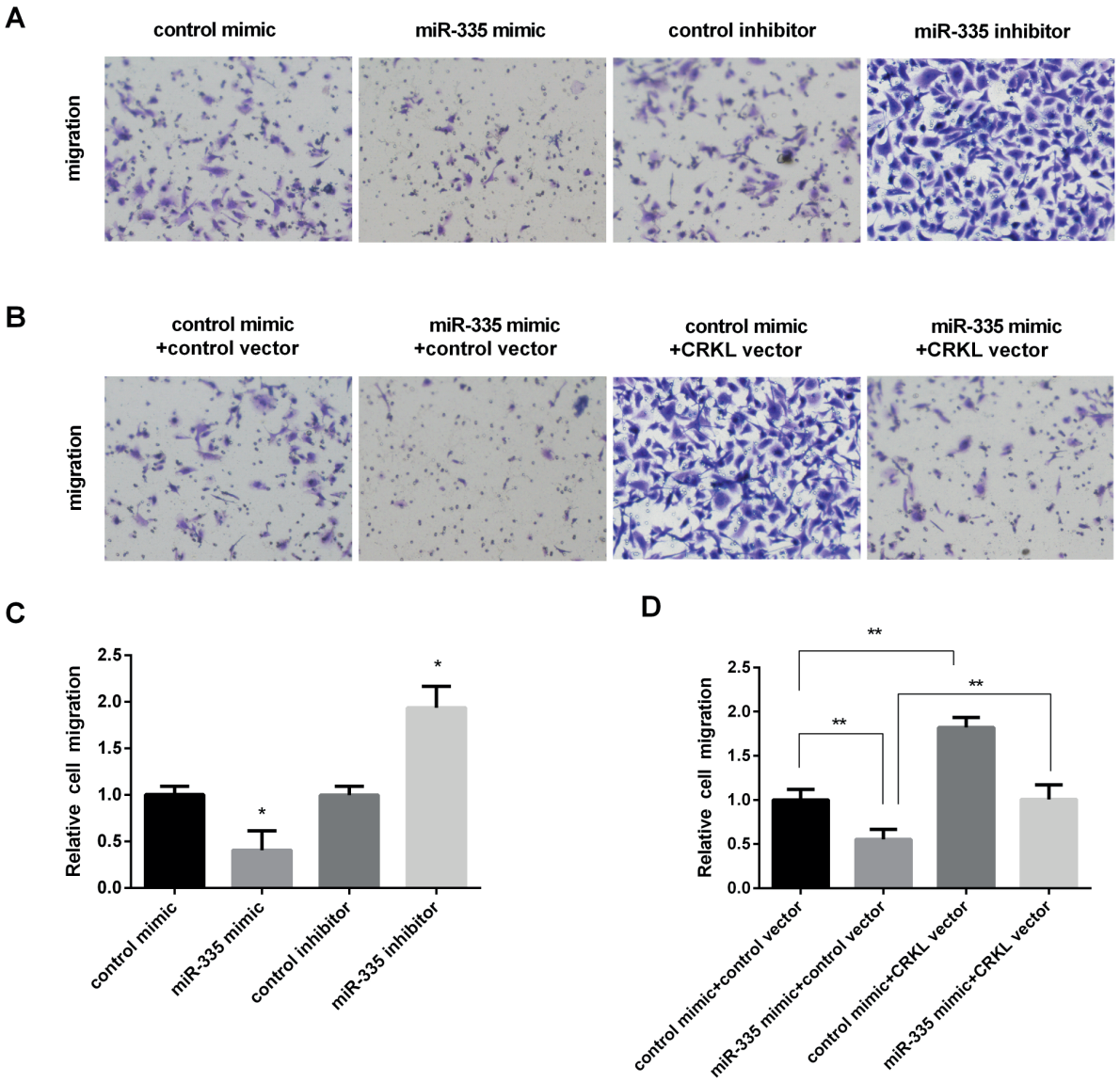

Figure 5. The suppression effect of miR-335 on BC cell migration by targeting CRKL. ***A,*** and ***C,*** Relative cell migration of T24 cells was examined by transwell assay after promoting or inhibiting miR-335. ***B,*** and ***D,*** Relative cell migration of T24 cells was examined by transwell assay in the same four groups. *$p<0.05$, **$p<0.01$

Conclusions

We showed that miR-335 could target CRKL to inhibit BC tumorigenesis. MiR-335 might therefore represent a novel therapeutically relevant cellular target for the treatment of BC patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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