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MiR-652-3p promotes bladder cancer migration and invasion by targeting KCNN3

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Abstract. – OBJECTIVE: Increasing evidence indicated that microRNAs (miRNAs) are crucial regulators for cancer development. Bladder cancer (BCa) is a major threat to human health. The aim of this study was to analyze the roles of miR-652-3p in BCa, and to explore the associated mechanisms.

MATERIALS AND METHODS: MiR-652-3p expression in BCa cell lines was explored using Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) method. MiR-652-3p expression level in BCa tissues was explored at StarBase. Cell Counting Kit-8 (CCK-8) assay, wound-healing assay, and transwell invasion assay were conducted to investigate the biological roles of miR-652-3p. The underlying mechanisms of miR-652-3p in NSCLC were investigated using luciferase activity reporter assay and rescue experiments.

RESULTS: We showed that miR-652-3p expression level was upregulated in both BCa tissues and cell lines. The knockdown of miR-652-3p significantly inhibited BCa cell proliferation, migration, and invasion *in vitro*. Moreover, we showed that potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3) was a functional target for miR-652-3p. Besides, the expression of KCNN3 in BCa tissues was negatively correlated with miR-652-3p.

CONCLUSIONS: Collectively, these results showed that miR-652-3p could promote BCa cell proliferation, migration, and invasion *via* directly regulating KCNN3, which may provide a novel therapeutic target for BCa treatment.

Key Words:
MiR-652-3p, KCNN3, Bladder cancer, Oncogene.

Introduction

Bladder cancer (BCa) is one of the most commonly occurred cancer type in urinary system[1]. Treatment methods for BCa nowadays, including surgical resection and chemotherapy, however, overall survival for BCa patients is still undesirable[2]. Hence, it is imperative to screen novel biomarkers to help BCa early diagnosis or targeted therapy.

MicroRNAs (miRNAs) are RNAs with the length of 18 to 24 nucleotides[3]. MiRNAs are able to bind with message RNA (mRNA) to promote the degradation of mRNA or hinder translation process[4]. MiRNAs are involved in many disease processes and played crucial roles in diseases, including cancer[5].

Numerous aberrantly expressed miRNAs in BCa have been identified[6]. Chen et al[7] found miR-101 expression level in serum was reduced in BCa patients in comparison with health controls. In addition, low miR-101 level was revealed to be closely associated with late tumor stage, worse lymph node metastasis, and was able to distinguish BCa patients from health controls. Li et al[8] recently reported miR-101-3p overexpression could promote the sensitivity of bladder urothelial carcinoma cell to cisplatin *via* silencing enhancer of zeste 2 polycomb repressive complex 2 subunits. Qin et al[9] revealed let-7i was able to inhibit BCa cell proliferation and migration by targeting high mobility group protein A1.

MiR-652-3p was a miRNA that abnormally expressed in human cancers. For instance, high miR-652-3p level in non-small cell lung cancer was found correlated with late tumor stages and poorer overall survival[10]. In addition, miR-652-3p was revealed to regulate non-small cell lung cancer proliferation, migration, invasion, and apoptosis *via* regulating lethal (2) giant larvae 1 (Lgl1)[10]. Moreover, miR-652-3p was shown to be decreased expression in pediatric acute lymphoblastic leukemia. Also, its overexpression could

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suppress cancer progression by promoting apoptosis or increasing the sensitivity to chemotherapeutic drugs[11].

In this study, we aimed to analyze biological roles of miR-652-3p and the associated mechanisms in BCa. Here, we showed miR-652-3p expression was elevated, while potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3) expression was decreased in BCa. Expression of miR-652-3p and KCNN3 was negatively correlated in BCa tissues. Furthermore, we showed miR-652-3p could promote BCa cell proliferation, migration, and invasion *via* regulating KCNN3.

Materials and Methods

Exploration MiR-652-3p and KCNN3 Levels in BCa Tissues Using StarBase

Expression level of miR-652-3p or KCNN3 in BCa cancer tissues and normal tissues was explored at StarBase, a database contains gene expression pattern in pancancer[12].

Cell Lines

BCa cell lines T24 and J82 and normal human cell SV-Huc-1 obtained at ATCC (Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) in supplement with 10% fetal bovine serum (FBS, Beyotime, Haimen, Jiangsu, China) at the temperature of 37°C containing 5 % of CO_2.

Cell Transfection

MiR-652-3p inhibitor and corresponding control (NC-miR) were synthesized by GeneChem (Shanghai, China). Small interfering RNA regulating KCNN3 (si-KCNN3) and the corresponding control (NC-siR) were also designed by GeneChem. Cell transfection was accompanied using Lipofectamine 6000 (Beyotime, Haimen, Jiangsu, China) according to the suitable protocols.

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

The cells were lysed to extract RNA using TRIzol reagent (Beyotime, Haimen, Jiangsu, China). Reverse transcription was performed with complementary DNA (cDNA) synthesis kit (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT-qPCR was conducted using SYBR Green Mix (Beyotime, Haimen, Jiangsu, China) with the following procedure: 1 cycle of 95°C for 10 min; 40 cycles of 94°C for 15 s, 60°C for 1 min. The primers used were as follows: miR-652-3p: forward, 5'-ACACTCCAGCTGGGCAACCCTAGGAGAGGGTGC-3' and reverse, 5'-GTGTCGTGGAGTCGGCAATTC-3'; U6 snRNA: forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5′-CGCTTCACGAATTTGCGTGTCAT-3'. Relative expression level of miR-652-3p was calculated with comparative cycle threshold method.

Western Blot

The cells were lysed with Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime, Haimen, Jiangsu, China) to isolate total proteins. After quantification with bicinchoninic acid (BCA) kit, equal amount of sample was isolated with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electric transferred to polyvinylidene difluoride (PVDF) membrane. Non-fat milk was used to block the non-specific targets in membranes. Then, membranes were incubated with primary antibodies (anti-KCNN3: ab28631, anti-GAPDH: ab181602, Abcam, Cambridge, MA, USA) at 4°C for overnight. The band was then incubated with secondary antibody (ab6721, Abcam, Cambridge, MA, USA) at room temperature for 4 h. Bands were visualized with BeyoECL kit (Beyotime, Haimen, Jiangsu, China) based on the provided protocols.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation rate was analyzed using CCK-8 reagent purchased from Beyotime (Shanghai, China). Cells were seeded to 96-well plate and incubated for the indicated time. Then, 10 µl CCK-8 solution was added to each well and further incubated for 4 h. At length, optical density at 450 nm was measured using microplate reader.

Wound-Healing Assay

The cells were incubated into 6-well plate and incubated until about 100% confluence. Then, scratch at cell surface was created using pipette tip and then washed with phosphate-buffered saline (PBS) to remove cell debris. At 0 h and 24 h after scratch creation, cell images were observed and captured under microscope.

Transwell Invasion Assay

The cells in serum-free medium were filled into upper chamber with a Matrigel pre-coated 8 µm insert. Dulbecco's Modified Eagle's Medium (DMEM) contains fetal bovine serum (FBS)

was filled into lower chamber. After 48 h of incubation, cells that did not pass through the insert were removed, while invasive cells were fixed, stained, and counted under a microscope.

Luciferase Reporter Assay

The targets for miR-652-3p were predicted at TargetScan. We found KCNN3 was a putative target. pMIR vector was used to build the wild-type KCNN3 (wt-KCNN3) luciferase construct by inserting the 3'-untranslated region sequence of KCNN3 into this vector. Then, mutant KCNN3 (mt-KCNN3) luciferase construct was built using site-direct mutagenesis kit (TaKaRa, Dalian, Liaoning, China). After transfection with miRNAs and luciferase vectors for 48 h, relative luciferase activity was measured using Dual-Luciferase reporter assay (Promega, Madison, WI, USA).

Statistical Analysis

Data were presented as mean ± standard deviation and analyzed with Student's *t*-test or One-way analysis of variance (ANOVA) and Tukey post-hoc test at Statistical Product and Service Solutions 19.0 (IBM, Armonk, NY, USA). Differences in groups were considered as significant when $p < 0.05$.

Results

MiR-652-3p Level Was Increased in BCa

We detected miR-652-3p expression level in BCa tissues and normal tissues at StarBase. We found miR-652-3p levels were significantly higher in tumor tissues than in normal tissues (Figure 1A). Moreover, we showed that miR-652-3p levels in BCa cell lines were also higher than in normal cell line (Figure 1B).

Knockdown of MiR-652-3p Inhibits BCa Proliferation, Migration, and Invasion

We investigated the biological roles of miR-652-3p using loss-of-function experiments. MiR-652-3p inhibitor transfection significantly decreased miR-652-3p level in BCa cells (Figure 2A). CCK-8 assay, wound-healing assay, and transwell invasion assay showed knockdown of miR-652-3p inhibits BCa proliferation, cell migration, and invasion *in vitro* (Figure 2B-2D).

MiR-652-3p Targets KCNN3 in BCa

We searched for targets for miR-652-3p using TargetScan. It was found KCNN3 contains a binding site for miR-652-3p in its 3'-UTR (Figure 3A). Luciferase activity reporter assay revealed relative luciferase activity was markedly increased in BCa cells transfected miR-652-3p inhibitor and wt-KCNN3 (Figure 3B). However, luciferase activity was not altered in cells with miR-652-3p inhibitor and mt-KCNN3 transfection (Figure 3B). Moreover, we found KCNN3 expression level was lower in tumor tissues compared with normal tissues (Figure 3C). Importantly, an inversely correlation between miR-652-3p and KCNN3 was observed in BCa tumor tissues (Figure 3D).

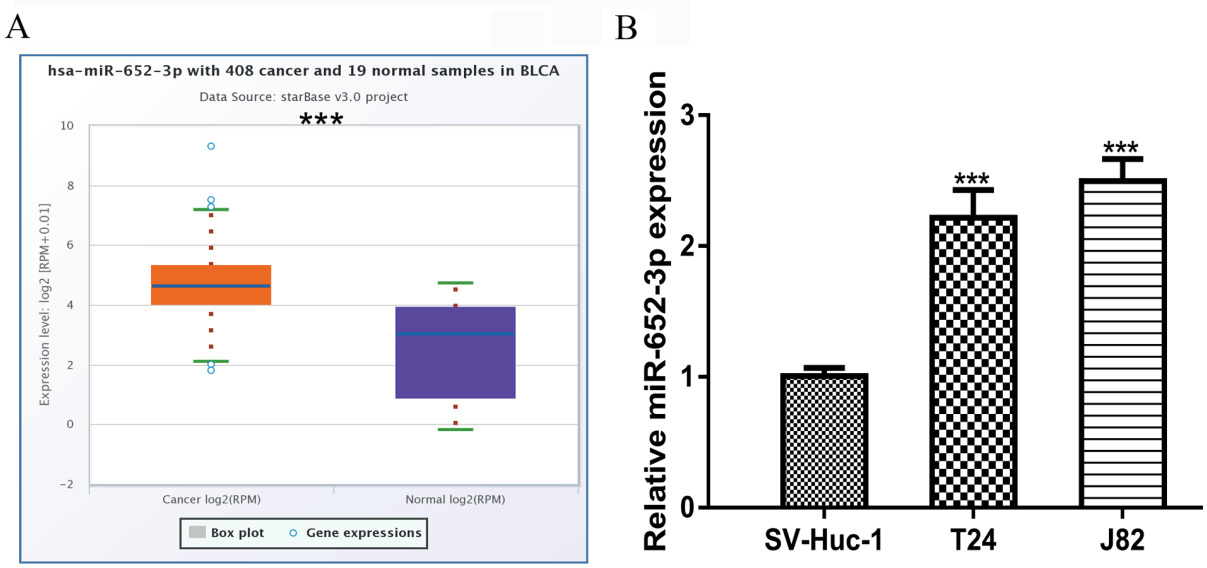

Figure 1. Expression of miR-652-3p was increased in BCa. **A**, MiR-652-3p expression level in BCa tissues and normal tissues. **B**, MiR-652-3p expression level in BCa cell lines and normal cell line. MiR-652-3p: microRNA-652-3p; BCa: bladder cancer.

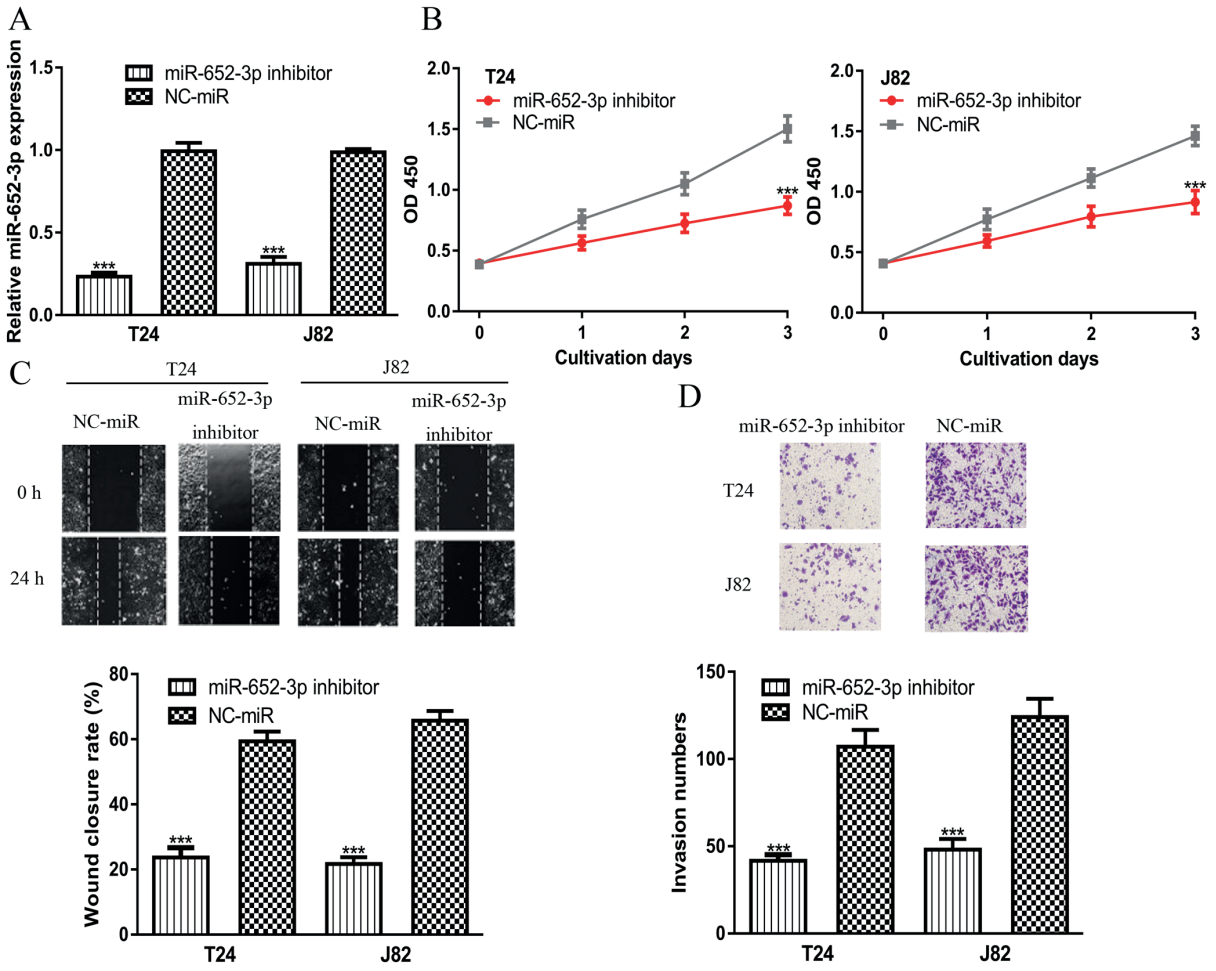

Figure 2. MiR-652-3p knockdown suppressed BCa cell proliferation, migration, and invasion. **A**, MiR-652-3p expression, **B**, CCK-8 assay was used to analyze cell proliferation, **C**, Wound-healing assay was used to analyze cell migration (magnification: 200x), **D**, Transwell invasion assay was utilized to analyze cell invasion (magnification: 200x) in BCa cells transfected with miRNAs. MiR-652-3p: microRNA-652-3p; BCa: bladder cancer; CCK-8: cell counting kit-8; NC-miR: negative control miRNA.

KCNN3 Reverses the Effects of MiR-652-3p on Proliferation, Migration, and Invasion of BCa Cells

To further validate whether KCNN3 was a functional target for miR-652-3p, rescue experiments were conducted. Results showed si-KCNN3 transfection significantly decreased the levels of KCNN3 in BCa cells (Figure 4A). *In vitro* functional assays showed knockdown of KCNN3 promoted BCa cell proliferation, migration, and invasion (Figure 4B-4D). Importantly, we showed the knockdown of KCNN3 could partially abrogated the biological functions of miR-652-3p in BCa (Figure 4B-4D).

Discussion

Our understanding regarding the roles of non-coding RNAs in human cancers is increasing due to the development in high-throughput sequencing technology[13-17]. For instance, Lyu et al[18] constructed a competing endogenous RNA (ceRNA) network related to the progression of muscle-invasive BCa consisted of 30 long non-coding RNAs (lncRNAs), 13 miRNAs, and 32 mRNAs. Also, they reported that lncRNA MIR137HG was correlated with the overall survival of patients with muscle-invasive BCa[18]. In addition, Jiang et al[19] identified 27 circle RNAs (circRNAs), 76 miR-

NAs, and 4744 mRNAs were aberrantly expressed in BCa tissues compared with normal tissues.

In this study, we explored the expression level of miR-652-3p in BCa tissues and cell lines. We showed miR-652-3p levels were significantly higher in BCa tissues and cell lines compared with normal tissue and cell line, respectively. However, the value of miR-652-3p on overall survival of BCa patients was not explored here and it should be validated in further studies. Functional assays showed knockdown miR-652-3p inhibits BCa cell proliferation, migration, and invasion *in vitro*. These results indicated miR-652-3p functions as oncogene in BCa, which is consistent with its role in non-small cell lung cancer[10]. Hence, targeting miR-652-3p may be a potential therapeutic measure for BCa but should be further validated using *in vivo* assays.

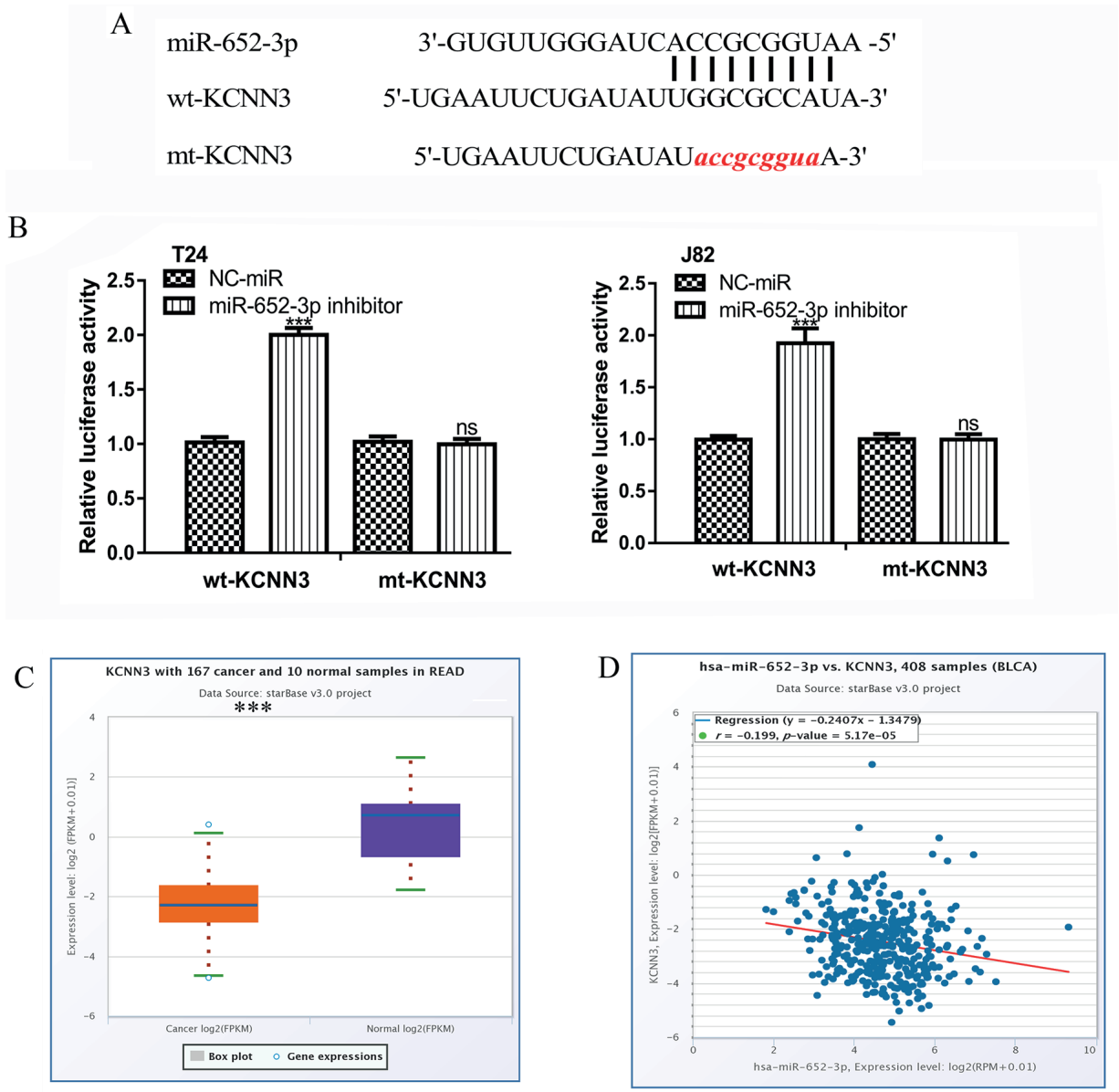

Figure 3. KCNN3 was a target for miR-652-3p in BCa. **A**, Binding region between miR-652-3p and KCNN3. **B**, Luciferase activity in cells with miRNAs or luciferase vectors transfection. **C**, KCNN3 expression level in BCa tissues and normal tissues. **D**, Correlation of miR-652-3p and KCNN3 in BCa tissues. MiR-652-3p: microRNA-652-3p; BCa: bladder cancer; KCNN3: potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3; wt: wild-type; mt: mutant; NC-miR: negative control miRNA.

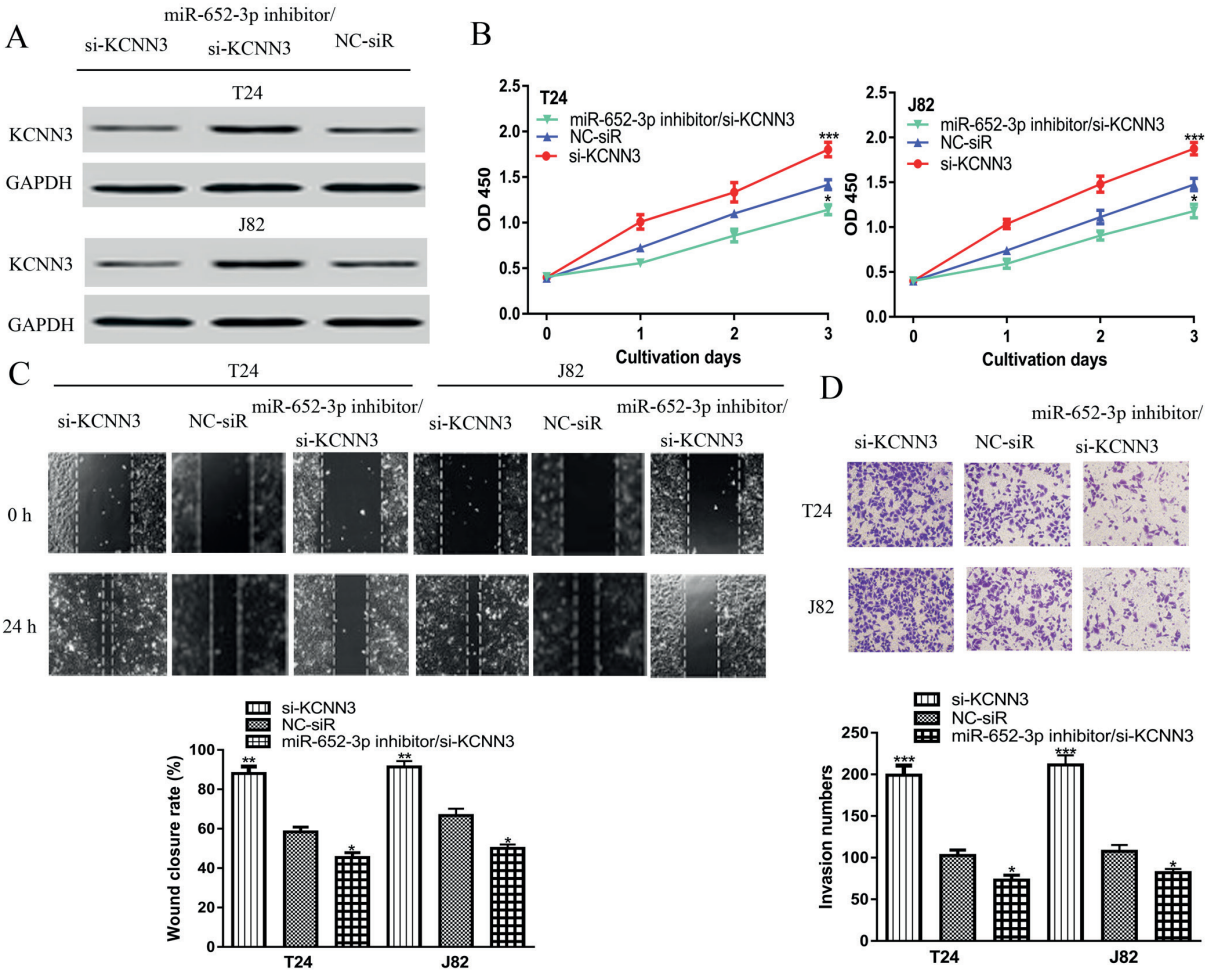

Figure 4. KCNN3 reverses the biological functions of miR-652-3p. **A**, KCNN3 expression, **B**, CCK-8 assay was used to analyze cell proliferation. **C**, Wound-healing assay was used to analyze cell migration (magnification: 200x). **D**, Transwell invasion assay was utilized to analyze cell invasion (magnification: 200x) in BCa cells transfected with si-KCNN3, NC-siR; si-KCNN3 and miR-652-3p inhibitor. MiR-652-3p: microRNA-652-3p; BCa: bladder cancer; CCK-8: cell counting kit-8; KCNN3: potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3; si-KCNN3: small interfering RNA targeting KCNN3; NC-siR: negative control small interfering RNA.

Lgl1 has been identified as a direct target for miR-652-3p in non-small cell lung cancer[10]. Here, for the purpose to understand the molecule involved in the miR-652-3p mediated cellular functions, targets for miR-652-3p were predicted by TargetScan. KCNN3, whose expression was found decreased expression in tumor tissues, was selected for further analyses. KCNN3 belongs to the Ca^{2+}-activated potassium channel family, was reported to have crucial roles in cancer progression[20]. For instance, KCNN3 was found decreased expression in ovarian cancer tissues and correlated with poorer overall survival of cancer patients[20]. In this work, we revealed miR-652-3p and KCNN3 was negatively correlated in BCa tissues. Furthermore, rescue experiments validated KCNN3 was a functional target for miR-652-3p.

Conclusions

MiR-652-3p directly regulated KCNN3 to affect BCa cell proliferation, migration, and invasion. These results presented advanced our understanding of the mechanisms behind BCa progression, hence providing novel potential treatment options for BCa.

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Conflicts of interest
The authors declare no conflicts of interest.

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