CircPVT1 Promotes Bladder Cancer Progression by Acting as a ceRNA for miR-140-3p to Target TRPS1

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Abstract

**Background:** Bladder cancer (BC) is one of the most malignancy tumor in the urinary system. Therefore, further studies are needed to revealed the molecular mechanism of BC progression and development. Previous study demonstrated that the deregulation of circRNAs can regulate cell biological functions in tumorigenesis and development. However, the roles of circPVT1 in BC have not yet been revealed.

**Materials and methods:** The expression level of circPVT1, miR-140-3p, and TRPS1 were measured by RT-PCR in BC tissues and cells; dual luciferase reporter and RIP assay showed that circRNA served as a sponge for miRNA, and miRNA could target mRNA. In vitro, effects of overexpression circPVT1 or sh-circPVT1 can regulate BC cells’ proliferation, migration, invasion were detected by CCK-8 assay, wound healing assay, and transwell assay.

**Results:** Our research demonstrated that the expression of circPVT1 was upregulated in BC tissues and cell lines, and increased in metastatic tissues compared to that of non-metastatic tissues. CircPVT1 sponging miR-140-3p to target TRPS1 was revealed by dual luciferase reporter and RIP assay. In addition, the expression level of miR-140-3p was reduced in BC tumor tissues, and TRPS1 was significantly increased in BC tumor tissues. Pearson correlation analysis showed that miR-140-3p with circPVT1 and TRPS1 as compare with miR-140-3p have a moderately negative correlation, and there was a moderately positive correlation between circPVT1 with TRPS1. Further, cytological studies found that circPVT1 enhance BC cells’ proliferation, migration, and invasion by targeting TRPS1 via miR-140-3p.

**Conclusion:** CircPVT1 plays a tumor enhancement role in BC and that can effectively promote cell proliferation, migration, invasion and EMT by targeting the miR-140-3p/TRPS1 axis. CircPVT1 may be a novel potential treatment and diagnosis biomarker in BC.

1. Background

Bladder cancer (BC) is a common malignant tumor of the urinary system. Globally, there will be an estimated 549,000 new cases diagnosed and BC has caused approximately 200,000 deaths in 2018, the incidence of BC in men is more than about four times that in women.\(^1\) Currently, surgery, radiotherapy and chemotherapy are still the main therapies for BC, Due to its high incidence, and recurrence and metastasis rates, the five-year survival rate for patients with BC remains low.\(^2, 3\) However, there are insufficient studies focused on early BC diagnosis and specific biomarkers.

CircRNA is an endogenous single-stranded circular non-coding RNA molecule without 5’ cap and 3’ tail that is formed by reverse splicing of pre-mRNA.\(^4, 5\) CircRNAs dysregulation can act as a tumor suppressor or be oncogenic and circRNAs can regulate cell biological functions including cell proliferation, migration, invasion, cell cycle, autophagy, and apoptosis in tumorigenesis and development.\(^6-10\) Recently, circPVT1 was shown that it could contributed to the progression of lung cancer,
hepatocellular carcinoma, and Osteosarcoma. [9-14] However, the roles of circPVT1 have not been elucidated in BC.

In our study, we demonstrate that circPVT1 is more highly expressed in BC tissues and cell lines than in the relevant controls. CircPVT1 directly targets miR-140-3p and regulate downstream the expression of TRPS1. Cytological studies found that circPVT1 regulates proliferation, migration, invasion, and (epithelial to mesenchymal transition) EMT in BC cells by targeting the miR-140-3p/TRPS1 axis. Taken together, these results suggest that circPVT1 may serves as a novel potential treatment and diagnosis biomarker in BC.

2. Materials And Methods

2.1 Bioinformatics analysis

We used online databases (circinteractome, starbase and circbank) to find potential miRNA of circPVT1 and the starbase databases was used to predict the target genes of miR-140-3p.

2.2 Clinical Specimens

39 pairs of BC clinical specimens and adjacent normal tumors were collected from the First Affiliated Hospital of Jiamusi University. None of them had undergone chemotherapy, radiotherapy or any anti-cancer treatment before surgery and all of the clinical specimens were immediately flash-frozen in liquid nitrogen during surgery until RNA extraction.

2.3 Cell culture and cell transfection

T24, UMUC3, EJ ,5637, J82, and SV-HUC-1 were obtained from the Cell Collection Committee of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated by RPMI-1640 medium (Gibco, USA) with 10% FBS (Gibco, Australia) at 37°C. CircPVT1 sh-RNA and circPVT1 over-expression plasmids were synthesized by GenePharma (Shanghai, China). MiRNAs inhibitors were purchased from RiboBio (Guangzhou, China) and cell transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s protocol.

2.4 RNA isolation, RNase R treatment and Real-Time PCR

The total RNA was extracted from 39 pairs of BC clinical specimens and adjacent normal specimens or BC cells using TRizol reagent (Invitrogen, CA, USA). 10 μg total RNA isolation was separated by 2 U/μg RNase R (Epicentre Technologies) 30 min at 37°C. Random primers and stem-loop primers were used to synthesize cDNA using the TaKaRa system (Takara, Dalian, China). RT-PCR primers were purchased from RiboBio. CircPVT1-Forward: ATCGGTGCCTCAGCGTTCGG, circPVT1-Reverse: CTGTCCTCGCCGTCACACCG; miR-140-3p-Forward: GCGCGTACCAGGGTAGAA, miR-140-3p-Reverse: AGTGCAGGGTCCGAGGTATT; TRPS1-Forward: GTATCCTGCATCGGGAGAAA, TRPS1-Reverse: AGCTTCTGCTAGAGG-CCACA; β-actin-Forward: CTAGTTGCGTTACACCCCTTCTTG, β-actin-Reverse:
CTGTCACCTTCACCGTTCCAGTTT; Real-time PCR was detected by the CFX96 Tm Real-Time System (Bio-
Rad, USA). The calculation method of relative expression was using the comparative Ct( $2^{-ΔΔCt}$) method.

### 2.5 CCK-8 assay

BC cells proliferation were determined by CCK-8 assay (Dojindo, Japan) following the manufacturer’s
instructions, a density of 1×10^4 T24 and EJ cells were plated into 96-well plates. After 24h, 10 µL of CCK-
8 solution was combined and incubated without light for 2h at 37°C. The absorbance was measured at a
wavelength of 450 nm using the BioTek (Winooski, USA) microplate spectrophotometer.

### 2.6 Wound healing assay

1×10^4 cells/well T24 cells or EJ cells was seeded into 6-well plates. When the cells reached 90%
confluency, the tip of a 200 µL pipette was used to scratch the cell monolayer and then fresh serous
medium was used to wash the plates three times. After 24 h, the wound width was calculated using
Image J software.

### 2.7 Transwell assay

1×10^4 cells/well T24 or EJ cells were seeded on the upper chambers with 2% serum medium and medium
containing 20% serum medium were added to the lower chambers. Then, the cells were incubated at
37°C for 24h. The migration cells number were counted to calculate the average number of migrated
cells per plate.

### 2.8 Dual-luciferase reporter assay

Cells were cotransfected with circPVT1-miR-140-3p and TRPS1-miR-140-3p into the luciferase gene(wild-
type or mutant-type), the specific operation was carried out according to the manufacturer’s protocol.
After 48h, luciferase activities were calculated for each well using the Dual Luciferase Reporter Assay
System (Promega).

### 2.9 RNA-binding protein immunoprecipitation (RIP)

RIP assay (Millipore, Billerica, MA) was carried out according to the manufacturer’s instructions. The RIP
lysate was obtained and centrifuged at 14,000 rpm for 10 minutes. Add 100 µL of the supernatant to the
RIP immunoprecipitation buffer containing the magnetic bead-antibody complex, then the RNA was
purified and obtained from TRIzol. Finally, analysis of immunoprecipitated RNA by RT-PCR.

### 2.10 Tumor xenografts

5×10^6 cells/well T24 were stably transfected with sh-circPVT1 plasmids or circPVT1 overexpression
plasmids or negative control vector were subcutaneously injected into the upper back of nude mice.
Tumor weight was detected every 7 days. The volume of tumors were calculated using the following
formula: \[ V = 0.5 \times L \times W^2 \]. One month later, all animals were scarified, and the tumor volume were recorded. The laboratory animals were approved by the medical laboratory animal ethics committee of Jiamusi University. Instructive notions with respect to caring for laboratory animals (which is released by the Ministry of Science and Technology of the People’s Republic of China in September 30th, 2006.) were followed for the welfare of the animals.

2.11 Western blot assay

Proteins were extracted from cells using RIPA with proteinase inhibitors (Sigma-Aldrich, USA) and the concentrations of proteins were measured using BCA Protein Assay kits (Thermo Scientific, MA). After separation by 10% SDS/PAGE, proteins were blocked with 10% non-fat milk for 1 h and immunoblotted with the primary antibodies at 4 °C overnight. Proteins were incubated with secondary antibodies for 1 hr. After TBST washing 3 times and protein levels were measured using the chemiluminescence image system (Bio-Rad, USA).

2.12 Statistic analysis

All data were analyzed by SPSS 20.0, \( P < 0.05 \) indicated statistical significant findings. Data is represented as means ± standard, and the differences of the two groups’ data were analyzed by Student’s t-test; Pearson correlation analysis was used to evaluate the correlation among circPVT1, miR-140-3p and TRPS1.

3. Results

3.1 circPVT1 is up-regulated in BC patients and cell lines

RT-PCR results showed that the expression of circPVT1 was higher in BC tissues compared to that in adjacent normal tumors(\( P<0.001 \), Fig. 1A), and was significantly increased in metastatic tissues compared to that of non-metastatic tissues(\( P<0.001 \), Fig. 1B). Besides, the expression of circPVT1 was significantly increased in T24, UMUC3, EJ, 5637 and J82 compared with SV-HUC-1(Fig. 1C). The linear mRNA PVT1 expression was significantly decreased, but circPVT1 expression did not have no significant change after the RNase R treatment(Fig. 1D). Then convergent primers and divergent primers were used to amplify linear or circRNA PVT1 by cDNA and genomic DNA (gDNA). RT-PCR results showed that divergent primers could amplify by cDNA but could not amplify by gDNA(Fig. 1E).

3.2 circPVT1 regulates BC cells proliferation, migration, and invasion

CircPVT1 over-expression plasmid could significantly increased the expression of circPVT1, and sh-circPVT1 could significantly decreased the expression of circPVT1 in EJ and T24 cells(Fig. 2A). However, there was no significant change PVT1 mRNA expression. CCK8 assay suggested that sh-circPVT1 induced the inhibition of cell proliferation and circPVT1 overexpression could promoted cell proliferation in EJ and T24 cells (Fig. 2B). Wound healing assay and transwell assay revealed that circPVT1
knockdown could suppressed invasion and migration, and circPVT1 overexpression could reversed the function of EJ and T24 cells,(Fig. 2C, D, E, F). BALB/c nude mice results showed that mice injected with T24 cells circPVT1 knockdown had smaller average volume than the control groups, whereas overexpressing circPVT1 significantly increased tumor xenografts(Fig. 2G, H).

3.3 circPVT1 can binding to miR-140-3p, and TRPS1 is a direct target of miR-140-3p in BC cell

We used three publicly online tools circinteractome (https://circinteractome.nia.nih.gov/), starbaseV3.0(http://starbase.sysu.edu.cn/) and circbank (http://www.circbank.cn/index.html) to predicted the possible binding miRNAs of circPVT1. We compared three online databases, and 16 potential miRNAs were selected(Fig. 3A, B). Dual-luciferase reporter assay revealed that 6 miRNAs including miR-140-3p, miR-361-3p, miR-384, miR-508-3p, miR-620, and miR-769-5p could decreased luciferase reporter activities more than 50% compared to that of the control group(Fig. 3C). In addition, we used biotin-labeled circPVT1 probe to perform RIP assay. RIP assay results demonstrated that biotin-coupled circPVT1 probe was copurified with miR-140-3p more than NC, and sh-circPVT1 probe was copurified with miR-140-3p less than NC.(Fig. 3D). The expression levels of miR-140-3p was up-regulated when transfected with sh-circPVT1 plasmid and decreased by circPVT1 over-expression plasmid in EJ and T24 cells(Fig. 3E).

We used starbase V3.0 databases (http://starbase.sysu.edu.cn/) to predict the target genes of miR-140-3p. Dual luciferase reporter assay confirmed that miR-140-3p considerably decreased the luciferase activity in the TRPS1-WT group but not in the TRPS1-MUT group when compared with the control group(Fig. 3F, G). Furthermore, the expression of TRPS1 transfected with circPVT1 over-expression plasmid was up-regulated, and The expression of TRPS1 was down-regulated cotransfected with miR-140-3p mimics in EJ and T24 cells, miR-140-3p mimics can reverse TRPS1 overexpression function by circPVT1 (Fig. 3H). RT-PCR revealed that the expression of miR-140-3p was reduced and TRPS1 was significantly increasedin BC tumor tissues compared with their controls (Fig. 3I, J).

In addition, Correlation analysis showed a moderately negative correlation between miR-140-3p with circPVT1 and TRPS1 with miR-140-3p, and a moderately positive correlation between circPVT1 with TRPS1(Fig. 3K).

3.4 circPVT1 inhibits bladder cells proliferation, migration, invasion and EMT by targeting TRPS1 via miR-140-3p

The study also investigated whether or not circPVT1 directly target TRPS1 via miR-140-3p to induce EMT in BC cells. The results showed miR-140-3p inhibition could reverse the function of BC cells’ proliferation, migration, and invasive by sh-circPVT1 (Fig. 4A,B,C), western blot results showed that the expression of TRPS1 protein had the similar results in BC cells. Furthermore, E-cadherin expression was significantly decreased, N-cadherin and Vimentin were increased by sh-circPVT1 and sh-TRPS1, and miR-140-3p inhibition could significantly decreased E-cadherin and increased N-cadherin, Vimentin expression(Fig.
4D), and showed that miR-140-3p inhibition could neutralize the expression of EMT biomarker by sh-circPVT1 in BC cells (Fig. 4D, E, F).

4. Discussion

BC is a heterogeneous disease, making it more difficult to study its diagnosis, treatment and pathogenesis. The overall efficacy of existing BC treatment options is limited,\(^\text{[15]}\) and the pathogenesis of BC remains unclear. Studies have reported that circular RNA is involved in the pathogenesis of BC.\(^\text{[16–18]}\) In our research, we found that circPVT1 expression was higher in BC tissues and cell lines and was significantly increased in metastatic tissues compared with non-metastatic tissues.

It is widely accepted that circRNA can binding to miRNA and miRNA can target mRNA were widely accepted. In gastric cancer, circSPECC1 regulates the growth and invasion by targeting miR-526b and the downstream KDM4A/YAP1 pathway.\(^\text{[19]}\) Non-small cell lung cancer progression is regulated by circ_0020123 by sponging miR-488-3p.\(^\text{[20]}\) It has been shown that circRNA can affect the migration, invasion and EMT in various tumors, including BC.\(^\text{[21–25]}\) Dual luciferase reporter and RIP assay results showed that circPVT1 could sponge miR-140-3p, and that miR-140-3p could target TRPS1. Further, Cytological studies showed that the lower circPVT1 expression can effectively inhibit cell proliferation, migration, invasion, and the EMT in BC cells by targeting TRPS1 via miR-140-3p.

Studies have reported that EMT is the driving force for cancer cells metastasis, the EMT is an important mechanism in the early stage of cancer metastasis, to study the molecular mechanism of EMT is the key to improve tumor diagnosis and treatment.\(^\text{[26,27]}\) The decreased expression of E-cadherin and Vimentin, N-cadherin and other proteins have increased which have been considered to be the most significant feature of EMT. The changes of these EMT markers have enabled cancer cells to obtain features that promote migration and invasion.\(^\text{[28]}\) Similarly, our results reveal that the over-expression of circPVT1 can regulate the E-cadherin/N-cadherin pathway to inhibit the EMT in BC cells.

5. Conclusion

In conclusion, this study demonstrated that circPVT1 was more highly expression in BC tissues and cell lines, and circPVT1 act as a sponge for miR-140-3p to regulate TRPS1. Moreover, cytological studies found that circPVT1 regulates cell proliferation, migration, invasion, and EMT by targeting TRPS1 via miR-140-3p in BC cells. Our results suggest that circPVT1 plays a tumor enhancement role in BC and that can effectively promote cell proliferation, migration, invasion and EMT by targeting the miR-140-3p/TRPS1 axis. circPVT1 may be a novel potential treatment and diagnosis biomarker in BC.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Jiamusi University. All BC patients provided written informed consent and the study abided by the right to privacy of human rights subjects.

**consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included

**Conflicts of interest**

The authors reports that they have no competing interests.

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**Authors’ contributions**

Bao-Jin Chi, Yao Sun Jin-tao zhao, and Sheng Bi performed the experiments, analysed the data and wrote the paper. Shu-Qiu Wang, Liang Huang conceptualized the study design, and contributed to data analysis and experimental materials. All authors read and approved the final manuscript.

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Figures
circPVT1 is up-regulated in BC patients and cell lines A, QRT-PCR analysis of the expression levels of circPVT1 in BC tissues compared with normal tissues. B, The expression levels of circPVT1 in BC tissues with lymph node metastasis compared with those without metastasis. C, QRT-PCR analysis of the expression levels of circPVT1 in BC cells SK-HEP-1, HepG2, in T24, UMUC3, EJ, 5637, and J82 and human immortalized uroepithelium cells (SV-HUC-1). D, QRT-PCR analysis of the expression of circPVT1
and mRNA PVT1 after RNase R treatment in EJ or T24 cells. E, The schematic diagram of convergent primers and divergent primers to amplify linear and circPVT1 by cDNA and gDNA by RT-PCR, and below is the result of agarose gel electrophoresis after qRT-PCR, respectively. Data represent mean ± SD. *P < 0.05, ***P < 0.001 compared with negative control.

Figure 2
circPVT1 regulates BC cells proliferation, migration, invasion. A, RT-PCR assay showed circPVT1 overexpression plasmid was significantly increased the expression of circPVT1, and sh-circPVT1 could significantly decreased the expression of circPVT1 in EJ and T24 cells. B, CCK8 assay showed that circPVT1 overexpression induced to promoted cell proliferation and sh-circPVT1 could inhibition of cell proliferation in EJ and T24 cells. C,D,E,F, Wound healing, transwell assays showed that over-expression of circPVT1 could significantly increased invasion, migration and circPVT1 knockdown significantly suppressed migration, invasion in EJ and T24 cells. G,H, BALB/c nude mice injected with T24 cells sh-circPVT1 had smaller volume and overexpressing circPVT1 could promoted tumor Xenografts. Data represent mean ± SD. *P < 0.05 compare with negative control.
CircPVT1 acts as a sponge for miR-140-3p, and TRPS1 is a direct target of miR-140-3p in BC cell A, bioinformatic analysis to search for miR-140-3p interact with circPVT1-MUT or circPVT1-WT. B, 16 miRNA mimics were co-transfected with the circPVT1 vector into T24 cells, the line means reduced at least half of the luciferase reporter activities. C, RNA pull-down assay for the luciferase activity of circPVT1-MUT or circPVT1-WT in T24 cells co-transfected with 6 miRNA mimics. D, RIP assay for the amount of circPVT1 and miR-140-3p in T24 cells transfected with circPVT1 overexpression or sh-circPVT1 or negative control. E, qRT-PCR analysis of expression levels of miR-140-3p in EJ and T24 cells transfected with circPVT1 overexpression or sh-circPVT1 or negative control. F, bioinformatic analysis to search for miR-140-3p interact with TRPS1-MUT or TRPS1-WT. G, Luciferase reporter assay for the luciferase activity of TRPS1-MUT or TRPS1-WT in T24 cells co-transfected with miR-140-3p. H, RT-PCR analysis of the expression levels of TRPS1 transfected with sh-circPVT1 plasmid was down-regulated and circPVT1 over-expression was up-regulated compare with their control in EJ and T24 cells, the expression of TRPS1 transfected with miR-140-3p mimics was down-regulated in EJ and T24 cells compared with their control. I, QRT-PCR analysis of the expression levels of miR-140-3p in BC tissues compared with normal tissues. J, The expression levels of TRPS1 in BC tumor tissues compared with adjacent normal tissues. K, Pearson correlation was used for correlation analysis between circPVT1, miR-140-3p and TRPS1 in BC patients. Data represent mean ± SD. *P < 0.05 compare with negative control, #P < 0.05 compare with circPVT1+miR-140-3p mimics.
Figure 4

CircPVT1 inhibits bladder cells proliferation, migration, invasion and EMT by targeting TRPS1 via miR-140-3p. A, CCK8 assay showed that negative control or sh-circPVT1 or sh-circPVT1+ miR-140-3p inhibition or miR-140-3p inhibition or sh-TRPS1 could regulated EJ and T24 cells’ proliferation. B, Wound healing assay showed that negative control or sh-circPVT1 or sh-circPVT1+ miR-140-3p inhibition or miR-140-3p inhibition or sh-TRPS1 could regulated EJ and T24 cells’ migration. C, Transwell assay showed that negative control or sh-circPVT1 or sh-circPVT1+ miR-140-3p inhibition or miR-140-3p inhibition or sh-TRPS1 could regulated EJ and T24 cells’ invasion. D, E, F Western blot analysis the expression levels of TRPS1, E-cadherin, N-cadherin, and Vimentin transfected with negative control or sh-circPVT1 or sh-circPVT1+ miR-140-3p inhibition or miR-140-3p inhibition or sh-TRPS1 in EJ and T24 cells. Data represent mean ± SD. *P < 0.05, #P < 0.05 compare with sh-circPVT1+ miR-140-3p inhibition.