Circular RNA hsa_circ_0075828 promotes bladder cancer cell proliferation through activation of CREB1

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Running Title: hsa_circ_0075828 promotes proliferation in BC

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Supplementary Table and figure legends

Fig. S1 Microarray analysis was performed in BC 5637, T24 and control SV-HUC-1. (A) Samples were normalized for further analysis. (B, C) Scatter diagrams between 5637/T24 and SV-HUC-1 were presented. (D, E) The heatmaps of circRNA expression between 5637/T24 and SV-HUC-1 were shown.
Fig. S2 The mice body weight was no difference after knockdown of \textit{circCASC15} during thirty days of feeding compared with the control.
Fig. S3 miR-1224-5p was the target of circCASC15 using luciferase assay. (A) Schematic of circCASC15 wild-type (WT) and mutant (Mut) luciferase reporter vectors is shown. (B, C) The luciferase activity was decreased or increased significantly after overexpression or knockdown of miR-1224-5p in 5637 and T24 cells, respectively. (D) Schematic of CREB1 wild-type (WT) and mutant (Mut) luciferase reporter vectors is presented. (E, F) The luciferase activity was inhibited or promoted remarkably after overexpression or knockdown of miR-1224-5p in 5637 and T24 cells, respectively. *p < 0.05 and **p < 0.01.
**Table S2** Correlation between *circCASC15* expression and clinicopathological characteristics of BC patients

<table>
<thead>
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<th>Parameters</th>
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<td></td>
<td></td>
<td></td>
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<td>Low</td>
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<td></td>
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<tr>
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<td>Pt2-T4</td>
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<td>Lymph nodes</td>
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<td>18</td>
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*P < 0.05 was considered significant (Chi-square test between 2 groups)
Supplementary Materials and Methods

Cell transfection

Cells were transfected with miRNA mimics, miRNA inhibitors or corresponding plasmids using Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. 2 μg/ml puromycin was used to select BC 5637 cells to construct stable cell line.

Microarray analysis

Total RNA was isolated from BC 5637, T24 and normal control SV-HUC-1 cells. Isolated RNA was amplified and transcribed into fluorescent cRNA using random primers, and then hybridized onto Arraystar Human circRNA Array (8×15K, Arraystar). After washed, the arrays were scanned by Agilent Scanner G2505C and analyzed with Agilent Feature Extraction. Quantile normalization and subsequent data processing were performed using R software. Differentially expressed transcripts with statistical significance between cancer cells group and SV-HUC-1 group were identified by threshold (fold change $\geq 2.0$).

Vector construction

The siRNA aimed at circCASC15 was synthesized by GenePharma (Shanghai, China). The sequence of siRNA targeting circCASC15 was
5’-TGGGCCCAAGGTGCTGCTTGTG-3’.

The shRNA against circCASC15 was synthesized and cloned into pLL3.7 vector. The sequence of shRNA targeting circCASC15 was 5’-TGGGCCCAAGGTGCTGCTTGTG TCAAGAGCATCCCCCATGGTCTTCTATTTTTT-3’. Sequence of circCASC15 was synthesized by Sangong (Shanghai, China) and cloned into pCD-ciR vector (Geenseed Biotech Co, Guangzhou, China) that possesses two elements termed as the front circular and the back circular frame. The wide-type circCASC15/CREB1 3’ untranslated region (UTR) sequence or circCASC15/CREB1 3’UTR sequence with mutation of miR-1224-5p binding site was subcloned into psiCHECK2 vector (Promega, Madison, WI). The CDS sequence of CREB1 was cloned into the plasmid vector pcDNA3.1. MiR-1224-5p mimics, inhibitors and its corresponding control were purchased from RiboBio (Guangzhou, China).

**Cell proliferation assays**

Cell proliferation was tested by Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Shanghai, China) and 5-ethynyl-20-deoxyuridine (EdU) assay kit (Ribobio, Guangzhou, China) following the manufacturer’s instructions. For CCK-8 assay, cells (3000 per well) were seeded into a 96-well plate and 10 μl of CCK-8 was added to each well. The cells were incubated for 30 minutes after a 24, 48 or 72 hour transfection. Absorbance was detected at a wavelength of 450 nm employing an ELISA microplate reader (Bio-Rad, Hercules, CA, USA). For EdU assay, EdU images were acquired and analyzed with an
Olympus FSX100 microscope (Olympus, Tokyo, Japan). EdU positive cells were visualized with red (Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit from Riobio) or green (Cell-Light™ EdU Apollo®567 In Vitro Imaging Kit from Riobio) fluorescence. Blue fluorescence represented Hoechst-stained cells. Cell proliferation activity was assessed by the rate of EdU positive cells/Hoechst-stained cells.

Subcellular fractionation location

NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA) was used to get cytoplasmic and nuclear fraction from BC 5637 and T24 cells. RNAs separated from each of the fractions were used for qRT–PCR analysis to measure the expression levels of cytoplasmic control transcript (GAPDH), nuclear control transcript (U6) and circCASC15.

RNA-binding protein immunoprecipitation (RIP) assay

BC 5637 cells were transfected with miR-1224-5p mimics or NC mimics and RIP was conducted after 48 hours transfection. The Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) was used for RIP assay according to the manufacturer’s instructions. Human anti-Argonaute2 (AGO2) antibody (Millipore, Billerica, MA, USA) or negative control mouse IgG (Millipore, Billerica, MA, USA) were used to pull down binding RNAs and then the immunoprecipitated RNAs were analyzed by RT-PCR or qRT-PCR to detect the expression of circCASC15.
Dual-luciferase reporter assay

BC cells were transfected with corresponding plasmids and miRNA mimics or inhibitors. After 48 hour transfection, dual-luciferase reporter assay kit (Promega, Madison, WI, USA) was used to detect luciferase and renilla signals. The renilla luciferase activity was normalized to luciferase activity.

Western blot analysis

20 μg proteins were split using 10% SDS/PAGE gels and then transferred onto polyvinylidene fluoride (PVDF) membranes and then incubated with primary specific antibodies including anti-CREB1 antibody (ab31387, ABCAM) and anti-Vinculin antibody (ab129002, ABCAM). The secondary antibody anti-rabbit IgG, HRP-linked (#7074) was bought from Cell Signaling Technology (Danvers, MA, USA; dilution, 1:10, 000). The signal intensities were quantified using Super Signal Chemiluminescence Substrate (Pierce, Thermo Scientific, Waltham, MA, USA).

Xenograft tumor model

Male immune-deficient BALB/c nude mice (5-6 weeks old) were purchased from Beijing Wei-tong Li-hua Laboratory Animals and Technology Ltd, Beijing, China. The experimental procedures were approved by the Institutional Ethics Review Board of Peking University Shenzhen Hospital. The shRNA plasmids targeting circCASC15 were
packed into lentivirus according to the manufacturer’s instructions using Lentiviral Packing Kit, SyngenTech, Beijing, China. T24 cells could not generate tumor in mice according to ATCC. Thus, 5637 cells were infected with lentivirus following the manufacturer’s protocols. $3 \times 10^6$ 5637 cells were suspended in 100 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and injected subcutaneously into the dorsal flank regions of mice. After half a month, tumor volumes were observed every 5 days during a 2-week period. Tumor volumes were calculated using the formula: $0.5 \times \text{length} \times \text{width}^2$. Mice were sacrificed and measured for tumor weight.