European Review for Medical and Pharmacological Sciences 2020; 24: 1081-1087

Circular RNA hsa_circ_0017247 acts as an oncogene in bladder cancer by inducing Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Bladder cancer (BLCA) is the most common genitourinary malignancy in the world. Recent studies have revealed that circular RNAs (circRNAs) are dysregulated in malignant tumors and participate in carcinogenesis. The purpose of our work is to uncover how hsa_circ_0017247 functions in BLCA.

PATIENTS AND METHODS: In this research, Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was conducted to measure hsa_circ_0017247 expression in BLCA tissue samples. Besides, proliferation assay, colony formation assay, and flow cytometry assay were performed in BLCA cells after hsa_circ_0017247 was knocked down. Meanwhile, the western blot assay was conducted to explore the target signaling pathway of hsa_circ_0017247. Furthermore, tumor formation and metastasis assay was also conducted *in vivo*.

RESULTS: Compared with the adjacent tissues, a significant upregulation in hsa_circ_0017247 expression was observed in BLCA samples. Functional assays showed that the inhibition of cell proliferation was induced by downregulating hsa_circ_0017247 in BLCA *in vitro*, while the promotion of cell proliferation was induced by downregulating hsa_circ_0017247 in BLCA *in vitro*. Moreover, the results of further experiments revealed that the targeted proteins in the Wnt/β-catenin signaling pathway were downregulated via knockdown of hsa_circ_0017247 in BLCA. In addition, tumor formation and metastasis of BLCA were inhibited by knockdown of hsa_circ_0017247 in nude mice.

CONCLUSIONS: We discovered a vital regulatory mechanism of hsa_circ_0017247 in BLCA which might serve as a new therapeutic intervention for BLCA patients.

Key Words:
Circular RNA, Hsa_circ_0017247, Wnt/β-catenin signaling pathway. Bladder cancer.

Introduction

Bladder cancer (BLCA) remains the second most common genitourinary malignancy which ranks the sixth most frequent cancer in the world. Incidence has been increasing with a stable rate in most countries[2]. Moreover, it has been estimated that 549,393 new BLCA patients were diagnosed with this disease in 2018 and 170,728 new cases would be expected to suffer from BLCA in 2040[3]. In addition, BLCA constituted 199,922 cancer-related deaths in 2018 and the number was predicted to double in 2040[4]. The 5-year survival rate for BLCA patients remains dismal because of the migration to surrounding organs and lymph nodes or distant organs[5]. Therefore, it's very important and urgent to figure out potential early detection markers and therapeutic targets of BLCA.

Circular RNAs (circRNAs) are a large class of non-protein-coding transcripts. Recently, circRNAs have caught much attention for key regulators of important biological processes during the development and progression of tumors. Circular RNAs (circRNAs) are tissue-specific, ubiquitously expressed noncoding RNAs. The majority of circRNAs are more stable than linear RNA due to their resistance to exonucleolytic degradation[6]. Serving as microRNA (miRNA) sponges is the first described function of cellular circRNAs. Recently, it has been reported that circRNAs participate in tumorigenesis in a variety of cancers. For example, by sponging miR-424-5p and modulating the expression of LATS1, circ _LARP4 suppresses cell proliferation and cell invasion in gastric cancer[7]. Circ VPS13C-has-circ-001567 is

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upregulated in ovarian cancer and promotes cell proliferation and cell invasion[8]. By regulating the expression of miR-29a, circ MYLK functions as an oncogene and promotes the progression of prostate cancer[9]. CircRNA 100146 functions as an oncogene and enhances cell proliferation and cell in non-small cell lung cancer by binding to miR-615-5pand miR-361-3p directly[10].

Hsa_circ_0017247 is a novel circRNA in numerous cancers. The fundamental role of hsa_circ_0017247 has been identified in tumor metastasis and proliferation. However, the role of hsa_circ_0017247 in BLCA is still unclear. In this report, we first discovered that hsa_circ_0017247 was involved in cell proliferation, apoptosis, and cell cycle by inducing Wnt/β-catenin signaling pathway in BLCA cells, which might offer new insights on the therapy of BLCA.

Patients and Methods

Clinical Samples

Human BLCA tissues and adjacent tissues were obtained from 50 BLCA patients at the To[illegible] Hospital, Shanghai Jiao Tong University of Medicine from April 2016 to Decembe[illegible]18. This research was approved at Hospital's Pi[illegible] tion of Human Subjects Committee of Tong[illegible] Hospital, Shanghai Jiao Tong Uni[illegible]rsity Scho[illegible] of Medicine required. All cas[illegible]iagnosed with BLCA by two inde[illegible]ent p[illegible]ologists without any controversy. [illegible]ten inf[illegible]ed con-sent was offered by each[illegible]t bef[illegible]

Cell Culture

Four BLCA cel[illegible]s (UM-UC3, [illegible]80, BIU, and J82) were p[illegible] from the Ame[illegible]n Type Culture Colle[illegible] (A[illegible] Manassas, VA, USA). The culture[illegible]edium Dulb[illegible] Modified Eagle's Medium [illegible]EM; Hyclone, [illegible] Logan, UT, USA) a[illegible]0% fe[illegible]al bovine ser[illegible]m (FBS; Gibco, Carls[illegible], CA, [illegible]) were used to incubate the cel[illegible]n inc[illegible]or cont[illegible]aining 5% CO_2 at 37°C.

Cell Tra[illegible]tion

[illegible]ransfe[illegible]virus expressing short-hairpin [illegible] (shRN[illegible]geting hsa_circ_0017247 was com[illegible]aded and then cloned to a pLenti-EF1a-EGFP[illegible]tor (Biosettia Inc., San Diego, CA, [illegible]). Hsa_circ_0017247 shRNA and negative control shRNA were used for transfection in J82 BLCA c[illegible]h lipofectamine 2000 (Invitrogen, Carlsbad, CA, [illegible]) according to the manufacturer's protocol.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-[illegible]

TRIzol reagent (Invitrogen, C[illegible]ad, [illegible] USA) was utilized to isolate tota[illegible]A from tissues and cells. SYBR green (R[illegible] Basel, Switzerland) was conducted to meas[illegible] relative expression levels by norm[illegible]ed to p[illegible] The primers were used as fo[illegible]s: hsa_circ_[illegible] forward: 3'-ACTGCC[illegible]AAAG[illegible]GTGTCC[illegible]', circ_0017247, rever[illegible]3'-TCC[illegible]GAATGAG-CCATCTGTCT-5'; [illegible]ard 5'-[illegible]ATG-GAAATCGTCA[illegible]GG[illegible]d reve[illegible]-TGG-CACTTAGTT[illegible]AATG[illegible] The[illegible]nal cycle was as follow[illegible] sec at 95°C, [illegible]40 cycles at 95°C, 35 s[illegible] The relative [illegible]xpression was calculat[illegible] per[illegible] the $2^{-\Delta\Delta CT}$ method.

Me[illegible]hiazolyl Tet[illegible]ium (MTT) Assay

[illegible]owing the manufacturer's protocol, 2 ×10[3] [illegible]sfected cells [illegible]ere seeded in 96-well plates [illegible] cell prolife[illegible]n was assessed by the Cell [illegible]iferation Re[illegible]t Kit I (MTT; Roche, Basel, S[illegible]land) at [illegible]4, 48, and 72 h. Absorbance at 4[illegible] [illegible]ssessed using an ELISA reader system (Multiskan Ascent, LabSystems, Helsinki, Finland).

Flow Cytometry Assay

Flow cytometry binding buffer (100 µL) was added after harvested cells were washed twice using ice-cold. A mixture containing 5 µL Annexin V/FICC (fluorescein isothiocyanate) and 5 µL propidium iodide (PI; BD, Franklin Lakes, NJ, USA) was used for staining these cells for 15 min in the dark. Then, they were added with 400 microliters binding buffer. FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was performed to analyze cell apoptosis.

2×10[5]/mL cells were diluted by RNase A in 75% ice-cold ethanol overnight. These cells were stained with propidium iodide (PI; 50 mg/mL; BD, Franklin Lakes, NJ, USA) in the dark for 30 min at 4°C. The distribution of the cell cycle was analyzed were measured with a flow cytometer (FACScan; BD Bioscience, CA, USA).

Western Blot Analysis

Cell samples were washed with precooled phosphate-buffered saline (PBS) and then lysed with cell lysis radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). The protein concentration was detected using bicinchoninic acid (BCA; Thermo Fisher Scientific, Waltham, MA, USA). The proteins were transferred on to

a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), blocked in Tris-Buffered Saline and Tween-20 (TBST; 25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and incubated for 2 h. The proteins were incubated with the primary antibody of target proteins including Wnt3a, β-catenin, C-myc, and Survivin (Abcam, Cambridge, MA, USA) in the Wnt/β-catenin signaling pathway and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, MA, USA) and incubated at 4°C overnight. After being washed (3 × 10 min) with TBST, the secondary antibody was added and incubated at room temperature for 1 h. Results were analyzed by Image J software (Media Cybernetics, Silver Springs, MD, USA).

Xenograft Model

For the tumor formation assay, transfected cells were subcutaneously injected into NOD/SCID mice (6 weeks old). Tumor diameters were detected every 5 days after inoculation. Tumor volume was calculated as the formula (volume= length × width^2 × 1/2). Mice were sacrificed and t[illegible]rs were extracted after 4 weeks. The animal[illegible] ments were approved by the Animal Ethics [illegible]m-mittee of Shanghai Jiao Tong University S[illegible]l of Medicine.

Statistical Analysis

Statistical Product and [illegible]rvice[illegible]olutions (SPSS) 18.0 (IBM Corp., A[illegible]nk, NY[illegible]A) was utilized to conduct the [illegible]cal a[illegible] tailed Student's *t*-test [illegible]as p[illegible] to analyze the significance. Wh[illegible]<0.05, [illegible]a were considered statistically [illegible]nificant.

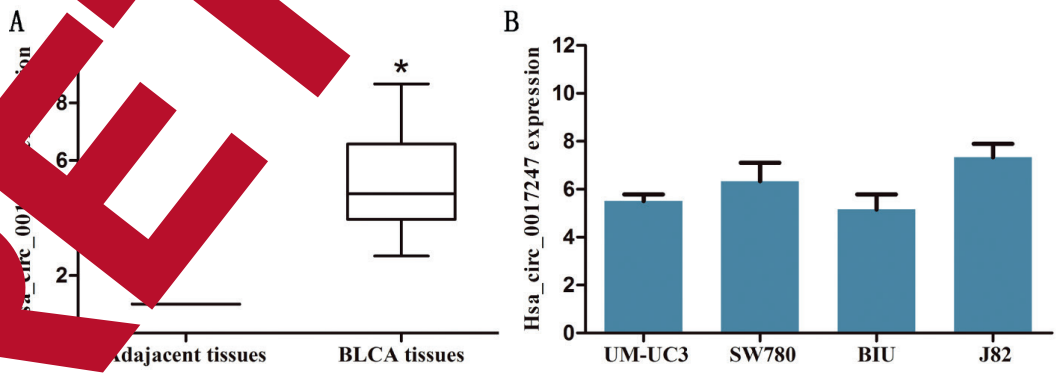
Results

Expression Level of Hsa_circ_[illegible]247 in Tissues and Cells of BLCA

To determine how hsa_ci[illegible]7247 functioned in the tumorigenesis of B[illegible]T-qPCR was conducted to detect [illegible]_circ_[illegible]7 expression in 50 patients'[illegible]es and fou[illegible] cells. As was shown i[illegible]gure 1[illegible] upregu[illegible] hsa_circ_0017247 w[illegible]oserve[illegible] BLCA tissue samples compared [illegible]hat[illegible]djacen[illegible]sues. Besides, as was [illegible]ow[illegible]ure 1B[illegible]e hsa_circ_0017247 [illegible]ession le[illegible]s t[illegible]ighest in J82 cells am[illegible]our BLCA ce[illegible]

Hsa_circ_0017247 [illegible]ckdown Re[illegible]d Cell Grow[illegible]bility i[illegible]CA Cells

To determine [illegible]hether hsa_circ_0017247 had [illegible]ital function[illegible]BLCA, J82 cells were cho-[illegible]for knockd[illegible] of hsa_circ_0017247. The h[illegible]_0017[illegible]shRNA and negative control shR[illegible]thetized and transduced into J82 cells. Then, the hsa_circ_0017247 expression was determined by RT-qPCR (Figure 2A). As was [illegible]Figure 2B, MTT assay results showed [illegible]at th[illegible]nhibition of cell viability in BLCA cells was induced by knockdown of hsa_circ_0017247. To further confirm the outcome of the MTT assay, we performed colony formation assays in BLCA cells. As was shown in Figure 2C, colony formation assay results showed that the number of colonies was reduced after hsa_circ_0017247 was knocked down. These results indicated hsa_circ_0017247 served as an oncogene in the proliferation of BLCA.



[illegible]re 1. Expression levels of hsa_circ_0017247 in BLCA tissues and cell lines. **A,** Hsa_circ_0017247 expression was sig-[illegible]y upregulated in the BLCA tissues compared with adjacent tissues. **B,** Expression levels of hsa_circ_0017247 relative to [illegible]were determined in the human BLCA cell lines by RT-qPCR. Data are presented as the mean ± standard error of the mean. [illegible]<0.05.

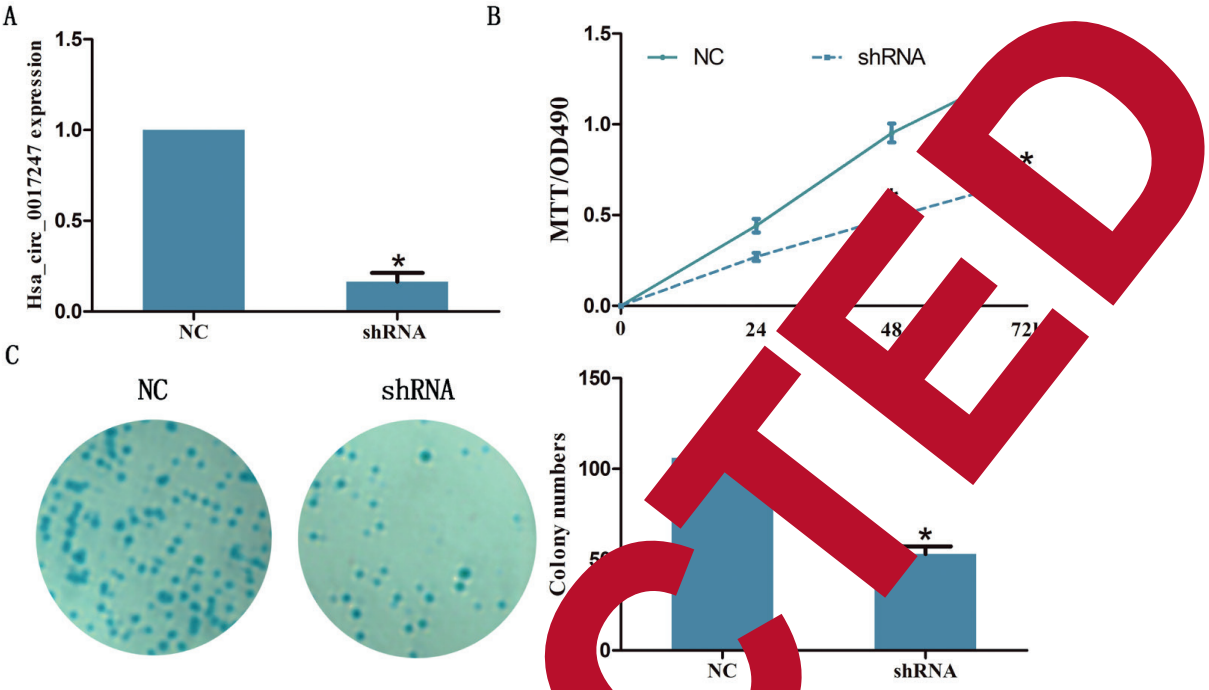


Figure 2. Knockdown of hsa_circ_0017247 inhibited BLCA cell pr[illegible]ion. **A,** Hs[illegible]_0017247 expression in BLCA cells transduced with negative control shRNA (NC) or hsa_circ_0017247 s[illegible]as detected by RT-qPCR. β-actin was used as an internal control. **B,** MTT assay showed that the inhibition of cel[illegible] BLCA cells was induced by knockdown of hsa_circ_0017247. **C,** Colony formation assay re[illegible]d that number of colonies was reduced after hsa_circ_0017247 was knocked down (magnification: 10×). The resu[illegible]age of three independent experiments (mean ± standard error of the mean). *p<0.05

Hsa_circ_0017247 Knock[illegible] Promoted Cell Apoptosi[illegible]d R[illegible]lated Cell Cycle in BLCA Cel[illegible]

To explore the effect[illegible] circ[illegible] the cell apoptosis and [illegible]ell [illegible]LCA cells, flow cytometry ass[illegible]was pe[illegible]d. As was shown in Figure [illegible]a significan[illegible]ase was viewed in the [illegible]e of cell ap[illegible]osis in BLCA cells [illegible]sfect[illegible]th hsa_circ_0017247 shRNA. As [illegible]was shown in[illegible]e 3B, the percentage of G0[illegible] cells was increa[illegible]d the percentage of [illegible]ls was reduced after [illegible]he downregulation [illegible]sa_circ[illegible]7247 in BLCA cells. These res[illegible]gges[illegible]t hsa[illegible]_0017247 functioned in reg[illegible]optosis[illegible] cell cycle of BLCA.

The [illegible]teractio[illegible]etween Wnt/β-Catenin Sig[illegible]ng Pathway and Hsa_circ_0017247 in[illegible]

[illegible] explore the underlying mechanism of [illegible]circ_0017247 function in BLCA, RT-qP-[illegible] Western blot assay were conducted to d[illegible]t the target proteins in Wnt/β-catenin signaling pathway such as Wnt3a, β-catenin, C-myc, and Survivin. As was shown in Figure 4A, the mRNA expression of Wnt3a, β-catenin, C-myc, and Survivin could be downregulated *via* the knockdown of hsa_circ_0017247. As was shown in Figure 4B, the protein level of Wnt3a, β-catenin, C-myc, and Survivin could be downregulated *via* the knockdown of hsa_circ_0017247. These results suggested that hsa_circ_0017247 participated in the regulation of the Wnt/β-catenin signaling pathway and further promoted BLCA development.

Hsa_circ_0017247 Knockdown Inhibited Tumor Formation In Vivo

The ability of hsa_circ_0017247 in tumor formation was further detected *in vivo*. As was shown in Figure 5A, the tumor size in hsa_circ_0017247 shRNA group was smaller than that in negative control shRNA group. As was shown in Figure 5B, the weight of dissected tumors in hsa_circ_0017247 shRNA group was smaller than that in negative control shRNA group.

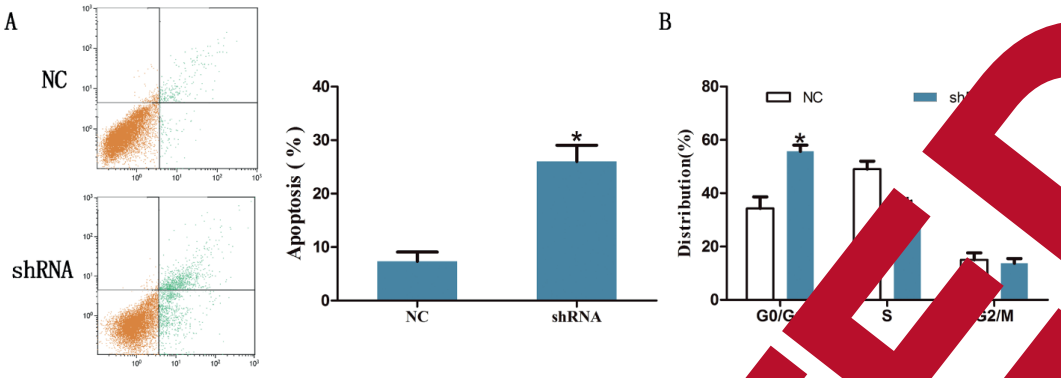
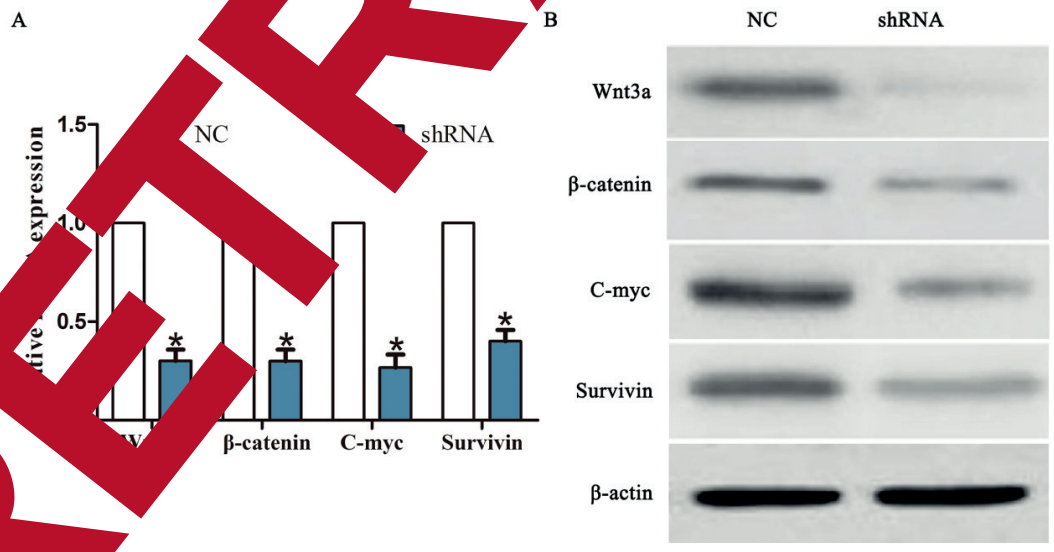


Figure 3. Knockdown of hsa_circ_0017247 promoted BLCA cell apoptosis and ...ted cell cy... ...nt increase viewed in the percentage of cell apoptosis in BLCA cells transfected with hsa_ci... 7247 shRNA. ...ge of G0/G1 cells was increased and the percentage of S cells was reduced after downregu... circ_0017247 ...LCA cells. The results represent the average of three independent experiments (mean ± stand... for ...ean). *p<0.05

Discussion

Evidence has proved that circRNAs participate in the regulation of BLCA development and are used to predict the treatment response, assess the disease state and clinical outcome. Serving as a competing endogenous RNA, circ-MYLK ...li... tates the progression of BLCA by modul... VEGFA/VEGFR2 signaling pathway[11]. T...gh the miR-31-5p/RAB27A axis, knockdown o... BPTF suppresses recurrence and tumor prog... sion of BLCA[12]. By suppressing RUNX2 a...

...ating miR-217expression, low expression ...sa_circ_0001...4 restrains the progression of ...CA[13]. CircP... promotes cell proliferation ...cell migratio... BLCA which may be a ther-ap... target a... novel potential biomarker for BLC... ...ous studies[15], circ_0017247 was initially discovered in osteosarcoma and promot-...d cell proliferation of osteosarcoma. Our study ...at the expression of hsa_circ_0017247 ...as up...gulated in BLCA tissues. Furthermore, after hsa_circ_0017247 was knocked down, the cell proliferation was suppressed, cell apoptosis



...e 4. Interaction between Wnt/β-catenin signaling pathway and hsa_circ_0017247 in BLCA. **A,** RT-qPCR results revealed ...the expression of target proteins in Wnt/β-catenin signaling pathway was downregulated in shRNA group compared with ...up. **B,** Western blot assay results revealed that the expression of target proteins in Wnt/β-catenin signaling pathway was do...lated in shRNA group compared with NC group. The results represent the average of three independent experiments. Data a...presented as the mean ± standard error of the mean. *p<0.05.

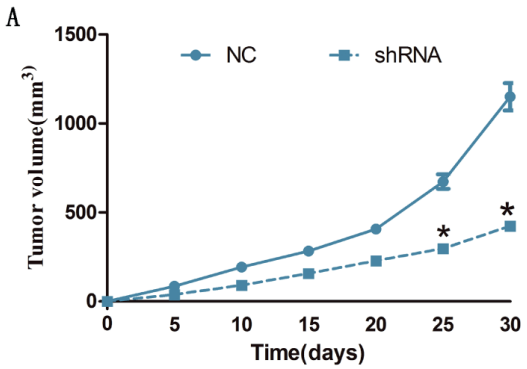


Figure 5. Knockdown of hsa_circ_0017247 inhibited tumor formation *in vivo*. A, Tumor size in shRNA group was smaller compared with NC group. **B,** Weight of dissected tumors in shRNA group was compared with NC group. The results represent the average of three independent experiments (mean ± standard error). *$p<0.05$, as compared with the control cells.

was promoted, and the cell cycle was regulated in BLCA cells. These data indicated that hsa_circ_0017247 functioned as an oncogene and promotes tumorigenesis of BLCA.

Recently, circRNAs have been reported to participate in the regulation of signaling pathway in tumorigenesis. The Wnt/β-catenin signaling pathway is one of the most important pathways which are vital processes of tumor proliferation and metastasis. Wnt proteins mediate a variety of processes during embryogenesis by modulating integrity of the stem cell, stem cell division and migration. Wnt/β-catenin in metastasis-initiating cells has been suggested to be an important regulatory factor in the progression of several cancers which may be therapeutic target. For instance, up-regulation of circ-ITCH inhibits cell proliferation and cell metastasis in triple-negative breast cancer by regulating the Wnt/β-catenin pathway through the downregulation of Wnt/β-catenin signaling and FZD4 expression induced by miR-516b, circRNA_100290 enhances the progression of colorectal cancer[17]. Circ_0067934 facilitates tumor growth and cell proliferation in hepatocellular carcinoma by modulating miR-1324/FZD5/Wnt/β-catenin axis[18]. Through the regulation of miR-135a-5p/MTDH/Wnt/β-catenin pathway, has_circ_0001946 promotes cell proliferation of lung adenocarcinoma *via* activation of Wnt/β-catenin axis[19].

To further experiments were used to identify the relationship between Wnt/β-catenin pathway and hsa_circ_0017247. After hsa_circ_0017247 was knocked down *in vitro*, target proteins in Wnt/β-catenin signaling pathway could be downregulated *via* the knockdown of hsa_circ_0017247. All the results above suggested that hsa_circ_0017247 might promote tumorigenesis of BLCA *via* activating the Wnt/β-catenin signaling pathway. To further verify the oncogenic role of hsa_circ_0017247 in BLCA, experiments were performed. We found that the inhibition of BLCA tumor formation was triggered by the knockdown of hsa_circ_0017247.

Conclusions

We showed that hsa_circ_0017247 is a new biomarker in the progression of BLCA and could enhance BLCA development and inhibit apoptosis through the activation of the Wnt/β-catenin signaling pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

This study was supported by the Research Fund of Shanghai Tongren Hospital, Shanghai Jiaotong University School of Medicine. No. TRYJ (LC) 201805.

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