European Review for Medical and Pharmacological Sciences 2019; 23: 9499-9505

Circular RNA circ_0067934 functions as an oncogene in breast cancer by targeting Mcl-1

J.-M. WANG[1,2], X.-J. LI[1], J. WANG[1]

[1]Department of Imaging and Nuclear Medicine, Shanxi Medical University, Taiyuan, China
[2]Department of Radiology, Shanxi Provincial People's Hospital, Taiyuan, China

Abstract. – OBJECTIVE: Breast cancer (BC) is one of the most ordinary malignant tumors. Recent studies have revealed that long noncoding RNAs (lncRNAs) play an important role in the progression of tumorigenesis. This work aims to identify how circ_0067934 functions in the progression of BC.

PATIENTS AND METHODS: Circ_0067934 expression of both 57 paired BC patients' tissue samples and cells was detected by Real Time-quantitative Polymerase Chain reaction (RT-qPCR). Moreover, the function of circ_0067934 was identified by performing proliferation assay, colony formation assay, cell cycle assay, and Ethynyl deoxyuridine (EdU) incorporation assay *in vitro*. Besides, the underlying mechanism was explored through Western blot assay and RT-qPCR.

RESULTS: In this study, circ_0067934 expression was significantly higher in BC tissues when compared with that in adjacent non-tumor s ples. Cell proliferation in BC was inhibite ter knockdown of circ_0067934 *in vitro*. M over, cell cycle in BC was regulated after kno down of circ_0067934 *in vitro*. Res furth experiments revealed that Mcl-1 nregu lated *via* the knockdown of ci 067 BC.

CONCLUSIONS: Our w st circ_0067934 enhances B ell pr ation and regulates BC cell cycle pregula Mcl-1.

Key Words:
Circular RNA, C 0067934, Br ancer, Mcl-1.

Introduction

Brea ncer (BC) remains a threat to women accountin one of the dominating reasons of cancer-related mortality in both in China and the world[1]. Although screening techniques have improved a lot and a higher prevalence of risk factors have been well-established in recent years, the morbidity of BC is notably rising[2]. It has been estimated that 246,660 new cases were diagnosed with BC and 40,450 cases died of BC in America in 2016[3]. There dy more than 3.1 million females were gnosed with BC by January 2018. ever, th ajority of the cases were dia sed dvanc stages, with the 5-year s val rate an 25%[4]. Thus, it is very i rtan have deep understanding of m lec aracte tics underlying BC progres and rsonalized medicine for es.

As the de ment of high-throughput sequ ng techno circRNAs (Circular RNAs) h been widely explored as new stars. Formed covalentl closed loop, circRNAs has been i ated to y an important role in various diseas ng tumorigenesis. For example, by activating the expression of TPX2 *via* restraining 8075, hsa_circRNA_101996 promotes cell pr iferation and invasion in cervical cancer[5]. Upregulation of hsa_circ_100395 significantly inhibits cell proliferation and reduces cell migration and invasion in lung cancer by targeting TCF21[6]. CircRNA_100269 is downregulated in GC which inhibits cell growth in gastric cancer tumor *via* targeting miR-630[7]. The down-regulation of circ_HIPK3 inhibits cell proliferation and cell invasion in osteosarcoma which may be a potential biomarker and therapeutic target of osteosarcoma[8].

Our research demonstrated that circ_0067934 was remarkably upregulated in BC tissues and cell lines. Moreover, the knockdown of circ_0067934 inhibited the proliferation of BC *in vitro*. The knockdown of circ_0067934 also regulated cell cycle of BC *in vitro*. Furthemore, we found that the function of circ_0067934 in BC was also associated with the promotion of Myeloid cell leukemia-1 (MCL-1), which was reported to be an oncogene in BC.

Corresponding Author: Jun Wang, Ph.D; e-mail: 332854663@qq.com

Patients and Methods

Tissue Specimens

57 paired tumor tissues and adjacent non-tumor tissues were sequentially gathered from BC patients who underwent surgery at the Shanxi Provincial People's Hospital. All cases were diagnosed with BC by two independent pathologists without any controversy. This investigation was approved by the Ethics Committee of Shanxi Provincial People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human BC cell lines (MCF-7, LCC9, T-47D, SKBR3) and normal human breast cell line (MCF-10A) were purchased from the Shanghai Cell Biochemical Institute (Shanghai, China). The culture medium consisted of 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM), as well as 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Be cells were cultured in an incubator contai CO_2 at 37°C.

Cell Transfection

After BC cells were cultured f 6-well plates, cells were transfected cDN oligonucleotides specifically targ circ (shRNA) and control (Gen angha China) using Lipofectam 3 itrogen, Carlsbad, CA, USA). Th ansfectio ncy was monitored by Rea me quantitati oly-merase Chain Reacti CR).

RNA Extracti nd RT-qPCR

Total RNA s ext cted from cultured BC cells or pati tum ssues by using TRIzol reagent (T Bi nc., C Shiga, Japan) and then, re scribe complementary deoxyribose nuc NAs) through the revers ription aKaRa, Otsu, Shiga, Japa mer sequences used for RT-qPCR we s fol 9967934 forward: 5′-TAG-C TG-3′ and reverse: 5′- TCCCATCATTCCC-3′; glyceraldehyde hate dehydrogenase (GAPDH), forward: CCGTCAAGGCTGAGAAC-3′ and reverse: 5′-TGGTGAAGACGCCAGTG-GA-3′. PCR was performed th he following sequence: pre-denat on at for 1 min, followed by 40 cycle 95°C for 15 °C for 30 s, and 72°C for 30 . The $2^{-\Delta\Delta Ct}$ meth was utilized to calculate the fo pressio

Cell Viability A y

The transfect BC c were replaced into 96-well plates by th l Counting Kit-8 assay (CCK ndo ecular Technologies, Kuma) was utilized to monito cell viabilit absorbance was chosen

C y Formation ay

C cells were placed in a 6-well plate for days. Then colonies were treated with 10% maldehyde fo min and stained for 5 min 0.5% cry violet. The Image-Pro Plus s, MD, USA) was used for data ana

Ethynyl Deoxyuridine (EdU) ation Assay

Kit (Roche, Mannheim, Germany) was ed for detecting cell proliferation of transfected ells. The representative photograph was taken y Zeiss Axiophot Photomicroscope (Carl Zeiss, berkochen, Germany).

Cell Cycle Assay

RNase A solution (250 µg/mL) was used to treat BC cells (2×10^5) in 90% methanol solution for 30 minutes at 37°C. Following were incubated with propidium iodide (PI) for another 15 min. FlowJo software (FACSCalibur; BD Biosciences, Detroit, MI, USA) was used to detect cell cycle.

Western Blot Analysis

Cell samples were washed with precooled phosphate buffered saline (PBS) and then lysed with cell lysis solution (RIPA; Beyotime, Shanghai, China). Protein concentration was detected using bicinchoninic acid (BCA; Thermo Fisher Scientific Inc., 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 (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 Mcl-1 and GAPDH (Abcam Inc., Cambridge, MA, USA) 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. The results were analyzed by Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

All statistical analyses were performed by Statistical Product and Service Solutions (SPSS) 21.0 (IBM Corp., Armonk, NY, USA). Independent-sample *t*-test was used to compare the difference between the two groups. Moreover, $p<0.05$ was considered to indicate a statistically significant difference.

Results

Expression Level of Circ_0067934 in Tissues and Cells of BC

RT-qPCR was conducted for det circ_0067934 expression in 57 patients' s. Circ_0067934 was significantly upregulat tumor tissue samples than that in adjacent sues (Figure 1A). Moreover, its expression four human BC cell lines and one human breast cell line (MCF-10A) was itored. Compared with the expression MCF-10A, the circ_0067934 level was sig ntly BC cells (Figure 1B). The re t that a high level of circ_006793 migh BC development.

Knockdown of Circ_0067 ed Cell Viability in BC Cell

In our study, we chos CF-7 and R3 cell lines for the knoc of circ_00 34. Then RT-qPCR was uti detect the circ_0067934 expre ion (Fig ex-plore how circ_00 34 affected iability of BC cells, CC nd c y forma on assay were performed s sho in Fi ures 2B and 2C, circ_0067 own r ed the cell viability of MCF-7 BR3 ls. The number of col s was re decreased after circ_006 was knocked n in MCF-7 and SKBR3 ure 2D).

K down of Ci 067934 ulated Cell Cycle and Cell liferation in BC Cells

To explore ho rc_0067934 affected cell cycle and cell pro ation of BC cells, cell cycle assay were performed. As shown in Figu d 3B, the percentage of G0/G1 cells was increased and the percentage of S cells was d after the knockdown of circ_0067934 and SKBR3 cells. EdU positive cells e reduced after knockdown of circ_0067934 MCF-7 and SKBR3 cells (Figure 3C).

irc_0067934 Knockdown Inhibited cl-1 in BC

Previous studies have reported that the key regulator Mcl-1, promotes cell proliferation in many cancers including BC. In our work, the interaction between Mcl-1 and circ_0067934 was studied. We found that circ_0067934 knock-

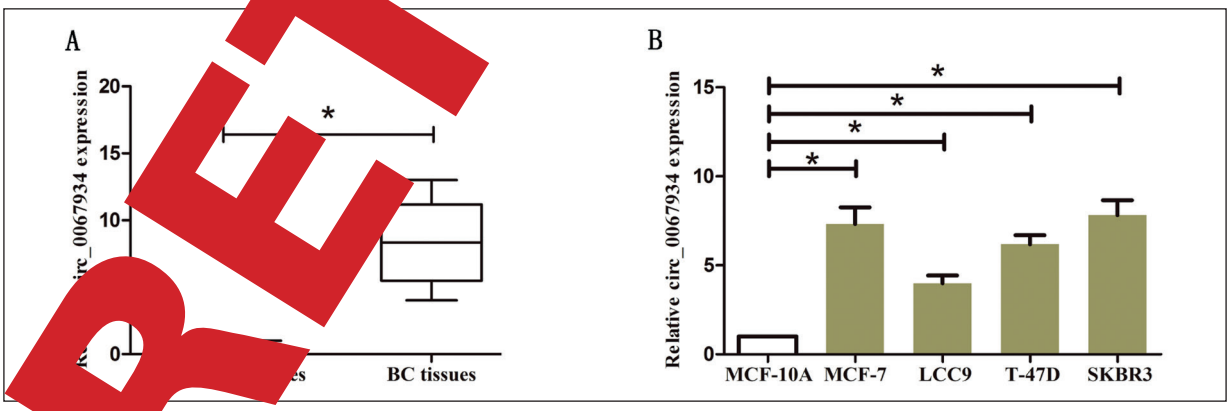

Figure sion level of circ_0067934 was increased in BC tissues and cell lines. **A,** Circ_0067934 expression was significantly d in the BC tissues compared with adjacent tissues. **B,** Expression levels of circ_0067934 relative to GAPDH were d ned in the human BC cell lines and MCF-10A by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

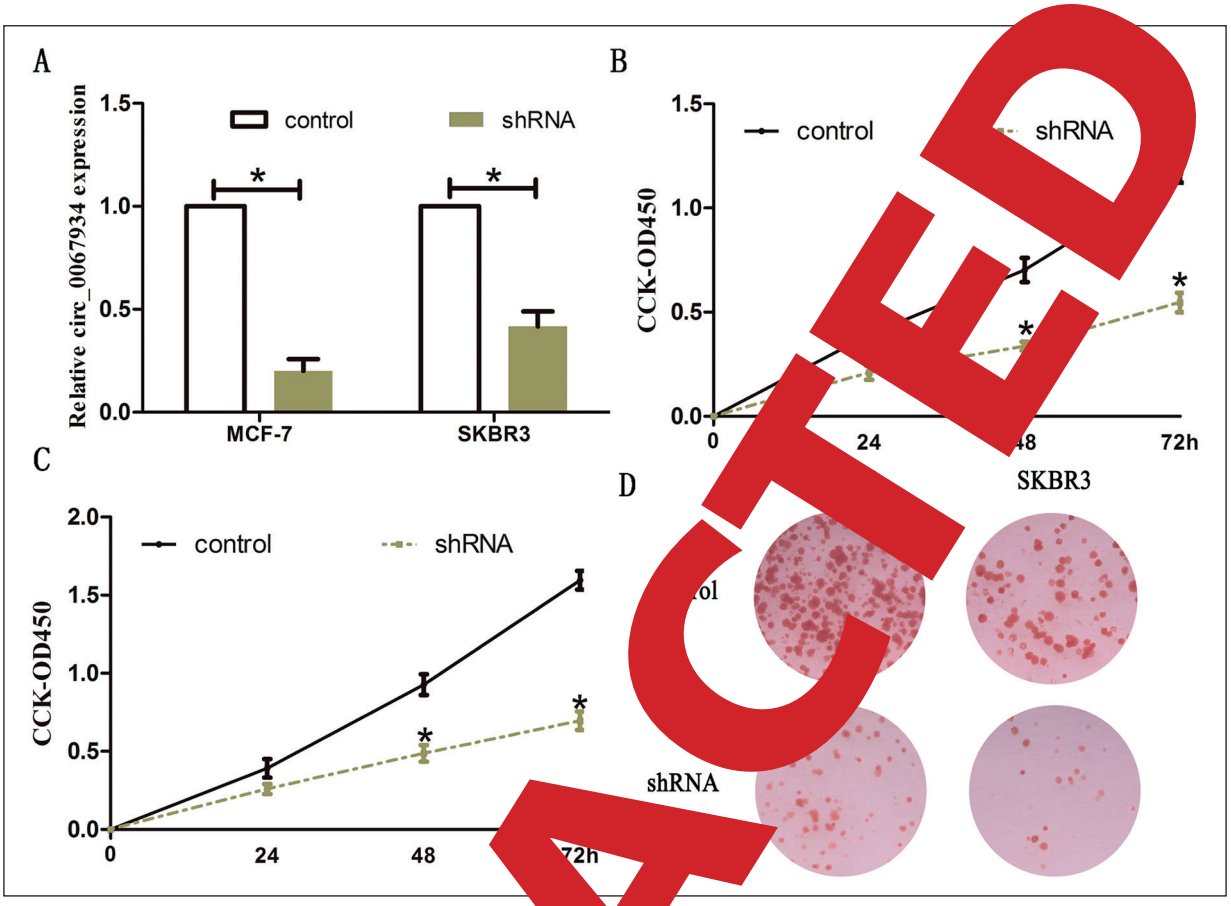

Figure 2. Knockdown of circ_0067934 inhibited BC ce[illegible]y. **A,** Circ_0067934 expression in BC cells transfected with negative control or circ_0067934 shRNA was detected by [illegible]R. GAPDH was used as an internal control. **B,** CCK-8 assay showed that knockdown of circ_0067934 [illegible]ntly repre[illegible]ll viability in MCF-7 cells. **C,** CCK-8 assay showed that knockdown of circ_0067934 significan[illegible]d cell viabi[illegible]n SKBR3 cells. **D,** Number of colonies was remarkably decreased after circ_0067934 was kn[illegible]d dow[illegible]C cells (magnification: 40×). The results represent the average of three independent experiments (mean ± s[illegible]rd erro[illegible] $p<0.05$, as compared with the control cells.

down reduced the mR[illegible] expression o[illegible]cl-1 in MCF-7 and SKBR[illegible]igure 4A). Moreover, circ_0067934 [illegible]ck[illegible]uced the protein level of Mcl-1 in MCF-7 [illegible]BR3 cells (Figures 4B an[illegible]2). Furthermor[illegible]e verified the interaction [illegible]ween Mcl-1 and circ_0067934 in BC tissu[illegible] Resul[illegible]howed that Mcl-1 was significantl[illegible]gu[illegible]d in [illegible] tissue samples than in adjac[illegible]s (Fig[illegible]4D). Correlation analysis demonst[illegible]-1 expression level positi[illegible]related [illegible]0067934 expression in B[illegible]Figure 4E).

Discussion

Evidence [illegible]uggested that circRNAs are crucial regulato[illegible]s in carcinogenesis of BC. For instance, the up-regulation of circ-ITCH inhibits cell proliferation and cell metastasis in triple-negative breast cancer by regulating the Wnt/β-catenin pathway[9]. Overexpression of CircRNA BARD1 inhibits the progression of BC through the miR-3942/BARD1 axis[10]. The knockdown of circRNACER restrains cell proliferation and cell migration in BC *via* modulating the activity of miR136/MMP13 signaling[11]. By sponging miR-1271, circ-ABCB10 promotes the tumorigenesis of BC through enhancing cell proliferation and inducing cell apoptosis[12].

Recently, circ_0067934 has been screened as a new topic in several cancers. For example, through the modulation of miR-1324/FZD5/Wnt/β-catenin axis, circ_0067934 facilitates tumor growth and cell metastasis in hepatocellular carcinoma[13]. Circ_0067934 functions as an

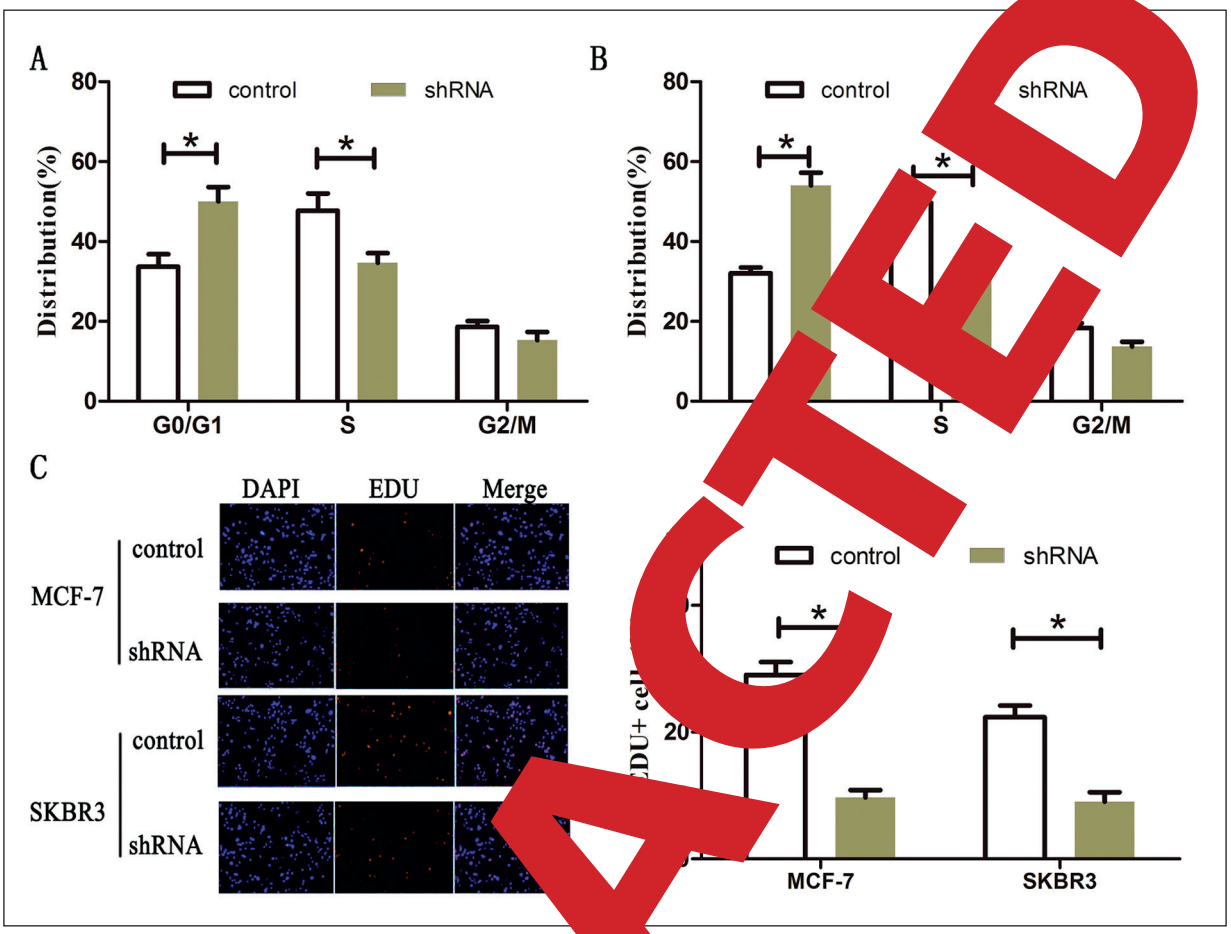

Figure 3. Knockdown of circ_0067934 [illegible] cell cycle [illegible] hibited BC cell proliferation. **A,** Cell cycle assay showed that the percentage of G0/G1 cells wa[illegible] after knoc[illegible]wn of circ_0067934 in MCF-7 cells. **B,** Cell cycle assay showed that the percentage of G0/G1 [illegible]s was [illegible] after knockdown of circ_0067934 in SKBR3 cells. **C,** Edu assay showed that EDU positive cells w[illegible]educed [illegible]wn of circ_0067934 in BC cells (magnification: 40×). The results represent the average of th[illegible] expe[illegible]ts (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

oncogene in cervica[illegible] regulating the miR-545/EIF3C a[illegible] C[illegible]934 is over-expressed in non-small cell lu[illegible]er which promotes cell p[illegible]eration and is re[illegible]ed to poor prognosis[15].

In the p[illegible]t re[illegible]ch, circ_0067934 was found to b[illegible]l[illegible] in b[illegible]BC tissues and cells. A signi[illegible]relati[illegible]was observed between circ_0067[illegible]n and tumor stage, the ly[illegible]de meta[illegible] Furthermore, after circ[illegible]was knocked down, the ability of cel[illegible]owth[illegible]nd invasion were sup-p[illegible] T[illegible]a[illegible]icated that circ_0067934 fun[illegible]s an oncogene and promoted the tumorig[illegible]f BC.

To exp[illegible] the related proteins of circ_0067934, bioinformatics analysis and experiments were performed. We discovered that the expression of MCL-1 was positively correlated with circ_0067934 in BC tissues. MCL-1 is a potent survival factor for both normal and malignant tissues which functions as a critical anti-apoptotic protein. Mcl-1 is an important contributor to bromodomains and extra-terminal inhibitors resistance in hepatocellular carcinoma[16]. Overexpression of Mcl-1 facilitates the progression of lung cancer by suppressing cell apoptosis[17]. By the regulation of Mcl-1, miR-320 inhibits the progression in cervical cancer which may offer a potential biomarker and therapeutic target for cervical cancer patient[18]. The downregulation of MCL-1 causes mitochondrial stress and induces autophagy-dependent necroptosis in oral cancer cells[19].

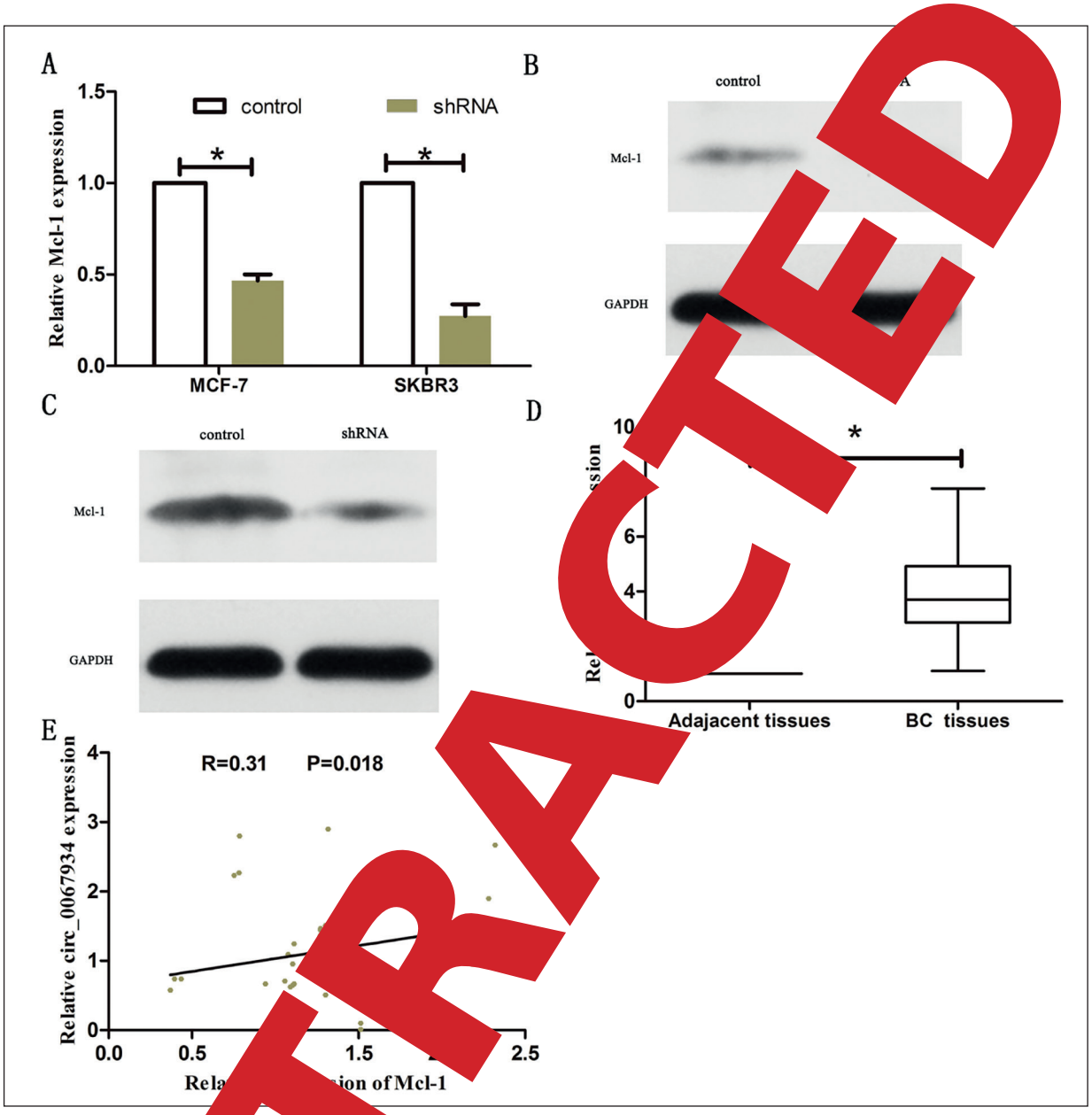

Figure 4. Circ_0067934 knockdown[illegible] MCL-1 in BC. **A,** RT-qPCR results showed that MCL-1 expression was decreased in circ_0[illegible]34 shRNA group [illegible]ared to control group. **B,** Western blot results showed that MCL-1 expression was decreased in [illegible]0067934 shRNA group compared to control group in MCF-7 cells. **C,** Western blot results showed that MCL-1 expressi[illegible]as dec[illegible]d in circ_0067934 shRNA group compared to control group in SKBR3 cells. **D,** Mcl-1 was significantly u[illegible]lated [illegible]C tissu[illegible]amples than that in adjacent tissues. **E,** Linear correlation between the expression level of MCL[illegible]/934 in [illegible]issues. The results represent the average of three independent experiments. Data are presented as the [illegible]ndard [illegible]of the mean. *p<0.05.

The[illegible]her explor[illegible]d how circ_0067934 int[illegible]cted [illegible]in BC. Results showed t[illegible]CL[illegible] was upregulated in BC tiss[illegible]ould be reduced by the knockdown of circ[illegible]*in vitro*. All these results indicated that [illegible]67934 might promote tumorigenesis of BC[illegible]y upregulating MCL-1.

Conclusions

It has been found that circ_0067934 could enhance BC cell proliferation and regulate the cell cycle by upregulating MCL-1. These findings implied that circ_0067934 could serve as a promising marker for BC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.
2) Keramatinia A, Mousavi-Jarrahi SH, Hiteh M, Mosavi-Jarrahi A. Trends in incidence of breast cancer among women under 40 in Asia. Asian Pac J Cancer Prev 2014; 15: 1387-1390.
3) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
4) Luo X, Song Y, Tang L, Sun DH, Ji DG. LncRNA SNHG7 promotes development of breast cancer by regulating microRNA-186. Eur Rev Med Pharmacol Sci 2018; 22: 7788-7797.
5) Song T, Xu A, Zhang Z, Gao F, Zhao L, Chen X, Gao J, Kong X. CircRNA hsa_circRNA_101996 increases cervical cancer proliferation and invasion through activating TPX2 expression by restraining miR-8075. J Cell Physiol 2019; 234: 14296-14305.
6) Chen D, Ma W, Ke Z, Xie F. CircRNA hsa_circ_100395 regulates miR-1228/TCF21 pa to inhibit lung cancer progression. Cel 2018; 17: 2080-2090.
7) Zhang Y, Liu H, Li W, Yu J, Li J, Shen Z, Ye G, Li G. CircRNA_100269 is downregulated in g tric cancer and suppresses tumor cell growth targeting miR-630. Aging (Albany NY) 2017; 9: 1585-1594.
8) Xiao-Long M, Kun-Peng Z, C ircular RNA circ_HIPK3 is down- lated presses cell proliferation tion sion in osteosarcoma. J C 9: 1856-1862.
9) Wang ST, Liu LB, Li XM ng YF, Xie F ng R, Wei Q, Kang YH, R, Feng XH. C CH regulates triple-ne st cancer progression through the pathway. Neoplasma 2019; 66: 2-239.
10) Zhao J, Zou H an C, Ma J, Zh ang J. Circular RNA RD1 (Hsa_circ_00 098) overexpression in breast canc TCDD treatment could promote miR-3942/BARD1 axis. Cell le 2018; 31-2744.
11) Qu Y, Dou P, Hu M, X Sun H. Cir AC-ER mediates malignant ion east cancer through ta ting the 3 axis. Mol Med Rep 9; 19: 3314-3
12) Liang HF, Zha , Liu Jia GT, Li L. Circular RNA cir CB10 motes breast cancer proliferatio ssion ugh sponging miR-127 cer P 017; 7: 1566-1576.
13) Zhu Q G, Luo Z, Gu Zhang D, Ni Y. CircRN 0067934 prom tumor growth and me epatocellular carcinoma through re tio 24/FZD5/Wnt/β-catenin axis. Bi hem Biop Commun 2018; 497: 626-
1 u C, Wang Y, Li A, Zhang J, Xue F, Zhu L. Overexpressed circ_0067934 acts as an oncogene to facilitate cervi cancer progression via the miR-545/EIF3C a J Cell Physiol 2019; 234: 9225-232.
T, Li B, Li G, Zhang L, Wang B, Sun ression of circ-0067934 is associated with increased cellular proliferation and the prognosis of non-small cell lung cancer. Oncol Lett 16: 5551-5556.
HP, Li GQ, Zhang Y, Guo WZ, Zhang JK, Li J, Lv JF, Zhang SJ. Upregulation of Mcl-1 inhibits JQ1-triggered anticancer activity in hepatocellular carcinoma cells. Biochem Biophys Res Commun 2018; 495: 2456-2461.
Feng C, Yang F, Wang J. FBXO4 inhibits lung cancer cell survival by targeting Mcl-1 for degradation. Cancer Gene Ther 2017; 24: 342-347.
18) Zhang T, Zou P, Wang T, Xiang J, Cheng J, Chen D, Zhou J. Down-regulation of miR-320 associated with cancer progression and cell apoptosis via targeting Mcl-1 in cervical cancer. Tumour Biol 2016; 37: 8931-8940.
19) Sulkshane P, Teni T. BH3 mimetic Obatoclax (GX15-070) mediates mitochondrial stress predominantly via MCL-1 inhibition and induces autophagy-dependent necroptosis in human oral cancer cells. Oncotarget 2016; 8: 60060-60079.