Am J Cancer Res 2023;13(8):3433-3448
www.ajcr.us /ISSN:2156-6976/ajcr0149072

Original Article

Circular RNA ATP2C1 (has_circ_0005797) sponges miR-432/miR-335 to promote breast cancer progression through regulating CCND1 expression

Caiping Chen[1], Jianju Lu[1], Wang Li[1,2], Xiang Lu[1]

[1]*Department of Breast Surgery, Affiliated Hospital of Jiaxing University (The First Hospital of Jiaxing), Jiaxing, Zhejiang, China;* [2]*School of Graduate, Bengbu Medical College, Bengbu, Anhui, China*

Received January 11, 2023; Accepted July 31, 2023; Epub August 15, 2023; Published August 30, 2023

Abstract: Breast cancer (BC) is the most frequently diagnosed malignancy in the world. Accumulating evidence has indicated that circular RNAs (circRNAs) play essential roles in BC. Here we investigated the biological functions of circATP2C as a competing endogenous RNA (ceRNA) in BC development. We found that circATP2C1 expression was upregulated in BC cells and tissues and was significantly associated with the poor overall survival in BC patients. CircATP2C1 is more resistant to RNase R exonuclease and Actinomycin D than is the linear mRNA of *ATP2C1*. CircATP2C1-knockdown inhibited the viability, colony proliferation and invasion abilities, while increasing the apoptosis rates of BC cells *in vitro*, as well as inhibiting tumor mass, size and weight *in vivo*. Upregulation of miR-432 and miR-335 inhibited CCND1 expression in BC cells. Both miR-432/miR-335 specifically bind to the 3'-UTR of circATP2C1 and *CCND1* (CyclinD1). The inhibition of the aggression of BC cells by circATP2C1-knockdown was rescued by co-transfection of miR-432/miR-335 inhibitors. In conclusion, circATP2C1 promotes BC oncogenesis and metastasis by sponging miR-432/miR-335 to abolish the inhibition of the target gene, CCND1. This study suggests that circATP2C1 has implications for BC diagnosis and treatment.

Keywords: Breast cancer, ceRNA, circATP2C1, miR-432/miR-335, CCND1

Introduction

Breast cancer (BC) ranks first among diagnosed malignancies and second in principal malignancy-related death in females worldwide, accounting for around 30% of diagnosed cases and 15% of mortality-to-incidence. Approximately 10% of BC patients are associated with genetic susceptibility or family history [1-3]. Early diagnosis and target therapies could be promising strategies to improve the survival rate of BC patients. For instance, some molecules have been identified as the markers of early diagnosis to improve the efficacy of treatment [3]. However, a large fraction of BC patients is still suffering from the recurrence and metastasis of tumors and drug resistance. Therefore, it is urgently required to discover novel biomarkers and elucidate potential molecular mechanisms.

Circular RNAs (circRNAs) represent a novel cluster of single-stranded endogenous non-coding RNAs (ncRNAs), which could be used as potential novel biomarkers for disease diagnosis and target therapies [4-6]. Increasing evidence reports that circRNAs are commonly expressed in human tissues, mainly serving as competing endogenous RNAs (ceRNAs) by sponging microRNAs (miRNAs) [7-12]. For example, circFOXK2 facilitates the oncogenesis of BC via the IGF2BP3/miR-370 axis [13]. CircTADA2As suppresses BC progression and metastasis by targeting the miR-203a-3p/SOCS3 axis [14]. Besides, circRNA DDX21 acts as a prognostic factor and sponges the miR-1264/QKI axis to suppress the progression of triple-negative breast cancer (TNBC) [15]. Downregulation of circATP2C1 inhibits endometrial cancer by modulating miR-298/CTNND1 signaling [16]. However, the function and associated molecu-

lar mechanisms of circATP2C1 in BC carcinogenesis and development have not been reported.

CircRNAs typically sponge miRNA regulate the expression of downstream target genes [7-9, 12, 17, 18], thereby modulating carcinogenesis and malignancy progression [5, 11, 19, 20]. For example, circ-TRIO promotes TNBC progression by regulating the miR-432-5p/CCDC58 axis [21]. Circ_0008039 promotes BC cell proliferation and migration by regulating the miR-432-5p/E2F3 axis [22]. Circular RNA 0007255 regulates BC progression through the miR-335-5p/SIX2 axis [23]. However, the function of miR-432-5p and miR-335 in BC progression has not been reported.

CCND1 belongs to the cyclin family, which is highly conserved and is characterized by periodic protein abundance throughout cell cycles [24]. CCND1 has been implicated in cancer progression. For example, AURKB promotes gastric cancer progression by increasing CCND1 expression [25]. CCND1 silencing suppresses the differentiation of liver cancer stem cells and overcomes the resistance to 5-Fluorouracil in hepatocellular carcinoma [26]. FGFR1 regulates the proliferation and metastasis of lung cancer by targeting CCND1 [27]. Reportedly CCND1 contributes to the malignant behavior of BC cells *in vitro* and *in vivo*. For instance, CCND1 expression was regulated by miR-374b to inhibit BC progression [28]. CircPSMA1 facilitates tumorigenesis, metastasis, and migration in TNBC through the miR-637/Akt1/β-catenin CCND1 axis [29]. However, how circRNAs regulate CCND1 expression in BC is unknown.

This study aimed to explore the biological functions of circATP2C1 in BC carcinogenesis and development, thus providing novel biomarkers to improve the survival rate of patients with BC in future clinical practices.

Materials and methods

Reagents

Dulbecco's modified Eagle medium (DMEM), RNase®, and fetal bovine serum (FBS) were obtained from Sigma-Aldrich Inc. (St. Louis, USA). Bestar qPCR RT kit and Bestar qPCR MasterMix kit were provided by DBI Bioscience Inc. (Beijing, China). The regents of RNA immunoprecipitation and RNA-binding protein immunoprecipitation were purchased from Millipore (Burlington, MA, USA). CCK-8 assay kit was provided by Dojindo Corp (Kyushu, Japan). RIPA Cell Lysis Buffer and PMSF were bought from Beyotime Biotechnology (Nanjing, China). Dual-luciferase reporter assay reagents and pmirGLO dual luciferase reporter plasmid were provided by Promega Inc. (Shanghai, China). Annexin V-FITC apoptosis kit was bought from Sizhengbai (Beijing, China). Antibodies against CCND1 (Cat #MA5-16356) and GAPDH (Cat #MA1-16757), Pierce™ Magnetic RNA-Protein Pull-Down Kit, RNAiMax, and Lipofectamine 3000 with Plus Reagent were bought from Thermo Fisher Scientific Inc. (Waltham, USA).

Patient tissue collection and ethics

The cancerous tissues and adjoining normal tissues were dissected from 60 BC patients during surgical treatment at Affiliated Hospital of Jiaxing University (the First Hospital of Jiaxing). No chemotherapies or radiotherapies had been performed on these patients before surgery. Tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C before use.

Current study was approved by the Ethics Committee of Affiliated Hospital of Jiaxing University (the First Hospital of Jiaxing) and performed following the 2013 revised Declaration of Helsinki. Each participant provided written informed consent.

Cells and cultures

The human BC cells (MDA-MB-453, MDA-MB-231, BT-549, and SUM-159) and the human mammary epithelial cells (MCF-10A) were bought from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 atmosphere in a humidified incubator.

Cell transfection

Small interfering RNA (siRNA) of circATP2C1 (si-circATP2C1), miR-432/miR-335 mimics, miR-432/miR-335 inhibitors, CCND1 overexpression plasmid (CCND1), and respective negative controls were provided by Shanghai

GenePharma Co., Ltd. (Shanghai, China). Cell transfection was performed using RNAiMax and Lipofectamine 3000 with Plus Reagent.

RNA stability examination

The stability of linear ATP2C1 mRNA and circATP2C1 were compared by quantifying the RNA levels using RT-qPCR after treatment with 2 μg/mL of actinomycin D for 0, 6, 12, and 24 h, or 5 U/μg RNase® for 20 minutes at 37°C.

RNA extraction and RT-qPCR assay

Cellular RNAs were extracted from BC tissues and cells by TRIzol. Bestar qPCRRT kit was used to synthesize cDNAs. RT-qPCR assay was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, USA). GAPDH (for circRNA and mRNA) and U6 (for miRNA) were adopted as the endogenous reference genes. Relative RNA expression was calculated with the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method [30].

Human circRNA microarray data collection and processing

The expression profiles of circRNAs in five pairs of BC and adjoining non-tumor breast tissues in the GEO database (accession number: GSE182471) (https://www.ncbi.nlm.nih.gov/geo) were determined by the platform of 074301 Arraystar Human CircRNA Microarray V2. Significantly differentially expressed circRNAs (DEcircRNAs) were then screened by using the limma package in the R software with a cut-off criterion of |logFC (foldchange)| > 1, $P < 0.05$.

Prediction of potential binding targets

The potential binding targets of circRNAs and miRNAs were predicted using online tools. Circular RNA interactome (https://circinteractome.nia.nih.gov/) was applied to predict the miRNAs bound by circATP2C1. Targetscan (http://www.targetscan.org/) was used to predict downstream mRNAs targeted by miR-432 or miR-335.

Luciferase assay

Relative luciferase activity was evaluated by a dual-luciferase reporter assay system. CircATP2C1 and *CCND1* reporter gene as well as the wild-type (wt) or mutant type (mut) of the 3' non-coding region (3'-UTR) of *CCND1* were synthesized and subcloned into the pmirGLO dual-luciferase reporter plasmid. Then luciferase activity was determined with a dual luciferase assay system in cells 48 h after the transfection of the wt or mut circATP2C1 or *CCND1* reporter plasmid and miR-432 or miR-335 mimics.

RNA-pulldown

Pierce™ Magnetic RNA-Protein Pull-Down Kit was applied for the enrichment of the miRNAs targeted by circATP2C1 following the manufacturer's instructions. The enriched miRNAs were eluted and subjected to RT-qPCR assay.

RNA immunoprecipitation (RIP) assay

The Magna RIP RNA-Binding Protein Immunoprecipitation reagent was used for RIP assay. Cell lysates were isolated by RIPA lysis buffer containing protease and RNase inhibitors and were then incubated with Argonaute-2 (AGO2) or control immunoglobulin G (IgG)-coated beads followed by treatment with proteinase K. RT-qPCR was performed to determine the abundance of immunoprecipitated RNAs.

Cell viability assay

Cells (5×10^3 cells/well) were seeded in 96-well plates and cultured at 37°C for 0, 24, 48, and 72 h. A CCK-8 assay kit was applied to check cell viability following the manufacturer's guidelines. A microplate reader was used to read the optional density (OD) value at 450 nm.

Colony proliferation ability assay

The proliferation ability of BC cells was evaluated by seeding the cells in a 6-well plate (3000 cells/well) and routinely cultured for two weeks. The colonies were photographed and counted under a microscope after fix in 4% paraformaldehyde and staining with crystal violet.

Apoptosis assay

The apoptosis rate was detected by the annexin V-FITC apoptosis kit following the manufacture's guideline. After double staining with PI and Annexin V-FITC, the apoptosis rate was analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, USA).

Transwell cell invasion assay

The cell invasion ability was determined using a 24-well Transwell (Corning Costar, Tewksbury, USA). In brief, 500 μL of medium containing 10% FBS was added in the lower well, and 2 × 10^5 cells in 300 μL of serum-free medium were applied to the removable chamber of the Transwell. After 24 h of cell culturing, the cells that migrated to the opposite side of the removable chamber were fixed in 4% paraformaldehyde and stained with crystal violet for counting and imaging under a microscope.

Western blot assay

Protein expression levels were determined by Western blot assay. Total proteins were isolated by the RIPA buffer containing 10% PMSF followed by separation by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then blotted to a PVDF membrane followed by blocking in 5% non-fat milk and incubation with the primary antibodies against CCND1 and GAPDH. Finally, the blot was developed with chemiluminescent substrates and photographed by the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, USA).

Nude mouse model for tumor formation evaluation

Twelve 4-week-old female BALB/c nude mice were bought from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were assigned to two groups (n = 6): (1) si-NC group injected with MDA-MB-231 cells; (2) si-circATP2C1 injected with MDA-MB-231 cells with circATP2C1 knockdown. Each experiment was conducted following the ethical rules for animal experiments in a pathogen-free laboratory in the animal center of Affiliated Hospital of Jiaxing University (the First Hospital of Jiaxing). 1 × 10^5 MDA-MB-231 cells in 100 μL of culture medium were injected subcutaneously into the right flank of each mouse. The tumor sizes were routinely measured once a week for 5 weeks, and the tumors were dissected from the model mice that were sacrificed by anesthesia and cervical dislocation. Finally, the tumors were photographed and weighed.

Statistical analysis

Data were reported as mean ± standard deviation. Statistical analyses were performed in SPSS 23.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to detect significance among multiple groups, and two-tailed Student's *t* test was used for two-groups. Kaplan-Meier curves were plotted to compare the overall survival rate of patients. $P < 0.05$ was statistically different.

Results

The circATP2C1 expression is up-regulated in BC cells and tissues

To investigate the circRNAs involved in BC progression, we analyzed the GSE182471 dataset from the GEO database to detect the differentially expressed circRNAs (DEcircRNAs) between cancerous and paracancerous tissues of 5 BC patients using the R language limma package. The analysis identified 119 circRNAs that were down-regulated and 372 circRNAs that were up-regulated in BC tissues (**Figure 1A**, **1B**). CircATP2C1 (has_circ_0005797), which is derived from the exons 2-7 of *APT2C1* (**Figure 1C**), was identified to be a significantly up-regulated circRNA in BC tissues (**Figure 1B**). This upregulation was validated by the higher expression level of circATP2C1 in cancerous tissues than in the normal tissues of 60 BC patients (**Figure 1D**). Furthermore, the association between the circATP2C1 expression with the clinical outcome of BC patients was explored by the Kaplan-Meier method. The results displayed that the patients exhibiting higher circATP2C1 expression showed significantly shorter overall survival time (**Figure 1E**), which suggested that the high circATP2C1 expression resulted in the poor prognosis of BC patients. Moreover, circATP2C1 expression levels in the BC cell lines (MDA-MB-453, MDA-MB-231, BT-549, and SUM-159) were significantly higher than in the human normal breast epithelial cell line, MCF-10A (**Figure 1F**).

To confirm the circular nature of circATP2C1, both circATP2C1 and the linear mRNA of *ATP2C1* were treated with RNase R exonuclease and Actinomycin D in MDA-MB-231 cells. The data showed that the circATP2C1 level was not significantly affected, whereas the linear mRNA level was dramatically decreased after treatment with RNase R exonuclease (**Figure 1G**) and Actinomycin D (**Figure 1H**). This result indicated that the circATP2C1 is more resistant to RNase R exonuclease and Actinomycin D

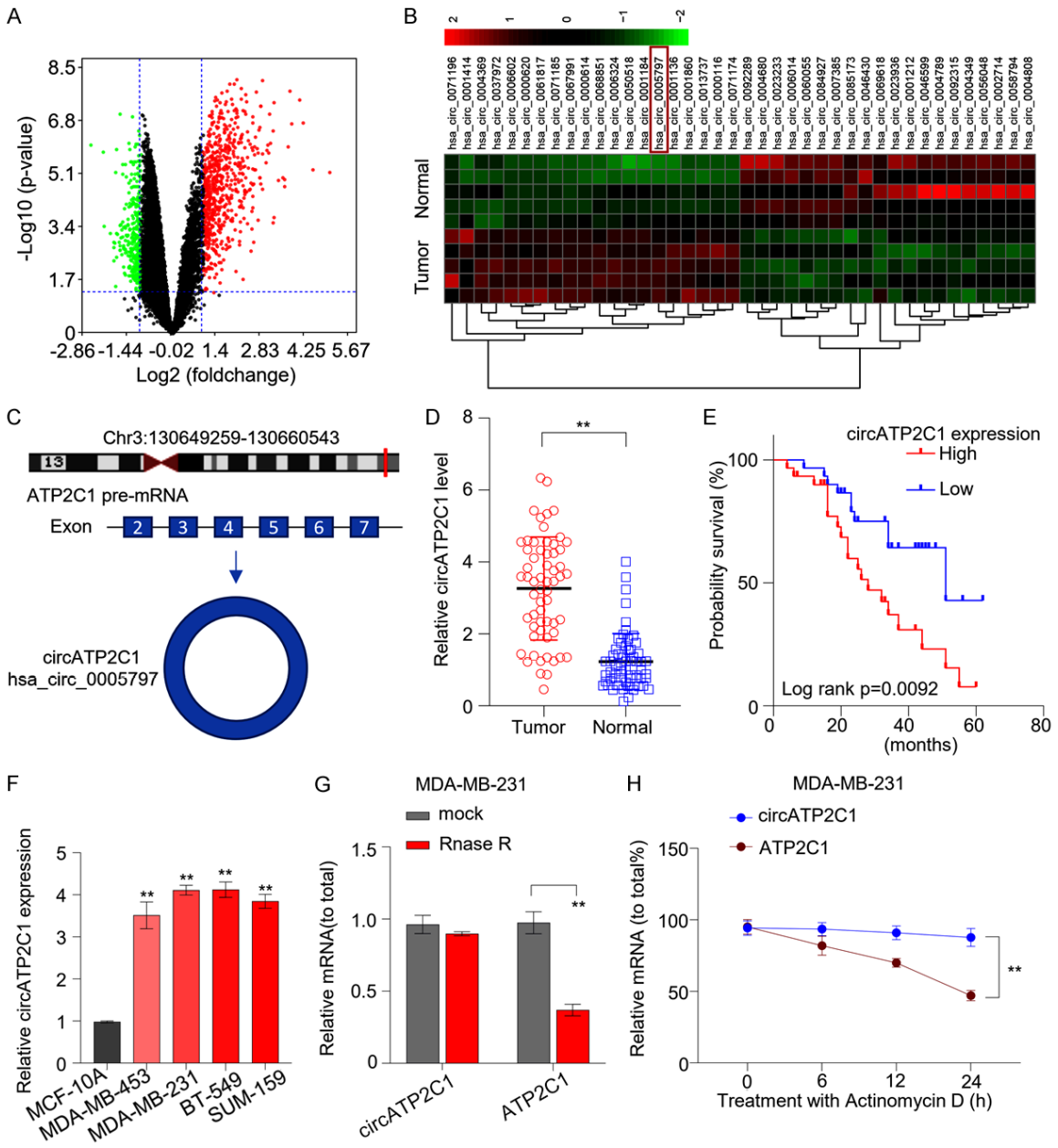


Figure 1. CircATP2C1 expression is upregulated in breast cancer tissues. (A, B) CircRNA expression was compared between 5 pairs of cancerous and paracancerous normal tissues from BC patients in the GEO database GSE182471 by the R language limma package. (A) Volcano and (B) Heatmap of the differently expressed circRNAs (DEcircRNAs). (C) CircATP2C1 is formed by the reverse splicing of the exons 2-7 of the host gene *APT2C1*. (D) RT-qPCR verified circATP2C1 expression in the cancerous and paracancerous tissues of 60 BC patients. (E) Kaplan-Meier analysis of the association of circATP2C1 expression with the overall survival time of patients. (F) RT-qPCR determined circATP2C1 expression in the BC cells (MDA-MB-453, MDA-MB-231, BT-549, SUM-159) and the human normal breast epithelial cells (MCF-10A). The levels of ATP2C1 linear mRNA and circATP2C1 were detected by RT-qPCR in total RNA extracted from MDA-MB-231 cells (G) treated with RNase R at 37°C for 30 minutes, and (H) treated with Actinomycin D for 0, 6, 12, and 24 h. $^{**}P < 0.01$.

than the linear mRNA, thereby confirming the circular structure of circATP2C1.

Together, the data suggest that circATP2C1 coregulation is significantly related to tumorigenesis and poor prognosis of BC patients.

Knockdown of circATP2C1 inhibits BC progression

To unravel the function of circATP2C1 in BC progression, circATP2C1 was knocked down in MDA-MB-231 and BT-549 cells by two specific

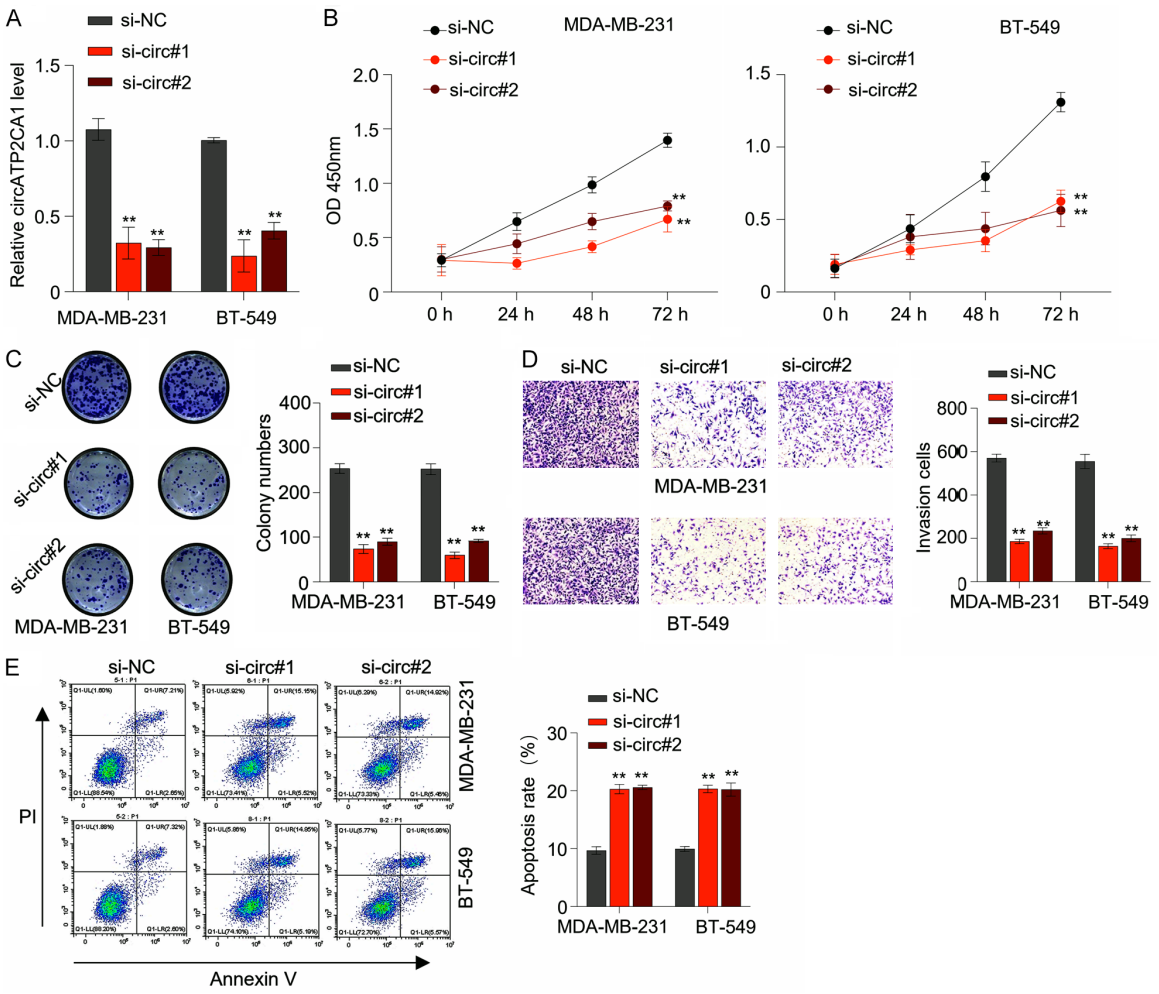


Figure 2. Knockdown of circATP2C1 inhibits BC progression. (A) RT-qPCR analysis confirmed that circATP2C1 was effectively silenced by si-circATP2C1 in the MDA-MB-231 and BT-549 cells. (B, C) Effect of circATP2C1-knockdown on the growth of MDA-MB-231 and BT-549 cells, (B) the viability assessed by CCK8 assay and (C) the colony proliferation determined by colony formation assay. (D) Effect of circATP2C1-knockdown on the invasion ability of MDA-MB-231 and BT-549 cells evaluated by Transwell with Matrigel. (E) Effect of circATP2C1-knockdown on the apoptosis of MDA-MB-231 and BT-549 cells evaluated by Flow cytometer.

si-circATP2C1 (si-circ#1 and si-circ#2). RT-qPCR showed that both si-circ#1 and si-circ#2 effectively knocked down circATP2C1 expression by more than 50% (**Figure 2A**). Next, the effect of circATP2C1-knockdown on cell growth was determined. CCK-8 assay discovered that circATP2C1-knockdown significantly reduced the viability of MDA-MB-231 and BT-549 cells (**Figure 2B**). Furthermore, colony formation test showed that circATP2C1-knockdown significantly reduced the proliferation ability of MDA-MB-231 and BT-549 cells (**Figure 2C**). Moreover, transwell test by Matrigel revealed that circATP2C1-knockdown significantly reduced the invasion ability of the two cell lines (**Figure 2D**). On the other hand, flow cytometer test showed that circATP2C1-knockdown significantly increased the apoptosis rates of the two cell lines (**Figure 2E**). The evidence indicates that circATP2C1-knockdown significantly inhibits the oncogenesis and progression of BC cells *in vitro*.

CircATP2C1 sponges miR-432 and miR-335 in BC cells

To identify the miRNAs targeted by circATP2C1, the circular RNA interactome (https://circinteractome.nia.nih.gov/) was used to predict the miRNAs that might specifically bind to circATP2C1 (**Figure 3A**). Then the specific bindings of circATP2C1 to each of the predicted miRNAs

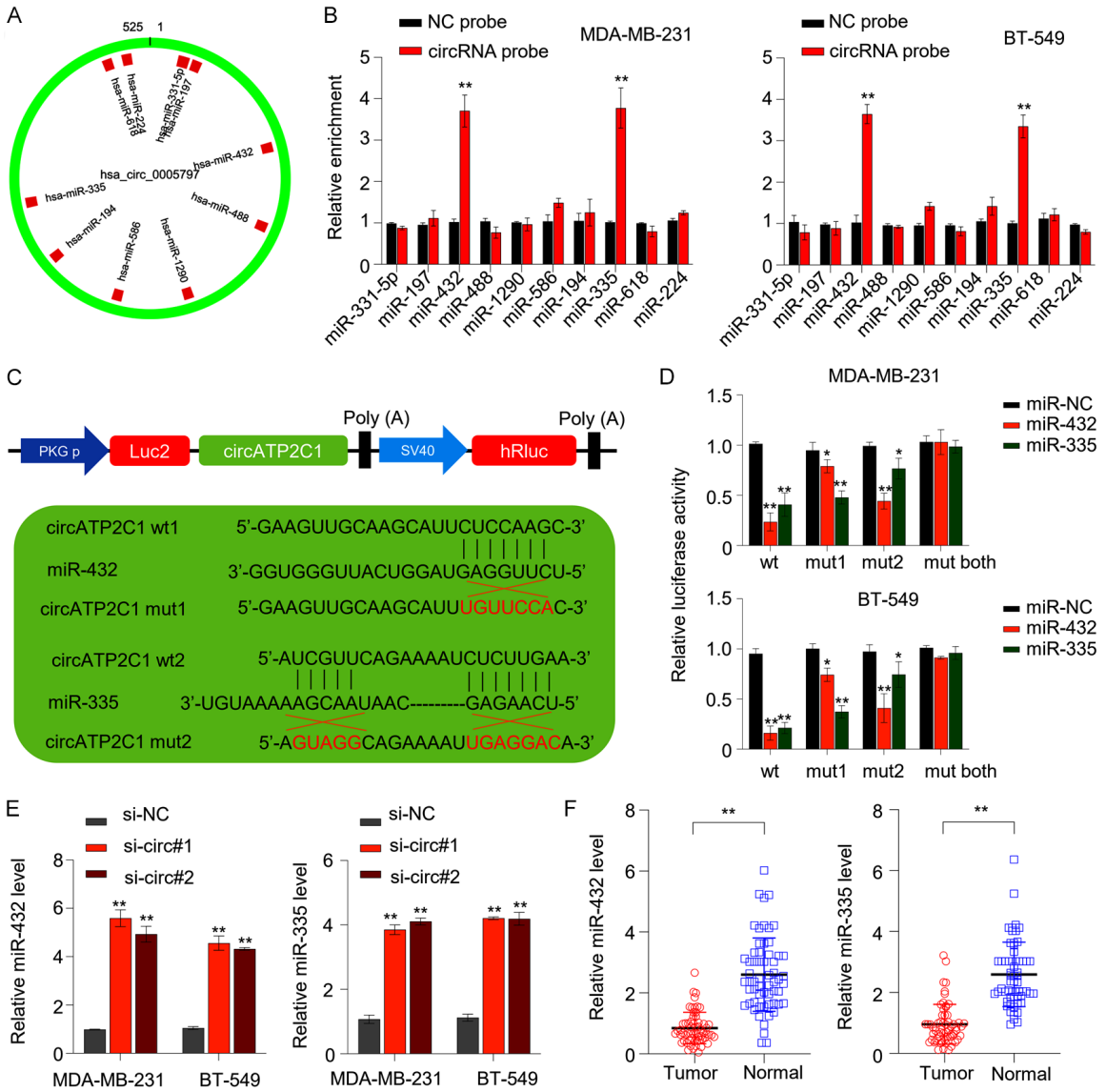


Figure 3. CircATP2C1 acts as a sponge of miR-432 and miR-335. A. miRNA binding sequences in circATP2C1 predicted by the circular RNA interactome (https://circinteractome.nia.nih.gov/). B. miRNA enrichment was determined by RT-qPCR after pulldown with nucleic acid probes of circATP2C1 in MDA-MB-231 and TB-549 cells. C, D. Dual-luciferase reporter assay in the MDA-MB-231 and BT-549 cells co-transfected with circATP2C1 wild-type (wt) or mutated reporter (mut) and miR-432/miR-335 mimics (miR-432/miR-335). E. Effect of circATP2C1-knockdown on the expressions of miR-432 and miR-335 in MDA-MB-231 and TB-549 cells. F. Expressions of miR-432 and miR-335 in the cancerous and paracancerous tissues of the 60 BC patients. $^{**}P < 0.01$.

were verified by pulldown experiments in MDA-MB-231 and TB-549 cells. RT-qPCR found that the circRNA probe effectively enriched both miR-432 and miR-335 (**Figure 3B**). Furthermore, dual-luciferase reporter test revealed that luciferase activity in the wt group transfected with wild-type circATP2C1 reporter gene and the mimics of miR-432 or miR-335 was significantly decreased (**Figure 3C** and **3D**), while was not when the miRNA binding site in circATP2C = was mutated (**Figure 3C** and **3D**). Moreover, the interaction between circATP2C1 and miR-432/miR-335 was examined by determining miR-432/miR-335 expression levels in BC cells (**Figure 3E**) with or without circATP2C1-knockdown, as well as in the cancerous and paracancerous tissues of 60 BC patients (**Figure 3F**). The data revealed that both miR-432 and miR-335 were significantly up-regulated when circATP2C1 was knocked down in

MDA-MB-231 and BT-549 cells (**Figure 3E**). Both miR-432 and miR-335 were statistically significantly down-regulated in cancerous tissues compared to the paired paracancerous normal tissues (**Figure 3D**). Together, these findings suggest that both miR-432 and miR-335 are targeted bycircATP2C1.

CCND1 (CyclinD1) is a target of miR-432 and miR-335 in BC cells

Having demonstrated that miR-432 and miR-335 are targets ofcircATP2C1, we set out to identify the target gene of these two miRNAs. We used TargetScan (http://www.targetscan.org/) to predict the genes containing potential binding sites of miR-432 or miR-335 and found prospective binding sites in the 3'UTR of *CCND1* (**Figure 4A**). Next, the dual-luciferase reporter test showed that luciferase activity in MDA-MB-231 and BT-549 cells that were transfected with wild-type (wt) CCND1 reporter gene and either of miR-432 and miR-335 was significantly inhibited, but was not when the predicted binding sites in the 3'UTR of *CCND1* were mutated (**Figure 4A** and **4B**). Furthermore, the AGO2 immunoprecipitation assay showed that the captured miR-335 and miR-432 were enriched in the CCND1 fraction rather than the IgG fraction (**Figure 4C**). Moreover, the mRNA and protein levels of CCND1 in MDA-MB-231 and BT-549 cells were significantly downregulated when miR-432 or miR-335 was overexpressed, while increased when miR-432 or miR-335 was knocked down (**Figure 4D** and **4E**). Consistently, CCND1 was significantly upregulated at both mRNA (**Figure 4F**) and protein (**Figure 4G**) levels in the cancerous tissues relative to the paired adjoining normal tissues of 60 BC patients. Taken together, our data suggest that CCND1 is a target of both miR-432 and miR-335 in BC cells.

CircATP2C1 promotes BC development by regulating the miR-432/miR-335/CCND1 axis

To further investigate whether circATP2C1 promotes BC progression via regulating the miR-432/miR-335/CCND1 signaling, we first detected CCND1 expression by RT-qPCR and Western blot assays in MDA-MB-231 and BT-549 cells that were transfected with siRNAs of circ ATP2C1 plusmiR-432/miR-335 inhibitors. The results showed that the mRNA and protein levels of CCND1 were significantly downregulated in MDA-MB-231 and BT-549 cells in which circATP2C1 was knocked down. The downregulation of CCND1 was fully restored by co-transfection of the inhibitor of either miR-432 or miR-335 (**Figure 5A** and **5B**). Furthermore, the colony formation and Transwell tests showed that the proliferation (**Figure 5C**) and invasion (**Figure 5D**) ability of MDA-MB-231 and BT-549 cells were significantly inhibited by circATP2C1-knockdown, which was almost fully rescued by co-transfection of either miR-432 inhibitor or miR-335 inhibitor (**Figure 5C** and **5D**). Meanwhile, the flow cytometer found that the apoptosis rate (**Figure 5E**) and the cells that were arrested at G0/G1 phase (**Figure 5F**) were significantly increased after circATP2C1-knockdown, which was almost fully rescued by co-transfection of either miR-432 inhibitor or miR-335 inhibitor (**Figure 5E** and **5F**). These findings suggest that circATP2C1 upregulates CCND1 level by sponging miR-432 and miR-335 in BC cells.

To validate the promoting role of circATP2C1 in BC development *in vivo*, we subcutaneously injected si-NC- or si-circATP2C1-transfected MDA-MB-231cells into the nude mice. We found that circATP2C1-knockdown significantly repressed the tumor formation of MDA-MB-231 cells *in vivo,* as evidenced by significantly reduced tumor masses (**Figure 6A**), tumor sizes (**Figure 6B**), and tumor weights (**Figure 6C**). Moreover, western blot was performed to detect the apoptosis, invasion, and proliferation markers in cancer tissues (**Figure 6D**). The results indicated that down-regulation of decreased BCL2 expression, while increasing the expression of Bax and caspase 9 significantly. In addition, the expression of the epithelial marker, E-cadherin, was increased significantly, but the expressions of the mesenchymal markers, N-cadherin and Snail, as well as the proliferate-related markers, PCNA and MMP9, were decreased dramatically when circATP2C1 was knocked down in cancer tissues. Together, these results indicated that circATP2C1-knockdown inhibited cell proliferation and invasion while promoting apoptosis *in vivo*.

Based on the results we described above, the molecular mechanism by which circATP2C1 regulates BC development was depicted in **Figure 7**.

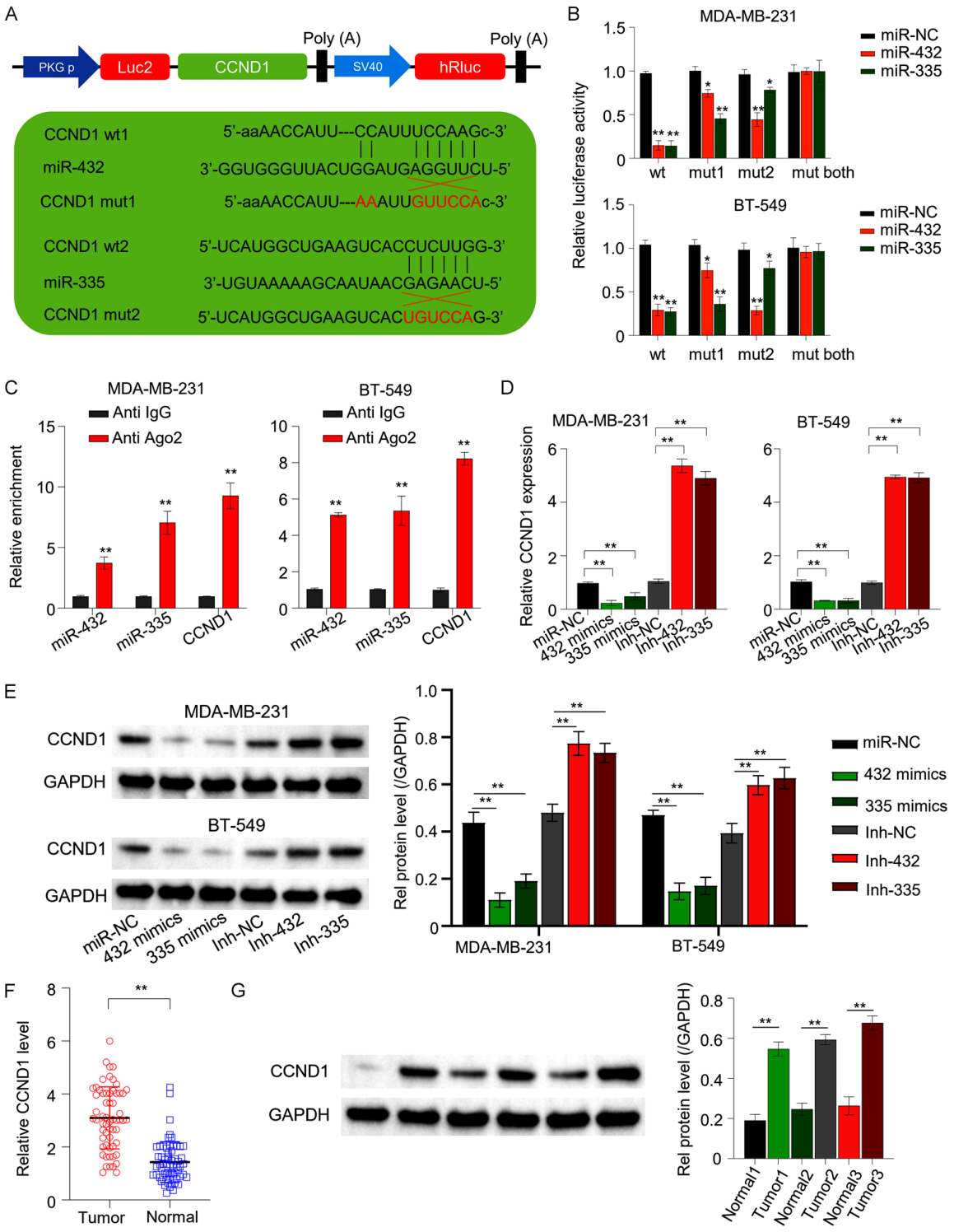
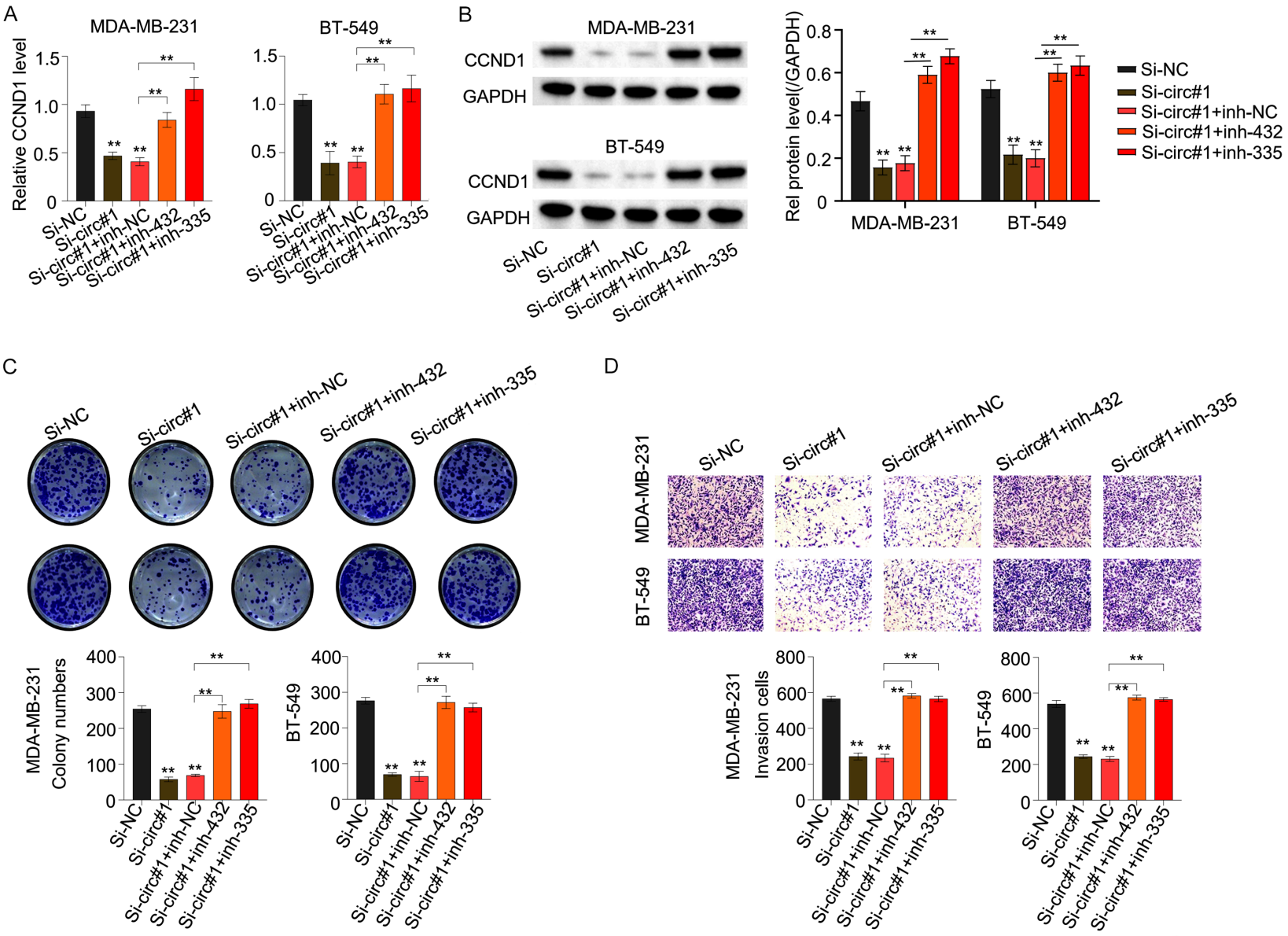


Figure 4. CCND1 is a common target of miR-432 and miR-335. (A) Potential binding sites of miR-432 and miR-335 in the *CCND1* 3'UTR predicted by TargetScan (http://www.targetscan.org/). (B) Dual-luciferase reporter assay in MDA-MB-231 and BT-549 cells co-transfected with CCND1 wild-type (wt) or mutated reporter (mut) and miR-432 or miR-335 mimics (miR-432, miR-335). (C) Enrichment of miR-335, miR-432, and CCND1 by ago2 in MDA-MB-231 and BT-549 cells determined by RIP-RT-qPCR. (D, E) Effect of the overexpression or knockdown of miR-432 or miR-335 in MDA-MB-231 and BT-549 cells on CCND1 expression. (F, G) RT-qPCR (F) and Western Blot (G) detected CCND1 expression in BC tissues. $^{**}P < 0.01$.


A
MDA-MB-231
BT-549
Relative CCND1 level
Si-NC
Si-circ#1
Si-circ#1+inh-NC
Si-circ#1+inh-432
Si-circ#1+inh-335
B
MDA-MB-231
CCND1
GAPDH
BT-549
Rel protein level(/GAPDH)
C
Colony numbers
D
Invasion cells
3442
Am J Cancer Res 2023;13(8):3433-3448

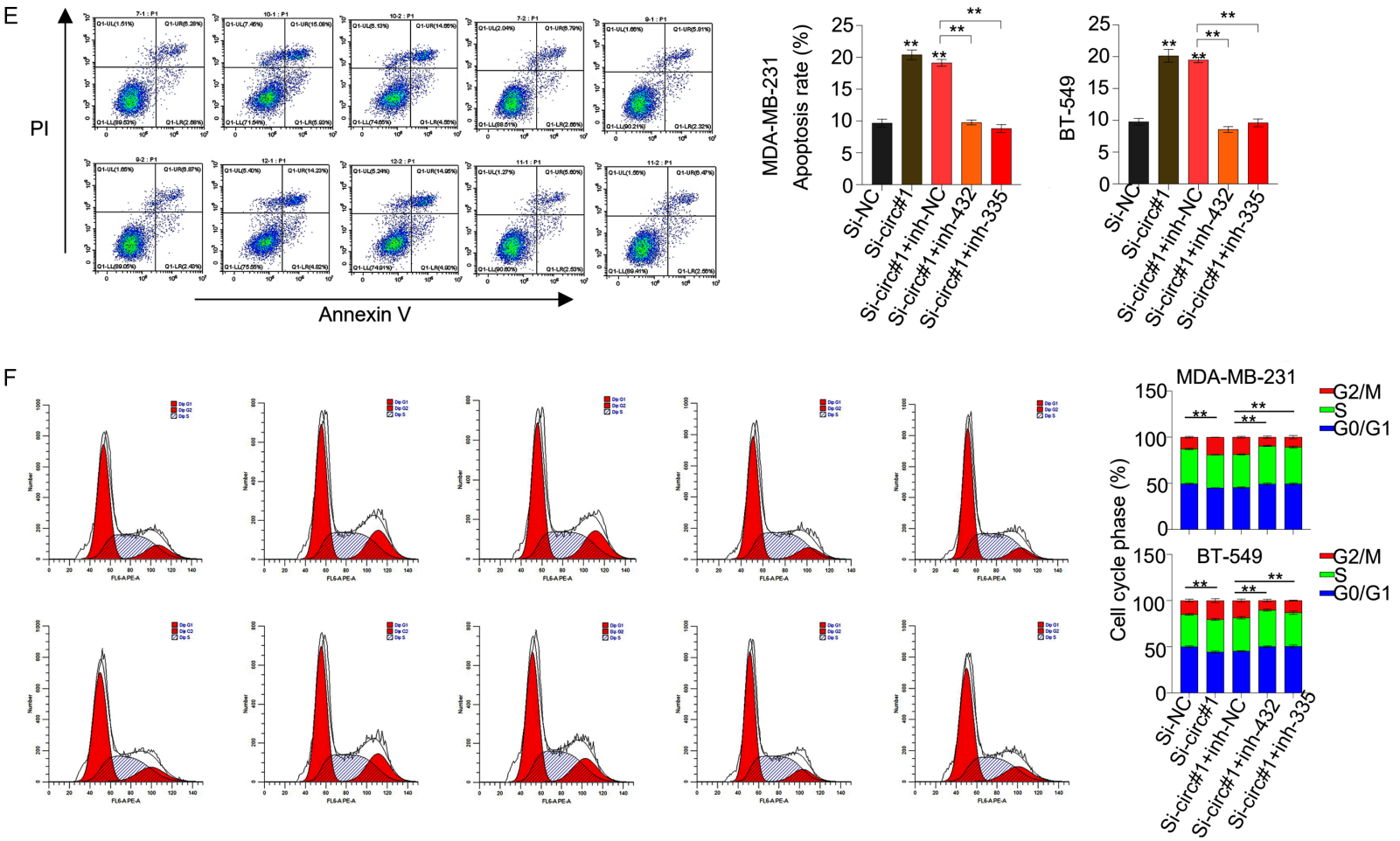


Figure 5. CircATP2C1 promotes BC cell progression via miR-432/miR-335/CCND1 signaling axis. (A) RT-qPCR and (B) Western blot determined CCND1 expression levels in MDA-MB-231 and BT-549 cells after circATP2C1-knockdown with or without co-transfection of miR-432 or miR-335 inhibitor (inh-432 or inh-335). (C) Colony proliferation ability by colony formation assay, (D) Invasion ability assessed by Transwell with Matrigel, (E) Apoptosis rate determined by Flow cytometer and (F) cell cycle detected by Flow cytometer of MDA-MB-231 and BT-549 cells after circATP2C1-knockdown with or without co-transfection of miR-432 or miR-335 inhibitor (inh-432 or inh-335). $^{**}P < 0.01$.

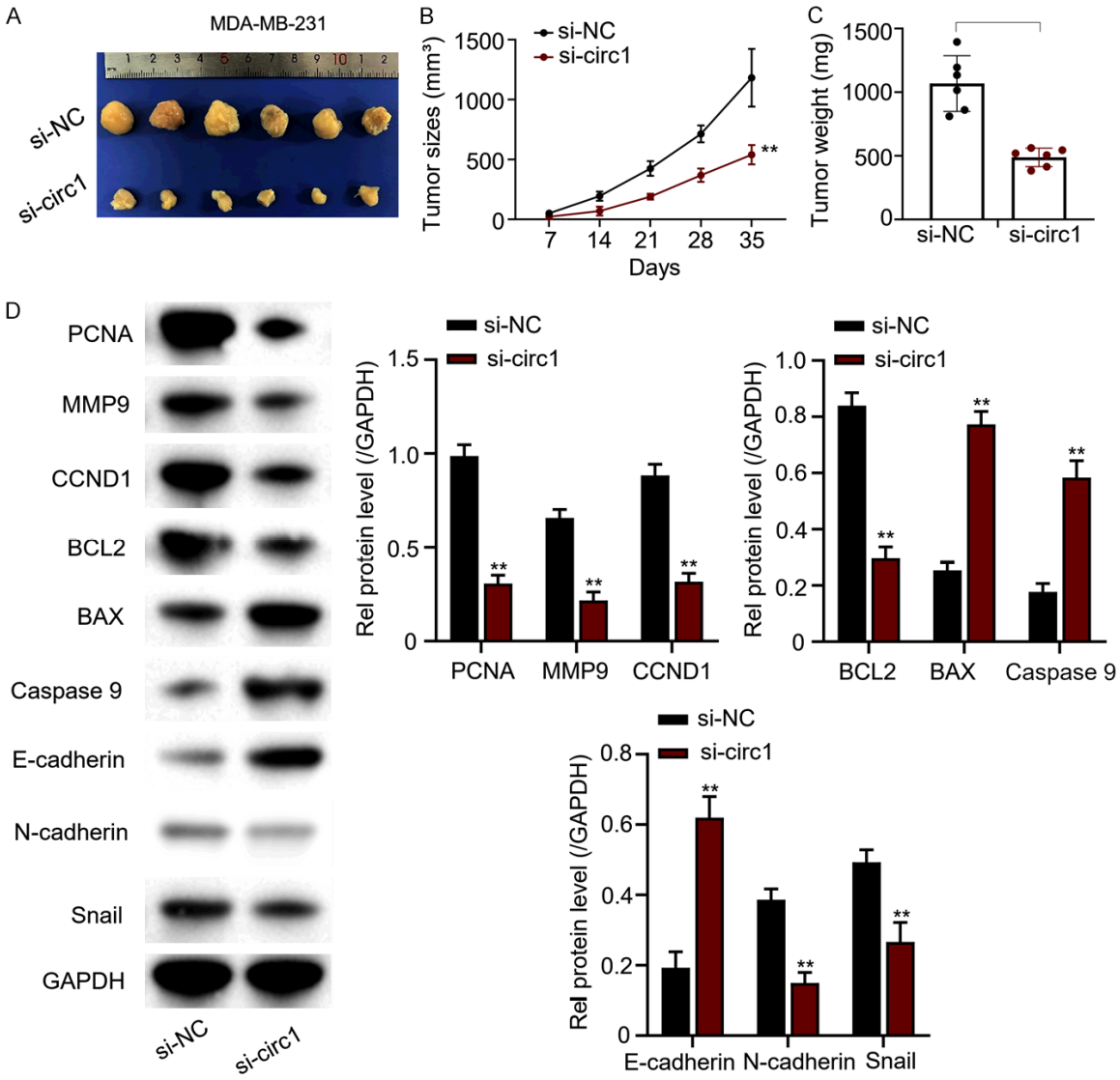


Figure 6. CircATP2C1 promotes BC cell progression *in vivo*. (A) Tumor mass, (B) tumor size, and (C) tumor weight in xenograft mouse model injected with MDA-MB-231 cells with or without circATP2C1-knockdown. (D) Expression of apoptosis, invasion, and proliferation markers in BC cancer tissue sections was detected by Western blot after circATP2C1-knockdown. $^{**}P < 0.01$.

Discussion

BC is the most frequently diagnosed malignancy in the world. The most updated statistics of global cancer burden estimated 2.26 million BC cases in 2020 [2, 3, 31]. Burgeoning evidence shows that dysregulation of circRNAs is closely associated with the apoptosis, proliferation, cell cycle arrest, invasion, and migration of cancer cells, thus playing key roles in the carcinogenesis, development, and recurrence of cancers [4, 5, 32]. Therefore, circRNAs are thought to be excellent biomarkers for diagnoses and the evaluation of prognosis and drug resistance in BC [4-7, 32]. Therefore, understanding how circRNAs regulate BC progression would be a promising approach to benefit the prognosis of BC patients.

Here, using the limma package in the R software to analyze the GSE182471 dataset, circATP2C1 was found to be up-regulated in BC tissues. This upregulation was confirmed in 60 BC patients and four different BC cell lines. Furthermore, the Kaplan-Meier plot identified that patients exhibiting higher circATP2C1

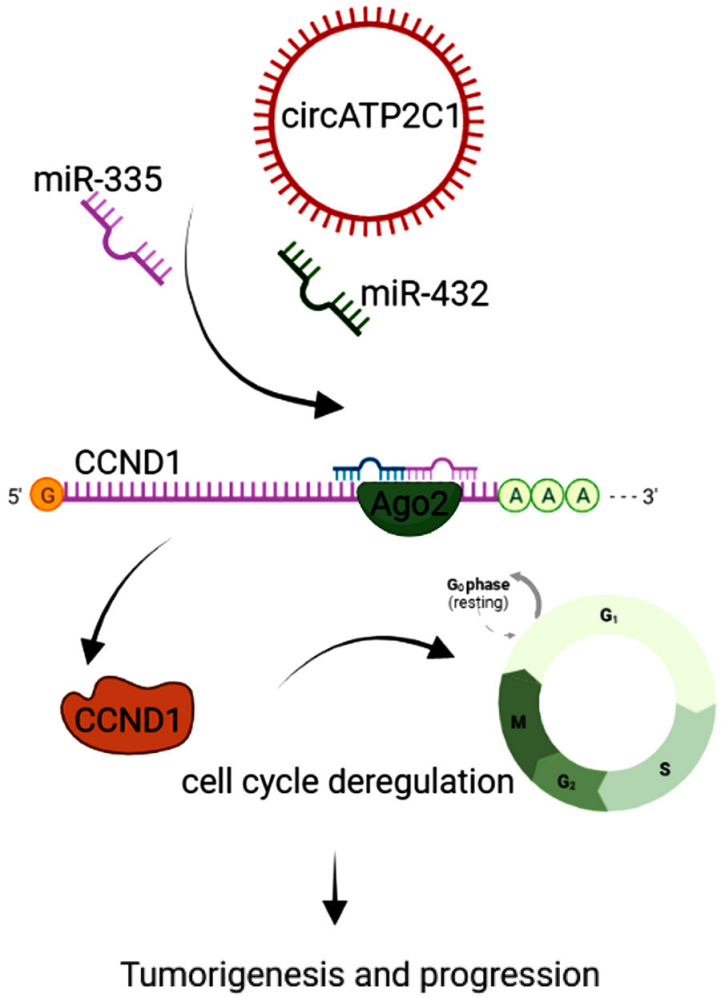


Figure 7. Schematic of the mechanism of circATP2C1 in regulating BC progression.

expressions had significantly shorter overall survival time, suggesting a significant association of circATP2C1 expression with the prognosis of BC patients.

Downregulation of circATP2C suppressed the viability, colony proliferation, and invasion of BC cells, induced cell cycle arrest and apoptosis, and inhibited BC growth *in vivo*. These results indicate that circATP2C1 acts as an oncogenetic factor in BC carcinogenesis, development, and metastasis, which potentiate circATP2C1 as a novel biomarker for early diagnosis and a therapeutic target for BC treatment.

As one class of ncRNAs, miRNAs regulate gene expression levels via binding to the 3'UTR of target genes [33-37]. It is well known that abnormal miRNA expressions can cause cancer and other diseases [37-41]. Here, using bioinformatics prediction, dual-luciferase reporter assay, and anti-AGO2 RIP, we demonstrated the circATP2C1 targeted miR-432 and miR-335 and inhibited their expressions.

It has been reported that both miR-432-5p and miR-335 are human cancer inhibitors [23, 33, 42-44]. For instance, downregulation of miR-432-5p by DRAIC enhanced BC growth [41]. Similarly, miR-432-5p was sponged by circZNF609, which upregulated LRRC1 expression and ultimately promoted [45]. LINC01783 promoted the growth and metastasis of non-small cell lung cancer via sponging miR-432-5p [46], and circ-TRIO facilitated TNBC development via regulating the miR-432-5p/CCDC58 pathway [21]. Moreover, LINC01087 facilitated BC metastasis by sponging miR-335-5p [47], and miR-335-5p prevents uterine leiomyoma development via targeting ARGLU1 [48]. Circ_103973 promoted cell proliferation in cervical cancer by sponging miR-335 [49]. In this study, we validated that miR-432 and miR-335 were significantly down-regulated in the cancerous tissues compared to the paired paracancerous normal tissues. Rescue experiments confirmed the direct binding between circATP2C1 and the two miRNAs, miR-432-5p and miR-335. These findings indicate that circATP2C1 acts as an oncogene in BC patients and functions by sponging miR-432 and miR-335.

CCND1 is a critical regulator of the cell cycle and oncogenesis of various cancers, including BC. Upregulation of CCND1 promoted BC progression and is associated with the poor clinical outcome, BC [50], and miR-93-5p regulates oncogenesis and cancer immunity by targeting PD-L1/CCND1 in BC [51]. The ceRNA hypothesis posits that circRNAs competitively bind to miRNAs to regulate the expression of downstream target genes. In the present study, bio-

informatics analyses combined with the dual-luciferase reporter assay, anti-AGO2 RIP and rescue tests showed that CCND1 was targeted by miR-432 and miR-335. CCND1 expression in MDA-MB-231 and BT-549 cells was significantly inhibited when miR-432 or miR-335 was overexpressed but was increased when either of the two miRNAs was knocked down. Moreover, CCND1 expression in MDA-MB-231 and BT-549 cells was downregulated by circATP2C1-knockdown, which was almost fully restored by co-transfection of either miR-432 inhibitor or miR-335 inhibitor. These findings were validated by checking the colony formation ability, invasion ability, apoptosis rates and cell cycle arrest in MDA-MB-231 and BT-549 cells. Taken together, our evidence demonstrates that circATP2C1 acts as a ceRNA to sponge both miR-432 and miR-335 to upregulate CCND1 expression, thus promoting BC development.

In conclusion, our current work highlighted the prominence of the circATP2C1/miR-432/miR-335/CCND1 axis in BC progression. Therefore, suppressing circATP2C1 expression in BC cells would be a strategy for treating BC.

Acknowledgements

This study was supported by the Jiaxing Key Discipiline of Medcine --Surgery Med (Mastopathy) (2023-FC-001), the Jiaxing Key Discipline of Medicine (Mastropathy, Innovation Subject, 2019-cx-04), and the Breast Cancer Precision Diagnosis and Treatment Center of the First Hospital of Jiaxing (2021-ZZZX-06).

Disclosure of conflict of interest

None.

Address correspondence to: Xiang Lu, Department of Breast Surgery, Affiliated Hospital of Jiaxing University (The First Hospital of Jiaxing), No. 1882 Zhonghuan South Road, Jiaxing 314000, Zhejiang, China. E-mail: 00187649@zjxu.edu.cn

References

[1] Siegel RL, Miller KD, Fuchs HE and Jemal A. Cancer statistics, 2021. CA Cancer J Clin 2021; 71: 7-33.

[2] Loibl S, Poortmans P, Morrow M, Denkert C and Curigliano G. Breast cancer. Lancet 2021; 397: 1750-1769.

[3] Giaquinto AN, Sung H, Miller KD, Kramer JL, Newman LA, Minihan A, Jemal A and Siegel RL. Breast cancer statistics, 2022. CA Cancer J Clin 2022; 72: 524-541.

[4] Zheng X, Huang M, Xing L, Yang R, Wang X, Jiang R, Zhang L and Chen J. The circRNA circSEPT9 mediated by E2F1 and EIF4A3 facilitates the carcinogenesis and development of triple-negative breast cancer. Mol Cancer 2020; 19: 73.

[5] Sang Y, Chen B, Song X, Li Y, Liang Y, Han D, Zhang N, Zhang H, Liu Y, Chen T, Li C, Wang L, Zhao W and Yang Q. circRNA_0025202 regulates tamoxifen sensitivity and tumor progression via regulating the miR-182-5p/FOXO3a axis in breast cancer. Mol Ther 2019; 27: 1638-1652.

[6] Xu X, Zhang J, Tian Y, Gao Y, Dong X, Chen W, Yuan X, Yin W, Xu J, Chen K, He C and Wei L. CircRNA inhibits DNA damage repair by interacting with host gene. Mol Cancer 2020; 19: 128.

[7] Ng WL, Mohd Mohidin TB and Shukla K. Functional role of circular RNAs in cancer development and progression. RNA Biol 2018; 15: 995-1005.

[8] Zhang ZJ, Zhang YH, Qin XJ, Wang YX and Fu J. Circular RNA circDENND4C facilitates proliferation, migration and glycolysis of colorectal cancer cells through miR-760/GLUT1 axis. Eur Rev Med Pharmacol Sci 2020; 24: 2387-2400.

[9] Xiao L, Ma XX, Luo J, Chung HK, Kwon MS, Yu TX, Rao JN, Kozar R, Gorospe M and Wang JY. Circular RNA circHIPK3 promotes homeostasis of the intestinal epithelium by reducing microRNA 29b function. Gastroenterology 2021; 161: 1303-1317, e3.

[10] Ma J, Li Q and Li Y. CircRNA PRH1-PRR4 stimulates RAB3D to regulate the malignant progression of NSCLC by sponging miR-877-5p. Thorac Cancer 2022; 13: 690-701.

[11] Yang W, Wu L and Jin M. Hsa_circ_0041268 promotes NSCLC progress by sponging miR-214-5p/ROCK1. J Clin Lab Anal 2022; 36: e24262.

[12] Fan Y, Wang Q, Shi M, Ju G, Lu H, Zheng L, Chen J, Zhou X, Xiao T and Chen S. Circ_0020123 promotes NSCLC tumorigenesis via up-regulating KIAA1522 expression through miR-940. Cell Cycle 2022; 21: 894-907.

[13] Zhang W, Liu H, Jiang J, Yang Y, Wang W and Jia Z. CircRNA circFOXK2 facilitates oncogenesis in breast cancer via IGF2BP3/miR-370 axis. Aging (Albany NY) 2021; 13: 18978-18992.

[14] Xu JZ, Shao CC, Wang XJ, Zhao X, Chen JQ, Ouyang YX, Feng J, Zhang F, Huang WH, Ying Q, Chen CF, Wei XL, Dong HY, Zhang GJ and Chen M. circTADA2As suppress breast cancer progression and metastasis via targeting miR-

203a-3p/SOCS3 axis. Cell Death Dis 2019; 10: 175.
[15] Huang R, Yang Z, Liu Q, Liu B, Ding X and Wang Z. CircRNA DDX21 acts as a prognostic factor and sponge of miR-1264/QKI axis to weaken the progression of triple-negative breast cancer. Clin Transl Med 2022; 12: e768.
[16] Liu Y, Yuan H and He T. Downregulated circular RNA hsa_circ_0005797 inhibits endometrial cancer by modulating microRNA-298/Catenin delta 1 signaling. Bioengineered 2022; 13: 4634-4645.
[17] Douglas MG. Hydrophobic and hydrophilic signals in protein sorting. Protein Eng 1987; 1: 80-81.
[18] Yang J, Qi M, Fei X, Wang X and Wang K. Hsa_circRNA_0088036 acts as a ceRNA to promote bladder cancer progression by sponging miR-140-3p. Cell Death Dis 2022; 13: 322.
[19] Han B, Chao J and Yao H. Circular RNA and its mechanisms in disease: from the bench to the clinic. Pharmacol Ther 2018; 187: 31-44.
[20] Wang J, Zhao X, Wang Y, Ren F, Sun D, Yan Y, Kong X, Bu J, Liu M and Xu S. circRNA-002178 act as a ceRNA to promote PDL1/PD1 expression in lung adenocarcinoma. Cell Death Dis 2020; 11: 32.
[21] Wang Z, Li Y, Yang J, Liang Y, Wang X, Zhang N, Kong X, Chen B, Wang L, Zhao W and Yang Q. Circ-TRIO promotes TNBC progression by regulating the miR-432-5p/CCDC58 axis. Cell Death Dis 2022; 13: 776.
[22] Liu Y, Lu C, Zhou Y, Zhang Z and Sun L. Circular RNA hsa_circ_0008039 promotes breast cancer cell proliferation and migration by regulating miR-432-5p/E2F3 axis. Biochem Biophys Res Commun 2018; 502: 358-363.
[23] Jia Q, Ye L, Xu S, Xiao H, Xu S, Shi Z, Li J and Chen Z. Circular RNA 0007255 regulates the progression of breast cancer through miR-335-5p/SIX2 axis. Thorac Cancer 2020; 11: 619-630.
[24] Maimaiti Y, Kamali A, Yang P, Zhong K and Abuduhadeer X. Hsa_circ_0008092 contributes to cell proliferation and metastasis in hepatocellular carcinoma via the miR-502-5p/CCND1 axis. Protein Pept Lett 2022; 29: 595-604.
[25] Nie M, Wang Y, Yu Z, Li X, Deng Y, Wang Y, Yang D, Li Q, Zeng X, Ju J, Liu M and Zhao Q. AURKB promotes gastric cancer progression via activation of CCND1 expression. Aging (Albany NY) 2020; 12: 1304-1321.
[26] Ding H, Wang Y and Zhang H. CCND1 silencing suppresses liver cancer stem cell differentiation and overcomes 5-Fluorouracil resistance in hepatocellular carcinoma. J Pharmacol Sci 2020; 143: 219-225.
[27] Yang Y, Lu T, Li Z and Lu S. FGFR1 regulates proliferation and metastasis by targeting CCND1 in FGFR1 amplified lung cancer. Cell Adh Migr 2020; 14: 82-95.
[28] Liu Y, Zhang A, Bao PP, Lin L, Wang Y, Wu H, Shu XO, Liu A and Cai Q. MicroRNA-374b inhibits breast cancer progression through regulating CCND1 and TGFA genes. Carcinogenesis 2021; 42: 528-536.
[29] Yang SJ, Wang DD, Zhong SL, Chen WQ, Wang FL, Zhang J, Xu WX, Xu D, Zhang Q, Li J, Zhang HD, Hou JC, Mao L and Tang JH. Tumor-derived exosomal circPSMA1 facilitates the tumorigenesis, metastasis, and migration in triple-negative breast cancer (TNBC) through miR-637/Akt1/beta-catenin (cyclin D1) axis. Cell Death Dis 2021; 12: 420.
[30] Zhou PL, Wu Z, Zhang W, Xu M, Ren J, Zhang Q, Sun Z and Han X. Circular RNA hsa_circ_0000277 sequesters miR-4766-5p to upregulate LAMA1 and promote esophageal carcinoma progression. Cell Death Dis 2021; 12: 676.
[31] Wilkinson L and Gathani T. Understanding breast cancer as a global health concern. Br J Radiol 2022; 95: 20211033.
[32] Huang P, Li F, Mo Z, Geng C, Wen F, Zhang C, Guo J, Wu S, Li L, Brunner N and Stenvang J. A comprehensive RNA study to identify circRNA and miRNA biomarkers for docetaxel resistance in breast cancer. Front Oncol 2021; 11: 669270.
[33] Hill M and Tran N. miRNA interplay: mechanisms and consequences in cancer. Dis Model Mech 2021; 14: dmm047662.
[34] Zhang M, Bai X, Zeng X, Liu J, Liu F and Zhang Z. circRNA-miRNA-mRNA in breast cancer. Clin Chim Acta 2021; 523: 120-130.
[35] Panda AC. Circular RNAs act as miRNA sponges. Adv Exp Med Biol 2018; 1087: 67-79.
[36] Cai Y, Yu X, Hu S and Yu J. A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics 2009; 7: 147-154.
[37] Kumar V, Kumar V, Chaudhary AK, Coulter DW, McGuire T and Mahato RI. Impact of miRNA-mRNA profiling and their correlation on medulloblastoma tumorigenesis. Mol Ther Nucleic Acids 2018; 12: 490-503.
[38] Gu J, Wang M, Wang X, Li J, Liu H, Lin Z, Yang X, Zhang X and Liu H. Exosomal miR-483-5p in bone marrow mesenchymal stem cells promotes malignant progression of multiple myeloma by targeting TIMP2. Front Cell Dev Biol 2022; 10: 862524.
[39] Gebert LFR and MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol 2019; 20: 21-37.
[40] Adam RS, Poel D, Ferreira Moreno L, Spronck JMA, de Back TR, Torang A, Gomez Barila PM, Ten Hoorn S, Markowetz F, Wang X, Verheul

HMW, Buffart TE and Vermeulen L. Development of a miRNA-based classifier for detection of colorectal cancer molecular subtypes. Mol Oncol 2022; 16: 2693-2709.
[41] Li S, Jia H, Zhang Z and Wu D. DRAIC promotes growth of breast cancer by sponging miR-432-5p to upregulate SLBP. Cancer Gene Ther 2022; 29: 951-960.
[42] Ramadan A, Hashim M, M Hassan N and Swellam M. Expression of miR-335 and its target metalloproteinase genes: clinical significance in breast cancer. Arch Physiol Biochem 2022; 128: 569-575.
[43] Song G, Ma Y, Ma Y, Liu P, Hou L, Xu Z, Jiang J, Shen Y, Cao Y and Zhao Y. miR-335-5p targets SDC1 to regulate the progression of breast cancer. Crit Rev Eukaryot Gene Expr 2022; 32: 21-31.
[44] Jin L, Luo C, Wu X, Li M, Wu S and Feng Y. LncRNA-HAGLR motivates triple negative breast cancer progression by regulation of WNT2 via sponging miR-335-3p. Aging (Albany NY) 2021; 13: 19306-19316.
[45] Guan C, Liu L, Zhao Y, Zhang X, Liu G, Wang H, Gao X, Zhong X and Jiang X. YY1 and eIF4A3 are mediators of the cell proliferation, migration and invasion in cholangiocarcinoma promoted by circ-ZNF609 by targeting miR-432-5p to regulate LRRC1. Aging (Albany NY) 2021; 13: 25195-25212.
[46] Deng Y, Zhang L and Luo R. LINC01783 facilitates cell proliferation, migration and invasion in non-small cell lung cancer by targeting miR-432-5p to activate the notch pathway. Cancer Cell Int 2021; 21: 234.
[47] She JK, Fu DN, Zhen D, Gong GH and Zhang B. LINC01087 is highly expressed in breast cancer and regulates the malignant behavior of cancer cells through miR-335-5p/Rock1. Onco Targets Ther 2020; 13: 9771-9783.
[48] Zhao W, Zhao Y, Chen L, Sun Y and Fan S. miR-335-5p inhibits progression of uterine leiomyoma by targeting ARGLU1. Comput Math Methods Med 2022; 2022: 2329576.
[49] Zhu Y, Jiang X, Zhang S, Wang L, Zhou Q and Jiang J. Hsa_circ_103973 acts as a sponge of miR-335 to promote cervical cancer progression. Onco Targets Ther 2020; 13: 1777-1786.
[50] Valla M, Klaestad E, Ytterhus B and Bofin AM. CCND1 amplification in breast cancer-associations with proliferation, histopathological grade, molecular subtype and prognosis. J Mammary Gland Biol Neoplasia 2022; 27: 67-77.
[51] Yang M, Xiao R, Wang X, Xiong Y, Duan Z, Li D and Kan Q. MiR-93-5p regulates tumorigenesis and tumor immunity by targeting PD-L1/CCND1 in breast cancer. Ann Transl Med 2022; 10: 203.