

Croton Alkaloids Suppresses Breast Cancer Cell Proliferation, Migration and Invasion through mediating Circular_0079593

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Yanjiang *et al.*: Effects of Croton Alkaloids on Breast Cancer Cells

To investigate the effects of croton alkaloids on malignant behaviors of breast cancer cells T47D and the possible mechanism. Different doses of croton alkaloids were exposed to T47D cells. T47D cells were transfected with small interfering-circular_0079593 and small interfering-negative control by liposome transfection, respectively. The plasmid cloning deoxyribonucleic acid-circular_0079593 transfected T47D cells were performed with 150 µg/ml croton alkaloids treatment. Circular_0079593 and microRNA-1299 were determined *via* qualitative reverse transcription polymerase chain reaction in breast cancer samples and cells. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide assay and colony formation assay for proliferation, scratch assay for migration and transwell assay for invasion were performed. Circular_0079593 and microRNA-1299 target relationship was examined using dual-luciferase reporter assay. Protein analysis was implemented through Western blot. Croton alkaloids increased cell proliferation inhibition rate and E-cadherin protein level ($p < 0.05$) while reduced colony formation number and invasive cells ($p < 0.05$), and croton alkaloids suppressed scratch healing rate and N-cadherin protein level ($p < 0.05$), and these effects were dose-dependent. Circular_0079593 up-regulation and microRNA-1299 down-regulation were detected in breast cancer tissues contrasted to adjacent samples ($p < 0.05$). Croton alkaloids induced circular_0079593 expression up-regulation ($p < 0.05$) and microRNA-1299 level inhibition ($p < 0.05$) in a dose-dependent manner. Circular_0079593 could targeted regulate the expression of microRNA-1299. Transfection of small interfering-circular_0079593 elevated microRNA-1299 level, and suppressed cell malignant behaviors. Circular_0079593 overexpression could weaken the regulation of croton alkaloids in T47D cell behaviors. Croton alkaloids could repress breast cancer cell proliferation, colony formation, migration and invasion *via* mediating circ_0079593/microRNA-1299 axis.

Key words: Breast cancer, croton alkaloids, circ_0079593, microRNA-1299, cell proliferation, migration, invasion

Breast Cancer (BC) is a commonly diagnosed tumor with high occurrence rate in female, and the age distribution is younger in recent years^[1]. With the advances of therapeutic technologies, survival prognosis has signally improved in BC patients^[2]. However, some patients still have high recurrence and metastasis to result in poor treatment effect^[3,4]. Hence, it is of great importance to discover safe and effective medicines in BC treatment. Some active ingredients isolated from plants appear anti-tumor function and can be used for BC treatment^[5,6]. Croton Alkaloids (CA) is extracted from croton in the Euphorbia family, and exerts

inhibiting effect on cell development in colorectal cancer and ovarian cancer^[7,8]. Nevertheless, the function of CA in BC has not been fully studied.

Circular RNA (circRNA) is a closed non-coding RNA (ncRNA) molecule with tissue specificity and stability, and possesses the characteristic as microRNA (miR)-sponge^[9,10]. circRNA exhibits

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aberrant expression in tumors and vital molecular function in accelerating or suppressing biological processes of tumor cells^[11]. circ_0038632 was identified as a promoter of BC through interacting with miR-520a-3p^[12]. circNCOR1 affected radiotherapy efficacy in BC cells *via* miR-638 binding^[13]. Yang *et al.*^[14] have shown that circ_0079593 overexpression promoted cell metastasis and proliferation of glioma. Level inhibition of miR-1299 was discovered in BC and miR-1299 up-regulation reduced BC cell growth^[15]. However, the relation of circ_0079593/miR-1299 in BC has not been clarified.

This study concentrated on the influences of CA on proliferation and motility of BC cells. Moreover, circ_0079593 was hypothesized to have a target relation with miR-1299. The regulatory mechanism of CA with circ_0079593/miR-1299 was elucidated.

MATERIALS AND METHODS

Materials and reagents:

BC and adjacent tissues (n=31 of each group) were collected after surgical resection in our hospital from May 2019 to July 2020, then immediately preserved at -80°. The age of BC patients was 50-68 (56.35±4.11) y old. Patients or close relatives have written informed consent forms, and this study was following the Declaration of Helsinki of the world medical association. CA (purity ≥99 %) was purchased from Google Biological Chemical Co., Ltd. (Wuhan, China). Human BC cell line T47D was purchased from Kebai Biology (Nanjing, China). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Gibco (United States of America (USA)). Trizol reagent, Lipofectamine2000 and circ_0079593 overexpression vector (plasmid cloning Deoxyribonucleic Acid (pcDNA)-circ_0079593) were provided by Invitrogen (USA). The complementary DNA (cDNA) synthesis and quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) kits were provided by Thermo Fisher (USA). The oligonucleotides contained miR-1299 mimic, mimic NC, si-circ_0079593 and si-NC (Ribobio, Guangzhou, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent and matrigel were bought from Solarbio (Beijing). Transwell chamber was purchased from Corning (USA). Rabbit antibody for E-cadherin, N-cadherin

and goat anti-Rabbit secondary antibody were obtained from CST (USA).

Grouping:

After cell culture (3×10³ cells/well) in 96-well plates overnight, T47D cells were disposed to increasing concentrations of CA for 24 h^[16], which was recorded as CA 50 µg/ml group, CA 100 µg/ml group and CA 150 µg/ml group, respectively. T47D cell line with normal culture was recorded as control group. Lipofectamine2000 was used for si-NC and si-circ_0079593 transfection according to the instruction book, which was denoted as si-circ_0079593 group and si-NC group. T47D cells with transfection of pcDNA-circ_0079593 were disposed with 150 µg/ml CA for 24 h, which was labeled as CA+pcDNA-circ_0079593 group.

RT-qPCR for circ_0079593 and miR-1299 quantification:

Total Ribonucleic Acid (RNA) was isolated employing Trizol reagent, and concentration detection was performed under an ultraviolet spectrophotometer. The reverse transcription into cDNA was administrated through cDNA synthesis kit, followed by PCR amplification with cDNA as the template. The reaction system was as follows; 10 µl SYBR Green Master Mix, 1 µl cDNA, 0.8 µl forward and reverse primer, and system was supplemented by double distilled Water (ddH₂O) to 20 µl. Reaction procedures included Predenaturation (95°, 2 min), denaturation (95°, 30 s), annealing (60°, 30 s), and extension (72°, 30 s) with 40 cycles. The relative expression was examined through ABI StepOnePlus fluorescence qPCR instrument.

MTT assay for cell proliferation:

The 96-well plates were inoculated with T47D cells with 3×10³ cells/well, then 20 µl/well MTT incubation was administrated at 37° for 4 h. Then the supernatant was discarded after cell centrifugation (3000 r/min, 5 min), followed by lucifugal incubation of 150 µl/well Dimethyl Sulfoxide (DMSO) for 5 min. OD value was examined *via* microplate reader and proliferation inhibition rate was calculated as follows:

$$\left(\frac{\text{Optical Density (OD)}_{\text{control group}} - \text{OD}_{\text{experimental group}}}{\text{OD}_{\text{control group}} - \text{OD}_{\text{blank group}}} \right) \times 100 \%$$

Colony formation assay for cell growth:

500 T47D cells of each well in 6-well plates were

cultivated for 2 w. Subsequently, culture medium needed to be changed every 2 d. The fastened (4 % paraformaldehyde, 30 min) and dyed (1 % crystal violet, 20 min) colonies were washed in Phosphate Buffer Solution (PBS) and counted.

Scratch assay for cell migration:

1×10^4 T47D cells/well in 6-well plates were cultured to 100 % confluence, followed by producing two straight scratches using a sterile pipette tip. PBS was added for cell washing and cell culture was performed in serum-free DMEM for 24 h. Following detection of scratch width, scratch healing rate was calculated.

Scratch healing rate = $(\text{scratch width}_{0\text{h}} - \text{scratch width}_{24\text{h}}) / \text{scratch width}_{0\text{h}} \times 100 \%$

Transwell assay for invasion examination:

The top chamber was coated with the diluted matrigel in pre-cooled medium for 4 h. Respectively, the top and bottom chambers were pipetted with 200 μl T47D cells (2.5×10^5 cells/ml) and 600 μl DMEM+10 % FBS medium. Transwell chamber was incubated for 24 h, then the transmembrane cells were administrated with fixation (paraformaldehyde, 20 min) and staining (crystal violet, 10 min). Eventually, invasive cells were numbered.

Dual-luciferase reporter assay for circ_0079593 and miR-1299 target detection:

Circular RNA interactive was employed for predicting circ_0079593 and miR-1299 binding region. The complementary sequence was mutated by gene mutation technique. The recombinant vectors wt-circ_0079593 and mut-circ_0079593 were obtained by cloning the complementary sequence and mutant sequence into pmirGLO vector, respectively. miR-1299 mimic or miR-NC and wt-circ_0079593 or mut-circ_0079593 were co-transfected into T47D cells by liposome transfection. 48 h later, cells were gathered for relative luciferase detection.

Western blot for protein analysis:

Radioimmunoprecipitation Assay (RIPA) lysis solution was utilized to extract total proteins, and concentration was examined *via* Bicinchoninic Acid (BCA) assay method. Protein sample was isolated through Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), followed by transferring membranes and blocking

for 2 h. The primary antibody for E-cadherin (1:800), N-cadherin (1:800) or Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (1:1000) was incubated for 24 h at 4°. After incubation with the secondary antibody (1:3000) at 25° for 1 h, gray values of bands were analyzed by ImageJ software after Enhanced Chemiluminescence (ECL) detection.

Statistical analysis:

Data were indicated as $\bar{x} \pm s$ in accordance with normal distribution, and Statistical Package for the Social Sciences (SPSS) 21.0 was utilized for data analysis. Independent sample t test was employed for comparison between two groups, and comparison among multiple groups was implemented *via* one-way Analysis of Variance (ANOVA). Statistically, the significant difference was represented as $p < 0.05$.

RESULTS AND DISCUSSION

Circ_0079593 expression was elevated ($p < 0.05$) but miR-1299 level was lessened ($p < 0.05$) in BC samples contrasted to adjacent samples, as shown in fig. 1.

Relative to control group, cell proliferation inhibition rate was gradually increased in three CA groups ($p < 0.05$). Cell colonies, invaded cells and scratch healing rate of three CA treatment groups were reduced in a dose-independent way ($p < 0.05$). As the results of CA treatment, E-cadherin was enhanced ($p < 0.05$) while N-cadherin protein level was lessened ($p < 0.05$). Difference among different dose groups was statistically significant ($p < 0.05$), as displayed in fig. 2 and Table 1.

Contrasted to control group, circ_0079593 level was reduced ($p < 0.05$) and miR-1299 was up-regulated ($p < 0.05$) in three CA treatment groups. The statistical difference was found in different groups ($p < 0.05$), as exhibited in Table 2.

Circ_0079593 and miR-1299 binding region by circular RNA interactive was revealed in fig. 3. In cell experiment with co-transfection of wt-circ_0079593 vector, the relative luciferase activity of miR-1299 group was suppressed by comparison to miR-NC transfection ($p < 0.05$). No statistical difference of luciferase activity was observed in miR-1299 and miR-NC groups with co-transfection of mut-circ_0079593, as shown in Table 3.

In contrast with control group and si-NC group, miR-1299 level in si-circ_0079593 group was increased ($p < 0.05$). Knocking down circ_0079593 increased cell proliferation inhibition rate ($p < 0.05$), while colony formation, invasive cells and scratch healing rate were suppressed ($p < 0.05$). circ_0079593 inhibition resulted in E-cadherin protein up-regulation ($p < 0.05$) and N-cadherin protein reduction ($p < 0.05$), as exhibited in fig. 4 and Table 4.

Compared with CA group, miR-1299 level down-regulation was detected in CA+pcDNA-circ_0079593 group ($p < 0.05$). Overexpression of circ_0079593 evoked reduction of cell proliferation inhibition rate ($p < 0.05$), as well as the promotion of cell colonies, invaded cells and scratch healing rate ($p < 0.05$). E-cadherin protein level was inhibited ($p < 0.05$) while N-cadherin was elevated ($p < 0.05$) with circ_0079593 up-regulation, as exhibited in fig. 5 and Table 5.

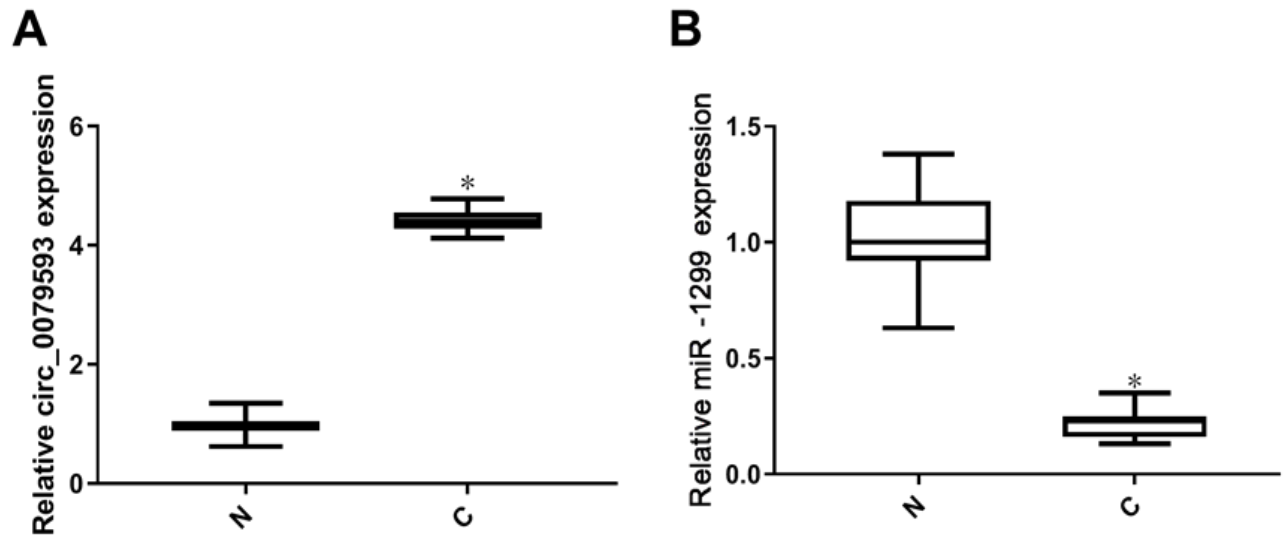


Fig. 1: Detection of circ_0079593 and miR-1299 expression, (A): circ_0079593 was up-regulated in BC tissues and (B): miR-1299 was down-regulated in BC tissues

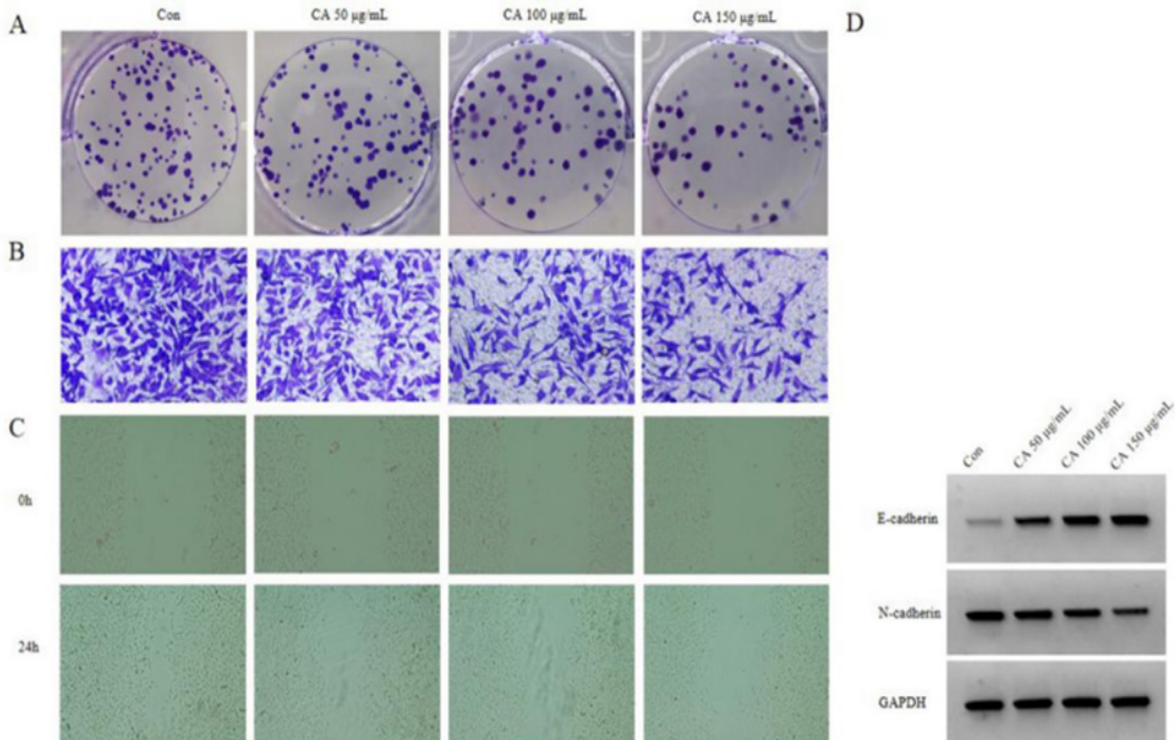


Fig. 2: CA inhibited T47D cell proliferation, migration and invasion, (A): CA inhibited T47D clone formation; (B): CA repressed T47D cell invasion; (C): CA reduced T47D cell scratch healing and (D): CA down-regulated N-cadherin and elevate E-cadherin protein level in T47D cells

TABLE 1: CA INHIBITED T47D CELL GROWTH AND MOTILITY (x±s, n=9)

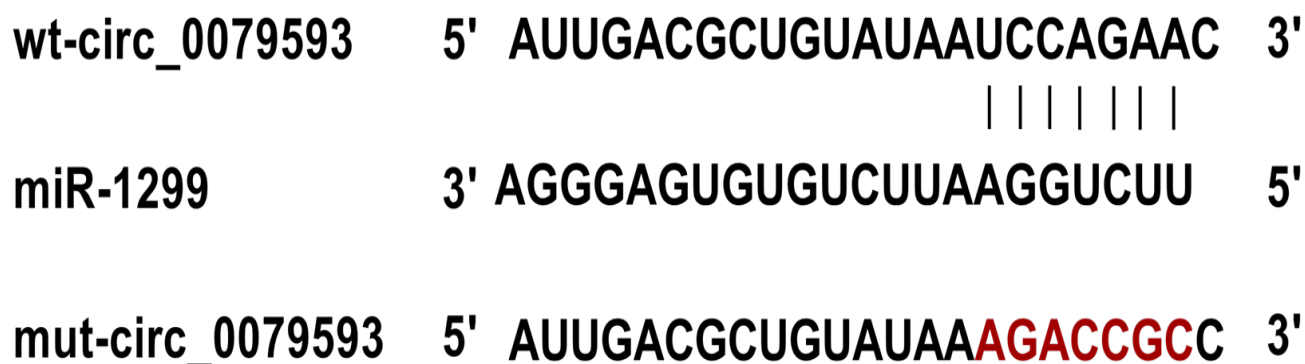
Group	Inhibition rate (%)	Colony formation (number)	Scratch healing rate (%)	Invaded cells (number)	E-cadherin	N-cadherin
Control	0.00±0.00	116.67±3.30	70.67±3.97	170.67±5.44	0.17±0.01	0.69±0.04
CA 50 µg/ml	14.45±0.71 ¹⁾	100.67±3.09 ¹⁾	60.92±2.79 ¹⁾	148.00±5.10 ¹⁾	0.33±0.02 ¹⁾	0.55±0.03 ¹⁾
CA 100 µg/ml	38.08±1.25 ¹⁾²⁾	78.33±2.05 ¹⁾²⁾	44.86±1.43 ¹⁾²⁾	109.67±3.86 ¹⁾²⁾	0.56±0.04 ¹⁾²⁾	0.38±0.02 ¹⁾²⁾
CA 150 µg/ml	55.19±2.77 ¹⁾²⁾³⁾	54.00±0.82 ¹⁾²⁾³⁾	32.56±0.73 ¹⁾²⁾³⁾	74.33±1.25 ¹⁾²⁾³⁾	0.80±0.04 ¹⁾²⁾³⁾	0.21±0.02 ¹⁾²⁾³⁾
F	746.868	352.491	131.192	300.583	244.865	157.424
p	0.000	0.000	0.000	0.000	0.000	0.000

Note: Contrasted to control group, ¹⁾p<0.05; contrasted to CA 50 µg/ml group, ²⁾p<0.05 and contrasted to CA 100 µg/ml group, ³⁾p<0.05

TABLE 2: CA DOWN-REGULATED circ_0079593 AND UP-REGULATED miR-1299 (x±s, n=9)

Group	circ_0079593	miR-1299
Control	1.00±0.00	1.00±0.00
CA 50 µg/ml	0.88±0.02 ¹⁾	1.53±0.06 ¹⁾
CA 100 µg/ml	0.57±0.04 ¹⁾²⁾	2.47±0.08 ¹⁾²⁾
CA 150 µg/ml	0.29±0.03 ¹⁾²⁾³⁾	3.67±0.10 ¹⁾²⁾³⁾
F	422.759	823.695
p	0.000	0.000

Note: Relative to control group, ¹⁾p<0.05; relative to CA 50 µg/ml group, ²⁾p<0.05 and relative to CA 100 µg/ml group, ³⁾p<0.05

**Fig. 3: Complementary sequences between circ_0079593 and miR-1299****TABLE 3: DETECTION OF RELATIVE LUCIFERASE ACTIVITY (x±s, n=9)**

Group	wt-circ_0079593	mut-circ_0079593
miR-NC	0.96±0.08	1.01±0.09
miR-1299	0.31±0.02 ¹⁾	0.95±0.08
t	13.653	0.863
p	0.000	0.437

Note: Contrasted to miR-NC group, ¹⁾p<0.05

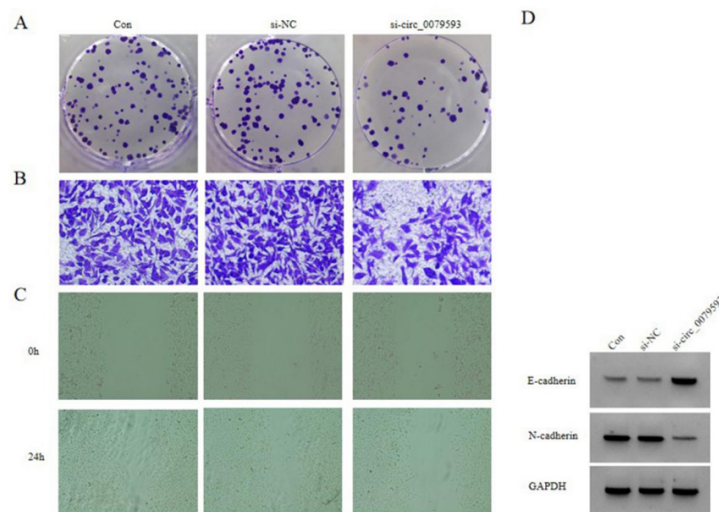


Fig. 4: Circ_0079593 down-regulation suppressed T47D cell processes, (A): Inhibition of circ_0079593 repressed T47D cell colony formation; (B): Inhibition circ_0079593 suppressed T47D cell invasion; (C): Inhibition circ_0079593 reduced T47D cell scratch healing and (D): Inhibition circ_0079593 decreased N-cadherin and promoted E-cadherin protein in T47D cells

TABLE 4: KNOCKDOWN OF circ_0079593 INHIBITED T47D CELL PROCESSES (x \pm s, n=9)

Group	Circ_0079593	miR-1299	Inhibition rate (%)	Colony formation (number)	Scratch healing rate (%)	Invaded cells (number)	E-cadherin	N-cadherin
Control	1.00 \pm 0.00	1.00 \pm 0.00	0.00 \pm 0.00	116.33 \pm 4.50	71.30 \pm 3.38	170.00 \pm 6.16	0.17 \pm 0.01	0.69 \pm 0.05
si-NC	0.99 \pm 0.03	1.02 \pm 0.02	0.01 \pm 0.01	118.00 \pm 4.32	70.77 \pm 4.23	172.00 \pm 6.48	0.16 \pm 0.02	0.68 \pm 0.05
si-circ_0079593	0.18 \pm 0.02 ¹⁾²⁾	4.33 \pm 0.06 ¹⁾²⁾	63.96 \pm 1.27 ¹⁾²⁾	45.33 \pm 0.47 ¹⁾²⁾	25.41 \pm 0.53 ¹⁾²⁾	63.33 \pm 1.25 ¹⁾²⁾	0.93 \pm 0.04 ¹⁾²⁾	0.13 \pm 0.01 ¹⁾²⁾
F	1533	25437	7607.39	395.752	211.011	426.848	836.143	181.235
P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: Contrasted to control group, ¹⁾p<0.05 and relative to si-NC group, ²⁾p<0.05

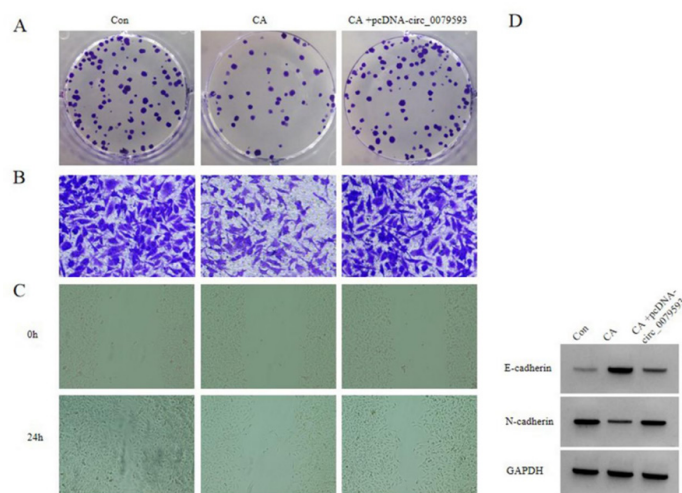


Fig. 5: Overexpression of circ_0079593 restored the regulation of CA in T47D cells, (A): Overexpression of circ_0079593 weakened the inhibited influence of CA on T47D cell colonies; (B): Overexpression of circ_0079593 attenuated the repressive regulation of CA in T47D cell invasion; (C): Overexpression of circ_0079593 abated the suppressive effect of CA on T47D cell scratch healing and (D): Overexpression of circ_0079593 reduced the inhibiting changes of CA on N-cadherin and E-cadherin protein levels in T47D cells

TABLE 5: OVEREXPRESSION OF circ_0079593 RESTORED THE REGULATION OF CA IN T47D CELLS (x±s, n=9)

Group	circ_0079593	miR-1299	Inhibition rate (%)	Colony formation (number)	Scratch healing rate (%)	Invaded cells (number)	E-cadherin	N-cadherin
Control	1.00±0.00	1.00±0.00	0.00±0.00	117.00±4.55	71.21±4.17	170.00±6.16	0.17±0.02	0.70±0.05
CA	0.29±0.03 ¹⁾	3.68±0.10 ¹⁾	55.78±3.21 ¹⁾	54.67±0.47 ¹⁾	32.80±1.04 ¹⁾	74.67±2.49 ¹⁾	0.79±0.05 ¹⁾	0.21±0.02 ¹⁾
CA +pcDNA-circ_0079593	0.92±0.05 ²⁾	1.27±0.06 ²⁾	8.04±0.37 ²⁾	106.33±3.68 ²⁾	65.10±2.89 ²⁾	163.33±4.11 ²⁾	0.24±0.02 ²⁾	0.62±0.04 ²⁾
F	400.324	1440.82	783.712	290.185	142.936	417.599	314.455	138.2
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: Contrasted with control group, ¹⁾p< 0.05 and relative to CA group, ²⁾p<0.05

Active ingredients extracted from plants can play anti-cancer roles in BC through multiple targets and pathways. For example, curcumin repressed migration and induced apoptosis in colorectal cancer cells by modulating circHNI^[17]. Astragaloside IV restrained cell growth and metastasis in BC *via* up-regulating long noncoding RNA (lncRNA) Thyrotropin Releasing Hormone Degrading Enzyme-Antisense RNA 1 (TRHDE-AS1)^[18]. circRNA with dysregulation in BC can induce the negative regulation of miRNA by serving as a miRNA sponge to participate in BC development^[19,20]. However, whether circRNA can act as a potential target in BC treatment with traditional Chinese medicine or active ingredients of plants needs further research.

CA was reported to reduce cell growth and induce cell apoptosis of lung cancer^[21]. Cell proliferation and apoptosis-related proteins were regulated by CA in gastric cancer^[22]. Also, CA treatment resulted in liver cancer cell growth inhibition and apoptosis acceleration^[23]. It is unknown about the effects of CA on BC cell malignant behaviors. The results during this study attested that proliferation inhibition rate was elevated and colony formation number was decreased with the concentration increase of CA, suggesting that CA could inhibit BC cell proliferation. Epithelial-Mesenchymal Transformation (EMT) is an essential biological process with transformation from polar epithelial cells to mesenchymal cells under certain conditions, consequently leading to cell migration and invasion^[24]. E-cadherin (a biomarker for epithelial cells) and N-cadherin (a biomarker for mesenchymal cells) dysregulation

can evoke EMT occurrence to partake in cell metastasis^[25]. Herein, scratch healing rate and invaded cells were hindered by increasing doses of CA. CA induced E-cadherin up-regulation and N-cadherin down-regulation, which certified that CA suppressed EMT process and BC cell motility. In a dose-dependent pattern, CA was implicated in the regulation of BC cellular behaviors.

The published studies indicated that circ_0079593 functioned as a pivotal molecule in cancer regulation *via* miRNA sponging effect. circ_0079593 exhibited high expression in melanoma, and promoted proliferation as well as invasion^[26]. The oncogenic function of circ_0079593 was associated with miR-516b/GRM3 or miR-433/EGFR axis^[27,28]. circ_0079593 was discovered to facilitate glioma cell motility and angiogenesis through sponging miR-324-5p to elevate XBP1 level^[29]. In the current study, aberrant up-regulation of circ_0079593 in BC samples hinted that circ_0079593 might have vital regulation in the pathological processes of BC. The further evidences affirmed that circ_0079593 interacted with miR-1299 and negatively regulated miR-1299 level. Anti-tumor role of miR-1299 has been elucidated in human cancer research. By acting as a target of circ_0006404, miR-1299 repressed cell viability and metastasis in prostate cancer^[30]. circ_0058608 contributed to lung cancer cell malignant progression and chemo resistance *via* absorbing miR-1299^[31]. Also, miR-1299 was down-regulated in BC and up-regulated miR-1299 expression inhibited BC progression^[32]. Also, circ_0001925 expedited cell processes in BC through miR-1299 sponging mechanism to mediate

YY1^[33]. Consistently, our expression detection suggested that miR-1299 was down-regulated in BC tissues. CA could reduce circ_0079593 level and enhance miR-1299 expression in BC. Moreover, circ_0079593 expression inhibition impeded BC cell progression and up-regulated miR-1299. Overexpression of circ_0079593 attenuated the cancer-inhibitory influences of CA on BC cells and sequestered miR-1299 level. It was suggested that CA suppressed cell malignant behaviors through down-regulating the expression of circ_0079593 to induce miR-1299 up-regulation.

In conclusion, CA exhibited anti-tumor regulation in BC cell proliferation, migration and invasion. Additionally, CA function was achieved by circ_0079593/miR-1299 sponging mechanism. circ_0079593/miR-1299 axis could participate in BC development, as a possible therapeutic target for CA in BC. However, this research remains some limitations. The downstream targets for circ_0079593/miR-1299 still need further exploration.

Authors contribution:

Li Yanjiang and Pei Liu have contributed equally to this study.

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Conflict of interests:

The authors declared no conflict of interests.

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