Circular RNA CNOT2 knockdown regulates twist family BHLH transcription factor via targeting microRNA 409-3p to prevent breast cancer invasion, migration and epithelial–mesenchymal transition

QunXue Su*a, Hao Shen*, Bei Gu, and Ning Zhu*

*Department of Pathology, Kunshan Second People’s Hospital, Kunshan City, China; †Department of Breast, Kunshan Second People’s Hospital, Kunshan City, JiangSu Province, 215300, China

ABSTRACT
Numerous studies have manifested that circular RNA (circRNA) is closely associated with the development of breast cancer (BC), but the specific mechanism has not been fully clarified. The purpose of this study was to investigate the effect of circCNOT2 on BC invasion, migration and epithelial mesenchymal transition (EMT) and its potential molecular mechanism. The results assured that circCNOT2 and Twist Family BHLH Transcription Factor (TWIST1) were elevated in BC, while microRNA (miR)-409-3p was reduced. CircCNOT2 was positively correlated with TWIST1 and negatively correlated with miR-409-3p. Elevated circCNOT2 is associated with poor prognosis of BC. Knockdown circCNOT2 or augmented miR-409-3p could promote apoptosis but repress proliferation, invasion, migration and EMT of BC cells. In addition, overexpression of circCNOT2 or TWIST1 accelerated BC invasion, migration and EMT, which could be reversed by simultaneous transfection of miR-409-3p-mimic. Further dual luciferase reporting and RNA-pull down assay clarified that circCNOT2 acted as a competing endogenous RNA of miR-409-3p to mediate TWIST1 expression. In conclusion, the results of this study suggest that circCNOT2 affects the biological behavior of BC via regulating the miR-409-3p/TWIST1 axis, and may be applied as a potential therapeutic target for BC later.

1. Introduction
Breast cancer (BC), the most universal cancer in females, has become a serious public health problem worldwide [1]. Studies have shown that its mortality accounts for 14% of the total among female cancer [2]. In recent years, BC incidence has an year-by-year increase. Since 2008, BC-related mortality has elevated by nearly 18% [3]. Although measures including drugs, radiation therapy and surgery can alleviate BC development, its survival rate is still low with large side effects [4]. Therefore, it is necessary to further probe into the molecular mechanism of BC pathogenesis to seek key targets for effective treatment.

Circular RNA (circRNA) is an endogenous RNA that has been re-recognized in recent years. As high-throughput sequencing and bioinformatics develop, increasing circRNAs have been identified in eukaryotic transcriptome. Via combining with miRNA, it plays a key role in regulating transcription and post-transcriptional gene expression [5]. A great many studies have supported that circRNA affects various diseases, like cardiovascular diseases, cancer and neurological diseases [6,7]. Recently, based on ever-growing studies, circRNA is crucial in treating and diagnosing BC [8,9]. CircCNOT2, a recently identified circRNA, is highly expressed in BC. Its knockdown is available to signally repress BCMCF-7 and BT474 cell viability [10]. But migration, invasion and EMT are vital factors for evaluating cancer metastasis [11]. Whether Circ_CNOT2 takes part in regulating BC migration, invasion and EMT and its specific mechanism has not been ascertained.

Thus, this study was implemented to probe into the changes in BC cell invasion, migration and EMT after silencing circCNOT2 via constructing siRNA that targets circCNOT2. Additionally, DLR and RNA-pull down experiments supported that downstream miRNA and target genes received impacts from circCNOT2 amid BC progression, thus providing a further theoretical basis for diagnosing and treating BC.
2 Methods and materials

2.1. Tissue sample collection

Total 44-pair BC tissue and adjacent normal tissue samples were gained from patients who received treatment from 2012–2014 in Kunshan Second People’s Hospital. Patients’ clinicopathological characteristics were collected for further evaluation. All patients in this study voluntarily signed written informed consent. Additionally, the study has got approval from the Human Research Ethics Committee of Kunshan Second People’s Hospital.

2.2. Cell culture

Human normal breast epithelial cell line (MCF-10A) and two human BC cell lines (MCF-7, SKBR-3) were bought from the Chinese Academy of Sciences (Shanghai, China). All cell lines were given culture in RPMI1640 medium (Gibco, U.S.) with 10% fetal bovine serum (FBS, Invitrogen), 100 IU/mL penicillin and 100 mg/mL streptomycin (Abcam, UK) at 37°C in a humid surrounding containing 5% CO₂.

2.3. Cell transfection

For circCNOT2 knockdown, small interfering RNA (siRNA) targeting circCNOT2 (si-CNOT2) was synthesized with si-negative control (NC) (GenePharma, Shanghai, China) as a NC. Additionally, overexpressed plasmids targeting circCNOT2 and TWIST1 (oe-CNOT2/TWIST1) and elevated plasmid NCs (oe-NC) were bought from GenePharma. Aiming to enrich miR-409-3p, miR-409-3p-mimic and mimic-NC were bought from Ribobio (Guangzhou). Cell transfection was done in BC cells via Lipofectamine 3000 (Invitrogen, U.S.) [12].

2.4. 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyltetrazolium bromide (MTT) determination

In reference to manufacturer’s request, transfected MCF-7 cells (2000 cells/well) proliferation was tested at different times (24, 48 and 72 h) using MTT cell proliferation assay kit (Sangon Biotech, China). Absorbance measurement was done at 490 nm under microplate reader (Thermo Fisher Scientific, Massachusetts, USA).

2.5. Flow cytometry

Via Annexin V Apoptosis Detection Kit (Sangon Biotech) MCF-7 cell apoptosis was studied, that is, these cells were treated with resuspension in 195 µL of binding buffer (4 × 10⁵ cells), later exposed to AV-FITC for 15 minutes, and treated with 10 µL of propidium iodide. At last, apoptotic cells were identified via flow cytometry (BD Bioscience, U.S.).

2.6. Transwell migration assay

The cell migration was detected as set forth [13]. Transwell chamber (Corning, U.S.) was applied in this assay. BC cells were subjected to seeding into upper chambers with serum-free Dulbecco’s Modified Eagle Medium (DMEM). Meanwhile, DMEM containing 20% FBS was added to lower chambers. After 24 hours, cell removal in upper chambers was done with cotton swabs, while cells in surface of lower chambers were subjected to fixation with 4% paraformaldehyde (PFA) to stain with 0.1% crystal violet for 15 minutes. After wash twice with PBS, the number of cells in five selected areas by random under microscopes was counted (Olympus, Japan).

2.7. Transwell invasion assay

Transwell upper chambers (Corning) coated with diluted Matrigel (BD Bioscience) and serum-free DMEM, were employed to seed MCF-7 cells, and later were treated with incubation in DMEM wells containing 10% FBS. After 24-hour incubation at 37°C, cells in inner chambers and remaining Matrigel were subjected to removal with cotton swabs, whereas cells on surface of lower chamber outsides were treated with fixation with 4% PFA to stain with 0.1% crystal violet for 15 minutes. After wash twice with PBS, the number of cells in five selected areas by random was counted under microscopes.
2.8. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

TriQuick Reagent (Solarbio, China) was applied to extract total RNA from BC tissue samples and cells. Via random primers (TaKaRa, China) for reverse transcription of circCNOT2 and TWIST1, and miRNA 1st cDNA synthesis kit (Vazyme, Nanjing, China) for that of miR-409-3p, qPCR was done on ABI 7500 RealTime PCR system (Applied Biosystems, U.S.) via SYBR Premix Ex Taq II (TaKaRa). Relative circRNA, mRNA and miRNA expression levels were subjected to calculation in reference to 2^−ΔΔCT. U6 was applied as the internal reference for miRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for mRNA. Each experiment was repeated three times, primer sequences detailed in Table 1.

2.9. Western blot

In short, Radio-Immunoprecipitation assay buffer (Solarbio) was employed to do total protein extraction from cells or tissues. Concentration of protein sample was evaluated via Pierce bicinchoninic acid protein detection kit (Thermo Scientific, U.S.). In equal amount, 30 μg protein sample separation was done via sulfate polyacrylamide gel electrophoresis to transfer to Polyvinylidene fluoride films that were later subjected to block in skim milk at room temperature for 2 h and incubated with primary antibodies: GAPDH (sc-32,233, 1:1000, Santa Cruz Biotechnology), E-cadherin (20,874-1-AP, 1:1000, Proteintech), N-cadherin (13,116, 1:1000, Cell Signaling Technology), Vimentin (5741, 1:1000: Cell Signaling Technology), Snail (ab53519, 1:1000, Abcam), TWIST1 (sc-81,417, 1:1000, Santa Cruz Biotechnology) overnight at 4°C. After the films were treated with incubation with goat anti-rabbit Immunoglobulin G-horseradish peroxidase secondary antibody (Abcam) at 37°C for 2 hours, protein signal visualization was done via eyoECL plus kit (Beyotime, China).

2.10. Double luciferase reporter (DLR) experiment

Through online database starbase (http://starbase.sysu.edu.cn/) for bioinformatics analysis, predicting the interaction was between miR-409-3p and circCNOT2 or TWIST1 to analyze their binding sites; Based on predicted sequence-WT of binding sites, sequence-MUT of circCNOT2 and TWIST1 mutating at binding sites were designed. Later, the two kinds of sequences were treated with amplification and clone into PGL4 reporter plasmids (Promega, U.S.), that is, circCNOT2-WT, circCNOT2-MUT, TWIST1-WT and TWIST1-MUT. Quickly, separated fusion plasmids and miR-409-3p-mimic or mimic-NC were treated with co-transfection into MCF-7 cells. After 48 hours, luciferase activity detection was done on dual luciferase assay system (Promega).

2.11. RNA-pull down

MiR-409-3p and mimic-NC labeled with biotin from Ribobio were named Biotin-miR-409-3p and Biotin-mimic-NC, respectively, and were treated with transfection into MCF-7 cells and 48-hour incubation, after which the cells were placed in lysis buffer. Pierce Magnetic RNA Protein Pulldown Kit (Thermo Fisher Scientific) was later employed for determination. RNA abundance was checked via RT-qPCR analysis.

2.12. Statistics

The data were expressed as mean ± standard deviation. The Student’s t test was applied to determine the statistical significance of the difference between the two groups, while the χ2 test to assess the correlation between circCNOT2 levels and clinical features. Prism Software 8.0 (GraphPad Software, USA) was employed to analyze and display the data. *P < 0.05 was considered

| Table 1. RT-qPCR primer sequences. |
|------------------------------------|
| Primer sequences (5′ – 3′)          |
| GAPDH                             |
| Forward: 5′-ATGGGGAAGTGAAAGTTCG-3′ |
| Reverse: 5′-TTACTCCTTGAGCAATGCTG-3′ |
| U6                                |
| Forward: 5′-CTGCGTTCCAGCAGCATATCT-3′ |
| Reverse: 5′-ACGCTTCAGAAATTTGCGTC-3′ |
| CircCNOT2                         |
| Forward: 5′-TCCACATCGTCAGAAAAAGAT-3′ | Reverse: 5′-GAGGAATCTAAATGAAACTGCTACT-3′ |
| miR-409-3p                        |
| Forward: 5′-GAATGTGCTGCTGTTGA-3′ | Reverse: 5′-GTGACGGTGTCGCAGCTG-3′ |
| TWIST1                            |
| Forward: 5′-GCCAGTATACAGCACTCTCTC-3′ | Reverse: 5′-TCCATCTCCAGACCGAAGG-3′ |
to be a statistically significant difference. At least three biological replicates were performed for all the results of this study, and three technical replicates for each biological sample.

3 Results

3.1. CircCNOT2 is up-regulated in BC

To explore the biological function of circCNOT2 in BC, CNOT2 expression was checked in BC. Vs. adjacent normal tissues, CNOT2 was signally up-regulated in BC tissues, which is consistent with previous findings (Figure 1a). For further confirmation, CNOT2 expression was analyzed in MCF-7 and SKBR-3 and MCF-10A. In the latter, CNOT2 level was signally higher (Figure 1b). In reference to the median of CNOT2 expression (3.82times, fold change to normal tissue), the patients were allocated into high expression group and low expression group, and correlation analysis of clinicopathological characteristics was done, finding that CNOT2 was highly correlated with TNM staging and tumor size (Table 2). Additionally, Kaplan–Meier survival curve of BC patients revealed that BC patients’ survival prognosis with high CNOT2 expression was visually worse than those with low (Figure 1c). These findings indicate that circCNOT2 expression elevates in BC, which is related to poor survival prognosis.

3.2. Knocking down circCNOT2 effectively prevents BC invasion, migration and EMT

Aiming to probe into how CNOT2 biologically functions in BC, MCF-7 cells was selected with the highest expression level for subsequent experiments. After transfecting si-CNOT2, CNOT2 level in MCF-7 was obviously reduced (Figure 2a). Later, the impacts of knocking down CNOT2 was examined on proliferation, apoptosis, migration, invasion and EMT in MCF-7 cells, finding that this knockdown effectively inhibited proliferation (Figure 2b). Flow cytometry revealed that such knockdown observably promoted apoptosis (Figure 2c). Additionally, Transwell assay implied that this knockdown reduced invasion and migration ability (Figure 2d). Based on western blot findings, knocking down CNOT2 also increased E-cadherin expression but inhibited N-cadherin, Vimentin and snail expressions (Figure 2e). These findings indicate that circCNOT2 knockdown effectively prevents BC from deterioration.

3.3. CircCNOT2 competitively bind to miR-409-3p

CircRNA usually functions in cancer as a miRNA sponge [14]. Next, the downstream miRNA binding to circCNOT2 was exploited. A miRNA was noticed namely miR-409-3p, has proved to be under-

![Figure 1](image-url)
Table 2. Correlation analysis of circCNOT2 and clinicopathological characteristics of BC patients.

| Characteristics | Groups | Cases | High expression group (n = 22) | Low expression group (n = 22) | P     |
|-----------------|--------|-------|-------------------------------|-------------------------------|-------|
| Age             | < 60   | 17    | 10 (45%)                      | 7 (31%)                       | 0.3530|
|                 | ≥ 60 or more | 27    | 12 (55%)                      | 15 (69%)                      |       |
| Gender          | Male   | 21    | 9 (40%)                       | 12 (55%)                      | 0.3652|
|                 | Female | 23    | 13 (60%)                      | 10 (45%)                      |       |
| Tumor size      | < 5 cm | 19    | 3 (13%)                       | 16 (72%)                      | <0.0001|
|                 | ≥ 5 cm or more | 25    | 19 (87%)                      | 6 (28%)                       |       |
| Histological grade | Moderate | 30    | 14 (63%)                      | 16 (72%)                      | 0.5174|
|                 | Low    | 14    | 8 (37%)                       | 6 (28%)                       |       |
|                 | I + II | 13    | 3 (13%)                       | 10 (45%)                      | 0.0207|
|                 | III + IV | 31    | 19 (87%)                      | 12 (55%)                      |       |

Expressed in BC and low-level miR-409-3p links with the poor prognosis of BC [15]. First check of its expression level was in BC. MiR-409-3p expression was significantly reduced in BC patients (Figure 3a). Additionally, the same findings existed in cell experiments. MiR-409-3p level in two BC cell lines was visually lower than that of MCF-10A cells (Figure 3b). Later, whether miR-409-3p is regulated by circCNOT2 was checked in BC. After knocking down or overexpressing circCNOT2, miR-409-3p level in MCF-7 cells was significantly elevated or lowered, respectively (Figure 3c). Consequently, it was speculated that miR-409-3p maybe target circCNOT2. Aiming to prove this conjecture, it was predicted that circCNOT2 and miR-409-3p have specific binding sites through bioinformatics website http://starbase.sysu.edu.cn/ (Figure 3d). DLR experiment showed that CNOT2-WT significantly reduced dual-luciferase activity of miR-409-3p-mimic group, whereas CNOT2-MUT had no obvious impact on it (Figure 3e). Additionally, RNA-pull down experiment revealed that biotinylated miR-409-3p successfully captured circCNOT2, indicating that miR-409-3p and circCNOT2 interacted with each other (figure 3f). What’s more, the correlation analysis on miR-409-3p expression and circCNOT2 revealed that they had a negative correlation (Figure 3g). These findings indicate that circCNOT2 competitively binds to miR-409-3p in BC.

3.4. Overexpressing miR-409-3p inhibits BC invasion, migration and EMT

Next, it was probed into how miR-409-3p biologically acts in BC. By transfecting miR-409-3p-mimic,
up-regulated miR-409-3p was in MCF-7 cells (Figure 4a) to checked its effects on MCF-7 cell proliferation, apoptosis, invasion, migration, and EMT. Overexpressing miR-409-3p signally inhibited proliferation and elevated apoptosis rate (Figure 4b and 4c). Transwell implied that overexpressing miR-409-3p effectively reduced invasion and migration (Figure 4d). Additionally, RT-qPCR and western blot findings supported that overexpressing miR-409-3p elevated E-cadherin expressions in MCF-7 cells but inhibited N-cadherin, Vimentin and snail expression (Figure 4e). These findings indicate that miR-409-3p as a tumor suppressor gene in BC effectively inhibits BC cell invasion, migration and EMT.

### 3.5. **TWIST1 is a target gene of miR-409-3p**

TWIST1 has proved to be up-regulated in BC, which links with BC EMT [16]. So it was checked whether it is a target gene of miR-409-3p. In BC patients’ cells, TWIST1 expression was visually elevated, which is consistent with previous findings (Figure 5a and 5b) [17]. Additionally, it was found that in MCF-7 cells, up-regulated miR-409-3p effectively inhibited TWIST1 expression (Figure 5c). Consequently, it was speculated that TWIST1 may be a potential target gene of miR-409-3p. Aiming to verify this conjecture, a prediction through bioinformatics website http://starbase.sysu.edu.cn/ was that TWIST1 and miR-409-3p had specific binding sites (Figure 5d). To further support our conjecture, DLR and RNA-pull down experiments were done, finding that TWIST1-WT effectively repressed the dual luciferase activity of miR-409-3p-mimic group. Additionally, biotinylated miR-409-3p-mimic successfully captured TWIST1 (Figure 5e and 5f). In correlation analysis, patient’s TWIST1 and miR-409-3p levels were negatively correlated (Figure 5g). These data indicate that TWIST1 may be a potential target gene of miR-409-3p.
3.6. Up-regulated TWIST1 promotes BC cell invasion, migration and EMT

Later, overexpressed TWIST1 was in MCF-7 cells (Figure 6a) to examine its impact on BC cell proliferation, apoptosis, invasion and migration and EMT. After up-regulating TWIST1, MCF-7 cell proliferation was signally elevated but its apoptosis rate was visually reduced (Figure 6b and 6c). Additionally, Transwell findings revealed that up-regulated TWIST1 signally promoted MCF-7 cell invasion and migration (Figure 6d). Based on RT-qPCR and western blot, up-regulated TWIST1 reduced E-cadherin expression in MCF-7 cells but elevated N-cadherin, Vimentin and snail expression (Figure 6e). This indicates that up-regulated TWIST1 promotes BC cell proliferation, invasion, migration and EMT, thus inhibiting apoptosis.

3.7. MiR-409-3p/TWIST1 axis is involved in circCNOT2’s regulating on BC

Finally, it was probed into whether miR-409-3p/TWIST1 takes part in the process of circCNOT2 regulating on BC. In BC patients, TWIST1 level and circCNOT2 expression were positively correlated (Figure 7a). Later, miR-409-3p was overexpressed while overexpressing circCNOT2, finding that the latter signally increased circCNOT2 and TWIST1 expression, whereas TWIST1 level was signally reversed after miR-409-3p-mimic treated with simutaneous transfection (Figure 7b). Subsequently, it was examined what impacts the co-transfection imposed on BC biological functions. Overexpressed circCNOT2 visually elevated the of MCF-7 cell proliferation but inhibited its apoptosis, whereas promoted its invasion and migration capabilities, reducing E-cadherin expression and promoting N-cadherin, Vimentin and snail expression (Figure 7c-f). Simultaneous transfection of miR-409-3p-mimic reversed the impact posed by overexpressing circCNOT2 on MCF-7 cell proliferation, apoptosis, invasion, migration and EMT. This indicates that circCNOT2 affects BC proliferation, apoptosis, invasion and migration and EMT via regulating miR-409-3p/TWIST1 axis.
4. Discussion

BC, among the most common cancers in women, is very aggressive with high mortality [18]. A great many studies have revealed that circRNA is involved in regulating post-transcriptional expression in human body, playing an essential role in cancer occurrence and development [19]. In this study, it was found that circCNOT2 affects BC cell proliferation, migration, invasion and EMT via regulating miR-409-3p/TWIST1 axis.

Based on previous studies, with high stability in tissues and blood, circRNA usually acts as a potential biomarker for cancer prognosis and diagnosis [20]. In this study, it was demonstrated that circCNOT2 expression was up-regulated in BC tissues and was extremely correlated with clinicopathologic features and poor postoperative

Figure 5. TWIST1 is a target gene of miR-409-3p. A: RT-qPCR to detect TWIST1 expression in BC tissue samples and adjacent normal tissues; B: RT-qPCR and western blot to detect TWIST1 expression in MCF-10A, MCF-7 and SKBR-3; C: RT-qPCR and western blot to detect how up-regulated miR-409-3p impacted TWIST1; D: Via http://starbase.sysu.edu.cn/ to predict the binding sites of TWIST1 and miR-409-3p; E-F: DLR and RNA-pull down experiments to detect the targeting relationship between TWIST1 and miR-409-3p, respectively; G: Correlation analysis on TWIST1 and miR-409-3p expressions. Values presentation was detailed in mean ± SD (n = 3). Via one-way ANOVA to calculate the significance of each group, the variance correction was done via Tukey’s test. *P < 0.05.
prognosis of BC. However, its expression in the serum of patients remains unclear. It is necessary to detect circCNOT2 expression in the serum of patients in subsequent experiments, which is crucial to determine whether circCNOT2 can be applied as an early biomarker for BC diagnosis. Recent studies have reported that various circRNAs are abnormally expressed in BC and participate in BC development. For example, Liu C et al. found 520 circRNAs that are abnormally expressed in BC, among which hsa_circ_0043278 expression is relative to patient’s lymph node metastasis and histological type. This study manifested that circCNOT2 is largely correlated with patient’s TNM staging and tumor size. Additionally, high-level circCNOT2 has a worse prognosis, which is consistent with previous findings. Smid M et al. also clarified that inhibiting CNOT2 is available to attenuate BC cell proliferation. Wang F et al. reported that high-level circNOL10 inhibits BC cell proliferation, migration and invasion in vitro [21]. This supports that high- or low-level circRNA can affect BC development. In this study, it was observed that silencing circCNOT2 inhibited BC proliferation via regulating the miR-409-3p/TWIST1 axis. Additionally, it was also noticed that BC cell migration, invasion and EMT process are also under regulation by circCNOT2. This suggests that circCNOT2 is a key oncogenic element in BC. It is worth noting that the biological function and molecular mechanism of circCNOT2 were only explored.
in vitro experiments in this study, and the effect of knockdown circCNOT2 on BC tumors in vivo is still unclear, which needs to be explored in subsequent studies. In addition, only MCF-7 cells were applied in this study, and the role of circCNOT2 in other BC cell lines may be different. This is a limitation of this study.

TWIST1 is a basic helix-loop-helix transcription factor encoded by the TWIST1 gene. It has proved that this factor is directly related to the pathogenesis of various diseases, like neurocognitive defects [22], intervertebral discs degeneration [23], osteoarthritis [24] and cancer [25,26]. Importantly, TWIST1 is usually seemed as a transcription factor of cancer EMT, playing an essential role in cancer metastasis [27]. Previous reports have revealed that the interaction between TWIST1 and various miRNAs take part in regulating cancer, like miR-16, miR-26b-5p, miR-1271, miR-539, miR-214, miR-200b/c, miR-335, miR-10b and miR-381, while TP73-AS1, LINC01638, ATB, NONHSAT101069, CASC15, H19, PVT1, LINC00339, LINC01385, TANAR, SNHG5, DANC, CHRF and TUG1 are positively regulating TWIST1 and affects long non-coding RNA in carcinoma development [28]. This study testified that overexpressing TWIST1 promotes BC cell proliferation, migration, invasion and EMT, which is consistent with previous reports. Additionally, a new circRNA/miRNA axis (circCNOT2/miR-409-3p) was found to target TWIST1 expression in BC. This will provide new insights into TWIST1’s involvement in cancer pathogenesis.

Figure 7. MiR-409-3p/TWIST1 axis is involved in the process of circCNOT2 regulating on BC. A: Correlation analysis on TWIST1 and circCNOT2 expressions in patients’ tissues; B: RT-qPCR and western blot to detect how co-transfection impacted circCNOT2 and TWIST1 expressions; C: MTT to detect the influence of co-transfection on MCF-7 cell proliferation; D: Flow cytometry to detect how co-transfection affected MCF-7 cell apoptosis; E: Transwell to detect the impact of co-transfection on MCF-7 cell invasion and migration; F: Western blot to detect how co-transfection impacted E-cadherin, N-cadherin, Vimentin and snail protein expression in MCF-7 cells. Values presentation was detailed in mean ± SD (n = 3). Via one-way ANOVA to calculate the significance of each group, the variance correction was done via Tukey’s test. *P < 0.05.
5. Conclusion

To conclude, it was found that circCNOT2 and TWIST1 are highly expressed in BC, while miR-409-3p is under-expressed. BC patients with high-level circCNOT2 have worse prognosis. It was demonstrated for the first time that silencing circCNOT2 inhibits the of BC cell growth, migration, invasion and EMT via regulating the miR-409-3p/TWIST1 axis, thus providing a better understanding of BC pathogenesis and a possible target for treating BC.

Disclosure Statement

No potential conflict of interest was reported by the author(s).

References

[1] Tao Z, Shi A, Lu C, et al. Breast cancer: epidemiology and etiology. Cell Biochem Biophys. 2015;72 (2):333–338.

[2] Henley SJ, Ward EM, Scott S, et al. Annual report to the nation on the status of cancer, part I: national cancer statistics. Cancer. 2020;126(10):2225–2249.

[3] Ghoncheh M, Pournamdar Z, Salehiniya H, et al. Incidence and mortality and epidemiology of breast cancer in the world. Asian Pac J Cancer Prev. 2016;17 (sup3):43–46.

[4] Carbine NE, Lostumbo L, Wallace J, et al. Risk-reducing mastectomy for the prevention of primary breast cancer. Cochrane Database Syst Rev. 2018;4: Cd002748.

[5] Xuan L, Qu L, Zhou H, et al. Circular RNA: a novel biomarker for progressive laryngeal cancer. Am J Transl Res. 2016;8:932–939.

[6] Lukiw WJ. Circular RNA (circRNA) in Alzheimer’s disease (AD). Front Genet. 2013;4:307.

[7] Guarnerio J, Bezzi M, Jeong JC, et al. Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations. Cell. 2016;165:289–302.

[8] Liu C, Han T, Shi Y, et al. The decreased expression of hsa_circ_0043278 and its relationship with clinicopathological features of breast cancer. Gland Surg. 2020;9(6):2044–2053.

[9] Wang X, Ji C, Hu J, et al. Hsa_circ_0005273 facilitates breast cancer tumorigenesis by regulating YAP1-hippo signaling pathway. J Exp Clin Cancer Res. 2021;40 (1):29.

[10] Smid M, Sm W, Uhr K, et al. The circular RNA of primary breast cancer. Genome Res. 2019;29:356–366.

[11] Wang X, Ren Y, Ma S, et al. Circular RNA 0060745, a novel circRNA, promotes colorectal cancer cell proliferation and metastasis through miR-4736 sponging. Onco Targets Ther. 2020;13:1941–1951.

[12] Huang X, Shen X, Peng L, et al. CircCNSN1G1 contributes to the development of colorectal cancer by increasing the expression of MYO6 via competitively targeting miR-455-3p. Cancer Manag Res. 2020;12:9563–9575.

[13] Du J, Xu J, Chen J, et al. circRAE1 promotes colorectal cancer cell migration and invasion by modulating miR-338-3p/TYRO3 axis. Cancer Cell Int. 2020;20 (1):430.

[14] Su Y, Lv X, Yin W, et al. CircRNA Cdr1as functions as a competitive endogenous RNA to promote hepatocellular carcinoma progression. Aging (Albany NY). 2019;11:8183–8203.

[15] Cao GH, Sun XL, Wu F, et al. Low expression of miR-409-3p is a prognostic marker for breast cancer. Eur Rev Med Pharmacol Sci. 2016;20:3825–3829.

[16] Xu Y, Qin L, Sun T, et al. Twist1 promotes breast cancer invasion and metastasis by silencing Foxa1 expression. Oncogene. 2017;36:1157–1166.

[17] Cai YJ, Ma B, Wang ML, et al. Impact of nischarin on EMT regulators in breast cancer cell lines. Oncol Lett. 2020;20:291.

[18] Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.

[19] Cheng D, Wang J, Dong Z, et al. Cancer-related circular RNA: diverse biological functions. Cancer Cell Int. 2021;21(1):11.

[20] Li Y, Zheng Q, Bao C, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. Cell Res. 2015;25(8):981–984.

[21] Wang F, Wang X, Li J, et al. CircNOL10 suppresses breast cancer progression by sponging miR-767-5p to regulate SOCS2/JAK/STAT signaling. J Biomed Sci. 2021;28(1):4.

[22] Yu M, Ma L, Yuan Y, et al. Cranial suture regeneration mitigates skull and neurocognitive defects in Craniosynostosis. Cell. 2021;184:243–256. e218.

[23] Qiu HB, Bian WG, Zhang LJ, et al. Inhibition of p53/p21 by TWIST alleviates TNF-α induced nucleus pulposus cell senescence in vitro. Eur Rev Med Pharmacol Sci. 2020;24:12645–12654.

[24] Tu J, Huang W, Zhang W, et al. TWIST1-MicroRNA -10a-MAP3K7 axis ameliorates synovitis of osteoarthritis in fibroblast-like synoviocytes. Mol Ther Nucleic Acids. 2020;22:1107–1120.

[25] Jao TM, Fang WH, Ciou SC, et al. PCDH10 exerts tumor-suppressor functions through modulation of EGFR/akt axis in colorectal cancer. Cancer Lett. 2021;499:290–300.

[26] Tang Q, Li W, Zheng X, et al. MEKL is an oncogenic kinase essential for metastasis, mitotic progression, and programmed death in lung carcinoma. Signal Transduct Target Ther. 2020;5(1):279.
[27] Liu T, Zhao X, Zheng X, et al. The EMT transcription factor, twist1, as a novel therapeutic target for pulmonary sarcomatoid carcinomas. Int J Oncol. 2020;56:750–760.

[28] Ghafouri-Fard S, Abak A, Bahroudi Z, et al. The interplay between non-coding RNAs and twist1 signaling contribute to human disorders. Biomed Pharmacother. 2021;135:111220.