Circ_0041732 regulates tumor properties of triple-negative breast cancer cells by the miR-149-5p/FGF5 pathway

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Abstract

Background: Triple-negative breast cancer (TNBC) is a subtype of breast cancers with a high recurrence and mortality. The important factors promoting the TNBC process have not been fully identified. In this research, the role of a TNBC-related circular RNA (circRNA), circ_0041732, was revealed in TNBC cell tumor properties.

Methods: The expression levels of circ_0041732, microRNA-149-5p (miR-149-5p) and fibroblast growth factor 5 (FGF5) were detected by quantitative real-time polymerase chain reaction. The protein expression was determined by Western blot analysis or immunohistochemistry assay. Cell proliferation was detected by cell counting kit-8 and cell colony formation assays. Cell apoptosis was analyzed by flow cytometry and caspase-3 activity assays. Cell migration and invasion were evaluated by wound-healing and transwell invasion assays. Cell angiogenic capacity was investigated by a tube formation assay. The targeting relationship between miR-149-5p and circ_0041732 or FGF5 was identified by dual-luciferase reporter and RNA immunoprecipitation assays. The impacts of circ_0041732 knockdown on tumor formation were determined by an in vivo assay.

Results: Circ_0041732 and FGF5 expression were significantly upregulated, whereas miR-149-5p was downregulated in TNBC tissues and cells compared with normal breast tissues and cells, respectively. Circ_0041732 silencing inhibited TNBC cell proliferation, migration, invasion, and tube formation, but induced apoptosis. Additionally, circ_0041732 regulated TNBC cell tumor properties by binding to miR-149-5p. MiR-149-5p also modulated TNBC cell tumor properties by targeting FGF5. Furthermore, circ_0041732 knockdown hindered tumor formation in vivo.

Conclusion: Circ_0041732 silencing suppressed TNBC cell tumor properties by decreasing FGF5 expression through miR-149-5p. This finding demonstrated that circ_0041732 had the potential as a therapeutic target for TNBC.

Keywords
TNBC, circ_0041732, miR-149-5p, FGF5

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Introduction

As one of the most common carcinomas, breast cancer is the second major cause of cancer-related deaths in women worldwide.1 Triple-negative breast cancer (TNBC), the most pernicious subtype of breast carcinomas, accounts for more than 10% of total clinical breast cancer cases. TNBC is resistant to conventional chemotherapy owing to the lack of three receptors: estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2.2,3 Despite the outstanding progress in improving breast cancer treatment, the prognosis of TNBC patients remains poor. Thus, an in-depth understanding of the molecular mechanism behind TNBC progression is necessary to identify more molecules for the therapy of TNBC.

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Circular RNA (circRNA) is an endogenous non-coding RNA that is more stable than linear RNA. An abundance of circRNAs have been discovered through microarray and high-throughput sequencing, and their roles and functions have been revealed in various human tissues. An increasing number of reports indicate that circRNAs play crucial parts in disease development via serving as the sponges of miRNAs. For example, circ_100395 upregulation inhibited cell proliferative and metastatic capacities via binding to miR-1228 in lung cancer. Bi et al. indicated that circ_0000799 absence hindered cell metastasis by interacting with miR-31-5p in bladder cancer. Additionally, circ_0000745 promoted cell growth in cervical cancer via binding to miR-1228 in lung cancer. Bi et al. indicated that circ_0000799 absence hindered cell metastasis by interacting with miR-31-5p in bladder cancer. Therefore, circ_0000745 and circ_0000799 were involved in breast cancer progression, such as circ_001783, circ_000167, and circ_0072309. In preliminary experiments, we found the high expression of circ_0041732—a novel circRNA—in TNBC tissues and cells; however, there were no data about the function of the circRNA in the TNBC process.

miRNA is a small 20-nucleotide RNA that mainly acts as a post-transcriptional regulator. Numerous studies indicate that miRNAs are involved in cell carcinogenesis in various cancers, including breast cancer. Considerable studies have confirmed that miRNA-149-5p (miR-149-5p)—a cancer-related miRNA—represses the progression of medullary thyroid carcinoma, gastric cancer, lung carcinoma, and cervical cancer. However, there are few data about the mechanism behind miR-149-5p regulating TNBC development. Fibroblast growth factor-5 (FGF5) belongs to the FGF family that plays vital roles in the transduction of signals, embryonic development, tissue maintenance, and wound healing. Researchers have found that breast cancer patients with low FGF5 expression have better clinical outcomes, suggesting the importance of FGF5 in breast cancer progression. Based on the bioinformatics predictions, we found that circ_0041732 possessed the binding sequence of miR-149-5p, and miR-149-5p carried the binding sites of FGF5. Thus, whether circ_0041732-mediated TNBC development involved miR-149-5p/FGF5 needed to be explored.

Herein, the roles of circ_0041732 in TNBC cell proliferation, migration, invasion, and apoptosis were analyzed. Whether TNBC-associated properties involved the circ_0041732/miR-149-5p/FGF5 axis was determined. Additionally, the impact of circ_0041732 absence on tumor growth in vivo were investigated.

**Materials and methods**

**Specimen collection and storage**

In accordance with the Helsinki Declaration, TNBC tissues (N = 57) and paracancerous normal breast tissues (N = 57) were collected from TNBC patients from Lianshui People’s Hospital. The TNBC patients signed the written informed consent. The Ethics Committee of Lianshui People’s Hospital approved this study. All obtained tissues were kept at ~8°C.

**Cell culture**

Human breast cancer cell lines (BT-549 and MDA-MB-231), human normal breast epithelial cell line (MCF-10A), and human umbilical vein endothelial cell line (HUVEC) were purchased from American Type Culture Collection (Manassas, VA, USA) within a month prior to investigation. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Procell, Wuhan, China), Dulbecco’s modified Eagle’s medium (DMEM; Procell) or EGM-2 (Lonza, Basel, Switzerland). Media were mixed with 10% fetal bovine serum (FBS; Procell) and 1% penicillin/streptomycin (Procell). These cells were grown at 37°C in a humid incubator with 5% CO2.

**Cell transfection**

The small interfering RNAs (si-RNAs) and short hairpin RNA (sh-RNA) against circ_0041732 (si-circ_0041732#1, si-circ_0041732#2, and sh-circ_0041732), the mimics of miR-149-5p (miR-149-5p), the inhibitors of miR-149-5p (anti-miR-149-5p), and the respective control groups (si-NC, sh-NC, NC, and anti-NC) were synthesized by GenePharma (Shanghai, China). The overexpression plasmids of circ_0041732 (circ_0041732) and FGF5, and control groups (circ-NC and vector) were provided by Geneseed (Guangzhou, China). Cell transfection was carried out based on the manufacturer’s instruction of TurboFect reagent (Thermo Fisher, Waltham, MA, USA). The synthesized sequences are listed below. The small interfering RNAs (si-RNAs) and short hairpin RNA (sh-RNA) against circ_0041732 (si-circ_0041732#1, si-circ_0041732#2, and sh-circ_0041732), the mimics of miR-149-5p (miR-149-5p), the inhibitors of miR-149-5p (anti-miR-149-5p), and the respective control groups (si-NC, sh-NC, NC, and anti-NC) were synthesized by GenePharma (Shanghai, China). The overexpression plasmids of circ_0041732 (circ_0041732) and FGF5, and control groups (circ-NC and vector) were provided by Geneseed (Guangzhou, China). Cell transfection was carried out based on the manufacturer’s instruction of TurboFect reagent (Thermo Fisher, Waltham, MA, USA). The synthesized sequences are listed below.

**Quantitative real-time polymerase chain reaction**

For quantitation analysis of circ_0041732, miR-149-5p and FGF5, quantitative real-time polymerase chain reaction (qRT-PCR) was performed. In brief, the RNA from tissues and cells was first isolated with an RNAsimple kit (Tiangen, Beijing, China). Complimentary DNA was synthesized with a FastKing RT Kit (Tiangen) or MicroRNA Reverse Transcription Kit (Thermo Fisher).
Then, the SuperReal PreMix Color kit (Tiangen) was used to detect gene expression in an IQ5 thermocycler (Bio-Rad, Hercules, CA, USA). Finally, the obtained data were assessed by the 2ΔΔCt method with β-Actin or U6 as a control gene reference. The sense and antisense sequences of primers are listed below. Circ_0041732 5′-ACATGCCCAACCCATTACG-3′ and 5′-TGCCCTTCTGTCCTCTC-3′; PICALM interacting mitotic regulator (FAM64A) 5′-CTTGGACCTGAGCCTTCTAT-3′ and 5′-TGAAGTGAAGCTGAGAGTCA-3′; miR-149-5p 5′-AGGTCCTAAGTGATTGCTCC-3′ and 5′-TTTCTGAAGTGTGGAGCTCC-3′; 18S ribosomal RNA (rRNA) 5′-GATGGTAGTCGCCGTGCC-3′ and 5′-GGCTTGCCTTCATTTCTC-3′; U6 5′-CCTGCTTCGTGGTCTTTCG-3′ and 5′-AACGCTTCACGAATTTGCGT-3′; PICALM-CT and 5′-AGTTCTTTGCGGATGTCCACGT-3′.

Cytoplasmic and nuclear RNA analysis

Cell nuclei were first separated from the cytoplasm using a PARiSTM Kit (Thermo Fisher). Briefly, the cultivated cells were harvested and placed on ice. Ice-cold cells were suspended in Cell Fractionation Buffer (Thermo Fisher) for 6 min. The cytoplasmic fraction was aspirated away from the nuclear fraction, and then lysed using Cell Disruption Buffer (Thermo Fisher) and Lysis Solution (Thermo Fisher). Finally, qRT-PCR was applied to quantify circ_0041732 expression. 18S rRNA and U6 served as control gene references.

RNase R resistance analysis of circRNA

The isolated RNA was incubated with RNase R (RNase R +; 3 U/μg RNA; Geneseeed) at 37°C for 20 min. RNA was purified with an RNaseasy cleaning kit (Qiagen, Valencia, CA, USA). Finally, circ_0041732 expression was determined by qRT-PCR with FAM64A mRNA as a control gene reference. The cells not incubated with RNase R (RNase R-) were employed as a negative control.

Cell counting kit-8 assay

After growing in 96-well plates for 14 h, BT-549 and MDA-MB-231 cells were transfected with si-circ_0041732#1, si-circ_0041732#2, si-NC, anti-miR-149-5p, anti-NC, miR-149-5p, NC, FGF5 or vector according to the defined purposes. The cells were cultured for 24, 48, or 72 h, respectively. Cell supernatant was removed, and then cell counting kit-8 (CCK-8) solution (Beyotime, Shanghai, China) was added to each well. After 2 h of incubation, cell viability was determined by a Varioskan LUX Multimode microplate reader (Thermo Fisher).

Cell colony formation assay

BT-549 and MDA-MB-231 cells mixed with RPMI-1640 (Procell) and DMEM (Procell) were cultured in 6-well plates for 10–14 days. The supernatant was discarded, and the cells were incubated with paraformaldehyde (Sigma, St. Louis, MO, USA) and crystal violet (Sigma), respectively. Finally, cell colony-forming ability was assessed by determining the number of colonies containing 50 or more cells.

Flow cytometry analysis

The cell apoptotic rate was assessed by an Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) double staining kit (Solarbio, Beijing, China). In brief, 1 × 10^5 cells for each experiment were collected and suspended in a binding buffer (Solarbio). After that, Annexin V-FITC (Solarbio) and PI (Solarbio) were used to incubate the cells in the dark. Finally, Attune NxT flow cytometer (Thermo Fisher) was used to analyze the samples.

Caspase-3 activity assay

Caspase-3 activity was detected with a caspase-3 activity assay kit (Beyotime). Briefly, BT-549 and MDA-MB-231 cells were collected after digestion using trypsin (Thermo Fisher). Single cells were obtained with a lysis buffer (Beyotime) on ice, and cell supernatant obtained by centrifugation was then incubated with acetyl-Asp-Glu-Val-Asp p-nitroanilide (Beyotime). Finally, caspase-3 activity was detected using a Varioskan LUX Multimode microplate reader (Thermo Fisher).

Wound-healing assay

The migratory ability of TNBC cells was determined by a wound-healing assay. Briefly, cells with different transfections were cultured in six-well plates at 37°C. Cells were detached by scratching when the confluence of cells reached about 100%. The cells were washed and cultured in FBS-free RPMI-1640 (Procell) or DMEM (Procell). After 24 h, results were determined by assessing the area occupied by the migrated cells under a CKX53 inverted microscope (Olympus, Tokyo, Japan) with 40× magnification.

Transwell invasion assay

Transwell chambers were initially coated with Matrigel (Corning, Madison, NY, USA). The cells diluted in FBS-free RPMI-1640 (Procell) or DMEM (Procell) were drop-wise added into the upper chambers. RPMI-1640 (Procell) and DMEM (Procell) containing 15% FBS (Procell) were added into the lower chambers. After 24 h, media were
removed and the cells were incubated with 4% paraformaldehyde (Sigma) and 0.1% crystal violet (Sigma), respectively. Finally, cell invasive capacity was analyzed by calculating the number of cells in the lower chambers under a CKX53 inverted microscope (Olympus) at a 100× magnification.

**Western blot analysis**

Tissues and cells were lysed with NP-40 buffer (Beyotime), and the lysates were mixed with loading buffer (Thermo Fisher). The separated protein bands were electro transferred onto nitrocellulose membranes (Membrane Solutions, Shanghai, China). The membranes were blocked using 5% non-fat dry milk (Solarbio) for 2 h. After that, the membranes were incubated with anti-matrix metallopeptidase 2 (anti-MMP2) (1:3000; Abcam, Cambridge, UK), anti-MMP9 (1:8000; Abcam), anti-FGF5 (1:1000; Affinity, Nanjing, China), anti-proliferating cell nuclear antigen (anti-PCNA) (1:1000; Abcam) or anti-β-Actin (1:1000; Abcam). The anti-IgG (H&L) secondary antibody (1:2500; Abcam) was used to incubate the membranes. Finally, protein bands were visualized with an eyoECL Plus Kit (Beyotime). β-Actin acted as a control gene reference.

**Tube formation assay**

Ninety-six-well plates were pre-coated with Matrigel (Corning) at 37°C for 30 min, and HUVECs were seeded in the Matrigel-coated plates with the conditioned medium, which was collected from the transfected TNBC cells after 24 h of incubation with a serum-free medium. After 24 h, the formation of capillary-like structures was assessed by calculating the number of branch points under a DP71 microscope (Olympus). A branch point contained at least three cells.

**Target prediction and dual-luciferase reporter assay**

The complementary sequence between miR-149-5p and circ_0041732 (3′-UTR) of FGF5 was assessed through circRNA Interactome online database (https://circinteractome.nia.nih.gov/api/v2/mirnasearch?circular_rna_query=hsa_circ_0041732&mirna_query=hsa-miR-149&submit=miRNA+Target+Search) or targetscan online database (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?r=ENST00000456523&taxid=9606&members=miR-149-5p&showcnc=1&shownc=1&subset=1). The wild-type (wt) plasmids of circ_0041732 (circ_0041732-wt) and FGF5 (FGF5-wt 3′UTR), and the mutant (mut) vectors of circ_0041732 (circ_0041732-mut) and FGF5 (FGF5-mut 3′UTR) were generated by Genesee Co., Ltd. For cell transfection, the luciferase reporter plasmids were first mixed with miR-149-5p or NC prior to treating with TurboFect reagent (Thermo Fisher). After 20 minutes, these mixtures were added into 24-well plates. After 48 h of transfection, firefly luciferase activity was detected using a GloMax® 20/20 Luminometer (Promega, Madison, WI, USA) at a wavelength of 560 nm with Renilla luciferase activity (at a wavelength of 465 nm) used as a reference.

**RNA immunoprecipitation assay**

RNA immunoprecipitation (RIP) assay was performed using a Magna RNA immunoprecipitation kit (Millipore, Billerica, MA, USA). In brief, BT-549 and MDA-MB-231 cells were collected and lysed with RIP lysis buffer (Millipore) possessing protease and RNase inhibitors (Millipore). Cell lysates were incubated with the antibodies against argonaute2 (Anti-Ago2; Abcam) and immunoglobulin G (Anti-IgG; Abcam) for 4 h, respectively. After that, the lysates were incubated with magnetic beads (Millipore) for 24 h. At last, qRT-PCR was used to quantify the expression of circ_0041732, miR-149-5p and FGF5 in the complexes.

**In vivo assay**

Charles River (Beijing, China) provided female BALB/c nude mice (5 weeks old, N = 12). The lentiviral genomic integrations stably expressing sh-circ_0041732 (LV-sh-circ_0041732) and the lentiviral expressing sh-NC (LV-sh-NC) were packaged by FulenGen company (Guangzhou, China). 5 × 10⁶ MDA-MB-231 cells carrying LV-sh-circ_0041732 or LV-sh-NC were diluted in 200 μL phosphate buffer solution (Thermo Fisher), and then subcutaneously injected into these nude mice. Then, tumor volume was measured every 5 days until the 30th day after injection. After 30 days later, these mice were euthanized using pentobarbital sodium (40 mg/kg), and the forming tumors were excised for tumor weight and gene expression analysis. In addition, circ_0041732 and miR-149-5p expression were determined by qRT-PCR, FGF5 and proliferating cell nuclear antigen (PCNA) protein levels were detected by Western blot analysis, and the positive expression rate of nuclear proliferation marker (Ki67) was analyzed by an immunohistochemistry (IHC) assay in the excised tumors. The Animal Care and Use Committee of Lianshui People’s Hospital agreed to this study.

**IHC for Ki67**

IHC assay was performed as shown previously. In brief, the tissues from the nude mice were immobilized with paraformaldehyde (Sigma), followed by dehydration with
ethanol (Millipore). The paraffin-embedded tissues were sectioned, dewaxed with xylene (Millipore) and rehydrated with ethanol. The tissues were rinsed, and then the antigen retrieval was performed by heating the sections immersed in sodium citrate (Millipore). The sections were incubated with anti-Ki67 (Abcam) and the biotinylated secondary antibody (Abcam), respectively. The stained images were captured with a TCS SP8 confocal microscope (Leica Microsystems, Mannheim, Germany).

**Statistical analysis**

Data derived from three independent duplicate tests were analyzed by SPSS software (IBM, Somers, NY, USA). Results were shown as means ± SD. Significant differences were compared with Spearman’s correlation test, two-tailed Student’s t-tests, Wilcoxon rank-sum test or one-way analysis of variance (ANOVA). Statistical significance was considered when the P value was < 0.05.

**Results**

**Circ_0041732 silencing repressed TNBC-associated properties in vitro and in vivo**

Circ_0041732 expression was first determined in TNBC tissues and cells. The data from qRT-PCR showed the overexpression of circ_0041732 in TNBC tissues and cells (BT-549 and MDA-MB-231 cells) compared with paracancerous normal breast tissues and MCF-10A cells, respectively (Figure S1(a) and (b)). Subsequently, we found that circ_0041732 expression was higher in the cytoplasm than in cell nuclei (Figure S1(c)). The high stability of circ_0041732 was confirmed by the RNase R resistance analysis of circRNA assay (Figure S1(d)). These data suggested that circ_0041732 might participate in the progression of TNBC.

We then explored whether circ_0041732 regulated TNBC-associated properties. Results initially showed the high efficiency of si-circ_0041732#1 and si-circ_0041732#2 in reducing circ_0041732 expression (Figure 1(a)). Subsequently, circ_0041732 silencing repressed cell viability and colony-forming ability (Figure 1(b) and (c)). In contrast, the apoptosis of BT-549 and MDA-MB-231 cells was promoted after circ_0041732 silencing (Figure 1(d)). In support, the data from Figure 1(e) showed that circ_0041732 absence promoted caspase-3 activity. Additionally, the migratory and invasive capacities of BT-549 and MDA-MB-231 cells were restrained after circ_0041732 silencing (Figure 1(f) and (g)). Meanwhile, the protein expression of MMP2 and MMP9 was downregulated after circ_0041732 depletion (Figure 1(h)). Tube formation assay displayed that circ_0041732 silencing repressed tube formation of HUVECs (Figure 1(i)). Comparatively, our data showed that circ_0041732 silencing reduced tumor volume and weight in vivo (Figure S2(a) and (b)). The high efficiency of circ_0041732 knockdown was shown in Figure S2(c). Further, the expression of the proliferation-related protein (PCNA) and the number of Ki67-positive cells were decreased after circ_0041732 silencing in the forming tumors (Figure S2(d) and (e)). Taken together, the above data demonstrated that circ_0041732 knockdown repressed TNBC cell tumor properties. si-circ_0041732 was employed for the subsequent study as it displayed a higher efficiency in decreasing circ_0041732 expression.

**Circ_0041732 acted as a sponge of miR-149-5p in BT-549 and MDA-MB-231 cells**

MiRNA(s) with the potential to bind to circ_0041732 were further explored. Our data showed that miR-149-5p expression was significantly downregulated, and was negatively related to miR-149-5p expression in TNBC tissues (Figure 2(a) and (b)). Also, miR-149-5p expression was reduced in BT-549 and MDA-MB-231 cells compared to MCF-10A cells (Figure 2(c)). Based on the above results, we hypothesized that circ_0041732 was associated with miR-149-5p. The circRNA Interactome online database was used to verify the hypothesis. As expected, circ_0041732 carried the potential binding sites of miR-149-5p (Figure 2(d)). Dual-luciferase reporter assay showed that the relative luciferase activity of circ_0041732-wt was significantly repressed after transfection of miR-149-5p, whereas the luciferase activity of circ_0041732-mut had no response to miR-149-5p overexpression (Figure 2(e) and (f)). Moreover, the RIP assay showed that both circ_0041732 and miR-149-5p were enriched by Anti-Ago2 compared with them in the Anti-IgG group (Figure 2(g)). Further, qRT-PCR was employed to determine the association between circ_0041732 and miR-149-5p expression. Data showed that circ_0041732 was effective in augmenting circ_0041732 expression based on the high expression of circ_0041732 in both the BT-549 and MDA-MB-231 cells transfected with circ_0041732 (Figure 2(h)). We then found that miR-149-5p expression was decreased after circ_0041732 overexpression, but increased after circ_0041732 silencing (Figure 2(i)). Thus, these data demonstrated that circ_0041732 was directly associated with miR-149-5p in BT-549 and MDA-MB-231 cells.

**Circ_0041732 regulated TNBC cell tumor properties by binding to miR-149-5p**

The study continued to explore whether circ_0041732 regulated TNBC cell tumor properties via interacting with miR-149-5p. Data first showed the high efficiency of anti-miR-149-5p in downregulating miR-149-5p expression (Figure 3(a)). Then, we found that miR-149-
5p expression was upregulated after circ_0041732 silencing, whereas this effect was attenuated by miR-149-5p inhibitors (Figure 3(b)). The data from Figure 3(c) and (d) showed that miR-149-5p inhibitors reversed the repressive impacts of circ_0041732 silencing on cell viability and colony-forming ability. The promoting effects of...
circ_0041732 silencing on cell apoptosis and caspase-3 activity were also hindered by miR-149-5p inhibitors (Figure 3(e) and (f)). Additionally, circ_0041732 knockdown repressed cell migration and invasion, but these effects were impaired by miR-149-5p inhibitors (Figure 3(g) to (i)). Similarly, the repressive impacts of circ_0041732 knockdown on the protein expression of MMP2 and MMP9 were restrained by miR-149-5p inhibitors.
inhibitors (Figure 3(j) and (k)). MiR-149-5p inhibitors also reversed the inhibitory effect of circ_0041732 absence on tube formation (Figure 3(l)). Therefore, the above evidence demonstrated that circ_0041732 regulated TNBC cell tumor properties by interacting with miR-149-5p.

**Circ_0041732 regulated FGF5 expression by associating with miR-149-5p**

The target gene of miR-149-5p was further validated. The expression of FGF5, a candidate, was first determined in TNBC tissues. Data showed that the mRNA and protein levels of FGF5 were significantly upregulated in TNBC tissues compared with paracancerous normal breast tissues (Figure 4(a) and (b)). Additionally, it was found that FGF5 expression was positively related to circ_0041732 (Figure 4(c)), but negatively correlated with miR-149-5p in TNBC tissues (Figure 4(d)). In contrast, the high expression of FGF5 was also found in BT-549 and MDA-MB-231 cells (Figure 4(e)). The above data suggested that FGF5 might be involved in miR-149-5p-mediated TNBC cell tumor properties. Next, the targetscan online database predicted that FGF5 3'-UTR carried the complementary sequence of miR-149-5p (Figure 4(f)). Also, miR-149-5p significantly repressed the luciferase activity of FGF5-wt 3'UTR rather than the luciferase activity of FGF5-mut 3'UTR (Figure 4(g)). It was found that FGF5 and miR-149-5p significantly enriched in the Anti-Ago2 group compared with the Anti-IgG group (Figure 4(h)). These data suggested that miR-149-5p directly targeted FGF5 in BT-549 and MDA-MB-231 cells. Further, Western blot analysis clarified that circ_0041732 knockdown downregulated FGF5 protein level, whereas this effect was attenuated after transfection of anti-miR-149-5p (Figure 4(i)), which implied that circ_0041732 could regulate FGF5 expression by binding to miR-149-5p.

**MiR-149-5p repressed TNBC cell tumor properties by targeting FGF5**

Given the binding relationship between miR-149-5p and FGF5, whether miR-149-5p mediated BT-549 and MDA-MB-231 cell tumor properties by binding to FGF5 was revealed in this part. Results first showed the high efficiency of miR-149-5p and FGF5 overexpression (Figure 5(a) and (b)). Subsequently, miR-149-5p mimics decreased FGF5 protein expression, which was reversed by FGF5 overexpression (Figure 5(c)). MiR-149-5p mimics repressed cell viability and colony-forming ability, whereas these effects were restored after transfection of FGF5 (Figure 5(d) and (e)). The promoting effects of miR-149-5p mimics on cell apoptosis and caspase-3 activity were also impaired by FGF5 overexpression (Figure 5(f) and (g)). Additionally, the migration and invasion of BT-549 and MDA-MB-231 cells were inhibited by miR-149-5p, but these impacts were attenuated by ectopic FGF5 expression (Figure 5(h) and (i)). Consistently, the repressive impacts of miR-149-5p mimics on the protein expression of MMP2 and MMP9 were mitigated after FGF5 introduction (Figure 5(j) and (k)). The decreased tube formation by miR-149-5p mimics was also impaired after transfection of FGF5 (Figure 5(l)). Collectively, these findings demonstrated that miR-149-5p could regulate BT-549 and MDA-MB-231 cell tumor properties by binding to FGF5.

**Discussion**

With the application of high-throughput sequencing technology, multiple circRNAs have been identified in various cancers. CircRNA is a covalently closed cyclic transcript that is abundant in various types of cells, thereby more suitable as a diagnostic marker or therapeutic target than linear RNA.27 Multiple reports have indicated that circRNAs regulate cancer progression. For example, circ_100395 repressed cell tumor properties of lung cancer5 and liver cancer.28 Circ_0048124 contributed to the tumorigenesis of papillary thyroid29 and lung cancer.30 In this paper, we provided evidence that the role of circ_0041732 in TNBC cell tumor properties was associated with miR-149-5p and FGF5.

Multiple circRNAs were involved in TNBC progression. For instance, Wang et al.31 indicated that overexpression of circRNA itchy E3 ubiquitin protein ligase (circ-ITCH) restrained the proliferation and metastasis of TNBC cells via the Wnt/β-catenin pathway. Circ_0005728 facilitated cell growth by modulating miR-512-3p in TNBC in vitro.32 Additionally, some circRNAs participated in tumor development by regulating tube formation, such as circ_000320433 and circ-protein arginine methyltransferase 5.34 In this research, we are the first to report that circ_0041732 promoted TNBC-associated properties. Our data showed that circ_0041732 was augmented in TNBC specimens and cells in comparison with normal breast tissues or cells. Circ_0041732 knockdown repressed TNBC cell tumor properties via regulating cell proliferation, migration, invasion, and apoptosis. Additionally, circ_0041732 contributed to tumor growth in vivo. Also, circ_0041732 silencing suppressed tube formation. These data indicated that circ_0041732 acted as an oncogene in TNBC.

Next, cytoplasmic and nuclear RNA analysis showed that circ_0041732 was chiefly located in the cytoplasm, which suggested that circ_0041732 functioned at the post-transcriptional level. Considering that circRNAs commonly served as sponges of miRNAs that functioned at the post-transcriptional level,5,35 we further analyzed the miRNAs that were interacted with circ_0041732. Data exhibited that miR-149-5p directly bound to
Previous evidence has revealed that miR-149-5p is associated with the chemoresistance of breast cancer cells to paclitaxel and trastuzumab. Some researchers also explained that circ_0072995 silencing inhibited cell growth, metastasis, and anaerobic glycolysis via binding to miR-149-5p in breast cancer, suggesting the inhibitory effect of miR-149-5p on breast cancer cell malignancy. In this paper, we also found that miR-149-5p acted as a repressor of TNBC cell line tumor features. Our data showed that miR-149-5p expression was reduced in TNBC samples and cells compared with normal breast tissues or cells. MiR-149-5p expression was inversely correlated with circ_0041732 in TNBC tissues. MiR-149-5p mimics restrained cell proliferation, migration, invasion, and tube formation, but upregulated cell apoptotic rate. Additionally, miR-149-5p inhibitors reversed the inhibitory impacts of circ_0041732 knockdown on TNBC cell tumor properties. This evidence demonstrated that circ_0041732 regulated TNBC-associated properties via binding to miR-149-5p.

To reveal the underlying mechanism by which miR-149-5p modulated TNBC-associated properties, the target gene of miR-149-5p was further analyzed. As a result, FGF5 was associated with miR-149-5p. Han et al. reported that FGF5 facilitated the proliferation of osteosarcoma cells through modulating mitogen-activated protein kinase (MAPK) pathway. Ghassemi et al. indicated that FGF5 overexpression promoted clonogenicity and invasion of melanoma cells. In addition, FGF5 enhanced the proliferative and metastatic capacities of hepatocellular cancer cells through associating with miR-188-5p. In the present study, we found that FGF5 was overexpressed in TbNC tissue samples, which was in line with the published data. Also, our results showed that FGF5 was upregulated in TNBC cells. FGF5 overexpression reversed the inhibitory impact of miR-149-5p on TNBC cell tumor properties.

Figure 3. Circ_0041732 silencing repressed the tumor properties of BT-549 and MDA-MB-231 cells by binding to miR-149-5p. (a) MiR-149-5p expression was determined by qRT-PCR in both the BT-549 and MDA-MB-231 cells transfected with anti-NC or anti-miR-149-5p. (B-K) Both BT-549 and MDA-MB-231 cells were transfected with si-NC, si-circ_0041732#1, si-circ_0041732#1 + anti-NC and si-circ_0041732#1 + anti-miR-149-5p, respectively. (b) MiR-149-5p expression was determined by qRT-PCR. (c) and (d) Cell viability and cell colony-forming ability were detected by CCK-8 and cell colony formation assays, respectively. (e) Cell apoptosis was detected by flow cytometry analysis. (f) Caspase-3 activity assay was performed to determine caspase-3 activity. (g) to (i) The migration and invasion of both BT-549 and MDA-MB-231 cells were detected by wound-healing and transwell invasion assays, respectively. (j) and (k) Western blot analysis was performed to detect the protein levels of MMP2 and MMP9. (l) The tube formation of HUVECs was investigated by a tube formation assay. *P < 0.05.

CCK-8: cell counting kit-8; HUVEC: human umbilical vein endothelial cell line; qRT-PCR: quantitative real-time polymerase chain reaction.
properties, which suggested that miR-149-5p could regulate TNBC-associated properties by targeting FGF5.

Based on the above results, we hypothesized that circ_0041732 regulated FGF5 expression through interacting with miR-149-5p. To validate this, we performed rescue experiments. Results showed that miR-149-5p depletion remitted the inhibitory effects of circ_0041732 absence on FGF5 protein expression. The above evidence manifested that circ_0041732 could regulate FGF5 via interacting with miR-149-5p. Additionally, the following fact should be considered when evaluating our new findings. The study was performed with non-tumorigenic MCF10A used as a control rather than with non-triple-negative-receptor-described cell lines. Thus, the results shown in this study might not be specific to the triple-receptor-negative tumor process.

Collectively, TNBC cell tumor properties were associated with circ_0041732. The underlying mechanism was that circ_0041732 knockdown led to the decrease of FGF5 expression by miR-149-5p, and the reduced expression of FGF5 inhibited cell proliferation, migration, invasion, and tube formation, and increased cell apoptosis.

**Figure 4.** Circ_0041732 modulated FGF5 expression via interacting with miR-149-5p in BT-549 and MDA-MB-231 cells. (a) and (b) The mRNA and protein levels of FGF5 were determined by qRT-PCR and Western blot analysis, respectively, in 57 pairs of TNBC tissues and paracancerous normal breast tissues. (c) and (d) Spearman correlation analysis was carried out to determine the linear relationship between FGF5 and circ_0041732 or miR-149-5p expression. (e) The protein level of FGF5 was determined by Western blot analysis in MCF-10A, BT-549 and MDA-MB-231 cells. (f) The binding sites of miR-149-5p for FGF5 were predicted by the targetscan online database. (g) and (h) Dual-luciferase reporter and RIP assays were conducted to demonstrate that miR-149-5p was directly associated with FGF5 in both BT-549 and MDA-MB-231 cells. (i) Western blot analysis was conducted to reveal the effects of circ_0041732 silencing and miR-149-5p inhibitors on FGF5 protein expression in BT-549 and MDA-MB-231 cells. *P < 0.05.

FGF5: fibroblast growth factor 5; mRNA: messenger RNA; qRT-PCR: quantitative real-time polymerase chain reaction; TNBC: triple-negative breast cancer.
This study supports the use of circ_0041732 as a therapeutic target for TNBC.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval and consent participate
Written informed consent was obtained from patients with approval by the Institutional Review Board in Lianshui People’s Hospital.

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