Circ_0072995 promotes cell carcinogenesis via up-regulating miR-149-5p-mediated SHMT2 in breast cancer

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Research

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Abstract

Background

Circ_0072995 is a novel identified circRNA and has been identified to involve in the metastasis of breast cancer. However, the detailed function and mechanism of circ_0072995 in the biological property of breast cancer cell remain vague.

Methods

The expression of circ_0072995, microRNA (miR)-149-5p and serine hydroxymethyltransferase 2 (SHMT2) mRNA was detected using quantitative real-time polymerase chain reaction. Western blot was used to detect levels of SHMT2, hexokinase-2 (HK-2), lactate dehydrogenase a chain (LDHA), glucose transporter 1 (GLUT1) and phosphoinositide 3-kinase (PI3K)/p-protein kinase B (AKT) pathway/mammalian target of rapamycin (mTOR) pathway-related protein. Cell proliferation, apoptosis, migration, and invasion were analyzed using cell counting kit-8 assay, flow cytometry, caspase-3 activity analysis, cell adhesion assay and transwell assay, respectively. Glucose metabolism was calculated by measuring glucose uptake, lactate production, and adenosine triphosphate (ATP) levels. The interaction between miR-149-5p and circ_0072995 or SHMT2 was confirmed by dual-luciferase reporter assay. In vivo tumorigenesis was performed using the murine xenograft model.

Results

Circ_0072995 and SHMT2 were up-regulated in breast cancer tissues and cell lines, and knockdown of circ_0072995 or SHMT2 suppressed cell malignant properties and anaerobic glycolysis; importantly, SHMT2 overexpression attenuated the anticancer action of si-Circ_0072995 in breast cancer. Besides, we also found miR-149-5p directly bound to circ_0072995 or SHMT2 in breast cancer cells, and circ_0072995 promoted the expression of SHMT2 by competitively binding to miR-149-5p. Moreover, circ_0072995 activated PI3K/AKT/mTOR pathway via elevating SHMT2 through miR-149-5p in vitro and in vivo. Additionally, xenograft tumors analysis showed circ_0072995 silence suppressed tumor growth via regulating SHMT2 and miR-149-5p.

Conclusion

This study demonstrated that circ_0072995 promoted cell malignant phenotypes and anaerobic glycolysis in breast cancer via up-regulating SHMT2 through sponging miR-149-5p, and activated PI3K/AKT/mTOR pathway via miR-149-5p/ SHMT2 axis, indicating a promising molecular target for breast cancer treatment.

Background

On a global scale, breast cancer is the most frequently diagnosed female malignancy, which seriously threatens the health of women in the word [1]. As the first leading cause among female malignancy, the overall occurrence and mortality of patients with breast cancer have not significantly reduced despite the great improvement in diagnosis and therapeutic technology [2, 3]. Hence, it is paramount to investigate the mechanism underlying the progression of breast cancer for managing the clinical treatments.

Circular RNAs (circRNAs) are a special subclass of endogenous noncoding RNA molecules with covalently closed loop structures, which improve the resistance to exonuclease degradation relative to their linear RNAs decay [4]. Previous studies have reported that circRNAs are important modulators in multiple cancer types via affecting various cellular processes, including cell proliferation, invasion, migration, drug resistance and metabolism [5–8], thus involving in the progression and development of cancers. In breast cancer, multiple functions of circRNAs have been identified to exert various influences on the tumorigenesis of cancer cells. For example, hsa_circ_0001982 interacted with miR-143 to promote tumor cell proliferation and invasion in breast cancer, thus contributing to the progression of cancer [9]. CircRNA hsa_circ_0052112 induced breast cancer cell migration and invasion by targeting miR-125a-5p [10]. CircRNA hsa_circRNA_002178 promoted cell viability, tube formation and energy metabolism via up-regulating COL1A1 through miR-328-3p [11]. circ_0072995 is a novel identified circRNA, a recent study found circ_0072995 was elevated in breast cancer cells, and promoted cell metastasis by binding to miR-30c-2-3p in vitro [12]. Nevertheless, the detailed function and mechanism of circ_0072995 in breast cancer remain vague.

Serine hydroxymethyltransferase 2 (SHMT2), a mitochondrial SHMT, can reversibly and simultaneously catalyze the conversions of L-serine to glycine, playing significant roles in cellular one-carbon pathways [13, 14]. An adequate supply of glycine is strongly responsible for rapidly proliferating cancer cells, and glycine consumption and catabolism contribute to the carcinogenesis and malignancy [15, 16]. Silencing of SHMT2 is shown to be a key target for therapeutic intervention of cancer.

However, little work has shown the role of SHMT2 in cell tumorigenesis in breast cancer.

In this study, we aimed to investigate the function and molecular mechanisms of circ_0072995 and SHMT2 in the malignant biological behaviors and glucose metabolism of breast cancer cells, explored whether SHMT2 involved in the action of circ_0072995 in the progression of breast cancer.

Materials And Methods

Clinical specimens

Tumor tissues and matched non-tumor tissue samples from 70 breast cancer patients who underwent surgical resection at The Central Hospital of Enshi Tujia and Miao Autonomous Prefecture were collected, and then immediately frozen in liquid nitrogen at -80℃ until subsequent analysis. All patients were diagnosed by histopathological assessment.

Cell Culture And Transfection

Human breast cancer cell lines (SKBR3, HCC38, MCF-7, MDA-MB-231 and BT-549) and MCF-10A nonmalignant breast epithelial cells were purchased from Shanghai Academy of Life Science (Shanghai, China). All cells were grown in the Dulbecco's modifed Eagle's medium (DMEM) harboring with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) with 5% CO_2 at 37℃.

The miR-149-5p mimic (miR-149-5p), mimic control (miR-NC), small interfering RNA (siRNA) targeting circ_0072995 (si-circ#1, si-circ#2, si-circ#3), siRNA targeting SHMT2 (si-SHMT2#1, si-SHMT2#2, si-SHMT2#3), siRNA negative control (si-NC), pcDNA3.1 circ_0072995 overexpression vector (circ_0072995), negative

plasmid (pcDNA), pcDNA3.1 SHMT2 overexpression vector (SHMT2), empty vector (vector), lentiviral particles stably expressing either short hairpin RNA (shRNA)-targeting circ_0072995 (sh-circ) or a scrambled control sequence (sh-NC) were designed and synthesized by Invitrogen (Carlsbad, CA, USA). Then, the transfection of oligonucleotides or vectors was performed using Lipofectamine 2000 (Invitrogen).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Whole-RNA extracts from tissues and cells were conducted by using TRIzol reagent (Invitrogen). Then extracted RNA was reversely transcribed into complementary DNA (cDNA) using the Prime Script RT Master Mix (Takara, Dalian, China), and the synthesized cDNA template was detected using SYBR Premix Ex Taq (Takara). After that, The expression levels were measured by the $2^{-\triangle\triangle Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GADPH) or U6 small nuclear B noncoding RNA (U6) using as an internal reference. The primer sequences were listed as follows: circ_0072995: F, 5'-AGAACAGCTATGCCCTCCAG-3' and R, 5'-CCCATCTCATAGCCAGGTGT-3'; miR-149-5p: F, 5'-TCTGGCTCCGTGTCTTCACTCCC-3' and R, 5'-TATGGTTGTTCTGCTCTCTGTGTC-3'; SHMT2: F, 5'- AGCATTGCTCATCAATTTGTTGCAACGAAC-3' and R, 5'-GTTCGTTGCAACAAATTGATAAGCAA-3'; GADPH: F 5'-GAGAAACCTGCCAAGTATGATGAC-3' and R 5'-GGAGTTGCTGTTGAAGTCAC-3', U6: F, 5'-CTCGCTTCGGCAGCACA – 3' and R, 5'-AACGCTTCACGAATTTGCGT-3'.

Western Blot

Total proteins were isolated using RIPA buffer (Beyotime, Shanghai, China). Subsequently, proteins were separated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto the polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk for 2 h, the membranes were incubated with the primary antibodies against SHMT2 (1:1000, ab180786, Abcam, Cambridge, MA, USA), hexokinase-2 (HK-2) (1:5000, ab104836, Abcam), lactate dehydrogenase A chain (LDHA) (1:3000, ab135366, Abcam), glucose transporter 1 (GLUT1) (1:10000, ab40084, Abcam), phosphoinositide 3-kinase (PI3K) (1:1000, ab40776, Abcam), phosphorylated(p)-PI3K (p-PI3K) (1:1000, ab182651, Abcam), protein kinase B (AKT) (1:1,000, 9272, Cell Signaling Technology, Boston, MA, USA), p-AKT (1:1,000, 9271, Cell Signaling Technology), mammalian target of rapamycin (mTOR) (1:2000, ab2732, Abcam), p-mTOR (1:5000, ab109268, Abcam) and then interacted with the secondary HRP-conjugated antibody (1:1000, ab9482, Abcam). Protein bands were visualized using electrochemiluminescence and normalized by β-actin (1:1,000, 4967, Cell Signaling Technology).

Cell Proliferation Assay

Transfected SKBR3 and BT-549 cells were seeded in 96-well cell culture plates for 24 h, then per well was incubated with 10 μL cell counting kit-8 (CCK-8) solution (Beyotime, Shanghai, China) for about 2 h. Subsequently, the optical density was measured by a spectrophotometric microplate reader at 450 nm.

Cell Apoptosis Analysis

Cell apoptosis was analyzed by flow cytometry with Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Transfected SKBR3 and BT-549 cells were collected and resuspended in binding buffer, followed by interaction with Annexin V-FITC (10 μL) and propidium iodide (PI) (10 μL) for 15 min. The apoptosis rate of cells were analyzed by flow cytometer.

Measurement Of Caspase-3 Activity

The caspase-3 activity were detected by a colorimetric assay kit (Sigma, St Louis, MO, USA) in SKBR3 and BT-549 cells following the standard procedure. Finally, the absorbance was measured by a microplate reader at 450 nm.

Cell Adhesion Assay

Transfected SKBR3 and BT-549 (1×10^5) were seeded into the 96-well culture plates pre-coated with 1:3 diluted Matrigel (Corning, NY, USA) for 30 min. After discarding the corresponding medium with non-adhered cells, cells were gently washed with PBS for two times to remove loosely adhered cells. After that, adhered cells were stained with 0.1% crystal violet, and then fixed with methanol for 30 minutes. Finally, adherent cells were counted by a microscope at 100 × magnification.

Cell Migration And Invasion Assay

Transwell chambers were used to detect cell migration and invasion. For invasion assay, the transwell chamber were pre-coated with Matrigel, while cell migration analysis didn't need Matrigel. Then, SKBR3 and BT-549 cells transfected with the assigned vector were seeded in the upper chambers with 200 μL serum-free DMEM, and 600 μL of medium fixed with FBS was added to the lower chambers. 24 h later, cells on the lower face of the membranes were fixed and stained, and counted using an inverted light microscope in five random fields.

Glycolysis Analysis

After transfection, SKBR3 and BT-549 cells were placed in a 6-well plate, then supernatants of cell culture media were collected to analyze the levels of glucose and lactate by using a glucose or lactate assay kit (Sigma). The relative glucose uptake and lactate production were assessed based on the standard curve and normalized by the percentage of the control group.

Levels detection of ATP in transfected SKBR3 and BT-549 cells were performed using an ATP Assay Kit (Sigma). Briefly, transfected cells were lysed, and then ATP reaction mix was added into the lysate and incubated for 30 min. Subsequently, the absorbance at 570 nm was detected by a microplate reader.

Dual-luciferase Reporter Assay

The circ_0072995 or SHMT2 3'UTR sequences with wild type (WT) or mutant (MUT) binding sites of miR-149-5p were separately cloned into the pRL-TK luciferase plasmids (Promega, Shanghai, China) to generate pRL-TK-WT-circ_0072995, pRL-TK-MUT-circ_0072995, pRL-TK-SHMT2 3'UTR-WT, or pRL-TK-SHMT2 3'UTR-MUT luciferase reporter, respectively. Then these constructed luciferase reporters with miR-149-5p mimic or miR-NC were transfected into SKBR3 and BT-549 cells using Lipofectamine 2000 (Invitrogen). 48 h later, luciferase activities were examined using a Dual-Luciferase reporter assay kit (Promega).

Xenograft experiments in vivo

Female BALB/c nude mice (5 weeks old, N = 6) were used to establish xenograft models. Six mice were divided into 2 groups for stable injection with BT-549 cells that were transfected with sh-NC or sh-circ. Tumor volume was recorded every week. At day 28, mice were killed and tumor masses were weighed and harvested for further molecular analysis.

Statistical analysis

All analyses were conducted on GraphPad Prism 7 software. Data from at least three experiments were expressed in terms of means ± standard deviation (SD). Statistical difference between different groups was analyzed by Student's t test or one-way analysis of variance (ANOVA), and a P-value less than 0.05 was considered statistically significant.

Results

The expression of circ_0072995 and SHMT2 is elevated in breast cancer

To detect the role of circ_0072995 and SHMT2 in breast cancer progression, the expression levels of them were first measured. Results showed that circ_0072995 and SHMT2 expression were higher in breast cancer tissues than that in paracancer tissues (Fig. 1A, B, D), and a positive correlation between circ_0072995 and SHMT2 expression in breast cancer tissues was observed (Fig. 1C). Similarly, levels of circ_0072995 and SHMT2 cells were also elevated in breast cancer cells (SKBR3, HCC38, MCF-7, MDA-MB-231 and BT-549) compared with MCF-10A nonmalignant breast epithelial cells (Fig. 1E-G). These results indicated that circ_0072995 and SHMT2 elevation might be associated with the initiation and development of breast cancer.

Circ_0072995 knockdown inhibits cell malignant phenotypes and anaerobic glycolysis in breast cancer

The detailed functions of circ_0072995 in the progression of breast cancer were investigated. Circ_0072995 was knocked down by transfecting with constructed si-circ_0072995 plasmid into SKBR3 and BT-549 cells. Subsequently, we found si-circ_0072995 of three forms, especially si-circ#3, significantly reduced the expression of circ_0072995 relative to the si-NC group in SKBR3 and BT-549 cells (Fig. 2A, H), and then si-circ#3 was selected for subsequent functional analysis. By contrast with si-NC group, circ_0072995 knockdown inhibited SKBR3 cell proliferation (Fig. 2B), but induced SKBR3 cell apoptosis evidenced by the increase of apoptotic cells and caspase-3 activity (Fig. 2C, D). Besides, cell adhesion assay showed that circ_0072995 knockdown attenuated cell adhesion ability (Fig. 2E), and transwell migration and invasion assays demonstrated that circ_0072995 down-regulation significantly inhibited both the cell migratory and invasive capabilities of SKBR3 cells (Fig. 2F, G). Similarly, the same effects were also discovered in BT-549 cells, circ_0072995 knockdown also inhibited BT-549 cell proliferation (Fig. 2I), migration, invasion (Fig. 2L-N) and promoted apoptosis (Fig. 2J, K).

Additionally, glucose metabolism was calculated, results showed silencing circ_0072995 decreased glucose uptake (Fig. 3A, E), lactate production (Fig. 3B, F), and ATP levels (Fig. 3C, G) in SKBR3 and BT-549 cells. Western blot analysis indicating levels of HK-2, LDHA, GLUT1 also were down-regulated by circ_0072995 knockdown in SKBR3 (Fig. 3D) and BT-549 cells (Fig. 3H). Thus, circ_0072995 silence inhibited cell anaerobic glycolysis in breast cancer. Taken together, circ_0072995 knockdown suppressed breast cancer progression.

SHMT2 knockdown inhibits cell malignant phenotypes and anaerobic glycolysis in breast cancer

The function of SHMT2 in the progression of breast cancer was then explored. We used si-SHMT2 to knock down the expression of SHMT2 in SKBR3 and BT-549 cells, and western blot analysis showed three forms of si-SHMT2, especially si-SHMT2 #2, significantly decreased the level of SHMT2 relative to the si-NC group in SKBR3 and BT-549 cells (Fig. 4A, L). Then functional experiments were conducted. Results displayed that SHMT2 knockdown mediated proliferation inhibition (Fig. 4B, M), apoptosis promotion (Fig. 4C, D, N, O), and suppression of adhesion (Fig. 4E, P), migration (Fig. 4F, Q) and invasion (Fig. 4G, R) ability in SKBR3 and BT-549 cells. Besides, we also found silencing SHMT2 repressed anaerobic glycolysis in SKBR3 and BT-549 cells, reflected by the decrease of glucose uptake (Fig. 4H, S), lactate production (Fig. 4I, T), and ATP levels (Fig. 4J, U), as well as levels of HK-2, LDHA, GLUT1 (Fig. 4K, V) in SKBR3 and BT-549 cells. These data suggested that SHMT2 knockdown also impeded the development of breast cancer.

SHMT2 overexpression reverses the anticancer function of si-Circ_0072995 in breast cancer cells

Given the negative correlation of circ_0072995 and SHMT2 expression in breast cancer, whether si-Circ_0072995 exerted anticancer effects via SHMT2 was investigated. First, SKBR3 and BT-549 cells were transfected with SHMT2 or vector, and western blot analysis showed SHMT2 transfection significantly elevated SHMT2 levels compared with vector transfection (Fig. 5A, L). Next, SKBR3 and BT-549 cells were transfected with si-NC, si-circ#3, si-circ#3 + vector, or si-circ#3 + SHMT2 to performed rescue assay. We found SHMT2 overexpression attenuated si-Circ_0072995-induced proliferation inhibition (Fig. 5B, M), apoptosis promotion (Fig. 5C, D, N, O), and suppression of adhesion (Fig. 5E, P), migration (Fig. 5F, Q) and invasion (Fig. 5G, R) ability in SKBR3 and BT-549 cells. Moreover, the suppression of anaerobic glycolysis in SKBR3 (Fig. 5H-K) and BT-549 (Fig. 5S-V) cells induced by circ_0072995 knockdown also was rescued by SHMT2 re-expression. Altogether, circ_0072995 contributed the progression of breast cancer by regulating SHMT2.

MiR-149-5p directly binds to circ_0072995 or SHMT2 in breast cancer cell

It has been reported that circRNAs can act as competitive endogenous RNAs (ceRNAs) for miRNAs, thus regulating the depression of miRNA targets [17]. Therefore, we hypothesized that circ_0072995 knockdown-mediated anticancer function may operate via a ceRNA mechanism. According to the prediction of CircInteractome and TargetScan program, the sequences of circ_0072995 or SHMT2 3'UTR containing binding sites of miR-149-5p were identified (Fig. 6A). Subsequently, the reduction of luciferase activity in SKBR3 and BT-549 cells co-transfected with WT-Circ_0072995 and miR-149-5p, or SHMT2 3'UTR-WT and miR-149-5p confirmed the interaction between miR-149-5p and circ_0072995 or SHMT2 (Fig. 6B, F). Besides, we also found circ_0072995 knockdown enhanced miR-149-5p expression in SKBR3 and BT-549 cells (Fig. 6C, G), and after elevating the expression of miR-149-5p by transfecting with miR-149-5p mimic (Fig. 6D, H), it was also discovered that miR-149-5p overexpression reduced the expression of SHMT2 in SKBR3 and BT-549 cells (Fig. 6E, I). In all, these results suggested that circ_0072995 was a sponge of miR-149-5p, and negatively regulated its expression; besides, miR-149-5p targetedly suppressed SHMT2 expression.

Circ_0072995 Activates PI3K/AKT/mTOR Pathway Via miR-149-5p/SHMT2 Axis

Given circ_0072995 bound to miR-149-5p, and SHMT2 was a target of miR-149-5p, we then detected whether circ_0072995 regulated SHMT2 via miR-149-5p in breast cancer. First, SKBR3 and BT-549 cells were transfected with pcDNA or circ_0072995, and qRT-PCR analysis showed circ_0072995 was obviously increased (Fig. 7A, D). Next, SKBR3 and BT-549 cells were transfected with pcDNA, circ_0072995, circ_0072995 + miR-NC, circ_0072995 + miR-149-5p, circ_0072995 + si-NC, or circ_0072995 + si-SHMT2 #2 to perform rescue assay. Results indicated that the up-regulation of SHMT2 mRNA and protein expression-induced by circ_0072995 was abated by miR-149-5p increase or SHMT2 decrease in SKBR3 (Fig. 7B, C) and BT-549 (Fig. 7E, F) cells. Thus, circ_0072995 positively regulated SHMT2 expression via miR-149-5p. Importantly, western blot analysis also showed miR-149-5p increase or SHMT2 decrease rescued the up-regulation of phosphorylation levels of PI3K, AKT and mTOR mediated by circ_0072995 in SKBR3 (Fig. 7C) and BT-549 (Fig. 7F) cells. These data suggest that circ_0072995 promoted the expression of SHMT2 by competitively binding with miR-149-5p, thus activating PI3K/AKT/mTOR pathway in breast cancer cells.

Circ_0072995 impedes the growth of xenograft tumors in vivo

Following on from the above findings, we further explored whether the miR-149-5p/SHMT2/PI3K/AKT/mTOR pathway was implicated in the anticancer effects of circ_0072995 knockdown in the murine xenograft model. As shown in the Fig. 8A, B, circ_0072995 knockdown suppressed the tumor growth in vivo, demonstrated by the smaller in size and weight of xenograft tumors in sh-Circ_0072995 groups compared with sh-NC groups. QRT-PCR analysis showed the level of circ_0072995 was decreased while miR-149-5p was increased in the tumors from sh-Circ_0072995 groups (Fig. 8C, D). In addition, western blot analysis showed levels of SHMT2, p-PI3K, p-AKT and p-mTOR were also suppressed in sh-Circ_0072995 groups compared with sh-NC groups (Fig. 8E). Collectively, circ_0072995 knockdown inhibited tumor growth and the activation of PI3K/AKT/mTOR pathway in vivo via miR-149-5p/SHMT2 axis.

Discussion

Up to date, there is increasing evidence emerging to reveal that circRNAs have important roles in the progression of cancers. For example, circHIPK3 up-regulated BDNF expression by competitively binding with miR-107, thus facilitating cell viability and migration in gastric cancer [18]. CircMAT2B induced the glycolysis and malignancy in hepatocellular carcinoma via regulating PKM2 through sponging miR-338-3p [19]. Therefore, circRNAs are involved in the regulation of cell malignant phenotypes and metabolism, thus affecting the occurrence and development of cancers.

The occurrence of breast cancer is a complex biological process with multiple genes and multiple factors. Recent evidence has identified the involvement of circRNAs in the progression and development of breast cancer [9, 20]. Circ_0072995 is a novel identified circRNA and has been found to induce cell metastasis in breast cancer in vitro [12]. Thus, this study further investigated the detailed function and mechanism of circ_0072995 in breast cancer progression. We found circ_0072995 was elevated in breast cancer tissues and cell lines, and knockdown of circ_0072995 inhibited cell proliferation, migration, and invasion while induced cell apoptosis in vitro; importantly, xenograft tumors analysis showed circ_0072995 knockdown also impeded tumor growth in vivo. Currently, increasing researches have indicated that most cancer cells prefer to take adenosine triphosphate (ATP) through aerobic glycolysis rather than oxidative phosphorylation to meet their

metabolic needs for sustained cell proliferation even in the presence of oxygen [21, 22]. Inhibition of aerobic glycolysis is an effective therapeutic strategy to hinder cancer malignancy [23]. Therefore, glucose metabolism was calculated in this study, and we found silencing circ_0072995 decreased glucose uptake, lactate production, ATP levels, enzymes of HK-2 and LDHA, as well as GLUT1, thereby suppressed aerobic glycolysis in breast cancer cells. Taken together, suppression of circ_0072995 could repressed breast cancer progression.

Recently, it was found that SHMT2 was highly expressed in breast cancer cells, SHMT2 was valuable independent prognostic markers in breast cancer, which high expression predicated poor overall survival [24]. Besides, SHMT2 expression was positively associated with breast cancer grade [25]. Li et al. discovered that inhibition of SHMT2, the enzyme the mitochondrial serine and one-carbon pathway, suppressed tumor growth and lung metastasis in metastatic breast cancer [26]. Thus, SHMT2 may be a potential target for the treatment and drug discovery of breast cancer. In this study, an elevation of SHMT2 expression was also observed in breast cancer tissues and cell lines, then functional experiments exhibited SHMT2 silence inhibited cell malignant phenotypes and anaerobic glycolysis in breast cancer. Thus, we confirmed that SHMT2 promoted breast cancer progression.

In our work, a negative correlation between SHMT2 and circ_0072995 expression in breast cancer was found, and SHMT2 up-regulation blocked the anticancer function of si-Circ_0072995 in breast cancer cells. Previous studies have reported that circRNAs can act as ceRNAs for miRNAs, thereby abolishing the function of the corresponding miRNA and regulating the depression of miRNA targets [17, 27]. Thus, whether circ_0072995 knockdown-mediated anticancer function may operate via a ceRNA mechanism was explored, and miR-149-5p was confirmed to directly bind to circ_0072995 or SHMT2. Moreover, circ_0072995 positively regulated SHMT2 expression by binding to miR-149-5p in vivo and in vitro. Therefore, a circ_0072995/miR-149-5p/SHMT2 network in the progression of breast cancer was identified. The PI3K/AKT/mTOR pathway is an intracellular signaling, and play significant role in regulating cellular function, such as cell cycle, cell proliferation, and metabolism [28, 29], thus, it is directly associated with the occurrence and development of many types of cancers, including breast cancer [30]. In this study, we demonstrated that circ_0072995 could activate PI3K/AKT/mTOR pathway via elevating miR-149-5p-mediated SHMT2 expression in vivo and in vitro, while whether PI3K/AKT/mTOR pathway involved in circ_0072995/miR-149-5p/SHMT2 axis-mediated carcinogenic effects in breast cancer still needs to prove.

Conclusion

This findings showed that circ_0072995 functioned as an oncogenic circRNA to promote breast cancer progression by inducing cell malignant phenotypes and anaerobic glycolysis through miR-149-5p/SHMT2 axis; besides, circ_0072995 could also activate PI3K/AKT/mTOR pathway via regulating miR-149-5p-mediated SHMT2 in breast cancer. This study suggested a new insight into the pathogenesis of breast cancer and provided potential targets for breast cancer treatment.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Central Hospital of Enshi Tujia and Miao Autonomous Prefecture and written informed consents were collected from all patients and hospitals.

The animal experiment was permitted by the Animal Research Committee of The Central Hospital of Enshi Tujia and Miao Autonomous
Prefecture and performed in accordance with the guidelines of the National Animal Care and Ethics Institution.

Consent for publication

Not applicable

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflicts of interest

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None

Authors' contributions

TL designed and supervised the study. CQ conducted the experiments and drafted the manuscript. XQ conducted the experiments and supervised the study. ZZ collected and analyzed the data. YW contributed the methodology and analyzed the data. QY operated the software and edited the manuscript.

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Figures

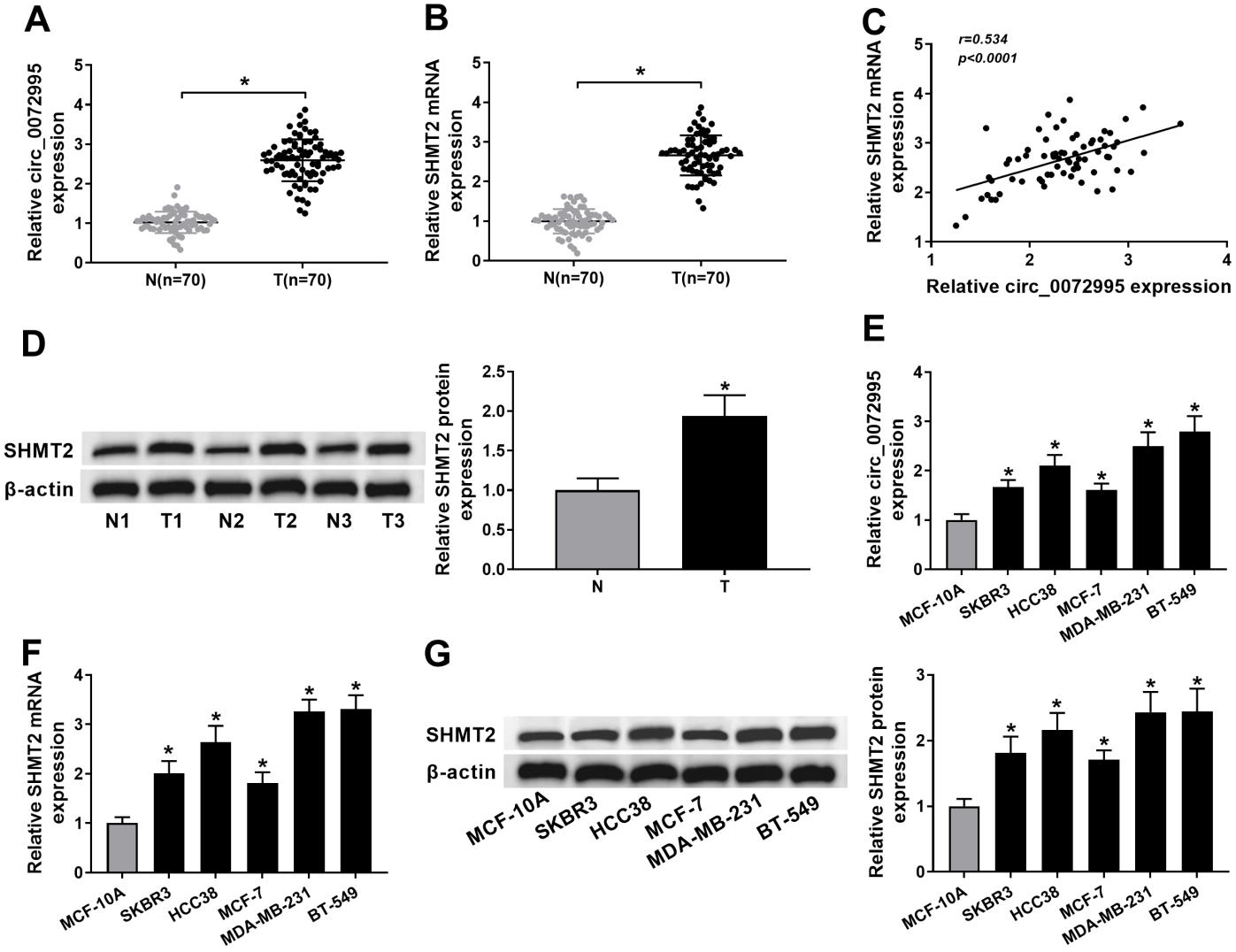

Figure 1

The expression of circ_0072995 and SHMT2 is elevated in breast cancer. (A, B) qRT-PCR analysis of circ_0072995 and SHMT2 expression in breast cancer tissues and paracancer tissues. (C) Correlation analysis of circ_0072995 and SHMT2 expression in breast cancer tissues using Pearson correlation analysis. (D) Western blot analysis of SHMT2 protein expression in breast cancer tissues and paracancer tissues. (E-G) Levels detection of circ_0072995 and SHMT2 in breast cancer cells (SKBR3, HCC38, MCF-7, MDA-MB-231 and BT-549) and MCF-10A nonmalignant breast epithelial cells using qRT-PCR or western blot, respectively. *P<0.05.

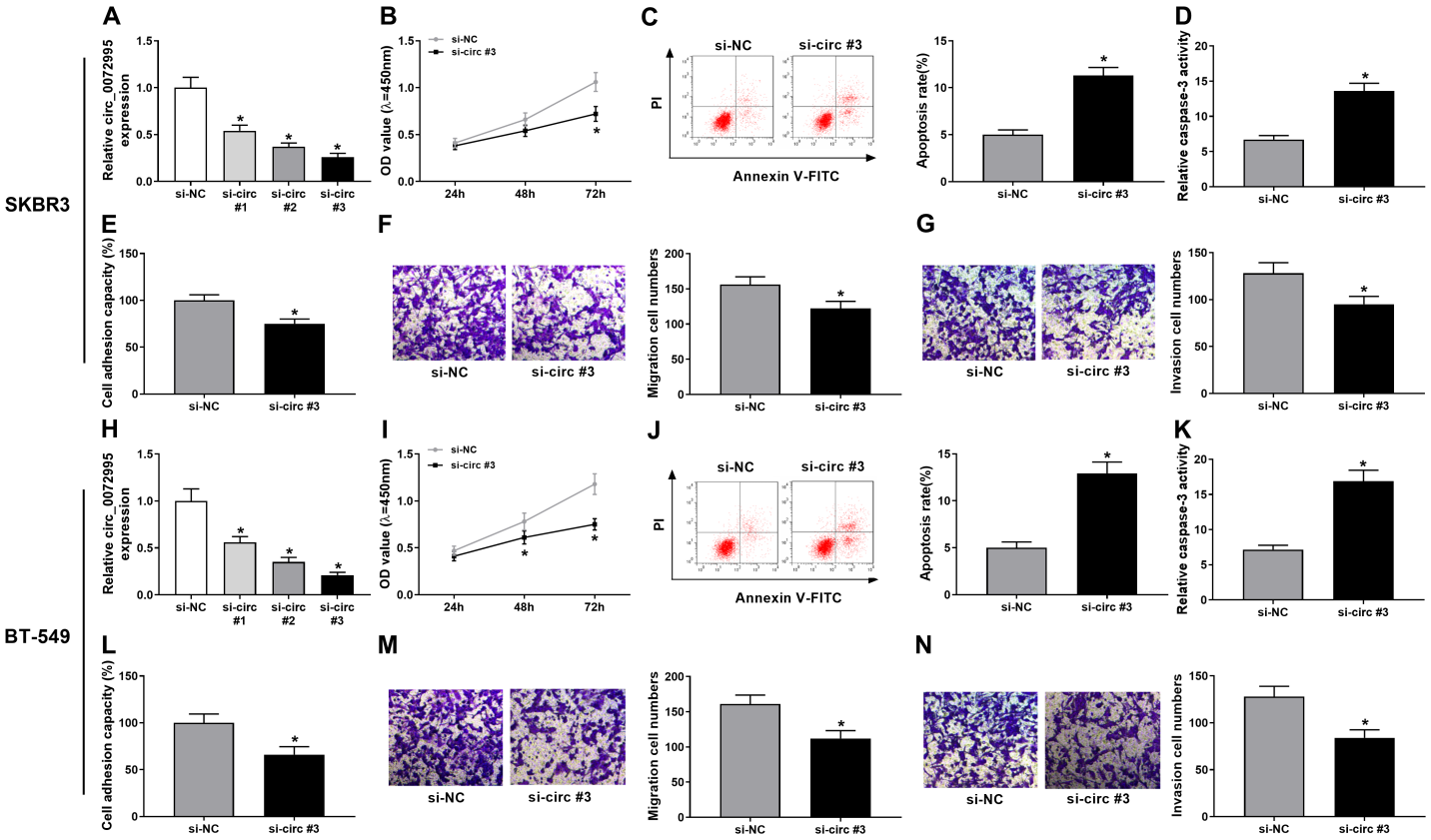

Figure 2

Circ_0072995 knockdown inhibits cell malignant phenotypes in breast cancer. SKBR3 and BT-549 cells were transfected with si-Circ_0072995 (si-circ#1, si-circ#2, and si-circ#3) or si-NC. (A, H) qRT-PCR analysis of circ_0072995 expression in SKBR3 and BT-549 cells after transfection. (B, I) The proliferation analysis of SKBR3 and BT-549 cells using CCK-8 assay. (C, J) Apoptosis analysis of SKBR3 and BT-549 cells using flow cytometry. (D, K) Detection of caspase-3 activity in SKBR3 and BT-549 cells using a colorimetric assay kit. (E, L) The analysis of cell adhesion ability in SKBR3 and BT-549 cells using cell adhesion assay. (F, G, M, N) Analysis of SKBR3 (F, G) and BT-549 (M, N) cell migration and invasion with transwell assay. *P<0.05.

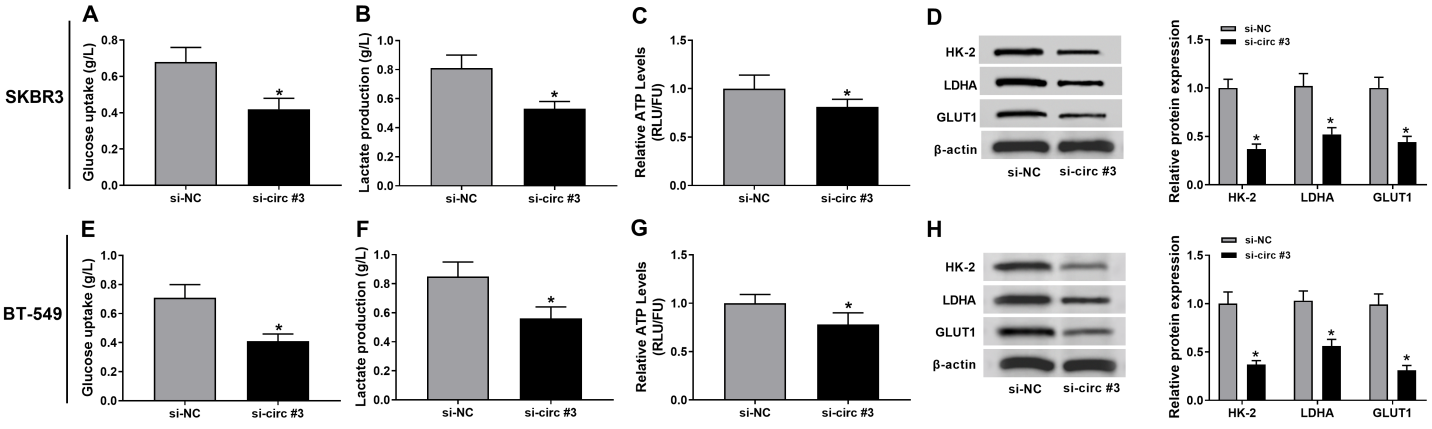

Figure 3

Circ_0072995 knockdown inhibits cell anaerobic glycolysis in breast cancer. (A, E) The glucose uptake, (B, F) lactate production, and (C, G) ATP levels analysis in SKBR3 and BT-549 using a glucose, lactate and ATP assay kit, respectively. (D, H) Levels detection of HK-2, LDHA, and GLUT1 protein using western blot in SKBR3 and BT-549 cells. *P<0.05.

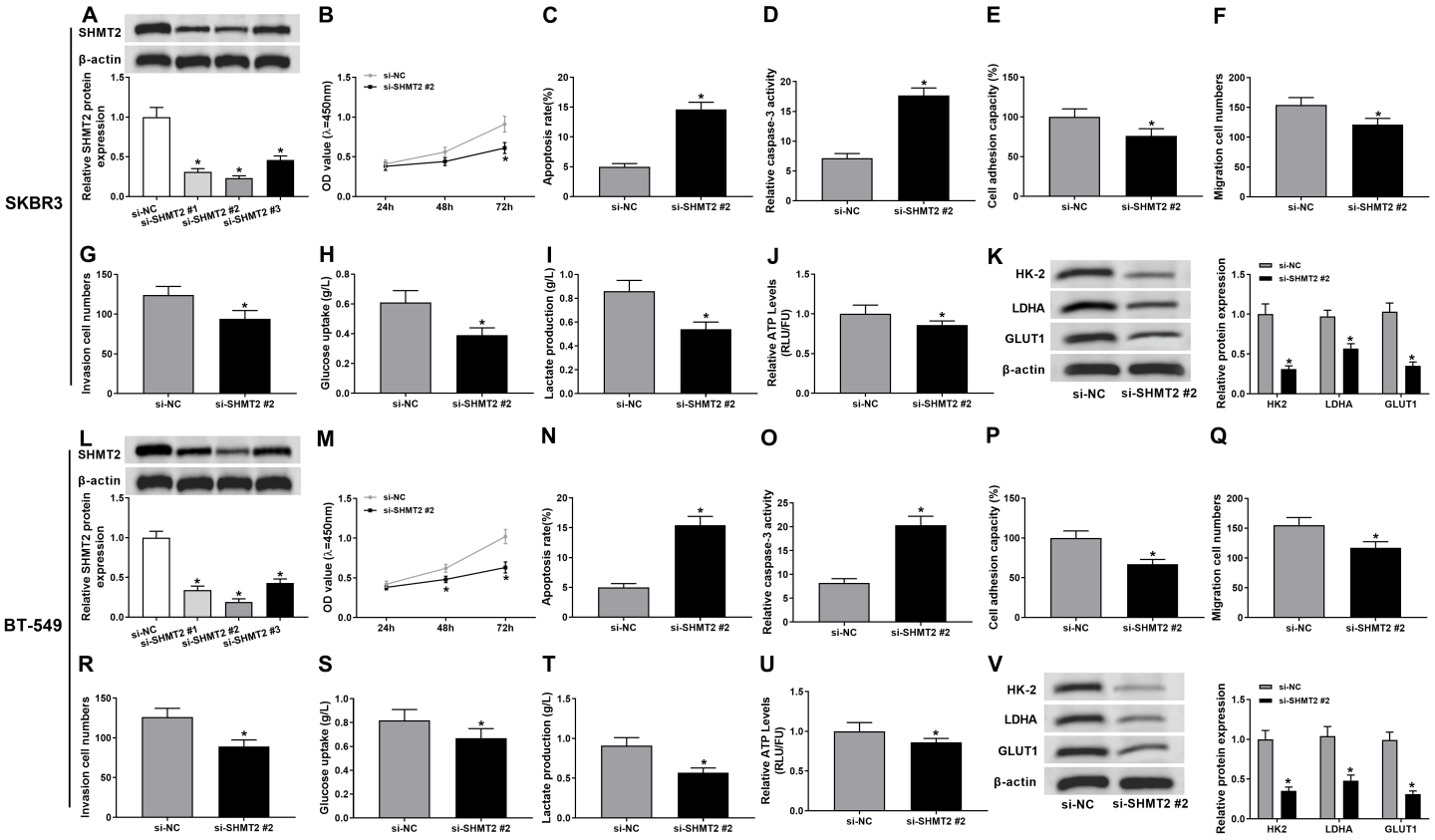

Figure 4

SHMT2 knockdown inhibits cell malignant phenotypes and anaerobic glycolysis in breast cancer. SKBR3 and BT-549 cells were transfected with si- SHMT2 (si-SHMT2 #1, si-SHMT2 #2, and si-SHMT2 #3) or si-NC. (A, L) Western blot analysis of SHMT2 expression in SKBR3 and BT-549 cells after transfection. (B, M) CCK-8 assay analysis of SKBR3 and BT-549 cell proliferation. (C, N) Apoptosis analysis of SKBR3 and BT-549 cells using flow cytometry. (D, O) Caspase-3 activity analysis of SKBR3 and BT-549 cells using a colorimetric assay kit. (E, P) The analysis of cell adhesion ability in SKBR3 and BT-549 cells using cell adhesion assay. (F, Q, G, R) The analysis of migratory and invasive capabilities of SKBR3 and BT-549 cells with transwell assay. (H, S) Detection of glucose uptake, (I, T) lactate production, and (J, U) ATP levels in SKBR3 and BT-549 using a glucose, lactate and ATP assay kit, respectively. (K, V) Levels detection of HK-2, LDHA, and GLUT1 protein using western blot in SKBR3 and BT-549 cells. *P<0.05.

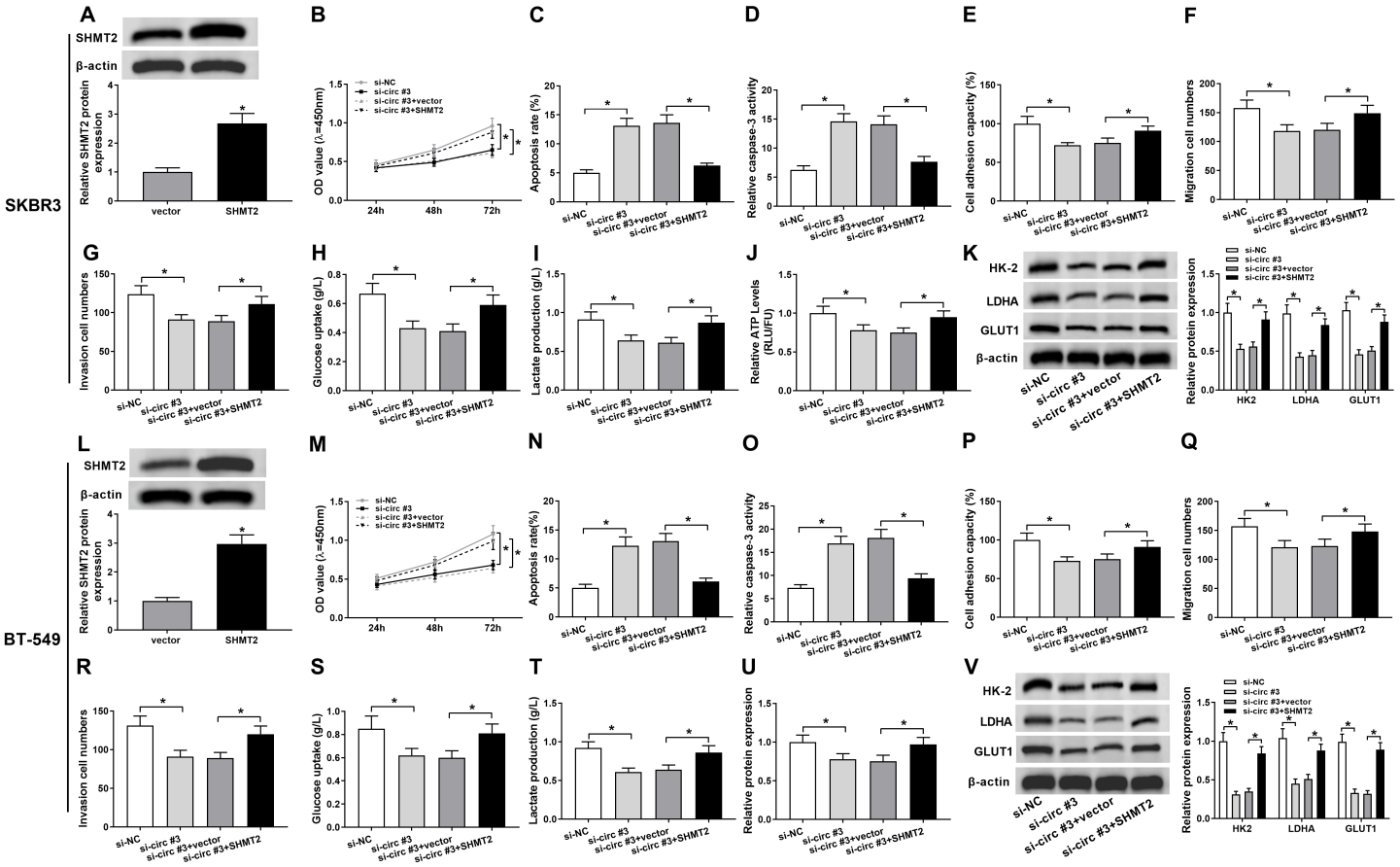

Figure 5

SHMT2 overexpression reverses the anticancer function of si-Circ_0072995 in breast cancer cells. (A, L) Western blot analysis of SHMT2 protein expression in SKBR3 and BT-549 cells transfected with SHMT2 or vector. SKBR3 and BT-549 cells were transfected with si-NC, si-circ#3, si-circ#3 + vector, or si-circ#3 + SHMT2. (B, M) CCK-8 assay analysis of SKBR3 and BT-549 cell proliferation. (C, N) The analysis of SKBR3 and BT-549 cell apoptosis using flow cytometry. (D, O) Caspase-3 activity analysis of SKBR3 and BT-549 cells using a colorimetric assay kit. (E, P) Cell adhesion ability analysis in SKBR3 and BT-549 cells using cell adhesion assay. (F, Q, G, R) The analysis of the migratory and invasive capabilities of SKBR3 and BT-549 cells using transwell assay. (H, S) Detection of glucose uptake, (I, T) lactate production, and (J, U) ATP levels in SKBR3 and BT-549 cells using a glucose, lactate and ATP assay kit, respectively. (K, V) Western blot analysis of HK-2, LDHA, and GLUT1 protein in SKBR3 and BT-549 cells. *P<0.05.

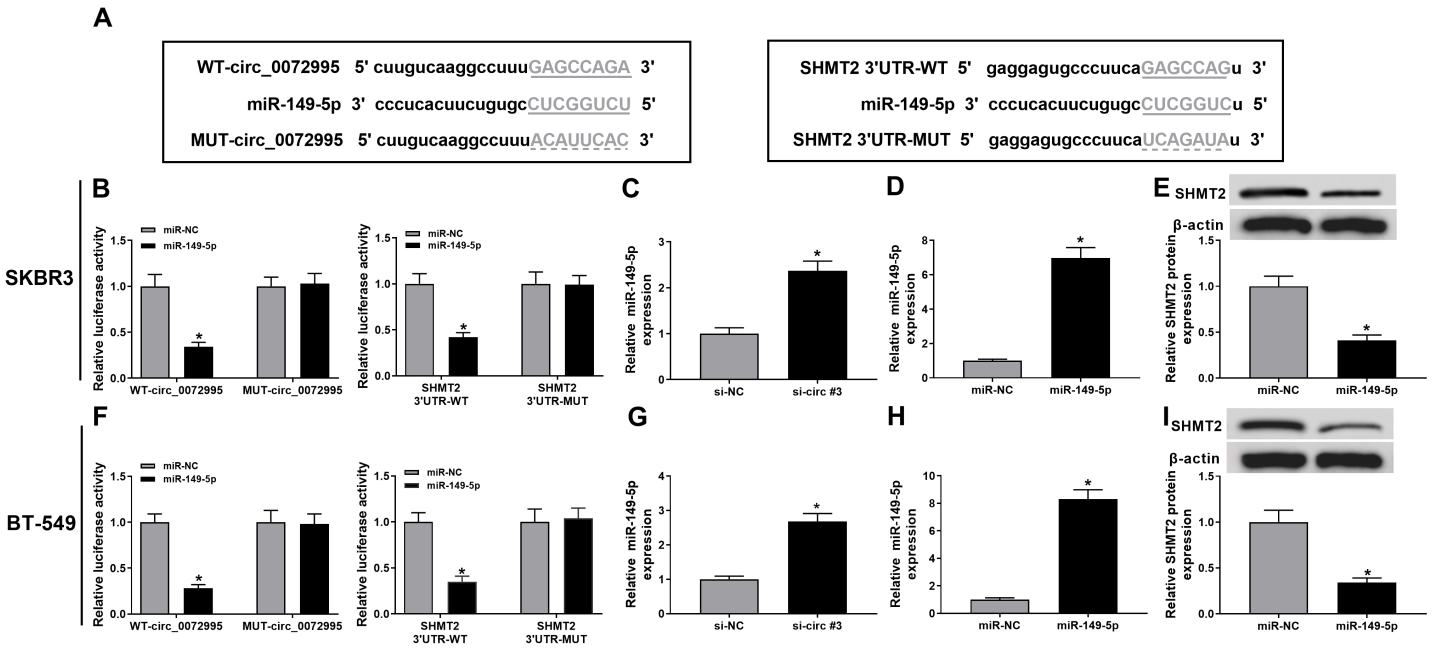

Figure 6

MiR-149-5p directly binds to circ_0072995 or SHMT2 in breast cancer cells. (A) The predicted binding sites of miR-149-5p on circ_0072995 or SHMT2 sequences. (B, F) Interaction analysis between miR-149-5p and circ_0072995 or SHMT2 in SKBR3 and BT-549 cells using the dual-luciferase reporter assay. (C, G) qRT-PCR analysis of miR-149-5p expression in SKBR3 and BT-549 cells transfected with si-NC or si-circ#3. (D, H) qRT-PCR analysis of miR-149-5p expression in SKBR3 and BT-549 cells transfected with miR-149-5p or miR-NC. (E, I) Western blot analysis of SHMT2 protein expression in SKBR3 and BT-549 cells transfected with miR-149-5p or miR-NC. *P<0.05.

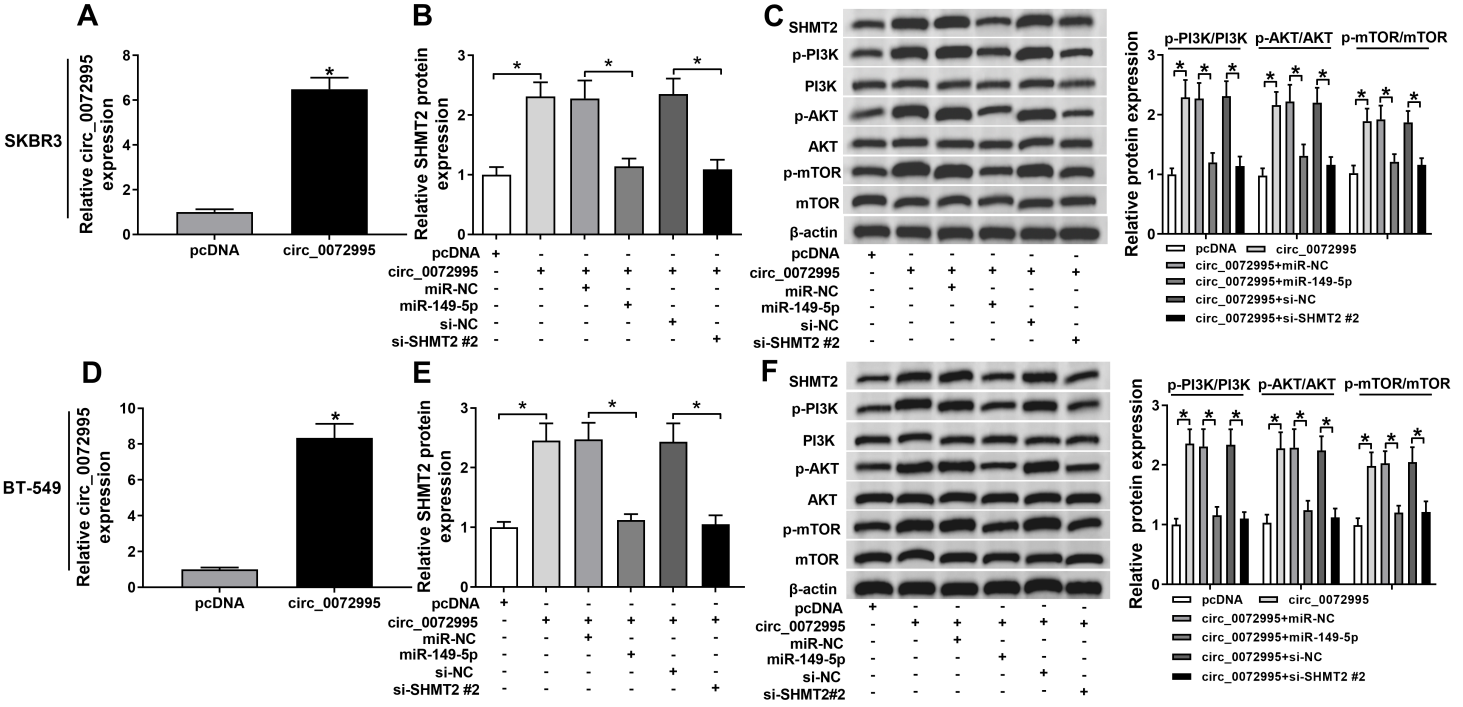

Figure 7

Circ_0072995 activates PI3K/AKT/mTOR pathway via miR-149-5p/SHMT2 axis. (A, D) qRT-PCR analysis of circ_0072995 in SKBR3 and BT-549 cells transfected with pcDNA or circ_0072995. SKBR3 and BT-549 cells were transfected with pcDNA, circ_0072995, circ_0072995 + miR-NC, circ_0072995 + miR-149-5p, circ_0072995 + si-NC, or circ_0072995 + si-SHMT2 #2. After transfection, (B, E) qRT-PCR analysis of SHMT2 mRNA in SKBR3 and BT-549 cells; (C, F) western blot analysis of SHMT2 and PI3K/AKT/mTOR pathway-related protein in SKBR3 and BT-549 cells. *P<0.05.

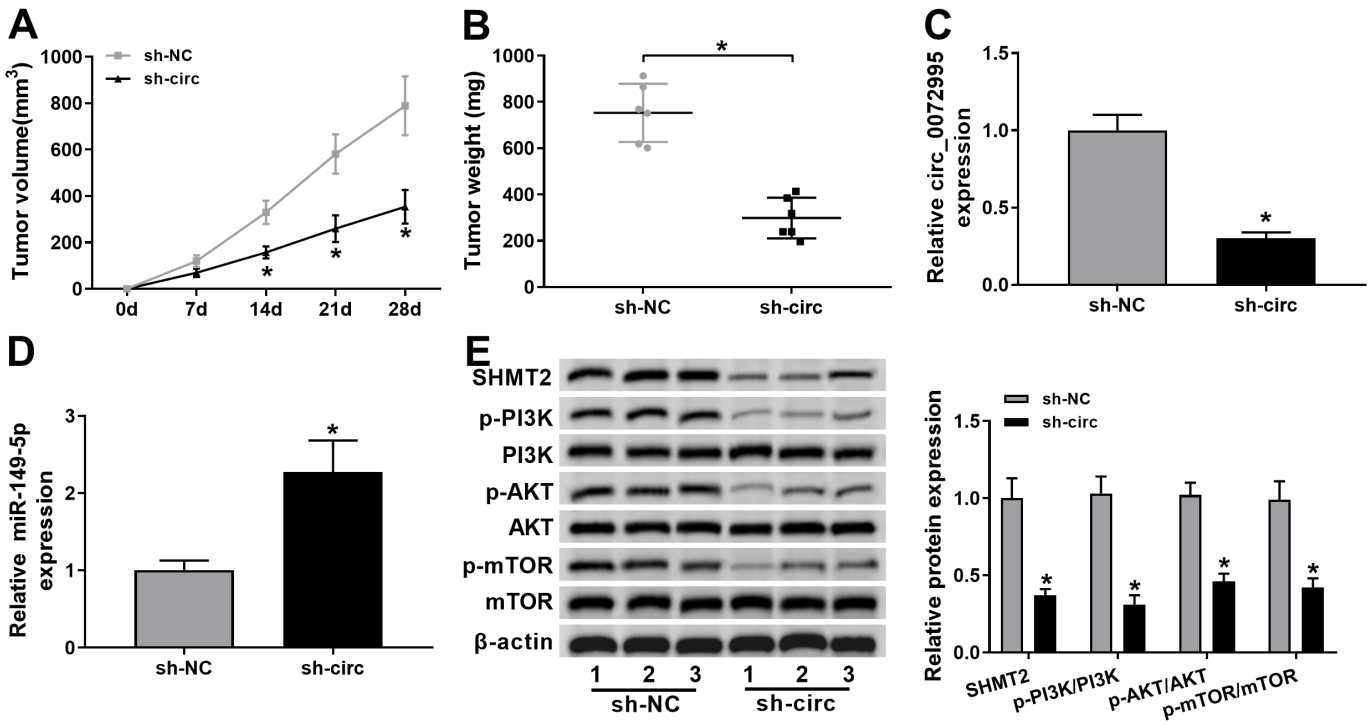

Figure 8

Circ_0072995 impedes the growth of xenograft tumors in vivo. (A, B) Measurement of the size and weight of xenograft tumors. (C, D) qRT-PCR analysis of circ_0072995 and miR-149-5p levels in tumor masses from each group. (E) Western blot analysis of SHMT2 and PI3K/AKT/mTOR pathway-related protein levels in tumor masses. *P<0.05.