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CircDDX17 acts as a competing endogenous RNA for miR-605 in breast cancer progression

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Abstract. – OBJECTIVE: Circular RNAs (circRNAs), a novel class of noncoding RNAs, are reported to be involved in the progression of various cancers. CircDDX17 was reported as a tumour suppressor in colorectal cancer. However, the expression and role of circDDX17 in breast cancer remain unclear.

PATIENTS AND METHODS: We used qPCR analysis to reveal the expression levels of circRNAs and miRNAs in breast cancer tissues and cell lines. The target relationship between circRNA and miRNAs was predicted using miRanda and then detected using a Luciferase reporter assay. The effects of circDDX17 and miR-605 on the growth of breast cancer were detected using MTT, colony formation assay and apoptosis analysis.

RESULTS: In this study, low circDDX17 expression was observed in breast cancer tissues and cell lines. Moreover, circDDX17 expression was inversely associated with the clinicopathological parameters of tumour grade and advanced TNM stage ($p<0.05$). Functionally, overexpressed circDDX17 inhibited cell proliferation and colony formation and promoted cell apoptosis in breast cancer. Mechanistically, circDDX17 directly bound to miR-605, which functions as an oncogene in breast cancer, and its expression was associated with low overall survival of breast cancer patients. Finally, we found that circDDX17 suppressed cell proliferation by regulating cell cycle-related factors (CDK1 and p21), and the effect was reversed by miR-605 mimics.

CONCLUSIONS: We identified the downregulation of circDDX17 in breast cancer, and circDDX17 acted as a tumour suppressor by inhibiting proliferation and promoting apoptosis through its function as a sponge of miR-605 in breast cancer, indicating that it serves as a potential biomarker and a therapeutic target for breast cancer.

Key Words:
Circular RNA, MiR-605, Breast cancer, CircDDX17.

Introduction

Breast cancer is one of the most common cancers and the leading cause of cancer mortality worldwide[1]. Although great improvements in surgical therapy and chemotherapies have been made, the survival of breast cancer patients remains very poor[2]. Therefore, it is urgent to elucidate the molecular mechanisms of breast tumourigenesis, which may lead to improvements in the therapy of breast cancer patients.

Circular RNAs (circRNAs), a class of widespread and diverse endogenous noncoding RNAs, are widely expressed in various cells and play an important role in various biological functions[3-5]. CircRNAs are dysregulated in several tumour tissues and are involved in the progression of cancer[6]. Notably, Tang et al[7] detected a lower expression of circ-KIAA1244 in gastric cancer using circRNA expression profiles, and they found that the low expression of circ-KIAA1244 was associated with clinicopathological parameters in gastric cancer patients. Tan et al[8] detected an upregulation of circular RNA F-circEA-2a in non-small cell lung cancer, and the function of this circRNA as a circRNA was reported to be involved in cell migration and invasion. Moreover, ribosomal-depleted RNA sequencing data identified at least 27,000 circRNA candidates, and most of them were dysregulated between the tumour tissue and normal tissue[9]. High-throughput RNA sequencing (RNA-seq) also identified 6,154 distinct circRNAs and 448 circRNAs that were significantly dysregulated in bladder cancer tissues[10]. In breast cancer, circRNA microarray analysis revealed 1705 dysregulated circRNAs in breast cancer tissues[11]. However, to our knowledge, the role and mechanism of circRNAs in breast cancer have not been well explored.

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MicroRNAs are a class of small noncoding RNAs that regulate gene expression by binding to the 3'UTR of mRNA[12]. Several reports[13,14] have shown dysregulated miRNAs in various tumour tissues and the involvement of these miRNAs in the progression of cancer. Li et al[10] showed that miRNAs in cancers could regulate circRNAs. Based on the presence of specific miR binding sites between circRNAs and miRNAs, circRNAs function as miR "sponges" and are involved in the progression of various cancers[10]. Hsa_circ_0061140 acts as a miR-370 sponge and is involved in the proliferation and metastasis of ovarian cancer cells[4]. The circRNA circHIPK3 clearly represses the progression of bladder cancer by sponging miR-558[10]. The circRNA circNT5E regulates the tumourigenesis of glioblastoma by functioning as a sponge of miR-422a[15]. CircDDX17 was first detected to be downregulated in colorectal cancer using high-throughput RNA sequencing (RNA-seq)[16]. However, the expression and role of circDDX17 in breast cancer remain unclear.

In this study, we found that circDDX17 was downregulated in breast cancer and that circDDX17 acted as a miR-605 sponge to suppress the proliferation of breast cancer.

Patients and Methods

Patients and Specimens

Breast cancer tissues and adjacent normal tissues were collected from patients from 2016 to 2018 at the Third Xiangya Hospital of Central South University. The tissues were stored immediately in liquid nitrogen. The experiments were approved by the Ethics Committee of The Third Xiangya Hospital of Central South University. Written informed consent was obtained from all patients before participation in this study.

Cell Culture and Transfection

Breast cancer cell lines (MCF-7, MCF-10A, BT474, BT549, and HCC2218) and normal human breast epithelial cells (HBL-100) were purchased from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Grand Island, NY, USA) with 10% FBS (Gibco, Grand Island, NY, USA) at 37°C.

To construct a circDDX17-overexpressing plasmid, we synthesized circDDX17 cDNA and cloned it into the pcD-ciR vector (Geneseed Biotech Co., Guangzhou, China). The circDDX17 plasmid was transfected into breast cancer cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). Then, the cells were selected with G418 for 4 weeks to obtain circDDX17-overexpressing cells.

miRNA mimics and inhibitors were synthesized by Gene-Pharma (Shanghai, China) and transfected into breast cancer cells using Lipofectamine RNAiMax (Life Technologies, Carlsbad, USA) as previously described[10].

Quantitative Real-Time PCR

Total RNA was obtained from breast cancer tissue and cells using TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). The expression levels of miR-605 and circDDX17 were detected using TB Green™ Premix Ex Taq™ II (TaKaRa, Dalian, China). 18S rRNA was used as an internal control.

Western Blots

Total protein was prepared using radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Waltham, MA, USA). The anti-CDK1 (1:1000, Sigma-Aldrich, St. Louis, MO, USA), anti-p21 (1:1000, Sigma-Aldrich), and anti-GAPDH antibodies (1:1,000, Proteintech, Rosemont, IL, USA) were incubated at 4°C overnight. CDK1 and p21 expression levels were analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Breast cancer cells were seeded into 6-well plates for colony formation assays. Then, 4% paraformaldehyde was used to fix the colonies for 15 min, and 0.1% crystal violet was used to stain the colonies for 15 min.

Cell Proliferation Assay

The cell proliferation ability was analysed using a CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) at 0, 24, 48 and 72 h, as previously described.

Dual-Luciferase Reporter Assay

We cloned DDX17 into the pGL3 Luciferase reporter vector (Promega, Madison, WI, USA). Mut-DDX17 pGL3 was obtained by site-directed mutagenesis. The DDX17-pGL3 or mut DDX17-pGL3 plasmid was cotransfected with miR-NC or miR-605 into breast cancer cells, and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to detect Luciferase activities.

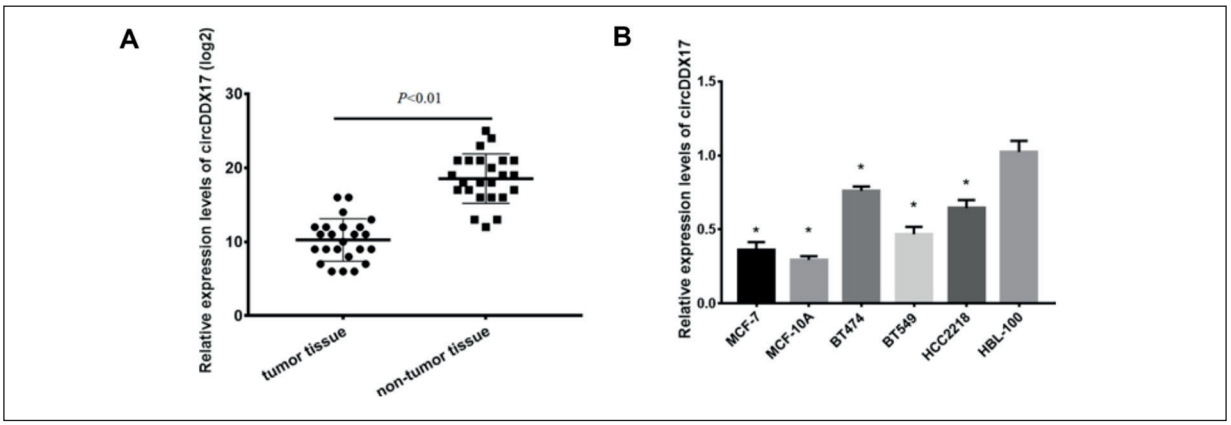

Figure 1. CircDDX17 expression in breast cancer. **A**, CircDDX17 expression in breast cancer tissue. Data are presented as the mean ± S.D. *, $p < 0.01$, compared to non-tumour tissues. **B**, CircDDX17 expression in breast cancer cell lines. Data are presented as the mean ± SD, *, $p < 0.01$, compared to HBL-100 cells.

Apoptosis Analysis

The Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) was used to detect apoptotic cells as previously described. Cell apoptosis was determined using a FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA), and FlowJo Version 10 software was used for data analysis.

RNA Fish

The oligonucleotide probe for hsa_circDDX17 was synthesized by Songan Biotech, and an *in situ* hybridization kit (RiboBio, Guangzhou, China) was used for FISH as previously described[17]. A Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to obtain confocal images.

Statistical Analysis

The data were analysed using SPSS 23.0 (SPSS Inc., Armonk, NY, USA). Student's *t*-test, one-way ANOVA, and chi-square test were used. A *p*-value <0.05 was considered statistically significant. A Kaplan-Meier plot was generated using the Cancer Genome Atlas (TCGA) data to explore the survival rate of breast cancer patients with low and high expression of miR-605.

Results

CircDDX17 Was Downregulated in Breast Cancer Tissue and Cell Lines

CircDDX17 is a novel circRNA reported to be involved in the progression of colorectal cancer[16]; however, the expression and potential role of circDDX17 in breast cancer remain unclear. Here, we used qRT-PCR analysis to detect circDDX17 expression in human breast cancer tissues compared to noncancerous tissues. As shown in Figure 1A, circDDX17 was clearly expressed at low levels in breast cancer tissues (Figure 1A). Moreover, circDDX17 expression was also reduced in breast cancer cell lines (MCF-7, HCC2218, BT474, BT549, and MCF-10A) compared with the normal breast epithelial cell line HBL-100 (Figure 1B). We next analysed the clinicopathological characteristics and found that circDDX17 expression was inversely associated with tumour grade, lymph node infiltration and advanced TNM stage (Table I). **Supplementary Figure 1** shows that circDDX17 was localized in the cytoplasm of

Table I. The relationship of circRNAs expression levels (ΔCt) in cancer tissues with clinicopathological factors of patients with breast cancer.

Characteristics	CircDDX17	
	Mean	*p*
Age		0.97
≤ 60	10.9 ± 0.8	
> 60	10.8 ±0.9	
Tumor size		0.0028
≤ 2.0 cm	9.3 ± 0.6	
> 2.0 cm	12.11 ± 0.5	
Lymph node infiltrated		0.0018
No	12.2 ± 0.55	
Yes	9.1 ± 0.58	
TNM staging		0.0025
I- II	12.1 ± .57	
III-IV	9.1 ± .61	

MCF-7 cells. In conclusion, these results demonstrate that circDDX17 is a tumour suppressor in breast cancer.

CircDDX17 Suppressed Tumour Growth of Breast Cancer Cells

To further reveal the biological function of circDDX17 in breast cancer, we transfected lentivirus-circDDX17 (LV-circDDX17) into breast cancer cells for subsequent functional analyses. As shown in Figure 2A, circDDX17 was increased in circDDX17-stable cell lines. We next performed CCK-8 and colony formation assays to detect the role of circDDX17 in the proliferation of breast cancer cells. We found that overexpressed circDDX17 reduced the proliferation of both breast cancer cell lines MCF-7 and MCF-10A (Figure 2B and 2C). The cell cycle results showed that overexpressed circDDX17 induced cell cycle arrest at G0/G1

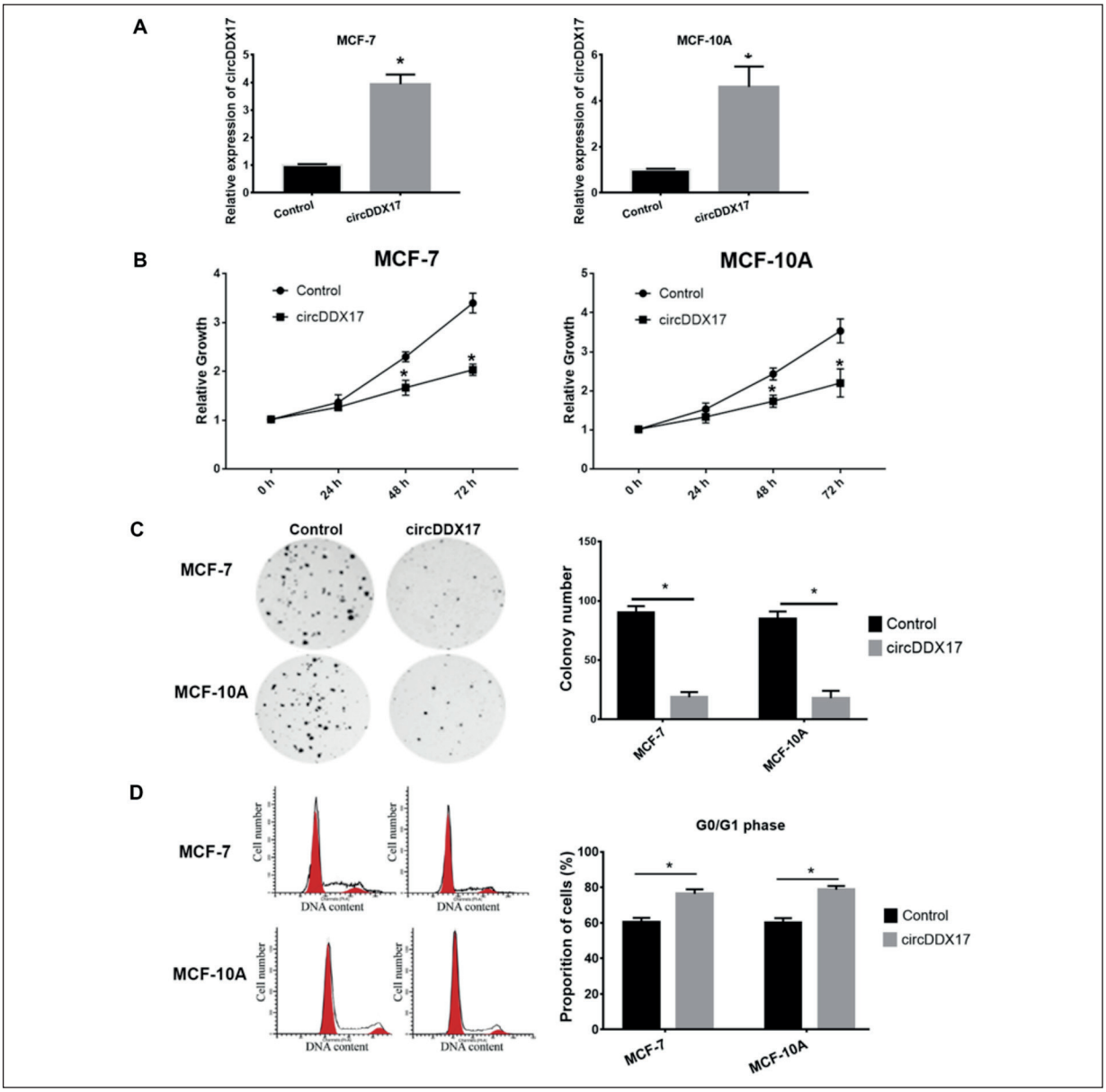

Figure 2. CircDDX17 suppressed tumour growth in breast cancer. **A**, LV-circDDX17 clearly upregulated the expression of circDDX17 in MCF-7 and MCF-10A breast cancer cells. **B**, Overexpressed circDDX17 reduced the proliferation of breast cancer cells. **C**, Overexpressed circDDX17 reduced the colony formation of breast cancer cells (magnifications 1×). **D**, Overexpressed circDDX17 induced cell cycle arrest at the G0/G1 phase in breast cancer cells. Data are presented as the mean ± SD, *, $p < 0.01$, compared to the control group.

phase in MCF-7 and MCF-10A cells (Figure 2D). These results indicated that circDDX17 suppressed tumour growth in breast cancer.

MiR-605 Is a Direct Target of CircDDX17 in Breast Cancer

It has been reported that circRNAs function as regulators of miRNAs in various cancers[10,18]. To determine whether circDDX17 could directly target miRNAs in breast cancer, we predicted candidate target miRNAs of circDDX17 using miRanda (Figure 3A) and then used a Dual-Luciferase reporter assay system to investigate the target relationship between circDDX17 and these miRNAs in breast cancer cells. As shown in Figure 3B, miR-605 significantly reduced the fluorescence activity in the circDDX17 group but did not affect the fluorescence activity in the mutant circDDX17 group. We next analysed the effect of circDDX17 on miR-605 expression and found low levels of miR-605 in cells stably overexpressing circDDX17 (Figure 3C).

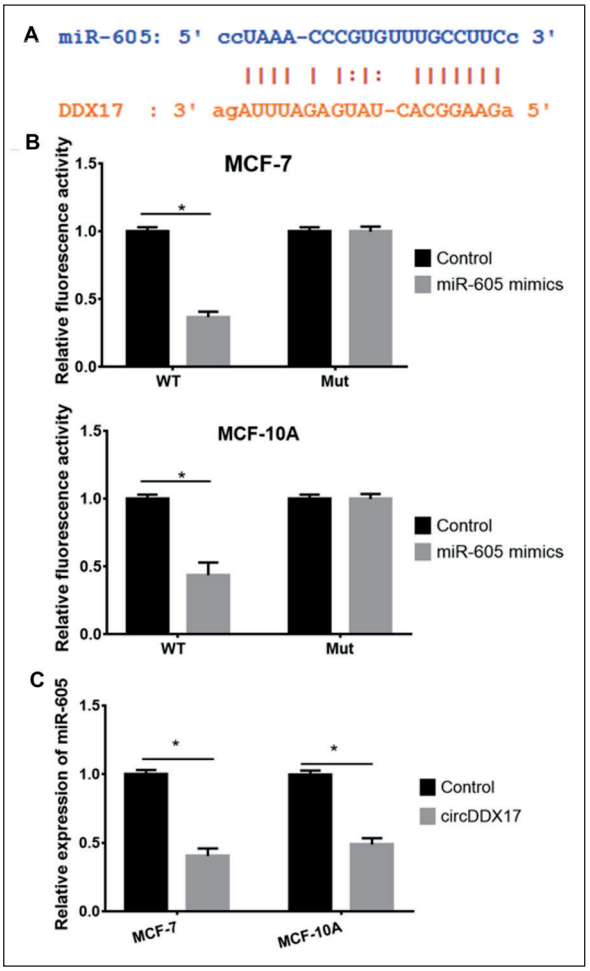

Figure 3. MiR-605 is a direct target of circDDX17 in breast cancer. **A**, Bioinformatics analysis predicted the putative target site of circDDX17 on miR-605. **B**, MiR-605 repressed the fluorescence activity of circDDX17 in breast cancer. **C**, CircDDX17 repressed miR-605 expression in breast cancer.

MiR-605 Expression Was Upregulated in Breast Cancer

We next detected miR-605 expression in breast cancer tissues and cell lines. Figure 4A shows that miR-605 expression was much higher in breast cancer tissues than in adjacent normal tissues. Moreover, miR-605 was also highly expressed in breast cancer cell lines compared with the HBL-100 cell line (Figure 4B). The Kaplan-Meier survival analysis revealed that patients with higher miR-605 levels showed worse overall survival (Figure 4C). Moreover, the miR-605 mimics clearly induced cell proliferation and suppressed cell apoptosis in both MCF-7 and MCF-10A cell lines (Figure 4D-F).

CircDDX17 Suppressed the Proliferation of Breast Cancer Cells Via MiR-605

To reveal the role of miR-605 in circDDX17-mediated suppression of tumour growth in breast cancer, we used miR-605 mimics to overexpress miR-605. As shown in Figure 5A, miR-605 mimics clearly induced miR-605 expression. MiR-605 mimics significantly induced cell growth and colony formation and inhibited the cell cycle in circDDX17-expressing stable cell lines (Figure 5A-C). To further investigate the underlying mechanisms of circDDX17 suppressing cell proliferation, proteins important for the cell cycle (CDK1 and p21) were detected as shown in Figure 5D. The results showed that circDDX17 clearly repressed CDK1 expression and increased p21 expression, and these effects were reversed by miR-605 mimics. In conclusion, these results indicated that circDDX17 suppressed the proliferation of breast cancer cells via miR-605.

Discussion

CircRNAs are a novel class of endogenous noncoding RNAs that are widespread in various organisms and conserved across species[5]. Some studies[11,19,20] have shown the involvement of circRNAs in the tumourigenesis of various cancers, including breast cancer. Lu et al[11] identified 1155

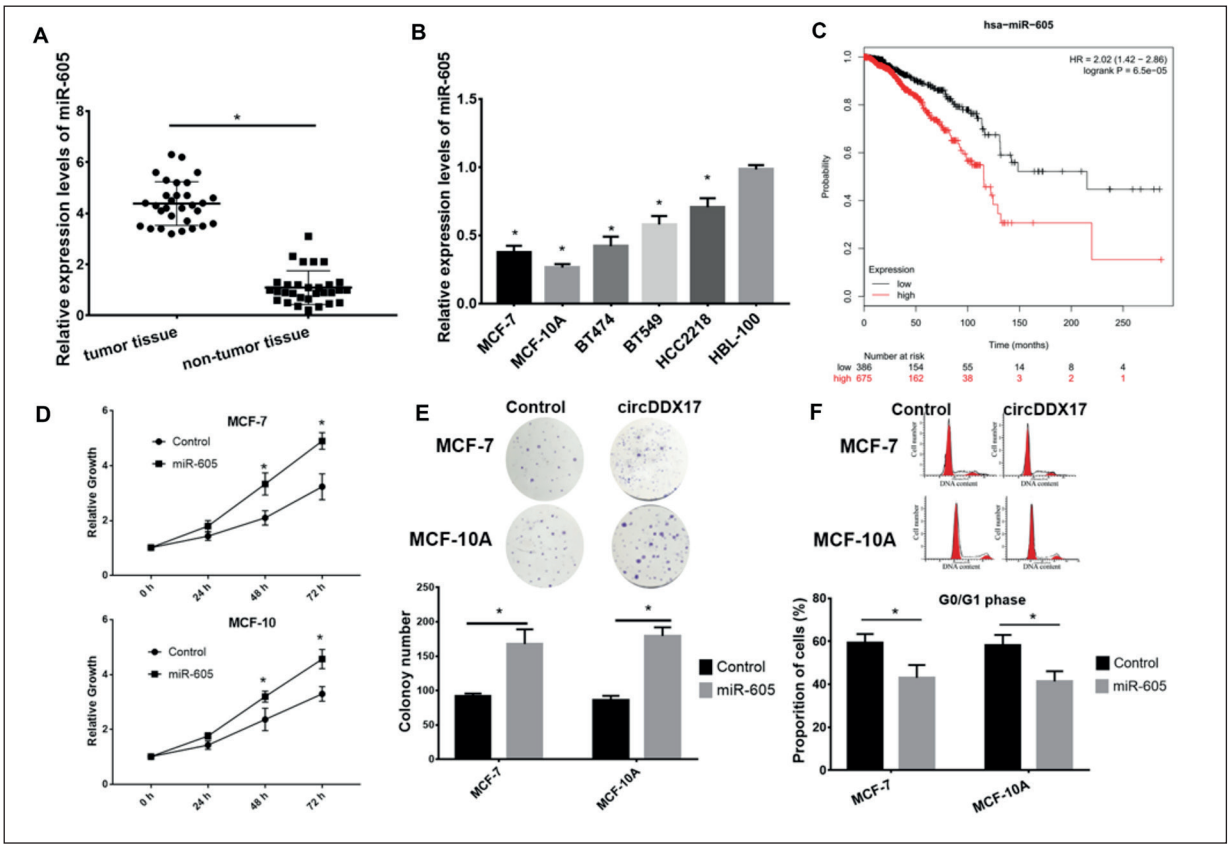

Figure 4. MiR-605 expression was upregulated in breast cancer. **A**, MiR-605 expression in breast cancer tissue. Data are presented as the mean ± SD, *, $p < 0.01$, compared to non-tumour tissues. **B**, MiR-605 expression in breast cancer cell lines. **C**, Kaplan-Meier analysis of overall survival curves for breast cancer patients with low *vs.* high expression of miR-605. Data are presented as the mean ± SD, *, $p < 0.01$, compared to HBL-100 cells. **D**, MiR-605 mimics increased the proliferation of breast cancer cells. **E**, MiR-605 mimics increased colony formation in breast cancer (magnifications 1×). **F**, MiR-605 mimics reduced the proportion of breast cancer cells in G0/G1 phase. Data are presented as the mean ± SD, *, $p < 0.01$, compared to the control group.

dysregulated circRNAs in breast cancer using a circRNA array. Gao et al[21] showed the significant upregulation of circRNA_0006528, which was associated with TNM stage and poor prognosis of breast cancer. Wu et al[22] showed that circIRAK3, a novel circRNA in breast cancer, is involved in the metastasis of breast cancer. CircDDX17 (also named hsa_circ_0002211) was reported to be clearly downregulated and associated with the clinicopathological characteristics of colorectal cancer patients[16]. However, the involvement of circDDX17 in the tumourigenesis and progression of breast cancer remains unclear. In this study, we found that circDDX17 was significantly downregulated in breast cancer tissue and cells. The expression of circDDX17 was associated with clinicopathological characteristics, as well as poor overall survival of patients. CircDDX17 was involved in the tumourigenesis and progression of breast cancer by sponging miR-605.

MiRNAs, as a class of small noncoding RNAs, have been reported to be involved in the tumourigenesis and progression of various cancers, including breast cancer. Chai et al[13] showed that miR-498 functions as an oncogene and promotes the proliferation and migration of breast cancer. MiR-27b, an oncogenic miRNA, was reported to deregulate cell metabolism in breast cancer[23]. MiRNA-605 is dysregulated and functions as a tumour suppressor in various cancers, including melanoma[24], intrahepatic cholangiocarcinoma[25] and prostate cancer[26]. In this study, miR-605 expression was significantly upregulated in breast cancer. Circular RNA acting as a competing endogenous RNA has been reported to be involved in the progression of bladder cancer by targeting miRNA[27-29]. In this study, circDDX17 was found to function as a miRNA sponge and directly targeted miR-605 in breast cancer cells. CircDDX17 suppressed cell proliferation by downregulating key proteins related to

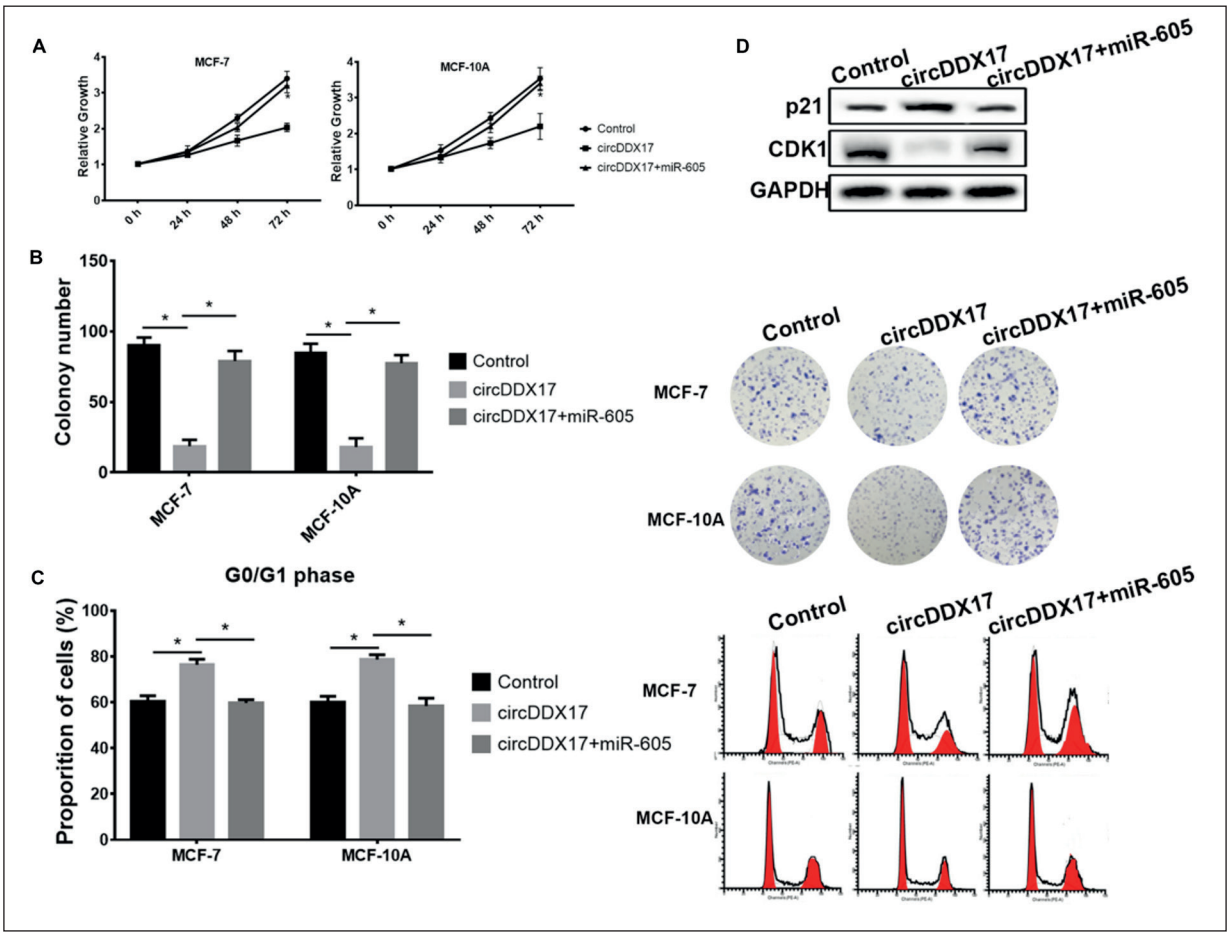

Figure 5. CircDDX17 suppressed the proliferation of breast cancer cells via miR-605. **A**, CCK-8 analysis. **B**, Colony formation assays (magnifications 1×) and **C**, cell cycle analysis revealed the cell proliferation ability of breast cancer cells. **D**, Western blot analysis revealed the expression levels of p21 and CDK1. Data are presented as the mean ± S.D. *, $p < 0.01$.

the cell cycle (CDK1 and p21), and this effect was reversed by miR-605 mimics. CircRNA circ-VANGL1 also functions as a competing endogenous RNA of miR-605 in bladder cancer[27].

Conclusions

We identified the downregulation of circDDX17 in breast cancer, and circDDX17 is involved in cell proliferation and apoptosis through the regulation of the expression of cell cycle genes (CDK1 and p21) by sponging miR-605 in breast cancer. These results offer new insights into circRNAs as promising prognostic markers and therapeutic targets for breast cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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