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Circ-ITCH regulates triple-negative breast cancer progression through the Wnt/β-catenin pathway

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Recent studies indicate that circular RNA (circRNA) is involved in tumorigenesis, but its role in triple-negative breast cancer (TNBC) remains largely unknown. In this study, we characterized the role of circ-ITCH in TNBC and found that circ-ITCH was significantly down-regulated in TNBC tissues and cell lines and closely associated with poor prognosis. We therefore constructed the MDA-MB-231 and BT-549 TNBC cell lines stably expressing circ-ITCH by lentiviral vectors to determine its underlying mechanisms in TNBC progression. Most importantly, over-expression of circ-ITCH remarkably inhibited TNBC proliferation, invasion and metastasis both *in vitro* and *in vivo*. Mechanistically, we found that circ-ITCH acts as a sponge for miR-214 and miR-17 to increase expression of its ITCH linear isoform, thereby inactivating Wnt/β-catenin signaling. Our combined results show for the first time that circ-ITCH is a tumor suppressor, a promising prognostic biomarker in TNBC and that its restoration could well be a successful strategy in TNBC.

Key words: CircRNA, Circ-ITCH, TNBC, prognosis, metastasis

Triple-negative breast cancer (TNBC) is aggressive with the absence of estrogen, progesterone and human epidermal growth factor receptors and a high risk of distant metastasis and recurrence [1]. Because of the lack of effective molecular targeted drugs, the prognosis of TNBC is poor compared with other breast cancer sub-types [2], and therefore elucidation of the exact pathogenesis underlying TNBC is essential in improving TNBC patient diagnosis, treatment and outcome.

Circular RNA (circRNA) is a class of non-coding RNA characterized by a covalently closed loop with high conservation and stability [3]. Emerging evidence indicates that circRNA has an important role in cancer development and progression with different biological functions, including acting as an miRNA sponge, protein interaction, regulation of gene transcription and translation of new proteins [4, 5]. "miRNA sponging" is the most studied function of circRNA and a large number of current studies have reported that circRNA can sponge miRNA long non-coding RNA in human cancers [6, 7], thus implying that the circRNA-miRNA regulatory network participates in cancer pathogenesis.

Circ-ITCH is proposed to be involved in cancer progression and it is markedly down-regulated in esophageal squamous cell carcinoma [8], colorectal cancer [9], lung cancer [10], glioma [11] and bladder cancer [12] This suggests that it is a tumor suppressor gene and it is reported to interact with miR-216b, miR-7, miR-214, miR-17 and miR-218 to increase the expression of its linear isoform ITCH, thus suppressing canonical Wnt signaling [8, 10, 11]. In addition, circ-ITCH can elevate expression of the popular p21 and PTEN tumor suppressors by sponging miR-17 and miR-224 and thus exert bladder tumor-inhibition [12]. While these confirm that circ-ITCH has a crucial role in human cancer as miRNA sponges, its role in TNBC remains unclear.

This study investigates the expression level of circ-ITCH in TNBC and assesses the clinical significance, biological function and underlying mechanism of circ-ITCH in TNBC

Materials and methods

Patient tissue specimens. A total of 275 breast cancer (52 Luminal A, 87 Luminal B, 45 HER-2 $^{+}$, 91 TNBC) and 68 adjacent normal tissues were collected from Affiliated Shaanxi Fourth People Hospital of Peihua University between January 2008 and December 2013. The histological type of breast cancer was classified according to 4th edition WHO classification: 83.3% of no special type (NST)/invasive ductal carcinoma (IDC), 7.6% special types of invasive breast

carcinoma and 9.1% of ductal carcinoma in situ (DCIS). The clinical-pathological features of these patients are summarized in Table 1. No patient received anti-tumor treatment before surgery, and we obtained informed consent of each patient before this study. We followed-up patients by phone or letter, and the deadline for follow-up was September 2017.

Cell culture. The MCF-10A, MCF-7, T47D, SK-BR-3, MDA-MB-231 and BT-549) cell lines used in this study were purchased from ATCC (Manassas, VA, USA). MCF-10A cells were cultured with DMEM/F12, and other cell lines were cultured with RPMI1640 or DMEM containing 10% fetal bovine serum. Cells were incubated at 37 °C with 5% CO_2.

Quantitative real-time polymerase chain reaction. Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) and its quality was determined by Micro-spectrophotometer. Detection of RNA expression was conducted by SYBR Premix Ex Taq Kit (Takara, Japan) and the ABI Prism 7500 sequence detection system. GAPDH and U6 were used as internal controls for circRNA/mRNA and miRNA; respectively. All experiments were in triplicate.

Establishment of circ-ITCH-over-expressing cell lines. The circ-ITCH cDNA sequence was synthesized and subcloned into pLO-ciR vector from Geenseed Biotech Company for its over-expression (Guangzhou, China). MDA-MB-231 and BT-549 cells were transduced with the lentiviral vector according to the manufacturer's instructions and selected by puromycin.

Table 1. Association of circ-ITCH expression with the clinical parameters in 91 triple-negative breast cancer patients.

Parameters	Total (n=91)	circ-ITCH expression		p-value
		Low (n=46)	High (n=45)	
Age (years)				
≤40	24	10	14	0.310
>40	67	36	31	
Menopause				
Yes	43	20	23	0.466
No	48	26	22	
Tumor size (cm)				
≤2	41	15	26	**0.016**
>2	50	31	19	
LN metastasis				
Negative	38	13	25	**0.008**
Positive	53	33	20	
TNM stage				
I	20	7	13	
II	46	19	27	**0.002**
III	25	20	5	
Histological Grade				
I	18	10	8	
II	47	20	27	0.267
III	26	16	10	

LN = Lymph node

Cell Counting Kit-8. 1×10^4 cells were seeded in a 96-well plate and then supplemented with 10 µl of CCK-8 solution from Dojindo Laboratory (Kumamoto, Japan). The absorbance at 450 nm was tested by microplate reader at the indicated time. The experiments were repeated three times.

Transwell migration and invasion assay. 2×10^5 cells were seeded in the upper chamber of a 24-well plate with matrigel to assess invasion and cells without migration (BD Biosciences, CA, USA) and 600 µl serum-containing medium was added into the lower chamber. Cells which did not pass through in 24 hours were omitted. The migrated or invaded cells were fixed by paraformaldehyde and then stained and counted.

Luciferase reporter assay. The sequences of circ-ITCH containing wild-type or mutant miR-214 and miR-17 binding site were synthesized and sub-cloned into pmirGLO luciferase reporter vector (Promega) and vectors were then co-transfected with miR-NC, miR-214 and miR-17 mimics into MDA-MB-231 and BT-549 cells, respectively. The luciferase activity was determined by the Dual Luciferase Reporter Assay Kit (Promega). The experiments were repeated three times.

Western blot. Protein was extracted from cell lines using RIPA buffer and then separated by SDS-PAGE and blocked by 5% skimmed milk. Membranes were incubated with corresponding primary and secondary antibody and visualized by multifunctional gel-imaging.

Immunohistochemistry. Immunohistochemistry (IHC) was conducted as described previously, and the IHC value was scored by the semi-quantitative H-score method [13].

Animal studies. A total of twenty 4–6 weeks old female BALB/c nude mice were divided into four groups (n=5). The vector and circ-ITCH-over-expressing MDA-MB-231 cells were respectively subcutaneously and tail vein injected into nude mice to establish xenograft and lung metastatic models. Four weeks later, all mice were euthanized and the tumors and lungs were collected for IHC and H&E staining.

Statistical analysis. The statistical significance between circ-ITCH expression and clinical data of TNBC patients was determined by chi-square test. Group comparisons were measured by unpaired Student's t-test or one-way ANOVA. The survival curve between low- and high-circ-ITCH expression groups was analyzed by Kaplan-Meier plot and the correlations of circ-ITCH with miR-214 and miR-17 were tested by Spearman correlation coefficients. $p<0.05$ was considered statistically significant.

Results

Circ-ITCH is significantly down-regulated in TNBC and correlates with poor TNBC patient outcome. In order to evaluate the expression of circ-ITCH in breast cancer, we collected 275 breast cancer (including 52 Luminal A, 87 Luminal B, 45 HER-2^+ and 91 TNBC) and 68 adjacent normal tissues. The qRT-PCR results showed that circ-ITCH

was down-regulated in breast cancer; especially in TNBC compared to normal tissues (Figure 1A). We then analyzed correlations between circ-ITCH expression and TNBC clinical parameters and found that low circ-ITCH expression was more prone to lymph node metastasis (p=0.008) (Figure 1B), larger tumor size (p=0.016) and advanced TNM stage (p=0.002, Table 1). Moreover, the Kaplan-Meier plot showed that TNBC patients with low circ-ITCH expression had shorter survival times than those with high circ-ITCH expression (p=0.01, Figure 1C). These results indicate that circ-ITCH is a tumor suppressor gene and is important in TNBC progression.

Over-expression of circ-ITCH inhibits TNBC proliferation, migration and invasion *in vitro*. We tested the expression of circ-ITCH in breast cancer cell lines and results showed that circ-ITCH was significantly down-regulated in breast cancer cells compared to normal breast epithelial MCF-10A cells. This was especially noted in TNBC cells and consistent with tissue qRT-PCR. We then established the MDA-MB-231 and BT-549 TNBC cell lines stably over-expressing circ-ITCH and verified their effects (Figure 2B). CCK-8 determined cell proliferation capacity, with ectopic circ-ITCH expression suppressing these cells' proliferation (Figures 2C–D). Figures 2E and F record the Transwell assay evaluation of the cells' ability to migrate and invade; MDA-MB-231 and BT-549 cells with circ-ITCH over-expression had stronger invasion and migration ability than those with the control vector.

Circ-ITCH sponges miR-214 and miR-17 in TNBC. Previous studies have reported that circ-ITCH acts as a sponge for miR-216b, miR-7, miR-214, miR-17 and miR-218 to inhibit cancer progression [8, 10, 11]. We therefore investigated if circ-ITCH can also sponge these miRNAs in TNBC. The expression levels of these five miRNAs were tested in MDA-MB-231 and BT-549 cells with and without circ-ITCH over-expression. The qRT-PCR results revealed that circ-ITCH over-expression dramatically decreases the expression of miR-214 and miR-17, but not miR-216b, miR-7 and miR-218 in both these cells (Figures 3A–B). These results were also confirmed by luciferase reporter assay; that over-expression of miR-214 and miR-17 remarkably reduced luciferase activity of the wild-type circ-ITCH vector, but not the mutant variety (Figures 3C–D). In addition, we found that circ-ITCH negatively correlated with miR-214 (r=–0.398, p=0.001) and miR-17 (r=–0.464, p<0.001) in TNBC tissues, (Figures 3E–F).

Circ-ITCH suppresses TNBC progression by regulating the miR-214/miR-17/ITCH/Wnt/β-catenin axis. To investigate whether miR-214 and miR-17 are involved in circ-ITCH-mediated tumor suppression, circ-ITCH-over-expressing MDA-MB-231 cells were transfected with miR-214 and miR-17 mimics. Figures 4A–C show that ectopic expression of miR-214 or miR-17 partly rescues the decreased proliferation, migratory and invasive ability caused by circ-ITCH over-expression. Previous studies revealed that binding miR-214 and miR-17 to the 3' UTR of ITCH to increase ITCH expression results in Wnt pathway activation [8]. Thus, circ-ITCH can up-regulate the expression of its linear isoform ITCH through sponging miR-214 and miR-17. Consistently, western blot results showed that while ITCH protein expression was markedly increased in circ-ITCH-over-expressing MDA-MB-231 cells compared to control cells, β-catenin protein expression had the opposite trend (Figures 4D–E). We also assessed the well-known downstream genes of Wnt/β-catenin signaling and found that over-expression of circ-ITCH decreased Axin2, c-Myc and Cyclin D1 protein expression (Figures 4D–E). Similarly, the above effects caused by circ-ITCH over-expression were

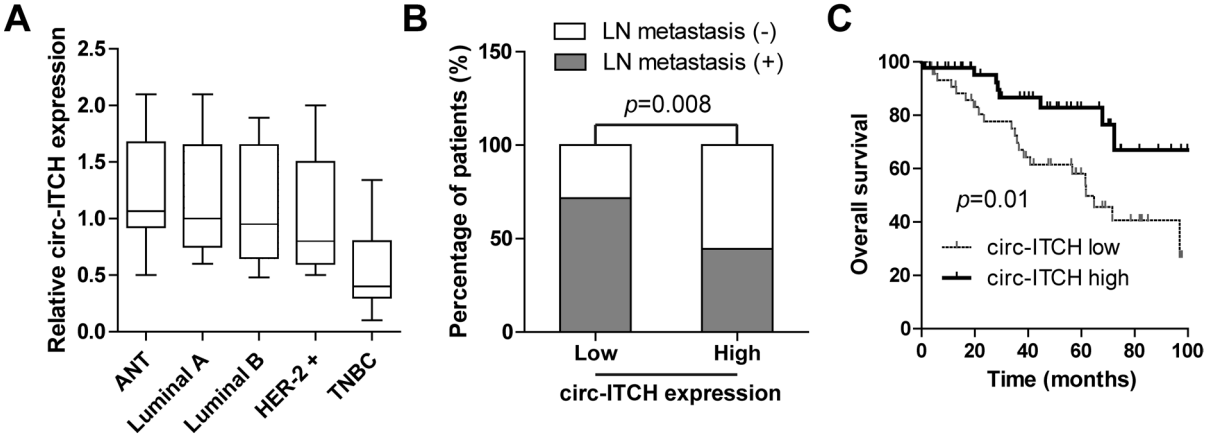

Figure 1. Circ-ITCH is significantly down-regulated in TNBC and correlated with poor prognosis. A) The expression levels of circ-ITCH in Luminal A (n=52), Luminal B (n=87), HER-2 + (n=45), TNBC (n=91) and adjacent normal tissues (n=68) were tested by qRT-PCR; B) The correlation between circ-ITCH expression and lymph node metastasis status in 91 TNBC tissues; C) The Kaplan-Meier plot of TNBC patients with low (n=46) and high (n=45) circ-ITCH expression, the median circZNF609 value was used as cutoff value.

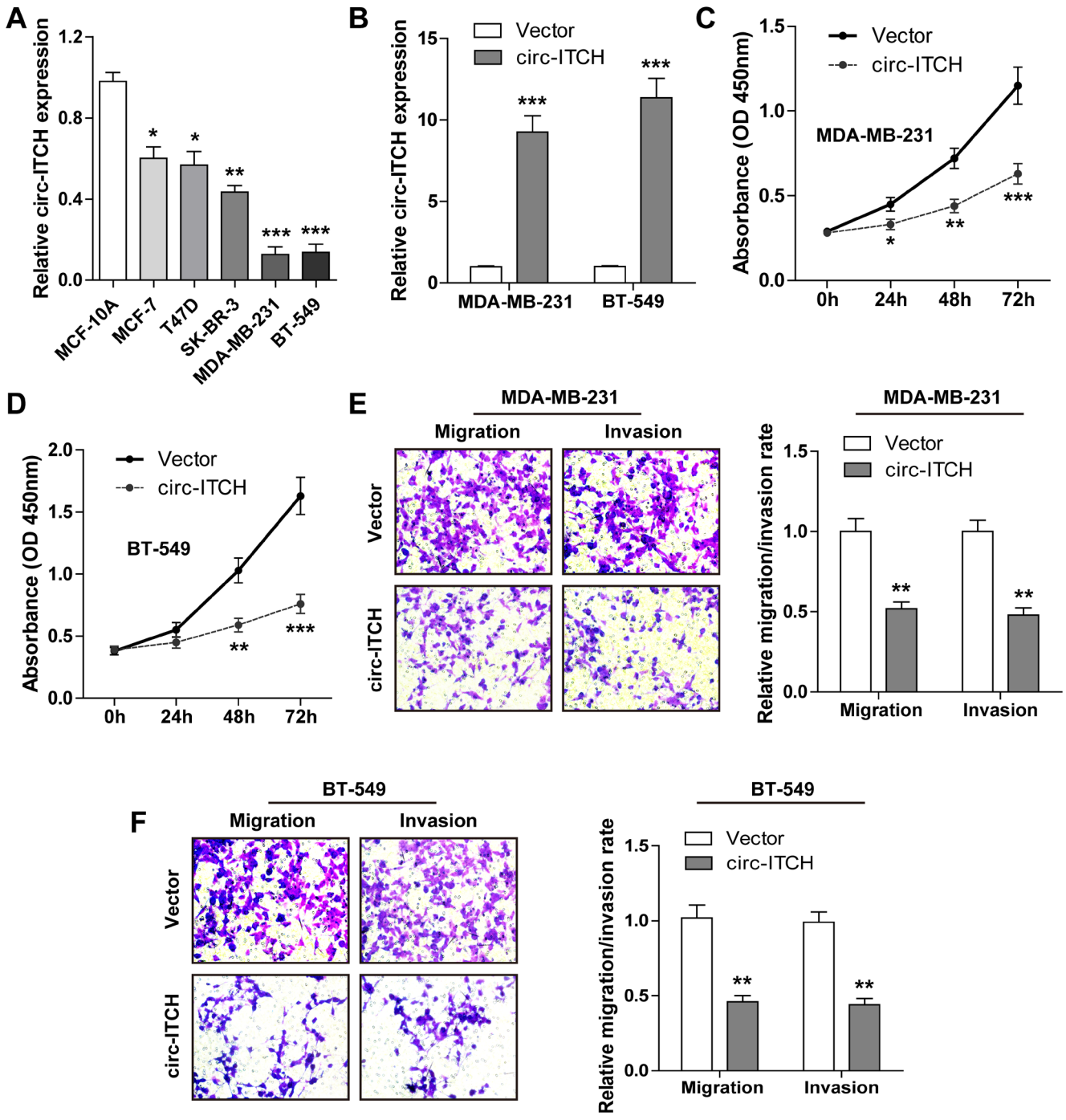

Figure 2. Overexpression of circ-ITCH inhibits TNBC proliferation, migration and invasion *in vitro*. A) The expression levels of circ-ITCH in breast cancer cell lines were measured by qRT-PCR; B) qRT-PCR for MDA-MB-231 and BT-549 cell lines with or without circ-ITCH overexpression; C and D) CCK-8 assay was used to evaluate proliferative ability of MDA-MB-231 and BT-549 cells with or without circ-ITCH overexpression; E and F) Transwell assays were conducted to test migratory and invasive ability of MDA-MB-231 and BT-549 cells with or without circ-ITCH overexpression. *p<0.05, **p<0.01, ***p<0.001.

partly rescued by over-expression of miR-214 or miR-17 (Figures 4C–D). These results suggest that circ-ITCH attenuates Wnt/β-catenin signaling by sponging miR-214 and miR-17 in TNBC.

Circ-ITCH over-expression inhibits TNBC growth and metastasis *in vivo*. We established xenograft and lung metastasis models described in Materials and methods to determine if circ-ITCH exerts its tumor suppressive effect *in vivo*.

The results showed that nude mice with circ-ITCH over-expression presented smaller tumors than those with the control vector (Figures 5A–B), and the Ki-67 IHC proliferation index provided confirmation (Figure 5C). Importantly, the average number of lung nodules was 26 and 7 in the control vector and circ-ITCH- over-expressing nude mice (Figure 5D). These findings indicate that circ-ITCH retards TNBC progression *in vivo*, and this is consistent with *in vitro* and clinical results.

Discussion

CircRNA is an emerging non-coding RNA and is now attracting greater attention. Considerable studies show that circRNA is widely expressed in eukaryotic cells with gene-regulatory potency [14, 15]. CircRNA has high stability, conservation and tissue/stage-specific expression and is therefore a potential cancer biomarker [16]. For example, circFARSA, circ-ANAPC7, circ-PVT1, circ-HIPK3 and circ-SMARCA5 were reported diagnostic and prognostic biomarkers in non-small cell lung cancer [17], acute myeloid leukemia [18], gastric cancer [19], colorectal cancer [20] and hepatocellular carcinoma [21]. In this study, we found that circ-ITCH is closely associated with lymph node metastasis, advanced TNM stage and TNBC prognosis, thus indicating that circ-ITCH is a promising prognostic biomarker for TNBC.

Circ-ITCH is derived from exons 6 to 13 of its linear ITCH isoform gene. Previous studies have reported that circ-ITCH is remarkably down-regulated in many cancers, including colorectal cancer, glioma, lung cancer, bladder cancer, and esophageal squamous cell carcinoma [8–12, 14]. Analogously, we found that circ-ITCH was significantly decreased in TNBC tissues and cell lines, implying that circ-ITCH is

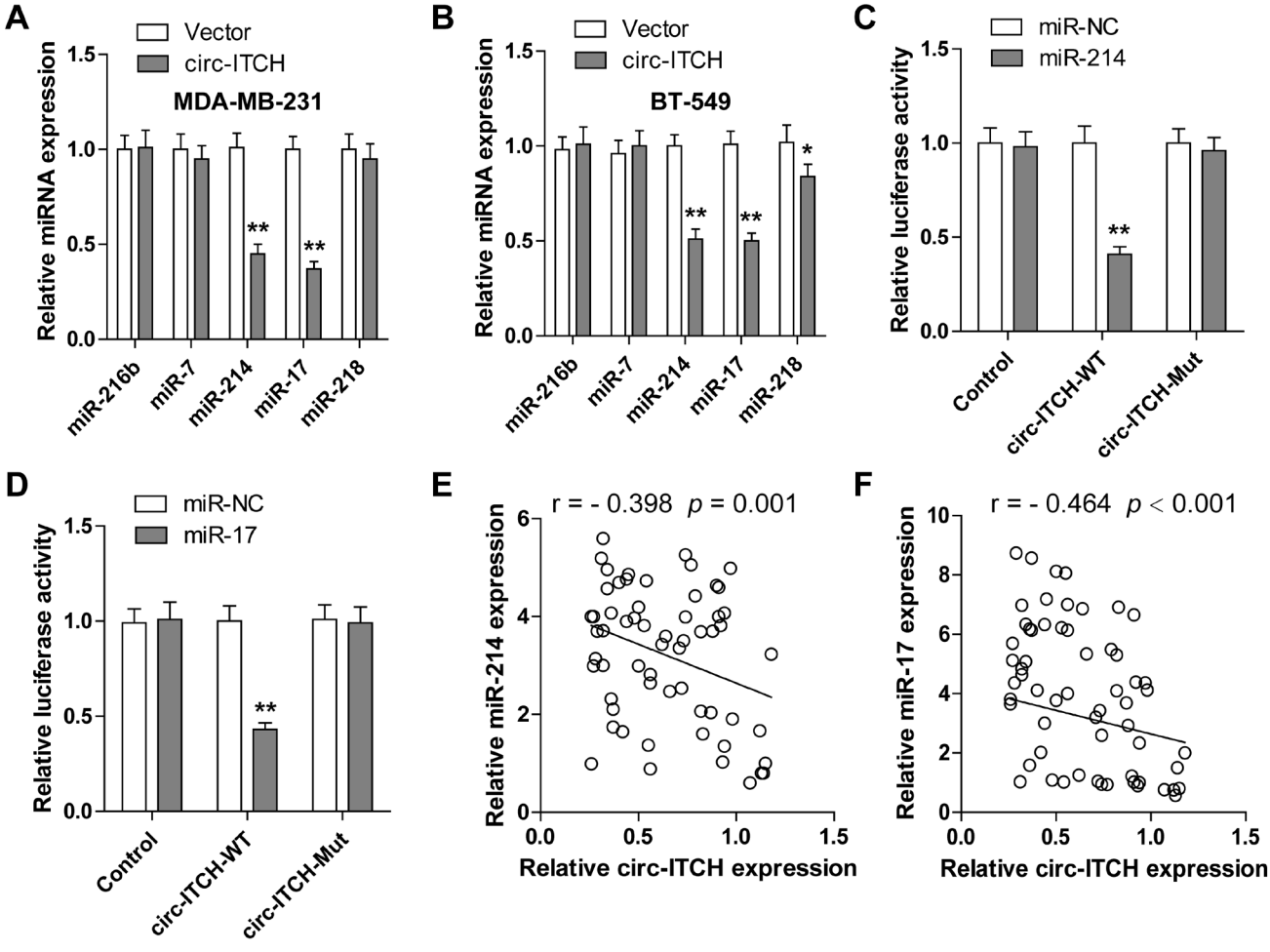

Figure 3. Circ-ITCH acts as a sponge for miR-214 and miR-17. The expression levels of the indicated miRNAs in MDA-MB-231 (A) and BT-549 (B) cells with or without circ-ITCH overexpression were determined by qRT-PCR; C and D) miR-214 and miR-17 mimics were co-transfected with wild type and mutant circ-ITCH expression vectors into MDA-MB-231 cells, 24h later, the luciferase activity was detected using the Dual Luciferase Reporter Assay Kit; E and F) The correlation between circ-ITCH and miR-214 or miR-17 expression in TNBC tissues. *p<0.05, **p<0.01

a tumor suppressor in most cancers. Although the circRNA action mechanism in cancers is not clarified, growing evidence shows that circRNA participates in many biological processes as competing endogenous RNA (ceRNA) [3, 6]. These include cell proliferation, apoptosis, invasion, senescence and pyroptosis, thus further suggesting that miRNA has a circRNA-mediated regulatory role. The most famous circRNA is circRS-7 which has many miR-7 binding sites [7, 22, 23], and the well-known circ-HIPK3 can bind to miR-124 [24], miR-558 [25], miR-193a [26] and miR-7 [20] to sequester these miRNAs from their respective targets. It is also reported that circ-ITCH sponges different miRNAs, including miR-216b, miR-7, miR-214, miR-17 and miR-218, to sequester and suppress their activities in different cancers [8–12, 14]. In our study, we found that circ-ITCH was involved in TNBC proliferation, migration and invasion. Mechanistic studies then showed that circ-ITCH interacts with miR-214 and miR-17, but not miR-216b, miR-7 and miR-218; thus revealing that circRNA different functions rely on the specific context. Both miR-214 and miR-17 are well-known oncogenes and are highly expressed in tumors [27, 28]. Our data also showed these two miRNAs were uniformly up-regulated and negatively correlated with circ-ITCH in TNBC tissues; miR-214 and miR-17 interacted with the 3' UTR of their common target ITCH to reduce its expression [8, 10]. ITCH is part of the Nedd4-like E3 family and it can degrade phosphorylated disheveled entities, thereby inactivating canonical Wnt signaling [29]. Thus, circ-ITCH can sponge and inhibit miR-214 and miR-17 activities to increase ITCH expression, thereby attenuating the activity of Wnt/β-catenin signaling to inhibit TNBC development and progression. This is confirmed in rescue assays, and it indicates that the circRNA-miRNA regulatory network has a key role in TNBC pathogenesis.

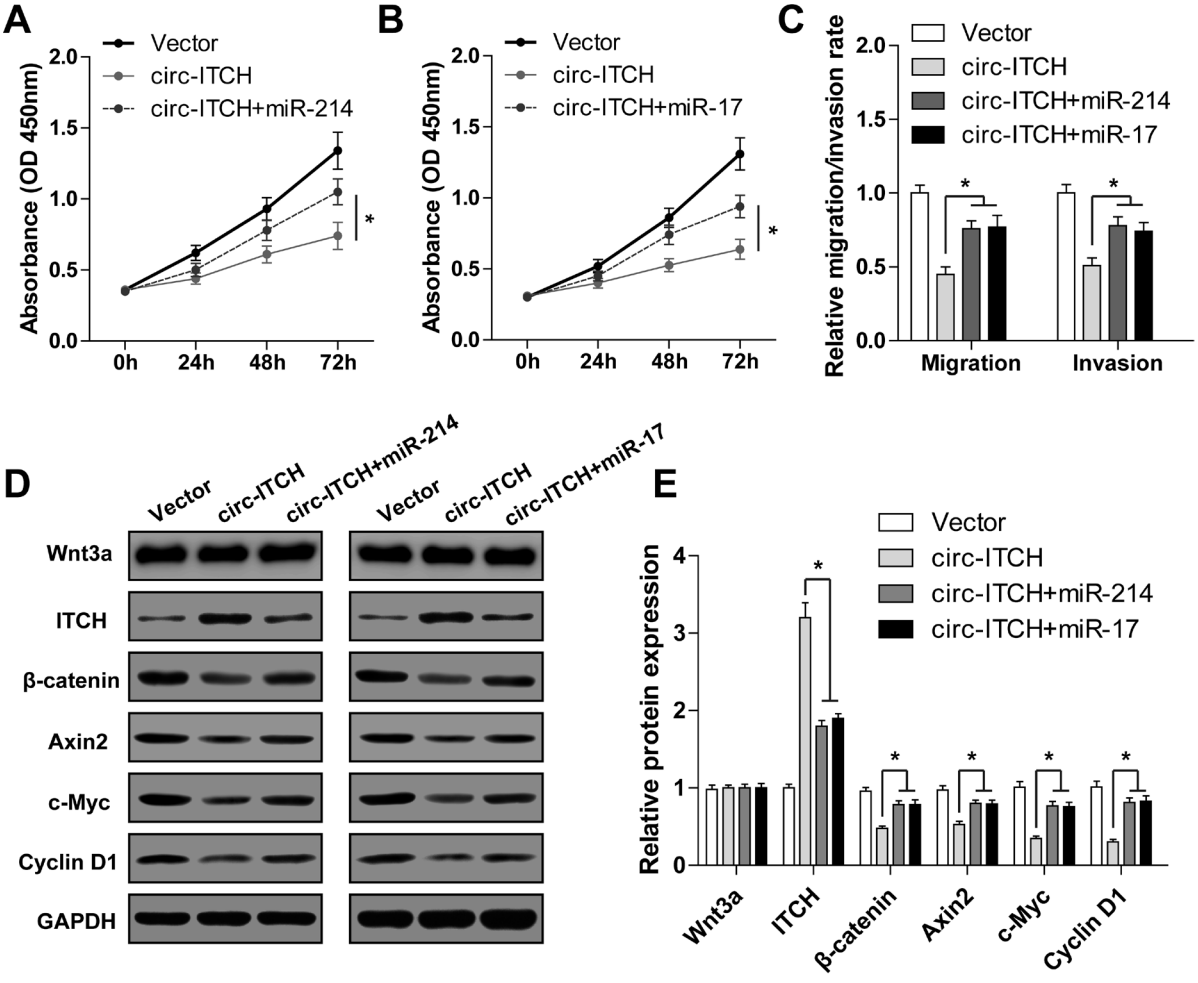

Figure 4. The tumor-inhibitory effects caused by circ-ITCH overexpression were partially rescued by overexpression of miR-214 or miR-17. A–C) The CCK-8 and transwell migration and invasion assays for circ-ITCH-overexpressing MDA-MB-231 cells transfected with miR-214 or miR-17 mimics; D and E) The protein expression levels of Wnt3a, ITCH and β-catenin, Axin2, c-Myc and Cyclin D1 in circ-ITCH-overexpressing MDA-MB-231 cells transfected with miR-214 or miR-17 mimics were measured by western blot. GAPDH was used as the internal control; *p<0.05.

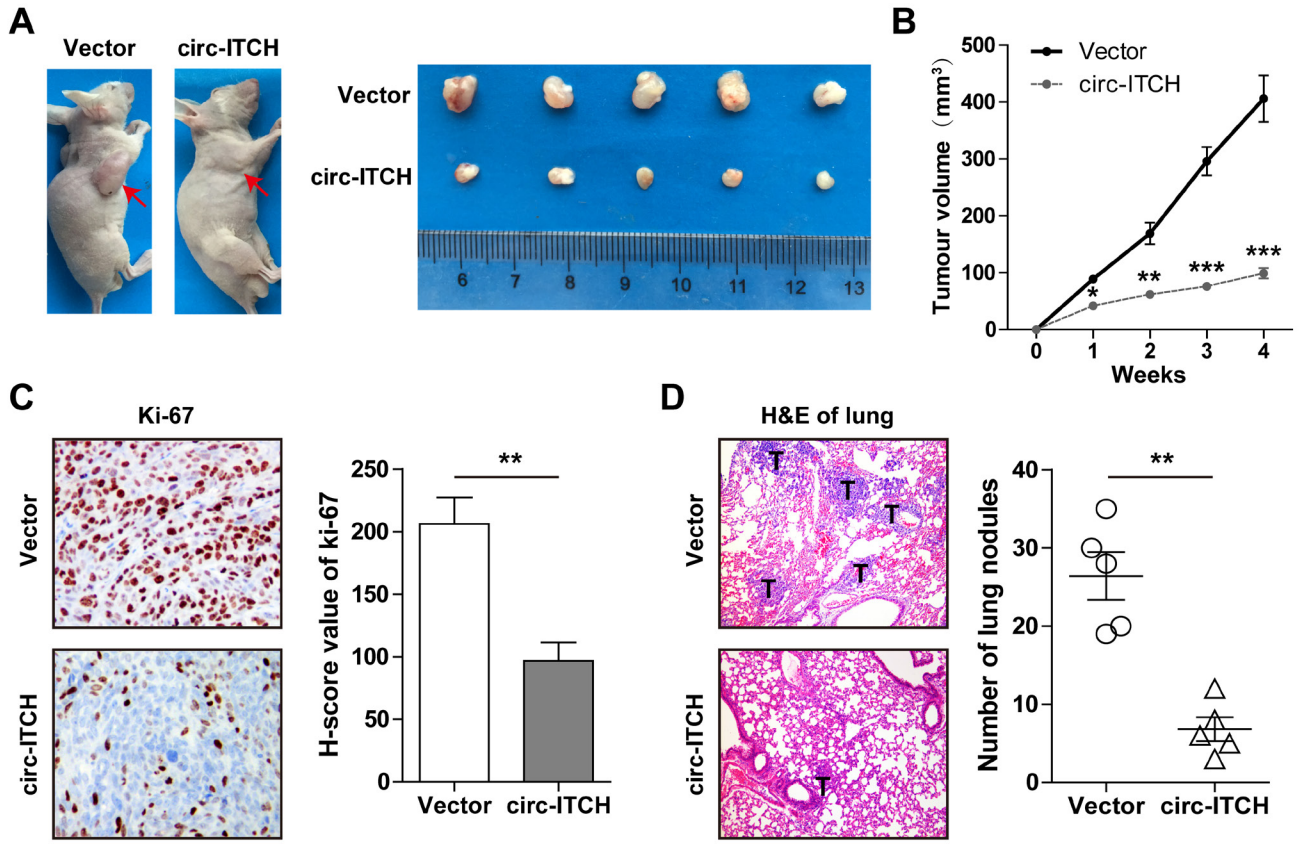

Figure 5. Overexpression of circ-ITCH suppresses TNBC growth and metastasis *in vivo*. A) The representative images of nude mice with or without circ-ITCH overexpression, the red arrows indicate tumors; B) The tumor volume of nude mice with or without circ-ITCH overexpression; C) The representative images of Ki-67 IHC of nude mice with or without circ-ITCH overexpression; D) The representative images of nude mice lung nodules with or without circ-ITCH overexpression. T indicates tumors. *p<0.05, **p<0.01, ***p<0.001.

To the best of our knowledge, this is the first study investigating circ-ITCH's role in TNBC. Our research established that circ-ITCH is a tumor suppressor and a promising prognostic biomarker for TNBC, and results also revealed that circ-ITCH inhibits TNBC progression by inactivating the Wnt/β-catenin pathway through regulation of the miR-214/ miR-17/ITCH axis. Further studies, however, are warranted to explore its role in other diseases.

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