# Original Article Circular RNA circ-ABCB10 promotes breast cancer proliferation and progression through sponging miR-1271

Hai-Feng Liang<sup>1\*</sup>, Xing-Zeng Zhang<sup>2\*</sup>, Bao-Guo Liu<sup>3</sup>, Guo-Tao Jia<sup>4</sup>, Wen-Lei Li<sup>3</sup>

<sup>1</sup>Department of Surgery, Tumour Hospital of Liaocheng, Liaocheng 252000, Shandong, P. R. China; Departments of <sup>2</sup>General Surgery, <sup>3</sup>Breast and Thyroid Surgery, <sup>4</sup>Pathology, Liaocheng People's Hospital, Liaocheng 252000, Shandong, P. R. China. \*Equal contributors and co-first authors.

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**Abstract:** Circular RNA (circRNA) is a key regulator in the development and progression of human cancers, however its role in breast cancer tumorigenesis is not well understood. The present study aims to investigate the expression profiles and potential modulation of circRNA on breast cancer carcinogenesis. Human circRNA microarray was performed to screen for abnormally expressed circRNA in breast cancer tissue. Results found circ-ABCB10, was significantly up-regulated in breast cancer tissue. And results were replicated in a larger sample size. *In vitro*, loss-of-function experiments showed circ-ABCB10 knockdown suppressed the proliferation and increased apoptosis of breast cancer cells. Bioinformatics prediction program predicted the complementary sequence within circ-ABCB10 and miR-1271, which was validated by luciferase reporter assay. Finally, miR-1271 rescued the function of circ-AB-CB10 on breast cancer cells, confirming the sponge effect of circ-ABCB10 on miR-1271. Overall, results identified a new functional circ-ABCB10 in breast cancer tumorigenesis, and reveal the important regulatory role of circ-ABCB10 through sponging miR-1271, providing a novel insight for breast cancer pathogenesis.

Keywords: Circular RNA, breast cancer, circ-ABCB10, miR-1271, proliferation

#### Introduction

Breast cancer is one of the most common malignant tumor groups in women worldwide [1, 2]. Although there are numerous therapies aimed at treating breast cancer, it is the second leading cause of cancer mortality in women [3]. Early diagnosis, radical surgery and adjuvant therapy have improved the survival rates and prognosis of breast cancer patients, however mortality rates remain high. In order to maximize the effectiveness of therapy, it is important to understand the molecular pathways involved in the pathogenesis of breast cancer that cause metastasis and chemotherapy resistance [4]. An important regulator in breast cancer development is non-coding RNA (ncRNA) [5].

NcRNA is a class of non-protein translation RNA, that includes: long non-coding RNA (IncRNA) more than 200 nucleotides in length, micro RNA (miRNA) 20-22 nucleotides in length, and circular RNA (circRNA), which are characterized by covalent closed loops. CircRNA are formed by back-splicing without 3'-end and 5'-end, unlike IncRNA [6].

CircRNA has been found to play an important role in the regulation of multiple diseases, including cardiovascular disease, diabetes mellitus and cancerous tumors [7, 8]. CircRNA, Cdr1as regulates insulin transcription and secretion in islet cells via miR-7 [9]. The exact role of circRNA in human disease is still unclear. and further research is needed. Like IncRNAs, the main role of circRNA at the molecular level is to act as a miRNA 'sponge' and absorb the functional miRNA, thereby decreasing the abundance of miRNAs in the cytoplasm. CircRNA has been found to take part in tumorigenesis. In hepatocellular carcinoma hsa\_circ0004018 is down-regulated and is involved in cancer-related pathways via interactions with miRNA [10].

The present study utilized human circRNA microarray analysis to screen circRNA expression profiles in breast cancer tissue. and discovered a significantly over-expressed circRNA hsa\_circ\_0008717 (circBase, www.circbase. org/). Hsa\_circ\_0008717 is located at chr1: 229665945-229678118 with 724 length in gene symbol ABCB10, thus, we nominate it as circ-ABCB10. Based on the microarray findings, a series of functional validation experiments were performed to explore the role of circ-ABCB10 in breast cancer cell progression.

### Materials and methods

### Clinical specimens and ethical statement

This study was approved by the Ethics Committee of Liaocheng People's Hospital. A total of 36 patients were recruited into the study between 2015 and 2016 at the Liaocheng People's Hospital. Informed consent was obtained from all enrolled patients. None of the enrolled patients received radiotherapy or chemotherapy prior to surgery. A total of 36 samples (cancer and adjacent noncancerous tissue) were obtained, including four specimens for microarray analysis and 32 specimens for RT-PCR. Specimens were immediately frozen in liquid nitrogen after resection and stored at -80°C until use.

### Cell lines and culture

Human breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-453) and normal human breast epithelial cells (MCF-10A) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Human breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) with 10% FBS (Gibco, Grand Island, NY, USA). Human breast epithelial cells (MCF-10A) were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco). All cells were cultured at 37°C and 5% CO<sub>2</sub>.

### Human circRNA microarray analysis

Total RNA was extracted from four pairs of breast cancer samples using NanoDrop ND-1000. CircRNA was enriched via digesting linear RNA with Rnase R (Epicentre, Madison, WI, USA). RNA was amplified using RT-PCR and labelled with Arraystar Human circRNA Array (8×15 K, Arraystar, Rockville, MD, USA). Labeled RNA were scanned using a Agilent Scanner G2505C (Jamul, CA, USA).

# Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was isolated from the tissue and cells using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. CDNA were reverse transcribed using SuperScript First-Stand Synthesis system (Invitrogen, Carlsbad, Calif, USA). The synthesized cRNA were used for real-time PCR with RT-PCR System (StepOneTM, Applied Biosystems, Darmstadt, Germany) and the conditions were performed according to manufacturer's instructions. The primers of U6 acted as internal loading control. All primers were shown as follows: circ-ABCB10 (divergent primers), forward, 5'-CTAAGGAGTCACAGGAAGACATC-3'; reverse, 5'-GTAGAATCTCTCAGACTCAAGGTTG-3'; miR-1271, forward, 5'-CTAGACGTCCAGATTGAATAGAC-3'; reverse, 5'-GTCCGAGCTTGGTCAGAATG-3'; U6, forward. 5'-AGCCCGCACTCAGAACATC-3' and reverse, 5'-GCCACCAAGACAATCATCC-3'. Samples were normalized to internal housekeeping genes followed by its control. All the values were standardized with  $2^{-\Delta\Delta CT}$  method.

### siRNA transfection

All oligonucleotide sequences were synthesized by GenePharma (Shanghai, China). A specific siRNA for circ-ABCB10 was designed to target covalent closed junction. Breast cancer cells (MCF-7, MDA-MB-231) were transfected with siRNAs at concentration of 50 nM oligonucleotides using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### Luciferase reporter assay

For luciferase reporter assay, pmirGLO Dualluciferase vectors (GenePharma) were used to construct dual luciferase reporter plasmids. Sequences of miR-1271 and circ-ABCB10 were separately cloned into the vectors. HEK-293T cells were co-transfected with wild-type pmir-GLO-circ-ABCB10 or mutated type and miR-



1271 mimics or negative control using Lipofectamine 2000 (Invitrogen). After induction for 48 h, luciferase activity was assessed using the dual-luciferase reporter kit (Promega, Madison, WI, USA). The relative firefly luciferase activity was normalized to Renilla luciferase activity.

### CCK-8 proliferation assay

Cells were plated at  $5 \cdot 10 \times 10^3$  cells per well in 96-well plates with serum-free 1640 medium and grown for 24 h. After incubation for indicated time, 10 µl of CCK-8 reagent (Dojindo Japan) was added to each well, and the assay was performed according to the manufacturer's protocol. The absorbance was measured at 450 nm using a spectrophotometer. All experiments were repeated a minimum of three times.

### Colony formation

MCF-7 and MDA-MB-231 cells were added into six well plates with 1640 medium containing 10% FBS. After 14 days in culture, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (Beyotime, Shanghai, China). Cells were washed with PBS and colonies containing >50 cells were manually counted.

Flow cytometry for apoptosis and cycle analysis

Annexin V-FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, Calif, USA) was used for



**Figure 2.** CircRNA circ-ABCB10 was significantly up-regulated in breast cancer tissue. A: The 5 candidate circRNAs were tested to be upregulated in 6 pairs of cancer tissue and noncancerous samples using RT-PCR. B: The 5 candidate circRNAs were tested to be downregulated in 10 pairs of cancer tissue and noncancerous samples using RT-PCR. C, D: CircRNA circ-ABCB10 expression was validated in 32 pairs of cancer tissue and noncancerous samples using RT-PCR. Circ-ABCB10 expression was significantly increased in cancer tissue compared to noncancerous samples with about 5-10 fold change. Data were expressed as mean ± SD. \*\*P<0.01 represents statistically difference.

apoptosis assay. Breast cancer cells (MCF-7, MDAMB-231) were digested and washed with cold PBS. Cells ( $1 \times 10^6$  cells/ml) were re-suspended in 100 µL binding buffer with Annexin V. PI was added for incubation at room temperature for 20 min in the dark. Apoptotic cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). Experiments were performed in triplicate.

For cell cycle assays, breast cancer cells (MCF-7, MDAMB-231) were washed with PBS three times and fixed with 80% ethanol. Subsequently, cells were incubated with RNase A and incubated with 20  $\mu$ g/ml Pl for 20 min at room temperature. The cell cycle was analyzed by the same flow cytometry as above.

### Statistical analysis

Data were presented as mean  $\pm$  S.E.M. Statistical analysis was performed using SPSS (18.0 vision) and GraphPad 5 and Student's t-test and one way ANOVA were used. *P* value <0.05 was considered as significant difference. All experiments were performed in triplicate.

### Results

# CircRNA expression profile in breast cancer tissue

Breast cancer tissue and paired adjacent noncancerous tissue was collected and screened for dysregulated circRNA using human circRNA microarray. A total of 2,587 circRNAs with 1.5 fold of upregulation or downregulation were analyzed. Box plots show the normalized intensities from the tumor and noncancerous tissue samples (Figure 1A). Scatter plots show the different expression of circRNA in breast cancer tissue and adjacent noncancerous tissue (Figure 1B). A heat map shows the selected ten upregulated and ten downregulated circRNA (Figure 1C). Initial analyzes screened the differences in expression of circRNAs in breast cancer tissue compared to adjacent noncancerous tissue, outlining the potential role of circRNA in breast cancer tumorigenesis. These dysregu-

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**Figure 3.** Circ-ABCB10 knockdown suppressed breast cancer cells proliferation and enhanced apoptosis *in vitro*. A: Expression of circ-ABCB10 in human breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-453) and normal human breast epithelial cell (MCF-10A) detected with RT-PCR. B: Specific siRNAs targeting circ-ABCB10 were transfected into MCF-7 and MDA-MB-231 cells. C: CCK-8 proliferation assay showed the proliferation in circ-ABCB10 knockdown group and control group. D: Colony formation assay showed the clone number in cells transfected with siRNAs or control. E, F: Cell cycle analysis detected by flow cytometry. Data were expressed as mean ± SD. \*P<0.05, \*\*P<0.01 represented statistically difference.



**Figure 4.** Circ-ABCB10 was targeted by miR-1271. A: Schematic model of circ-ABCB10 covalent closed loop. B: The putative sequences of miR-1271 and circ-ABCB10 with 8 paired nucleotides. C: Luciferase reporter assay showed the luciferase intensity of MCF-7 and MDA-MB-231 cells transfected with circ-ABCB10 (wild/mutant) and miR-1271 (control/mimics). D: Expression of miR-1271 in breast cancer tissue and adjacent non-tumor tissue. E: Expression of miR-1271 in MCF-7 cells transfected with or not si-circ-ABCB10. Data were expressed as mean ± SD. \*P<0.05, \*\*P<0.01 represented statistically difference.

lated circRNAs may play an important role for tumor genesis and development.

CircRNA circ-ABCB10 (hsa\_circ\_008717) was identified to be significantly up-regulated in breast cancer

CircRNA microarray revealed the differences in the expression of circRNA in breast cancer tissue compared to adjacent noncancerous tissue. These dysregulated circRNA may provide a better understanding of the role of circRNA in breast cancer tumorigenesis. Among screened up-regulated and down-regulated circRNA, five candidate circRNA were chosen to validate the expression using RT-PCR in six pairs of breast cancer tissue and adjacent noncancerous tissue. Results showed the ten candidate circRNA were significantly up-regulated or down-regulated in cancer tissue and noncancerous samples (Figure 2A, 2B). To further test the expression level of the dysregulated circRNAhsa\_ circ\_008717 was selected as a target. Hsa\_ circ\_0008717 is located at chr1:229665945-229678118 with 724 length in gene symbol ABCB10, thus, it was labeled circ-ABCB10. Expression of circ-ABCB10 was validated in 32 pairs of cancer tissue and noncancerous tissue samples (including previous six cases). Results showed circ-ABCB10 expression was significantly increased in cancer tissue compared to noncancerous tissue samples with about five to 10 fold change (**Figure 2C, 2D**). Overall, results validate the up-regulation of circ-ABCB10 in breast cancer.

# Circ-ABCB10 knockdown suppressed breast cancer cells proliferation and enhanced apoptosis

Circ-ABCB10 had been tested to be up-regulated five to ten fold in breast cancer tissue. To investigate this further, the expression of circ-ABCB10 in breast cancer cells in *vitro* was measured. Results showed circ-ABCB10 expression was significantly up-regulated in breast cancer cells (**Figure 3A**). Specific siRNA targeting circ-ABCB10 were transfected into breast cancer cells (MCF-7 and MDA-MB-231), resulting in a significant decrease in circ-ABCB10 expression

(Figure 3B). Cellular functional validation experiments were performed in MCF-7 and MDA-MB-231 cells, including CCK-8 proliferation assay, colony formation assay, cell cycle analysis and apoptosis assay. CCK-8 proliferation assay showed circ-ABCB10 knockdown suppressed breast cancer cell proliferation compared to the control group (Figure 3C). Colony formation assay showed circ-ABCB10 knockdown inhibited the number of clones in cells transfected with siRNAs (Figure 3D). Cell cycle analysis showed the cells at GO/G1 phase were significantly increased in circ-ABCB10 knockdown cells, indicating the cycle arrested at GO/ G1 phase (Figure 3E, 3F). Apoptosis assay showed circ-ABCB10 knockdown could enhance apoptosis in breast cancer cells (Figure 3G, 3H). Results indicate circ-ABCB10 knockdown suppressed proliferation and enhanced apoptosis.

### Circ-ABCB10 sponged miR-1271

The oncogenic role of circ-ABCB10 was confirmed using functional validation experiments in vitro. Following this, the miRNA 'sponge' function of circ-ABCB10 and its role in downstream regulation was examined. Bioinformatics analysis predicted the potential target was miR-1271, which shares complementary binding sites with circ-ABCB10. Figure 4A shows the schematic model of the circ-ABCB10 covalent closed loop. MiR-1271 contains eight paired nucleotides with circ-ABCB10 (Figure 4B). Luciferase reporter assay showed the luciferase intensity of miR-1271 binding with circ-ABCB10 wild type was decreased (Figure 4C). In breast cancer tissue, miR-1271 expression was significantly decreased compared to adjacent noncancerous tissue (Figure 4D). Moreover, miR-1271 expression in MCF-7 cells transfected with si-circ-ABCB10 was significantly increased compared to a negative control group (Figure 4E). Taken together, results indicate the direct binding of miR-1271 targeting circ-ABCB10.

# MiR-1271 inhibitor rescued the function of circ-ABCB10 knockdown

In order to verify specific targeting of miR-1271, rescue experiments in MCF-7 cells were performed. Firstly, miR-1271 expression was significantly decreased in several breast cancer cell lines, particularly in MCF-7 cells (**Figure**  **5A**). There was a significant decreased in miR-1271 expression by miR-1271 inhibitor, compared with that in si-circ-ABCB10 transfected MCF-7 cells (**Figure 5B**). Colony formation and CCK-8 assay both revealed miR-1271 inhibitor reversed the proliferation induced by si-circ-ABCB10 (**Figure 5C, 5D**). Moreover, cycle analysis and apoptosis assay detected by flow cytometry showed miR-1271 inhibitor rescued the suppression role of si-circ-ABCB10 (**Figure 5E-H**). Taken together, results demonstrate miR-1271 can reverse the function of circ-ABCB10 on MCF-7 cells, suggesting the role of the miRNA 'sponge' mechanism in tumorigenesis.

## Discussion

Multidisciplinary research investigating circRNA is increasing [11, 12] given their vital role as regulators and valuable diagnostic markers for disease, including cardiovascular disease, diabetes mellitus and multiple tumors [6]. In the present study, circRNA expression profiles were screened using circRNA microarray, and found circ-ABCB10 to be up-regulated in breast cancer tissue and cells. Further analyses were conducted to determine the preliminarily biological function of the newly identified circ-ABCB10.

Like other ncRNA, circRNA are expressed in multiple tissues with great variability [13-15]. In the primary phase of the study, circRNA microarrays were performed to screen the profiles in four pairs of breast cancer tissue. Results found a mass of dysregulated circRNA, and a new up-regulated, circRNA was identified: circ-ABCB10. In follow-up RT-PCR validation, the significant up-regulation of circ-ABCB10 was confirmed in a larger sample size and cell line. In vitro, cellular loss-of-function experiments indicated circ-ABCB10 silencing induced by circRNA-specific interfering RNA targeting covalently closed junctions, could significantly suppress the proliferation, and induce GO/G1 phase arrest and apoptosis of breast cancer cells. Above results suggest circ-ABCB10 acts as an oncogenic circRNA in breast cancer tumorigenesis, revealing the potential function of circRNA in breast cancer occurrence and development.

Recent studies indicate circRNAs plays an important role in multiple cancerous tumors



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[16, 17]. For example, circZKSCAN1 was significantly lower in HCC samples compared with matched adjacent noncancerous tissue, and mediated several cancer-related signaling pathways, providing a useful biomarker for the diagnosis of HCC [18]. In human gastric cancer, hsa\_circ\_0001895 expression was significantly downregulated in tumor tissue and cell lines. and was closely correlated with cell differentiation and tissue carcino-embryonic antigen expression, suggesting the potential biomarker for clinical prognosis prediction [19]. In colorectal cancer, hsa\_circ\_0000069 is up-regulated in tumor tissue and hsa\_circ\_0000069 knockdown can inhibit cell proliferation, migration and invasion and induce GO/G1 phase arrest of the cell cycle in vitro [20]. These findings suggest the important regulatory role of circRNA in different tumorigenesis.

The function of circRNA in human disease is still unclear, and research is in early stages [21, 22]. Like IncRNA, the main role of circRNA at the molecular regulation level is to act as a miR-NAs 'sponge' to absorb functional miRNA, and decrease the abundance of miRNA in the cytoplasm [23, 24]. For example, Cdr1as has approximately 70 miR-7 binding sites, making it a huge 'reservoir' for miR-7 [12, 25, 26]. The powerful adsorption of circRNA to miRNA can significantly decrease miRNA levels and further release the functional gene mRNA suppressed by miRNA. For example, circRNA MTO1 suppresses hepatocellular carcinoma progression by acting as a miRNA sponge harboring miR-9 to promote p21 expression [27].

The differences in circRNA expression in breast cancer tissue are associated with diverse pathogenesis. The present study identified circ-ABCB10 in breast cancer tissue, and it's role in acting as a miR-1271 sponge. In summary, the role of circ-ABCB10 in breast cancer carcinogenesis via sponging miR-1271 provides a novel insight for therapy and prevention in breast cancer.

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### Disclosure of conflict of interest

None.

### Abbreviations

circRNA, circular RNA; miRNA, micro RNA; ncRNAs, non-coding RNAs; IncRNAs, long noncoding RNAs.

Address correspondence to: Wen-Lei Li, Department of Breast and Thyroid Surgery, Liaocheng People's Hospital, Dongchang West Road, No.67, Liaocheng 252000, Shandong, P. R. China. E-mail: liwenlei\_ nice@126.com

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