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CircRNA CircABCB1 Diminishes the Sensitivity of Breast Cancer Cells to Docetaxel by Sponging MiR-153-3p

Jiaqi Liu,[1] Linna Kong,[1] Wenqing Bian,[2] Xiaona Lin,[1] Feifei Wei[3] and Jun Chu[1]

[1]Department of Breast and Thyroid Surgery, Zibo Central Hospital, Zibo, Shandong, China
[2]Maternal and Child Health Hospital of Zibo, Zibo, Shandong, China
[3]Weifang Maternal and Child Health Hospital, Weifang, Shandong, China

Resistance to docetaxel is a major problem to the success of docetaxel-based therapies for breast cancer. The present study was to identify the role of circABCB1 in altering the docetaxel resistance properties. Reverse transcription-quantitative PCR (qRT-PCR) was performed to quantify circABCB1 and miR-153-3p. The effects of circABCB1 on the viability, apoptosis and migration/invasion of docetaxel-resistant and -sensitive cells were investigated by cell function experiments, including Cell Counting Kit-8 and Transwell assays. Correlation between circABCB1 and the docetaxel-treated outcome was analyzed by multivariate Cox regression analysis, in addition to Kaplan-Meier analysis of time to treatment failure (TTF). The targeting relationship between circABCB1 and miR-153-3p was predicted and verified by dual-luciferase reporter assay and RNA immunoprecipitation. CircABCB1 was highly expressed in cancerous tissues, as well as the docetaxel-sensitive group and cells. The overexpression of circABCB1 contributed to cell viability, docetaxel-resistance and migration/invasion, but inhibited apoptosis. CircABCB1 can sponge miR-153-3p. CircABCB1 contributed to the docetaxel resistance of breast cancer, maybe via the miR-153-3p.

Keywords: breast cancer; ceRNA; circABCB1; docetaxel resistance; prognosis
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Introduction

In 2020, there were 19.29 million new cancer cases in the world, of which 2.26 million were breast cancer. Breast cancer has surpassed lung cancer (2.2 million) to become the largest cancer in the world for the first time (Siegel et al. 2022). Among the 4.43 million female cancer deaths, 680,000 were caused by breast cancer, ranking first (Wilkinson and Gathani 2022). In China, according to the data of the China urban cancer registry, the overall cancer incidence rate has increased by 20%-30% in the past 30 years and has increased by 3%-5% every year (Lei et al. 2021). This growth rate is significantly higher (1.5%) than the average world growth rate. The choice of treatment for breast cancer is usually based on lymph node metastasis and stage (Maughan et al. 2010). Choice for early-stage (I and II) breast cancers is usually breast-conserving surgery and radiation therapy (Curigliano et al. 2017). Chemotherapy and endocrine therapy are usually considered for node-positive breast cancer. Induction chemotherapy is used to treat stage III breast cancer to down-size the tumor. Treatment options for women with recurrent or metastatic (stage IV) should consider the benefits of life length and the harm of pain (Akram et al. 2017). Notably, drug resistance to targeted and systemic therapies is one of the causes of breast cancer-related mortality (Rivera and Gomez 2010). The development of chemoresistance remains a major bottleneck in the treatment of cancer patients.

In the past two decades, our understanding of the role of noncoding RNA (ncRNA) in gene regulation and cell biology has been steadily improving. NcRNA contains various types of RNAs, such as microRNAs (miRNAs) and circular RNAs (circRNAs). The discovery of circRNAs has brought our understanding of the important role of these RNA molecules to a new stage. First, lncRNAs can play a role in many biological processes, such as cell proliferation/differentiation/growth, development, metabolism, aging,

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Correspondence: Jun Chu, Department of Breast and Thyroid Surgery, Zibo Central Hospital, 54 Gongqingtuan West Road, Zibo, Shandong 255020, China.
e-mail: junchu_dr@163.com

and cell death (Li et al. 2020; Ghafouri-Fard et al. 2021). Secondly, circRNAs can act as key regulators in cancer, such as oncogenes or tumor suppressors, which may affect the development, metastasis and chemoresistance of many different types of cancers, including breast cancer (Lei et al. 2020; Li et al. 2020). In addition, lncRNAs can act as bait to competitively bind to miRNAs and thus affect the expression and function of downstream genes, which are defined as competing endogenous RNAs (ceRNAs) (Tay et al. 2014; Li et al. 2020). Some circRNAs are related to the prevalence of drug resistance phenotype and the recovery of drug sensitivity of cancer cells (Li et al. 2020). For instance, dysregulation of circKDM4C and hsa_circ_0006528 can mitigate or contribute, respectively, to doxorubicin resistance in breast cancer (Yu et al. 2015; Liang et al. 2019). At present, docetaxel is a common Taxus drug, which plays an important role in the chemotherapy of breast cancer. CircCYP24A1, circDPP4, and circ-XIAP have been proven to promote the development of docetaxel resistance in cancers (Zhang et al. 2021; Gu and Duan 2022; Yin et al. 2022). Therefore, targeting circRNAs may become a new strategy for the treatment of drug-resistant cancers.

CircABCB1 has been screened as a dysregulated circRNA in docetaxel-resistant breast cancer cell sublines (Huang et al. 2021). In this study, we further investigated the expression levels of circABCB1 in normal tissues and docetaxel-resistant/sensitive breast cancer tissues. Subsequently, the biological function, function mechanism, and clinical value of circABCB1 in breast cancer were explored based on clinical specimens, pathological information, and *in vitro* experiments.

Materials and Methods

Patients

The patients in the Zibo Central Hospital between March 2019 and March 2020 with locally advanced or metastatic breast cancer received docetaxel as a first-line chemotherapy drug, not combined with other drugs. The patient who had relapsed cancer or received neoadjuvant chemotherapy before inclusion were excluded. A total of 90 women were included in the study who met the Response Evaluation Criteria in Solid Tumors (Eisenhauer et al. 2009). The surgical material from patients was collected upon written informed consent. Docetaxel-resistant (DOCR) tissue samples (n = 40) and docetaxel-sensitive (DOCS) tissue samples (n = 50) were acquired from patients. The procedures used in this study adhere to the tenets of the Declaration of Helsinki. The present study was approved by the Ethics Committee of Zibo Central Hospital, and informed consent was obtained from all subjects involved in the study. Time to treatment failure (TTF) was defined as the period from the beginning of treatment to disease progression, loss of follow-up or replacement, or interruption of treatment for any reason.

Cell culture

Docetaxel-resistant human breast cancer cell line, MDA-MB-231/DOCR, was purchased from Ybio (Shanghai, China). Its parental cell line, MDA-MB-231, along with BT-474, SK-BR-3, and MCF7, was obtained from National Biomedical Experimental Cell Resource Bank (Beijing, China). These cell lines were cultured in DMEM (Gibco, Palo Alto, CA, USA) supplemented with 10% fetal calf serum (FCS) (Gibco). MDA-MB-231/DOCR cells were maintained in a medium with 65 nM docetaxel. The cultures were kept in a humidified atmosphere with 5% carbon dioxide at 37°C.

Cell transfection

An appropriate amount of cells were inoculated into cell culture dishes. After the cells adhered to the wall, the cells were washed, and the culture medium without FCS was added. The pcDNA, siRNA, and miRNA mimics used for transfection were designed and synthesized by RIBO Biology. Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) was used for transfection. After 8 h of transfection, the medium containing FCS was added to replace the previous medium. After 24 h of transfection, the follow-up cell function test was started; At the same time, a small number of cells were taken to extract RNA, for verification of the transfection efficiency by qRT-PCR.

Total RNA purification and quantification of circABCB1 and miR-153-3p

The cell culture dish was taken out, and then the culture medium was discarded. The patient's tumor tissues and their adjacent normal tissues were transferred to eppendorf tube, and ground. Total RNA of cells and tissues was isolated and purified using the RNAiso Plus (TAKARA, Kyoto, Japan). When circABCB1 was reverse transcribed, PrimeScript RT Master Mix kit was used; When miR-153-3p was reverse transcribed, the Mir-X miRNA First Strand Synthesis kit was used. The target molecular expression levels were detected using SYBR green Primeix ExTaq II kit according to the product manual. GAPDH served as an endogenous control for circABCB1, while U6 for miR-153-3p. The relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ approach.

Detection of IC50

The IC50 values of docetaxel for MDA-MB-231 and MDA-MB-231/DOCR cells were determined. Using 96-well plates (Sigma-Aldrich, Sigma-Aldrich, MO, USA), MDA-MB-231 cells were seeded at 5×10^4 cells per well; MDA-MB-231/DOCR, at 3×10^4 cells per well. After 24 h, cells were exposed to relevant docetaxel solutions of different concentrations (0, 1, 2, 4, 6, 8, 10, and 15 μg/mL) for 24 h. CCK-8 kit was used to determine the corresponding optical density values according to the instructions from the manufacturer. IC50 values were calculated by non-linear regression.

Cell migration and invasion

The transfected MDA-MB-231 and MDA-MB-231/DOCR cells were counted, washed twice, and resuspended in FCS-free medium at concentrations of 9×10^4/200 μL and 5×10^4/200 μL, respectively. On the 24-well cell plate, a complete medium containing 20% FCS (700 μL per hole) was added. The transwell inserts with (for cell invasion) or without (for cell migration) Matrigel were put into the 24 well plates and filled with the prepared cell suspensions. The culture time of MDA-MB-231 was 36 h, and that of MDA-MB-231/DOCR was 20 h. After culture, the cells were washed twice, fixed with methanol, and dyed with 0.1% crystal violet. After removing the cells in the upper chamber, the migrated cells were counted then.

Cell apoptosis analysis

According to the product manual, Annexin V-FITC Propidium Iodide (PI) apoptosis detection kit was used to analyze apoptosis. MDA-MB-231 and MDA-MB-231/DOCR cells were transfected for 24 h. Cell suspension was prepared using Annexin V Binding Solution. 5 μL Annexin V/FITC solution/5 μL PI solution was made to incubate the cells darkly at room temperature for 15 min. FACS Calibur was used for the experiment, and Flowjo 7.6.1 analyzed the data of apoptosis experiment.

Bioinformatics analysis

The targeting miRNAs for circABCB1 were predicted by Circular RNA Interactome (https://circinteractome.nia.nih.gov/index.html) database. The predicted miRNA with the highest 'context+ score percentile' was selected for verification.

Dual-luciferase reporter assay

For verification of the targeting relationship, dual-luciferase reporter assay was used. The vectors were used to construct wild-type reporter plasmid of circABCB1 (wt-circ) and mutant plasmid of circABCB1 (site 1 mut, site 2 mut, and site 1 + 2 mut). Next, MDA-MB-231/DOCR cells were co-transfected with miR-153-3p mimic or its negative control (NC) and the reporter plasmids using Lipofectamine 3000 reagent (Invitrogen). After 48 h, the relative luciferase activity was determined by a Dual Luciferase Reporter Gene Assay Kit.

RNA binding protein immunoprecipitation (RIP) assay

Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore, Burlington, MA, USA) was obtained to determine the connectivity between circABCB1 and miR-153-3p. Briefly, the cell pellet was re-suspended in an equal pellet volume of complete RIP Lysis Buffer. The Argonaute2 monoclonal antibody (Ago2) was immunoprecipitated to RNA-binding protein (RBP) of interest with protein A/G magnetic beads. The beads-bounding complexes were immobilized with a magnetic magnet and the unbound materials were washed off. RNAs were extracted and subjected to qRT-PCR.

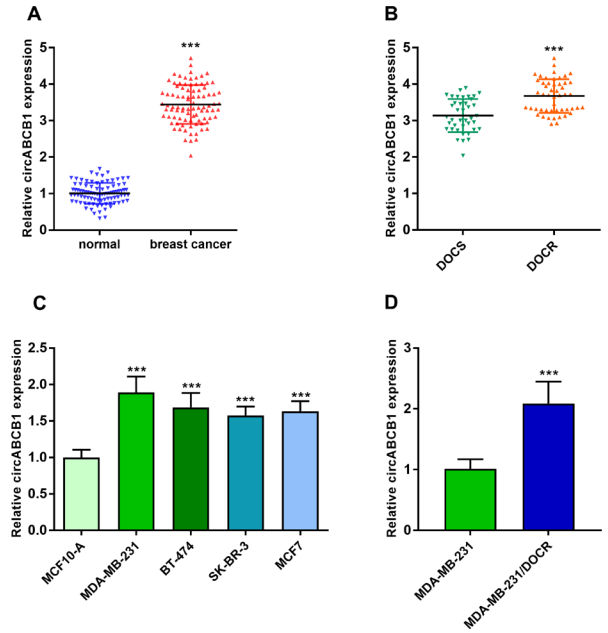

Fig. 1. Expression of circABCB1 in tissues and cells. (A, B) The expression of circABCB1 was quantified in normal breast tissues and cancerous tissues, including docetaxel-resistant and -sensitive groups. (C, D) The expression of circABCB1 was quantified in normal mammary epithelial cells and cancerous cells, including docetaxel-resistant and -sensitive groups. DOCR, docetaxel-resistant; DOCS, docetaxel-sensitive. ***$P < 0.001$.

Statistics

All analyzed data were from triplicate or more experiments. According to whether the data conformed to the Gaussian distribution, the data between the two groups were compared by unpaired t-test or Mann Whitney U test. For multiple-group comparisons, analysis of variance (ANOVA) followed by Dunnett method was employed. To test the association between two quantitative variables, the Pearson correlation was used. The nonrandom association of co-occurrence between two categorical variables was tested by the Fisher's exact test. Multivariate Cox regression and Kaplan-Meier survival analyses were utilized to identify prognostic significance of circABCB1 for time to treatment failure (TTF). P value < 0.05 was the boundary of significance or not.

Results

Docetaxel-resistant tissues and cells showed increased expression of circABCB1 compared to docetaxel-sensitive ones

To evaluate the expression of circABCB1, we used qRT-PCR in normal and cancerous tissues. We found that circABCB1 was highly expressed in cancerous tissues compared to normal tissues (Fig. 1A). Further, we got an

Table 1. Association of the circABCB1 expression with clinical characteristics in patients with advanced breast cancer.

Variables	Cases (n = 90)	CircABCB1 ≤ median	CircABCB1 > median	*P*
Age				
≤ 48	46	24	22	0.673
> 48	44	21	23	
HER2				
negative	65	29	36	0.099
positive	25	16	9	
HR				
negative	69	35	34	0.803
positive	21	10	11	
Metastasis sites				
none	52	32	20	0.028
viscera	23	9	14	
others	15	4	11	
TNM stage				
III	50	29	21	0.090
IV	40	16	24	
Histological grade				
I	56	25	31	0.247
II	26	14	12	
III	8	6	2	

HER2, human epidermal growth factor receptor 2; HR, hormone receptor (estrogen receptor or progesterone receptor).

Table 2. Multivariable analysis of time to treatment failure.

Variables	HR (95%CI)	*P*
CircABCB1 (> median vs. ≤ median)	2.892 (1.352-6.189)	0.006
Age (≤ 48 years vs. > 48 years)	1.442 (0.760-2.738)	0.263
HER2 (negative vs. positive)	1.213 (0.576-2.551)	0.612
HR (negative vs. positive)	1.115 (0.518-2.400)	0.780
Metastasis sites		
Viscera vs. none	2.159 (0.933-4.995)	0.072
Others vs. none	1.895 (0.727-4.936)	0.191
TNM stage (IV vs. III)	1.610 (0.758-3.420)	0.216
Histological grade		
II vs. I	2.657 (1.296-5.448)	0.008
III vs. I	4.074 (1.515-10.956)	0.005

HER2, human epidermal growth factor receptor 2; HR, hormone receptor (estrogen receptor or progesterone receptor).

upregulation of the expression level of circABCB1 in docetaxel-resistant tissues when compared to the docetaxel-sensitive ones (Fig. 1B). In breast cancer cell lines, circABCB1 also exhibited a higher expression than the normal cells (Fig. 1C). Given that MDA-MB-231 presented the highest level of circABCB1, we chose MDA-MB-231 and its docetaxel-resistant cell line (MDA-MB-231/DOCR) for subsequent experiments. As expected, circABCB1 expression exhibited significant differences between the docetaxel-sensitive (lower) and -resistant (higher) cells (Fig. 1D). These results indicate that circABCB1 was upregulated in breast cancer, especially docetaxel-resistant breast cancer.

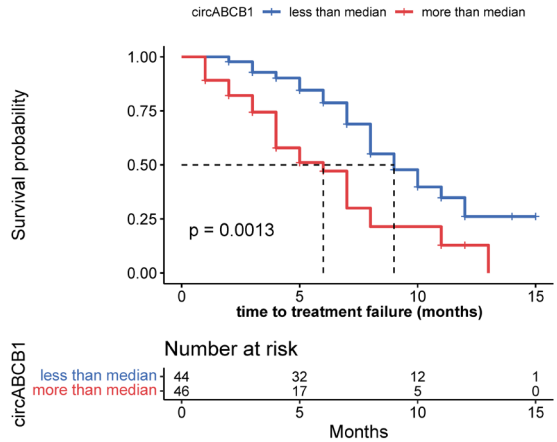

Fig. 2. CircABCB1 was related to a shorter time to treatment failure (TTF).
The figure shows circABCB1 expression split into two expression categories using the median expression value and its relationship with TTF in a Kaplan-Meier graph (log-rank test).

CircABCB1 levels were associated with TTF

We next assessed whether circABCB1 expression was associated with the outcome of patients who received docetaxel therapy. To this end, we evaluated the contribution of circABCB1 using multivariate Cox regression analysis and Kaplan-Meier survival analysis. In the Pearson correlation analysis, the high expression of circABCB1 (> median value) was associated with metastasis (Table 1). In the Cox regression analysis, we evaluated the contribution of circABCB1 in a multivariate model, and circABCB1 was associated with the periods of TTF (HR = 2.892, 95% CI 1.352-6.189, $P = 0.006$) (Table 2). The association of circABCB1 with TTF was visualized by a Kaplan-Meier plot after dividing our cohort into circABCB1-high and -low subgroups based on the median expression level (Fig. 2). These results indicated that circABCB1 was an independent predictive marker for poor TTF.

CircABCB1 regulated the viability of docetaxel-resistant and -sensitive MDA-MB-231 cells

To identify circRNAs related to docetaxel sensitivity or resistance, MDA-MB-231 cells were transfected with

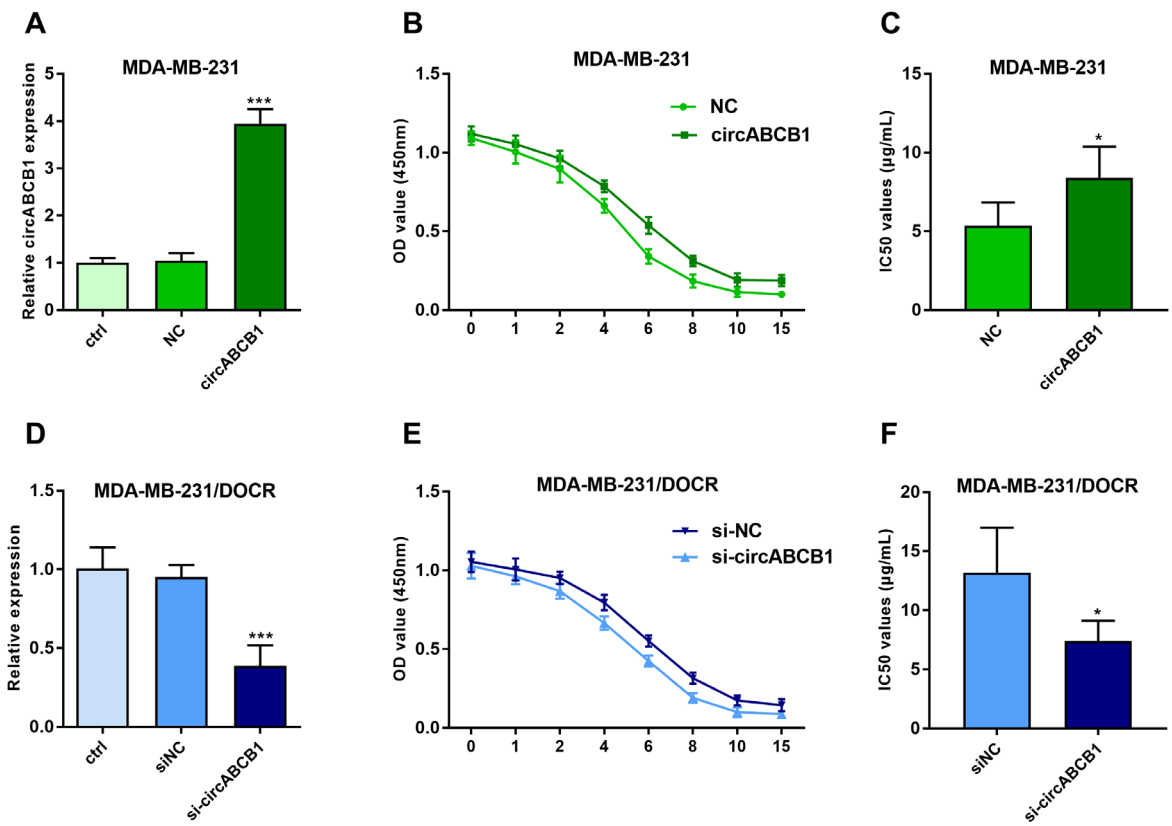

Fig. 3. CircABCB1 influenced the viability of docetaxel-resistant and -sensitive cells.
(A) qRT-PCR analysis of circABCB1 levels in transfected MDA-MB-231 cells. (B) CCK-8 viability assay was carried out in the circABCB1-transfected MDA-MB-231 cells. (C) IC50 was determined for MDA-MB-231 cells. (D) qRT-PCR analysis of circABCB1 levels in transfected MDA-MB-231/DOCR cells. (E) CCK-8 viability assay was carried out in the circABCB1-knockdown MDA-MB-231/DOCR cells. (F) IC50 was determined for MDA-MB-231/DOCR cells. DOCR, docetaxel-resistant. *$P < 0.05$, ***$P < 0.001$.

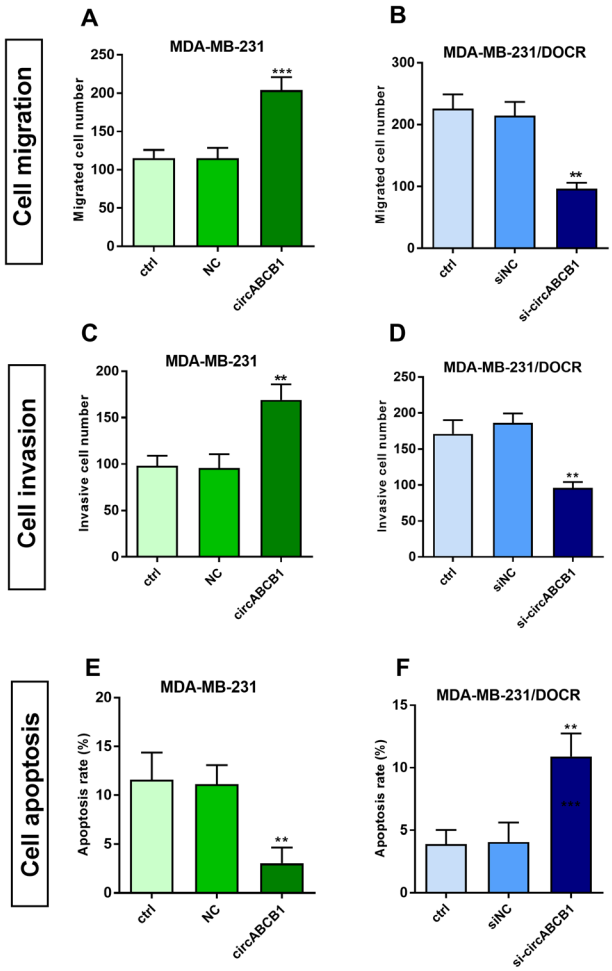

Fig. 4. CircABCB1 affects the apoptotic and migratory capacities of docetaxel-resistant and -sensitive MDA-MB-231 cells.
(A, B) The cell migration assay. (C, D) The cell invasion assay. (E, F) The cell apoptosis assay. DOCR, docetaxel-resistant. $^{**}P < 0.01$, $^{***}P < 0.001$.

circABCB1 overexpression plasmid, and MDA-MB-231/DOCR cells were transfected with circABCB1 siRNA (si-circABCB1). qRT-PCR results confirmed the transfection efficiency in MDA-MB-231 cells (Fig. 3A), determining an increase of circABCB1 in cells transfected by circABCB1 overexpression plasmid. The CCK-8 assay indicated that overexpressed circABCB1 enhanced the viability of parental MDA-MB-231 cells (Fig. 3B) and increased the IC50 value (Fig. 3C). The transfection of MDA-MB-231/DOCR was also successful (Fig. 3D). The silencing of circABCB1 significantly reduced the viability of MDA-MB-231/DOCR cells (Fig. 3E) and decreased the IC50 value (Fig. 3F).

CircABCB1 affected the apoptotic and migratory capacities of docetaxel-resistant and -sensitive MDA-MB-231 cells

It was demonstrated in Transwell migration assay that the overexpression of circABCB1 distinctly increased the number of migratory MDA-MB-231 cells (Fig. 4A), while knockdown of circABCB1 reduced the number of migratory MDA-MB-231/DOCR cells (Fig. 4B). Similarly, an increased number of invasive MDA-MB-231 cells was observed after the overexpression of circABCB1 (Fig. 4C), whereas a suppression after the silencing of circABCB1 (Fig. 4D). The apoptotic assay demonstrated that the overexpression of circABCB1 decreased the percentage of apoptotic parental MDA-MB-231 cells (Fig. 4E), whereas the silence of circABCB1 promoted apoptosis of the docetaxel-resistant MDA-MB-231/DOCR cells (Fig. 4F).

Prediction of the interactions of circABCB1 with miR-153-3p

Given the fact that circRNA can bind miRNA through their miRNA response elements (MREs), we predicted the targeting miRNA of circABCB1. CircABCB1 contained two MREs for miR-153-3p (Fig. 5A). MiR-153-3p was listed first in candidate target miRNAs with 'context+ score percentile' of 96 and 86. MiR-153-3p was lowly expressed between the normal tissues and cancerous tissues (Fig. 5B), as well as the docetaxel-sensitive and -resistant cell groups (Fig. 5C). So was the expression in cell lines (Fig. 5D, E). The expression levels of circABCB1 and miR-153-3p appeared to be a negative association (Fig. 5F). Subsequently, the results of the dual luciferase reporter assay (Fig. 5G) and RIP assay (Fig. 5H) verified that circABCB1 directly targeted miR-153-3p.

Discussion

Taxanes (including docetaxel and paclitaxel) have become the first-line treatment drugs for breast cancer (Sachdev and Jahanzeb 2016). However, genetic or acquired drug resistance hinders their effectiveness, and there are few biomarkers for predicting Taxanes resistance (Murray et al. 2012). As a chemotherapy drug for patients with breast cancer, docetaxel is widely used in clinical practice; however, more and more patients produce docetaxel resistance (Sekino and Teishima 2020). Many studies have explored the mechanism of the docetaxel-resistance. Among them, the study on the mechanism of epigenetic modification occupies a place in drug resistance-related mechanisms (Jia et al. 2019; Garcia-Martinez et al. 2021). Epigenetic modification is a heritable genetic change that affects DNA expression without changing gene nucleotide sequence. It includes DNA methylation, histone modification, and regulation of various ncRNAs (Miranda Furtado et al. 2019). CircRNA is recognized as a new class of ncRNA that plays an important role in the occurrence and progression of breast cancer (Tang et al. 2021). But its role in docetaxel resistance of breast cancer patients is less studied. In this study, we first measured circABCB1 in docetaxel-resistant breast cancer tissues. The results showed that the expression of circABCB1 in docetaxel-resistant tissues was significantly higher than that in

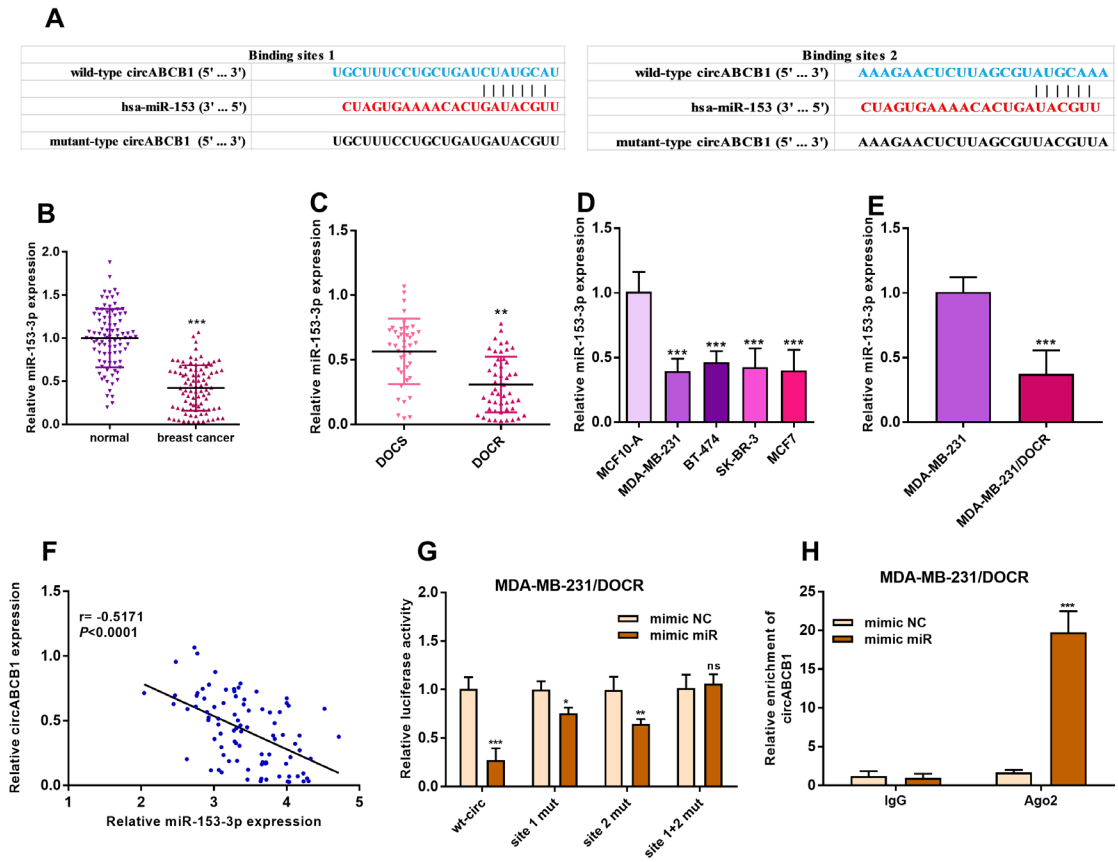

Fig. 5. CircABCB1 interacted with miR-153-3p.
(A) Target prediction analysis of circABCB1 and miR-153-3p. (B-E) The relative expression level of miR-153-3p in tissues and cells. (F) The correlation between circABCB1 expression and miR-153-3p expression in cancerous tissues. (G) Luciferase reporter assay. (H) RNA Binding Protein Immunoprecipitation (RIP) Assay. Ago2, Argonaute2 monoclonal antibody; DOCR, docetaxel-resistant; DOCS, docetaxel-sensitive; ns, not significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

docetaxel-sensitive tissues. The expression level of MDA-MB-231/DOCR in drug-resistant cell line was also significantly higher than that in parent cell line. We also found that the expression level of circABCB1 in tissue samples was positively correlated with the tumor metastasis of patients through the correlation analysis between the expression of circABCB1 in samples and clinical pathological parameters of patients. Based on the follow-up information, we found that patients with high circABCB1 expression showed shorter TTF. Through overexpression and interference experiments, we found that circABCB1 has the biological functions of increasing IC50 of docetaxel, promoting tumor cell migration/invasion, and inhibiting apoptosis. These results suggest that circABCB1 not only participates in the regulation of chemotherapy resistance of docetaxel in breast cancer patients, but also may play an important role in the occurrence and development of tumors as a tumor-promoting factor.

It has been reported that the most common mechanism of circRNA in tumor cells is to act as a "sponge" for miRNA, regulating the expression and activity of its downstream target genes by adsorbing miRNA (Han et al. 2018). CiRS-7 is the first reported circRNA with miRNA "sponge" function, which has more than 70 miR-7 binding sites (Hansen et al. 2013; Weng et al. 2017). Subsequent studies found that miR-7 was adsorbed through the "sponge" mechanism of circRNA molecules (Weng et al. 2017). CiRS-7 could reduce the activity of miR-7 and improve the expression of a series of downstream target genes, such as *EGFR*, *RAF1*, *E2A*, *NF-κB*, and *HOXB13*, and then perform biological functions. In addition to cirS-7, many other circRNAs also play an important role in different malignant tumors through the above mechanism. CircRNA-100290 promotes the occurrence and development of oral squamous cell carcinoma by adsorbing miR-378a (Chen et al. 2019); circHIPK3 sensitizes paclitaxel-resistant breast cancer cells to paclitaxel therapy at least partly via the regulation of the miR-1286 (Ni et al. 2021); CircLARP4 has been proved to inhibit the proliferation and invasion of tumor cells by adsorbing miR-424-5p in gastric cancer (Zhang et al. 2017). In this subject, we found that circABCB1 can bind to miR-153-3p and affect its expression through bioinformatics

prediction, RIP and double luciferase reporter gene detection experiments. Previous studies have confirmed that miR-153-3p plays an anti-tumor role in breast cancer and inhibits the proliferation and migration of breast cancer cells (Sun et al. 2020). It is worth noting that overexpression of miR-153-3p can inhibit the drug resistance of triple-negative breast cancer cells to paclitaxel by inducing G2/M phase (Wang et al. 2020). This suggests that miR-153-3p may be a potential therapeutic target for paclitaxel-resistant patients. Therefore, the above results suggest that miR-153-3p, as an important functional target gene of circABCB1, may mediate the promotion of circABCB1 in breast cancer and docetaxel resistance in breast cancer.

To sum up, this study shows that circABCB1 plays an important role in promoting breast cancer and participates in the regulation of docetaxel drug sensitivity. *In vitro* experiments confirmed that circABCB1 overexpression can significantly inhibit cell proliferation/migration, and increase the proportion of apoptosis and sensitivity to tamoxifen. Therefore, the above experimental results suggest that circABCB1 may be a new therapeutic target for breast cancer patients, especially those who use docetaxel as a first-line drug. In addition, its regulatory effect on docetaxel resistance suggests that circABCB1 can also be used as a new molecular marker to predict docetaxel resistance in breast cancer patients, which has important clinical significance. However, as for the application of circABCB1 in clinical practice, further patient follow-up data should be collected and analyzed with a larger sample size.

Conflict of Interest

The authors declare no conflict of interest.

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