CircRNA_0044556 regulates paclitaxel resistance in triple-negative breast cancer by targeting miR-665

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Abstract

Background

CircRNAs have been significantly implicated in the development and resistance of triple-negative breast cancer (TNBC). However, the association between circRNA_0044556 and TNBC paclitaxel (PTX) resistance is still limited. Therefore, the purpose of this study was to investigate the effect of circRNA_0044556 on the biological function and PTX resistance of TNBC cells.

Methods

PTX-resistant TNBC cells (MDA-MB-231/PTX) were obtained by continuously exposing MDA-MB-231 cells to increased paclitaxel levels. First, the expression levels of circRNA_0044556 and miR-665 were measured by qRT–PCR. Then, the regulatory relationship between miR-665 and circRNA_0044556 was verified by biological information website analysis and double luciferase reporter gene detection experiments. Finally, MTT assay, clonogenesis assay, flow cytometry and Western blot analysis were used to evaluate the influence of cell biological function.

Results

Elevated circRNA_0044556 was present in TNBC, and paclitaxel increased the expression of circRNA_0044556 in TNBC cells. In TNBC, circRNA_0044556 acts as a ceRNA for miR-665. In addition, low expression of circRNA_0044556 combined with miR-665 inhibited the proliferation of TNBC cells and paclitaxel-resistant TNBC cells while inducing cell death.

Conclusions

Our study demonstrated that downregulation of circRNA_0044556 inhibited the malignant progression of TNBC cells and paclitaxel resistance via miR-665. Thus, circRNA_0044556 may be a potential therapeutic target for TNBC paclitaxel resistance.

Introduction

Triple-negative breast cancer (TNBC), the most dangerous subtype of breast cancer, accounts for 15–20% of the global occurrences [1, 2]. A high degree of malignancy, easy occurrence of tumor progression, recurrence and metastasis, and worse prognosis of patients compared to other types are all unique biological behaviors and clinical characteristics of TNBC, which have become the most difficult link in the treatment of TNBC [3, 4]. At present, paclitaxel (PTX) is currently the main chemotherapeutic drug used for TNBC treatment of TNBC. However, the problem of drug resistance to PTX chemotherapy remains an important problem in the clinical treatment of TNBC [5]. Therefore, to create potential solutions for TNBC
clinical PTX chemotherapy resistance treatment, it is urgently necessary to further investigate the molecular mechanisms underlying TNBC incidence, development, and PTX chemotherapy resistance, as well as new therapeutic targets for TNBC.

As a unique group of noncoding RNAs, circRNAs are derived from the reverse splicing of a single pre-mRNA and have a covalent closed-loop structure [6, 7]. These advantages, compared to lncRNAs and miRNAs, have the potential to be ideal biomarkers for the treatment of cancer diseases [8]. Many studies have demonstrated that abnormal expression of circRNAs is inextricably linked to tumorigenesis and chemical resistance in TNBC [9, 10]. For example, Zheng et al reported that CircGFRA1 affects the sensitivity of TNBC cells to paclitaxel [10]. Similarly, Li et al showed that silencing hsa_circ_0000199 enhances the chemical sensitivity of TNBC[11]. This evidence strongly demonstrates the importance of circRNAs in the treatment of TNBC chemotherapy-resistance. CircRNA_0044556 (circCOL1A1) is a newly discovered circRNA. CircBase database analysis revealed circRNA_0044556 originated from the exon of the COL1A1 gene on chromosome 17q21.33, and the length of the spliced mature sequence was 699 bp. Importantly, our study showed that the expression of circRNA_0044556 was upregulated in TNBC tissues [12]. However, the effect of circRNA_0044556 on PTX resistance in TNBC remains largely unknown.

CircRNAs interacts with miRNAs and plays an indispensable role as a miRNA sponges in tumor progression and chemical resistance [13]. At present, miRNAs have been confirmed to be related to TNBC progression, prognosis and chemotherapy resistance [14, 15]. Among these, miR-665 has been shown to be associated with the progression of multiple cancers and chemotherapy resistance. More importantly, miR-665 was found to be underexpressed in TNBC[16]. In a previous study, the prediction results of circRNA_0044556 targeting miRNAs showed that miR-665 has a targeting relationship with circRNA_0044556. However, the exact role of circRNA_0044556 in sensitivity to paclitaxel chemotherapy in TNBC by regulating miR-665 expression is unclear.

Based on this background, we hypothesized that circRNA_0044556 promotes the malignant behavior of TNBC cells and PTX resistance by targeting miR-665 expression. Here, we investigated the expression, action and mechanism of circRNA_0044556 activity in PTX resistance in TNBC. These results may provide a new perspective on the antitumor mechanism of PTX in TNBC.

Methods

Cell culture

The TNBC cell line (MDA-MB-231) and non-TNBC cell line (MCF-10A) were purchased from the Type Culture Cell Bank of the Chinese Academy of Sciences(Shanghai, China). The cells were removed from the -80 °C freezer and immediately placed in a water bath. Then, a complete growth medium consisting of DMEM plus 10%(v/v) fetal bovine serum and 1%(v/v) penicillin−streptomycin was added, the cells were resuspended, and the suspension was centrifuged at 37°C at ~300xg for 5-10 minutes. The supernatant was discarded and the cells were transferred to a culture bottle and cultured at 37 °C in 5% CO₂.
Construction of PTX-resistant cells

MDA-MB-231 cells were subjected to PTX resistance through continuous exposure to increased paclitaxel (PTX) levels. Briefly, MDA-MB-231 cells were exposed to 5 nM PTX medium for a long period of time to maintain PTX resistance. The PTX concentration increased in each generation, and the PTX concentration eventually reached 20 nM, inducing PTX-resistant TNBC cell lines (MDA-MB-231/PTX).

Experimental grouping

Short hairpin RNA and pcDNA 3.1 overexpression plasmid vectors targeting circRNA_0044556, miR-665 mimics, miR-665 inhibitor and negative control were purchased from GenePharma company and named sh-circRNA_0044556, sh-NC, pcDNA 3.1, pcDNA 3.1-circRNA_0044556, miR-665 mimics, NC mimics, in-miR-665 and in-NC. Transfection of RNA and plasmids was performed in MDA-MB-231 and MDA-MB-231/PTX cells by using Lipofectamine 3000. The experiment was divided into 8 groups: sh-NC group, sh-circRNA_0044556 group, pcDNA 3.1 group, pcDNA 3.1-circRNA_0044556 group, miR-665 mimics group, NC mimics group, sh-circRNA_0044556+in-NC group, and sh-circRNA_0044556+in-miR-665 group.

RT–qPCR

TRIzol (Beyotime) was used to extract total RNA from the MDA-MB-231 and MDA-MB-231/PTX cells. The PrimeScript RT reagent kit (Vazyme) and miRNA 1st Strand cDNA Synthesis Kit (Vazyme) were used for reverse transcription of circRNA and miRNA, respectively. RT–qPCR was performed using a SYBR Green kit (Vazyme). U6 and GAPDH were selected as the internal reference genes for miRNA and circRNA, respectively. The primer sequences are shown in Table 1.

Table 1 Primer sequences

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>Hsa_circ_0044556</td>
<td>Forward 5'-GTCGTCCCGGTGAAGCTGGTCTGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCTCCTCGCTTTCCTCTCCTCC-3'</td>
</tr>
<tr>
<td>hsa-miR-665</td>
<td>Forward 5'-CGCGACCAGGGAGGCTGAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGTGCAGGGTCCGAGGTATT-3'</td>
</tr>
<tr>
<td>U6</td>
<td>Forward 5'-CGACAAGACGATCCGGGTAAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GGTTGAGGAGTGCTCTCAAGAG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-GGTCTCCTCTGACTTTCAACA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTGAGGGTCTCTCCTCCCTC-3'</td>
</tr>
</tbody>
</table>

Note: F, forward; R, reverse.
Western blot analysis

Proteins were extracted from the cells using a radioimmunoprecipitation (RIPA, Beyotime) buffer containing protease inhibitors. An equivalent amount of protein (20 μg) was loaded onto an SDS-PAGE gel and then transferred to a nitrocellulose membrane. After the membrane was sealed in 5% buttermilk for 1 h, it was diluted with TBST solution containing 5% BSA according to the manufacturer’s instructions (BAX, Proteintech, 50599-2-Ig, 1:1000; Bcl2, Proteintech, 68103-1-Ig, 1:1000; Ki67, Abcam, ab197547, 1:1000; GAPDH, bsm-33033M,1:1000), and incubated with the membrane overnight at 4 °C. The membrane was then incubated with secondary antibody for 2 h. Tanon ECL was used for chemiluminescence detection. Images were captured using a Tanon 5200 chemiluminescence imager, and ImageJ software was used for analysis.

MTT assay

When the confluence of TNBC cells reached 70%–90%, the cells (1×10^3 cells/well) were inoculated into 96-well plates. Transfected cells were treated with 100 μl of sterile MTT dye (5 mg/ml; Beyotime, C0009S) and stained at 37 °C for 4 h. The medium was then removed, and 100 μl of formazan solution was added (Beyotime, C0009S). Absorbance was measured at 570 nm using an enzyme-labeled instrument.

Clone formation assay

Transfected cells were seeded into 6-well plates at a density of 1×10^3 cells/well. After seven days of culture in the incubator, the cells were fixed with 4% formaldehyde for 10 min and stained with 5% crystal violet for 10 min. Finally, the cell clone count was determined using a low-power microscope.

Flow cytometry (FCM)

Cells in the logarithmic growth phase were seeded in a 24-well plate at a density of 1 × 10^5 cells/well, with a final volume of 500 μL. After transfection, cells were digested with 0.25% trypsin, washed with PBS, and centrifuged. The supernatant was discarded, and the density was adjusted by resuspension in 400 μL 1× annexin-binding buffer. Next, 100 μL was absorbed and transferred to a sterile centrifuge tube. Then, 5 μL Annexin V-FITC and 5 μL PI were added successively, mixed well, and incubated in the dark for 15 min. Analysis of the mixture was performed using FCM.

Luciferase activity assay

Lipofectamine 3000 reagent (Invitrogen) was used to cotransfect the pGL3-basic vector with circRNA_0044556-wild type (WT)/mutant one (MUT) and miR-665 mimics or NC mimics into TNBC cells. Luciferase activity was detected using a dual-luciferase detection system.

Data analysis

Data were analyzed and mapped using Graphpad Prism9 (Version9.5.0). Photoshop was used to organize the images. All maps are presented as the mean ±SD, and significant differences between
groups were tested using the t-test. A P value less than 0.05 was considered significant (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Results

CircRNA_0044556 was highly expressed in TNBC, while miR-665 was low expressed

First, we investigated the levels of circRNA_0044556 and miR-665 in TNBC cells by qRT‒PCR analysis. We found that the level of circRNA_0044556 was higher in MDA-MB-231 cells than in MCF-10A cells (P<0.001), while miR-665 expression was downregulated (P<0.01) (Fig 1A-B). To further investigate their relationship with paclitaxel resistance in TNBC, we cultured PTX-resistant cells and named them MDA-MB-231/PTX. The results showed that paclitaxel increased the expression of circRNA_0044556 in MDA-MB-231 cells (P<0.001) but decreased miR-665 expression (P<0.05) (Fig 1A-B).

Downregulated circRNA_0044556 inhibited the malignant phenotype and paclitaxel resistance of TNBC cells

Next, to study the influence of circRNA_0044556 on the biological function of TNBC cells and the sensitivity to paclitaxel, we conducted a functional knockout experiment. Transfection efficiency was confirmed by qRT-PCR, and as shown in Fig 1A, the level of circRNA_0044556 was inhibited after sh-circRNA_0044556 treatment. The results of MTT and clonogenesis experiments showed that knockdown of circRNA_0044556 reduced cell viability and clonal colony number (Fig 1B-C). FCM analysis showed that circRNA_0044556 knockdown increased the apoptosis rate of the cells (Fig 1D). Bax (apoptotic protein), Bcl-2 (anti-apoptotic protein), and Ki67 (proliferative protein) are some of the most important indicators of apoptosis and proliferation and are closely related to the occurrence, development, and prognosis of tumors. WB analysis confirmed that the knockdown of circRNA_0044556 depressed the expression levels of Ki-67 and Bcl-2 proteins in cells, but enhanced the expression levels of Bax protein (Fig 1E-G). The above data suggested that downregulation of circRNA_0044556 suppressed the malignant phenotype and paclitaxel resistance of TNBC cells.

CircRNA_0044556 competitively adsorbs miR-665

Next, we explored the relationship between circRNA_0044556 and miR-665 expression. The bioinformatics website found that miR-665 has a targeted binding site for circRNA_0044556 (Fig 3A-B). The circRNA_0044556-WT/MUT luciferase reporter vector was designed based on the predicted binding sites. Dual luciferase reporter assays showed that circRNA_0044556 targeted miR-665 (Fig 3C). Subsequently, we analyzed the regulatory mode of circRNA_0044556 on miR-665 expression. As shown in Fig 3D, miR-665 expression increased and decreased in MDA-MB-231 cells with low and overexpression of circRNA_0044556, respectively. These data indicate that miR-665 is the downstream miRNA of circRNA_0044556 in TNBC, and that the effect of circRNA_0044556 on TNBC paclitaxel resistance may be related to miR-665.
CircRNA_0044556 affects the malignant phenotype and paclitaxel resistance of TNBC cells by regulating miR-665 expression

To observe the modulatory mechanism of circRNA_0044556 and miR-665 in TNBC, we co-transfected sh-circRNA_0044556 with in-NC or in-miR-665. qRT-PCR analysis showed that transfection with sh-circRNA_0044556 upregulated miR-665 expression, whereas co-transfection with miR-665 inhibitors significantly eliminated this reaction (Fig 4A). The results of biological function experiments showed that compared to cells transfected with sh-circRNA_0044556, cells co-transfected with sh-circRNA_0044556 and in-miR-665 showed increased cell proliferation and decreased apoptosis (Fig 4B-D) (P<0.01). WB analysis further confirmed these results (Fig 4E-G). In summary, circRNA_0044556 inhibits the malignant phenotype and paclitaxel resistance of TNBC cells, and these biological functions are achieved by sponging miR-665.

Discussion

Paclitaxel is a promising chemotherapeutic drug for the treatment of advanced TNBC [17]. However, most advanced TNBC patients only respond to paclitaxel treatment in the initial stage, and acquired paclitaxel resistance occurs frequently in the later stage of treatment, resulting in no significant improvement in the 5-year survival of TNBC patients treated with paclitaxel [18]. Therefore, acquired resistance to paclitaxel is undoubtedly the biggest obstacle in improving the overall response and survival of patients with TNBC. In recent years, owing to the stability and abundance of circRNAs, an increasing number of circRNAs have been identified as abnormally expressed in TNBC; as important regulators of TNBC cancer progression, circRNAs are of great significance for the resistance of TNBC [9, 19]. However, research on circRNAs associated with paclitaxel resistance in TNBC remains limited. Therefore, we investigated the expression, action, and mechanism of circRNA_0044556 activity in PTX resistance in TNBC. The results showed that circRNA_0044556 promotes the malignant behavior of TNBC cells and PTX resistance by sponging miR-665.

This is the first study to verify the expression pattern of circRNA_0044556 in TNBC cells. circRNA_0044556 was upregulated in TNBC cells compared to normal cells. Subsequently, we constructed PTX-resistant TNBC cells and measured the levels of circRNA_0044556. PTX increased circRNA_0044556 expression in MDA-MB-231 cells, suggesting that circRNA_0044556 may be related to paclitaxel resistance. As a circRNA encoded by COL1A1, previous studies have shown that circRNA_0044556 plays a vital role in the occurrence and development of tumors, including colorectal cancer [20, 21], gastric cancer [22] and TNBC[12]. The infinite malignant proliferation of tumor cells and inhibition of apoptosis are the core factors in tumor development and change [23, 24]. Therefore, we performed a series of experiments in this study. We found that low expression of circRNA_0044556 can restrict the proliferation of TNBC cells and induce cell apoptosis. In previous studies, we confirmed that circRNA_0044556 reduced the sensitivity of triple-negative breast cancer cells to adriamycin by acting as a sponge for miR-145 and regulating NRAS [12]. However, the effect of circRNA_0044556 on PTX sensitivity in TNBC is unclear. Therefore, we further observed the effect of circRNA_0044556 knockdown...
on PTX-resistant TNBC cells, and found that low expression of circRNA_0044556 restricted the cell proliferation of PTX-resistant TNBC cells in vitro and promoted cell apoptosis. In summary, circRNA_0044556 can be used as a therapeutic target for TNBC, and downregulation of circRNA_0044556 has a beneficial effect on slowing down the progression of TNBC and PTX resistance by reducing cell proliferation and promoting cell apoptosis.

The development of circRNAs as ceRNA competitive binding miRNAs to regulate TNBC has been demonstrated in many studies [25]. Previous studies have confirmed that the main regulatory mechanism of circRNA_0044556 is that ceRNA, which acts as a downstream miRNA, plays a role in tumors [22]. Therefore, we screened miRNAs significantly enriched with circRNA_0044556 through the bioinformatics website and found that circRNA_0044556 showed highly specific enrichment of miR-665. To further verify the relationship between circRNA_0044556 and miR-665, we confirmed that circRNA_0044556 acts as a ceRNA for miR-665 in TNBC through luciferase reporter gene analysis. In addition, circRNA_0044556 actively regulated miR-665 expression in TNBC, leading us to propose a mechanism by which circRNA_0044556 may contribute to blocking TNBC progression by sponging miR-665.

Previous studies have confirmed that miRNAs play an important role in many cell biological processes, such as proliferation, apoptosis and drug resistance [26]. In recent years, a number of studies have been conducted on the role of miR-665 in tumorigenesis and development, but the results vary, and different studies have reached different conclusions regarding the role of miR-665 as a tumor suppressor or tumor promoter, and this contradictory role seems to be determined by the cell type and the localization of miR-665. Currently, miR-665 has been demonstrated as a tumor suppressor in some cancer cells, such as bladder cancer [27], hepatocellular carcinoma [28], and gastric cancer [29], whereas miR-665 has been described as a tumor promoter in lung cancer [28] and ovarian cancer [30]. Importantly, recent studies have found that miR-665 is significantly downregulated in TNBC, and the upregulation of miR-665 is positively correlated with the survival time of patients with TNBC [16]. In addition, miR-665 is inextricably associated with paclitaxel resistance in cervical cancer cells [31]. However, the mechanism of miR-665 resistance to paclitaxel in TNBC remains unknown. Therefore, in this study, sh-circRNA_0044556 and IN-NC or In-miR-665 were transfected into MDA-MB-231 and MDA-MB-231/PTX cells for the functional salvage experiments. The results showed that knockdown of circRNA_0044556 increased the cell viability and colony number of MDA-MB-231 and MDA-MB-231/PTX cells and induced cell apoptosis, but these effects were reversed by an miR-665 inhibitor. We can also explain that circRNA_0044556 depletion attenuated the effects of low miR-624-3p levels in TNBC and paclitaxel-resistant cells.

In summary, our study confirmed our hypothesis that circRNA_0044556 inhibits TNBC cell resistance to PTX by regulating miR-665 to reduce cell activity and proliferation capacity, and induce cell apoptosis. In this study, we identified a novel regulatory mechanism for TNBC. Nevertheless, this study has some limitations that should be further explored. For example, mechanistic studies are carried out in cells, and studies on other cell lines need to be further explored. Second, further studies are needed to determine which targets and signaling pathways miR-665 regulates TNBC progression and PTX resistance.
addition, future multicenter trials and animal studies are required to verify the diagnostic and therapeutic effects of circRNA_0044556.

**Conclusion**

Our results suggest that circRNA_0044556 is an "oncogene" in TNBC. Highly stable circRNA_0044556 promotes the malignant phenotype and PTX resistance of TNBC cells through miR-378a-3p. Our findings not only explain the mechanism of circRNA_0044556 in regulating TNBC cell progression and PTX resistance but also provide a potential biomarker and therapeutic target for TNBC.

**Declarations**

**Author contributions**

Jingjing Chen, Peng Shi: Conceptualization, Methodology, Experiments; Zhichao Cui, Nan Jiang, Jie Ma: Data collection and analysis; Jinghua Zhang: Supervision, manuscript reviewing and Editing. All authors have written and approved the final manuscript.

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**Data availability statements**:

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

**Compliance with Ethical Standards**

**Disclosure of potential conflicts of interest**:

The authors declare that they have no competing interests.

**Ethical approval**:

This article does not contain any studies with human participants or animals performed by any of the authors.

**Acknowledgments**

Not applicable.

**References**


Figures
CircRNA_0044556 was highly expressed in TNBC, while miR-665 was low expressed

A: The level of circRNA_0044556 in cells was analyzed by qRT‒PCR.

B: The level of miR-665 in cells was analyzed by qRT‒PCR.

**$P < 0.01$, ***$P < 0.001$ vs. MCF-10A, N = 3. *$P < 0.05$, ***$P < 0.001$ vs. MDA-MB-231, N = 3.
Figure 2

Down-regulated circRNA_0044556 inhibited the malignant phenotype and paclitaxel resistance of TNBC cells

A: qRT–PCR was used to assess the endogenous expression of circRNA_0044556 after transfection of cells.

B: MTT assays were used to assess cell proliferation.

C: Clonal formation assays were used to assess cell proliferation.

D: Apoptosis was measured by FCM.

E-H: WB analysis (E) of the expression levels of Bax (F), Bcl-2 (G) and Ki67 (H) proteins.

**P < 0.01, ***P < 0.001 vs. sh-NC, N = 3.
**Figure 3**

CircRNA_0044556 competitively adsorbs miR-665

A-B: Predicted binding sites of circRNA_0044556 and miR-665.

C: Dual luciferase reporting experiment verified the targeting relationship between circRNA_0044556 and miR-665.

D: The expression of miR-665 in MDA-MB-231 cells with circRNA_0044556 knockdown or overexpression was analyzed by qRT–PCR.

**P < 0.01, vs. NC mimics, N = 3. ***P < 0.001, vs. sh-NC, N = 3. **P < 0.01, vs. pcDNA 3.1, N = 3.**
Figure 4

CircRNA_0044556 affects the malignant phenotype and paclitaxel resistance of TNBC cells by regulating miR-665 expression

A: qRT–PCR was used to assess the endogenous expression of circRNA_0044556 after transfection of cells.

B: MTT assays were used to assess cell proliferation.

C: Clonal formation assays were used to assess cell proliferation.

D: Apoptosis was measured by FCM.

E-H: WB analysis (E) of the expression levels of Bax (F), Bcl-2 (G) and Ki67 (H) proteins.

*P<0.05, **P< 0.01, ***P < 0.001 vs. sh-circRNA_0044556+in-NC, N = 3.