Original Article
YY1 as a promoter regulating the circ_0001946/miR-671-5p/EGFR axis to promote chemotherapy resistance in breast cancer cells

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Abstract: Objective: To investigate the mechanism of circ_0001946 activated by transcription factor Yin Yang 1 (YY1), targeting miR-671-5p to regulate epidermal growth factor receptor (EGFR) and thereby participating in the chemotherapy resistance of breast cancer (BC) cells. Methods: Circ_0001946, which is differentially expressed in BC, was screened using gene expression omnibus. Dual luciferase assay and RNA immunoprecipitation were conducted to verify the relationship among circ_0001946/miR-671-5p/EGFR. A ChiP test confirmed that YY1 can be used as a transcription factor of circ_0001946 to specifically bind to its promoter. The expression of circ_0001946/miR-671-5p/EGFR regulatory axis in BC tissues and cell lines were evaluated using qRT-PCR. As for in vitro experiments, tamoxifen was used to establish a drug-resistant BC cell model. The effects of the regulatory axis on the proliferation, invasion and apoptosis of BC cells were studied using CCK-8, Transwell invasion assay and Annexin V-FITC/PI staining, so as to evaluate its effect on the sensitivity of BC cells to tamoxifen. Results: Circ_0001946 showed an abnormally high expression in BC tissues and tamoxifen resistant cells and was up-regulated in an IC₅₀ dependent manner (both P<0.05). Circ_0001946 was activated by YY1 in drug-resistant BC cells. Knockdown of circ_0001946 significantly inhibited the proliferation, invasion and promoted apoptosis of drug-resistant BC cells (all P<0.05). Overexpression of circ_0001946 promoted the proliferation and invasion of drug-resistant BC cells and hindered their apoptosis, which could be partially reversed by miR-671-5p mimics (all P<0.05). EGFR has been proven to be a downstream target gene of miR-671-5p. A knockdown of EGFR improved the malignant biological behavior of drug-resistant BC cells, which could be partially eliminated by overexpression of circ_0001946 (all P<0.05). Conclusion: Circ_0001946 absorbs miR-671-5p to target EGFR to promote the growth and malignant invasion of drug-resistant BC cells, thereby increasing the resistance to tamoxifen. This effect of circ_0001946 may be achieved by transcriptional activation of YY1.

Keywords: circ_0001946, miR-671-5p, epidermal growth factor receptor (EGFR), tamoxifen

Introduction

Breast cancer (BC) is the most common malignant tumor in women worldwide [1]. Though prominent advancement has developed in the detection and treatment and of BC recently, patients with metastatic BC still have a low survival rate [2]. Most patients with metastatic BC develop resistance to tamoxifen, and the metastasis of BC and chemotherapy resistance are the most common causes of clinical treatment failure, disease recurrence and death [3, 4]. Therefore, knowing the underline mechanism of tamoxifen resistance may create a new insight for the treatment of BC.

As closed circular non-coding RNAs, circRNAs are stable in structure, not affected by RNA exonuclease and widely present in eukaryotes [5]. CircRNAs participate in the progress of BC at the transcription, post-transcription and translation levels, and can act as a sponge of miRNA to affect the biological activity and function of miRNA target genes [6]. This study, based on previous integrated comprehensive gene expression data and bioinformatics analy-
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sis, revealed that circ_0001946 was highly expressed in the drug-resistant strain LCC9 cell line. It has been proven that hsa_circ_0001946 showed abnormally high expression in tissue samples of patients with BC [7]. However, the regulatory role and mechanism of circ_0001946 in BC have not been fully explored. In addition, circ_0001946 has also been found to play a promotion role in colorectal cancer [8]. Circ_0001946 may be a biomarker for the diagnosis and prediction of cisplatin chemotherapy sensitivity in patients with non-small cell lung cancer [9]. We also confirmed that the expression of circ_0001946 could be induced by the Yin Yang 1 (YY1) transcription factor. YY1 can promote the malignant progression of BC by activating miRNAs or LncRNAs [10]. So, it is speculated that YY1 may mediate circ_0001946 to affect the chemotherapy resistance of BC cells. The current research aimed to investigate the possible role and the mechanism of circ_0001946 in the sensitivity of chemotherapy drugs for BC.

As a type of short-chain non-coding regulatory RNA, miRNAs are instrumental in various biological processes, including the proliferation, metastasis, and chemotherapy resistance of triple-negative BC [11]. MiR-671-5p as a targeted downstream miRNA of circ_0001946, it has been proven to reduce DNA repair ability by regulating the FOXM1 gene to promote the sensitivity of BC cells to ultraviolet light and chemotherapy [12]. Our study subsequently screened the downstream target genes of miR-671-5p, which was confirmed to regulate the epidermal growth factor receptor (EGFR) gene at a post-transcriptional level. EGFR has been instrumental in physiological processes such as cell growth, proliferation and differentiation [13]. It is confirmed that EGFR is overexpressed in glial cells, as well as in multiple cancer tissues (kidney, lung, prostate and pancreas). Besides, the expression level of EGFR in BC is also elevated [14]. MiR-7 can inhibit EGFR and promote the sensitivity of BC cells to Adriamycin [15]. Therefore, this study selected EGFR for subsequent analyses.

This study analyzed the process by which YY1 transcription activated circ_0001946 to regulate the miR-671-5p/EGFR axis and to affect the biological function and the resistance to chemotherapeutic drugs of BC cells. We intended to reveal the potential biological relationship between the process and the drug sensitivity of BC cells as well as the specific mechanism, to provide a mature theoretical basis for biomolecular therapy for BC.

Methods and materials

Ethics statement

This experiment was reviewed and approved by the Ethics Committee of China-Japan Union Hospital of Jilin University. Patients knew about their tissue use in this study and provided written informed consents before operation.

Bioinformatics analysis

We searched for BC-related gene expression microarrays with the keyword “Breast cancer” from the Gene Expression Omnibus database of the National Center for Biotechnology Information Search database (NCBI) website. The data set GSE159980 related to BC chemotherapy drug sensitivity was obtained. This data set contains differentially expressed circRNAs in MCF-7 (sensitive to tamoxifen) and LCC9 cell lines (insensitive to tamoxifen). The differential analysis of the microarray expression profile was conducted, and a volcano plot of the differential expression was drawn. The differential screening conditions were adj. P. Value <0.05 and |LogFoldChange|>1. The top 5 circRNAs with up-regulated expression were presented on the heat map. Target miRNAs that specifically bind to circRNA were predicted using circinteractome (https://circinteractome.nia.nih.gov/) and StarBase (http://starbase.sysu.edu.cn/). Downstream target genes of miRNA were predicted using TargetScan (http://www.targetscan.org/vert_72/), miRDB (http://mirdb.org/index.html), RNA22 (https://cm.jefferson.edu/rna22/Interactive/) and StarBase. A Venn diagram of the intersection of the prediction results was obtained using the online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). The KOBAS (http://kobas.cbi.pku.edu.cn/anno_iden.php) was used to perform pathway enrichment analysis of Kyoto Encyclopedia of Genes and Genomes. The risk genes of BC (C2938924) were obtained from the DisGeNET database (https://www.disgenet.org/). STRING (https://string-db.org) was used for the correlation analysis of protein-pro-
tein interaction between the genes screened out by KEGG and the BC risk genes. JASPAR (http://jaspar.genereg.net) and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) were used to obtain transcription factors and their binding sites that can highly and specifically bind to the promoters of circRNAs. The IC_{50} values of tamoxifen on different BC cell lines were obtained from the GDSC database (https://www.cancerrxgene.org/).

**Tissue samples**

We collected 56 pathological sections of BC tissues and 56 sections from the para-carcinoma tissues (over 2 cm from the outer edge of the tumor) during operation from patients diagnosed with BC from December 2019 to June 2020. The diagnosis was confirmed by two pathologists in the Galactophore Department of our hospital according to the pathological diagnostic criteria [16]. The mean age of the subjects was (47.28±11.8) years old (24 to 65 years old). The TNM staging of patients was assigned according to the *American Joint Committee on Cancer Classification, 8th edition*, http://www.cancerstaging.org. It was shown that 16 patients were in stage I, 33 in stage II, and 7 in stage III. Among them, 18 cases were accompanied with a lymph node metastasis, and 38 cases were not. The diagnosis was confirmed in all tissues by pathological examination. All patients did not have a history of BC and did not receive other preoperative anti-cancer treatments such as surgical puncture, immunity enhancement, chemotherapy or radiotherapy.

**Cell culture**

The BC cell lines MDA-MB-231 (catalog number: TCHu227), MDA-MB-468 (TCHu136), MCF7 (TCHu74) were all purchased from the National collection of authenticated cell cultures. MDA-MB-231 was cultivated in Leibovitz’s L-15 medium (PM151010, Procell, China) with 15% fetal bovine serum (FBS). MDA-MB-468 was cultivated in Leibovitz’s L-15 medium (PM151010B, Procell, China) with 10% FBS. MCF7 was cultured in minimum essential medium (PM150411B, Procell, China) containing 0.01 mg/mL insulin and 10% FBS. All media was mixed with 100 U/mL penicillin streptomycin and the 5% CO_{2} incubator was keep humidified at 37°C. Subculture was conducted when 80% confluence was reached. In order to construct the drug-resistant cell line MCF7/R, MCF7 cells in the logarithmic growth phase were exposed to high-concentration in a short time before its inoculation into a culture dish. After the growth was stable, tamoxifen (ICI 47699, T5648, Sigma-Aldrich, USA) at a final concentration of 1×10^{-6} mol/L was added. The culture solution was changed every three days, and an equal concentration of tamoxifen was added when changing the medium. The cells were continuously incubated for 1 month in a humidified condition at 37°C and in 5% CO_{2} to construct stable MCF7/R cells.

**Cell transfection**

The third generation of logarithmic growth phase cells were digested with trypsin, added with serum-free medium to starve the cells for 2 h, and transfected using LipofectamineTM 3000 (L3000001, Thermo Fisher Scientific, USA). Transfection plasmids included Blank (without transfection), OE-circ_0001946 (over-expression of circ_0001946), OE-NC (over-expression of circ_0001946 negative control), si-circ_0001946 (knockdown of circ_0001946 negative control), si-NC (knockdown of circ_0001946 negative control), miR-671-5p mimic (with miR-671-5p mimic), mimic-NC (with miR-671-5p mimic negative control), pc-EGFR (overexpression of EGFR), pc-EGFR-NC (overexpression of EGFR negative control), pc-YY1 (overexpression of YY1), pc-YY1-NC (overexpression of YY1 negative control), si-YY1 (knockdown of YY1) and si-YY1-NC (knockdown of YY1 negative control). The specific transfection steps were carried out according to the kit instructions to construct stable cell lines. After 6 hours, the complete medium containing serum and double antibodies was replaced, and the incubation was continued for another 48 h in a 5% CO_{2} incubator at 37°C for subsequent experiments. The overexpressed plasmids and their siRNA interference plasmids, miRNA mimics, inhibitors and their controls were all designed and synthesized by GenePharm, Shanghai, China. See the transfection sequences in **Table 1**.

**qRT-PCR**

RNA was extracted from tissues and cells with TRizol. The RNA was re-dissolved with DECP water (693520, Sigma-Aldrich, USA), and deter-
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mined for concentration and purity using Nanodrop UV spectrophotometer (ND-1000, Thermofisher Scientific, USA). Total RNA (1 μL) was reverse transcribed to synthesize the first strand of cDNA using the reverse transcription kit (11141ES10, Shanghai Yisheng, China). The cDNA was inactivated at 80°C. Sequences of circ_0001946, miR-671-5p and EGFR were searched for. The sequence of miR-671-5p is short, so the forward and reverse primers were designed by tailing reaction. All the primers were synthesized by Shanghai Gema, China. See Table 2. SYBRPremix EX Taq kit (11198ES03, Shanghai Yisheng, China) was used to conduct RT-PCR synthesis and Q-PCR (ABI7700, Thermo Fisher, USA). The 10 μL reaction system was as follows, 0.2 μL of forward primer, 0.2 μL of reverse primer, 0.2 μL of ROX, 3.4 μL of DEPC water, 1.0 μL of cDNA sample and 5.0 μL of 5×SYBR Green qRT-PCR solution. The reaction conditions of qRT-PCR were 5 min at 95°C, 30 s at 95°C, 30 s at 60°C, 30 s at 72°C and 30 s at 72°C, 40 cycles in total. U6 was the internal reference for miR-671-5p, and GAPDH for the rest. StepOne qRT-PCR analysis software was used to analyze the Ct value of each sample during amplification. \[ \Delta Ct = Ct_{target} - Ct_{internal control} \]
\[ \Delta \Delta Ct = (Ct_{target in transfection group} - Ct_{internal control in transfection group}) - (Ct_{target in control group} - Ct_{internal control in control group}) \]
Relative expression level of mRNA or miRNA =2^\Delta \Delta Ct (average value of 3 repeated measures).

Western blot

Logarithmic-phase cells were washed with pre-cooled PBS, added with RIPA lysis buffer (P0013C, Shanghai Beyotime, China) to be lysed on ice for 15 min and then centrifuged at 12,000 rpm in a low-speed freezing centrifuge. The supernatant was added with 5× loading buffer, boiled for 5 min for denaturation, then measured for the protein concentration using BCA kit (A53227, Thermo Fisher, USA) and the absorbance of each well at A490 wavelength. Protein concentration was calculated according to the standard curve. Thereafter, the sample was added 5× SDS loading buffer, pipetted to mix, and boiled for 10 min to fully denature it. After protein electrophoresis, the sample was transferred to membrane (LC2002, Thermo Fisher, USA), rinsed in TBST 2 to 3 times, 5 min/time, blocked in 5% skim milk for 2 h, and rinsed again with TBST. EGFR (1:1000, ab52894, Abcam, UK) was diluted with TBST solution containing 5% BSA, then added to the membrane with GAPDH (1:1000, ab181602, Abcam, UK) primary antibody and incubated overnight at 4°C. On the next day, the membrane was washed with TBST solution. Corresponding secondary antibody IgG (1:500, ab133470, Abcam, UK) diluted with TBST solution containing 5% BSA and was added and incubated at room temperature for 2 h. We developed the color by chemiluminescence in a dark room and determined the gray value and ratio of the protein band of target genes and the internal control (GAPDH) with Image J.

RNase R digestion and fluorescence in situ hybridization (FISH)

RNase R digestion test was used to verify the ring structure of circ_0001946, and the procedures were according to the instruction of the reagent (RNR07250, Epicentre, USA). Firstly, the HS tissues were fixed for 6 h, dehydrated with decreasing concentrations of ethanol, embedded in paraffin, sliced and baked. Secondly, the slices were then dewaxed according to the instructions of the FISH kit (Bse1002, BerisinBio, Guangzhou, China), added with citric acid buffer solution dropwise, incubated for 20 min, then rinse with PBS, digested with proteinase K, and rinsed again with PBS. Thirdly, the slices were added with pre-hybridization solution drops and removed after 1 h. The hybridization solution was added with the Cy3-labeled circ_0001320 probe and incubated
with the cells overnight at 37°C in dark. Fourthly, the hybridization solution was washed off with SSC working solution next day. The nuclei were counter-stained with DAPI and incubated in dark for 10 min. Anti-fluorescence quencher was added dropwise to seal the slices. Pictures were taken with an inverted microscope. Fifthly, the slices were soaked in the fixative for 10 min, then in ice ethanol with a volume fraction of 70%, 90%, and 100% for 5 min, respectively. Sixthly, 50 mL of 50% formamide was added into a wet box and preheated. The pre-hybridization buffer was prepared and reacted with the sample at 55°C for 1 h. The circ_100395 probe was dissolved in hybridization buffer and incubated with the sample overnight at 37°C in the wet box. The cells were washed, added with Hoechst to stain the nucleus, and added with anti-fluorescence quencher dropwise to seal the slices. Lastly, the localization of circ_100395 expression in cells was observed under a fluorescence microscope.

**Dual-luciferase reporter assay**

The predicted specific binding sites between circ_0001946 and miR-671-5p, between miR-671-5p and EGFR were amplified by PCR and cloned into dual luciferase reporter plasmids (11402ES60, Yeasen, Shanghai, China), and the sequence was named circ-WT. Mutation kit was used for site-directed mutation, and the genes were cloned into the reporter plasmids to generate circ-MUT. Circ-WT and circ-MUT were co-transfected into HEK293T cells with miR-671-5p mimic and mimic-NC, respectively, and cultured for 48 h for corresponding luciferase activity analysis. The relative luciferase activity was normalized to Renilla Luciferase activity.

**Chromatin immunoprecipitation (ChIP)**

ChIP kits (P-2026-48, Epigentek, USA) were used for the experiment. The cells were cross-linked with 1% formaldehyde for 10 min at room temperature, added with 125 mM of glycine for ending, placed for 5 min and discarded the cross-linked compound in the culture dish. After washing with pre-cooled PBS for 2 to 3 times, the cells were transferred to ice, suspended with 1 mL of pre-cooled PBS, PMSF and protease inhibitor, centrifuged at 10,000 rpm and 4°C for 5 min and disrupted with ultrasound. Subsequently, the cells were added with elution buffer solution and NaCl solution, incubated at 65°C for 2 h, added with proteinase K, incubated for 1 h, added with phenol chloroform to extract DNA, and then detected by 1.5% agarose gel electrophoresis. Magnetic beads were prepared, mixed and centrifuged with antibodies at 3000 rpm for 2 min. The supernatant was discarded to obtain the sample which was washed with RIPA lysis solution, and then added with the ultrasonicated chromatin and RIPA solution. After RNA-binding protein immunoprecipitation, DNA was purified, and the input DNA was analyzed by qRT-PCR.

**Cell counting kit-8 (CCK-8)**

Cells were inoculated into 96-well plates at 1×10^5 cells/mL, and pre-cultured in a 5% CO₂ incubator at 37°C for 24 h. Each well was added with 1, 5, 10, 20, 30, 40, 50 μM of tamoxifen, respectively. After treated for 24 h, each well was added with 10 mL of CCK-8 solution (HY-K0301, MedChemExpress, USA) carefully to avoid generating bubbles, mixed gently, and incubated for 2 h. The absorbance at 450 nm was measured with a multi-function microporous reader (Spectrophotometer, Thermo Fisher, MA, United States).

<table>
<thead>
<tr>
<th>Table 2. qRT-PCR primers</th>
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<tr>
<td>Gene</td>
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<tr>
<td>circ_0001946</td>
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<tr>
<td>miR-671-5p</td>
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<tr>
<td>EGFR</td>
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<tr>
<td>CD163</td>
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<td>TNFA</td>
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<td>U6</td>
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<td>GAPDH</td>
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Note: EGFR: epidermal growth factor receptor.
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Annexin V-FITC/PI staining

The cells of each transfection group were inoculated into a 96-well plate. When the cell confluence reached 60-80%, PBS buffer was added to adjust the concentration. After the cell suspension was prepared, cells were stained according to the instruction of Annexin V-FITC/PI cell apoptosis detection kit (40302ES20, Yeasen, China). After being added with 5 μL of propidium iodide and 5 μL of annexin V-FITC, the solution was mixed well and incubated in dark for 15 min. The apoptosis was detected with the use of flow cytometry.

Transwell

The cells were starved with serum-free Opti medium for 2 h, washed with PBS, trypsinized, centrifuged, resuspended and counted. The Transwell chamber (3422, Corning, USA) was coated with Matrigel (354230, BD Matrigel, USA) and moistened with serum-free medium. Transwell was placed in a 24-well plate, and 500 μL of DMEM medium containing 10% FBS was added to the lower chamber, 200 μL of cell suspension to the upper chamber. The cells were then cultured in a 37°C incubator for 24 h. After the removal of the culture medium and the cells that did not pass the membrane, the cells that passed through were fixed with methanol, stained with crystal violet (both upper and lower chambers), and protected from light. After rinsing with PBS, the cells were naturally dried and observed under a microscope. Ten fields of view were selected randomly to count the number of cells.

Statistical analyses

SPSS 23.0 statistical software was used for data processing. Quantitative variables were expressed as mean ± standard deviation, and the ones conforming to the normal distribution were compared (intergroup) using t test and one-way analysis of variance. For the ones not conforming to normal distribution, Mann-Whitney U test was performed for the intergroup comparison, and Bonferroni correction for the pairwise comparisons between multiple groups. Correlation analysis of expressions was performed by Pearson’s correlation coefficient. A difference of P<0.05 was considered statistically significant.

Results

Circ_0001946 had a high expression in patients with BC and could be used as an effective diagnostic indicator of BC

By searching BC-related differential expression microarrays, GSE159980 data set in the gene expression omnibus database was selected for study. We analyzed the differentially expressed circRNAs in drug-resistant and non-resistant cell lines. Differentially expressed circRNAs are shown in a volcano plot (Figure 1A). We selected the highly expressed circRNAs in BC for study. The top 5 highly expressed circRNAs in the LCC9 cell line are presented in the heat map (Figure 1B). Detection of the expression of circ_0001946 showed that the expression of circ_0001946 in BC tissues was significantly up-regulated compared with that in the normal group (P<0.05, Figure 1C). It is suggested that the expression of circ_0001946 in BC tissue has a certain diagnostic value (AUC=0.8978, P<0.0001, Figure 1D). To confirm the relationship of circ_0001946 with the sensitivity of BC cells, we obtained the IC50 value of tamoxifen on BC cells from the GDSC database (Figure 1E). The results of qRT-PCR showed that circ_0001946 was highly expressed in BC cell lines with a higher IC50 value but showed decreased expression in BC cell lines with a lower IC50 value (all P<0.05, Figure 1F). It is preliminarily indicated that circ_0001946 may play a promoting role in the occurrence of BC and chemotherapy resistance.

Effect of circ_0001946 on the proliferation, invasion and apoptosis of drug-resistant BC cells

In order to illustrate the role of circ_0001946, this study evaluated the effect of circ_0001946 on the biological characteristics of BC cells in vitro. qRT-PCR results showed that the cells transfected with the si-circ_0001946 plasmid showed a low but stable expression of circ_0001946 compared with the si-NC group (all P<0.05, Figure 2A). The CCK-8 cytotoxicity test results showed that the cell viability reduced with the increase of tamoxifen concentration. Compared with the si-NC group, the cell viability of the si-circ_0001946 group decreased significantly. After knocking down circ_0001946, the IC50 value was significantly reduced (all P<0.05, Figure 2B). The results of
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Transwell and Flow cytometry showed that compared with the si-NC group, the si-circ_0001946 cells showed decreased invasion ability and increased apoptosis (all P<0.05, **Figure 2C, 2D**). It is indicated that knockdown of circ_0001946 inhibits the growth of BC cells by inhibiting cell proliferation, invasion, and inducing apoptosis, and that knockdown of circ_0001946 can also improve the drug sensitivity of BC cells to tamoxifen.

**MiR-671-5p had specific binding sites with circ_0001946 and dysregulated expression in BC**

In order to explore the specific mechanism of circ_0001946 regulating the BC, the circ_0001946 ring structure was determined by the RNase R digestion test, which showed that RNase R did not affect the expression of circ_0001946 (**Figure 3A**). We applied FISH to locate the expression position of circ_0001946 and found that circ_0001946 was mainly expressed in the cytoplasm (**Figure 3B**). We obtained four miRNAs after intersecting Circinteractome with the miRNAs queried in the Starbase: hsa-miR-1270, hsa-miR-944, hsa-miR-671-5p and hsa-miR-620 (**Figure 3C**). Previous research has revealed that miR-671-5p is down-regulated in BC and can be used as a target for diagnosis and treatment of BC [12]. Therefore, in this study, miR-671-5p was selected for further study. The specific binding sites of circ_0001946 and miR-671-5p are shown in **Figure 3D**. The Dual-luciferase reporter assay further proved the targeted binding relationship between circ_0001946 and miR-671-5p. MiR-671-5p mimic could significantly reduce the relative luciferase activity of circ_0001946...
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The results of qRT-PCR showed that compared with the normal group, the expression of miR-671-5p was down-regulated in the BC group (all P<0.05), and was negatively correlated with the level of circ_0001946 (r=-0.8347, P<0.0001). See Figure 3F, 3G. In addition, miR-671-5p showed abnormally low expression in MDA-MB-231 and MDA-MB-436 cell lines with higher IC_{50} values, but higher expression in MCF7 cells with lower IC_{50} values (P<0.05, Figure 3H). These results preliminarily indicate that the abnormally low expression of miR-671-5p adsorbed by circ_0001946 in BC is related to the occurrence of BC and chemotherapy resistance.

Circ_0001946 promoted the proliferation and invasion and inhibited the apoptosis of drug-resistant BC cells by adsorbing miR-671-5p

The biological functions of circ_0001946 and miR-671-5p were then explored. The transfection efficiency was evaluated by qRT-PCR, which showed stable transfection of both miR-671-5p mimic and OE-circ_0001946 (both P<0.05). See Figure 4A. Circ_0001946 significantly inhibited the level of miR-671-5p, and the inhibitory effect can be partially rescued by miR-671-5p mimic (all P<0.05). See Figure 4B. The results of CCK-8, Transwell and Flow cytometry showed that overexpression of miR-671-5p...
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A

B

C

D

E

F

G

H

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Figure 3. MiR-671-5p is the downstream target of circ_0001946. A: RNase R digestion test; B: Localization of subcellular expression of circ_0001946 by FISH (×400); C: Intersection of Circinteractome and the target miRNAs from Starbase; D: E: Specific binding sites of circ_0001946 and miR-671-5p and the results of dual-luciferase reporter assay; F: G: Expression of miR-671-5p in BC tissues and its correlation with circ_0001946; H: Expression of miR-671-5p in different drug-resistant cell lines. Compared to RNase R group, *P<0.05; compared to mimic-NC group, †P<0.05; compared to Normal, #P<0.05; compared to MCF7 group, %P<0.05; Compared to MDA-MB-231 group, &P<0.05. FISH: fluorescence in situ hybridization; BC: breast cancer.
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**Figure 4.** circ_0001946 promoted the proliferation and invasion and inhibited the apoptosis of drug-resistant BC cells by adsorbing miR-671-5p. A: Transfection efficiency of miR-671-5p mimic and OE-circ_0001946; B: Expression level of miR-671-5p was regulated by circ_0001946; C: Results of Transwell invasion test; D: Results of Transwell invasion test (×200); E: Apoptosis detected by annexin V-FITC/PI double labeling. Compared to OE-NC group, *P<0.05; compared to mimic NC group, @P<0.05; compared to OE-circ_0001946+mimic-NC group, %P<0.05. CCK-8: Cell Counting Kit-8.

Partially reversed the decrease in tamoxifen sensitivity, the increase in cell invasion, and the decrease in apoptosis caused by OE-circ_0001946 (all P<0.05). See Figure 4C-E. These results indicate that up-regulation of miR-671-5p can partially reverse the biological function of overexpressed circ_0001946 on BC cells. Circ_0001946 may promote the malignant progression of BC cells by adsorbing miR-671-5p, thereby increasing the resistance of BC cells to tamoxifen.

**EGFR was the downstream target of miR-671-5p**

We obtained the downstream target genes of miR-671-5p from miRDB, TargetScan, RNA22 and StarBase and took the intersection (Figure 5A). Then, KEGG pathway enrichment analysis was performed using the KABOS online prediction website. The terms with P<0.05 were plotted (Figure 5B). We found that the target genes of miR-671-5p were significantly enriched in the term of the cancer pathway (hsa05200: Pathways in cancer). The risk genes of BC (C2938924) were obtained from the DisGeNET database, and the top 5 genes (FGF2, ESR1, CYP19A1, ERBB2, and PIK3CA) were selected according to the GDA Score. STRING online tools was used to predict the interaction between genes enriched in cancer pathways and risk genes of BC, and the results showed that EGFR was closely related to other genes (Figure 5C). See Figure 5D for the specific binding sites of miR-671-5p and EGFR. The targeted regulation relationship between the two was verified by dual-luciferase reporter assay. MiR-671-5p mimic significantly inhibited the relative luciferase activity of wild-type EGFR (P<0.05, Figure 5E). qRT-PCR results compared with the normal group, revealed the expression of EGFR in the BC group was up-regulated (all P<0.05). Correlation analysis showed that the level of EGFR mRNA in the BC group was positively correlated with circ_0001946 (r=0.7337, P=0.0001), and negatively correlated with miR-671-5p (r=0.7836, P=0.0001). See Figure 5F, 5G. Additionally, mRNA and protein levels of EGFR showed abnormally high expression in MDA-MB-231 and MDA-MB-436 cell lines with higher IC<sub>50</sub> values, but lower expression in MCF7 cells with lower IC<sub>50</sub> values (P<0.05, Figure 5H). These results indicate that circ_0001946 up-regulates the expression of EGFR in BC by adsorbing miR-671-5p, thereby promoting the occurrence of BC and chemotherapy resistance.

**Knockdown of EGFR inhibited chemotherapy resistance of BC cells**

The biological functions of circ_0001946/miR-671-5p/EGFR were explored subsequently. Western blot and qRT-PCR and results showed that circ_0001946 significantly up-regulated the expression level of EGFR, and this promotion effect can be partially rescued by miR-671-5p mimic (both P<0.05, Figure 6A). The results of CCK-8, Transwell and flow cytometry showed that knocking down EGFR significantly promoted the sensitivity of BC cells to tamoxifen, inhibited their invasion and apoptosis. Those effects could be partly eliminated by OE-circ_0001946 (all P<0.05, Figure 6B-D). These results indicate that circ_0001946 promotes the expression of EGFR by adsorbing miR-671-5p to aggravate the malignant biological behavior of BC cells, and promote the resistance of BC cells to tamoxifen.

**Circ_0001946 was activated by YY1 in BC cells**

JASPAR and PROMO online tools showed that YY1 could specifically bind to the promoter of circ_0001946 (Figure 7A). Results of qRT-PCR found that the expression of YY1 in the BC group was significantly higher than that in the Normal group (all P<0.05; Figure 7B). In addition, compared with MCF7 cells, YY1 showed significantly higher expression in MDA-MB-231 and MDA-MB-436 cells (all P<0.05, Figure 7C). To explore whether the dysregulation of YY1 could affect the expression of circ_0001946, we transfected si YY1 or pc YY1 into BC cells and found that si YY1 inhibited the expression of YY1 and circ_0001946 in the cells, while transfection of pc YY1 showed the opposite
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Figure 5. EGFR was the downstream target of miR-671-5p. A: Intersection of target genes from miRDB, TargetScan, RNA22 and StarBase; B: KEGG pathway enrichment analysis; C: Protein-protein interaction; D: Specific binding site of miR-671-5p and EGFR; E: Targeted binding relationship between miR-671-5p and EGFR verified by dual-luciferase reporter assay; F, G: Expression of EGFR in BC tissues and correlation analysis of EGFR with circ_0001946 and miR-671-5p; H: Expression levels of EGFR in different drug-resistant cell lines measured by qRT-PCR and Western blot. Compared to Normal group, *P<0.05; compared to mimic-NC group, @P<0.05; compared to MCF7 group, %P<0.05; compared to MDA-MB-231 group, &P<0.05. EGFR: epidermal growth factor receptor.

trend (all P<0.05, Figure 7D, 7E). ChIP results showed obvious YY1 binding activity in the endogenous circ_0001946 promoter region (P<0.05; Figure 7F). According to the luciferase report analysis, YY1 could significantly increase the relative fluorescence activity of circ_0001946 (P<0.05, Figure 7G). These findings indicate that YY1 participates in the occurrence and progression of BC by regulating the transcription of the non-coding gene circ_0001946.

Discussion
The combined treatment of standard chemotherapy after surgical resection of BC has sub-
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Figure 6. Down-regulation of miR-671-5p significantly promoted the chemotherapy resistance of BC cells, which was partially eliminated by knocking down EGFR. A: Expression of EGFR measured by qRT-PCR and Western blot; B: CCK-8 cytotoxicity test; C: Results of Transwell invasion test (×200); D: Apoptosis detected by annexin V-FITC/PI double labeling. Compared to OE-NC group, $^a$P<0.05; compared to OE-circ_0001946+mimic-NC group, $^b$P<0.05; compared to si-EGFR-NC group, $^c$P<0.05; compared to si-EGFR+OE-NC group $^\land$P<0.05. EGFR: epidermal growth factor receptor; CCK-8: Cell Counting Kit-8.

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stastically improved the overall survival rate of patients in the past few decades [17, 18].

However, due to the metastasis of BC and drug resistance, the number of deaths caused by
Figure 7. circ_0001946 was activated by YY1 in BC cells. A: Prediction of transcription factors by JASPAR and PROMO; B: Expression of YY1 in patients with BC identified by qRT-PCR; C: Expression of YY1 in different cell lines; D: Transfection efficiency of YY1; E: Influence of YY1 on the expression of circ_0001946; F: Results of ChIP; G: Results of dual-luciferase reporter assay (P1 and P2 are the gene expression boxes of the two deletants constructed by cutting the normal promoter P). Compared to Normal group, *P<0.05; compared to MCF7 group, †P<0.05; compared to MDA-MB-231 group, ‡P<0.05; compared to si-YY1-NC group, §P<0.05; compared to pc-YY1-NC group, ¶P<0.05; compared to Anti-IgG group, ‰P<0.05; compared to circ_0001946 group, ∆P<0.05. BC: breast cancer; YY1: Yin Yang 1; ChIP: chromatin immunoprecipitation.

The cancer is still rising [19]. Highly metastatic BC cells may be sensitive to chemotherapy, especially to cytotoxic drugs such as anthracyclines and statins, but once the cells have chemotherapy resistance, they become more aggressive and metastatic [20]. We discussed
YY1 regulating the circ_0001946/miR-671-5p/EGFR axis to promote drug resistance

Figure 8. Mechanism of YY1 as a promoter regulating the circ_0001946/miR-671-5p/EGFR axis to promote chemotherapy resistance in breast cancer cells. YY1: Yin Yang 1; EGFR: epidermal growth factor receptor.

The role and mechanism of circ_0001946 in the process of tamoxifen resistance. Our results showed that high level of circ_0001946 after YY1 transcriptional activation can promote BC cells to be resistant to tamoxifen through miR-671-5p/EGFR.

The regulatory mechanisms of circRNAs on the drug-resistance of BC are complex. One of the common mechanisms is that circRNAs act as competitive endogenous RNAs to adsorb miRNAs and mediate target genes to participate in the progression of BC [21]. Circ_0092276 is highly expressed in doxorubicin-resistant BC cells. It promotes the autophagy of BC cells to resistant doxorubicin by regulating the miR-348/ATG7 axis [22]. The upregulation of circ_0025202 can increase the sensitivity of BC cells to tamoxifen, suggesting that the molecular targeting of circRNAs has become a potential therapeutic strategy for patients with BC to overcome chemotherapy resistance [3]. This study combined the results of bioinformatics analysis and clinical data. We speculated that circ_0001946 may be instrumental in promoting the resistance of BC cells to tamoxifen. This speculation was verified by testing the levels of circ_0001946 in the non-tamoxifen-resistant cell line MCF7 and the tamoxifen-resistant cell lines MDA-MB-231 and MDA-MB-468. We also found that circ_0001946 enhanced the sensitivity of MCF7/R to tamoxifen, promoted the malignant invasion of MCF7/R cells and inhibited their apoptosis. We believe that circ_0001946 can act as an oncogene and an inducer of tamoxifen resistance in BC. Besides, we found that YY1 could specifically bind to the promoter of circ_0001946, YY1 has been found to act as a transcription factor to activate gene transcription and then promote malignant progression of cancer. For example, YY1 acts as a transcription factor in ovarian cancer to regulate the network in cell proliferation and migration. It promotes the malignant progression of ovarian cancer by activating the long-chain non-coding RNA DSCR8 [23]. There are also a number of studies on YY1 influencing malignant progression of BC [24, 25]. For instance, YY1 can activate the long non-coding RNA LINC00673 to regulate miR-515-5p, then activate the Hippo signaling pathway and promote the malignant proliferation of BC cells [10]. This study further discovered that YY1 could bind to the promoter of circ_0001946 and participated in the progression of BC. MiR-671-5p as the downstream target of circ_0001946, can mediate BC epithelial-mesenchymal transition by targeting FOXM1 gene, thereby hindering the development of metastatic BC [12]. We found that miR-671-5p which is adsorbed by circ_0001946, relieved the inhibition to downstream gene EGFR. In terms of the mechanism, CHIP and the luciferase reporter assay have both shown that YY1 binds to the circ_0001946 promoter to enhance its transcription, and adsorbs miR-671-5p to up-regulate the expression of EGFR. It is confirmed that the EGFR family has become an important signaling pathway for the occurrence, development and metastasis of BC [26]. It’s abnormally high expression in BC tissues is significantly related to tumor hypertrophy, malignant degree, poor prognosis and high resistance to tamoxifen [27]. The results of the rescue experiment in this study also proved that the circ_0001946/miR-671-5p/EGFR axis was involved in the resistance of BC cells to tamoxifen.

In this experiment, we revealed and proved a new mechanism of tamoxifen resistance.
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Circ_0001946 is transcriptionally activated by YY1 and specifically binds to miR-671-5p to promote the expression of EGFR as well as the proliferation and invasion of drug-resistant BC cells, thereby inhibiting drug sensitivity. Therefore, circ_0001946 may become a targeted tumor gene and biomarker in drug therapy for BC. The knockdown of circ_0001946 is expected to become an effective strategy to inhibit the drug resistance in patients with BC.

There are also some limitations in this study. First, we did not conduct in vivo functional experiments. Second, we only proved the regulation of circ_0001946/miR-671-5p/EGFR but did not put YY1 in the molecular network to further explore its function. So, further research is needed in the future. The mechanism of this research is shown in Figure 8.

Disclosure of conflict of interest

None.

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