Circ_0081143 Contributes to Gastric Cancer Malignant Development and Doxorubicin Resistance by Elevating the Expression of YES1 by Targeting miR-129-2-3p

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Background/Aims: The increased mortality of gastric cancer (GC) is mainly attributed to the development of chemoresistance. Circular RNAs, as the novel type of biomarkers in GC, have attracted wide attention. The purpose of this study was to investigate the functional role of circ_0081143 in GC with doxorubicin (DR) resistance and its potential action mechanism.

Methods: The expression of circ_0081143, miR-129-2-3p and YES proto-oncogene 1 (YES1) in GC tissues and cells was measured by quantitative real-time polymerase chain reaction. The half maximal inhibitory concentration value was calculated based on the MTT cell viability assay. Cell proliferation and apoptosis were monitored by MTT and flow cytometry assays. Transwell assays were employed to check cell migration and invasion. The protein levels of YES1 and apoptosis-related proteins were detected by western blotting. The interaction between miR-129-2-3p and circ_0081143 or YES1 was verified by dual-luciferase reporter and pull-down assays. A tumorigenicity assay was conducted to verify the role of circ_0081143 in vivo.

Results: Circ_0081143 was highly expressed in DR-resistant GC tumor tissues and cells. Depletion of circ_0081143 reduced DR resistance and inhibited DR-resistant GC cell proliferation, migration and invasion. Circ_0081143 targeted miR-129-2-3p and inhibited the role of miR-129-2-3p. In addition, YES1 was a target of miR-129-2-3p, and its function was suppressed by miR-129-2-3p. Importantly, circ_0081143 positively modulated the expression of YES1 through mediating miR-129-2-3p. Circ_0081143 knockdown weakened the DR-resistant GC tumor growth in vivo.

Conclusions: Circ_0081143 knockdown weakened DR resistance and blocked the development of DR-resistant GC by regulating the miR-129-2-3p/YES1 axis. Our data suggest that circ_0081143 is a promising target for the treatment of GC with DR resistance. (Gut Liver 2022;16:861-874)

Key Words: Circ_0081143; miR-129-2-3p; YES1; Stomach neoplasms; Doxorubicin

INTRODUCTION

Gastric cancer (GC) has developed into the fifth common malignancy around the world and the third primary cause of cancer-linked deaths. The high mortality of GC attributes to the lack of early clinical symptoms, absence of screening, and ineffective treatment. Therefore, most patients are diagnosed with advanced tumors. The incidence and mortality of GC have declined nowadays due to multimodal treatment, including surgical resection and chemotherapy. However, metastasis and recurrence are frequent, and the patients' survival rate is still unsatisfactory within 5 years. Besides, the generation of drug resistance is a fearful threat to the prognosis of chemotherapy and the survival rate of patients. Therefore, the underlying mechanism of GC development and drug resistance needs to be further explored.

Circular RNAs (circRNAs), produced by back-splicing of precursor messenger RNA (mRNA), are a type of closed circRNA molecules. CircRNAs are highly conserved
and stable because there are no polar 3’ and 5’ ends.\textsuperscript{10,11} In recent years, with the boom of cell biology and bioinformatics, an increasing number of circRNAs has been identified, which has attracted wide attention of researchers. Studies have shown that circRNAs are drawn into the development of some malignancies, and several novel targets of circRNAs have been obtained.\textsuperscript{12} The involvement of circRNAs in GC has also been mentioned much. For example, circOSBP1L10 (hsa_circ_0008549) was forcefully expressed in GC tissues, and deficiency of circOSBP1L10 significantly inhibited the development of GC via targeting miR-136-5p. CircAKT3 (hsa_circ_0000199) showed a higher level in GC cells resistant to cisplatin than that in cells without cisplatin resistance, and circAKT3 enhanced the cisplatin resistance and DNA damage repair but blocked the apoptosis of GC cells.\textsuperscript{13} These data indicate that circRNA dysregulation is linked to the initiation, development, and drug-resistance of GC. Circ_0081143, derived from collagen type I alpha 2 chain (COL1A2), is poorly investigated in GC. In this paper, we attempted to disclose its role and underlying action mechanism in the development of doxorubicin (DR)-resistant GC.

Recently, circRNAs have been confirmed to serve as competitive endogenous RNAs to mediate microRNAs (miRNAs), leading to the inhibition of expression and functions of miRNAs.\textsuperscript{14} MiRNAs post-transcriptionally control gene expression via combining with the 3’ untranslated region (UTR) of the target mRNAs.\textsuperscript{15} Among these miRNAs, miR-129-2-3p has been documented to be crucial for the development of numerous cancers. For instance, miR-129-2-3p expression was weakened in breast cancer, and miR-129-2-3p upregulation significantly depleted the growth and survival of breast cancer cells.\textsuperscript{16} Whereas, the study of miR-129-2-3p in GC is scanty yet, particularly in GC with drug resistance.

YES proto-oncogene 1 (YES1) has been documented to participate in numerous cancers, and YES1 has significant impacts on tumor growth, including prostate cancer,\textsuperscript{17} breast cancer,\textsuperscript{18} and thyroid cancer.\textsuperscript{19} YES1 is a kinase, belonging to the SRC family. Generally, YES1 regulates the transcription activity of YES-associated protein 1, resulting in the amplification of YES1-related cancers.\textsuperscript{20} However, the function of YES1 as an oncogene in GC with DR resistance has not been elucidated.

Here, we monitored the expression of circ_0081143 in DR-resistant cells of GC. The role of circ_0081143 was investigated in DR-resistant GC cells. Besides, the interplays between miR-129-2-3p and circ_0081143 or YES1 were verified. The aim of our study was to address a novel mechanism for the implication of circ_0081143 in GC resistant to DR.

### MATERIALS AND METHODS

#### 1. Tissues collection

A total of 66 paired GC tumor tissues and adjacent normal tissues from GC patients were obtained from Affiliated Hospital of Guangdong Medical University. No local or systemic treatment was undertaken in these patients before surgery. The patients were assigned into DR-resistant group (n=35) or DR-sensitive group (n=31) according to the therapeutic effects. Each subject provided informed consent before surgery. This research acquired the permission of the Ethics Committee of Affiliated Hospital of Guangdong Medical University (approval number: YJ2017-059-03). The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

#### 2. Cell lines and culture

Gastric epithelium cell line GES1 and GC cell lines, including HGC-27 and AGS, were purchased from Key-GEN Biotech (Nanjing, China). DR-resistant GC cell lines, including HGC-27/DR and AGS/DR, were established by gradually treating with 0.5, 1, and 2 µg/mL DR, which was accomplished by the commissioning of KeyGEN Biotech. GES1 cells were cultured in 90% Dulbecco modified Eagle medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma). HGC-27, AGS, HGC-27/DR and AGS/DR cells were kept in 90% RPMI 1640 (Sigma) containing 10% FBS (Sigma). In order to maintain the resistance phenotype, HGC-27/DR and AGS/DR cells were exposed to 1 µg/mL DR (Sigma). All cells were placed at 37°C atmospheres with 5% CO₂.

#### 3. Oligo and vector transfection

Small interference RNA against circ_0081143 (si-circ_0081143) or YES1 (si-YES1) and matched negative control (si-NC) were assembled by GenePharma (Shanghai, China). MiR-129-2-3p mimic (miR-129-2-3p), miR-129-2-3p inhibitor (anti-miR-129-2-3p), and controls (miR-NC and anti-miR-NC) were bought from RiboBio (Guangzhou, China). The vector pCD5-ciR was used for circ_0081143 overexpression, and fusion vector pCD5-ciR+circ_0081143 (circ_0081143 and empty vector (vector)) were also synthesized by GenePharma. Cell transfection was carried out with the application of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

#### 4. Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen) was adopted for RNA isolation. The assay of complementary DNA synthesis was
implemented using Transcriptor First Strand complementary DNA Synthesis Kit (Roche, Basel, Switzerland) for circ_0081143 and YES1 or using the miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) for miR-129-2-3p. Then, qRT-PCR analysis was performed with the use of a Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen). Relative expression normalized by GAPDH or U6 was calculated by the $2^{-\Delta\Delta Ct}$ method. We listed the sequences of primers below:

- Circ_0081143: 5’-GGCAATATTGGTCCCGTTGT-3’ (forward) and 5’-ACCAGTTTCACCACCGTTTCC-3’ (reverse);
- YES1: 5’-GGAGGTGGAGGAGGGAGAG-3’ (forward) and 5’-GCTCTCATGAGTGCTGCTA-3’ (reverse);
- miR-129-2-3p: 5’-CGAAGCCCTTACCCCAA-3’ (forward) and 5’-AGTGCAGGGTCCGAGGTATT-3’ (reverse);
- GAPDH: 5’-AAGTATGACAACAGCCTCAAAG-3’ (forward) and 5’-CACACACCTCTTGATGTCATCA-3’ (reverse);
- U6: 5’-CAGCACAATATACAAATTGGAGACG-3’ (forward) and 5’-ACGAATTTCGCTGTACCC-3’ (reverse).

5. MTT assay
The experimental cells were cultured in 96-well plates (3×10^4 cells per well). Then 10 µL MTT solution (Beyotime, Shanghai, China) was pipetted to challenge cells for another 4 hours at 37 ℃. After that, upper medium was discarded, and dimethyl sulfoxide (Beyotime) was used to remove formazan. Last, the absorbance at 490 nm was measured using the Multiskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA) at 24, 48 and 72 hours to value cell proliferation.

6. Cell viability and DR resistance assay
Cells in 96-well plates (3×10^4 cells/well) were challenged with DR at different concentrations (0.1, 1, 5, 10, 20, 40, 80 µM). After 48 hours, cells were administered with MTT to detect absorbance. The survival curve at different concentrations was charted to calculate the half maximal inhibitory concentration (IC_{50}) value.

7. Flow cytometry assay
Cells with transfections were resuspended with 195 µL binding buffer (2×10^5 cells/mL) from the Annexin V-FITC Apoptosis Detection Kit (Invitrogen). Then, 5 µL Annexin V-FITC was pipetted into each well for incubation. Next, 10 µL propidium iodide (20 µg/mL) was pipetted in each well for incubation. Finally, the apoptotic cells were sorted using FACSCalibur (BD Biosciences, San Jose, CA, USA).

8. Western blot
RIPA buffer (Beyotime) was adopted for protein extraction. Then, 30 µg total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred onto polyvinylidene fluoride membranes. Next, the membranes were experienced with 5% skim milk and then exposed to the specific primary antibodies at 4 ℃ overnight. The subsequent incubation at next day was performed with the secondary antibodies. The ECL kit (Beyotime) was utilized to display protein bands. The antibodies used were anti-YES1 (ab109744; 1:1,000; Abcam, Cambridge, MA, USA), anti-B-cell lymphoma-2 (Bcl-2) (ab185002; 1:1,000; Abcam), anti-Bcl-2 associated X protein (Bax) (ab32503; 1:1,000; Abcam), anti-cleaved caspase-3 (C-caspase3) (ab32042; 1:1,000; Abcam), anti-pro-caspase-3 (ab184787; 1:2,000; Abcam), anti-MMP2 (ab92536; 1:2,000; Abcam), anti-MMP9 (ab76003; 1:5,000; Abcam), anti-GAPDH (ab9485; 1:2,500; Abcam) and the secondary antibody (ab205718; 1:5,000; Abcam).

9. Transwell analysis
Transwell chambers with or without Matrigel coating were used for cell invasion or migration detection. Simply put, the transfected cells were maintained for 24 hours and then collected in serum-depleted culture medium. Cells in serum-depleted medium were added into the top of chambers, and the lower chambers were supplemented with cell medium containing 20% FBS. Cells were induced to migrate or invade at 37 ℃ conditions with 5% CO_2 for 24 hours. After that, the migrated or invaded cells were fixed with methanol and then dyed with crystal violet. The migratory and invasive capacities were evaluated by the average number of cells in five randomly selected areas under a light microscope (Nikon, Tokyo, Japan) with a magnification of 100×.

10. Dual-luciferase reporter assay
The binding between miR-129-2-3p and circ_0081143 or YES1 was predicted by online software starBase v3.0 (http://starbase.sysu.edu.cn/). The wild-type sequence of circ_0081143 harboring miR-129-2-3p binding site and the mutant sequences of circ_0081143 were synthesized and constructed onto pGL4 (Promega, Madison, WI, USA) reporter plasmid by Hanbio Biotechnology Co., Ltd. (Shanghai, China), naming as circ_0081143 WT and circ_0081143 MUT. HGC-27/DR and AGS/DR cells were co-transfected with miR-338-3p and circ_0081143 WT or circ_0081143 MUT, using miR-NC as the control. Dual-Luciferase assay system (Promega) was next employer to examine luciferase activity at 48 hours post-transfection. The analysis method of YES1 and miR-129-2-3p was the same as above. Recombinant vectors were constructed containing YES1 3’ UTR wild-type sequences and YES1 3’
UTR mutant-type sequences, naming as YES1 3’ UTR WT and YES1 3’ UTR MUT.

11. Pull-down assay
Biotinylated miR-129-2-3p (Bio-miR-129-2-3p) and its control (Bio-NC) were provided by Ribobio. HGC-27/DR and AGS/DR cells were experienced with Bio-miR-129-2-3p or Bio-NC transfection and then lysed using lysis buffer (Invitrogen). Cell lysates were co-cultured with streptavidin magnetic beads (Invitrogen). The RNA compounds were eluted and detected by qRT-PCR.

12. Tumorigenicity assay in vivo
BALB/c mice (6-week-old, male) were bought from Shanghai Lab. Animal Research Center (Shanghai, China) and divided into two groups (n=5). Lentiviral vector (Lenti-short hairpin sh-circ_0081143) for stable circ_0081143 downregulation was obtained from Genechem (Shanghai, China). AGS/DR cells infected with sh-circ_0081143 or sh-NC were subcutaneously injected into the left dorsal flanks of the mice (2×10⁶ cells per mouse). After 1 week of feeding, tumor volume was detected using a vernier caliper every one week based on the formula: 1/2×length×width². After 35 days, the mice were killed, and all tumor nodes were removed for further biological analyses. The animal experiments were authorized by the Animal Care and Use Committee of Affiliated Hospital of Guangdong Medical University.

13. Statistical analysis
All quantitative data from experiments repeated at least three times were analyzed by SPSS 21.0 (IBM Corp., Armonk, NY, USA) and shown as mean±standard deviation. The differences were analyzed by unpaired Student t-test or analysis of variance. The correlation of the expression between two groups was identified by the Pearson correlation analysis. p<0.05 was deemed as statistical significance.

RESULT

1. Circ_0081143 was upregulated in DR-resistant GC tissues and cells
To monitor the expression level of circ_0081143 in GC, qRT-PCR analysis was performed. As depicted in Fig. 1A, circ_0081143 was significantly overexpressed in tumor tissues (n=66). Besides, circ_0081143 expression was aberrantly higher in DR-resistant tumor tissues (n=35) relative to DR-sensitive tumor tissues (n=31) (Fig. 1B). circ_0081143 expression was prominently enhanced in HGC-27, HGC-27/DR, AGS, and AGS/DR cells relative to GES1 cells, and its expression was higher in DR-resistant GC cells than that in parental cells (Fig. 1C). The data suggested that circ_0081143 showed higher expression in DR-resistant GC tissues and cells.

2. Circ_0081143 depletion inhibited DR-resistant GC cell proliferation, migration and invasion
To determine the role of circ_0081143 in DR-resistant GC cells, the endogenous expression of circ_0081143 was impaired by transfecting with si-circ_0081143. Circ_0081143 was pronouncedly down-regulated in HGC-27/DR and AGS/DR cells with si-circ_0081143 transfection (Fig. 2A and B). MTT assay figured that circ_0081143 knockdown weakened IC₅₀ of DR and thus reduced DR chemoresistance in HGC-27/DR and AGS/DR cells (Fig. 2C-F). Besides, MTT assay also indicated that the proliferative capacity of HGC-27/DR and AGS/DR cells was reduced by circ_0081143 knockdown (Fig. 2G and H). Moreover, the result from flow cytometry elucidated that the apoptosis of HGC-27/DR and

Fig. 1. Circ_0081143 was upregulated in doxorubicin (DR)-resistant gastric cancer (GC) tumor tissues and cells. (A) The expression of circ_0081143 in normal tissues (n=66) and tumor tissues (n=66) was measured by quantitative real-time polymerase chain reaction (qRT-PCR). (B) The expression of circ_0081143 in DR-sensitive (n=31) and DR-resistant (n=35) tumor tissues was measured by qRT-PCR. (C) The expression of circ_0081143 was detected in GC cells (HGC-27 and AGS), DR-resistant GC cells (HGC-27/DR and AGS/DR) and gastric epithelium cells (GES1) by qRT-PCR. *p<0.05.
Fig. 2. Circ_0081143 knockdown inhibited doxorubicin (DR) resistance and blocked the proliferation, migration and invasion of DR-resistant gastric cancer (GC) cells. HGC-27/DR and AGS/DR cells were transfected with si-circ_0081143 or si-NC. (A, B) The efficiency of circ_0081143 silencing was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). (C, D) Cell viability was detected by MTT assay. (E, F) The half maximal inhibitory concentration (IC50) value was calculated based on the cell viability. (G, H) Cell proliferation was assessed by MTT assay. (I, J) Cell apoptosis was monitored by flow cytometry assay. (K, L) The protein levels of Bcl-2, Bax, and cleaved caspase-3 were quantified by Western blotting. (M, N) Cell migration and cell invasion were determined by Transwell assay. *p<0.05.

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AGS/DR cells was notably induced by circ_0081143 knockdown (Fig. 2I and J). Meanwhile, the levels of several apoptosis-related proteins were examined. As depicted in Fig. 2K and L, the level of Bcl-2 was strikingly declined, while the levels of Bax and C-caspase3 were heightened in HGC-27/DR and AGS/DR cells after circ_0081143 silencing. In addition, transwell assay presented that circ_0081143 downregulation markedly suppressed HGC-27/DR and AGS/DR cell migratory and invasive capacities (Fig. 2M and N). These analyses indicated that circ_0081143 knockdown could reduce DR resistance to DR and block the development of DR-resistant GC cells. We also transfected si-circ_0081143 into HGC-27 and AGS cells (Supplementary Fig. 1A) to explore the effect of circ_0081143 on parental cell behaviors. We determined that circ_0081143 knockdown repressed HGC-27 and AGS cell malignant phenotypes (Supplementary Fig. 1B-F), verifying that circ_0081143 knockdown also blocked HGC-27 and AGS cell malignant behaviors.

3. Circ_0081143 interacted with miR-129-2-3p and antagonized miR-129-2-3p expression

To establish the regulatory network of circ_0081143 in GC, miRNAs targeted by circ_0081143 were verified. The binding site between circ_0081143 and miR-129-2-3p was analyzed by starBase. (A) The efficiency of miR-129-2-3p mimic was measured by quantitative real-time polymerase chain reaction (qRT-PCR). (B, C) The interaction between circ_0081143 and miR-129-2-3p was confirmed by dual-luciferase reporter assay. (E) The expression of miR-129-2-3p in gastric cancer normal tissues (n=66) and tumor tissues (n=66) was measured by qRT-PCR. (F) The expression of miR-129-2-3p in cell lines was detected by qRT-PCR. (G) The correlation between miR-129-2-3p expression and circ_0081143 expression was analyzed by Pearson correlation coefficient. (H) The relationship between circ_0081143 and miR-129-2-3p was confirmed by pull-down assay. (I, J) The expression of miR-129-2-3p in cells transfected with si-circ_0081143 or circ_0081143 was detected by qRT-PCR.

WT, wild-type; MUT, mutant-type; NC, negative control; DR, doxorubicin. *p<0.05.
**Fig. 4.** Circ_0081143 overexpression inhibited the effects of miR-129-2-3p enrichment. HGC-27/DR and AGS/DR cells were introduced with miR-129-2-3p and miR-129-2-3p+circ_0081143, respectively, using miR-NC or miR-129-2-3p+vector as the matched control. 

**A** The expression of miR-129-2-3p in cells was measured by quantitative real-time polymerase chain reaction (qRT-qPCR). 

**B, C** Cell viability was detected by MTT assay. The half maximal inhibitory concentration (IC_{50}) value was calculated based on the cell viability. 

**E, F** Cell apoptosis was monitored by flow cytometry assay. 

**H, I** The levels of Bcl-2, Bax, and cleaved caspase-3 were quantified by Western blotting. 

**J, K** Cell migration and cell invasion were assessed by Transwell assays. 

NC, negative control; DR, doxorubicin; OD, optical density. *p<0.05.
3p was provided by starBase (Fig. 3A). Fig. 3B exhibited that miR-129-2-3p mimic could promote the expression of miR-129-2-3p in HGC-27/DR and AGS/DR cells. Luciferase activity was notably repressed in HGC-27/DR and AGS/DR cells with miR-129-2-3p and circ_0081143 WT cotransfection (Fig. 3C and D). MiR-129-2-3p was conspicuously down-regulated in GC samples (n=66) (Fig. 3E). The data from starbase v3.0 public database also showed the downregulation of miR-129-2-3p in stomach adenocarcinoma (Supplementary Fig. 2A). Besides, miR-129-2-3p expression was pronouncedly decreased in GC cells compared to GES1 cells, and its expression was lower in DR-resistant GC cells in contrast to parental cells (Fig. 3F). The Pearson correlation coefficient proved that miR-129-2-3p expression showed notably negative correlation with circ_0081143 expression in GC tumor tissues (n=66) (Fig. 3G), while their correlation in normal tissues was not significant (Supplementary Fig. 2C). Additionally, circ_0081143 abundance could be enriched by Bio-miR-129-2-3p compared to Bio-NC in pull-down assay (Fig. 5).

Fig. 5. YES1 is a target of miR-129-2-3p. (A) The binding site between YES1 and miR-129-2-3p was analyzed by starBase. (B, C) The relationship between YES1 and miR-129-2-3p was confirmed by dual-luciferase reporter assay. (D, E) The expression of YES1 in tumor tissues (n=66) and normal tissues (n=66) was measured by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. (F) The correlation between YES1 expression and miR-129-2-3p expression in tumor tissues was analyzed by Pearson correlation coefficient. (G, H) The expression of YES1 was measured by qRT-PCR and Western blot in cell lines. (I-K) The expression of YES1 in HGC-27/DR and AGS/DR cells transfected with miR-129-2-3p or anti-miR-129-2-3p at mRNA and protein levels was quantified by qRT-PCR and Western blotting.

YES1, YES proto-oncogene 1; UTR, untranslated region; WT, wild-type; MUT, mutant-type; NC, negative control; DR, doxorubicin; mRNA, messenger RNA. *p<0.05.
Fig. 6. MiR-129-2-3p inhibition suppressed the effects of YES1 knockdown. HGC-27/DR and AGS/DR cells were introduced with si-YES1 and si-YES1+anti-miR-129-2-3p, respectively, using si-NC or si-YES1+anti-miR-NC as the control. [A-C] The expression of YES1 in these cells was measured by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot at mRNA and protein levels, respectively. [D, E] Cell viability was detected by MTT assay. [F] The half maximal inhibitory concentration (IC50) value was calculated based on the cell viability. [G, H] Cell proliferation was observed by MTT assay. [I] Cell apoptosis was monitored by flow cytometry assay. [J] The protein levels of Bcl-2, Bax, and cleaved caspase-3 were assessed by Western blotting. [K, L] Cell migration and cell invasion were monitored by Transwell assays.

YES1, YES proto-oncogene 1; si-NC, siRNA negative control; si-YES1, siRNA targeting YES1; DR, doxorubicin; mRNA, messenger RNA. *p<0.05.
3H). Moreover, we discovered that miR-129-2-3p expression was enhanced by circ_0081143 knockdown but declined by circ_0081143 overexpression in HGC-27/DR and AGS/DR cells (Fig. 3I and J). Above data confirmed that miR-129-2-3p was targeted by circ_0081143.

4. Circ_0081143 overexpression suppressed the function of miR-129-2-3p

The analysis of qRT-PCR showed that miR-129-2-3p abundance was enhanced in HGC-27/DR and AGS/DR cells with miR-129-2-3p transfection but weakened in cells with miR-129-2-3p+circ_0081143 cotransfection (Fig. 4A). In terms of function, miR-129-2-3p restoration-inhibited cell viability and IC_{50} value of HGC-27/DR and AGS/DR cells were largely recovered by circ_0081143 reintroduction (Fig. 4B-D). The proliferative capacity of HGC-27/DR and AGS/DR cells was depleted by alone miR-129-2-3p overexpression but restored after the overexpression of circ_0081143 (Fig. 4E and F). The apoptosis rate was elevated by miR-129-2-3p restoration but repressed by combined circ_0081143 overexpression (Fig. 4G). The level of Bcl-2 was reduced in cells harboring miR-129-2-3p but strengthened in cells harboring miR-129-2-3p+circ_0081143, while the levels of Bax and C-caspase3 were opposite to level of Bcl-2 in these cells (Fig. 4H and I). In addition, miR-129-2-3p restoration-blocked the migratory and invasive capacity of HGC-27/DR and AGS/DR cells were strengthened by circ_0081143 reintroduction (Fig. 4J and K). These data deemed that circ_0081143 overexpression inhibited miR-129-2-3p functions.

5. YES1 was a target of miR-129-2-3p

To further clear the regulatory mechanism of circ_0081143 in GC, the target mRNAs of miR-129-2-3p were further screened and identified. The binding site between YES1 3’ UTR and miR-129-2-3p was obtained from starBase (Fig. 5A). The cotransfection of miR-129-2-3p and YES1 3’ UTR WT significantly reduced luciferase activity in HGC-27/DR and AGS/DR cells, while miR-129-2-3p and YES1 3’ UTR MUT cotransfection did not affect the luciferase activity (Fig. 5B and C). In addition, YES1 abundance was strikingly elevated in tumor tissues (n=66) of GC relative to normal tissues (n=66) (Fig. 5D and E). The data from starbase v3.0 public database also showed the upregulation of YES1 in stomach adenocarcinoma tissues (Supplementary Fig. 2B). Pearson correlation coefficient presented that YES1 expression showed negative correlation with miR-129-2-3p expression in GC tumor samples (Fig. 5F), while their correlation was not significant in normal tissues (Supplementary Fig. 2E). Besides, the expression of YES1 at both mRNA and protein levels was greatly enhanced in HGC-27/DR and AGS/DR cells in contrast to their parental cells, respectively (Fig. 5G and H). Additionally, the expression of YES1 was impaired in HGC-27/DR and AGS/DR cells with miR-129-2-3p overexpression but reinforced in cells with miR-129-2-3p inhibition at both mRNA and protein levels (Fig. 5I-K). Collectively, YES1 was a direct target of miR-129-2-3p.

6. miR-129-2-3p inhibition abolished the effects of YES1 knockdown

The expression of YES1 was noticeably reduced in HGC-27/DR and AGS/DR cells by si-YES1 transfection.
but restored by si-YES1+anti-miR-129-2-3p transfection (Fig. 6A-C). Then, MTT assay illuminated that the YES knockdown-depleted cell viability and IC50 value were largely recovered by combined miR-129-2-3p inhibition (Fig. 6D-F). Besides, cell proliferation capacity was suppressed by si-YES1 but restored by si-YES1+anti-miR-129-2-3p in HGC-27/DR and AGS/DR cells (Fig. 6G and H). The apoptotic rate of HGC-27/DR and AGS/DR cells was stimulated by YES1 knockdown but suppressed by additional miR-129-2-3p inhibition (Fig. 6I). The level of Bcl-2 was reduced in cells harboring si-YES1 but strengthened in cells harboring si-YES1+anti-miR-129-2-3p, while the levels of Bax and C-caspase3 were opposite to the level of Bcl-2 (Fig. 6J). In addition, HGC-27/DR and AGS/DR cell migratory and invasive abilities repressed by YES1 knockdown were substantially promoted by miR-129-2-3p absence (Fig. 6K and L). The result claimed that miR-129-2-3p inhibition overturned the impacts of YES1 down-regulation in DR-resistant GC cells.

7. YES1 was regulated by circ_0081143 through miR-129-2-3p

Furthermore, we discovered from the Pearson correlation analysis that YES1 expression displayed a positive correlation with circ_0081143 expression in GC tumor tissues (n=66) (Fig. 7A), while their correlation was not significant in normal tissues (Supplementary Fig. 2D). The expression of YES1 was notably declined in HGC-27/DR and AGS/DR cells harboring si-circ_0081143 transfection but considerably restored in HGC-27/DR and AGS/DR cells harboring si-circ_0081143+anti-miR-129-2-3p transfection (Fig. 7B-D). The data declared that circ_0081143 positively regulated the expression of YES1 through mediating miR-129-2-3p.

8. Circ_0081143 knockdown inhibited DR-resistant GC development in vivo

To test the role of circ_0081143 in vivo, xenograft tumor models were established in nude mice. We found that AGS/DR cells infected with sh-circ_0081143 led to smaller...
tumor volume and tumor weight (Fig. 8A and B). Moreover, circ_0081143 expression markedly declined, while miR-129-2-3p expression was strongly reinforced in the removed tumor nodes from the sh-circ_0081143 group (Fig. 8C and D). Besides, the expression of YES1 was remarkably reduced in the sh-circ_0081143 group (Fig. 8E and F). Additionally, the expression of C-caspase3 was remarkably elevated, while MMP2 and MMP9 levels were markedly reduced in sh-circ_0081143-administered tumor tissues (Fig. 8G and H). In short, circ_0081143 downregulation largely repressed DR-resistant GC cell malignant development in vivo via regulating miR-129-2-3p and YES1 expression.

**DISCUSSION**

GC is a malignant tumor with high mortality. Unfortunately, drug resistance often develops during the course of treatment. Existing reports have claimed that circRNAs play crucial roles in GC. However, little is known about drug resistance of circRNAs in GC. In this study, our data manifested that circ_0081143 was forcefully expressed in GC, particularly in DR-resistant GC. Knockdown of circ_0081143 weakened the IC_{50} of DR, attenuated DR-resistant GC cell growth in vitro, and impeded DR-resistant GC tumor tumorigenesis in vivo. Mechanism analysis concluded that circ_0081143 exerted its role in GC by upregulation of YES1 and downregulation of miR-129-2-3p and YES1 expression.
3p. Our study provided a theoretical basis for circ_0081143 functioning in GC with DR resistance.

Owing to the wide application of sequencing technology, a large number of circRNAs have been screened and identified. Nevertheless, the biological function of certain circRNA is not fully understood. Our study mentioned that a circRNA circ_0081143, dating from COL1A2, was aberrantly expressed in GC. COL1A2, as a novel therapeutic target of GC, was concluded in several reports. They also emphasized that high expression of COL1A2 was implicated with poor overall survival. Interestingly, a previous study elucidated that circ_0081143 was abundantly expressed in GC tissues by the analysis of microarray and qRT-PCR. Besides, downregulation of circ_0081143 inhibited viability, invasion, and cisplatin resistance, which was accomplished by the circ_0081143/miR-646/CDK6 regulatory pathway. Consistent with the previous study, we also noticed that circ_0081143 expression was increased in DR-resistant GC samples. Functional analysis claimed that circ_0081143 knockdown triggered the suppression of cell proliferation and DR resistance and thereby enhanced cell apoptotic rate. Above evidence suggested that circ_0081143 was a key regulator in GC development and drug resistance. Therefore, the antagonism of circ_0081143 will partly help to control the development of GC.

For circ_0081143 mechanism analysis, miR-129-2-3p was identified to be a direct target of circ_0081143. Yu et al. observed that miR-129-2-3p, along with other members of miR-129 family, were all expressed with a low level in GC cells. Besides, miR-129-2-3p mimics arrested cell growth and cell cycle. Yu et al. also pointed out that miR-129-2-3p abundance was reduced in gastric juice from GC relative to healthy groups. In agreement with these views, we clarified that miR-129-2-3p was weakly expressed in GC samples and negatively correlated with circ_0081143 expression. Besides, miR-129-2-3p enrichment promoted DR sensibility, blocked the ability of proliferation, and expedited apoptosis in GC cells. More importantly, the effects of miR-129-2-3p enrichment could be inhibited by circ_0081143 overexpression. These data manifested that miR-129-2-3p served as a tumor inhibitor in the progression of GC.

YES1 was verified as a downstream target of miR-129-2-3p. A previous study found that YES1 was notably activated in afatinib-resistant GC cell lines. Fang et al. confirmed that YES1 was interacted by miR-140-5p, and YES1 up-regulation retrieved the miR-140-5p-modulated suppression of cell growth and metastasis in GC. Moreover, YES1 provided chemotherapeutic resistance to cetuximab in non-small cell lung cancer cell lines. In accordance with the existing research, we found that YES1 was highly expressed in GC samples, and its expression was positively correlated with circ_0081143 expression but negatively correlated with miR-129-2-3p expression. Moreover, YES1 knockdown played the same role as circ_0081143 knockdown in GC, while these impacts were partially abolished by miR-129-2-3p inhibition. Expression analysis presented that YES1 level was reduced in cells after circ_0081143 depletion but recovered in cells with additional miR-129-2-3p inhibition, indicating that YES1 was a downstream cytokine regulated by circ_0081143/miR-129-2-3p pathway.

To summarize, we discovered that the abundance of circ_0081143 was heightened in DR-resistant GC, and circ_0081143 knockdown elevated DR sensibility, suppressed proliferation and accelerated apoptosis of GC cells. Additionally, our analyses characterized that circ_0081143 functioned in GC by positively regulating YES1 expression via sequestering miR-129-2-3p. Our study provides a new mechanism for circ_0081143 in the progression of DR-resistant GC, suggesting that circ_0081143 may be a prospective biomarker for the treatment of GC.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology: L.L., R.C. Formal analysis, data curation: R.C., Q.X., C.Z. Validation, investigation: W.O., L.L. Writing - original draft preparation: W.O., L.L., R.C. Writing - review and editing: W.O., L.L., R.C. Approval of final manuscript: all authors.

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