

Circle RNA PLEC Acts as a Sponge of MicroRNA-198 to Promote Gastric Carcinoma Cell Resistance to Paclitaxel and Tumorigenesis

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Abstract

Gastric carcinoma (GC) is one of the most frequent type of malignancy all over the world. The resistance of Paclitaxel (PTX) has become the obstacle of the prognosis of GC, and the underlying mechanism of is not clear. Previous study showed that GC-related circRNAs have been identified via microarray analysis and bioinformatics analysis, and we discovered that circPLEC (hsa_circ_0085923) remarkably upregulated in GC cells. The molecular mechanism of circPLEC in PTX-resistance GC cells still needs to explore. The expression of circPLEC in GC cells, PTX-resistant GC cells and GC tissues was analyzed by qRT-PCR. CircPLEC was knocked down in GC cells by transfecting shRNA, and then we used the CCK-8 assay, transwell, and FCM analysis to verify the effect in circPLEC in PTX-resistant GC cells. Additionally, we used luciferase reporter assays to confirm the relationship among circPLEC, miR-198 and MUC19. In present study, qRT-PCR exhibited that circPLEC were upregulated in PTX-resistant GC tissues and cells, indicating that circPLEC boost the PTX resistance of GC. circPLEC downregulation could weaken the GC resistance to PTX and the ability of tumorigenesis, migration and invasion, and promote the apoptosis of PTX-resistant GC cells. miR-198 inhibitor could revise the effect of circPLEC downregulation in PTX-resistant GC cells, and MUC19 downregulation could weaken the GC resistance to PTX and the tumorigenesis, and improve the apoptosis of PTX-resistant GC cells. In summary, circPLEC acts as a sponge of miR-198 to promote the PTX resistance and tumorigenesis of GC cells by regulating MUC19 expression.

Background

Gastric carcinoma (GC) is one of the most common type of malignancy all over the world, which is commonly associated with poor survival rates and accounts for a significant ratio of universal cancer mortality(1–3). Following the progress of surgical treatment and the mature application of chemotherapy and radiotherapy, the prognosis of GC is improved, but still awful(4, 5).

Paclitaxel (PTX), one of the most extensively used chemotherapeutic drug, is applied to treat different types of malignant cancer(6–8). However, as the front-line chemotherapeutic drug of GC, PTX have become resistant in some individuals(9, 10). The resistance of PTX have become the obstacle of the prognosis of GC, and the underlying mechanism of is not clear.

Additional treatment such as molecular therapy may benefit for the patients with GC(11). However, as one of potential treatments, molecular therapy has little evidence in clinical trials to prove the effectiveness(12, 13). Thus, it is urgently needed to research the novel molecular pathway of GC to lay the foundation of the new therapeutic target.

Circular RNAs (circRNAs) are a type of endogenous noncoding RNAs, which have been first discovered in 1976 (14). circRNAs have the feature of low-level expression and specific patterns associated with cell types or tissues(15). Previous studies demonstrated that circRNAs take an essential part in pathogenesis as biomarkers for cancer, such as lung cancers, glioma, and (16–18). The mechanism of circRNAs might

serve on miRNA sponges or competing endogenous RNA, bind and seclude proteins, and regulate splicing(19).

Previous study showed that GC-related circRNAs have been identified via microarray analysis and bioinformatics analysis, and we discovered that circPLEC (hsa_circ_0085923) remarkably upregulated (20). In this study, we identified the expression and effect of circPLEC in PTX-resistance GC cells, and explored the molecular mechanism of circPLEC in PTX-resistance GC cells, aiming to make the foundation of the novel pathway of GC treatment.

Materials And Methods

Cell culture and establishment of paclitaxel-resistant cell line

GES-1 stomach mucosa epithelium cells, and human GC cell lines of SNU-5, MGC-803, HGC-27, SGC7901, and AGS cells were kindly provided by Cell Bank of Chinese Academy of Sciences. RPMI 1640 medium were selected to culture the GES-1 and GC cells except the AGS cells, which should be cultured in F12K medium. The ratio of fetal bovine serum (Gibco, USA) in culture medium was 10%, and streptomycin (100 U/mL) and penicillin(100 U/mL) (Gibco, USA) were essential to supplement in culture medium. Cells were incubated at a 37 °C, 5% CO₂. PTX-resistant GC cells were established via consecutive intervention to incremental concentrations of PTX (Sigma-Aldrich, Germany). SGC-7901 and AGS cells were incubated in respective medium containing PTX (1 µg/L), and then the cells were sub-cultured each 2 weeks with incremental (25% per 2 weeks) concentrations of PTX. When the concentration of PTX was 100 µg/L, PTX-resistant SGC-7901 and AGS cells were achieved(21, 22).

Clinical samples

Between June 2014 and September 2020, we collected 40 GC tissue samples and corresponding normal stomach mucosa tissues samples from GC patients who were definitely diagnosis GC at Affiliated Tumor Hospital of Xinjiang Medical University.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from GC tissues and cells using TRIzol reagent (Invitrogen, USA) in the light of the instruction. Reverted First Strand cDNA Synthesis (Lihan Biotech, China) was applied to compound cDNA from RNA. qRT-PCR was completed via a SYBR Premix Ex TaqII (Maokang Biotech, USA) in an ABI 7500 PCR instrument. U6 served as the control of standardize circRNA expression. The primers (Table 1) were purchased from GenePharma.

RNA transfection

Transfection was enforced by using Lipofectamine 3000 reagent (Invitrogen, USA) in the light of the instruction. Transfection of sh-circPLEC, miR-198 mimics or inhibitors (GenePharma, China) was enforced by Lipofectamine RNAiMAX (Invitrogen, USA).

CCK-8 assay

CCK-8 kit (Biosharp, China) was applied to analysis the IC₅₀ of PTX and cell viability in GC cells. After different concentration of PTX incubation or 0, 24, 48, 72h incubation, the cells were washed by 1 × PBS at least 3 times, and then the cells added the culture medium including 10 μL CCK-8 solution and incubated for 2 h at 37°C.

Transwell assay

The ability of migration and invasion was detected by transwell. 100μL serum-free medium adding Matrigel (BD Biosciences) (5:1) was put into the transwell chamber (0.8μm, Corning, USA), and the incubated at 37°C for 4h until the mixed became solid, cell suspension was prepared at the density of 1×10⁵/mL, added 100μL cell suspension to each well in a 24-well plate (Corning, USA), 500 μL culture medium with 20% FBS was added in the bottom of chamber. Methanol was used to immobilize the cells in the membrane and stained with crystal violet. The results were collected via microscope.

Flow cytometry (FCM)

PTX-resistant GC cells were cultured in 24-well plates and incubated at 37°C for 12h, and then rinsed by 1 ×PBS at least 3 times. The cells were resuspended with 250μl Binding Buffer, and took 100 μL cell suspension into 5 mL flow tube, added 5 μL Annexin V-PE and 10 μL 7-AAD solution (Solarbio, China). The apoptosis rate of PTX-resistant GC cells was measured by the FACSCalibur flow cytometer (BD Biosciences).

Luciferase reporter assay

GC cells (1 × 10⁵ /well) were prepared in a 12-well plate for the 200 ng pGL3/circPLEC WT or pGL3/circPLEC Mut and 100 nmol miRNA mimics transfection. After 48 h, the transfected cells were ready to detect, and luciferase activities were detected by the Dual-Luciferase Reporter Assay System (Promega, USA).

Statistical analysis

The data were described by the mean ± standard deviation (SD) and were analyzed via one-way analysis of variance and Tukey's post hoc multiple comparison tests. All data of this study were analyzed via SPSS 25.0. Values were reported statistically significant when P-values were < 0.05.

Results

CircPLEC is upregulated in PTX-resistant GC cells and tissues

We cultured the GES-1 stomach mucosa epithelium cells and human GC cell lines of SNU-5, MGC-803, HGC-27, SGC7901, and AGS cells to explore the circPLEC expression. The level of circPLEC expression in

GC cells was meaningfully increased than in GES-1 cells (**Fig. 1A**). Then we selected the SGC-7901 and AGS cells, which circPLEC expression level in highest, to establish the PTX-resistant cells, the result indicated that the expression of circPLEC was upregulated in PTX-resistant SGC-7901 and AGS cells, compared with the SGC-7901 and AGS cells (**Fig. 1B**). qRT-PCR of GC tissues and normal stomach mucosa tissues exhibited that the relative expression of circPLEC in GC tissues was remarkably higher than in normal stomach mucosa tissues (**Fig. 1C**). In the different stage of GC, the relative expression of circPLEC in T4 stage(n=9) was significantly higher than T3 (n=13) and T1-T2(n=18) stages (**Fig. 1D**). Moreover, circPLEC was more enriched in the PTX-resistant GC tissues(n=13) than the PTX-sensitive GC tissues(n=27) (**Fig. 1E**).

CircPLEC promote the GC resistance to PTX and tumorigenesis

To explore the relationship between circPLEC and GC resistance to PTX, we transferred the sh-circPLEC plasmid into SGC-7901 PTX-resistant cells and AGS PTX-resistant cells. The RNase R assay showed that circPLEC was enrich in PTX-resistant SGC-7901 and AGS cells, and circPLEC was more stable than PLEC mRNA (**Fig.S1**). qRT-PCR identified the effect of plasmid and showed that circPLEC was downregulated in PTX-resistant SGC-7901 and AGS cells, however the PLEC mRNA was undifferentiated (**Fig. 2A, B**). The IC₅₀ of PTX in PTX-resistant GC cells were decreased when circPLEC was downregulated (**Fig. 2C, D**), the cell numbers of PTX-resistant SGC-7901 cells were reduced compared with the control group after 72h of sh-circPLEC transferred (**Fig. 2E**), and the cell numbers of AGS PTX-resistant cells was decreased after 48h and 72h of sh-circPLEC transferred (**Fig. 2F**). The result of FCM showed that the cell apoptosis rate of sh-circPLEC group in PTX-resistant SGC-7901 and AGS cells is higher than the control group (**Fig. 2G**). The result of tranwell assay revealed that the circPLEC downregulation weakened the ability of migration and invasion of PTX-resistant GC cells (**Fig. 2H**). these results above demonstrated that circPLEC downregulation could weaken the GC resistance to PTX and the ability of tumorigenesis, and improve the apoptosis of PTX-resistant GC cells.

CircPLEC acts as a sponge of miR-198

To research the relative miRNAs of circPLEC, we have made the miRNA predictions via Target Scan v7.2(http://www.targetscan.org/vert_72/), and we discovered that miR-198 have the potential binding sites with the circPLEC (**Fig. 3A**). The result of luciferase reporter assay, which might prove the interaction between circPLEC and miR-198, presented that application of the miR-198 mimic remarkably weakened the luciferase activity of circPLEC wild type (WT) confronted with the transfection of negative control (NC). However, the miR-198 mimic did not influenced the luciferase activity of circPLEC mutant (**Fig. 3B, C**). The data of RIP assay showed that the circPLEC and miR-198 in the anti-Ago2 group was upregulated in PTX-resistant GC cells, indicated that circPLEC and miR-198 were combined by Ago-2 (**Fig. 3D, E**). The miR-198 in GC tissues (n=40) was overexpressed than in normal stomach mucosa tissues(n=40) (**Fig. 3F**). Among the 40 GC tissues, miR-198 was down expressed in the PTX-resistant GC tissues (n=13), in contrast to the PTX-sensitive GC tissues(n=27) (**Fig. 3G**). Moreover, the relative expression of miR-198 in SGC-7901 and AGS cells was lower than GES-1 cells, and then the PTX-resistant SGC-7901 and AGS cells

was lower than normal cells (**Fig. 3H**).when the circPLEC was downregulated, the expression of miR-198 was upregulated in PTX-resistant SGC-7901 and AGS cells (**Fig. 3I**).

miR-198 inhibited the effect of circPLEC promote the GC resistance to PTX.

To detect the further mechanism of miR-198 and circPLEC in GC cells, we transfected sh-circPLEC and miR-198 inhibitor into PTX-resistant GC cells, and divided into 4 groups: sh-control group, sh-circPLEC group, sh-circPLEC+NC group, and sh-circPLEC+inhibitor group. CCK-8 assay showed that IC₅₀ of PTX-resistant GC cells in sh-circPLEC and sh-circPLEC+NC group decreased compared with the sh-control and sh-circPLEC+inhibitor group (**Fig. 4A, B**). The cell number of PTX-resistant SGC-7901 and AGS cells after 72h of transferring in sh-control group was lower than sh-circPLEC+ NC group, and the cell number in sh-circPLEC group was lower than sh-circPLEC+ inhibitor group (**Fig. 4C, D**). FCM showed that the apoptosis rate in sh-circPLEC group was increased in contrast to sh-control group, and the apoptosis rate in sh-circPLEC+ NC group was remarkably increased than sh-circPLEC+inhibitor group (**Fig. 4E, F**). The result of tranwell assay revealed that the sh-circPLEC+ inhibitor group enhanced the ability of migration and invasion than sh-circPLEC+ NC group in PTX-resistant SGC-7901 and AGS cells (**Fig. 4G, H**). The results above predicted that miR-198 inhibitor reversed the effect of circPLEC downregulation in PTX-resistant GC cells, and proved that miR-198 could inhibit the improve effect of circPLEC in GC resistance to PTX and tumorigenesis.

Downstream gene MUC19 promoted the the GC resistance to PTX and tumorigenesis

Downstream gene of miR-198 have been forecasted by Target Scan v7.2(http://www.targetscan.org/vert_72/), MUC19 have the potential binding sites with the miR-198(**Fig. 5A**). the result of luciferase reporter assay indicated that the miR-198 mimic meaningfully moderated the luciferase activity of MUC19 wild type (WT)compared with the negative control (NC). However, the miR-198 mimic did not influenced the luciferase activity of MUC19 mutant (**Fig. 5B**). The expression of MUC19 was downregulated when the circPLEC was knocked down, and the expression of MUC19 was upregulated when the circPLEC was knocked down and miR-198 was inhibited (**Fig. 5C**). MUC19 in GC tissues (n=40) was overexpressed than normal stomach mucosa tissues(n=40) (**Fig. 5D**). Among the 40 GC tissues, the relative expression of MUC19 was downregulated in the PTX-resistant GC tissues (n=13), in comparison with the PTX-sensitive GC tissues(n=27) (**Fig. 5D**). Furthermore, the relative expression of MUC19 in SGC-7901 and AGS cells was higher than GES-1 cells, and then the PTX-resistant SGC-7901 and AGS cells was increased than normal cells (**Fig. 5E**). When the sh-MUC19 was transfected, the expression of MUC19 was downregulated in PTX-resistant GC cells (**Fig. 5E**). CCK-8 assay showed that IC₅₀ of PTX-resistant GC cells decreased when MUC19 was knocked down (**Fig. 5F**), and the cell viability of PTX-resistant SGC-7901 and AGS cells after 72h of transfection significantly increased when MUC19 was knocked down (**Fig. 5G**). FCM showed that the cell apoptosis rate in PTX-resistant GC cells increased when MUC19 was downregulated (**Fig. 5H**). Tranwell assay showed that the MUC19 knocked down weakened the ability of migration and invasion of PTX-resistant GC cell (**Fig. 5I**). The results above

revealed that MUC19 downregulation could weaken the GC resistance to PTX and the ability of tumorigenesis, and improve the apoptosis of PTX-resistant GC cells.

Discussion

With rapid progress of bioinformatics technology, variety of circRNAs were identified in different diseases, including GC(23, 24). More and more evidence proved that circRNAs take a crucial part in GC tumorigenesis. For instance, circ-SERPINE2 promotes the progress of GC via the regulation of miR-375/YWHAZ(25); circNRIP1 acts as a tumour promotor in GC by modulating AKT1(26); circPVT1 have the potential of proliferative factor of GC by regulating miR-125(27). PTX is greatly valid as a chemotherapeutic drug to treat GC (28, 29), but the appearance of PTX resistance is a challenge for the favorable prognosis of GC patients(30). Obviously, circRNAs have been verified to be nearly association with the PTX resistance in various of cancers(31–33). Previous study has verified that circPLEC was upregulated in GC cells, the potential mechanism of circPLEC in PTC resistance is worth further studied.

In the present study, firstly the expression of circPLEC was analyzed in GC cells, GC tissues, the data revealed that the expression of circPLEC were noticeable raised in PTX-resistant GC cells and tissues confronted with their respective control groups, indicating that circPLEC enhanced the PTX resistance of GC. And then sh-circPLEC was applied to knock down the expression of circPLEC, the results of CCK-8 demonstrated that circPLEC downregulation could inhibit the PTX resistance and weaken the cell viability; the data of FMC and transwell proved that circPLEC downregulation improved the GC cell apoptosis and decreased the migration and invasion, revealing that circPLEC not only promote the PTX resistance but also boost tumorigenesis of GC. Nevertheless, the molecular mechanism is still unknown.

MiRNAs were known to make gene silence by binding to mRNAs, and competing endogenous RNA (ceRNA) hypothesis demonstrated that non-coding RNAs such as circRNAs and lncRNAs can regulate gene expression by competitively binding to miRNAs, and finally influenced the physiological and pathological processes (34, 35). And it has been proved that ceRNAs take part in the cancer progress(36, 37), the lncRNAs, circRNAs and pseudogenes might act as miRNA “sponges” to regulate gene expression(38–40). In our study, we assumed that circPLEC acts as a sponge of miRNA, which inhibited the GC cells tumorigenesis, and then miR-198 have been predicted to have the binding site to circPLEC, and observed that miR-198 can inhibit the GC progress and the expression of miR-198 in GC was negative correlated with circPLEC, which supported that circPLEC acts as a sponge for miR-198 in GC cells. Additionally, the downstream gene MUC19, which promotes the PTX resistance and progress of GC cells, was proved to be the target of miR-198 and circPLEC improved MUC19 expression via regulating miR-198. To date, circPLEC have been proved to promote PTX resistance and tumorigenesis of GC cells via the circPLEC/miR-198/ MUC19 axis.

However, this study still has some limatation to be considered when comprehending the results and should be supplied in future researches. Firstly, only 40 GC tissues samples were involved in this research, and more GC tissues are required to verify the role of circPLEC by high-throughput sequencing in future

study. Secondly, the studies in vivo should be performed to further explore the function of circPLEC. Finally, the different miRNAs related to circPLEC should be identified to explore more pathways and targets associated with GC.

In summary, we illustrated that circPLEC is substantially upregulated in human GC tissues and cells, and circPLEC acts as a sponge of miR-198 to promote the PTX resistance and tumorigenesis of GC cells. We also demonstrated that circPLEC might promote the PTX resistance and tumorigenesis of GC cells via the regulating MUC19. CircPLEC has the potential of the biomarker of GC diagnosis, and inhibition of circPLEC will be a novel therapeutic target in PTX-resistant GC in the future.

Declarations

Acknowledgements

Not applicable.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Ning Zhou performed statistical analysis and wrote the manuscript. Wei Wang and Chunlei Xu were involved in the manuscript preparation. Wenyan Yu were responsible for the study design and data collection. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All patients provided written informed consent and the study protocol for the use of clinical samples was approved by the Affiliated Tumor Hospital of Xinjiang Medical University Institutional Review Board

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Primers used for qPCR in this study

Primer	Sequence(5'to3')
circPLEC	Forward: TCTGGACAATCATTCTGCA
	Reverse: ATCATTCCTGATGTTCCACC
miR-198	Forward: TCAAGTGCCTCAACCCATCT
	Reverse: ATCTCCTCCTCTGTCTGGGT
MUC19	Forward: GAAGAGGAGGAAGAGGACGAGGAG
	Reverse: GGACCACCAGGCACAAGAACATC

Figures

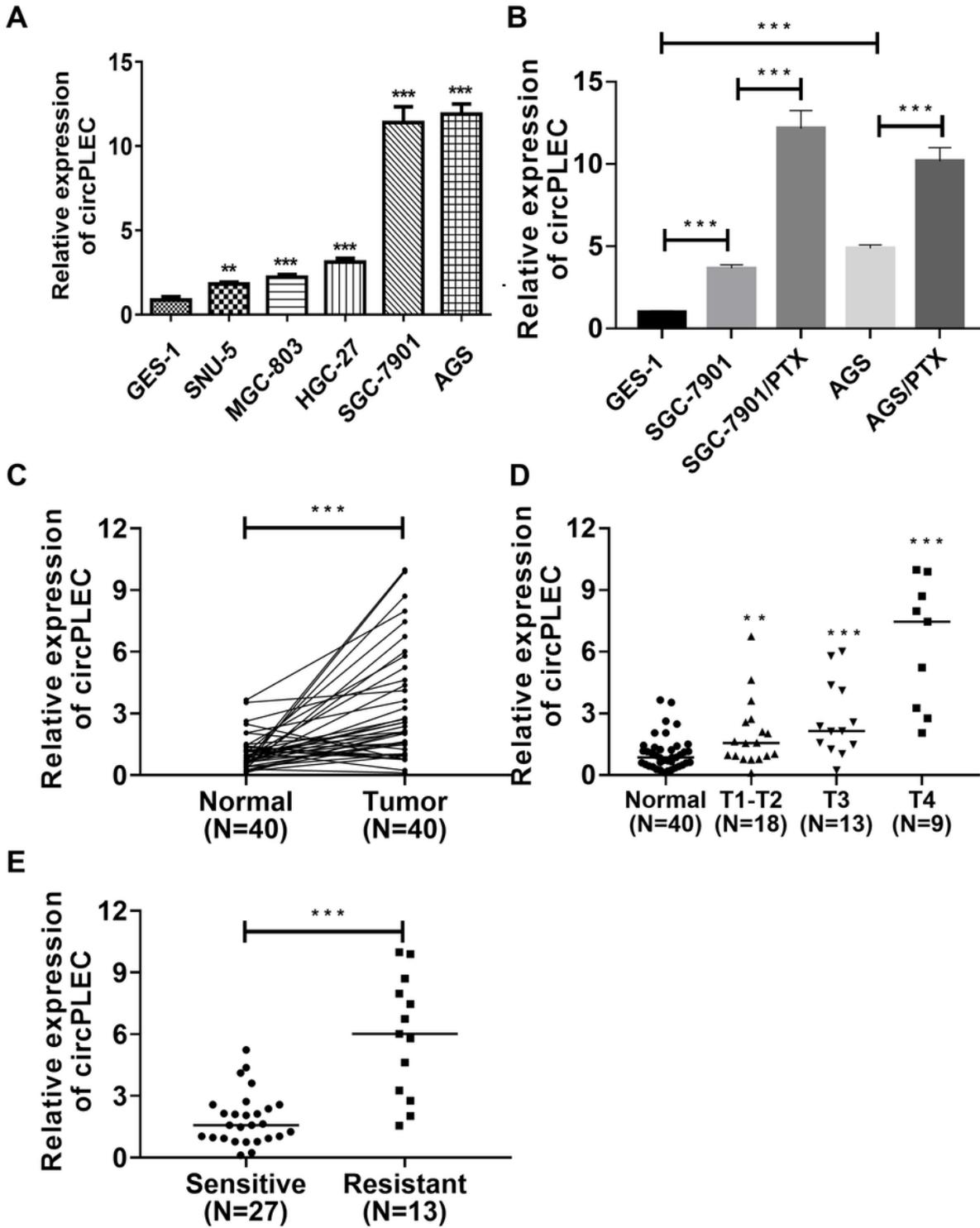


Figure 1

The relative expression of circPLEC in GC cells and tissues. A) The relative expression of circPLEC in GC cells and GES-1 stomach mucosa epithelium cells by qRT-PCR; B) The relative expression of circPLEC in PTX-resistant GC cells by qRT-PCR; C) The expression of circPLEC in GC tissues and normal tissues y qRT-PCR; D) The expression of circPLEC in GC tissues in different stage. E) The expression of circPLEC in PTX-sensitive and PTX-resistant GC tissues. (** $P < 0.01$, *** $P < 0.001$)

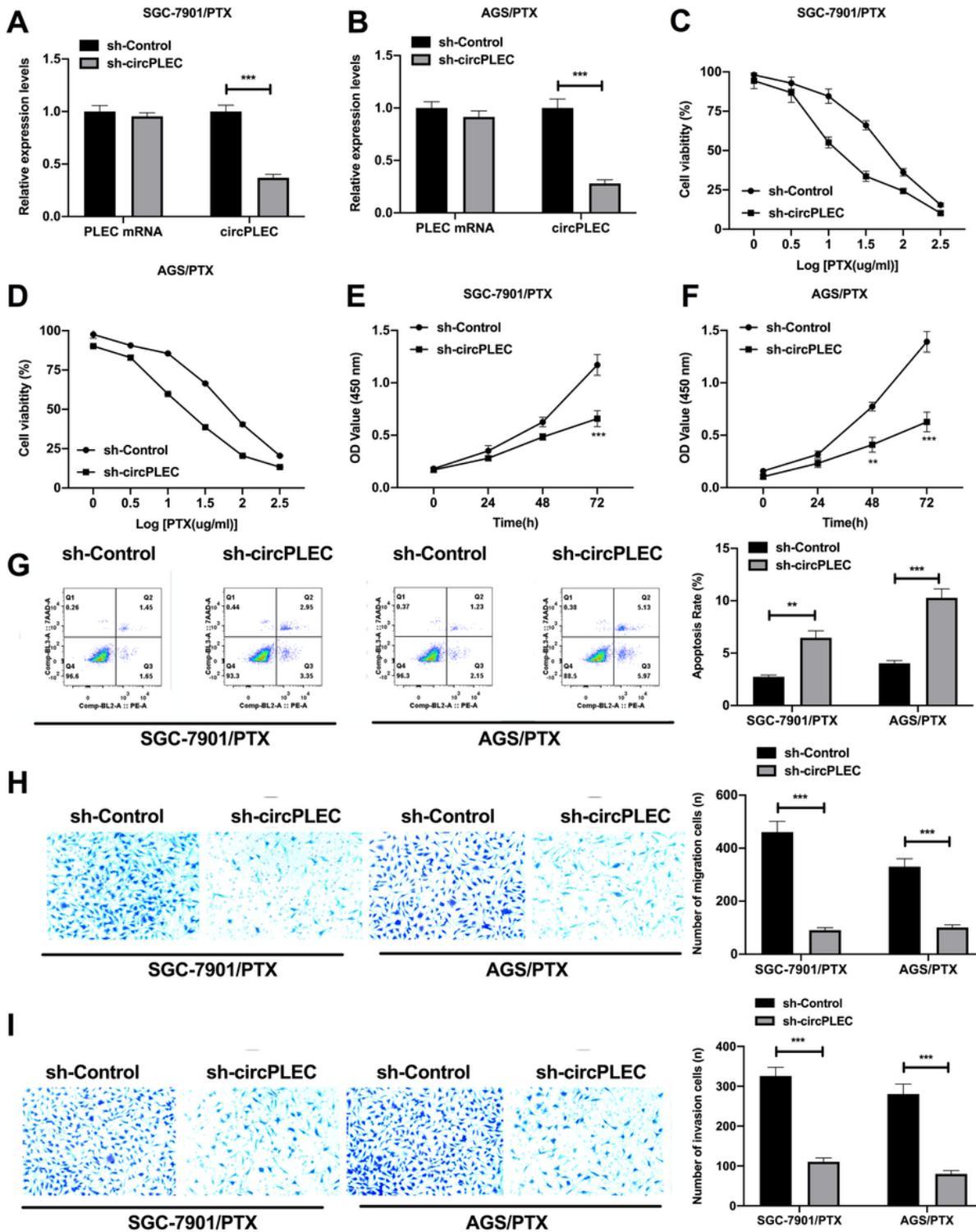


Figure 2

CircPLEC knock-down inhibited the proliferation, migration, invasion and induced apoptosis of PTX-resistant GC cells. The PTX-resistant SGC-7901 and AGS cells were transfected by sh-circPLEC and sh-control. A-B) The expression of PLEC and circPLEC in PTX-resistant GC cells via qRT-PCR; C-D) The value of IC50 in PTX-resistant GC cells by CCK-8 assay; E-F) The cell viability of PTX-resistant GC cells by CCK-8

assay; G) Cell apoptosis rate of PTX-resistant GC cells by FMC analysis; H-I) The ability of migration and invasion of PTX-resistant GC cells measured by transwell. (scale bar=50 μ m) (** $P < 0.01$, *** $P < 0.001$)

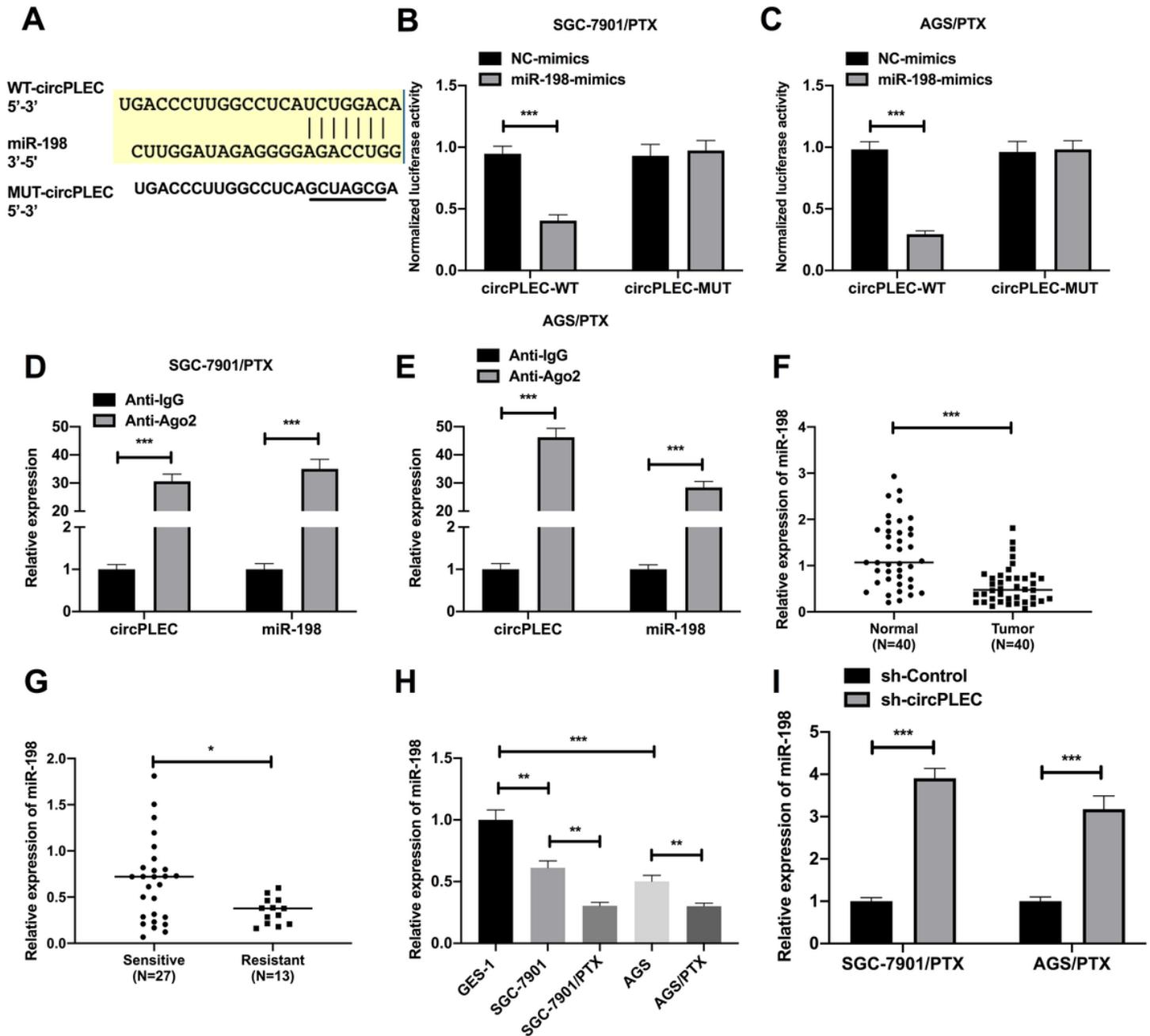


Figure 3

CircPLEC negatively regulates the expression miR-198. A) The binding sites of circPLEC and miR-198 predicted by Target Scan Database; B-C) The luciferase reporter assay to detect the relationship between circPLEC and miR-198 in PTX-resistant GC cells; D-E) RIP assay to detect the relationship between circPLEC and miR-198; F) the expression of miR-198 in GC tissues by qRT-PCR; G) The expression of miR-198 in PTX-sensitive and PTX-resistant GC tissues; H) The relative expression of miR-198 in GC cells and GES-1 stomach mucosa epithelium cells by qRT-PCR; I) The relative expression of miR-198 in PTX-resistant GC cells by qRT-PCR; (** $P < 0.01$, *** $P < 0.001$, * $P < 0.05$)

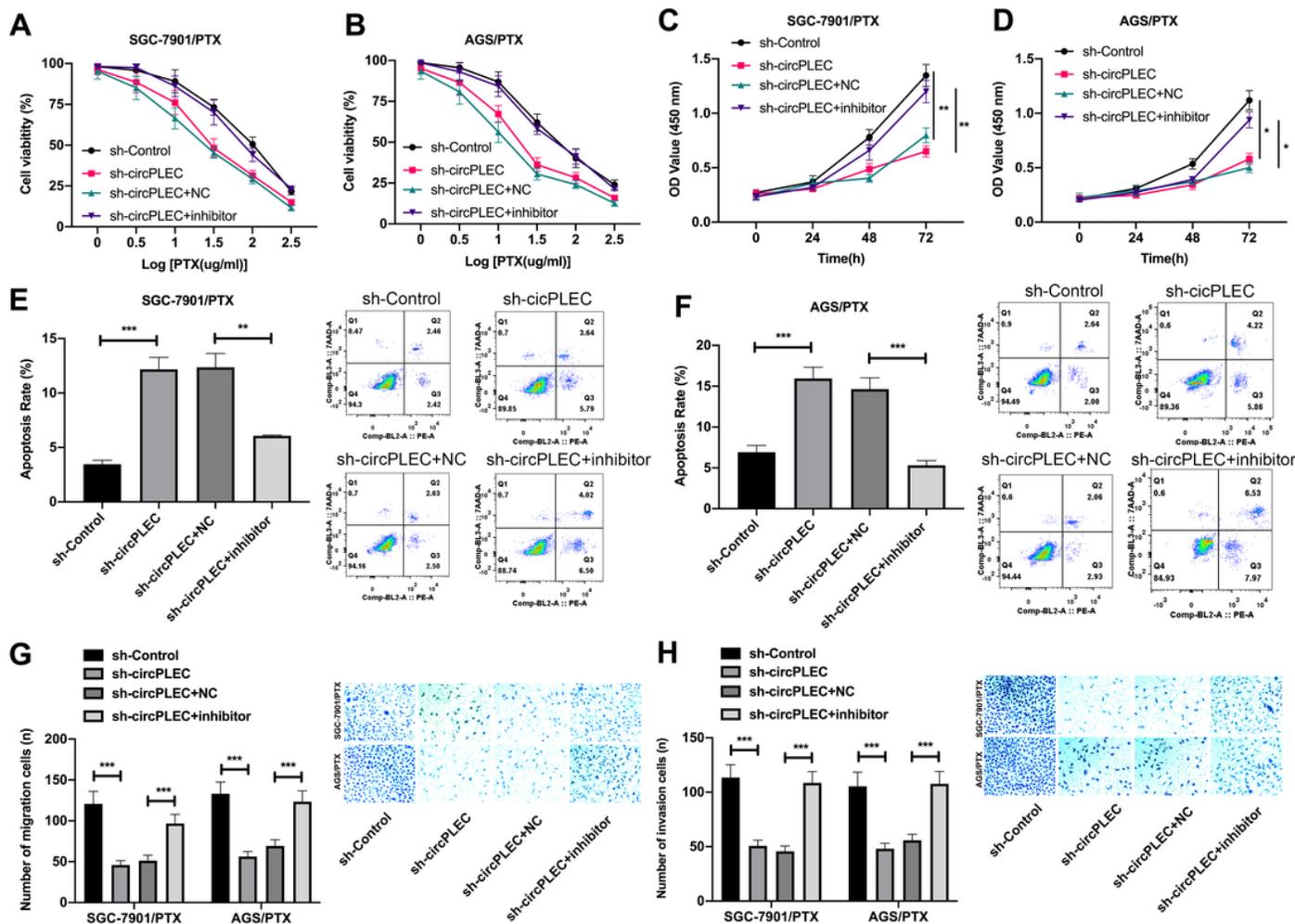


Figure 4

miR-198 inhibited the effect of circPLEC promote the GC resistance to PTX. The PTX-resistant SGC-7901 and AGS cells were transfected by sh-control, sh-circPLEC, sh-circPLEC+NC inhibitor, and sh-circPLEC+miR-198 inhibitor. A-B) The value of IC₅₀ of PTX-resistant GC cells in four groups by CCK-8 assay; C-D) The cell viability of PTX-resistant GC cells in four groups by CCK-8 assay; E-F) Cell apoptosis rate of PTX-resistant GC cells in four groups by FMC analysis; G-H) The ability of migration and invasion of PTX-resistant GC cells in four groups measured by transwell. (scale bar=50 μm) (**P < 0.01, ***P < 0.001, * P < 0.05)

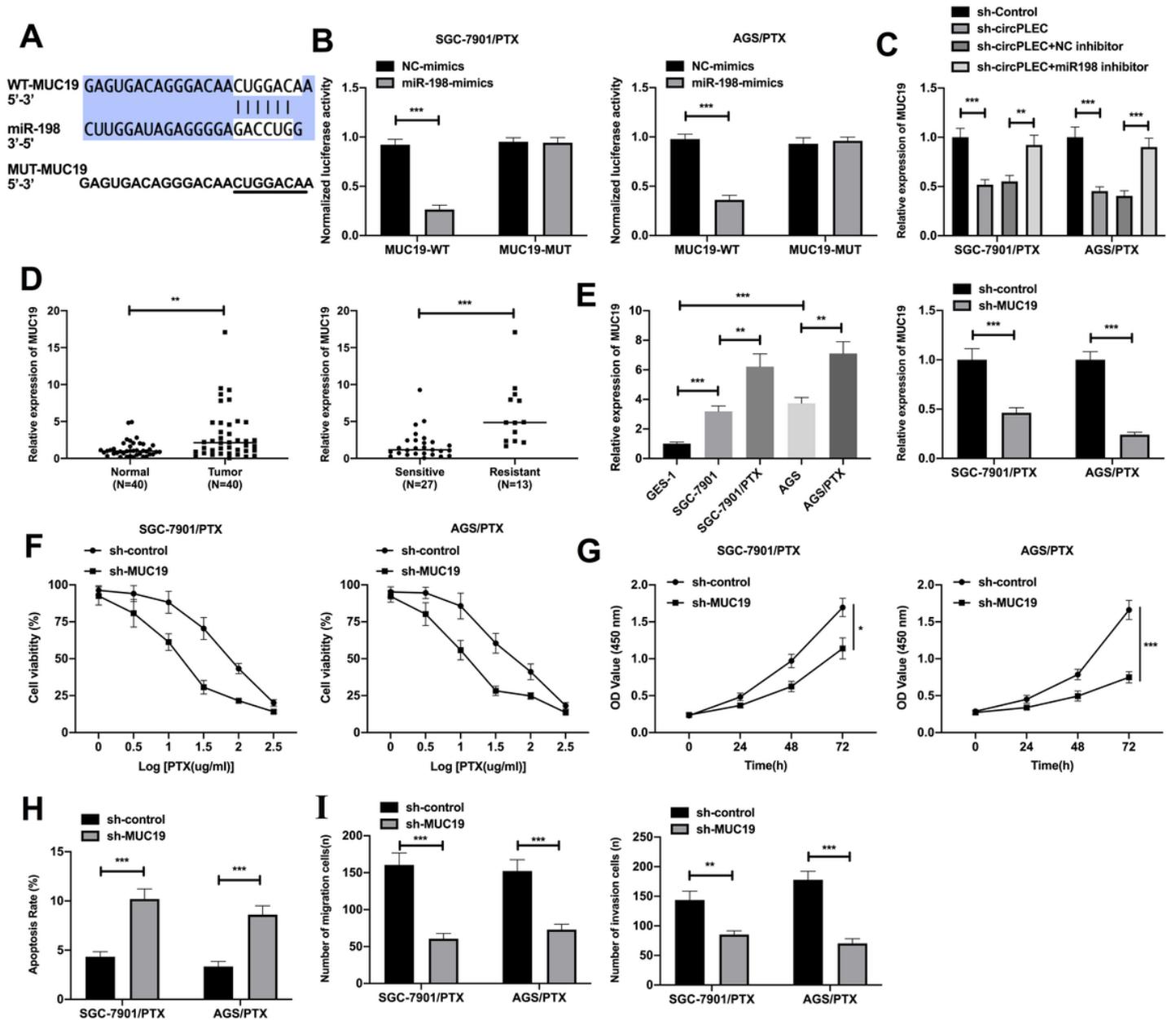


Figure 5

MUC19 promoted the the GC resistance to PTX and tumorigenesis. A) The binding sites of MUC19 and miR-198 predicted by Target Scan Database; B) The luciferase reporter assay to detect the relationship between MUC19 and miR-198 in PTX-resistant GC cells; C) The relative expression of MUC19 in PTX-resistant GC cells in four groups by qRT-PCR; D) The expression of MUC19 in PTX-sensitive and PTX-resistant GC tissues; E) The relative expression of MUC19 in GC cells ,GES-1 cells and PTX-resistant GC cells by qRT-PCR;F) The value of IC₅₀ in PTX-resistant GC cells by CCK-8 assay; G) The cell viability of PTX-resistant GC cells in four groups by CCK-8 assay; H) Cell apoptosis rate of PTX-resistant GC cells by FMC analysis; I) The ability of migration and invasion of PTX-resistant GC cells measured by transwell. (scale bar=50 μ m) (***P* < 0.001, ** *P* < 0.01, * *P* < 0.05)

Supplementary Files

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- [FigureS1.tiff](#)