European Review for Medical and Pharmacological Sciences 2019; 23: 9907-9914

MiR-187 influences cisplatin-resistance of gastric cancer cells through regulating the TGF-β/Smad signaling pathway

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Abstract. – OBJECTIVE: To explore the effect of micro-ribonucleic acid (miR)-187 on cisplatin (DDP) resistance of gastric cancer cells by regulating the transforming growth factor-β (TGF-β)/Smad signaling pathway.

MATERIALS AND METHODS: DDP-sensitivities in GES-1, SGC7901, and SGC7901/DDP cells were detected via Cell Counting Kit-8 (CCK-8) assay. The differential expression of miR-187 of these cell lines was detected by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR). DDP-resistant gastric cancer cells SGC7901/DDP were divided into control group (blank control), miR-187 inhibitor group (SGC7901/DDP cells transfected with miR-187 inhibitor), and miR-187 mimic group (SGC7901/DDP cells transfected with miR-187 mimic). The protein expressions of miR-187, TGF-β1, p-Smad4, excision repair cross-complementation group 3 (ERCC3), and ERCC4 were determined through RT-qPCR, immunohistochemistry, and Western blotting. The apoptosis in each group was detected by flow cytometry.

RESULTS: MiR-187 level had a negative correlation with DDP-resistance of GES-1, SGC7901, and SGC7901/DDP cells, and among them, the GES-1 cells had the lowest DDP-resistance and the highest expression of miR-187. CCK-8 assay revealed that compared with that in the control group, DDP-resistance significantly declined in the miR-187 mimic group, while it was significantly enhanced in miR-187 inhibitor group ($p<0.01$). According to the results of flow cytometry, after treatment with 100 nM DDP for 12 h, the apoptotic rate in miR-187 mimic group enhanced, while it was markedly reduced in the miR-187 inhibitor group ($p<0.01$). Western blotting and immunohistochemistry results showed that expressions of TGF-β1 and p-Smad4 were significantly downregulated in the miR-187 mimic group, while they were upregulated in the miR-187 inhibitor group ($p<0.01$). Besides, compared with the control group, ERCC3 and ERCC4 were downregulated in the miR-187 mimic group, while upregulated in miR-187 inhibitor group ($p<0.01$).

CONCLUSIONS: The overexpression of miR-187 alleviates DDP-resistance in gastric cancer cells by inhibiting the TGF-β/Smad signaling pathway.

Key Words:
MiR-187, TGF-β/Smad, Gastric cancer, Cisplatin resistance.

Introduction

Gastric cancer is a very common malignant tumor that occurs in the digestive tract with a high morbidity rate. The detective rate of early-stage gastric cancer is relatively low, and 70% of patients have already been in the middle-advanced stage, with a 5-year survival rate of ≤20%[1]. Chemotherapy is the major therapy for gastric cancer, and cisplatin (DDP) is often adopted as a first-line drug in the treatment of gastric cancer. To inhibit the tumor deoxyribonucleic acid (DNA) replication and avoid cell variation during DNA replication and recombination, DDP-DNA drugs are synthesized, achieving satisfactory therapeutic effects. However, the low inhibitory rate of chemotherapeutic drugs on tumor cells easily cause chemotherapy failure. The occurrence of DDP-resistance has become a major problem leading to chemotherapy failure[2,3]. Micro-ribonucleic acids (miRNAs) are non-coding endogenous RNAs, and their involvement in cancer has been widely studied. MiRNAs are extensively involved in the regulation of cellular behaviors, as well as pathophysiological processes. Some miRNAs may inhibit or promote cancer cells, and they also play an important role in the drug resistance of cancer by altering the downstream gene expressions. In gastric cancer cells, it has been found that some miRNAs can regulate the resistance of a variety

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of chemotherapeutic drugs through different signaling pathways. MiR-187 plays a non-negligible role in various malignant tumors[4]. Moreover, miR-187 also has an inseparable correlation with transforming growth factor-β (TGF-β)/Smad. Once TGF-β binds to the receptor and the cytoplasmic protein Smads, it translocates into the nucleus, where it regulates the transcription of the response genes. The TGF-β/Smad signaling pathway is a critical pathway in tumor diseases. In this paper, the effect of miR-187 on DDP-resistance of gastric cancer cells by regulating the TGF-β/Smad signaling pathway was explored, so as to provide new ideas for the clinical treatment of DDP-resistance of gastric cancer[5,6].

Materials and Methods

Laboratory Materials and Reagents

DDP-resistant gastric cancer cells (SGC7901/DDP), gastric cancer cells (SGC7901), and normal human gastric mucosal cells (GES-1) were purchased from Shanghai Huiying Biotechnology Co., Ltd. (Shanghai, China); miR-187 primers from Sangon Biotechnology (Shanghai, China), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, penicillin-streptomycin solution (double antibody), and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA); M-MLV microRNA reverse transcription kit was purchased from Suzhou Yuheng Biotechnology Co., Ltd. (Suzhou, China); Western blotting antibody diluent was purchased from Shanghai Westang Biotechnology Co., Ltd. (Shanghai, China); 2-D Quant Kit from Amersham Biosciences (Thermo Fisher Scientific, Waltham, MA, USA), protease inhibitor phenylmethylsulfonyl fluoride (PMSF), protein radioimmunoprecipitation assay (RIPA) lysis buffer (strong), and bicinchoninic acid (BCA) working solution for protein concentration quantification from Shanghai GenePharma Co., Ltd. (Shanghai, China), and RNA extracting solution were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Laboratory Instruments

The cell incubator (HERAcell 150i) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), the microscope (IX51) and fluorescence microscope (IX73) from Shanghai Yongke Optical Instrument Co., Ltd. (Shanghai, China), the cell culture plates (6-well, 12-well and 96-well plates) from Corning (Corning, NY, USA), and the fluorescence quantitative polymerase chain reaction (qPCR) instrument (LightCycler 96 System) from Roche (Basel, Switzerland).

Cell Counting Kit-8 (CCK-8) Assay

The cells in the logarithmic growth phase were uniformly inoculated into a 96-well plate (1×10^4/well) and cultured in an incubator for 24 h. After 24-h DDP induction, 180 μL of fresh medium containing 20 μL of CCK-8 reaction solution (Dojindo Laboratories, Kumamoto, Japan) was replaced, followed by incubation in the dark at 37°C for 2 h. The cells were gently vibrated on a micro-vibrator for 3 min, and the absorbance was measured at a wavelength of 450 nm using a microplate reader.

RT-qPCR

The cells were incubated in 200 μL of chloroform and TRIzol mixture, placed at room temperature for 15 min, and centrifuged at 12,000 rpm and 4°C for 15 min. Then, the supernatant was aspirated into another centrifuge tube, applied with isopropanol (0.7-1-fold volume of the supernatant), placed at room temperature for 10-30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was discarded, and the RNAs were precipitated in the bottom. Next, 75% ethanol (1 mL of 75% ethanol/mL TRIzol) was added into the centrifuge tube, gently shaken to suspend the precipitates, centrifuged at 12,000 rpm and 4°C for 5 min. The supernatant was discarded as far as possible, and the precipitates were blown dry on a super clean bench for 10-20 min and dissolved using 10-50 μL of diethyl pyrocarbonate-treated ddH_2O. The concentration was detected using the OneDrop micro-spectrophotometer. RT reaction was performed using 4.5 μL of RNase-free ddH_2O, 2 μL of 5×RT reaction buffer, 0.5 μL of random primers, 0.5 μL of Oligo dT, 0.5 μL of reverse transcriptase, and 2 μL of RNAs. The cDNA samples diluted at 1:20 were subjected to PCR amplification via 5% agarose gel electrophoresis. The relative levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method and analyzed by LabWorks 4.0 image acquisition and analysis software. The primer sequences used were shown in Table I.

Cell Transfection and Grouping

GES-1, SGC7901, and SGC7901/DDP cells were cultured in RPMI-1640 medium containing 10% FBS, 100 μg/mL streptomycin and 100 UI penicillin in the incubator with 5% CO_2 at 37°C.

Table I. Primer sequences.

Gene	Primer	Sequence
MiR-187	F	5'-TCGTGTCTTGTGT-TGCAGC-3'
	R	5'-GTGCAGGGTCCGAGGT-3'
U6	F	5'-CTCGCTTCGGCAGCACA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'

SGC7901/DDP cells were uniformly inoculated into the 6-well plate (1.2×10^6/well), and cultured in RPMI-1640 medium containing 10% FBS. The cells were divided into control group, miR-187 mimic group (treated with 50 nmol/L miR-187 mimic), and miR-187 inhibitor group (treated with 50 nmol/L miR-187 inhibitor). After culture for 24 h, miR-187 mimic or miR-187 inhibitor was diluted in 250 μL of serum-free Opti-MEM, placed at room temperature for 5 min, and mixed with 10 μL of diluted Lipofectamine 2000. Then, the mixture was applied in the cells. After 6 h, the fresh medium was replaced. The transfected cells for 48 h were used for the following experiments.

Immunohistochemistry

SGC7901/DDP cells were washed with PBS, fixed in 4% polyformaldehyde for 20 min and incubated with 0.5% Triton X-100 for another 20 min. Subsequently, the cells were treated with H_2O_2 for 20 min, and washed with PBS for 3 times, followed by serum blockage at 37°C for 30 min. The cells were then incubated with the corresponding primary and secondary antibodies. DAB exposure and hematoxylin counterstain were conducted. Finally, the staining was observed and photographed under a light microscope to detect the positive expressions of TGF-β1 and Smad4.

Western Blotting

The cells were lysed in an appropriate amount of RIPA lysis buffer containing protease inhibitor PMSF (RIPA:PMSF=100:1). After centrifugation at 14000 rpm at 4°C for 30 min, the protein supernatant was collected, quantified, and subjected to a heating bath at 95°C for 10 min for protein denaturation. The protein sample was loaded on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under the constant pressure of 80 V for 2.5 h. Then, the protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a semi-dry transfer method. The PVDF membranes were immersed in Tris-Buffered Saline and Tween-20 (TBST) containing 5% skim milk powder and shaken slowly for 1 h. Then, the protein was incubated with the primary antibody diluted with 5% skim milk powder, rinsed with TBST for 3 times (10 min/time), and incubated again with the secondary antibody at room temperature for 2 h. Finally, the protein was detected using the enhanced chemiluminescence (ECL) reagent, followed by exposure in a dark room. The relative expression of the protein was analyzed using the Image-Pro Plus v6 (Media Cybernetics, Silver Spring, MD, USA), with β-actin as an internal reference.

Flow Cytometry

The changes in the apoptosis of the three kinds of cells after treatment with 100 nM DDP for 12 h were detected using the Annexin V/propidium iodide (PI) double-staining kit. 5×10^5 cells were

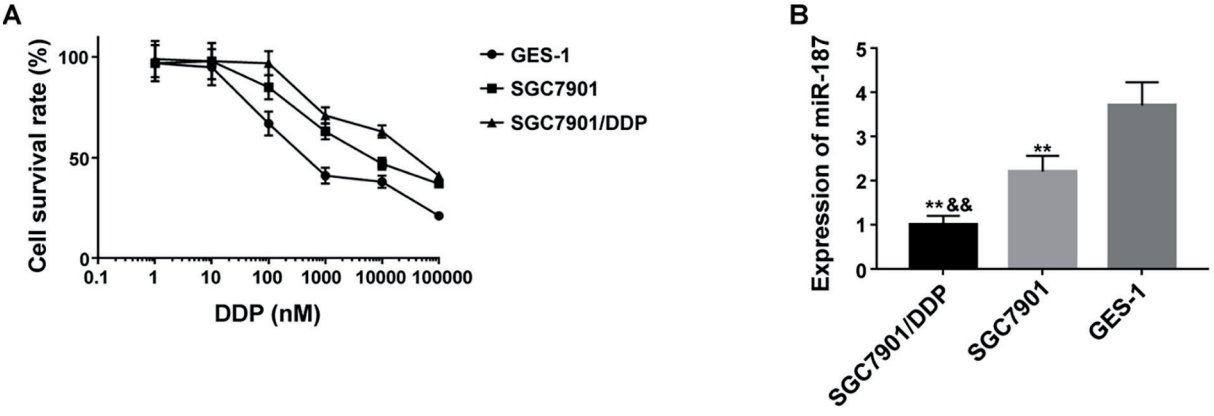

Figure 1. The expression level of miR-187 has a negative correlation with the DDP-resistance of GES-1, SGC7901, and SGC7901/DDP cells. **A,** Drug resistance of the three kinds of cells detected via CCK-8 assay. **B,** Expression of miR-187 detected via RT-qPCR. $^{**}p<0.01$ *vs.* GES-1 cells, $^{\&\&}p<0.01$ *vs.* SGC7901 cells.

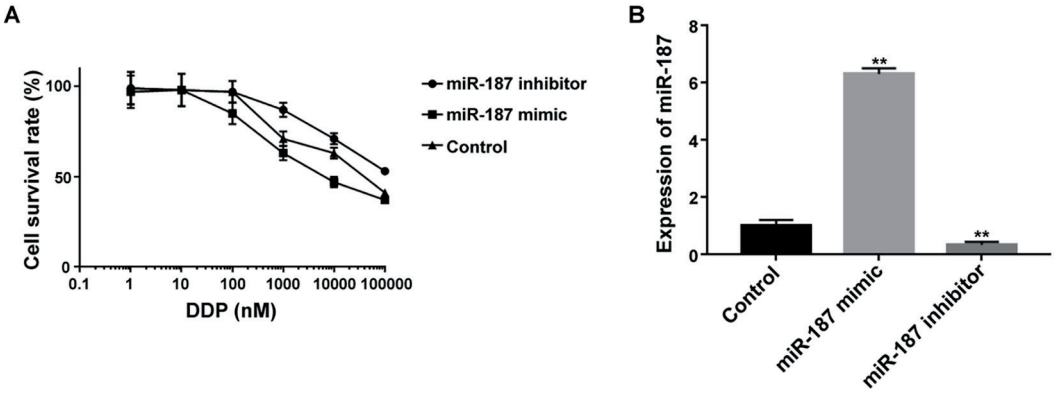

Figure 2. Increased expression of miR-187 significantly reduces the DDP-resistance of SGC7901/DDP cells. **A,** Difference in miR-187 expression in the three kinds of cells detected via RT-qPCR. **B,** DDP-resistance of the three kinds of cells detected via CCK-8 assay. $^{**}p<0.01$ *vs.* control group.

digested with trypsin and rinsed twice with PBS at 4°C. Then, the cells were centrifuged and resuspended in 500 μL of staining buffer. The cells were incubated in 5 μL of Annexin V-FITC and 5 μL of PI in a dark place at 37°C for 15 min. Finally, apoptosis was detected using the Guava flow cytometer.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The data were expressed as mean ± standard deviation. The *t*-test was used to analyze the measurement data. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using the One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Drug Resistance and MiR-187 Level in Three Cell Lines

The results of the CCK-8 assay showed that the DDP-resistance significantly increased in the SGC7901 cells compared with that in GES-1 cells ($p<0.01$), in which it was higher in SGC7901/DDP cells ($p<0.01$) (Figure 1A). The results of RT-qPCR showed that the expression of miR-187 significantly declined in SGC7901 cells compared with that in GES-1 cells, in which it was lower in SGC7901/DDP cells ($p<0.01$) (Figure 1B). The above results suggested that the expression level of miR-187 had a negative correlation with DDP-resistance of GES-1, SGC7901, and SGC7901/DDP cells.

MiR-187 Significantly Reduced DDP-Resistance of SGC7901/DDP Cells

To further explore the effect of miR-187 on DDP-resistance of SGC7901/DDP cells, miR-187 mimic or miR-187 inhibitor were transfected into cells. The results of the CCK-8 assay revealed that, compared with that in the control group, the overexpression of miR-187 reduced the DDP-resistance of SGC7901/DDP cells, and conversely, the knockdown of miR-187 yielded the opposite trend ($p<0.01$) (Figure 2).

Apoptosis Influenced by MiR-187 in SGC7901/DDP Cells

According to the results of flow cytometry, after treatment with 100 nM DDP for 12 h, the apoptotic rate increased in SGC7901/DDP cells overexpressing miR-187, and was reduced in those with miR-187 knockdown ($p<0.01$) (Figure 3). The above findings indicated that miR-187 significantly accelerated the apoptosis of SGC7901/DDP cells.

MiR-187 Evidently Decreased Expressions of TGF-β1 and p-Smad4

Compared with control group, protein expressions of TGF-β1 and p-Smad4 were remarkably reduced in miR-187 mimic group ($p<0.01$), while

they were significantly upregulated in miR-187 inhibitor group (p<0.01) (Figure 4).

Expressions of TGF-β1 and p-Smad4 Detected Using Immunohistochemistry

The results of immunohistochemistry showed that the positive expressions of TGF-β1 and p-Smad4 evidently decreased in the miR-187 mimic group and increased in miR-187 inhibitor group (Figure 5).

MiR-187 Remarkably Downregulated Expressions of ERCC3 and ERCC4

According to the results of the Western blotting, compared with the control group, ERCC3 and ERCC4 were downregulated in the miR-187 mimic group, while they were upregulated in miR-187 inhibitor group (p<0.01) (Figure 6). The above results demonstrated that after enhanced DDP-sensitivity in gastric cancer cells overexpressing miR-187, they may be related to downregulated ERCC3 and ERCC4.

Discussion

The mortality of gastric cancer ranks second in China, and it is characterized by genetic heterogeneity. Current examinations[7] on gastric cancer at the molecular level are limited. Because of insufficient early screening and diagnosis methods, plenty of gastric cancer patients are already progressed into advanced stages at the initial diagnosis, leading to a relatively poor prognosis[8]. Therefore, it is necessary to study the pathogenesis and molecular biology of gastric cancer, so as to develop the most effective individualized therapy to improve the clinical outcomes. The multidrug resistance of tumor cells is now the leading cause of chemotherapy failure[9]. The potential mechanism underlying multidrug resistance of tumor cells should be urgently clarified[10].

In the present study, DDP-resistance significantly increased in the SGC7901 cells compared with that in the GES-1 cells, which was more pronounced in SGC7901/DDP cells. The results of

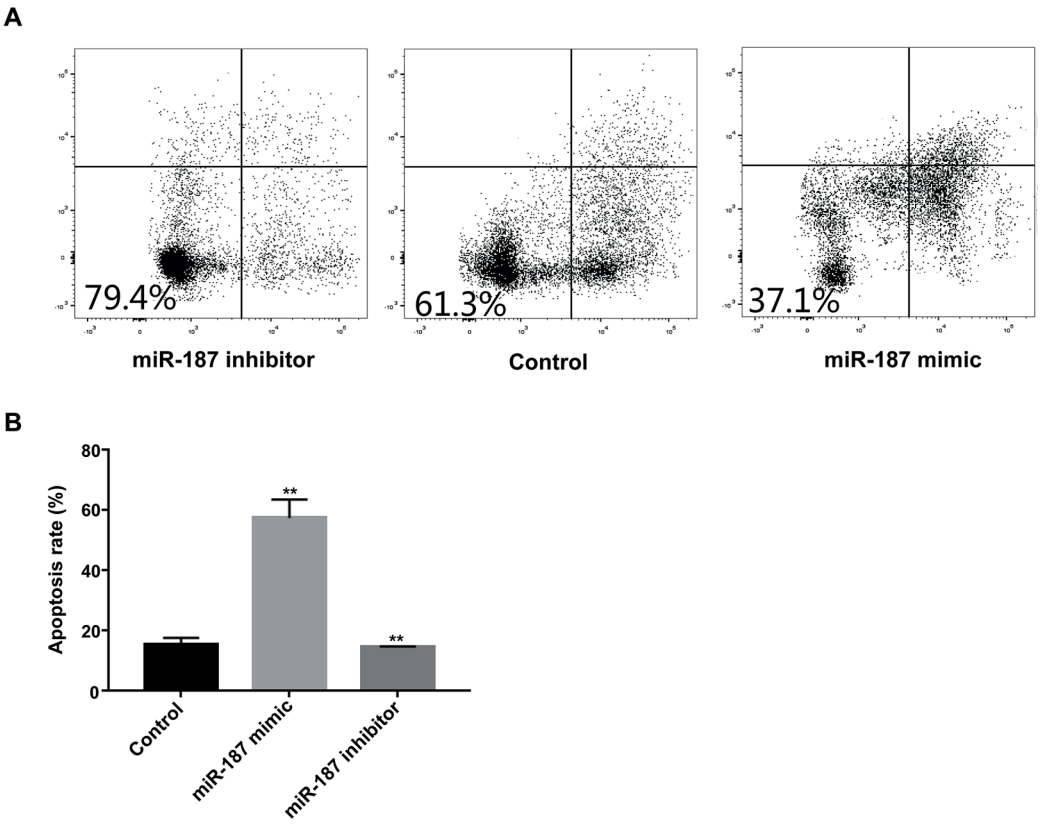

Figure 3. Increased expression of miR-187 markedly reduces the DDP-resistance of SGC7901/DDP cells. **A,** Resistance of the three kinds of cells to DDP detected via flow cytometry. **B,** Quantitative analysis of flow cytometry results. $^{**}p$<0.01 *vs.* control group.

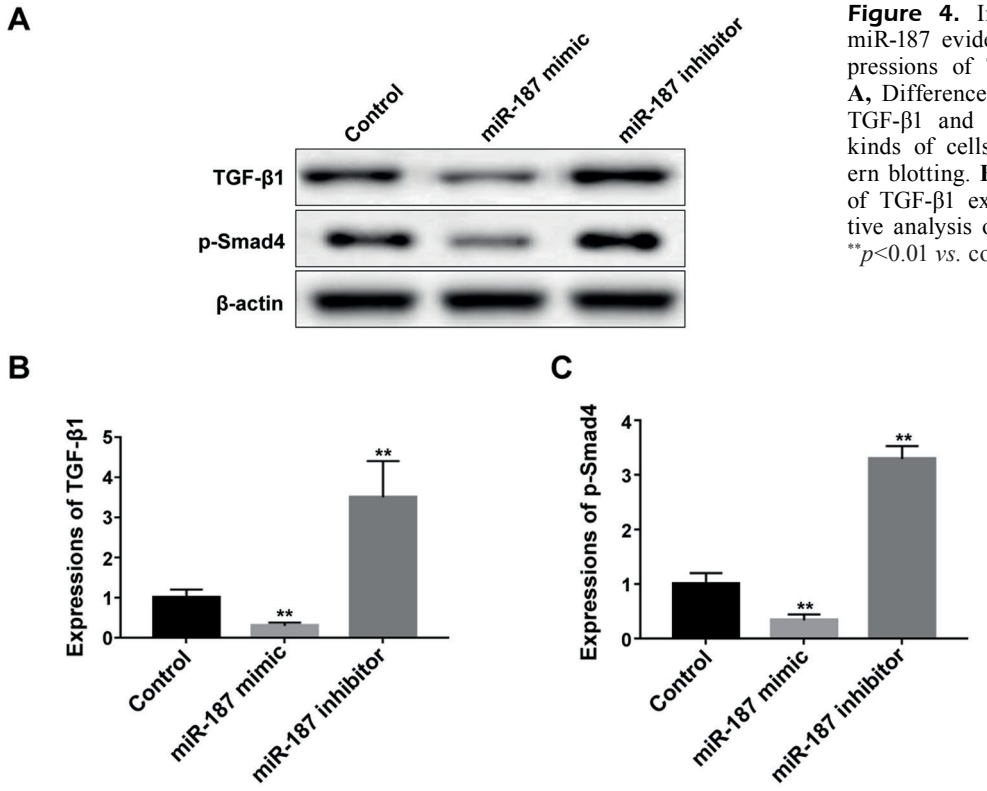

Figure 4. Increased expression of miR-187 evidently decreases the expressions of TGF-β1 and p-Smad4. **A,** Differences in the expressions of TGF-β1 and p-Smad4 in the three kinds of cells detected using Western blotting. **B,** Quantitative analysis of TGF-β1 expression. **C,** Quantitative analysis of p-Smad4 expression. $^{**}p<0.01$ *vs.* control group

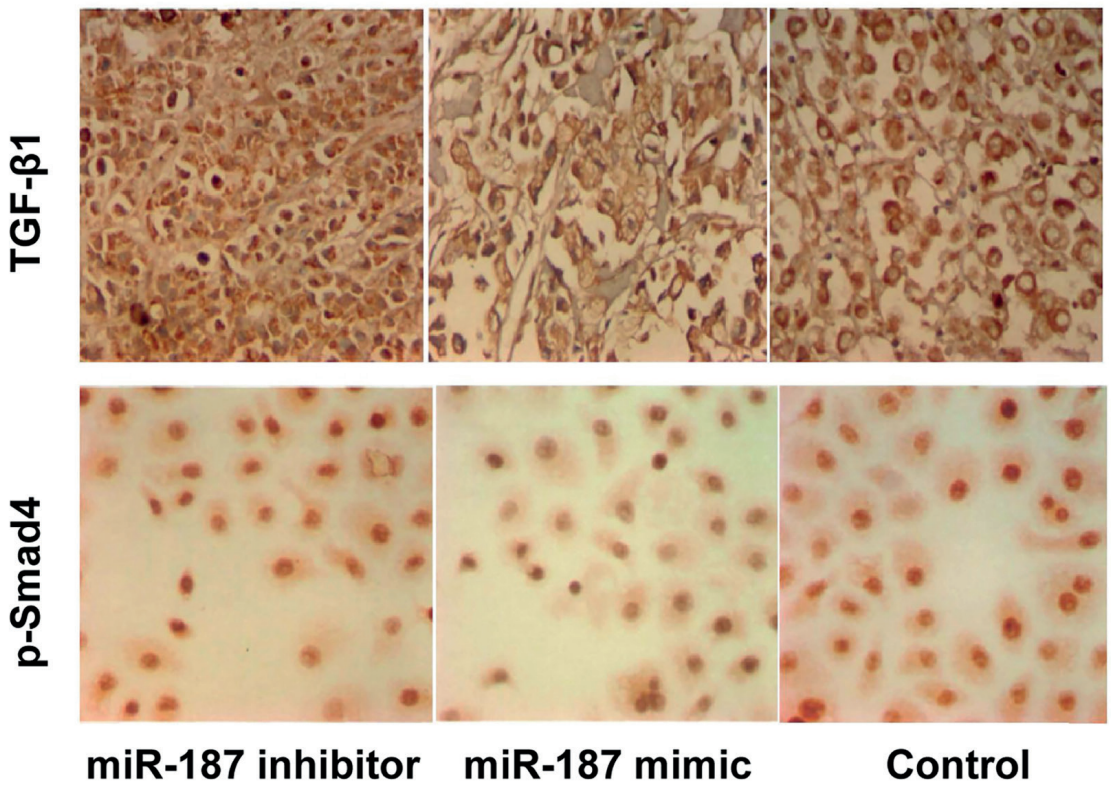

Figure 5. Increased expression of miR-187 evidently decreases the expressions of TGF-β1 and p-Smad4. The differences in the expressions of TGF-β1 and p-Smad4 in the three kinds of cells are determined using immunohistochemistry (magnification × 40).

RT-qPCR showed that the expression of miR-187 remarkably declined in SGC7901 cells compared with that in GES-1 cells, while it also remarkably declined in SGC7901/DDP cells compared with that in the SGC7901 cells. The above results suggested that the expression level of miR-187 may be negatively correlated with DDP-resistance in gastric cancer cells. Moreover, the overexpression of miR-187 was able to accelerate apoptosis in SGC7901/DDP cells.

According to the results of Western blotting and immunohistochemistry, the overexpression of miR-187 downregulated TGF-β1, p-Smad4, ERCC3, and ERCC4 in gastric cancer cells, suggesting that the involvement of TGF-β1 pathway in miR-187-regulated the DDP-resistance. Herr et al[11] showed that the downstream target of TGF-β signal is a key factor regulating cell growth cycle, and the overactivity of these genes can promote the growth of tumor cells. As one of the most important pathways for epithelial-mesenchymal transition, the TGF-β/Smad signaling pathway plays an important role in the invasion, metastasis, and recurrence of tumors. Some investigations[12,13] have demonstrated that TGF-β/Smad facilitates the development of malignant tumors and reduces the sensitivity of gastric cancer cells to DDP. Other studies[14,15] have shown that the overexpression of miR-187 can suppress the expressions of the target proteins ERCC3 and ERCC4 in the pathway, thus leading to the decline in the DDP-resistance of cancer cells. Loboda et al[16] observed that the overexpression of miR-187 can promote apoptosis of gastric cancer cells, thereby reducing the multidrug resistance of gastric cancer cells. Tachibana et al[17] have proved that miR-187 plays a non-negligible role in the DDP-resistance of gastric cancer, and it may become a new target for reversing the DDP-resistance of gastric cancer. Moreover, Simpson et al[18] proved that the overexpression of miR-187 can inhibit the TGF-β/Smad signaling pathway and weaken the drug-resistance of tumors, which offers a new reference for the targeted therapy of gastric cancer and other tumors[19].

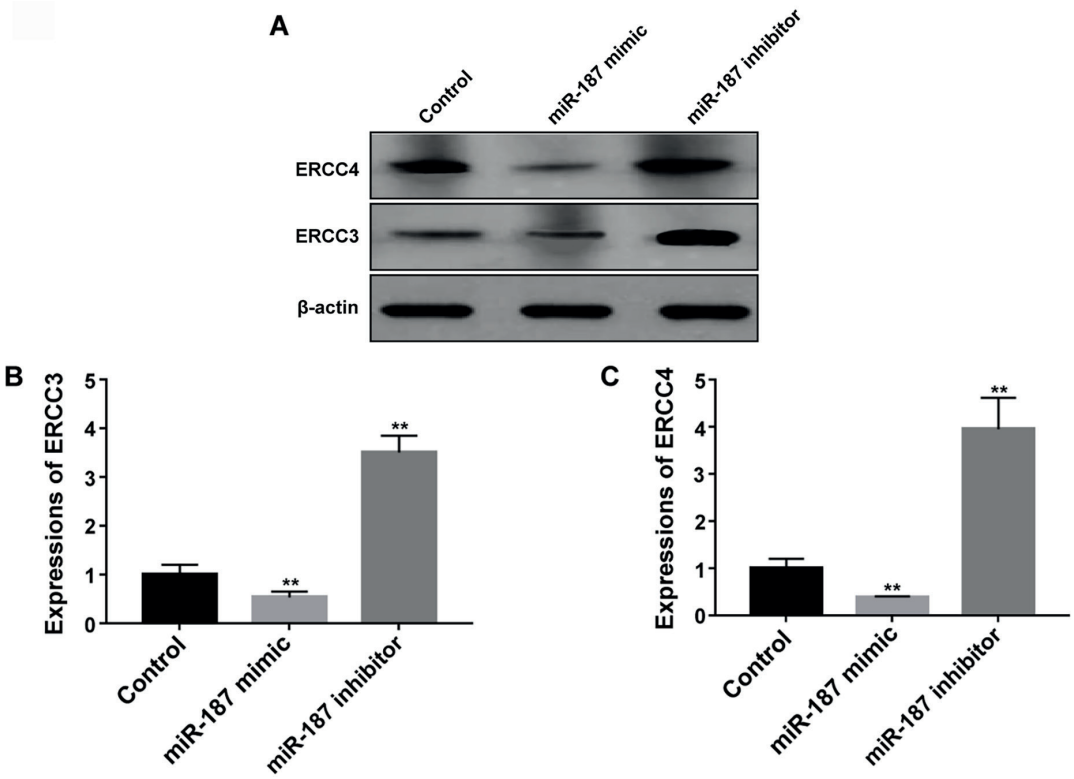

Figure 6. Increased expression of miR-187 remarkably lowers the expressions of ERCC3 and ERCC4. **A,** Differences in expressions of ERCC3 and ERCC4 in the three kinds of cells detected using Western blotting. **B,** Quantitative analysis of ERCC3 expression. **C,** Quantitative analysis of ERCC4 expression. $^{**}p<0.01$ *vs.* control group.

Conclusions

This study indicates that miR-187 plays an important role in DDP-resistance of gastric cancer cells by inhibiting the TGF-β/Smad signaling pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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