



Regulation of miR-596 on the Apoptosis of Gastric Cancer Cells through Its Targeting Inhibition of BCL-2

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Abstract

Background: We aimed to investigate the relationship between miR-596, BCL-2, and apoptosis of gastric cancer cells, and to explore the mechanism of miR-596 in gastric cancer. Besides, this study aimed to find the target of miR-596 and explore the mechanism of action of miR-596 in gastric cancer.

Methods: Eighteen samples of gastric cancer tissues and 18 samples of corresponding tumor-adjacent tissues were collected from 18 gastric cancer patients (aged from 40 to 55 yr) admitted to Zhuji People's Hospital, Zhuji, China from March 2017 to May 2018. The expression levels of miR-596 and BCL-2 were detected to verify the regulation of miR-596 on the apoptosis and proliferation of gastric cancer cell lines MKN-45 and HGC-27 and its effect on BCL-2 expression.

Results: The expression level of miR-596 was notably lower in gastric cancer tissues than in adjacent tissues, and BCL-2 level was notably higher in gastric cancer tissues than in adjacent tissues. After the up-regulation of miR-596 expression, the proliferation of MKN-45 and HGC-27 cells was significantly decreased, the level of apoptosis was significantly increased ($P < 0.05$), and the expression of BCL-2 was decreased. The dual-luciferase report showed that miR-596 had a targeting inhibition of BCL-2. Gastric cancer cells with up-regulated miR-596 and BCL-2 had significantly higher proliferation and lower apoptosis than cells with up-regulated miR-596.

Conclusions: miR-596 can inhibit the proliferation of gastric cancer cells and promote the apoptosis through its targeting inhibition of BCL-2 expression.

Keywords: Apoptosis; BCL2 proteins; Correlation; Gastric cancer; miR-596; Proliferation

Introduction

The incidence of gastric cancer varies widely across the globe, with the highest in East Asia, Eastern Europe, and South America, and a lower incidence in North America and Western Europe (1). The incidence and mortality of gastric cancer rank the second in malignant tumors worldwide. In 2012, the number of new cases of gastric cancer and death cases in the world was 951 600 and 723,100. In 2015, the worldwide number of gastric cancer deaths was 760,000. Gastric cancer has been one of the leading causes of cancer death in men (2-4). Despite great progress in the

therapeutic strategies for gastric cancer, the prognosis of patients treated with existing therapies is still disappointing. Chemotherapy resistance and metastasis are the main causes of death in gastric cancer patients, especially in poorly differentiated adenocarcinoma patients (5, 6). The search for new targets of drug treatment is important for improving the prognosis of patients with gastric cancer.

MicroRNAs (miRNAs) are a class of 22 nt-long non-coding short-stranded RNAs that bind to the 3'UTR area of its target gene by complemen-

tary pairing to form a complex regulatory network that participates in life activities, including carcinogenesis (7, 8). miR-596, located at 8p23.3, is recently reported to be abnormally lowly expressed in cancers such as melanoma (9), glioma (10), and gastric cancer (11). miR-596 has a tumor-suppressive effect in gastric cancer and is capable of inhibiting gastric cancer cell proliferation, migration, and invasion via peroxidase redoxin 1(11). However, the effect of miR-596 on apoptosis of gastric cancer cells and its mechanism are still unclear. miR-596 can target myeloid leukemia sequence 1 (BCL2-related) and BCL2-like protein 1 to regulate the apoptotic pathway and promote the apoptosis of melanoma cells (9). We hypothesized that miR-596's mechanism of regulation on BCL2 may also be applied to its regulation on the apoptosis of gastric cancer cells. We investigated the relationship between miR-596, BCL-2, and apoptosis of gastric cancer cells, and explored the mechanism of miR-596 in gastric cancer, hoping to provide an experimental basis for the clinical search for therapeutic targets of gastric cancer.

Methods

Research subjects

Eighteen samples of gastric cancer tissues and 18 samples of corresponding tumor-adjacent tissues were collected from 18 gastric cancer patients (aged from 40 to 55 yr) admitted to Zhuji People's Hospital, Zhuji, China from March 2017 to May 2018.

Inclusion criteria were Patients with complete medical records and diagnosed with gastric cancer by postoperative pathological diagnosis. Exclusion criteria were Patients with recurrent gastric cancer, previous presence of other tumors; pregnant or lactating women.

This study was approved by the Ethics Committee of Zhuji People's Hospital. All patients and their families gave informed consent through the telephone or letter.

Source of cells

Human normal gastric mucosal epithelial cells NGEC and human gastric cancer cells SGC-

7901, MKN-45, BGC-823, HGC-27, and MFC were purchased from BeNa Culture Collection (item numbers: BNCC341561, BNCC100674, BNCC337682, BNCC337689, BNCC338546, BNCC100581). The normal cell culture medium was composed of BEBM medium (Thermo Fisher Scientific, item number: No. 12767019) + 10% fetal bovine serum (Thermo Fisher Scientific, item number: No. 16250086). The gastric cancer cell strain medium was composed of RPMI-1640 medium (Thermo Fisher Scientific, item number: No. 61870044) +10% fetal bovine serum + penicillin 100 U/ml and streptomycin 100 µg/ml. Cells were incubated in a constant temperature incubator at 37 °C, with 5% CO₂. The cells were passaged for 2-3 generations before use.

Construction and transfection of the expression vector

miR-596 mimics (overexpression sequence), BCL-2 mRNA mimic, and blank vector miR-NC were constructed by Thermo Fisher Scientific (China). The blank group, miR-596 mimic, and co-transfection group (miR-596 mimic + BCL-2 mRNA mimic) were set. The gastric cancer resistant cell lines were digested by trypsin 24 hours before the transfection. The transfection of expression vector was performed when the cells reached 80% confluence in strict accordance with the kit instructions. The incubator was cultured at 37 °C, 5% CO₂ for 48 hours, and the medium was changed every 6 hours. Transfection results were detected by qRT-PCR. The Lipofectamine™ 2000 Transfection Kit was purchased from Invitrogen, USA (item number No. 35050).

qRT-PCR detection

The total RNA was extracted from collected cells using the TRIzol kit (Invitrogen, USA, 15590618). The purity, concentration, and integrity of total RNA were detected by UV spectrophotometer and agarose gel electrophoresis. The A260/A280 value between 1.8 and 2.1 was considered to be meeting the experimental requirements. qRT-PCR detection was conducted after the completion of RNA extraction. The reverse transcription reaction system was as follows: 2µL

of 5*PrimerScript Buffer, 0.5 μ L of PrimerScript RT Enzyme Mix, 0.5 μ L of Random 6 mers (100 μ M), 0.5 μ L of Oligo dT Primer (50 μ M), 2 μ g of total RNA, and enough RNase-free distilled water to make up the system to 10 μ L. The condition for reverse transcription reaction was as follows: 37 °C for 15 min (reverse transcription reaction), 85 °C for 5 sec (deactivate reactions of reverse transcriptase), and 4 °C (the termination of reaction). The completion of reverse transcription was followed by PCR amplification. PCR amplification system was as follows: 4 μ L of cDNA template, 25 μ L of SYBR Green Mix (2x), 1 μ L of forward primer, 1 μ L of reverse primer, 1 μ L of Reference Dye (optional), and enough

double distilled water to make up the system to 50 μ L. The condition for reverse transcription reaction was as follows: 30 cycles of pre-denaturation at 95 °C for 3 min, 95 denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 60 sec, and extension at 72 °C for 5 min after the completion of 30 cycles. U6 was used as the reaction internal reference. The results were analyzed by the 2- Δ Ct method. The qRT-PCR and reverse transcription kits were purchased from TransGen Biotech, Beijing, China (item numbers: AQ201-01, AQ202-01). The primer sequences were designed and synthesized by Hepeng (Shanghai) Biotechnology Co., Ltd (Table 1).

Table 1: Primer sequences

	<i>Forward primer</i>	<i>Reverse primer</i>
miR-596	5'-AAGCCTGCCCGGCTCCT-3'	5'-GCTGTCAACGATACGCTACGT-3'
U6	5'-GCCGCTCGTGAAGCGTTC-3'	5'-GTGCAGGGTCCGAGGT-3'

Western Blot detection

The total protein was extracted by RIPA lysis method, and its concentration was determined by BCA method. Then the protein concentration was adjusted to 4 μ g/ μ L and the protein was separated by 12% polyacrylamide gel electrophoresis. The initial voltage was 90V, and then the voltage was increased to 120V to move the sample to the appropriate position of the separation gel. After the accomplishment of electrophoresis, the membrane was transferred (100V constant pressure for 100 min and 37 °C for 60 min). The transferred membrane was then blocked in 5% skim milk and the immune reaction was carried out. The membrane was incubated with the primary antibody (1:1000) overnight at 4 °C. On the next day, the membrane was washed for three times with PBS (5 min for each time) and then incubated with secondary antibody (1:1000) for 1 hour at room temperature. Following that, color development and fixation were performed using ECL luminescence reagent. Statistical analysis was performed on the band after film scanning using Quantity One software. Protein relative expression level = gray value of band/gray value

of the internal reference. RIPA kit, BCA protein kit, ECL luminescence kit, and trypsin were purchased from Thermo Scientific™ (item number: 89901, 23250, 35055, 90058). The rabbit anti-BCL-2 monoclonal antibody and goat anti-rabbit IgG secondary antibody were purchased from the Abcam (the United States, item number: ab185002, ab6721)

Apoptosis detection

The cells were digested with 0.25% trypsin and then washed twice with PBS. Next, 100 μ L of binding buffer was added to prepare a suspension at a density of 1*10⁶ cells/mL, followed by addition of Annexin V-FITC and PI to perform the incubation at room temperature in the dark. Finally, the FC500MCL flow cytometry detection was performed for 3 times to obtain the mean value. The Annexin V/PI Apoptosis Detection Kit was purchased from Invitrogen (the USA, item number: V35113).

Cell proliferation assay

Set at a density of 3*10⁴ cells/well, the cells were routinely seeded in a 96-well plate and incubated

at 37 °C. At each time points (24, 48, 72, 96 h), 20 µL of MTT solution (5 µmg/mL) was added and the wells were inoculated at 37 °C for 4 hours, and then 150 µL of dimethyl sulfoxide was added to each well. Finally, the OD value of each group was measured using a microplate reader at a wavelength of 490 nm.

Dual -reporter assay

Target scan 7.2 was used to predict miR-596 downstream target genes. The pmirGLO-BCL2-3'UTR wild type (Wt), pmirGLO-BCL2-3'UTR mutant type (Mut), miR-596-mimics, and miR-NC were transferred into HEK293T cells using Lipofectamine™ 2000 kit. The luciferase activity was determined using a dual-luciferase reporter assay kit. The primers were designed by Thermo Fisher Scientific (China).

Statistical methods

Statistical analysis was performed using SPSS19.0 (Asia Analytics Formerly SPSS China). The

measurement data were expressed as mean ± standard deviation (mean ± sd). The comparison between the two groups was performed by independent sample t-test, the comparison between multiple groups was analyzed by one-way ANOVA, and the post hoc test was performed using the LSD test. Pearson correlation was used to analyze the correlation between miR-596 and BCL-2 expression. A statistical difference was recognized when $P < 0.05$.

Results

Expression levels of miR-596 and BCL-2 in gastric cancer tissues

The expression level of miR-596 was notably lower in gastric cancer tissues than in adjacent tissues ($P < 0.05$), and BCL-2 level was notably higher in gastric cancer tissues than in adjacent tissues ($P < 0.05$). More details are shown in Fig. 1.

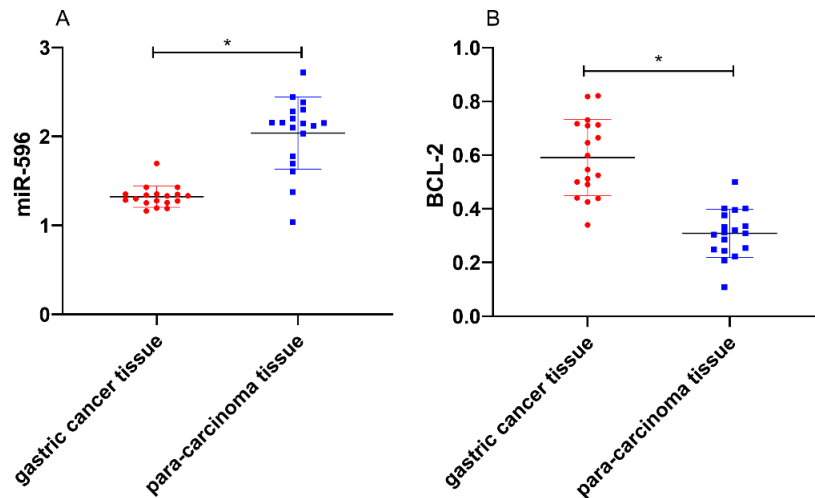


Fig. 1: Expression levels of miR-596 and BCL-2 in gastric cancer tissues. **(A)** Expression levels of miR-596 in gastric cancer tissues. **(B)** Expression levels of BCL-2 in gastric cancer tissues. * indicates $P < 0.05$

Expression levels of miR-596 and BCL-2 in gastric cancer cell lines

The expression level of miR-596 was significantly lower in SGC-7901, MKN-45, BGC-823, HGC-27, and MFC cells than in NGEK cells ($P < 0.05$),

while the expression level of BCL-2 was significantly higher in SGC-7901, MKN-45, BGC-823, HGC-27, and MFC cells than in NGEK cells ($P < 0.05$). More details are shown in Fig. 2.

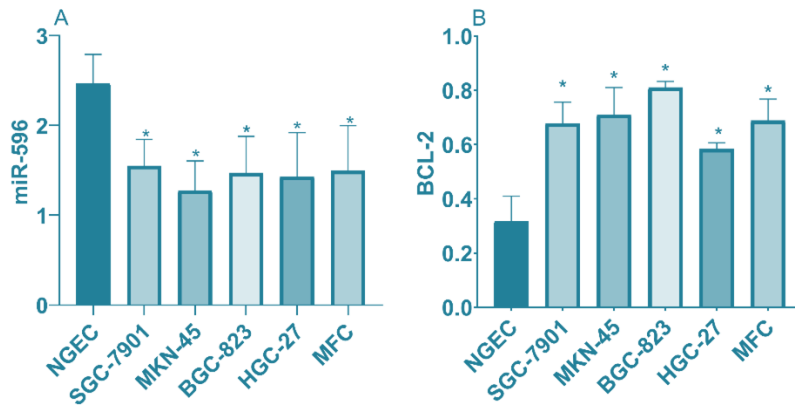


Fig. 2: Expression levels of miR-596 and BCL-2 in gastric cancer cell lines. **(A)** Expression levels of miR-596 in gastric cancer cell lines. **(B)** Expression levels of BCL-2 in gastric cancer cell lines. * indicates $P < 0.05$ when compared with NGEc cells

Effects of the up-regulation of miR-596 on proliferation and apoptosis of gastric cancer cells

After transfection with miR-596 mimic, the expression of miR-596 in MKN-45 and HGC-27 cells in the miR-596 mimic group was significant-

ly higher than that in the blank group ($P < 0.05$). The proliferation of MKN-45 and HGC-27 cells in miR-596 mimic group was lower than that in the blank group ($P < 0.05$), and the apoptosis level was higher than that in the blank group ($P < 0.05$). More details are shown in Fig. 3.

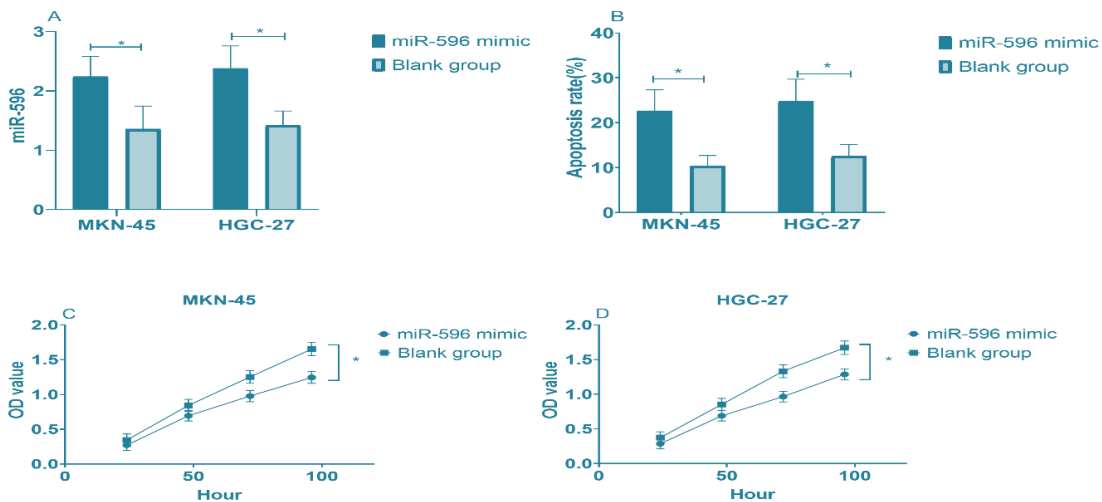


Fig. 3: Effects of the up-regulation of miR-596 on proliferation and apoptosis of gastric cancer cells. **(A)** Results after the up-regulation of miR-596. **(B)** Effects of the up-regulation of miR-596 on apoptosis of gastric cancer cells **(C)** Effects of the up-regulation of miR-596 on the proliferation of MKN-45 cells. **(D)** Effects of the up-regulation of miR-596 on the proliferation of HGC-27 cells. * indicates $P < 0.05$

Effects of the up-regulation of miR-596 on the BCL-2 expression

After transfection of miR-596 mimic, the expression of BCL-2 in MKN-45 and HGC-27 cells in

miR-596 mimic group was significantly lower than that in the blank group ($P < 0.05$). Pearson analysis demonstrated that the expression level of miR-596 was negatively correlated with BCL-2

expression ($P < 0.05$). The dual-luciferase report showed a targeted regulation relationship be-

tween miR-596 and BCL-2 ($P < 0.05$). More details are shown in Fig. 4.

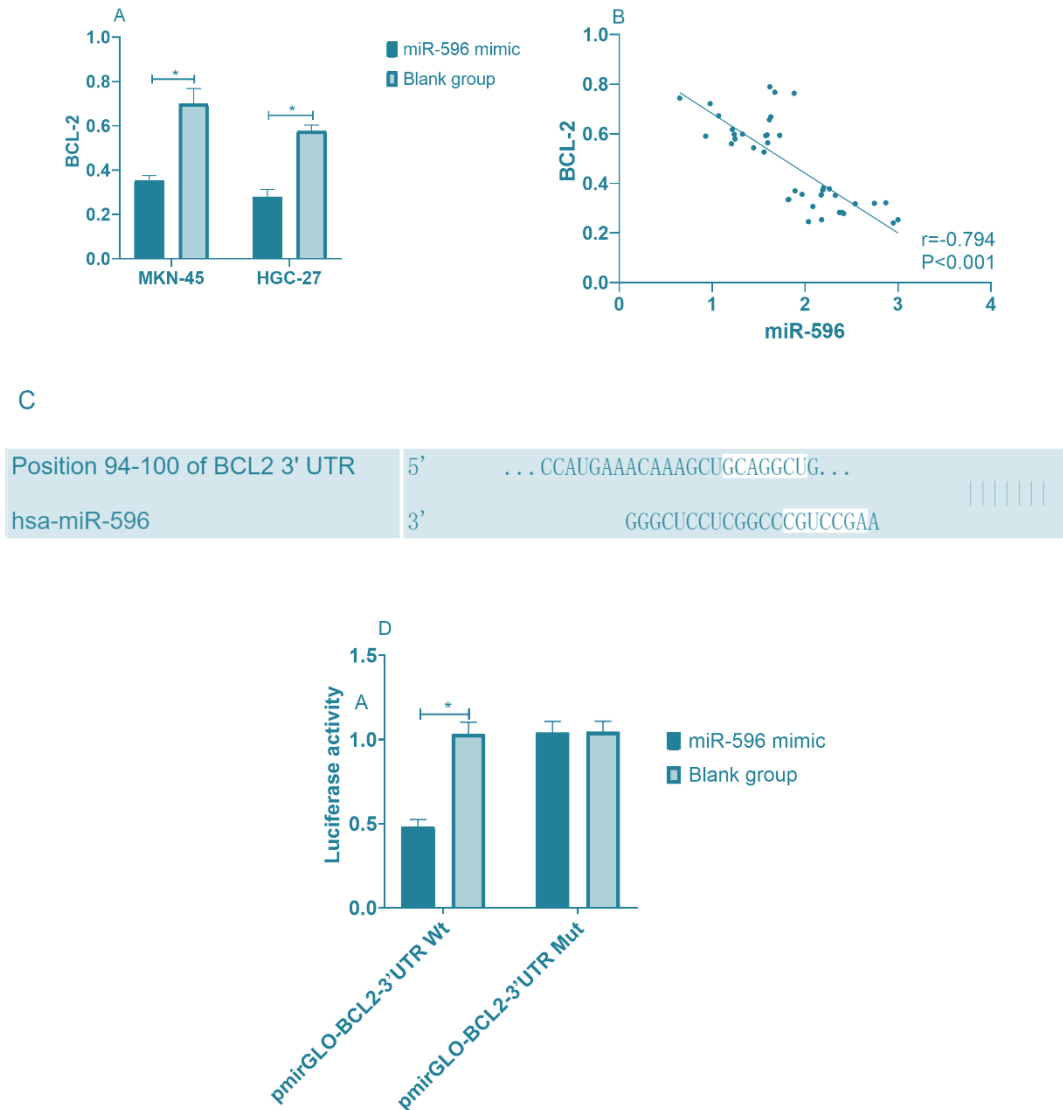


Fig. 4: Effects of the up-regulation of miR-596 on the BCL-2 expression. **(A)** Changes in BCL-2 expression after the up-regulation of miR-596. **(B)** Correlation analysis of miR-596 and BCL-2 expression. **(C)** Prediction of miR-596 targets by Targetscan 7.2. **(D)** Verification of the regulatory relationship between miR-596 and BCL-2 by dual-luciferase reporter. * indicates $P < 0.05$

Rescue experiment

After co-transfection of miR-596 mimic + BCL-2 mRNA mimic, the expression of BCL-2 in MKN-45 and HGC-27 cells in the co-transfection group was significantly higher than that in miR-596 mimic group ($P < 0.05$), while no significant difference was detected in the expres-

sion of miR-596 between the two groups. The proliferation of MKN-45 and HGC-27 cells in miR-596 mimic group was lower than that in the co-transfection group ($P < 0.05$), and the apoptosis level was higher than that in the co-transfection group ($P < 0.05$). More details are shown in Fig. 5.

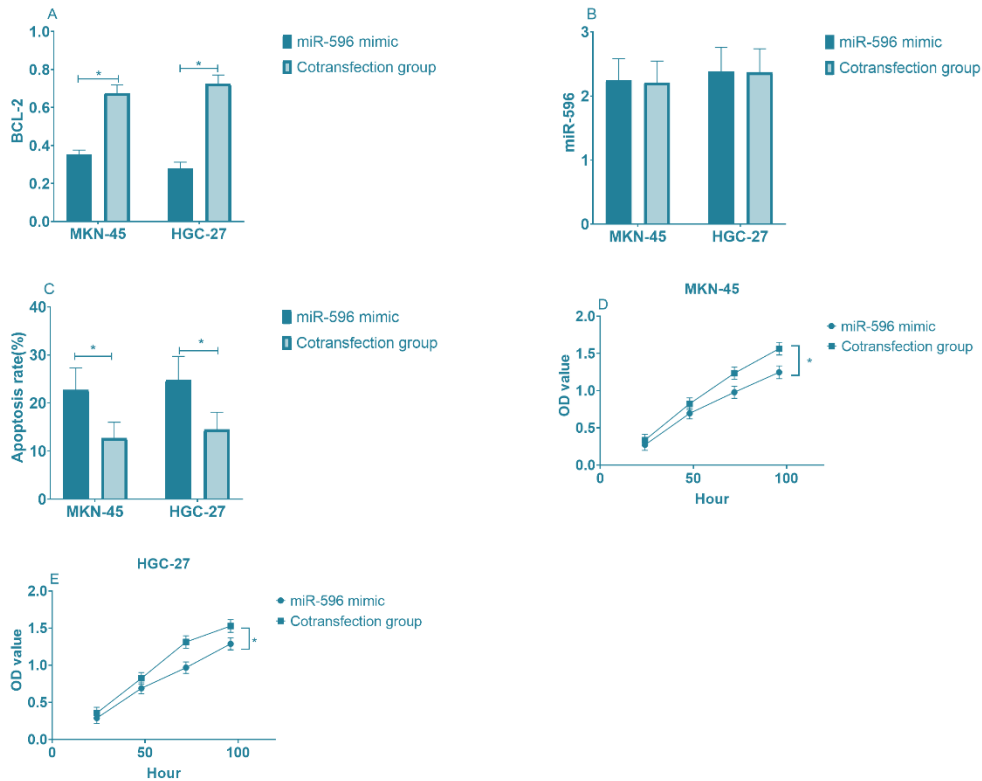


Fig. 5: Rescue experiment. **(A)** BCL-2 test results after co-transfection. **(B)** miR-596 test results after co-transfection. **(C)** Changes in the apoptosis of gastric cancer cells after co-transfection. **(D)** Changes in the proliferation of MKN-45 cells after co-transfection. **(E)** Changes in the proliferation of HGC-27 cells after co-transfection. * indicates $P < 0.05$

Discussion

Gastric cancer is the fifth most common cancer in the world and the third leading cause of cancer-related death (4). Gastric resection and lymphadenectomy are the only potential cures for gastric cancer. Generally, patients are already with advanced gastric cancer at the time of diagnosis, not suitable for surgery. Besides, the current chemotherapeutic drugs are limited by treatment-related toxicity (12, 13). In recent years, the value of targeted therapy in gastric cancer has been proved by a large number of phase III studies (14, 15). This study explored the role of miR-596 in the regulation of gastric cancer cell apoptosis, trying to find out the target of gastric cancer treatment.

In this study, miR-596 was down regulated in gastric cancer tissues and cell lines, while BCL-2 expression was up regulated in gastric cancer tissues and cell lines. SGC-7901, MKN-45, BGC-823, HGC-27, and MFC cells had significantly lower miR-596 and significantly higher BCL-2 expression than NGEc cells. The up-regulated miR-596 expression led to markedly lower proliferation and higher apoptosis of MKN-45 and HGC-27 cells, suggesting the role of miR-596 as a tumor suppressor gene in gastric cancer. At the same time, this study observed a significant decrease in BCL-2 expression after the up-regulation of miR-596. Pearson analysis showed a negative correlation between miR-596 and BCL-2 expressions. Dual-luciferase reporter demonstrated that miR-596 could target the inhibition of

BCL-2 expression. We speculate that the inhibition of BCL-2 may be the mechanism of how miR-596 inhibits the proliferation of gastric cancer cells and promotes the apoptosis. The rescue experiment showed that the proliferation of gastric cancer cells with up-regulated miR-596 and BCL-2 expression was significantly higher than with up-regulated miR-596 alone, but the apoptosis was lower. Such findings confirm our conjecture. The results of this study prove that miR-596 can inhibit the proliferation of gastric cancer cells and promote the apoptosis through its targeting inhibition of BCL-2 expression.

So far few reports have been published on miR-596 and gastric cancer. miR-596 can target LGALS3BP/Mac-2 BP/90K signal axis to inhibit the growth of oral cancer cells, and stated that the use of dsRNA mimicking miR-596 may be a potential method for treating oral cancer (16). miR-596 can predict the recurrence of early colon cancer (17). Its methylation is a specific marker for detecting liver cancer and poor prognosis (18). The above-mentioned studies revealed the close relationship between miR-596 and tumors. Impaired apoptosis is the key trigger of tumorigenesis and the main obstacle to effective treatment. The initiation of apoptosis depends on the interaction between three members of BCL-2 protein family: BH3, BCL-2, and Bax (19). miRNAs can regulate tumor cell apoptosis through the targeting inhibition of BCL-2, miR-148a can perform a targeting inhibition of BCL-2 to promote colorectal cancer apoptosis (20), and miR-143 can perform a targeting inhibition of BCL-2 to promote apoptosis in breast cancer cells (21). miRNAs could inhibit breast cancer proliferation and migration by targeting BCL-2 (22) and to boost the sensitivity of gastric cancer cells to chemotherapy drugs (23). We will further supplement the role of miR-596 and BCL-2 in gastric cancer cells in future studies. Currently little is known on the relationship between miR-596 and BCL-2, this study marks the first to report that miR-596 has a targeting inhibition of BCL-2 expression to promote apoptosis in gastric cancer cells.

However, this study is subject to certain limitations. For example, although we examined the expression of miR-596 and BCL-2 in multiple gastric cancer cell lines, and used two cell lines to verify the effect of miR-596 on apoptosis and proliferation of gastric cancer cells and the mechanism of action, in vivo experiment was in lack so the results were unable to be further verified because in vitro cell experiments cannot simulate the complex tumor microenvironment of the body. Besides, the effects of miR-596 on the sensitivity of chemotherapy drugs in gastric cancer cells and its application value in clinical diagnosis and prognosis evaluation were not explored.

Conclusion

miR-596 can inhibit the proliferation of gastric cancer cells and promote the apoptosis through its targeting inhibition of BCL-2 expression.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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There is no financial source of this study.

Conflict of interests

The authors have no conflict of interest.

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