

# MicroRNA-197 promotes proliferation and inhibits apoptosis of gallbladder cancer cells by targeting insulin-like growth factor-binding protein 3

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## Abstract

**Background.** Gallbladder cancer (GBC) is one of the common malignant tumors of the biliary tract. There is no report that miR-197 is involved in GBC.

**Objectives.** The relationship between miR-197 expression and survival time of GBC patients was analyzed. Furthermore, the role and mechanism of miR-197 in GBC was explored.

**Materials and methods.** A total of 39 GBC patients (21 males, 18 females; average age  $56.1 \pm 8.5$  years) were included from December 2013 to November 2014. All patients were admitted to our hospital for surgical treatment (excluding patients with preoperative chemotherapy). The expression of miR-197 in GBC tissues was examined, and the relationship between miR-197 and patient survival time was analyzed. Cell Counting Kit-8 (CCK-8) and colony formation assays were used to detect cell proliferation. Flow cytometry and TUNEL staining were used to detect apoptosis. Expressions of proteins related with proliferation and apoptosis were detected. The target of miR-197 was predicted through bioinformatics website and verified using the dual luciferase reporter gene assay. The target gene was interfered to so that the effect of miR-197 on the regulation of GBC cell proliferation and apoptosis could be observed.

**Results.** MiR-197 was highly expressed in GBC tissues, and the expression was closely related to the poor prognosis of GBC. Downregulation of miR-197 inhibited the proliferation and promoted the apoptosis of GBC cells; it also decreased the expressions of proliferation-related proteins p-ERK1/2 and p-AKT, and increased that of apoptosis pathway-related proteins Bax/Bcl-2 and c-caspase-3. The upregulation of miR-197 induced an opposite trend. MiR-197 directly regulated *IGFBP3*.

**Conclusions.** Our study proved that the expression of miR-197 is closely related to the poor prognosis of GBC. The miR-197–*IGFBP3* axis regulates the proliferation and apoptosis of GBC cells. Downregulation of miR-197 inhibited the proliferation and promoted the apoptosis of GBC cells, indicating potential therapeutic effects.

**Key words:** apoptosis, proliferation, gallbladder carcinoma, *IGFBP3*, miR-197

## Background

Gallbladder cancer (GBC) is the most common malignant tumor of the biliary tract, and ranked 6<sup>th</sup> among all digestive system cancers by incidence.<sup>1</sup> It is highly malignant and prone to both recur and metastasize, with a five-year survival rate between 5% and 15%.<sup>2,3</sup> Therefore, it is crucial to clarify the pathogenesis of GBC. It is derived from the gallbladder mucosa, and studies have shown that the occurrence and development of GBC are closely related to the activation of the proliferation and inhibition of apoptosis pathways.<sup>4</sup> At present, surgical resection remains an effective method to treat GBC; however, due to the poor prognosis of GBC most patients do not have the opportunity to undergo radical resection.<sup>5</sup> Therefore, further research focusing on the molecular mechanism is urgently needed to develop more effective therapeutic approaches for GBC treatment.

Recent studies have shown that microRNAs (miRs) are integrally involved in the onset and development of tumors. The miRs function as critical regulators of cancer occurrence, proliferation and apoptosis and certain miRs; namely, miR-20a, miR-182 and miR-155 have been described as potential GBC biomarkers.<sup>6</sup> Furthermore, recent literature has revealed that miR-197 has various patterns of expression and effects in different tumors. For example, miR-197 is downregulated in osteosarcoma, colorectal cancer and breast cancer, while being upregulated in lung, ovarian and pancreatic cancer, and hepatocellular carcinoma.<sup>7</sup> Through bioinformatics analysis, we found that miR-197 was significantly upregulated in GBC tissues,<sup>8</sup> but the mechanism remains unclear.

## Objectives

This study intends to explore the underlying mechanism of miR-197 in regulating GBC.

## Materials and methods

### Clinical samples

A total of 39 GBC patients from The Second Affiliated Hospital of Anhui Medical University, Hefei, China, were included from December 2013 to November 2014, with an average age of  $56.1 \pm 8.5$  years (21 males and 18 females). All patients were admitted to our hospital for surgical treatment (patients with preoperative chemotherapy were excluded). The overall survival (OS) time of patients was followed for up to 5 years. Tumor samples and adjacent normal tissue biopsies were taken during the operation. This study was approved by the institutional Ethics Committee and all patients gave informed consent prior to undergoing any procedure.

This study was approved by Ethics Committee of The Second Affiliated Hospital of Anhui Medical University. All patients provided written informed consent.

### Differential expression analysis of microarray data in GBC

The GBC expression microarray, GSE104165, which contains 40 GBC and 8 normal gallbladder tissue samples, was extracted from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>).<sup>8</sup> Differential expression analyses were performed using the R language “limma” package (<https://www.r-project.org/>), with log fold change (FC) >2 and p-value <0.05 regarded as the threshold of differentially expressed genes.

### Cell culture

Human gallbladder epithelial cells (HGBEC) and 5 gallbladder cancer cell lines (GBC-SD, EH-GB1, NOZ, SGC-996, and MZ-CHA-1; purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) with 10% fetal bovine serum (FBS), 1% penicillin (100 U/L) and streptomycin (100 mg/L) in a 5% CO<sub>2</sub> incubator at 37°C. Sub-culture was performed every 2–3 days.

### Cell transfection

The synthetic miR-197 mimic (miR-197) and miR-197 inhibitor (in-miR-197), together with corresponding mimic and inhibitor controls, were purchased from GenePharma Inc. (Shanghai, China). The cells were seeded in six-well plates and transfected with miR-197, in-miR-197 or their negative controls using Lipofectamine<sup>®</sup> 2000 transfection reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, USA), based on the manufacturer's guidelines. Briefly, 100 pmol of mimic or inhibitor was added to the mixture of 250  $\mu$ L of Opti-MEM (Gibco, Thermo Fisher Scientific, Inc.) and 5  $\mu$ L of Lipofectamine<sup>®</sup> 2000. The mixture was then added into cells ensuring the final concentration of miR-197, in-miR-197 or corresponding controls is 50 nM, and the culture medium was changed 6 h after transfection. After a 48-hour period, the cells were collected for subsequent experiments.

Insulin-like growth factor binding protein 3 (IGFBP3) siRNA (siIGFBP3) and its corresponding negative control were constructed using GENECHAM and siIGFBP3 transfection was performed. In brief, the medium was removed from the six-well plate, and 2 mL of serum-containing medium was added without antibiotics into each well. Then, 100  $\mu$ L of Opti-MEM<sup>®</sup> (Gibco, Thermo Fisher Scientific, Inc.) + 2000 ng of siIGFBP3 plasmid + 10  $\mu$ L of transfection reagent was added (Invitrogen, Thermo Fisher Scientific, Inc.) to each well of the plate, the plate was vortexed and incubated at room temperature for 10 min. Next,

the transfection mixture was added to a six-well plate and continued to incubate in a 5% CO<sub>2</sub> incubator. The mixture was added to the cells, and the medium changed after 6 h. After 48 h, the cells were collected for further experiments.

## Dual luciferase reporter gene assay

We applied the biological database ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)) to confirm the relationship between miR-197 and its putative binding sequence of the *IGFBP3* gene. The outcome indicates that the *IGFBP3* gene is one of the target genes of miR-197. Fragments from *IGFBP3* and flanking sequences on each side were synthesized with a short extension containing cleavage sites for XhoI (5'-end) and NotI (3'-end), while the second fragment, containing mutated binding site sequences, was also synthesized. The 2 constructs were termed as WT (wild-type gene) and MT (mutant genes). The fragments were cloned into the psiCHECK<sup>TM</sup>-2 vector (Promega, Madison, USA). Then, 293 T-cells (purchased from Cell Bank of the Shanghai Institute of Biochemistry & Cell Biology) were seeded in a six-well plate (Corning Inc., Corning, USA) at a density of  $1 \times 10^5$  cells per well, and the wild-type *IGFBP3* UTR or mutant *IGFBP3* UTR plasmids (Hanyin, Shanghai, China) with miR-197 mimic using Invitrogen<sup>TM</sup> Lipofectamine 2000 (Invitrogen) were transfected according to the manufacturer's instructions. After 24 h of incubation, the luciferase activity of the WT and mutated *IGFBP3* UTR was detected using the dual-luciferase reporter assay system (Promega).

## Quantitative real-time PCR

Total RNA was extracted from GBC tissues and cells using an RNA extraction kit (Invitrogen); PrimeScript reverse transcription (RT) kit was applied to reverse-transcribe RNA into cDNA. Fluorescent quantitative polymerase chain reaction (qPCR) was performed with SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> II solution kit using the ABI PRISM<sup>®</sup> 7300 system (Applied Biosystems, Foster City, USA). The primers for real-time PCR (RT-PCR) are shown in Table 1. *U6* was used as the internal reference for miR-197, and *GAPDH* was applied as the endogenous control for mRNA. The data was analyzed using the  $2^{-\Delta\Delta C_t}$  method.<sup>9</sup>

Table 1. Primers (mRNA) for real-time PCR

Name	Sequences
miR-197	5'-TTCACCACTTCTCCACCCAGC-3' (forward)
	5'-TGCACTGGTCCAAGCTCTAACC-3' (reverse)
U6	5'-CTCGCTTCGGCAGCACA-3' (forward)
	5'-AACGCTTCACGAATTTG CGT-3' (reverse)
IGFBP3	5'-GAGGGCGACACTGCTTTTTC-3' (forward)
	5'-CCAGCT CCAGGAAATGCTAG-3' (reverse)
GAPDH	5'-GAAGGTGAAGGTCGGAGTC-3' (forward)
	5'-AAGATGGTATGGGATTTC-3' (reverse)

## Western blotting analysis

SGC-996 and MZ-CHA-1 cells were washed twice with ice-cold PBS, then incubated in lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 2 mg/mL of aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) for 30 min. The supernatants were centrifuged at  $12,000 \times g$  for 15 min at 4°C, and retained for protein extraction. The protein (30 µg) was subjected to sodium dodecyl sulfate salt – polyacrylamide gel electrophoresis (SDS-PAGE 10% (w/v) acrylamide gel) and blotted onto polyvinylidene difluoride (PVDF) membranes, which were washed for 1 h with a blocking solution. The membranes were incubated overnight at 4°C with anti-IGFBP3 (1:1000; ab220429; Abcam Inc., Cambridge, USA), anti-ERK1/2 (1:1000; 9102; Cell Signaling Technology (CST), Beverly, USA), anti-p-ERK1/2 (1:1000; 4370; CST), anti-AKT (1:1000; 4691; CST), anti-p-AKT (1:1000; 4060; CST), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000; ATA29666; Zhongshan Golden Bridge Biotechnology, Beijing, China) antibodies. The next day, membranes were incubated with a secondary anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:1000; Zhongshan Golden Bridge Biotechnology). Finally, the relative expression levels of protein bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, USA).

## TdT-mediated dUTP nick-end labeling (TUNEL) staining

Based on the instructions of the Biyuntian TUNEL Apoptosis Detection Kit, genomic DNA was cut, and on the exposed 3'-OH end, a dUTP-GFP base was fixed that could be directly viewed under a microscope. The specific steps were as follows: cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 for 2 min. The TUNEL reaction mixture was added to react for 60 min, and then with Converter-POD for 30 min. After washing, the cells were stained in 100 µL of DAPI nuclear staining reagent (Sigma-Aldrich, St. Louis, USA) for 10 min. The stained cells were observed under the Olympus BX-53 fluorescence microscope and the images were captured using a digital camera (Olympus Corp., Tokyo, Japan) with a  $\times 10$  magnification. Nine fields per slide were then randomly selected. The number of TUNEL-positive cells was counted and averaged on each field of view. The results were expressed as mean  $\pm$  standard error of the mean (SEM).

## Colony formation assay

The colony formation assay is conducted in a six-well plate in which cells were seeded at a density of  $8 \times 10^2$  cells/well and cultured for 2 weeks, and then the colonies were

fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich). The number of colonies was counted, and the experiment was independently repeated 3 times to ensure accuracy.

## Flow cytometry

The cells of each group were suspended, collected and washed twice with saline. After that, the cells were fixed with 70% ethanol. Then, 100  $\mu$ L of cell suspension was added to a 5-mL culture tube, and subsequently 5  $\mu$ L of FITC-labeled Annexin-V (1:20, C1062S; Biotechnology, Shanghai, China) and 5  $\mu$ L of propidium iodide (PI) (1:20, C1062S) were added, mixed and incubated in the dark at room temperature for 15 min. After incubation, 400  $\mu$ L of 1X binding buffer was added, ensuring precise and accurate movements the whole procedure. Next, cell apoptosis was detected using flow cytometry (BD FACS Canto II; Becton Dickinson, Franklin Lakes, USA).

## Cell Counting Kit-8 assay

The transfected cells were seeded into 96-well plates ( $1 \times 10^3$  cells/well) with added Cell Counting Kit-8 (CCK-8; Invitrogen) according to the manufacturer's protocol. Cell proliferation was detected through the optical density (OD) value (570 nm).

## Statistical analyses

The IBM SPSS v. 21.0 software (IBM Corp. Armonk, USA) was utilized for data processing. The Kolmogorov-Smirnov test was used to check whether data was distributed normally. Data are presented as mean  $\pm$  standard deviation (SD). Comparisons between 2 groups were analyzed using a Student's t-test and comparisons among

multiple groups were analyzed using one-way or two-way analysis of variance (ANOVA), followed by the Tukey's multiple comparisons test. Kaplan–Meier method was used for survival analysis. The p-value was calculated using a two-tailed test and  $p < 0.05$  indicated a statistically significant difference.

## Results

### MiR-197 was highly expressed in GBC tissues

To date, there is no study that focuses on whether miR-197 regulates the occurrence and development of GBC. Through a bioinformatics analysis (<http://www.ncbi.nlm.nih.gov/geo/>), we found that miR-197 was highly expressed in GBC tissues (Fig. 1A). Furthermore, there is a significant upregulation of miR-197 in tumor tissues ( $p < 0.05$ ) when compared to healthy gallbladder tissue (Fig. 1B). Then, according to the median expression of miR-197, the 39 patients examined in this study were categorized into a high ( $\geq$ median miR-197 expression;  $n = 19$ ) or low expression group ( $<$ median miR-197 expression;  $n = 20$ ), and the baseline characteristics of different groups were compared (Table 2). The results showed that the expression of miR-197 was significantly correlated with T classification, also known as the depth of tumor invasion ( $p < 0.05$ ). Kaplan–Meier analysis showed that the expression of miR-197 was closely related to the poor prognosis of the GBC patients (Fig. 1C). Additionally, compared with normal gallbladder cells (HGBEC), GBC cell lines (EH-GB1, NOZ, SGC-996, and MZ-CHA-1) had higher expression of miR-197 ( $p < 0.05$ ), and of these cell lines, the upregulated expression of miR-197 in SGC-996 and MZ-CHA-1 cells was statistically significant (Fig. 1D).

**Table 2.** Correlation between clinicopathological characteristics and miR-197 expression in 39 patients with GBC

Characteristics		miR-197 expression (median expression 8.374)		p-value
		low	high	
Age	<56	11 (28.2%)	9 (23.1%)	0.634
	>56	9 (23.1%)	10 (25.6%)	
Gender	male	12 (30.8%)	9 (23.1%)	0.429
	female	8 (20.5%)	10 (25.6%)	
T classification	1–2	14 (35.9%)	6 (15.4%)	0.016
	3–4	6 (15.4%)	13 (33.3%)	
N classification	0	11 (28.2%)	11 (28.2%)	0.855
	1–2	9 (23.1%)	8 (20.5%)	
Distant metastasis	no	10 (25.6%)	9 (23.1%)	0.870
	yes	10 (25.6%)	10 (25.6%)	
Pathologic stage	1–2	9 (23.1%)	10 (25.6%)	0.634
	3–4	11 (28.2%)	9 (23.1%)	

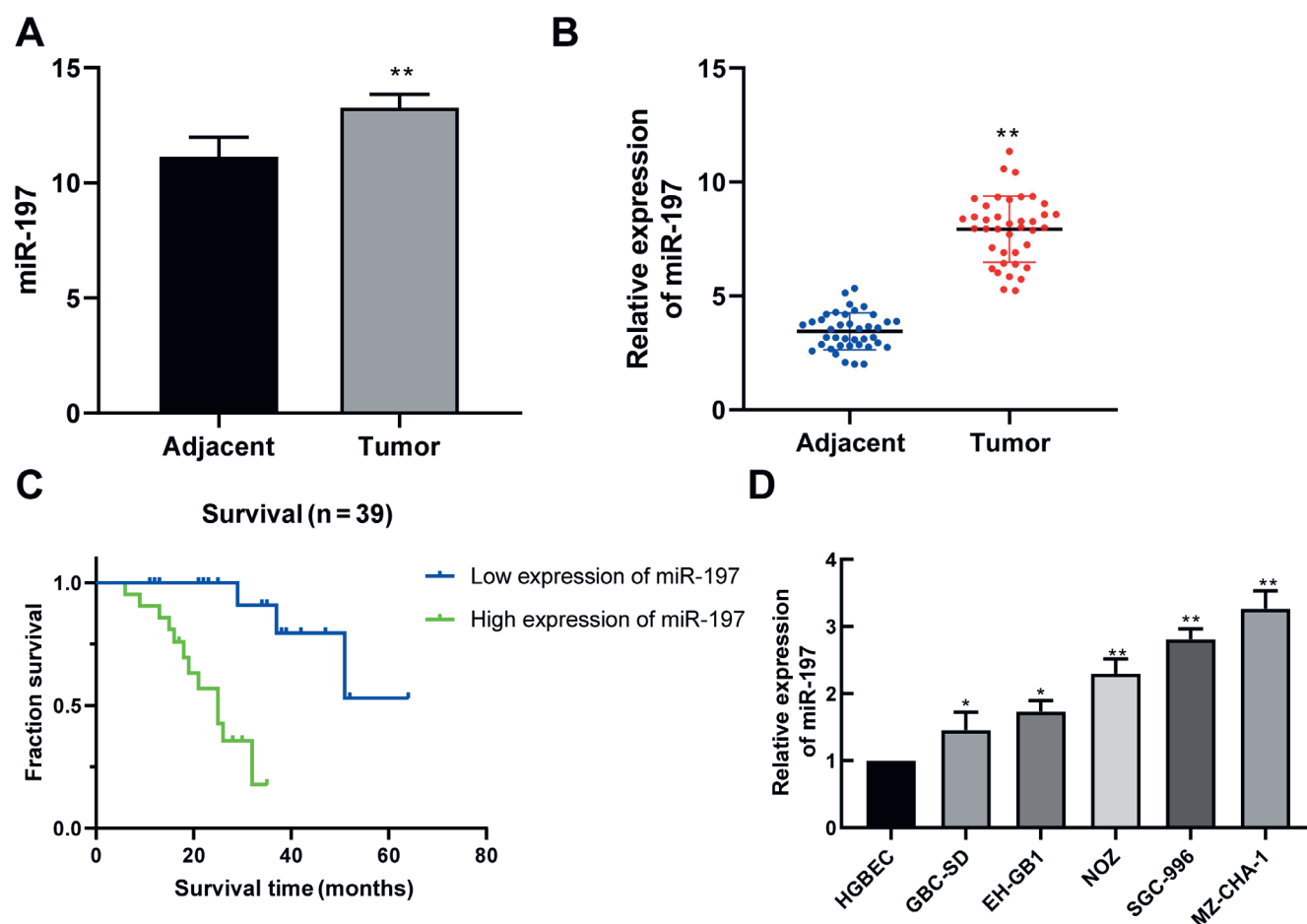


Fig. 1. MiR-197 is highly expressed in GBC tissues and cells. A. Bioinformatics analysis (<http://www.ncbi.nlm.nih.gov/geo/>) of the expression of miR-197 in GBC. \*\*compared with the adjacent group,  $p < 0.01$ ; B. The expression of miR-197 in GBC tissues and adjacent normal tissues was detected using qRT-PCR. U6 was used as endogenous control. \*\*compared with the adjacent group,  $p < 0.01$ ; C. Kaplan–Meier analysis observed the correlation between expression of miR-197 and survival; D. The expression of miR-197 in HGBEC, GBC-SD, EH-GB1, NOZ, SGC-996, and MZ-CHA-1 cell lines was detected using qRT-PCR, and U6 was used as endogenous control. \*compared with HGBEC group,  $p < 0.05$ . Data represent the mean  $\pm$  SD ( $n = 3$ )

## Downregulation of miR-197 impeded cell proliferation and promoted apoptosis of GBC cells

To study the role of miR-197 in GBC cells, the miR-197 inhibitor in-miR-197 was applied to GBC cells, while a random sequence was used as a negative control (in-NC), and transiently transfected into SGC-996 and MZ-CHA-1 cell lines. After 48 h, miR-197 expression was significantly reduced in SGC-996 and MZ-CHA-1 cells (both  $p < 0.01$ ; Fig. 2A). Then, CCK-8 and plate cloning experiments were used to test the effect of miR-197 on cell proliferation. These results demonstrated that in-miR-197 significantly inhibited cell proliferation compared with cells in the in-NC group (Fig. 2B), with a concomitant significant reduction in the number of clones (both  $p < 0.01$ ; Fig. 2C). We then observed reduced miR-197 in SGC-996 and MZ-CHA-1 cells resulted in a significant increase in both the apoptosis rate (both  $p < 0.01$ ; Fig. 2D), and the number of TUNEL-positive cells (both  $p < 0.01$ ; Fig. 2E). The above results showed that downregulation of miR-197 inhibited GBC cell proliferation and promoted apoptosis.

## MiR-197 regulated the expressions of proliferation pathway and apoptosis pathway-related proteins

As mentioned above, downregulation of miR-197 inhibited the proliferation of GBC cells and promoted apoptosis. This prompted us to explore the regulatory role of miR-197 in proliferation/apoptosis signaling pathways. It was demonstrated that miR-197 was significantly elevated in MZ-CHA-1 cells after transfection with miR-197 (Supplementary Fig. 6A), causing a subsequent upregulation of p-ERK1/2, p-AKT and Bcl-2, and downregulated the expression of c-caspase-3 and Bax (Fig. 3A–C). Inhibition of miR-197 with in-miR-197 significantly downregulated the expression of p-ERK1/2, p-AKT and Bcl-2, and upregulated the expression of c-caspase-3 and Bax (Fig. 3A,B,D). From the data above, it is reasonable to conclude that upregulation of miR-197 stimulated ERK and AKT pathways, and inhibited the Bax/Bcl-2 and caspase-3 pathways, while downregulation of miR-197 showed the opposite effect.



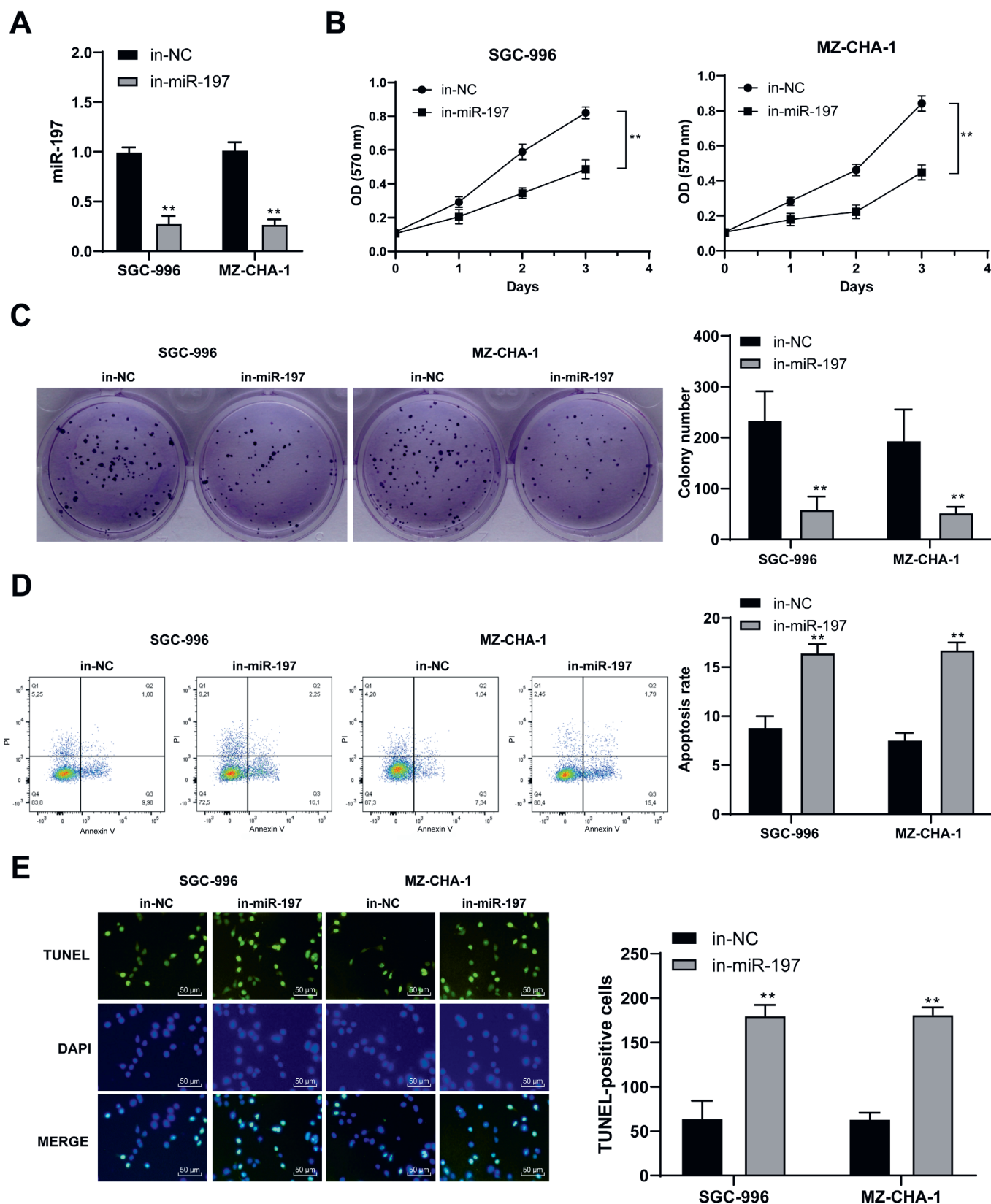


Fig. 2. Downregulation of miR-197 impedes cell proliferation and promotes apoptosis in GBC cells. SGC-996 and MZ-CHA-1 cells were transfected with in-miR-197. A. MiR-197 expression was detected using qRT-PCR; B. OD value (at 570 nm) was detected on day 1–5; C. Cloning number was counted; D. Apoptosis rate was observed with flow cytometry; E. Apoptosis-positive cells were observed using TUNEL experiment. Data represent the mean  $\pm$ SD (n = 3)

\*compared with in-NC,  $p < 0.05$ , \*\*compared with in-NC,  $p < 0.01$ .

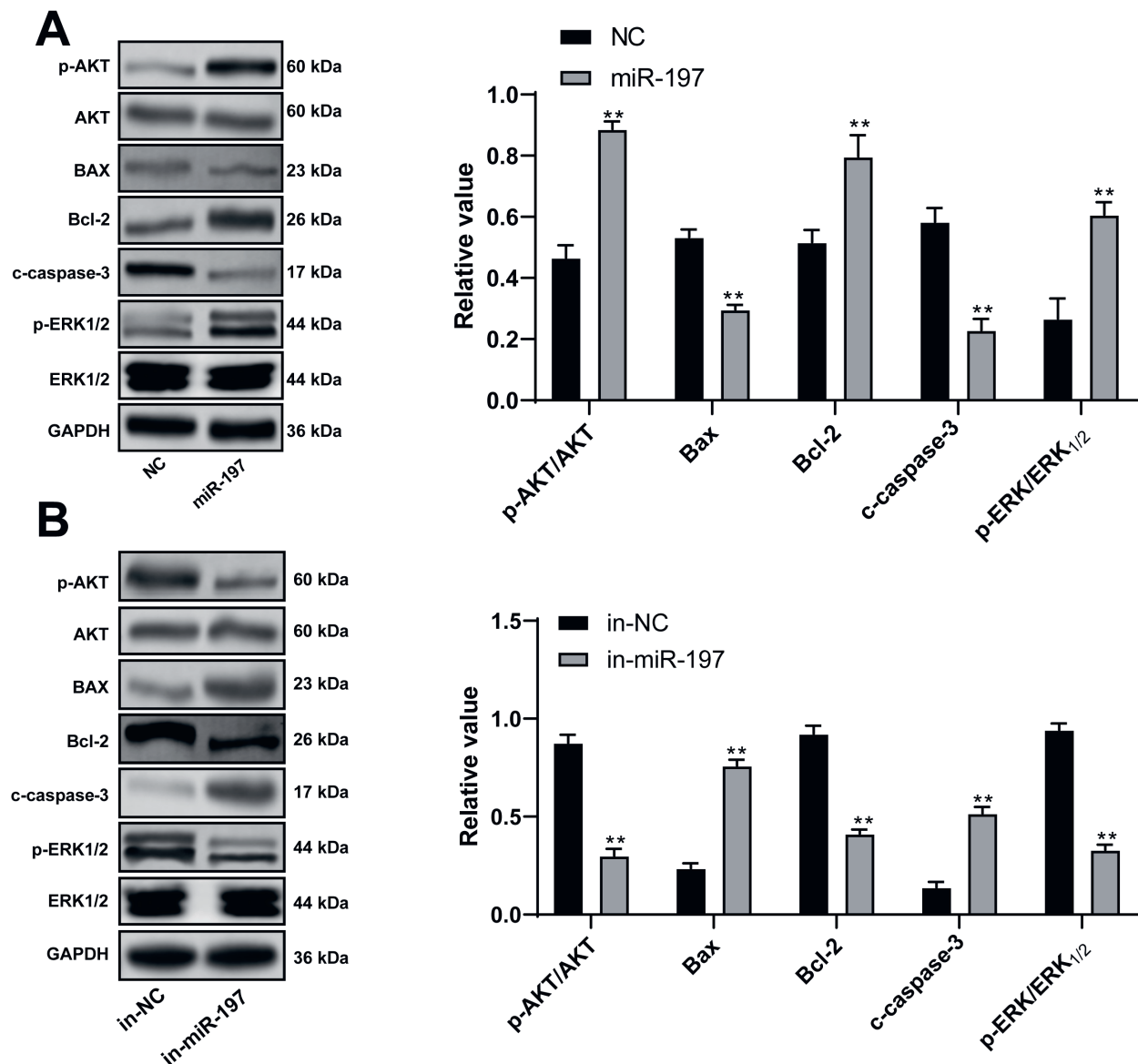


Fig. 3. MiR-197 regulates the expression of proliferation pathway proteins p-ERK1/2 and p-AKT and apoptosis pathway proteins Bax/Bcl-2 and c-caspase-3. A and B. MZ-CHA-1 cells were transfected with miR-197/in-miR-197, and western blotting analysis was used to detect the expression of p-ERK1/2 (42KD), p-AKT (60KD), Bax (21KD), Bcl-2 (26KD), and c-caspase-3 (17KD); C and D. Statistical analysis. Data represent the mean  $\pm$  SD (n = 3)

\*compared with NC/in-NC,  $p < 0.05$ .

### MiR-197 inhibited the expression of *IGFBP3*

MiRNA regulates cell proliferation and apoptosis by regulating its downstream target genes. Therefore, we predicted which mRNA may be targeted by miR-197 using an online database ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)) to further explore the mechanism of miR-197 regulation of GBC cell proliferation and apoptosis. The predicted results revealed that the *IGFBP3* gene, which is closely related to tumor proliferation, is one of the target genes of miR-197. The binding sites of *IGFBP3* mRNA and miR-197 are shown in Fig. 4A. We used a dual luciferase reporter gene assay to verify this relationship. When co-transfected with the *IGFBP3* WT reporter plasmid, miR-197 significantly reduced luciferase activity compared with the NC group,

and the mutated reporter plasmid eliminated miR-197-mediated inhibitory effect on the luciferase activity (both  $p < 0.05$ ) (Fig. 4B). These results demonstrated that miR-197 and *IGFBP3* mRNA closely interact.

In order to show whether miR-197 can affect the expression of *IGFBP3* in GBC, the SGC-996 cell line was transfected with miR-197, in-miR-197 and their NCs; then, the expression of *IGFBP3* at the mRNA and protein levels was examined. After transfection with miR-197, the mRNA level of *IGFBP3* in SGC-996 cells was significantly reduced, while transfection with in-miR-197 caused a significant increase in the mRNA level of *IGFBP3* (all  $p < 0.05$ ; Fig. 4C). Upregulation of miR-197 caused a decrease in *IGFBP3* protein expression, while downregulation of miR-197 exerted the reverse effect (all  $p < 0.05$ ; Fig. 4D). Compared with

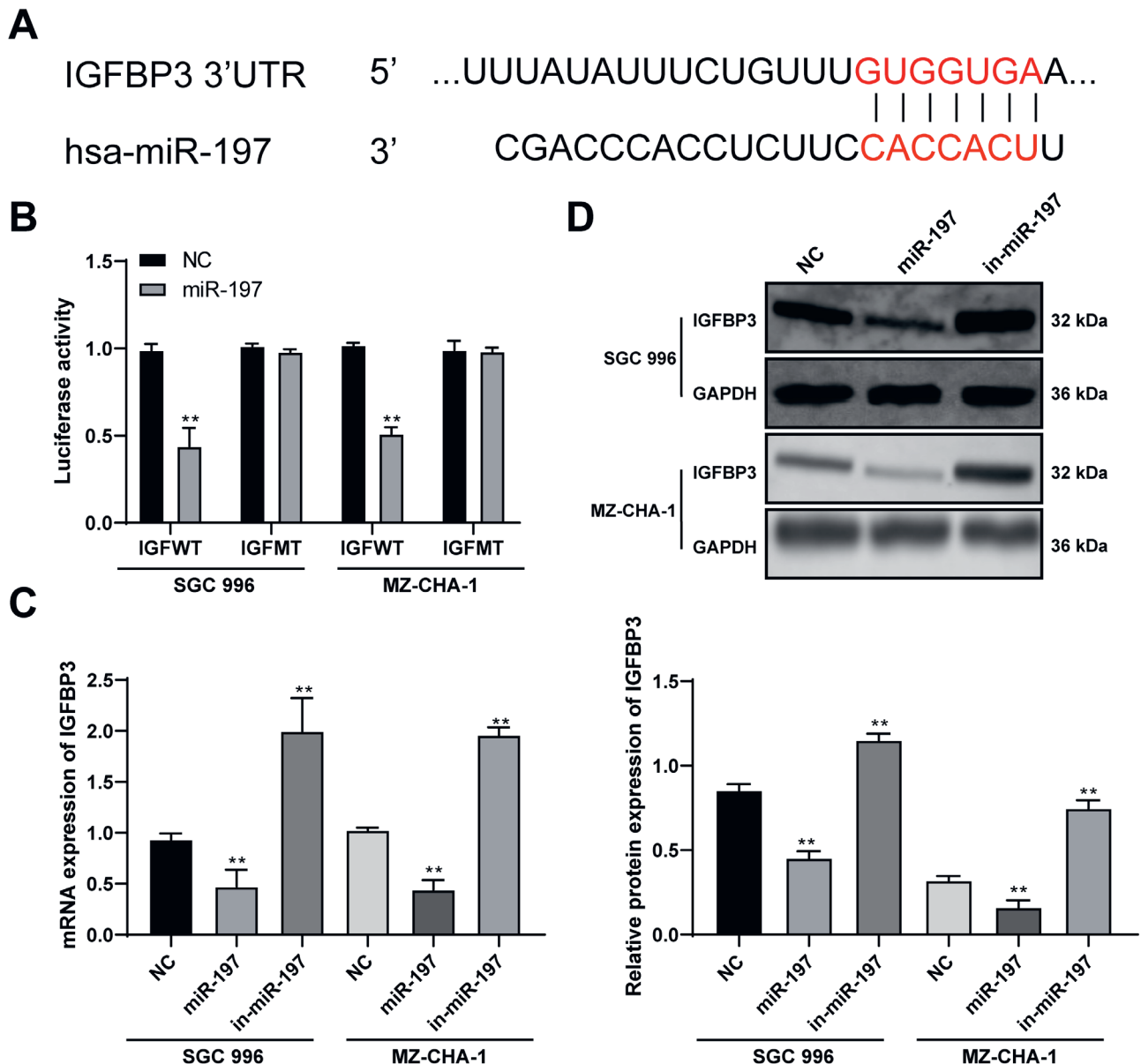


Fig. 4. MiR-197 inhibits the expression of *IGFBP3*. A. The miR-197 target sequence predicted in the 3'UTR of *IGFBP3*; B. SGC-996 and MZ-CHA-1 cells were co-transfected with miR-197 and *IGFBP3* WT or MT, and the luciferase activity was determined; C and D. SGC-996 and MZ-CHA-1 cells were transfected with miR-197/in-miR-197, and the expression of *IGFBP3* (32KD) was detected using qRT-PCR and western blotting analysis. Data represent the mean  $\pm$ SD (n = 3)

\*compared with NC,  $p < 0.05$ ; \*\*compared with NC,  $p < 0.01$ .

the adjacent tissues, the *IGFBP3* expression in tumor tissues was significantly reduced (Supplementary Fig. 6B). These results indicated that *IGFBP3* was one of the target genes of miR-197 in GBC cells.

### The siIGFBP3 could partially reverse the effect caused by miR-197 downregulation

The above results indicated that *IGFBP3* may participate in the miR-197-regulated proliferation and apoptosis of GBC cells, which prompted us to further explore its role. Firstly, we were able to effectively silence siIGFBP3 in MZ-CHA-1 cells (Fig. 5A,B). Then, transfection of the cells

with miR-197 inhibitor combined with siIGFBP3 (in-miR-197+siIGFBP3) increased the number of cell clones (Fig. 5C) when analyzed by the OD value (570 nm) (Fig. 5D). This group (in-miR-197+siIGFBP3) also had a lower apoptosis rate compared with the in-miR-197 group (Fig. 5E), suggesting that siIGFBP3 can partially inhibit the elevated apoptosis induced by miR-197 downregulation.

## Discussion

Investigation on the pathophysiology of GBC is critical due to its high recurrence rate and poor prognosis. To date, there is no report on the role of miR-197 during the disease



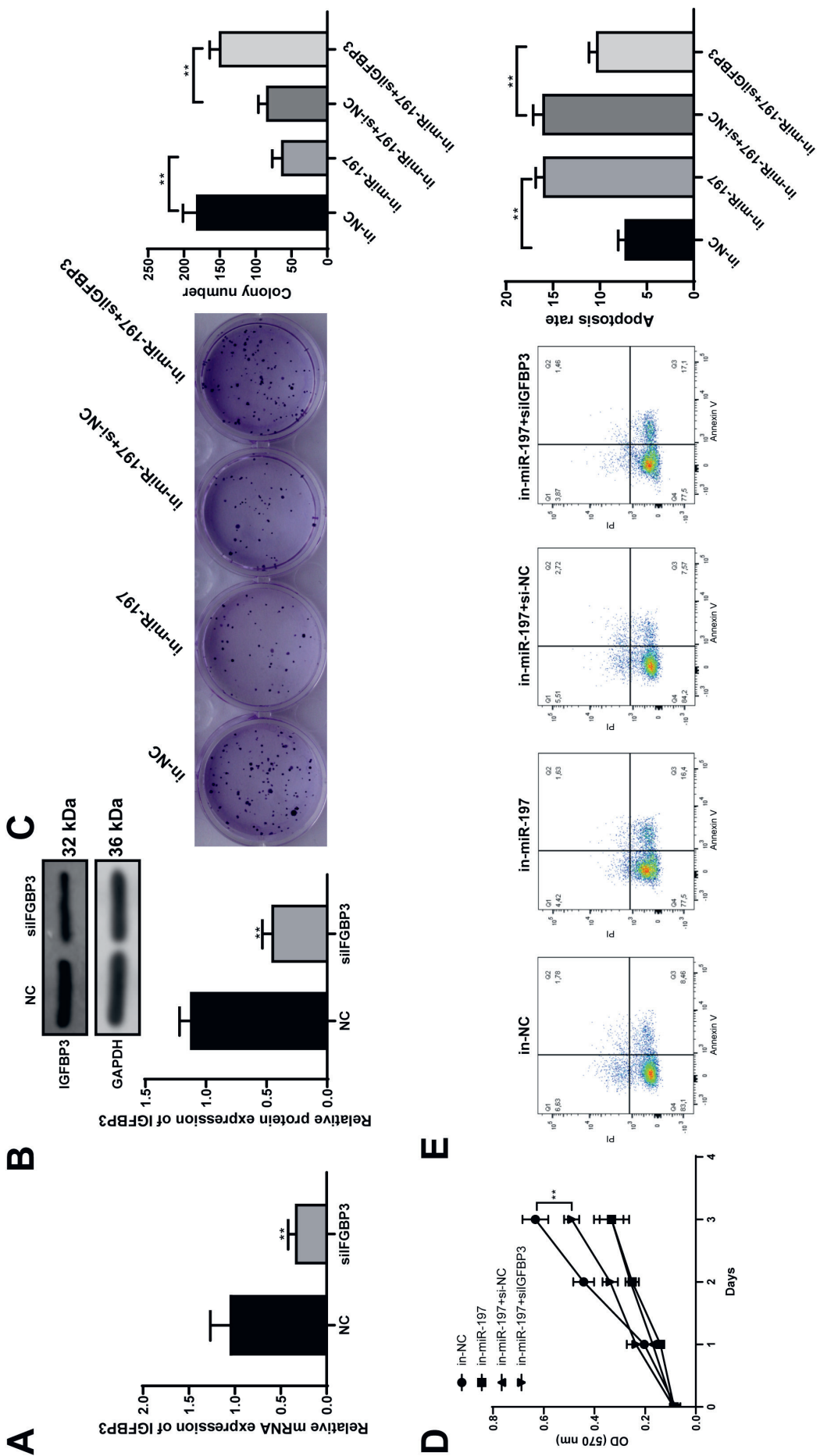


Fig. 5. siIGFBP3 partially inhibits the decrease in proliferation and the increase in apoptosis induced by miR197 downregulation. MZ-CHA-1 cells were transfected with in-miR-197 and/or siIGFBP3. A and B. The qRT-PCR and western blotting analysis were used to detect the expression of IGFBP3 (32KD); C and D. Colony formation assay and CCK-8 assay were conducted; E. Flow cytometry was used to detect the apoptosis rate. Data represent the mean  $\pm$ SD (n = 3)

\*compared with NC, p < 0.05; \*\*compared with NC, p < 0.01.

process. In this study, we found that miR-197 was highly expressed in GBC tissues, and the expression of miR-197 was closely related to the poor prognosis of GBC. MiR-197 was highly expressed in GBC cells, and downregulation of miR-197 inhibited GBC cell proliferation and promoted apoptosis. It acts through decreasing the expression of proliferation-related proteins p-ERK1/2 and p-AKT, while increasing expression of apoptosis pathway-related proteins Bax/Bcl-2 and c-caspase-3. Upregulation of miR-197, on the other hand, induced an opposite trend. MiR-197 directly regulated *IGFBP3*, as shown by silencing the gene using siIGFBP3, which partially inhibited the decrease of proliferation and the increase of apoptosis induced by downregulation of miR-197.

The function of miR-197 in oncogenesis and development is very complicated. Fiori et al. reported that miR-197 promotes tumor progression by inhibiting the apoptosis of p53 WT cells in non-small cell lung cancer (NSCLC).<sup>10</sup> However, it also exerts a tumor suppressor function in multiple myeloma.<sup>11</sup> Moreover, miR-197 inhibits the proliferation of glioma cells,<sup>12</sup> but promotes the progression of stomach,<sup>13</sup> pancreas,<sup>14</sup> colorectal tumors,<sup>15</sup> and hepatocellular carcinoma.<sup>16</sup> Clinical studies have shown that miR-197 is of great clinical significance, and it might serve as a promising prognostic biomarker to be used to monitor several types of malignant tumors. For example, miR-197 relates to the poor outcome and distant metastases in patients with NSCLC while its expression is also relevant to the size of the tumor and histopathological classification.<sup>17</sup> A meta-analysis confirmed that compared with a single miRNA, a group of multiple miRNAs may bear greater predictive ability.<sup>18</sup> Recent studies demonstrated that compared with the normal population, serum miR-197 expression in NSCLC patients is significantly higher, and the expression of miR-145 is remarkably reduced. These changes are related to the clinical stages and pathological classifications of NSCLC patients, and the combination of miR-197 and miR-145 has a high sensitivity for diagnosing NSCLC.<sup>19</sup> To date, there is no prior research of the function of miR-197 in GBC. In this study, the expression of miR-197 in the tumor tissues of GBC patients was found to be significantly higher in GBC tissues, which was consistent with the results of bioinformatics analysis.<sup>8</sup> Subsequently, in 2 different GBC cell lines, downregulation of miR-197 inhibited the proliferation of GBC cells and promoted apoptosis, while upregulation induced the opposite effect. Therefore, it is possible to elucidate the biological activity of miR-197 in GBC and its regulatory mechanism for the first time.

In the present study, a target of miR-197 was shown to be *IGFBP3*, which plays an important role in tumor progression through different molecular pathways. On the one hand, *IGFBP3* attenuates the signal transduction downstream of IGF1 induced by IGF1 by interfering with the binding between IGF1 and IGF1R.<sup>20</sup> The IGF1/IGF1R

axis can stimulate epithelial to mesenchymal transition and promote the metastasis of various tumors.<sup>21,22</sup> On the other hand, *IGFBP3* can also inhibit tumor progression in an independent manner. For example, *IGFBP3* inhibits head and neck squamous cell carcinoma by blocking the c-jun and c-fos pathways.<sup>23</sup> It also inhibits the progression of prostate cancer by degrading the NF- $\kappa$ B signaling.<sup>24</sup> However, the function of *IGFBP3* is still controversial. Chan et al. indicated that high expression of *IGFBP3* is associated with an increased incidence of colorectal cancer in elderly males, which is independent of IGF1.<sup>25</sup> It is suggested that *IGFBP3* may have differential actions in different tumors. To date, however, no studies have reported whether *IGFBP3* is involved in the progression of GBC. Our results indicated that upregulation of miR-197 caused *IGFBP3* inhibition, that miR-197 downregulation led to *IGFBP3* overexpression, and finally silencing *IGFBP3* could partly reverse the effect induced by miR-197 suppression. Hence, it can be concluded that *IGFBP3* plays a role in inhibiting GBC progression. In the current study, miR-197 upregulation caused a decrease in the expression of *IGFBP3*, which promoted the upregulation of p-ERK1/2, p-AKT and Bcl-2 in GBC cells, and downregulation of Bax and caspase-3, all of which could be reversed after downregulation of miR-197.

Previous studies have found that *IGFBP3* plays an important role in regulating tumor cell proliferation and apoptosis. Chen et al. found that *IGFBP3* is an indicator of poor prognosis in patients with glioma, and reducing its expression can inhibit tumor growth by inducing apoptosis of glioma cells.<sup>26</sup> Day et al. found that using *IGFBP3* siRNA to treat NSCLC cells with TNFAIP8 knockdown can upregulate pAKT levels and promote cell proliferation.<sup>27</sup> By blocking the IGF1 signal cascade, it was possible to confirm to a certain degree that overexpression of *IGFBP3* can induce apoptosis in NSCLC cells in vitro and enhance cellular response to cisplatin.<sup>28</sup> In lung adenocarcinoma and hepatocellular carcinoma, testis-specific protein Y-linked 1 (TSPY1) can directly bind to the promoter of *IGFBP3* to inhibit its expression. Downregulation of *IGFBP3* activates the PI3K/AKT and RAS/RAF/MEK/ERK signaling pathway, thereby promoting tumor cell proliferation.<sup>29</sup> Overall, the mechanisms of *IGFBP3* on tumor cell regulation varies significantly, and that this difference is likely tumor-specific.

## Clinical implications

The results of the five-year survival analysis of patients recruited to the present study suggest that miR-197 expression is associated with poor prognosis in GBC. This implies that increased expression of miR-197 can be used clinically as a prognostic marker. Since the downregulation of miR-197 inhibited proliferation and promoted apoptosis of GBC cells, it is tempting to speculate that the miR-197-IGFBP axis might serve as a therapeutic target in patients with GBC.

## Limitations


In this study, patients with miR-197 overexpression in tumor tissues had short OS, but OS was not consistent with clinicopathological characteristics. The reason could be attributed to the past surgical history of the patients. Therefore, there was a lack of diversity of clinical stages amongst that recruited patients in the 2 groups of high or low miR-197 expression. In addition, the sample size of this study is relatively small, which also diminishes the quality of this study. The best way to determine the impact of miR-197 on GBC cell lines would be to determine the *IGFBP3* function in cell proliferation. Therefore, further research could focus on identifying the effect of *IGFBP3* on cell proliferation. Moreover, detection of miR-197 levels in patients' peripheral blood can also be performed, which would further support miR-197 as a promising clinical marker.


## Conclusions

Our study demonstrated that the miR-197/*IGFBP3* axis regulates the proliferation and apoptosis of GBC cells. Downregulation of miR-197 inhibits the proliferation and promotes the apoptosis of GBC cells, indicating its potential use as a molecular target in the treatment of GBC.

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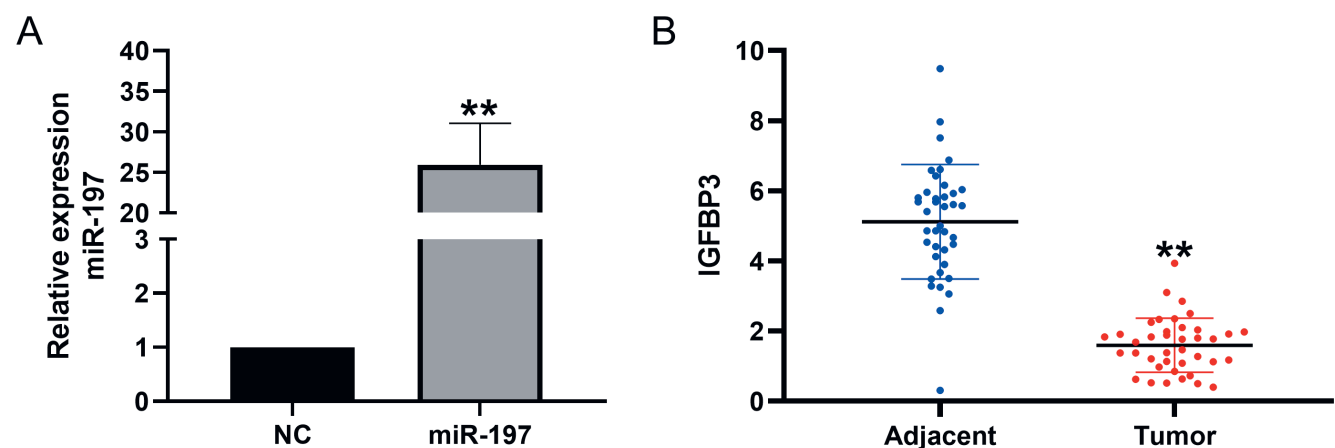
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Supplementary Fig. 6. A. After miR-197 transfection, the expression of miR-197 in MZ-CHA-1 cells increased significantly (\*\*compared with NC,  $p < 0.01$ ); B. The expression of *IGFBP3* in GBC tissues and adjacent normal tissues was detected using qRT-PCR (\*\*compared with the adjacent group,  $p < 0.01$ )

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