

Original Article

MiR-194 suppresses human gastric cancer cell proliferation and induces apoptosis by targeting GGCT

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Abstract: Background: Gamma-glutamylcyclotransferase (GGCT) has been confirmed to be involved in many kinds of cancers, while the biological function of GGCT in gastric cancer is still largely unknown. Methods: The expression level of miR-194 was detected in gastric cancer tissues and the adjacent non-tumor tissues as well as the gastric cancer cell lines. The proliferation and apoptosis of MGC-803 cells were detected after transfected with miR-194 mimics or miR-194 mimic control. qRT-PCR and Western blot were performed to detect the expression of related proteins in MGC-803 cells. The target gene of miR-194 was investigated by luciferase assay and Western blot. Results: The expression level of miR-194 was markedly decreased in gastric cancer patients compared to the non-tumor people. Up regulation of miR-194 significantly inhibited the proliferation and induced apoptosis of MGC-803 cells. What's more, the luciferase assay demonstrated that GGCT was a target gene of miR-194, restoration of GGCT could reverse the inhibiting effect of miR-194 on tumor cells proliferation. Conclusions: MiR-194 suppresses tumor development by regulating GGCT expression in gastric cancer and may thus be a potential prognostic marker and a therapeutic target in gastric cancer.

Keywords: Gastric cancer, miR-194, proliferation, apoptosis, GGCT

Introduction

Gastric cancer, as a common cancer, is the fourth common malignant tumor and the second leading cause of cancer-related death worldwide, especially in East Asia [1-3]. About 951,600 new gastric cancer cases and 723,100 deaths came about in 2012 [4]. The prevalence of gastric cancer is generally about twice as high in men than those in women and varies widely all over the countries. Despite great efforts have been made to improve early diagnosis rate and afford synthesized and advanced treatment methods for patients with gastric cancer, the prognosis of patients with gastric cancer is still very poor as they often experience post-treatment cancer relapses and metastasis [5, 6]. Therefore, a better understanding of the molecular mechanisms underlying gastric cancer development and metastasis is essential for finding novel therapeutic strategies.

MicroRNAs, a kind of small endogenous non-coding RNA molecules with 20-22 nucleotides

that negatively regulate the post-transcriptional regulation of target genes through their binding to 3'-untranslated regions (UTRs) [7-9]. Currently, many miRNAs has been verified to play critical roles in regulating the development of tumor processes and tumor metastasis [10-12]. Previous studies reported that miR-194 was dramatically decreased in gastric cancer tissues, and up-regulation of miR-194 could significantly inhibit migration, invasion and proliferation of gastric cancer cells by targeting RBX1 [13]. While, a single miR may have many targets, whether there have other targets need ed further study.

GGCT, as an important enzyme in the regulation of a γ -glutamyl cycle by regulating glutathione degradation, can specifically change γ -Glu-AA into pyroglutamate (pyroGlu) [14]. Some studies have been suggested that GGCT was over-expressed in many kinds of cancers, such as breast, bladder, esophagus, stomach and lung cancers [15-17]. Therefore, we speculated that GGCT may act as a critical role in gastric cancer development.

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In this study, we investigated the effect of miR-194 on gastric cancer cell proliferation and apoptosis. In addition, we also found that GGCT is a target gene of miR-194.

Materials and methods

Clinical tissues and cell culture

We recruited 52 gastric cancer patients (47±12 years, 46% males) who underwent D2 radical resection surgery at our hospital from 2014 to 2015. None of them had received radiotherapy or chemotherapy prior to surgery. The use of tissues for this study has been approved by the ethics committee of our hospital and all of the patients agreed to participate in this study and gave written informed consent. Once the clinical samples surgically collected from patients, they were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. The human gastric cancer cell lines, NCI-N87, SGC-7901, MGC-803 were purchased from American Type Culture Collection (ATCC) and human gastric epithelial GES-1 cell line were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were incubated at 37°C in 5% CO₂ and at saturation humidity in RPMI-1640 medium containing 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively).

Plasmid transfection

The miR-194 mimics and miR-194 mimics control were synthesized from Gene-Chem (Gene-Chem, Shanghai, China). Human GGCT gene was constructed into pcDNA3.1+HA empty vector by Life Technologies (Invitrogen, CA) and the empty vector were acted as the negative control. Cells were transfected by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocols.

Cell proliferation assay

The proliferation of cells was detected by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) following the manufacturer's instruction. MGC-803 cells were seeded at a density of 5×10³ cells per well in 96-well plates and cultured for various periods of time (0 h, 24 h, 48 h, 72 h). The

absorbance at 450 nm was measured using an electroluminescence immunosorbent assay reader (Thermo Fisher Scientific, Waltham, MA).

Apoptosis of gastric cancer cells was detected by flow cytometry

Cells were collected and washed twice with cold phosphate-buffered saline solution (PBS) to remove floating cells then labeled with Annexin V-FITC (BD Biosciences, San Jose, CA). Apoptosis were evaluated with a flow cytometry analyzer. Data were analyzed by Flowjo 7.6 software.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) (Roche, Shanghai, China) assay has been used to detect the late stages of apoptosis. Cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with the TUNEL detection kit from Roche at 37°C for 1 hour. After that, the samples were mounted in mounting media containing DAPI. Fluorescent images were captured using fluorescence microscope at 20× magnification. The total number of DAPI positive cells and total number of TUNEL positive cells were counted.

RNA extraction and real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from cell lines and clinical samples by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the operating instructions. RNA was quantified by using UV absorbancies at 260 and 280 nm (A₂₆₀/A₂₈₀). Subsequently the RNA was reverse-transcribed into cDNA using reverse transcription system (Thermo Scientific, CA, USA). The expression level of miR-194 was detected by the ABI PRISM 7500 Sequence Detection System (ABI) using the TaqMan MicroRNA assay kits (Applied Biosystems, California, USA). U6 small nuclear RNA (snRNA) was used as the control normalize. The expression level of GGCT also analyzed by SYBR Green and normalized to GAPDH. The judgment of primer sequences' specificity was based on dissociation curve, 2^{-ΔΔCt} (cycle threshold) was used to calculate the relative gene expression levels.

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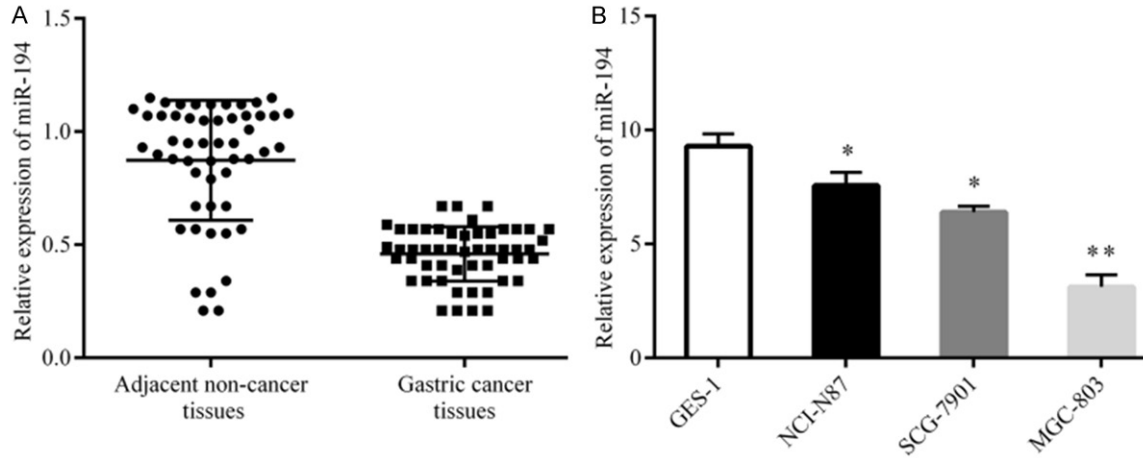


Figure 1. MiR-194 is down-regulated in gastric cancer tissues and cell lines. A. The expression level of miR-194 in gastric cancer tissues are decreased when compared with the adjacent non-cancer tissues. B. The expression of miR-194 was significantly decreased in gastric cancer cell lines (* $P < 0.05$ when compared with the GES-1, ** $P < 0.01$ when compared with the GES-1).

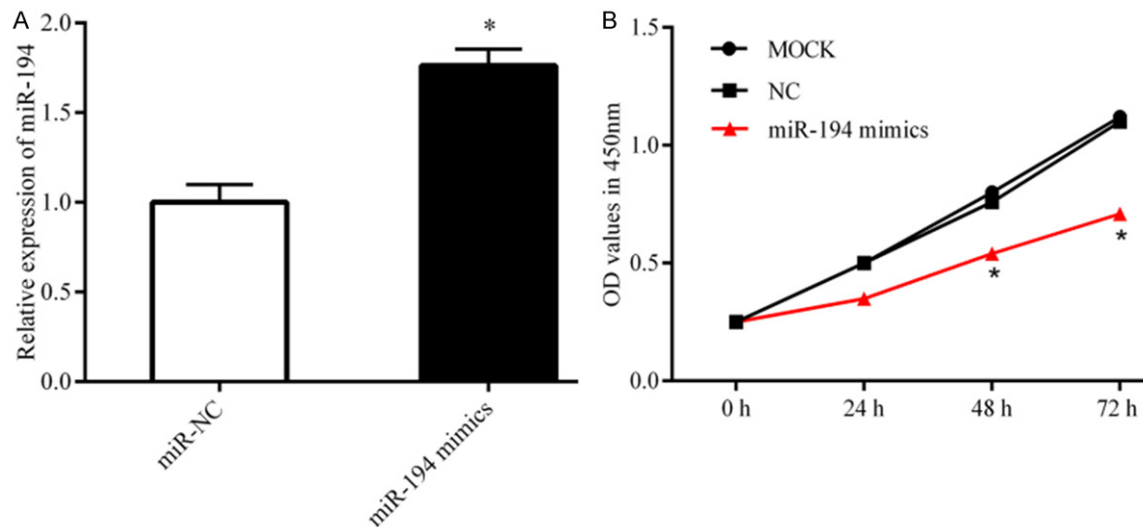


Figure 2. Up-regulation of miR-194 suppresses the proliferation of MGC-803 cells. A. The expression of miR-194 was detected in the MGC-803 cells after treatment with the miR-194 mimics. B. Over-expression of miR-194 inhibited the MGC-803 cell proliferation (* $P < 0.05$ when compared with the miR-NC).

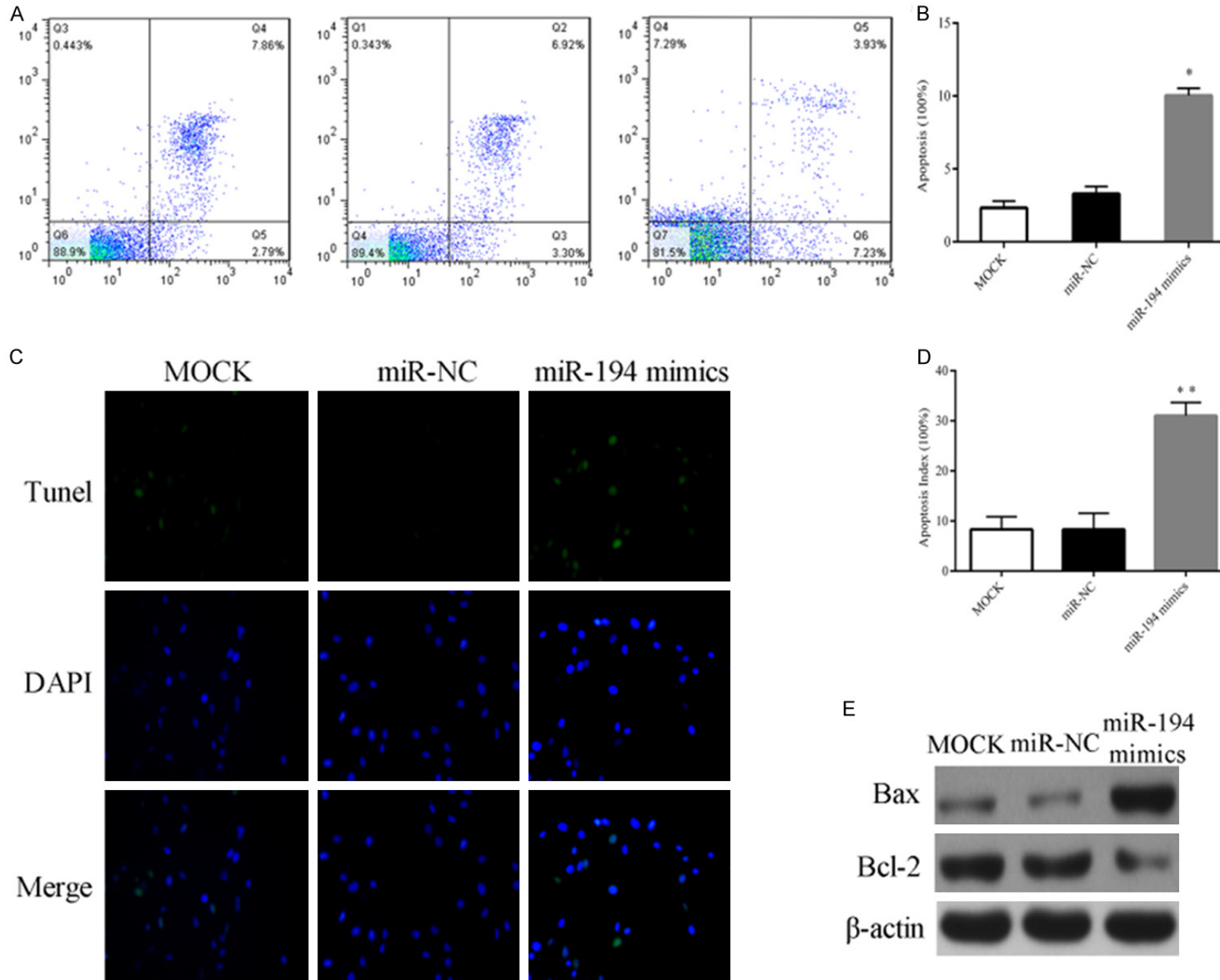
Western blot

Protein was collected by using RIPA buffer which contain a protease inhibitor cocktail and phosphatase inhibitors (Sigma, St. Louis, MO, USA) according to the operating instructions. 30 μg of protein samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF, Millipore, Bedford, MA, USA) membranes using the Bio-Rad transfer system. The protein levels were detected with an ECL kit (Thermo Scientific, CA, USA) following the manufacturer's instructions.

Lentivirus transfection and luciferase assays

The miR-194 mimics and miR-194 mimics control were purchased from Gene-Chem (Gene-Chem, Shanghai, China). Cells were transfected by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocols. Cells were incubated in the 24-well plate for 24 h before transfection. The GGCT 3'UTR of GGCT cDNA including putative site for the miR-194 was synthesized and inserted into the Renilla luciferase plasmid (Promega, Madison, WI, USA). Cells

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Figure 3. Up-regulation of miR-194 induces apoptosis of MGC-803 cells. A and B. Flow cytometry showed that the apoptosis of MGC-803 cells increased after miR-194 upregulated. C and D. TUNEL assay revealed that the apoptosis of MGC-803 cells increased after miR-194 upregulated. E. The protein expression levels of Bcl-2 and Bax. (* $P < 0.05$, ** $P < 0.01$ when compared with the miR-NC).

were co-transfected with miR-194 mimics or mimic control and WT 3'UTR or MUT 3'UTR for 48 h and detected by the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's instruction.

Statistical analysis

All the results analyses were performed by SPSS 19.0. Dates were presented as the means \pm SD (standard deviation). One-way ANOVA test and Student's t-test were used to measure the differences between the groups. $P < 0.05$ was considered to be statistically significant differences.

Results

Expression level of miR-194 was down-regulated in gastric cancer tissues and cells

In order to investigate the role of miR-194 in tumor metastasis and growth, we subsequently researched the level of miR-194 in gastric cancer tissues. As shown in **Figure 1A**, miR-194 expression level was down-regulated in gastric cancer tissues when compared with the adjacent normal tissues. In addition, the expression level of miR-194 in gastric cancer cell lines was lower than in human gastric epithelial GES-1 cell line (**Figure 1B**).

Upregulation of miR-194 suppresses the proliferation of MGC-803 cells

As shown in the **Figure 2A**, the expression level of miR-194 was obviously increased in MGC-803 cells after treatment with miR-194 mimics ($P < 0.05$). CCK8 results demonstrated that upregulation of miR-194 could significantly suppress the proliferation ability of MGC-803 cells at 48 h and 72 h ($P < 0.05$), while when compared with the control there was no significant difference at 24 h ($P > 0.05$) (**Figure 2B**).

Upregulation of miR-194 promotes MGC-803 cells apoptosis

Flow cytometry results showed that the apoptosis rates of MGC-803 cells were significantly

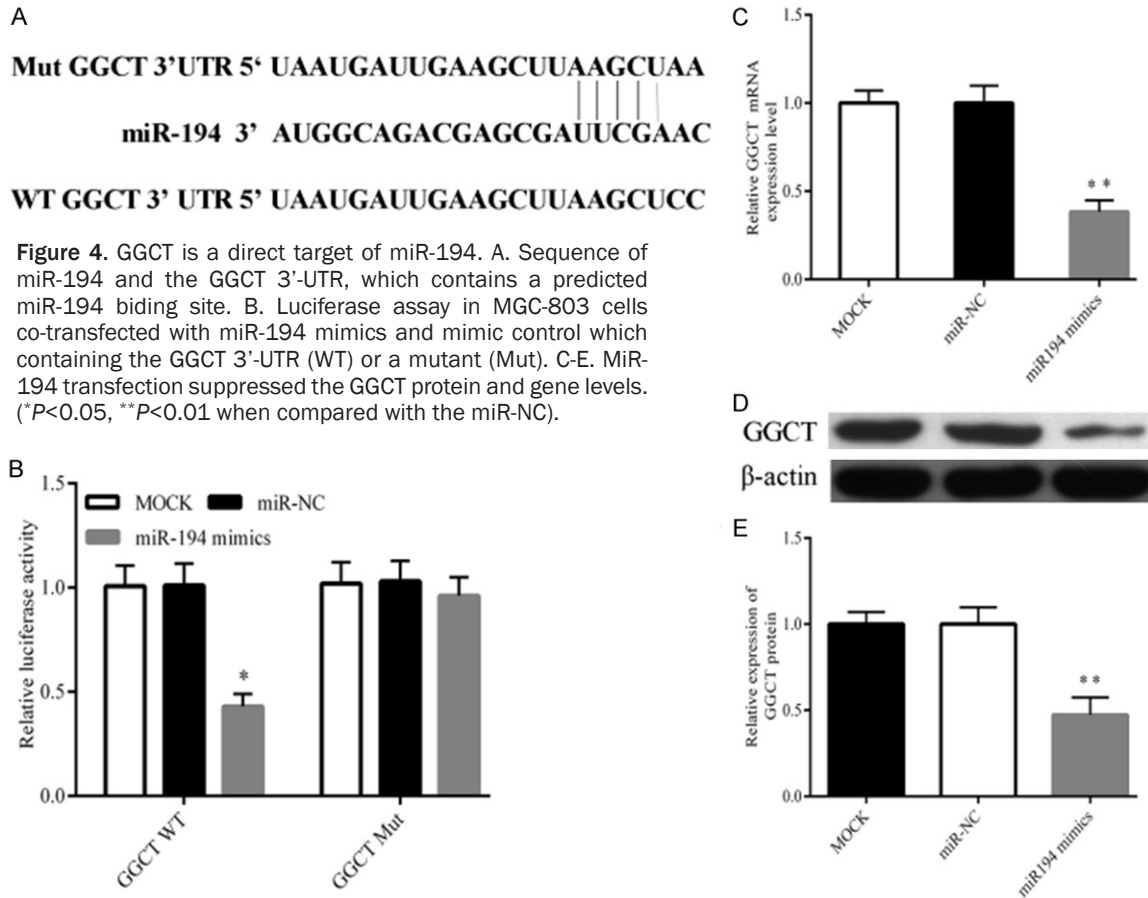
increased when the expression of miR-194 was up-regulated (**Figure 3A** and **3B**). The apoptosis of MGC-803 cells were also detected by using TUNEL assay, over-expression the level of miR-194 could dramatically increase percentage of TUNEL positive cells when compared with the control group (**Figure 3C** and **3D**). At the same time, the protein expression levels of Bcl-2 decreased and Bax markedly increased in MGC-803 cells after transfected with miR-194 mimics (**Figure 3E**). All these results illustrated that over-expression of miR-194 facilitate gastric cancer cells apoptosis.

GGCT is a direct target gene of miR-194

Targetscan was used to search for target genes of miR-194 in human cells. Among mRNAs involving miR-194 recognition sites in their 3'-UTRs, we focused on GGCT (**Figure 4A**). Then GGCT wild-type (WT) or mutant 3'-UTR was sub-cloned into a luciferase reporter vector and co-transfected with miR-194 mimics or mimics control into MGC-803 cells. Results demonstrated that miR-194 dramatically inhibited the luciferase activity of the wild type (WT) but not the mutant 3'-UTR of GGCT (**Figure 4B**). Furthermore, both the qPCR and Western blot analyses discovered that over-expression of miR-194 markedly decreased the expression level of GGCT in MGC-803 cells (**Figure 4C-E**). These results revealed that GGCT is a direct target gene of miR-194 in gastric cancer cells.

GGCT contributes to miR-194 increased proliferation of MGC-803 cells

In order to increase the expression of GGCT in MGC-803 cells pcDNA3.1+HA-GGCT was transfected into the cells which stable over-expressed miR-194. Western blot and qPCR revealed that the expression level of GGCT was significantly gained in the cells transfected with pcDNA3.1+HA-GGCT when compared with the empty vector (**Figure 5A-C**). The **Figure 5D** showed that over-expression of miR-194 significantly inhibit MGC-803 cells proliferation, while GGCT restoration reversed the anti-proliferation of miR-194.



GGCT contributes to miR-194 suppressed apoptosis of MGC-803 cells

The pro-apoptosis role of miR-194 also reversed by GGCT restoration. As shown in **Figure 6A-D**. Both the Flow cytometry and TUNEL analysis revealed that the apoptosis of MGC-803 cells were markedly decreased when transfected with miR-194 mimics+pcDNA3.1+HA-GGCT. What's more, the levels of Bcl-2 increased and Bax level declined in MGC-803 cells after transfected with miR-194 mimics+pcDNA3.1+HA-GGCT (**Figure 6E**).

Discussion

Previous studies have demonstrated that miRNAs can be act as a tumor regulator, either as a cancer suppressor or an oncogene [18, 19]. The functions of miR-194 have been confirmed in a variety of kinds of human cancer. For instance, Zhang et al illustrated that miR-194 inhibits migration, proliferation and invasion by

targeting RAP2B in human bladder cancer [20]. Zhao et al found that miR-194 functioned as a prognostic marker and regulated the development of HCC through directly suppressing the expression level of MAP4K [21]. Even more important, Li et al revealed that Exogenous expression of miR-194 inhibited cell migration, invasion, and the epithelial-mesenchymal transition phenotype in gastric cancer cells and that miR-194 acted as a tumor inhibitor through targeting FoxM1 [22].

In our present study, we illustrated that miR-194 was dramatically downregulated in gastric cancer tissues when compared with the adjacent normal tissues in vivo. Moreover, we also discovered that miR-194 markedly decreased in gastric cancer cell lines in vitro. These finding is consistent with the previous research showing that miR-194 is downregulated in many kinds of cancers [23, 24]. In addition, we studied the role of miR-194 in gastric cancer cells. First, we demonstrated that up-regulation the

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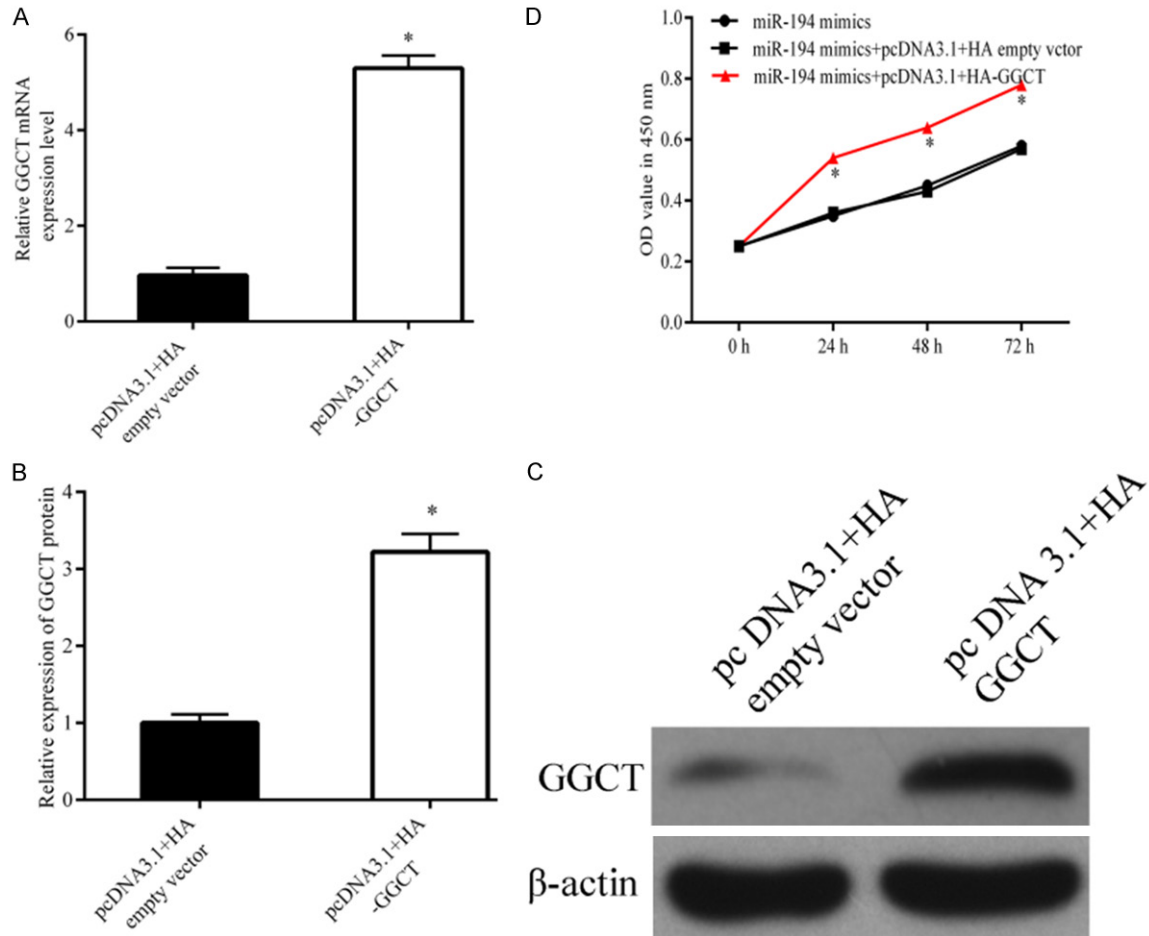


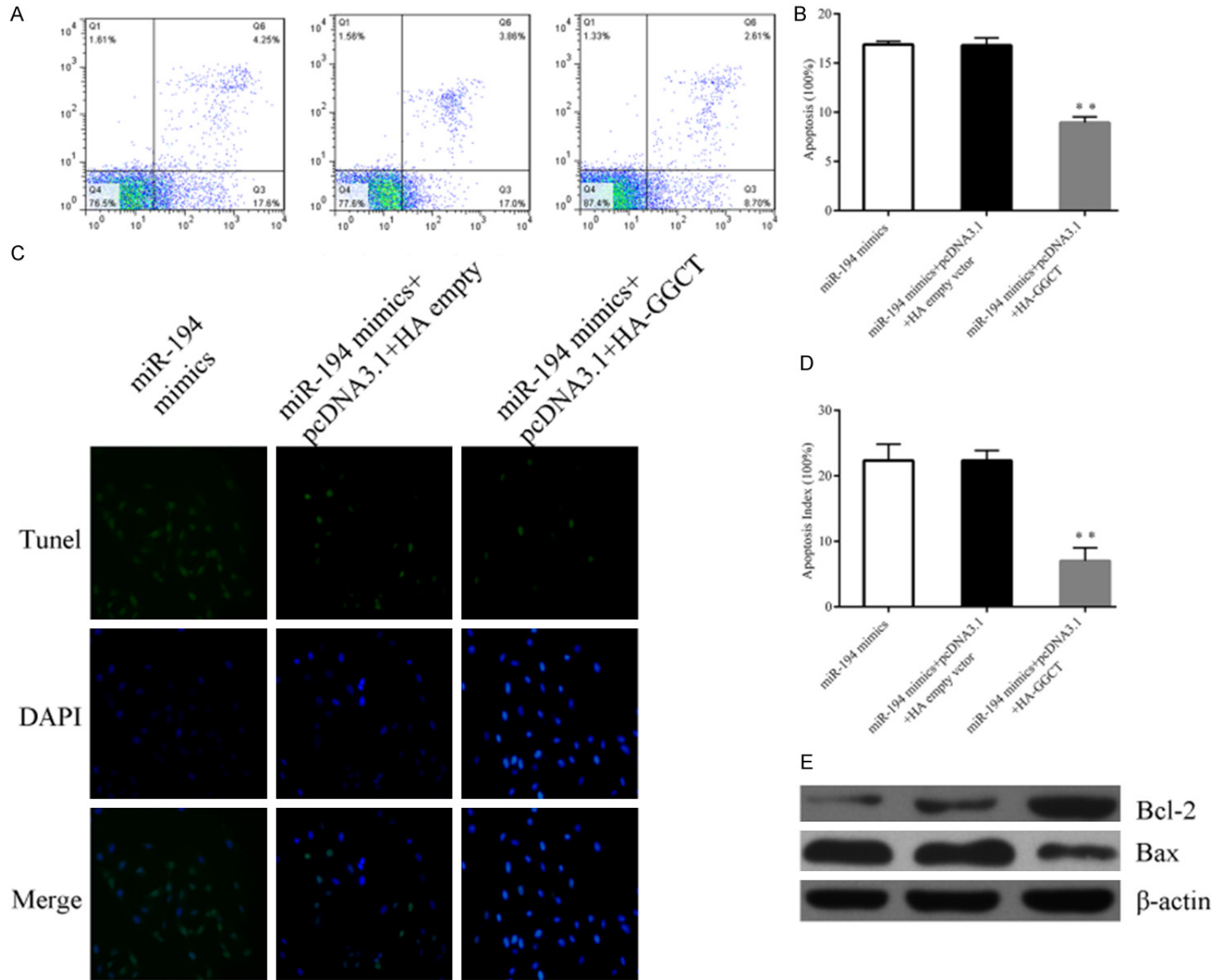
Figure 5. GGCT contributes to miR-194 increased proliferation of MGC-803 cells. A-C. The expression levels of GGCT mRNA and protein after transfected with miR-194 mimics+pcDNA3.1+HA-GGCT. D. Cell proliferation ability was detected by CCK8 assay in MGC-803 cells transfected with miR-194 mimics or miR-194 mimics+pcDNA3.1+HA-GGCT or miR-194 mimics+pcDNA3.1+HA empty vector. (* $P < 0.05$ when compared with the miR-194 mimics).

level of miR-194 significantly suppressed gastric cancer cell proliferation and induced apoptosis than the cells transfected with miR-194 mimic control. Moreover, we used luciferase activity assay and western blot showed that GGCT is a potential target gene of miR-194. Then both the western blot and qPCR showed that the expression level of GGCT can be negatively regulated by miR-194, which was play the role by binding with a site in the GGCT 3'-UTR. Additionally, recovery of GGCT partly changed the function of miR-194 in MGC-803 cells. So we concluded that GGCT may be involved in miR-194 regulated gastric cells proliferation and apoptosis. In previously, KS et al found that knockdown of GGCT dramatically suppressed the growth of many kinds of cancer cells including lung, prostate, bladder, and breast cancer cells [16]. Zhang et al also demonstrated that

knockdown of GGCT inhibits cell proliferation and induces late apoptosis in human gastric cancer [25]. These findings are consistent with our results. Bcl-2 and Bax are apoptosis-related genes. In this study, we found that when recovery of GGCT, it regulates Bcl-2 family members, promoting the BCL-2 resistance to apoptosis and inhibition Bax promoting apoptosis, which is consistent with the previous research.

According to our results, we showed that the expression level of miR-194 was down-regulated in gastric cancer patients and in the cell lines. Over-expression of miR-194 significantly inhibited proliferation and induced apoptosis of gastric cancer cells by targeting GGCT. Our research provided a better understanding of miR-194 in gastric cancer development, which

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Figure 6. GGCT contributes to miR-194 inhibited apoptosis of MGC-803 cells. A and B. Flow cytometry showed that the apoptosis of MGC-803 cells decreased after treated with miR-194 mimics+pcDNA3.1+HA-GGCT. C and D. TUNEL assay demonstrated that the apoptosis of MGC-803 cells decreased after treated with miR-194 mimics+pcDNA3.1+HA-GGCT. E. The protein expression levels of Bcl-2 and Bax. (** $P < 0.01$ when compared with the miR-194 mimics).

may also be benefit for the process of miRNA controlled diagnostic and therapeutic against gastric cancer.

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Disclosure of conflict of interest

None.

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