MicroRNA-19b inhibits proliferation of gastric cancer cells by targeting B-cell CLL/lymphoma 3

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Abstract. Previous studies reported that the aberrant expression of miR-19b in gastric cancer tissues and circulating miR-19b is a potential biomarker to indicate progression of gastric cancer. However, the prognostic significance of miR-19b, and its role and underlying mechanisms in gastric cancer remain poorly investigated. In the present study, we demonstrated that the expression of miR-19b was aberrantly downregulated in both gastric cancer tissues and cell lines. Clinical association analyses disclosed that the reduced expression of miR-19b was significantly associated with adverse clinicopathological characteristics including poor differentiation, large tumor size and advanced tumor-node-metastasis (TNM) stage. Gastric cancer patients with low expression of miR-19b had prominent shorter overall survival and disease-free survival. Gain-of-function studies indicated that miR-19b overexpression inhibited cell proliferation and cell cycle progression in MGC-803 cells. While miR-19b silencing promoted cell proliferation and cell cycle progression in SGC-7901 cells. Furthermore, *in vivo* experiments showed that miR-19b overexpression suppressed the tumor growth of MGC-803 cells. Notably, miR-19b inversely regulated B-cell CLL/lymphoma 3 (BCL3) abundance in gastric cancer cells. BCL3 was identified as a direct target of miR-19b using luciferase reporter assays. Moreover, BCL3 knockdown abolished the effects of miR-19b knockdown on gastric cancer cells. In conclusion, our data suggest that miR-19b may potentially serve as a novel prognostic biomarker and therapeutic target for gastric cancer.

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Introduction

Gastric cancer is currently one of the most common malignancies and the second leading cause of cancer-related deaths worldwide (1). Although multiple processes have been implicated in the treatment of gastric cancer, the prognosis of gastric cancer patients remains poor with 5-year survival below 24% (2). No typical symptoms for patients in early stage, postoperative recurrence and systemic metastasis are main reasons for the unsatisfactory prognosis of gastric cancer patients (3). Therefore, more studies need to be performed to disclose the molecular mechanisms involved in the initiation and progression of gastric cancer, which may contribute to screening out other alternative tumor biomarkers and development of more effective therapeutic targets for gastric cancer.

MicroRNAs (miRNAs) are a group of endogenous short non-coding RNAs with a length of ~22 nt, which regulate the expression of its target genes by precisely complementary pairing or repressing its translation through non-precisely complementary pairing within the 3'-untranslated region (3'-UTR) of target mRNAs (4,5). It has been reported that miRNAs play crucial roles in various cellular processes, including cell proliferation, apoptosis, differentiation and mobility (6-9). Notably, increasing studies have confirmed that miRNAs function as oncogenic or tumor suppressive factors in the initiation and progression of gastric cancer (10-12). However, the expression status, clinical significance and the biological function of each miRNA in gastric cancer remains largely unknown.

miR-19b-3p was previously found to be one member of the miR-17-92 cluster (miR-17-3p, miR-17-5p, miR-18, miR-19a, miR-19b-3p, miR-20a and miR-92a) frequently recognized as an oncomiR in cell or tissue (13). Wu *et al* reported that miR-19b was gradually reduced during the differentiation of gastric cancer stem cells (GCSCs) (14). Furthermore, miR-19b could sustain the self-renewal function of GCSCs and also promote the proliferation of gastric cancer cells (14). Otherwise, miR-19a/b was found to be overexpressed in gastric cancer tissues and significantly associated with the metastasis of gastric cancer patients (15). However, circulating miR-19b-3p was downregulated in gastric cancer cases and the downregulation degree was correlated with the progression of the

gastric cancer cases from the early stage to the advanced stage, indicating that the significant downregulation of miR-19b-3p may be a solid indicator of the severe progression of gastric cancer (5). In fact, miR-19 was also found to be decreased in clinical tissues of gastric cancer (16,17), suggesting that the exact role of miR-19b remains to be investigated.

In the present study, the expression of miR-19b was aberrantly reduced in human gastric cancer tissues and cell lines. Decreased expression of miR-19b was associated with adverse clinicopathological features and poor prognosis of gastric cancer patients. Functionally, miR-19b inhibited cell proliferation and cell cycle progression by suppressing B-cell CLL/lymphoma 3 (BCL3).

Materials and methods

Clinical specimens and cell culture. Tumor and the adjacent non-cancer tissues were obtained from 90 gastric cancer patients who received surgical resection at The First Affiliated Hospital of Wenzhou Medical University. All clinical specimens were frozen and stored at -80˚C. The patients did not receive any chemotherapy or radiotherapy before operation. The demographic and clinicopathological features of the patients are presented in Table I. All protocols involving clinical samples were approved by the Ethics Committee of Wenzhou Medical University according to the Declaration of Helsinki (as revised in Tokyo 2004) (18).

Human gastric cancer cell lines (SGC-7901, MGC-803, MKN-28 and BGC-823) and a normal gastric epithelium cell line (GES-1) were obtained from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank. All cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (both from Invitrogen, Carlsbad, CA, USA) at 37˚C with 5% CO_2.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the gastric cancer tissues and cells using TRIzol (Life Technologies) reagent. The TaqMan Human MiRNA Assay kit (Applied Biosystems, Foster City, CA, USA) and a SYBR® Premix Ex Taq™ II kit (Takara Bio, Shiga, Japan) were used for the PCR amplification. Primers for miR-19b-3p were: forward, 5'-TGA TAA TTA GCA AGC AGG ATT A-3' and reverse, 5'-ACC AAC ATT ACG CGG CAT CAT TA-3'; BCL3 forward, 5'-GAA AAC AAC AGC CTT AGC ATG GT-3' and reverse, 5'-CTG CGG AGT ACA TTT GCG-3'; U6 forward, 5'-CTC GCT TCG GCA GCA CA-3' and reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'; GAPDH forward, 5'-CAA GCT CAT TTC CTG GTA TGA C-3' and reverse, 5'-CAG TGA GGG TCT CTC TCT TCC T-3'. U6 was used as internal control for the relative expression level of miR-19b, while GAPDH was used as the internal control for the relative expression of BCL3.

Cell transfection. The plasmids and siRNA used in the present study include miR-19b (HmiR0203-MR04) and control vector (CmiR0001-MR04), miR-19b inhibitor (HmiR-AN0295-AM04) and negative control vector (CmiR-AN0001-AM04) (all from GeneCopoeia, Guangzhou, China), BCL3 siRNA (SR300415) and scrambled negative control siRNA (SR30004) (both from OriGene, Beijing, China). These vectors were transfected into the gastric cancer cells with Lipofectamine 2000 (Invitrogen) based on the manufacturer's protocol.

Table I. The clinical significance of miR-19b expression in gastric cancer.

Clinicopathological features	Total no. of pts., n=90	No. of patients: Low miR-19b	No. of patients: High miR-19b	P-value
Age (years)				0.832
<65	49	25	24	
≥65	41	20	21	
Gender				0.655
Male	60	29	31	
Female	30	16	14	
Histology				0.020[a]
Well, moderate	41	15	26	
Poor, signet	49	30	19	
Size (cm)				0.033[a]
<5	38	14	24	
≥5	52	31	21	
Depth				0.310
T_1	20	12	8	
T_2-T_4	70	33	37	
Lymph node metastasis				0.664
Absent	34	16	18	
Present	56	29	27	
Lymphatic invasion				0.120
Absent	31	12	19	
Present	59	33	26	
Venous infiltration				0.114
Absent	61	27	34	
Present	29	18	11	
TNM stage				0.003[a]
I,II	48	17	31	
III,IV	42	28	14	

T1, mucosa (m), submucosal (sm); T2-T4, muscularis propria (mp), subserosa (ss), serosa exposed (se), serosa infiltrating (si); TNM, tumor-node-metastasis; pts., patients. [a]Statistically significant.

Western blot analysis. RIPA buffer was used to extract the total protein from gastric cancer cells, and 20-30 μg of isolated protein was separated by 10% SDS-PAGE and transferred onto 0.22 μm NC membranes (Sigma, St. Louis, MO, USA). After membrane transfer, the membranes were incubated with BCL3 (sc-185; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Ki-67 (#9449) and GAPDH antibody (#5174) (both from Cell Signaling, Danvers, MA, USA) overnight at 4˚C. Then, the membranes were incubated with the secondary goat

anti-mouse or anti-rabbit IgG antibody (ZSGB-BIO, Beijing, China). GAPDH was used as control.

BrdU incorporation and MTT assay. For proliferation, 5-bromodeoxyuridine (BrdU)-labeling and immunofluorescence was used. Cells grown on coverslips (Fisher, Pittsburgh, PA, USA) were incubated with BrdU for 1 h and stained with anti-BrdU antibody (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. For cell viability, gastric cancer cells ($4x10^3$) were seeded into 96-well plates and stained with sterile MTT (Sigma, St. Louis, MO, USA) for 4 h at 37˚C, following which the culture medium was discarded and an extra 150 μl dimethyl sulfoxide (DMSO) (Sigma) were then added into each well 24, 48 and 72 h after transfection. The absorbance at 490 nm was examined.

Immunohistochemical staining. Immunohistochemistry was performed on paraformaldehyde-fixed paraffin sections. Ki-67 (1:100; Cell Signaling) antibody was used in immunohistochemistry using a streptavidin peroxidase-conjugated (SP-IHC) method. Detailed procedure of immunohistochemistry was performed as previously reported (19).

Cell cycle assays. For cell cycle analysis, 48 h after transfection, gastric cancer cells were collected, washed with phosphate-buffered saline (PBS) and fixed with 80% ethanol overnight at 4˚C. Then, the cells were incubated with RNaseA for 30 min at 37˚C, followed by incubation with propidium iodide (Sigma) for 20 min at room temperature. Then, the cells were subjected to flow cytometry analysis using a FACSCalibur (BD Biosciences, Bedford, MA, USA).

Tumor formation assay in a nude mouse model. Four-to-six week-old female BALB/c nude mice were used to establish the nude mouse xenograft model. MGC-803 cells transfected with miR-19b or miR-control vectors were suspended in 100 μl of PBS and were injected subcutaneously into the flank of a nude mouse. Tumor volumes were determined by measuring two of its dimensions with calipers every 3 days, and the equation for calculating the tumor volume was $V = 0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). All nude mice were sacrificed at 3 weeks after the injection of MGC-803 cells. The *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

Luciferase reporter assay. The wild-type 3'-UTR sequence of BCL3 or the mutated sequence within the predicted target sites was synthesized and inserted into the pGL3 control vector (Promega, Madison, WI, USA), to construct the wt BCL3-3'-UTR or mt BCL3-3'-UTR, respectively. Then, SGC-7901 cells were seeded into 24-well plates, and were cultured in OptimMEM reduced serum media (Life Technologies), and were co-transfected with corresponding vectors using FuGENE (Promega, Madison, WI, USA). Forty-eight hours after transfection, SGC-7901 cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega). Results were obtained from three independent experiments performed in triplicate.

Statistical analysis. Data are presented as the mean ± SEM. GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used to for statistical analysis. The differences between two or more groups were compared with a two-tailed Student's t-test or ANOVA. Chi-square test was used to analyze the clinical correlation. To compare the difference of the patients survival between two groups, the Kaplan-Meier method and the log-rank test were performed. Differences were considered statistically significant at P<0.05.

Results

miR-19b is aberrantly downregulated in gastric cancer tissues and cell lines. First, we measured the expression level of miR-19b in gastric cancer and matched tumor-adjacent tissues with qRT-PCR. The results showed that miR-19b expression in gastric cancer tissues was significantly lower than that in the non-tumor tissues (P<0.05; Fig. 1A). As compared with GES-1 cells, miR-19b was downregulated in a panel of gastric cancer cell lines (SGC-7901, BGC-823, MKN-28 and MGC-803) (P<0.05, respectively; Fig. 1B). These observations suggest that miR-19b probably plays a tumor suppressive role in the initiation and progression of gastric cancer.

Reduced expression of miR-19b confers adverse clinicopathological features and poor prognosis of gastric cancer patients. The gastric cancer patients were divided into two groups (miR-19b high or low expression group) based on the median expression level of miR-19b. As shown in Table I, low expression of miR-19b was associated with poor histological features (P=0.020), large tumor size (P=0.033) and advanced tumor-node-metastasis (TNM) stage (P=0.003). Furthermore, Kaplan-Meier analysis showed that low expression of miR-19b was significantly associated with shorter overall survival (P=0.002; Fig. 1C) and disease-free survival (P=0.012; Fig. 1D). These data indicate that miR-19b can serve as novel prognostic biomarker for the survival of gastric cancer patients.

miR-19b inhibits gastric cancer cell proliferation and cell cycle progression. To investigate the role of miR-19b in gastric cancer cells, miR-19b expression vector was transfected into MGC-803 cells. Transfection of miR-19b mimics significantly increased the level of miR-19b in MGC-803 cells (P<0.05; Fig. 2A). BrdU incorporation assays showed that miR-19b overexpression decreased the proliferative ability of MGC-803 cells (P<0.05; Fig. 2B). Furthermore, western blot analysis indicated that miR-19b overexpression markedly reduced the level of Ki-67 in MGC-803 cells (P<0.05; Fig. 2C). MTT assays indicated that miR-19b overexpression reduced cell viability of MGC-803 cells (P<0.05; Fig. 2D). Cell cycle analysis revealed that upregulation of miR-19b increased the percentage of cells in the G1 phase while decreased the percentage of cells in the S phase (P<0.05; respectively, Fig. 2E). Inversely, we downregulated the expression of miR-19b with corresponding inhibitors in SGC-7901 cells (P<0.05; Fig. 3A). Notably, miR-19b silencing resulted in significantly increased cell proliferation (P<0.05; Fig. 3B), Ki-67 expression (P<0.05; Fig. 3C), cell viability (P<0.05; Fig. 3D) and cell cycle

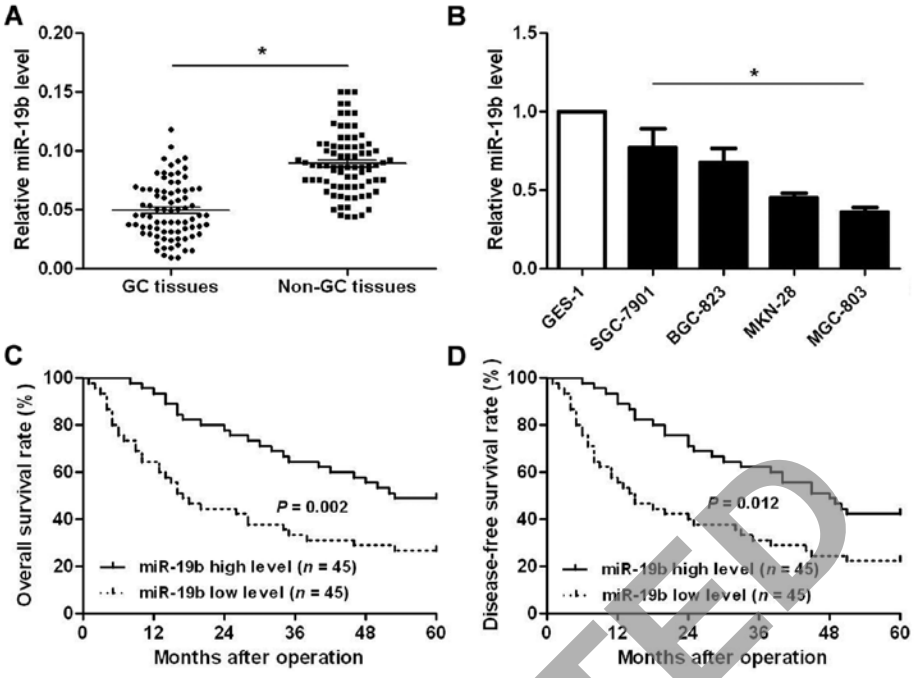

Figure 1. The expression status and prognostic value of miR-19b in gastric cancer. (A) Relative expression of miR-19b in gastric cancer (GC) and matched tumor-adjacent tissues were determined by qRT-PCR; n=90; *P<0.05 by t-test. (B) The expression of miR-19b in gastric cancer cells (SGC-7901, BGC-823, MKN-28 and MGC-803) was significantly decreased as compared to GES-1 cells; n=3; *P<0.05 by ANOVA. (C and D) Gastric cancer patients with low expression of miR-19b had poorer overall and disease-free survival.

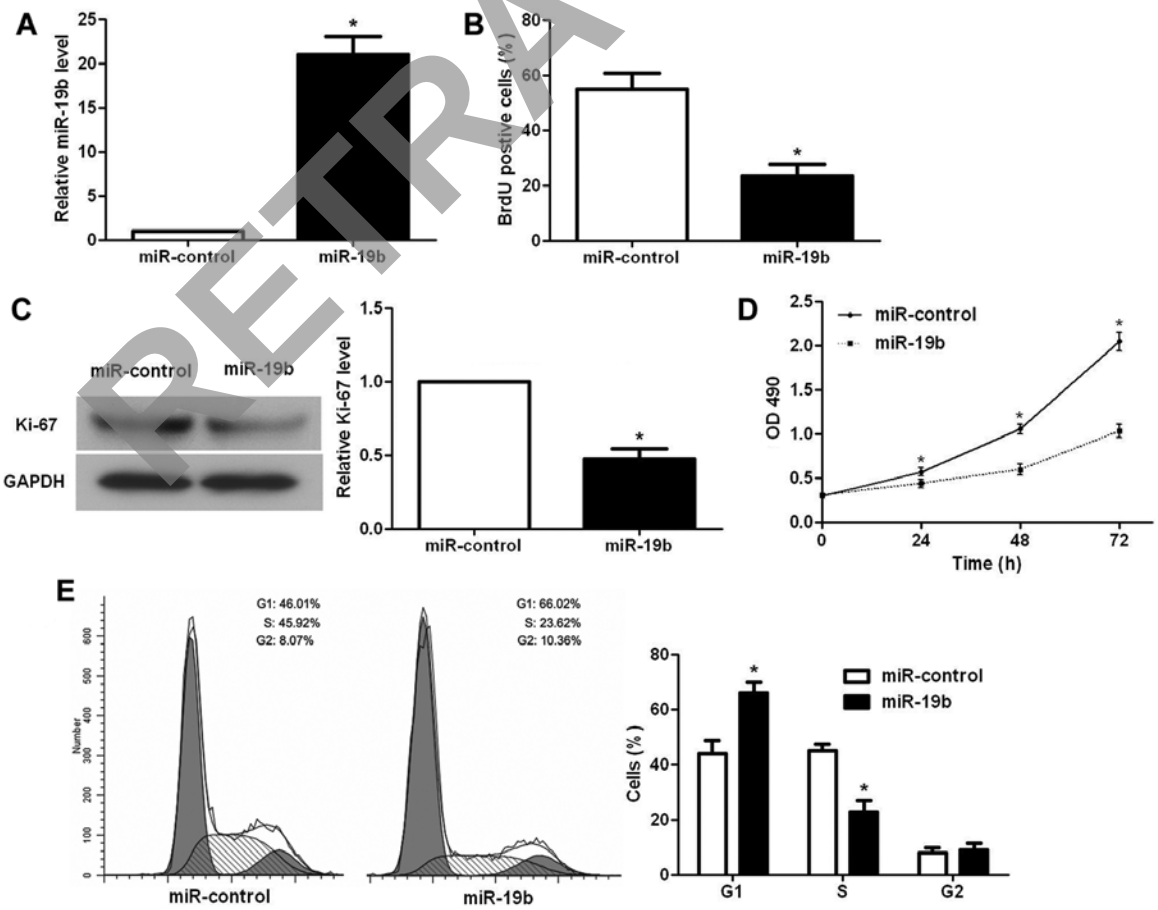

Figure 2. miR-19b overexpression inhibits cell proliferation and cell cycle progression in MGC-803 cells. (A) MGC-803 cells that were transfected with miR-19b and miR-control vectors were subjected to qRT-PCR; n=3; *P<0.05 by t-test. (B) The percentage of BrdU-positive MGC-803 cells was significantly decreased after miR-19b overexpression; n=3, *P<0.05 by t-test. (C) MGC-803 cells that were transfected with miR-19b and miR-control vectors were subjected to immunoblotting for Ki-67; n=3; *P<0.05 by t-test. (D) MTT assays indicated that miR-19b overexpression reduced cell viability of MGC-803 cells; n=3; *P<0.05 by ANOVA. (E) Effects of miR-19b overexpression on the cell cycle progression of MGC-803 cells; n=3; *P<0.05 by t-test.

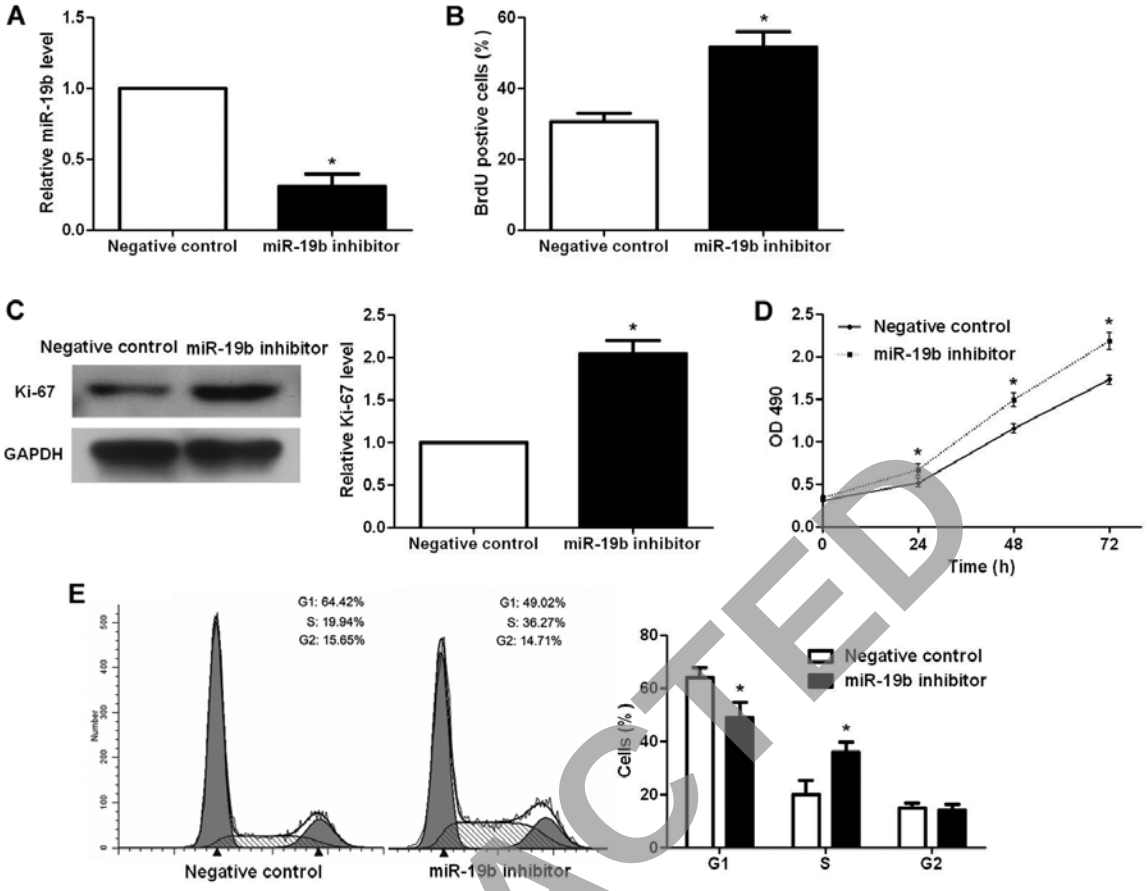

Figure 3. miR-19b silencing decreases cell proliferation and cell cycle progression in SGC-7901 cells. (A) SGC-7901 cells that were transfected with miR-19b inhibitor and negative control vectors were subjected to qRT-PCR; n=3; *P<0.05 by t-test. (B) The percentage of BrdU-positive SGC-7901 cells was significantly increased after miR-19b silencing; n=3; *P<0.05 by t-test. (C) SGC-7901 cells that were transfected with miR-19b inhibitor and negative control vectors were subjected to immunoblotting for Ki-67; n=3; *P<0.05 by t-test. (D) MTT assays indicated that miR-19b knockdown increased cell viability of SGC-7901 cells; n=3; *P<0.05 by ANOVA. (E) Effects of miR-19b knockdown on the cell cycle progression of SGC-7901 cells; n=3; *P<0.05 by t-test.

progression (P<0.05; Fig. 3E). To further confirm these functional effects of miR-19b on gastric cancer cells, we performed tumor formation assay in nude mouse model. miR-19b overexpression significantly decreased the tumor growth of MGC-803 cells in nude mice (P<0.05; Fig. 4A). Furthermore, we also performed immunohistochemistry for Ki-67 in the xenografted tissues. Consistent with our *in vitro* data, miR-19b overexpression inhibited proliferation *in vivo* (P<0.05, Fig. 4B). Taken together, these data indicate that miR-19b inhibits the tumor growth of gastric cancer cells both *in vitro* and *in vivo*.

BCL3 is a direct target of miR-19b in gastric cancer cells. To disclose the molecular mechanisms by which miR-19b inhibits tumor growth in gastric cancer, predicted target genes of miR-19b were retrieved and analyzed using publicly available databases (TargetScan 7.0 and miRanda). Significantly, BCL3, which was known to promote cell proliferation and cell cycle progression by targeting cyclin D1 (20), was predicted as one of the targets of miR-19b. MGC-803 cells that were transfected with miR-19b and miR-control vectors were subjected to qRT-PCR and western blotting for BCL3 expression. Both BCL3 mRNA and protein levels were significantly reduced by upregulation of miR-19b in MGC-803 cells (P<0.05, respectively; Fig. 5A and B). To further demonstrate that BCL3 is directly targeted by miR-19b in gastric cancer cells, we investigated whether the miR-19b directly interacted with the 3'-UTR of BCL3 mRNA using a dual-luciferase reporter assay. As expected, miR-19b significantly inhibited the luciferase activity of BCL3 containing a wild-type (wt) 3'-UTR but did not suppress activity of BCL3 with a mutant (mt) 3'-UTR (P<0.05; Fig. 5C and D). When miR-19b inhibitor was transfected, an increase in luciferase activity of wt BCL3 3'-UTR was observed. However, with the mt BCL3 3'-UTR constructs, there was no relative increase in activity (P<0.05; Fig. 5C and D). Thus, our data strongly suggest that BCL3 is a target of miR-19b in gastric cancer.

BCL3 is a functional mediator of miR-19b in gastric cancer cells. As BCL3 is a direct downstream target of miR-19b, we further evaluated whether BCL3 mediated the biological function of miR-19b in gastric cancer cells. BCL3 siRNA significantly reduced the protein level of BCL3 in miR-19b downregulating SGC-7901 cells (P<0.05; Fig. 6A). Knockdown of BCL3 abrogated the effects of miR-19b silencing on SGC-7901 cells with decreased cell proliferation (P<0.05; Fig. 6B), Ki-67 level (P<0.05; Fig. 6C), cell viability (P<0.05; Fig. 6D) and cell cycle progression (P<0.05; Fig. 6E). These data indicate that BCL3 is a functional mediator of miR-19b in gastric cancer cells.

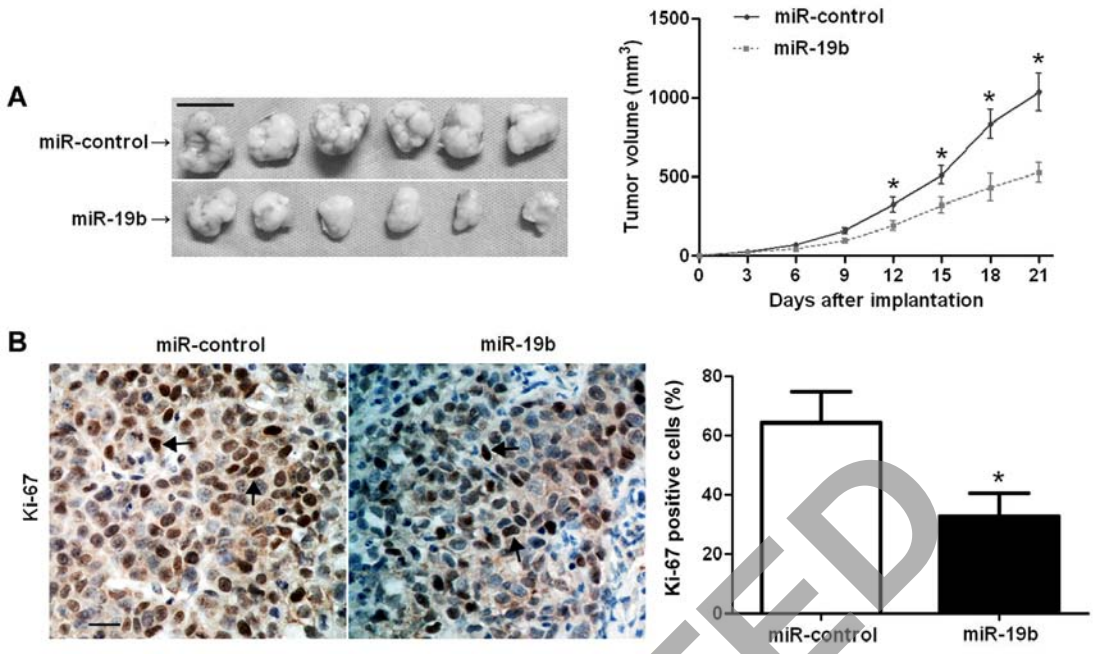

Figure 4. miR-19b overexpression inhibits the tumor growth in nude mice. (A) miR-control or miR-19b-expressing MGC-803 cells were implanted into nude mice via subcutaneous injection. Tumor growth curves indicated that miR-19b overexpression inhibited tumor growth in mice; n=6; *P<0.05 by ANOVA; scale bar, 1 cm; (B) The average percentage of Ki-67-positive cells in tumors arising from the miR-19b group was significantly lower than those in the control group; n=6; *P<0.05 by t-test; scale bar, 50 μm. Black arrows indicate positive cells.

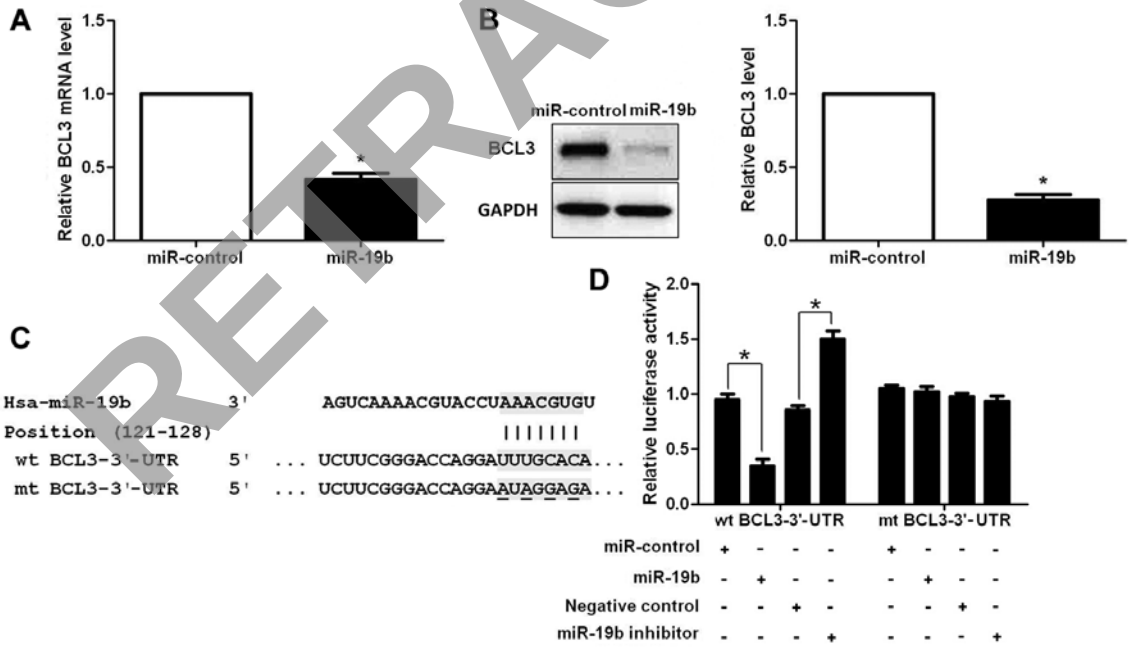

Figure 5. BCL3 is a direct target of miR-19b in gastric cancer cells. (A) qRT-PCR and (B) western blot analysis of BCL3 expression in MGC-803 cells with miR-19b or miR-control vector transfection; n=3; *P<0.05 by t-test. (C) miR-19b and its putative binding sequence in the 3'-UTR of BCL3. The mutant miR-19b binding site was generated in the complementary site for the seed region of miR-19b (wt, wild-type; mt, mutant type). (D) miR-19b significantly suppressed the luciferase activity that carried wt but not mt 3'-UTR of BCL3. miR-19b inhibitor led to a noticeable increase in luciferase activity of wt 3'-UTR of BCL3; n=3; *P<0.05 by t-test.

Discussion

Accumulating studies demonstrate that aberrant expression and dysfunction of miRNAs play critical roles in the development of various human cancers (21,22), including gastric cancer (21). Exploration of the expression status, biological function and underlying mechanisms for specific miRNA in gastric cancer contribute to identification of novel biomarker and therapeutic targets in human gastric cancer. In the present study, we found the expression of miR-19b was significantly

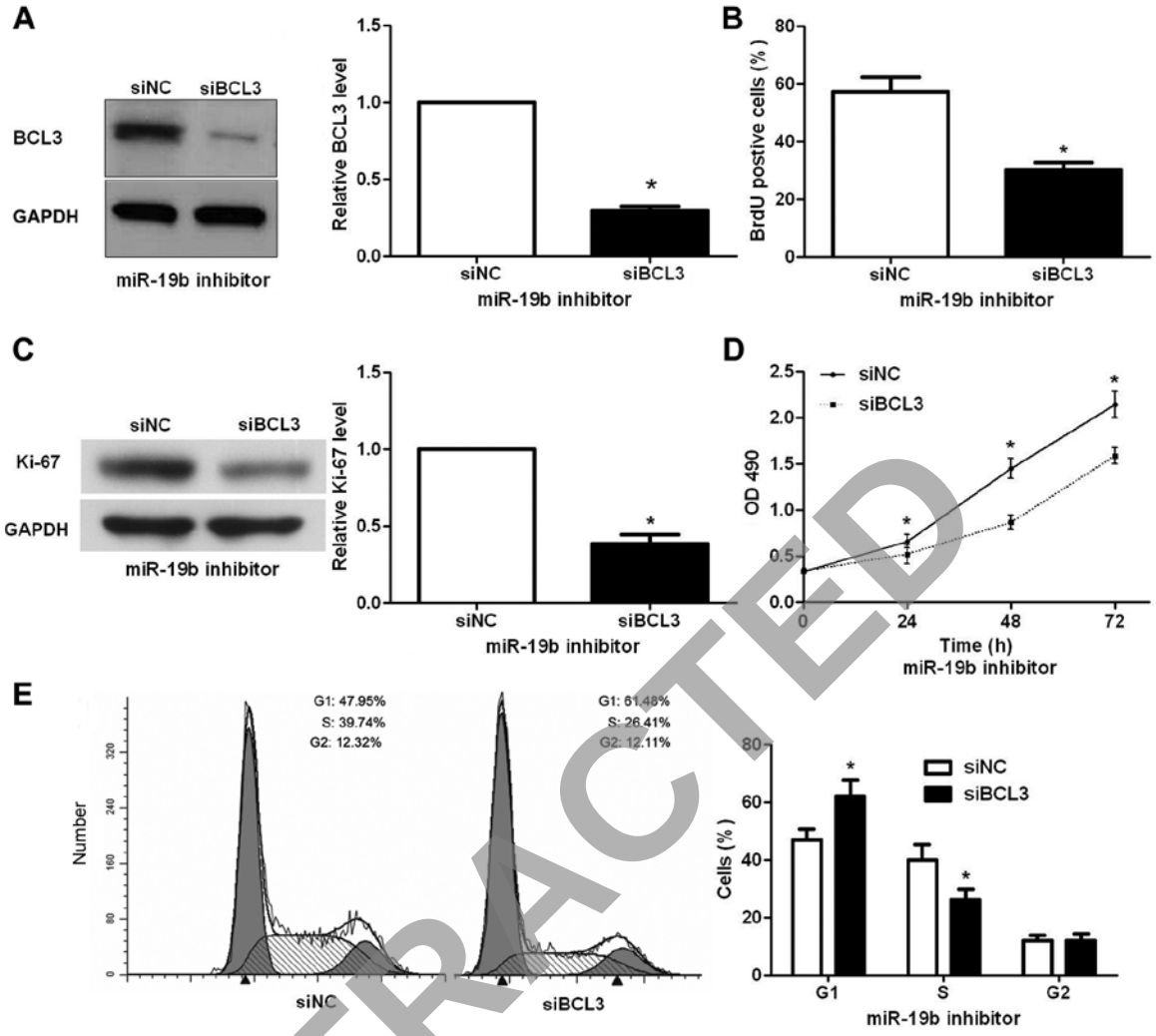

Figure 6. BCL silencing abolishes the effects of miR-19b inhibitor on cell proliferation and cell cycle progression. (A) miR-19b inhibitor transduced SGC-7901 cells that were transfected with BCL3 siRNA (siBCL3) and scrambled siRNA (siNC), respectively, were subjected to western blotting; n=3; *P<0.05 by t-test. BCL3 knockdown abrogated the effects of miR-19b inhibitors on (B) cell proliferation, (C) Ki-67 expression, (D) cell viability and (E) cell cycle progression; n=3; *P<0.05 by t-test.

decreased in gastric cancer tissues as compared to matched tumor-adjacent tissues. Significant downregulation of miR-19b was also observed in gastric cancer cells. Clinical analysis showed that reduced expression of miR-19b was significantly correlated with adverse clinicopathological features, including poor histological differentiation, large tumor size and advanced TNM stage. Moreover, survival analysis showed that low level of miR-19b was associated with poorer overall survival and disease-free survival of gastric cancer patients. These data indicate that miR-19b can serve as novel indicator of the prognosis of gastric cancer patients.

Increased proliferative ability and cell cycle progression are important functional foundation for the initiation and progression of human cancers (23). The functional assays in the present study showed ectopic expression of miR-19b inhibited cell proliferation and cell cycle progression in gastric cancer cells, while inhibition of miR-19b increased these cellular processes of gastric cancer cells. Therefore, these results demonstrate that miR-19b functions as a tumor suppressive miRNA by suppressing cell proliferation and cell cycle progression in gastric cancer. BCL-3 is an atypical member of the IkB family (24) and can bind NF-κB homodimeric complexes of p50 or p52, which switches the transcriptional properties of the homodimers from a repressive to an activating state (25). The mRNA and protein expressions of BCL-3 have been reported to be overexpressed in breast cancer (25,26), nasopharyngeal carcinoma (27), endometrial cancer (28), hepatocellular carcinoma (29) and colorectal cancer (30). Functionally, BCL-3 was found to regulate the colony formation and cell cycle progression by regulating ubiquitination-mediated degradation of c-Myc in colorectal cancer (31). In the present study, we presented solid data to show that BCL3 was a novel functional target of miR-19b in human gastric cancer cells. First, miR-19b significantly reduced the expression level of BCL3 mRNA and protein in gastric cancer cells. Second, the 3'-UTR of BCL3 contained the complementary sequence of miR-19b. Third, miR-19b significantly inhibited the luciferase activity of the wt 3'-UTR of BCL3 while had no influence on that of mt 3'-UTR of BCL3. Furthermore, the present study showed that BCL3 knockdown could reverse the effects of miR-19b silencing on cell proliferation and cell cycle progression. Notably, one miRNA has more than one target in cancer cells.

Thus, further studies need to be performed to disclose other targets for miR-19b in gastric cancer.

In conclusion, we demonstrated that miR-19b is down-regulated in gastric cancer tissues and cell lines. miR-19b plays an important role in the progression of gastric cancer by modulating the expression of BCL3 in gastric cancer cells. Therefore, the present study indicates that miR-19b can potentially serve as a promising prognostic factor and therapeutic target for gastric cancer.

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