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Original Article

MicroRNA-181b blocks gensenoside Rg3-mediated tumor suppression of gallbladder carcinoma by promoting autophagy flux via CREBRF/CREB3 pathway

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Abstract: Background: Gallbladder cancer (GBC) is the seventh most common gastrointestinal cancer. Suppression of autophagy contributes to cell death of gallbladder cancer. Gensenoside Rg3 sensitizes tumor cells to chemotherapeutic agents through autophagy inhibition. However, its role mechanism on the progression of GBC remains vague. The present study is aimed to explore the functional action of Rg3 on GBC progression. Methods: Expression of miR-181b and CREBRF in human gallbladder carcinoma specimen were determined by western blotting and qRT-PCR. Biological character of tumor cells were assessed by FACS, CCK8 and xenograft assays, respectively. Dual luciferase assay was employed to explore the targeting site of miR-181b. Autophagy flux was detected by IF staining. Results: MiR-181b expression was increased, while CREBRF expression was reduced in GBC specimens compared to adjacent normal tissues. Based on Catalogue of Somatic Mutations in Cancer (COSMIC) database (408 GBC samples), there was negative correlation between hsa-miR-181b-5p/-3p and CREBRF which was a direct targeting of miR-181b. miR-181b mimic promoted cell proliferation and autophagy, restrained cell apoptosis by regulating CREBRF/CREB3 pathway. As an anti-tumor agent, gensenoside Rg3 inhibited cell proliferation and tumor growth, while promoted cell apoptosis by inhibiting autophagy. However, exogenous miR-181b blunted Rg3-evoked anti-tumor effect possibly by inhibiting CREBRF/CREB pathway. Conclusion: Collectively, these data indicates that miR-181b possibly mediates the pathologic progression of GBC by CREBRF/CREB3 signaling pathways and impairs anti-tumor effects of Rg3 on GBC development, which suggests that miR-181b might be an key switch in the process of Rg3-mediated tumor cytotoxicity in the progression of GBC.

Keywords: Gallbladder cancer, autophagy, gensenoside Rg3, miR-181b, CREBRF

Introduction

Gallbladder carcinoma (GBC) is one of the most common malignancy of biliary tract [1]. Since early clinical diagnosis and prognostic markers are limited, primary gallbladder carcinoma detection presents intermediate-aggressive stage, which results in a higher mortality in GBC than other types of tumors [2]. Currently, treatment strategies for GBC mainly depend on radical surgery combined with chemotherapeutic agents, such as Cisplatin and Gemcitabine [3, 4], but the prognosis for aggressive gallbladder carcinoma is very poor [5]. Advanced autophagic flux provides substrates for metabolism and contributes to the progression of many types of cancer [6]. And targeting autophagy has been proposed as a potential approaches to improve the efficacy of conventional therapies [7]. However, the mechanism of autophagy involvement in GBC have not been fully revealed and need to be further investigated.

Autophagy recognizes as a conserved self-adaptive cellular response and promotes intensely aggressive cancer cell survival in the face of nutrient depletion, hypoxia and the presence of cytotoxic drugs [8]. It is confirmed that autophagy associated protein beclin-1 is overexpressed in gallbladder carcinoma patients and has a significant association with TNM stage and prognosis [9]. Chloroquine (CQ), an inhibitor of autophagy, can powerful enhance anti-tumor effects of 5-FU on GBC [10]. How-

ever, 5-FU can induce chemoresistance of gastrointestinal cancer cells by activating autophagy process [11]. Therefore, autophagy process is a key regulator in the progression of GBC. Ginsenoside Rg3 (Rg3), extracted from the root of the Ginseng plant, exerts an antitumor effects on numerous malignant tumor such as colorectal cancer and lung cancer by regulating tumor angiogenesis or epithelial-mesenchymal transition (EMT) [12, 13]. Rg3-mediated autophagy inhibition sensitizes hepatocarcinoma cell line to doxorubicin in vitro [14]. However, as a potential autophagy inhibitor, whether Rg3 acts as an anti-cancer agent in the development of GBC remains unclear.

CREB3 regulatory factor (CREBRF), also known as Luman regulatory factor (LRF), is able to recruit nuclear CREB3 (cAMP responsive element binding protein 3) to discrete nuclear foci, which represses the transactivation activity of CREB3 and accelerates the degradation of CREB3 protein [15]. CREBRF is highly expressed in primary gastric cancer (GC) tissues and involved in cell proliferation by activating AKT signaling pathway [16]. CREBRF can also promote endometrial epithelial cells (EECs) proliferation by activating autophagy flux [17]. Additionally, hypoxia contributes to malignant glioma progression by activating miR-155-3p-CREBRF-CREB3-ATG5 signaling-mediated the induction of autophagy, whereas miR-155-3p inhibitor significantly inhibited autophagy on human glioma cells by directly targeting CREBRF [18]. Thus, autophagy process is required in the course of CREBRF-mediated tumorigenesis.

Herein, we observed that miR-181b was negative correlation with CREBRF in GBC patients. miR-181b aggravated tumor biologic characteristics of GBC-SD cells by targeting CREBRF and subsequently enhancing CREB3 levels. Rg3 mediated anti-tumor effects by autophagy flux inhibition via blocking miR-181b/CREBRF/CREB3 pathway. However, exogenous enforced miR-181b blunted the cytotoxicity of Rg3 on GBC. Therefore, Rg3-launched tumor-suppressor activity in GBC possibly through inhibiting miR-181b-evoked autophagy process.

Materials and methods

Clinical samples

93 cases of gallbladder carcinoma tissues and the paired para-carcinoma tissue were collected from 93 individual GBC patients who underwent surgical operation. Tumor extent of these tissue were evaluated by tumor, lymph node, and metastasis (TNM) staging system. Clinicopathological data from 93 Gallbladder cancer patients was presented in **Table 1**. This study was approved by the ethics committee of The First Affiliated Hospital of Zhejiang Chinese Medical University and all the patients signed the informed consent before the experiment. Fresh tumor and the paired para-carcinoma tissues were cut into 0.1 cm^3 pieces and stored at -80°C for further study.

Cell culture and transfection

GBC-SD cells were purchased from Shanghai Fuxiang Biotechnology and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in 37°C incubator with 5% CO_2. For cell transfection, GBC-SD cells were seeded at 6-well plate at the density of 5 × 10^4 cells/well. After 12 h incubation, negative control miRNAs, miR-181b mimic, or miR-181b inhibitor (Genepharma, Shanghai, China) were transfected in GBC-SD cells by using Lipofectamine 3000 (Thermo Scientific, US). Transfection efficiency were determined by qRT-RCR after transfection for 48 h.

Stable-transfected cell line of miR-181b

Briefly, the sequence of miR-181b was amplified by PCR amplification and inserted into the lentiviral vector PGMLV-6395 (Genomeditech, Shanghai, China). 293T cells were prepared for the transfection of vector or PGMLV-CMV-hsa-miR-181b plasmids combined with the packaging plasmids. After 48 hours, the virus were collected, purified and added into GBC-SD cells with Polybrene (8 μg/ml, H9268, Sigma, USA). Infection efficiency was determined by qRT-PCR in virus-infected GBC-SD cells after infection for 48 h. The primers of miR-181b were as follows: forward, 5'-CCGGAATTCGAGACTGGGGAATACACATGAGCC-3'; reverse, 5'-CCG GGATCC GGTTTGGAACATGGTTCATAAGCCC-3'.

Xenograft model

6-8 weeks old BALB/c nude mice (20-22 g) were provided by Changzhou Cavans Laboratory Animal. All the mice were housed under standard conditions (20-22°C) and free access to purified rodent diet and water. All animal experiments were approved by the ethics com-

Table 1. Clinicopathological data from 93 Gallbladder cancer patients

Clinicopathological data	Numbers (93)
Age	
<60	39
≥60	54
Gender	
Male	27
Female	66
TNM stage	
I+II	18
III+IV	75
Lymph node metastasis	
Yes	67
No	26
Liver metastasis	
Yes	43
No	50
Differentiated degree	
Well	23
Moderate/poor	70

mittee of The First Affiliated Hospital of Zhejiang Chinese Medical University. For xenograft experiments, control or miR-181b overexpressing cells (2×10^7 cells/mouse) were subcutaneously injected into the right posterior flank (n=6/group). After 3 days injection, tumor size was measured with Vernier calipers every 3 d. When tumor size reached to 200 mm^3, half of the tumor-bearing mice were given Rg3 (20 mg/kg) or isovolumetric PBS by gavage once a day, respectively. At the 21th day after originally subcutaneous injection, tumors were removed for the next experiments. Tumor volume was calculated as 0.5 × length × $width^2$.

Cell viability assay

Transfected GBC-SD cells were seeded in 96-well plate at the density of 2×10^3 cells/well. After incubation overnight, cells viability were detected by CCK-8 kit according to the manufacture's instruction (CA1210, Solarbio, China) after transfection for 0, 24 and 48 hours. The absorbance was measured at the wavelength of 450 nm by a Multiscan plate reader (SynergyTM H1, BioTek, USA).

Cell apoptosis assay

Transfected GBC-SD cells were cultured at 6-well plate at the density of 2×10^4 cells/well. 24 h later, cells were digested with 0.25% trypsin without EDTA. For drug exposed experiment, Rg3 (100 μM) or Solvent control were administrated in GBC-SD cells for 24 h. Then cells were stained with Annexin V-FITC/PI according to the direction of Apoptosis Detection Kit (CA1020, Solarbio, China). The proportion of cell apoptosis was assessed by flow cytometry (CytoFLEX, BECKMAN, USA).

Western blotting

Total protein were extracted by using RIPA protein lysate (P0013B, Beyotime, China), and its concentration was determined using a BCA kit (P0009, Beyotime, China). 30 μg total protein were separated by 10% SDS-PAGE, then transferred to an activated PVDF (IPVH00010, Millipore, Thermo Scientific, USA) and blocked in 1x TBST with 5% fat-free milk for 1 h. Membranes were incubated with objective primary antibodies overnight at 4°C. After washed with TBST, membranes were incubated with Goat Anti-Rabbit IgG (H+L)/AffiniPure secondary antibody or Goat Anti-Mouse IgG (H+L) (1:10000, Jackson Immuno Research, US) secondary antibody at room temperature for 1 h. Finally, protein bands were captured by using C-DiGit Blot Scanner (LI-COR) after incubated with ECL (NCI5079, Thermo Scientific, US). Primary antibodies information were as follows: CREBRF (ab26262, Abcam, USA), CREB3 (ab78182, Abcam, USA), LC3 (PM036, MBL, USA), atg5 (ab228668, Abcam, USA), Cleaved Caspase-3 (ab2302, Abcam, USA), β-Actin (ab8224, Abcam, USA). All samples were performed at least 3 independent experiments.

RNA extraction and qRT-PCR

Total RNA was extracted using Trizol kit (H10318, Transgen Biotech, China) and were reverse-transcribed into cDNA by using cDNA reverse transcription kit (no. 4368813, Applied Biosystems, USA) according to the manufacturer's protocol. Real-time quantitative PCR (qRT-PCR) was performed to measure mRNAs expression of target genes by using SYBR Green (AQ131-01, Transgen Biotech, China). All data were normalized to the control of U6 or GAPDH. Primer sequences were as follows: U6: 5'-CGCAAGGATGACACGCAAATTC-3'; Has-mir-181b-5p: 5'-AACATTCATTGCTGTCGGTG-3'; hCREBRF: forward: 5'-ACCCACTTCAAGCACACAAAT-3', reverse: 5'-GGGTTGATCTTTACCTTTG-

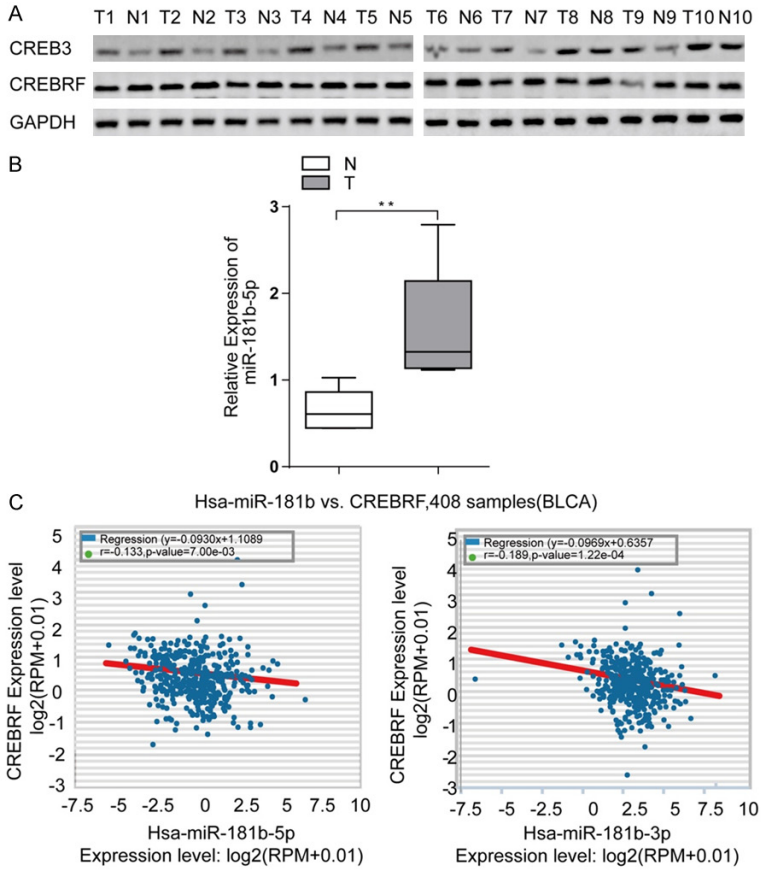

Figure 1. The relationship of miR-181b, CREBRF and CREB3 in human gallbladder carcinoma samples. A. CREBRF and CREB3 protein levels in 10 pairs of GBC tissue (T) and para-carcinoma tissue (N) determined by Western blotting. n=10/group. B. The mRNA expression of miR-181b in tumor and para-carcinoma tissue analyzed by qRT-pCR. n=10/group. C. Correlation analysis of CREBRF and hsa-miR-181b-5p/-3p in COSMIC Database. **Indicates tumor tissue (T) vs nontumor tissue (N). Data represent the means ± SD of three experiments, each performed in triplicate. **, $P<0.01$.

CCT-3': hGAPDH: 5'-GCCTTCCGTGTCCCCACT-GC-3'.

Immunofluorescence

In brief, cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized by 0.5% Triton X-100 for 20 min at room temperature. The fixed cells were blocked with 5% goat serum at room temperature for 30 min, and then incubated with LC3 antibody (1:1000, PM036, MBL, China) at 4°C overnight followed by secondary antibody (#5366, Cell Signaling Technology, USA) for 1 h at room temperature. Finally, cells were washed by PBS for 5 min × 3 times, and then added with DNA intercalating dye (DAPI) to visualize the cell nucleus. The LC3-positive cells were observed under a laser confocal microscope (C2, Nikon, Japan).

Dual-luciferase assay

The human 3'UTR containing miR-181b binding site and mutation 3'UTR of the CREBRF were synthesized by PCR amplifcation and inserted into the pYr-MirTarget basic vector. 293T cells were plated in 24-well plates at a density of 2×10^5 cells/well. After incubation overnight, cells were transfected with 100 nM miR-181b mimic or negative controls, followed by co-transfection with WT or mutant 3'UTR of CREBFR plasmids. Luciferase activities were performed with Luciferase Reporter Detection kits (E1910, Promega, USA) at 48 h post-transfection. Each experiments were duplicated at least 3 times.

Statistical analyses

All experiments were performed at least 3 times. Association analysis was performed by Pearson's coefficient using SPSS software. Numerical data are presented as means ± SEM, and these data were statistically analyzed by a one-tailed Student's t test or one-way ANOVA by using GraphPad Prism 5.0 software. Statistically significant differences were accepted at $P<0.05$.

Results

CREBRF is negative correlation with miR-181b and CREB3 in GBC

CREBRF is a potent tumor suppressor by regulating autophagy process. To ascertain the role of CREBRF on GBC, we firstly evaluated its expression pattern in GBC patients. As shown in **Figure 1A**, protein levels of CREBRF was vividly reduced in GBC tissue compared to paired-para-carcinoma tissues (8 of 10, **Figure 1A**, middle bands). As a negative regulatory target of CREBRF, CREB3 showed a significant increase in most of tumor tissues (8 of 10) (**Figure 1A**, upper bands). Interestingly, the expression

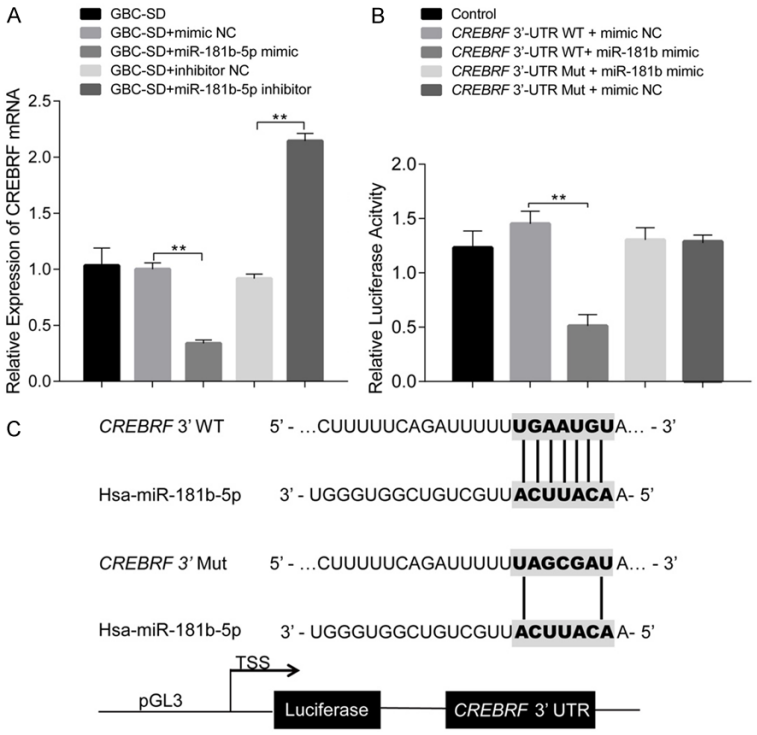

Figure 2. Effect of miR-181b on transcription activity of CREBRF. A. The expression of CREBRF mRNA determined by qRT-pCR after treated with miR-181b mimic or miR-181b inhibitor in GBC-SD cells. GBC-SD: control group. GBC-SD-mimic NC: mimic negative control group. GBC-SD-miR-181b mimic: cells transfected with miR-181b mimic. GBC-SD-inhibitor NC: inhibitor negative control group. GBC-SD-miR-181b inhibitor: cells transfected with miR-181b inhibitor. B. Luciferase reporter plasmids were constructed as described in the materials and methods, and relative luciferase activity was analyzed after co-transfection of the wild-type and mutant plasmids or a mock reporter plasmid into 293 T cells that were infected with miR-181b mimic or negative control. C. The binding sites of wild type and mutated sequences of CREBRF with has-miR-181b-5p. TSS, transcriptional start site. **, $P<0.01$.

pattern of miR-181b presented a contrary tendency with CREBRF, showing a notable increase in 93 GBC patients in contrast to that in para-carcinoma tissues (**Figure 1B**). Next, combined online cancer database, we further explored the correlation between CREBRF and miR-181b. The results showed that CREBRF was negatively correlated with both hsa-miR-181b-5p and hsa-miR-181b-3p in 408 GBC clinical samples of COSMIC (Catalogue of Somatic Mutations in Cancer) database (**Figure 1C**). Therefore, abnormal expression of miR-181b, CREBR and CREB3 possibly participate in the progression of GBC, and there are significant negative correlation between CREBRF and hsa-miR-181b in GBC patients.

CREBRF is a direct target of miR-181b

To explore the direct relationship between miR-181b and CREBRF, we measured the transcription level of CREBRF in GBC-SD cells transfected with miR-181b mimic and inhibitor for 48 h. qRT-PCR results showed that relative mRNA expression of CREBRF was notably declined in miR-181b mimic group while miR-181b inhibitor significantly enhanced the expression of CREBRF compared with control groups (**Figure 2A**). Besides, we discovered that there had the potential binding sites between miR-181b and CREBRF. We then performed luciferase reporter assay with plasmids of wild-type (WT) or mutant 3'UTR of CREBRF in the presence of miR-181b mimic or miR-181b mimic negative control (miR-181b mimic NC). As shown in **Figure 2B**, miR-181b mimic transfection significantly decreased the transcription activity of CREBRF in 3'-UTR-WT plasmid transfected group compared with miR-181b mimic NC group. In contrast, there showed no any inhibition effect on the reporter gene activity of CREBRF in cells exposed with 3'-UTR-Mut reporter plasmid (**Figure 2B** and **2C**). Thus, the data indicates that miR-181b downregulated CREBRF expression by directly targeting CREBRF.

MiR-181b enhanced cell viability and inhibited cells apoptosis in gallbladder carcinoma cells

Previous study have demonstrated that miR-181b accelerates cell survival and represses apoptosis by targeting adenylyl cyclase 9 (AC9) or large tumor suppressor (LATS2) in cervical cancer and ovarian cancer, respectively [19, 20]. However, the biological functions of miR-181b on GBC have not been reported currently.

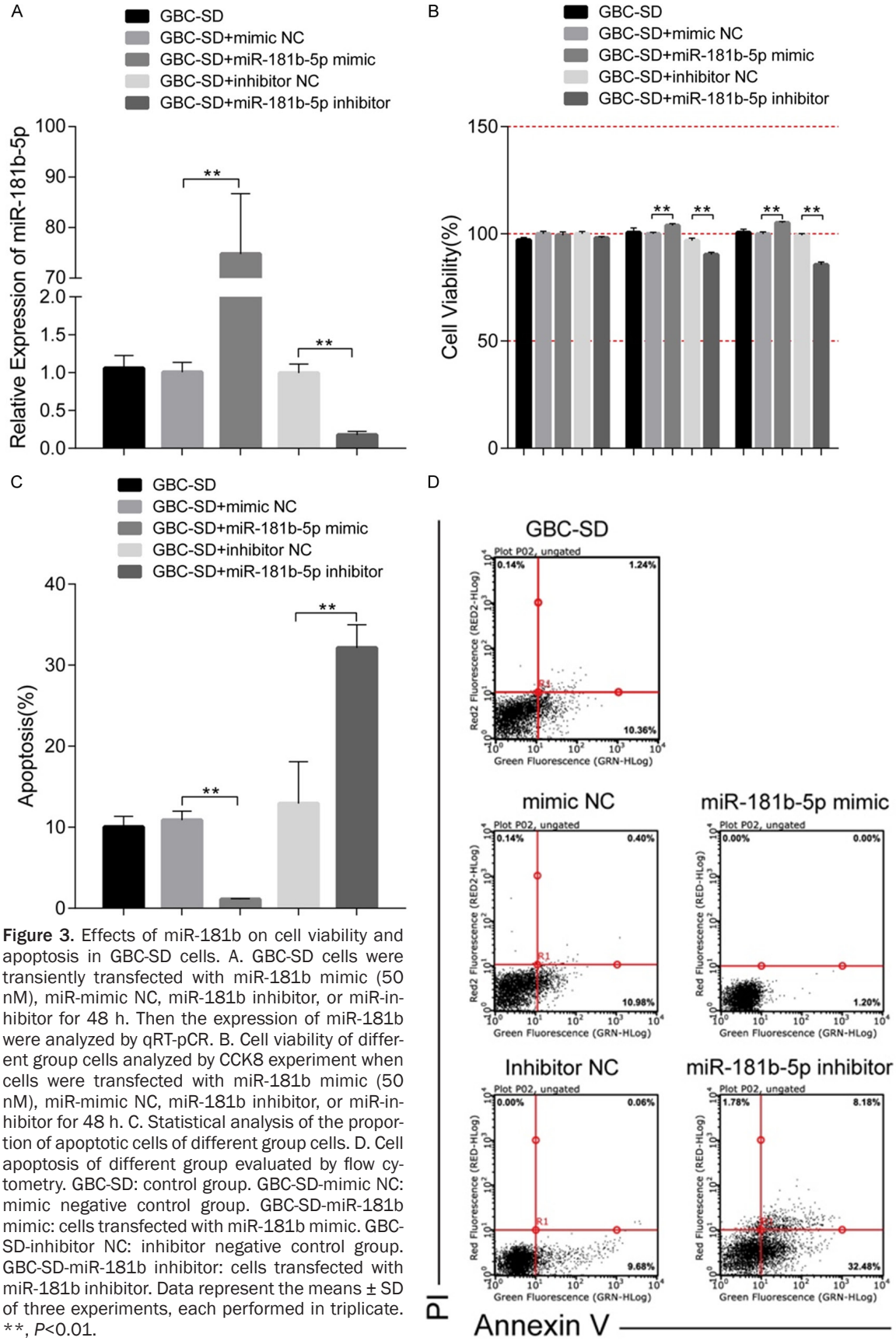

Figure 3. Effects of miR-181b on cell viability and apoptosis in GBC-SD cells. A. GBC-SD cells were transiently transfected with miR-181b mimic (50 nM), miR-mimic NC, miR-181b inhibitor, or miR-inhibitor for 48 h. Then the expression of miR-181b were analyzed by qRT-pCR. B. Cell viability of different group cells analyzed by CCK8 experiment when cells were transfected with miR-181b mimic (50 nM), miR-mimic NC, miR-181b inhibitor, or miR-inhibitor for 48 h. C. Statistical analysis of the proportion of apoptotic cells of different group cells. D. Cell apoptosis of different group evaluated by flow cytometry. GBC-SD: control group. GBC-SD-mimic NC: mimic negative control group. GBC-SD-miR-181b mimic: cells transfected with miR-181b mimic. GBC-SD-inhibitor NC: inhibitor negative control group. GBC-SD-miR-181b inhibitor: cells transfected with miR-181b inhibitor. Data represent the means ± SD of three experiments, each performed in triplicate. **, $P<0.01$.

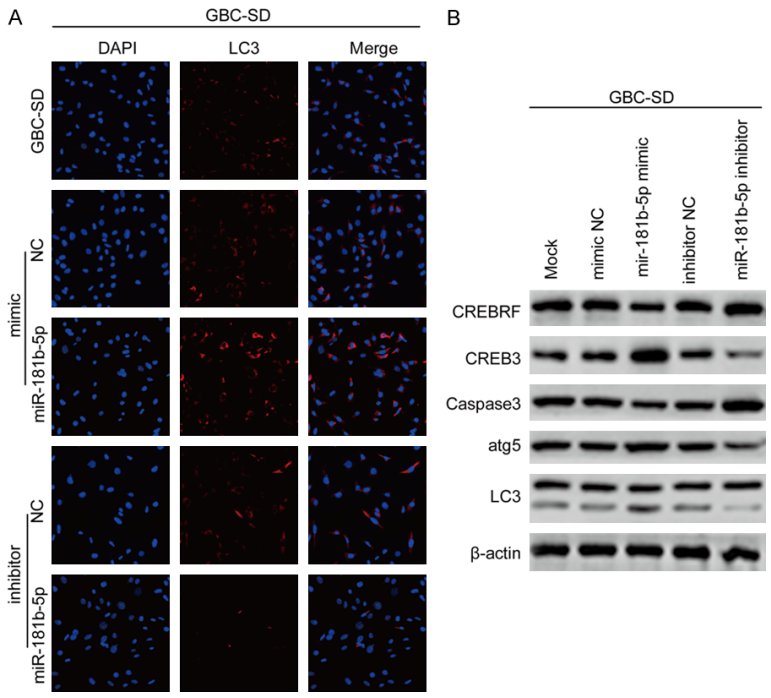

Figure 4. Effects of miR-181b on autophagy in GBC-SD cell. A. LC3 protein expression in different groups determined by immunofluorescence assay. GBC-SD cells were treated with miR-181b mimic (50 nM), miR-mimic NC, miR-181b inhibitor, or miR-inhibitor for 48 h. B. Protein expression of CREBRF, CREB3, atg5, LC3, and caspase 3 measured by western blotting. β-actin was used as a loading control. GBC-SD: control group. GBC-SD-mimic NC: mimic negative control group. GBC-SD-miR-181b mimic: cells transfected with miR-181b mimic. GBC-SD-inhibitor NC: inhibitor negative control group. GBC-SD-miR-181b inhibitor: cells transfected with miR-181b inhibitor. Multiple images were taken and representative one was presented. Scale bar: 20 μm.

Here we tried to investigate the effectives of miR-181b on tumor biologic characteristics of GBC-SD cells. Firstly, transfection efficiency of miR-181b mimic and miR-181b inhibitor were evaluated by qRT-PCR (**Figure 3A**). Through CCK8 assay, we observed that growth ability of GBC-SD cells transfected with miR-181b mimic showed a significant acceleration compared with control mimic at 24 h and 48 h post-transfection. However, miR-181b inhibition markedly restrained cell viability of GBC-SD cells compared with control cells (**Figure 3B**). Furthermore, we determined the impact of miR-181b on cell apoptosis of GBC-SD cells. After incubation with miR-181b mimic 48 h, the proportion of apoptotic cells were vividly reduced, which was significantly enhanced in cells exposed with miR-181b inhibitor compared with control groups (**Figure 3D**). Quantitative analysis on cell apoptosis rate further confirmed that miR-181b upregulation protected the GBC-SD cells from apoptosis (**Figure 3C**). These observations indicate that miR-181b might facilitate the carcinogenesis in the development of GSC.

MiR-181b promotes autophagy by regulating CREBRF/CREB3 pathway

Autophagy plays an important role in numerous cellular process including cell proliferation and apoptosis [21, 22]. Thus we next assessed the alteration of autophagy flux in the process of miR-181b-mediated proliferation disorder. In immunofluorescence assay, we detected that increased expression of miR-181b markedly promoted the protein level of microtubule-associated protein 1A/1B-light chain 3 (LC3) which was an adaptor protein for selective autophagy [23]. However, miR-181b reduction significantly inhibited the expression of LC3 compared with control GBC cells (**Figure 4A**). Immunoblotting results also showed that miR-181b mimic significantly enhanced, while miR-181b inhibitor reduced the expression of autophagy associated proteins atg5 and LC3, suggesting that miR-181b launched autophagy flux of GBC-SD cells. Accompanied the alteration of autophagy, miR-181b mimic transfection markedly decreased, but miR-181b inhibition accelerated the level of apoptosis-related protein caspase 3. Besides, cells exposed with miR-181b mimic inhibited the expression of CREBRF, and miR-181 inhibitor promoted its expression. However, the role of miR-181b on CREB3 showed the opposite trend with CREBRF (**Figure 4B**). These data indicates that miR-181b-mediated autophagy possibly was associated with the apoptosis inhibition effect of miR-181b.

MiR-181b blunts the anti-tumor effect of Ginsenoside Rg3 by upregulating autophagy

Ginsenoside Rg3 has been widely used in the treatment of various cancers by modulating

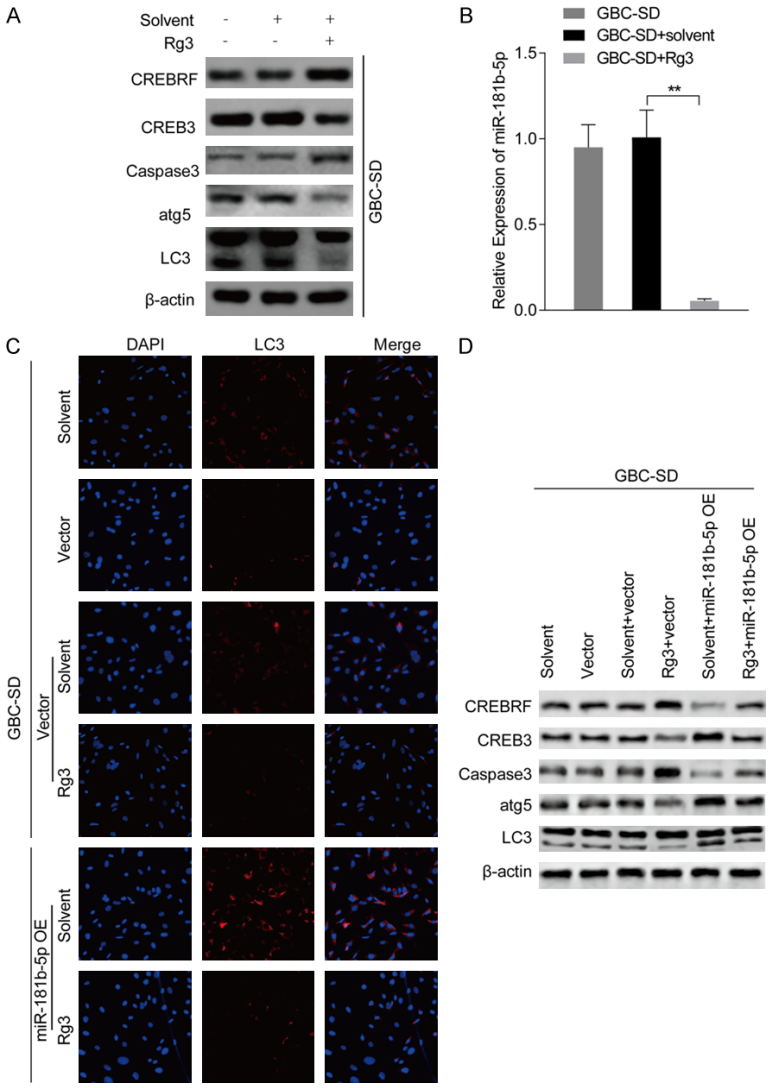

Figure 5. Effects of miR-181b on Rg3-induced cytotoxic in GBC-SD cell. A. Cells were pretreated with Rg3 and protein expression of CREBRF, CREB3, atg5, LC3, and caspase 3 were evaluated by western blotting. B. The expression of miR-181b after pre-treated with Rg3. C. LC3 protein expression from control and miR-181b overexpressing were determined by IF staining. Cells were pretreated with or without Rg3 (100 μM) for 48 h. D. The expression of CREBR, CREB, caspase3, atg5, and LC3 in GBC cells with normal or overexpression miR181b was analyzed by Western blotting after Rg3 exposure. β-Actin was used as loading control. GBC-SD: control group. GBC-SD-mimic NC: mimic negative control group. GBC-SD-miR-181b mimic: cells transfected with miR-181b mimic. GBC-SD-inhibitor NC: inhibitor negative control group. GBC-SD-miR-181b inhibitor: cells transfected with miR-181b inhibitor. Multiple images were taken and representative one was presented. Scale bar: 20 μm.

autophagy [24]. In this study, we found that Rg3 exposure significantly inhibited cells viability by promoting cell apoptosis of GBC-SD cells (Figure S1A-C). Additionally, treatment with Rg3 notably reduced the fluorescence intensity of LC3 proteins compared to control GBC-SD cells (Figure S1D). Western blotting further demonstrated that Rg3 effectively inhibited autophagy flux and promoted apoptosis by decreasing the protein expression of LC3 and atg5 and enhancing the activity of cleaved caspase 3. What was strikingly noticeable were the enhancement of CREBRF and the decline of CREB3 levels in the cells exposed with Rg3 (**Figure 5A**). Importantly, Rg3 administration also reduced the level of miR-181b, which implied that Rg3-mediated the increase of CREBRF was potentially through inhibiting miR-181b expression (**Figure 5B**). To verify the key role of miR-181b in that process, cells were pretreated with miR-181b overexpressing plasmid. As shown in Figure S2, Rg3-mediated the inhibition effect on cell viability and the promotion role on cell apoptosis were both blunted by miR-181b upregulation (Figure S2A-C).

We had confirmed that miR-181b-mediated carcinogenesis involvement with the alteration of autophagy. Thus, we also evaluated the changes of autophagy in cells transfected exogenous miR-181b in the presence of Rg3. As indicated in **Figure 5C**, control GBC-SD cells presented a moderate expression of LC3 in cytoplasm. Once treated with Rg3, little LC3-positive cells were observed (**Figure 5C**). However, miR-181b overexpression significantly boosted the level of LC3 compared with control group and partly rescued Rg3-mediated the inhibition on LC3 expression (**Figure 5C**). Besides, western blotting assay also demonstrated that Rg3-launched the decline of atg5, LC3 and CREB3, and the augment of CREBRF

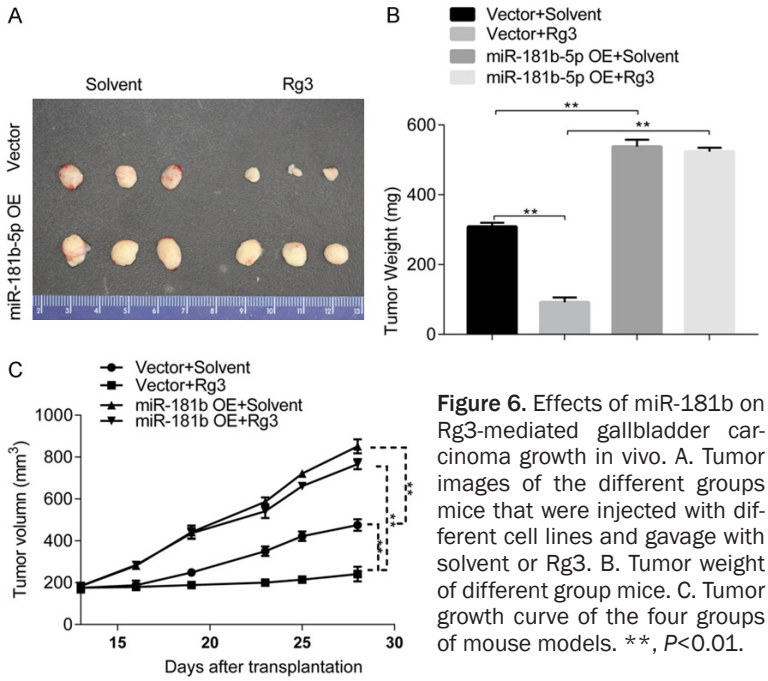

Figure 6. Effects of miR-181b on Rg3-mediated gallbladder carcinoma growth in vivo. A. Tumor images of the different groups mice that were injected with different cell lines and gavage with solvent or Rg3. B. Tumor weight of different group mice. C. Tumor growth curve of the four groups of mouse models. **, $P<0.01$.

and caspase 3 were blocked by miR-181b overexpression (**Figure 5D**). Based on our above results, we thought that Rg3-evoked cytotoxic effect on GBC-SD cells might be involved with miR-181b/CREBRF/CREB3 signaling pathway.

Exogenous miR-181b impairs Rg3-mediated tumor growth inhibition

In addition to *in vitro* study of cell lines, xenograft model was also been employed to investigate the role of miR-181b on Rg3-mediated the anti-tumor effect. Overexpression of miR-181b showed a higher weight than vector control. After administration with Rg3, tumor weight was obviously declined compared to control mice. However, miR-181b upregulation almost completely blocked the cytotoxicity of Rg3 on GBC tumor (**Figure 6A**, **6B**). Tumor growth curve also showed that cell grew faster in miR-181b upregulation group than control cells. Rg3 administration sharply declined the tumor growth rate, which was reversed by exogenous miR-181b (**Figure 6C**). These results indicate that overexpression of miR-181b powerfully promotes the progression of gallbladder carcinoma, and resist the anti-tumor effects of Rg3.

Discussion

Autophagy, a highly conserved mechanism of lysosome-mediated protein and organelle degradation, provides enough of valuable resource for tumor cells proliferation and defends cell apoptosis [25]. Targeting autophagy has been considered as a crucial therapeutic strategy for enhancing anti-tumor effect of clinical drugs. In the present study, we found a novel autophagy associated molecule CREBRF which might be served as a new potential target for therapy of GBC by modulating autophagy process.

CREBRF acting as an inhibitor of autophagy significantly inhibits the development of glioblastoma through CREB3/ATG5 pathway [26]. A research of malignant glioma showed that CREBRF were inhibited by miR-155-3p and significantly inhibited autophagy on human glioma cells [18]. In this study, we found that the expression of CREBRF in GBC tumor tissues is lower than para-carcinoma tissue. miR-181b was negative correlate with CREBRF, which implied that CREBRF may be regulated by miR-181b. Thus, miR-181b/CREBRF pathway possibly participated in the development of GBC. Oncogene roles of miR-181b have been reported in colorectal cancer (CRC) [27], B-cell acute lymphoblastic leukemia (B-ALL) [28], non-small cell lung cancer (NSCLC) [29], breast cancer [30, 31], glioma [32], and esophageal cancer [33]. miR-181b promoted cell survival, clone formation, migration and invasion of HCC cells by binding metalloprotease 3 (TIMP3) [34]. Increased miR-181b enlarged cell proliferation and migration and suppressed apoptosis of CRC cells by targeting Programmed cell death 4 (PDCD4) [35]. But the role of miR-181b in the progression of GBC have not been reported previously. Here, we identified the oncogenic role of miR-181b by using GBC-SD cell lines. Overexpressed miR-181b promoted proliferation, autophagy, xenograft tumor growth in nude mice, and inhibited cell apoptosis by directly targeting CREBRF/CREB3. Based on this, miR-181b/CREBRF/CREB3 axis-launched autophagy flux promoted the progression of GBC.

Rg3 exerts antitumor effects on several types of tumor [36-38]. For instance, Rg3 significantly inhibited cell viability, and accelerated apop-

tosis rate of lung cancer cells by inhibiting PI3K/Akt signaling pathways [39]. Rg3 inhibited the development of ovarian cancer by targeting miR-145 [40]. Warburg effect in ovarian cancer cells were blocked by Rg3 through activating miR-603 [41]. In GBC, Rg3 activated endoplasmic reticulum stress in GBC-SD cells, leading to apoptosis and cell proliferation inhibition [42, 43]. However, the underlying regulatory mechanism of Rg3 on gallbladder cancer remains understood. In this study, we demonstrated that in addition to effects of Rg3 on cells growth and cell apoptosis, it also reduced autophagy flux through inhibiting miR-181b/CREBRF/CREB3 pathway. However, anti-tumor effects of Rg3 were blocked in the presence of exogenous miR-181b in *in vitro* and *in vivo*. These results demonstrate that miR-181b mediates the anti-tumor effects of Rg3 on GBC.

Conclusions

In summary, this study revealed that miR-181b plays an oncogenic role in the progression of gallbladder carcinoma by promoting autophagy via CREBRF/CREB3 signaling pathways. miR-181b acted as a key switch in the process of Rg3-evoked anti-tumor effects of GBC *in vitro* and *in vivo*. Collectively, miR-181b, as an crucial moderator for autophagy in the progression of GBC is the key factor for Rg3-medaited cytotoxic.

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Disclosure of conflict of interest

None.

Abbreviations

GBC, Gallbladder cancer; COSMIC, Catalogue of Somatic Mutations In Cancer; CQ, Chloroquine; Rg3, Ginsenoside Rg3; EMT, epithelial-mesenchymal transition; CREB3, cAMP responsive element binding protein 3; CREBRF, CREB3 regulatory factor; LRF, Luman regulatory factor; GC, gastric cancer; EECs, endometrial epithelial cells; CRC, colorectal cancer; B-ALL, B-cell acute lymphoblastic leukemia; NSCLC, non-small cell lung cancer; PDCD4, Programmed cell death 4; TIMP3, metalloprotease 3.

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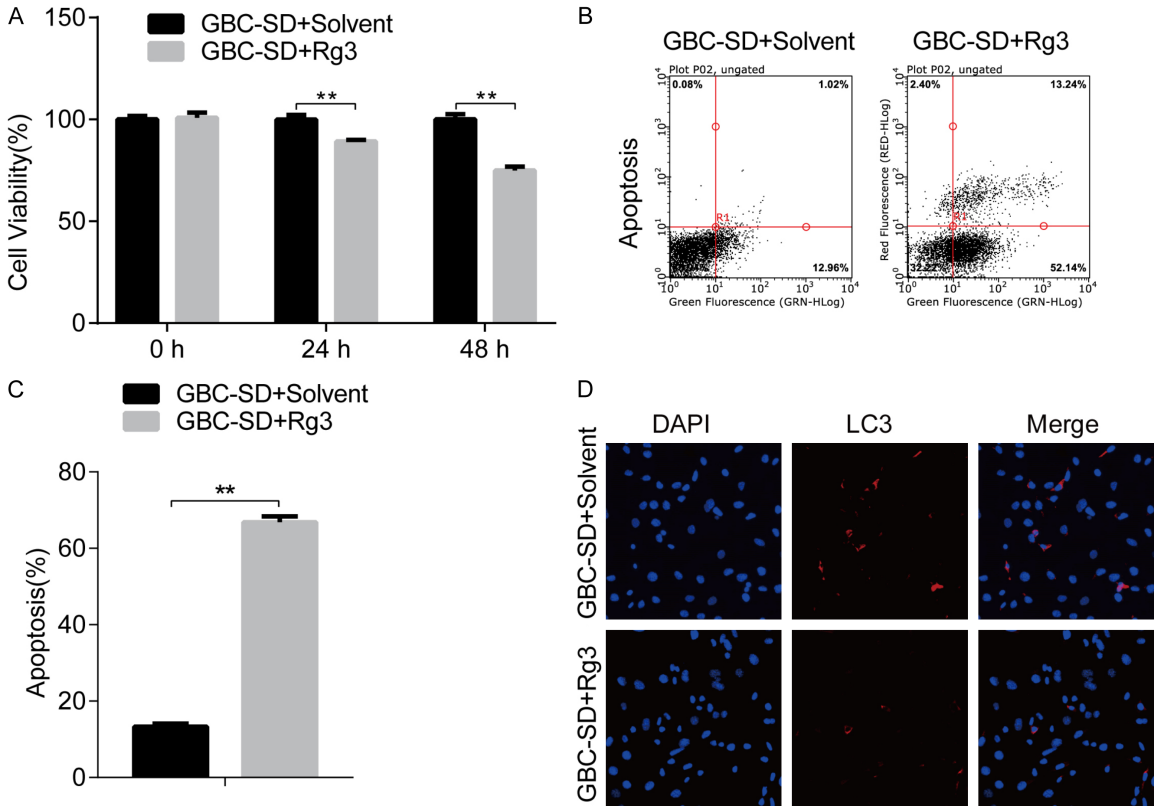

Figure S1. Effects of Rg3 on the cell viability, apoptosis and autophagy of GBC-SD cells. A. Cell viability measured by CCK8 assay in GBC-SD cells exposed with solvent or Rg3. B. Cell apoptosis of different GBC-SD cells analyzed by FACS. C. Quantitative analysis of the proportion of apoptotic cells after treatment with solvent or Rg3. D. LC3 expression in GBC-SD cells treated with solvent or Rg3 was determined by IF staining. Scale bar: 20 μm. **, $p<0.01$.

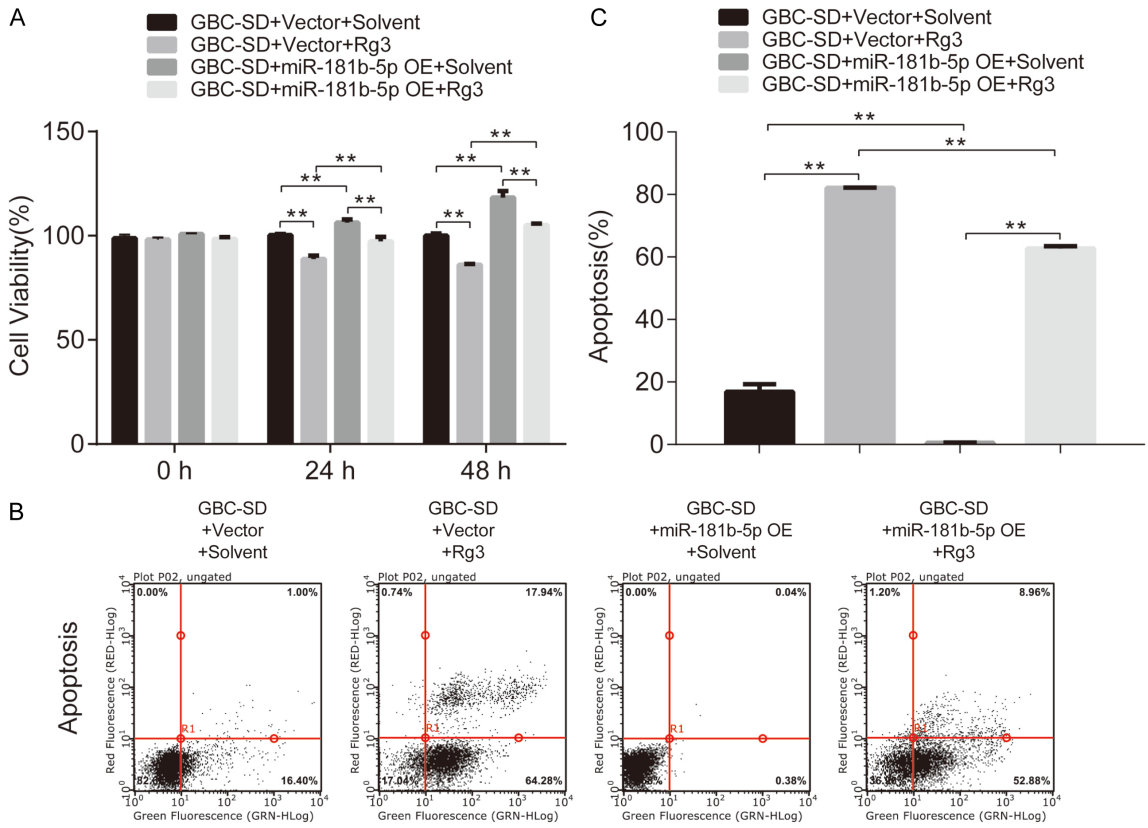

Figure S2. The role of miR-181b upregulation on Rg3-mediated proliferation inhibition and apoptosis enhancement. (A) GBC-SD cells transfected with NC or miR181b were treated with solvent or Rg3 (100 μM) for 48 h and then cell viability was detected by CCK-8 assays. (B) Cell apoptosis analyzed by flow cytometry in the cell groups of (A). (C) Quantitative analysis the percentage of apoptotic cells after different treatment. **, $p<0.01$.