

Original Article

MiR-148a induces apoptosis by upregulating BIM expression in gastric cancer cells

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Abstract: Background and objective: Gastric cancer is one of the most common malignant diseases worldwide. MicroRNAs (miRNAs) play crucial roles in the regulation of apoptosis. However, the regulation of gastric cancer (GC) apoptosis by miRNAs has not been intensively investigated. To address this issue, the effect of miR-148a on the cell proliferation of gastric cancer SGC7901 cells was characterized in the present study. Methods: We analyzed the expression of miR-148a in gastric cancer SGC7901 cell lines and normal gastric mucosal epithelial GES-1 cell. We examined the effect of miR-148a mimics on the apoptosis and growth of SGC7901 cells *in vitro* by flow cytometry (FCM) assays, cell cycle analysis, MTT cell viability assay and colony formation assay. Nude mouse xenograft model was used to determine whether miR-148a is involved in tumorigenesis of gastric cancer. The miR-148a target of BIM was identified by western blot analysis and immunohistochemistry. Cell apoptosis was detected by TUNEL assay. Results: MiR-148a was significantly downregulated in SGC7901 cells compared with GES-1 cells. Overexpression miR-148a with miR-148a mimics (miR-148a) initiated G0/G1 cell-cycle arrest, apoptosis and inhibited growth of SGC7901 cells *in vitro*, and miR-148a could significantly inhibit tumorigenicity of SGC7901 by increasing the apoptosis proportion of cancer cells *in vivo*. Moreover, BIM was identified as the potential target of miR-148a. Silencing of BIM was able to photocopy the effect of miR-148a overexpression on apoptosis regulation of cancer cells, indicating BIM is potentially involved in miR-148a-induced apoptosis on cancer cells. Conclusions: MiR-148a may function as a novel tumor suppressor gene in gastric cancer by targeting BIM and regulating the apoptosis of cancer cells. MiR-148a could serve as a potential biomarker and therapeutic target against gastric cancer.

Keywords: Gastric cancer, apoptosis, miR-148a, BIM

Introduction

Gastric cancer (GC), with high mortality, was one of the most malignant cancers in Asian countries. Its occurrence was a process with multi-factors and multi-steps, involved in alterations in many molecules, including activation of proto-oncogenes, inactivation of tumor-suppressor genes, alterations in cell cycle related proteins and so on [1]. In the past decade, a great number of proto-oncogenes and tumor-suppressor genes have been found. In spite of the sizable number of genes already described, new genes with oncogenic potential or tumor-suppressing activities are still being identified. However, the molecular mechanism of gastric carcinogenesis remains unclear.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs of about 19-25 nucleotides.

Through specifically pairing with complementary sites in 3' untranslated regions (UTRs) of target mRNAs, they mediate post-transcriptional silencing. MicroRNAs have been implicated in many physiological processes including proliferation, differentiation, development, apoptosis, and metabolism [2, 3]. In recent years many studies have revealed that the aberrant expression of miRNA is closely related to oncogenesis and is now an intense field of study.

In recent years, studies have indicated that aberrantly expressed miRNAs contributed to the initiation and progression of GC [4]. MiR-148a was significantly downregulated in GC cell-lines and in GC tissue samples, and thus served as a tumor suppressor [5, 6]. The down-regulated expression of miR-148a was significantly associated with tumor size, TNM stage and lymph node metastasis, progression and

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prognosis [7, 8]. Joshi et al has reported that enforced expression of miR-148a sensitized cells to TRAIL and reduced lung tumorigenesis in vitro and in vivo through the down-modulation of matrix metalloproteinase 15 (MMP15) and Rho-associated kinase 1 (ROCK1) [9].

In bladder cancer cells, overexpression of miR-148 leads to reduced cell viability through an increase in apoptosis rather than an inhibition of proliferation [10]. In GC cells, ectopic expression of miR-148a inhibited tumor cell proliferation and migration in vitro, and inhibited tumor formation in vivo [11]. However, the mechanisms of how miR-148a affects cell proliferation and apoptosis of GC cells is not clear.

Bcl-2 (B-cell lymphoma 2 gene) is an important molecule that can inhibit apoptosis as well as being able to promote oncogenesis. MiR-148a can induce tumor cell apoptosis by the targeted inhibition of Bcl-2 in CC and PC [12, 13]. Bcl-2-like protein 11 (BIM) is a pro-apoptotic factor that belongs to the Bcl-2 protein family BH3 group. BIM has been identified as a critical pro-apoptotic factor in solid tumors [14]. Kim et al. found that upregulated mir-148a could suppress BIM, which led to inhibition of glioma apoptosis, while downregulated mir-148a could increase BIM expression to induce apoptosis [15].

In the present study, we assessed the effect of miR-148a on apoptosis and growth of GC cells in vitro and vivo, and to explore its molecular mechanisms. Our findings demonstrate that miR-148a inhibits growth and induces apoptosis of GC cells at least in part via activation of the BIM. This study reveals a novel mechanism of miR-148a on growth and apoptosis of GC cells and may also provide a novel therapeutic target.

Materials and methods

Cell line and culture

Human gastric adenocarcinoma cell line SGC-7901 and normal gastric mucosal epithelial cell GES-1 were obtained from Shanghai Cell Bank (Shanghai, China) and preserved in our institute. The cells were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C with 5% CO₂ in a humidified incubator (Forma Scientific, Marietta, OH).

Transient transfection

MiR-148a mimics (MiR-148a) and negative control were purchased from Gene Pharma (Shanghai, China). SGC7901 cells in logarithmic growth phase were trypsinized, counted, and seeded in 6-well plates to ensure 50% cell confluence on the next day for transfection. Transfection of cells with oligonucleotides was performed using Lipofectamine™ 2000 Reagent in line with the manufacturer's instructions (Invitrogen) at a final concentration of 100 nM. To detect the effect of BIM in MiR-148a-induced apoptosis, siRNA plasmids of BIM were co-transfected into SGC7901 cells with MiR-148a or negative control for 48 h using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Stable miR-148a transfection

The genomic region that included the primary transcript of miR-24 was cloned into the EcoRI-XhoI site of the modified pMSCV-GW-RfA-PGK-EGFP retroviral vector. Negative control vectors had no insert. For each cultured 293 T plate (10 cm), a plasmid mixture containing 10 µg of miR-24 retroviral vector, 10 µg of gag/pol vector and 10 µg of VSVG vector was co-transfected with 90 µl FuGENE6 transfection reagent (Roche, Basel, Switzerland) added directly to 0.6 ml of serum-free medium. The plasmid/medium/FuGENE6 mix was added drop-wise to the 293 T plate. After 12 h, 15 ml viral collection medium was added to the transfected cells. We then harvested the virus twice a day for two days. Infections of SGC-7901 cells were performed in the presence of 8 µg/mL of polybrene in each well of a 6-well plate. SGC-7901 cells were spin infected at 1500 rpm for 0.5 h at room temperature and the virus-containing supernatant was removed after 2 h. Positive cells were selected by GFP expression by FACS and named as miR-24 or miR-control. Expression of miR-24 was confirmed by qRT-PCR.

MiRNA quantification by qRT-PCR

Total RNA (including miRNA) was isolated from cells or tissues using Trizol-based method (Invitrogen, Carlsbad, CA, USA). RNA concentrations were determined by NanoDrop 2000 (Wilmington, DE, USA). U6 RNA (U6F, 5'-CTCG-CTTCGGCAGCACATATACT-3'; U6R, 5'-ACGCTTCCGAATTTGCGTGTC-3'; U6 Taqman probe, 5'-

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FAM-CTTGCGCAGGGGCCATGCTAA-MGB-3') was used as an internal control of miRNA-148a. Specific reverse transcription primer of miRNA-148a and U6R primer were respectively used for reverse transcription with TaqMan® MicroRNA Reverse Transcription Kit in a total volume of 10 µl. Each reaction contained 1 µg RNA as template. Real-time PCR was performed respectively using specific PCR primers for miRNA-148a, primer pair U6F, and U6R for U6 RNA in TaqMan Universal PCR Master Mix according to the manufacture instruction with Light-Cycler® 480 System (Roche Applied Science, Mannheim, Germany). Each reaction was run in triplicate. The miRNA-148a abundance in each sample was normalized to U6 RNA reference. The expression of miRNA-148a was calculated with the $\Delta\Delta C_t$ method.

Western blot assay

After transfection, protein lysates were separated on NuPAGE 4-12% Bis-Tris gels (Thermo Fisher Scientific) and transferred onto PVDF membranes. Immunoblotting was conducted with diluted monoclonal anti-BIM antibodies (Abcam, Shanghai, China) and with diluted anti-GAPDH antibodies (1:5000, MAB374; Chemicon, Temecula, CA, USA). The membrane was washed and then incubated with goat anti-rabbit or mouse IgG (H+L)-HRP conjugate (Bio-Rad, Hercules, CA, USA). Specific complexes were visualized with an echo chemiluminescence (ECL) detection system (GE Health-care, Little Chalfont, UK).

MTT cell viability assay

Cells were seeded in 96-well plates (2000 cells per well). After cultured for 12 h, cells were transiently transfected with miR-148a or/and BIM siRNA or control for 2 days. Then, the cells were incubated in the medium containing a final concentration of 0.5 mg/ml of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma, Aldrich, MO, USA) solution at 37°C for 4 h. After removal of the supernatant, 150 µl DMSO (Thermo Scientific) was added to dissolve the crystals. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony formation assay

The stable miR-24 or miR-control transfected SGC7901 cells were trypsinized, counted and replated at a density of 500 cells/6 cm dish. 14

days later, colonies resulting from the surviving cells were fixed with 3.7% methanol, stained with 0.1% crystal violet and counted. Colonies containing at least 50 cells were scored. Each assay was performed in triplicates.

Flow cytometry assay

Apoptosis was detected using an FITC Annexin-V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. The cells were digested with 0.25% trypsin, washed with cold phosphate-buffered saline (PBS) twice, and resuspended in binding buffer (1×10^6 cells/ml). Then 100 µl of the cell suspension (1×10^5 cells) was incubated with 5 µl of Annexin-V FITC and 5 µl of propidium iodide (PI) for 15 min at room temperature in the dark. The population of apoptosis cells was analyzed by flow cytometry (BD FACSCalibur, Becton Dickinson, San Jose, CA, USA).

For cell cycle analysis, the cells were fixed with 70% ethanol and stored at 4°C overnight. The following day, the fixed cells were washed with PBS, treated with 2 µl of RNase A (50 µg/ml), and stained with 20 µl of Propidium Iodide (50 µg/ml) for 30 min in the dark at 37°C, with rocking every 5 min. The stained cells were analyzed by flow cytometry (FACSCalibur). At least 10000 cells in each sample were analyzed to obtain a measurable signal.

Tumorigenicity in nude mice

Tumor formation was carried out to assess the effects of miR-24 on tumorigenicity in vivo. BALB/c nude mice of 4 to 6 weeks were provided by Shanghai Cancer Institute and housed in microisolator cages under positive air pressure, and maintained at a constant temperature (22°C) and humidity for the tumorigenicity study. Approximately 3×10^6 cells (stably miR-24 or miR-control transfected SGC7901 cells) at log phase were collected and injected subcutaneously into the upper back of BALB/c nude mice. During a six-week follow-up period, the sizes of tumors were measured weekly. Mice were sacrificed at 42 days post-injection. Tumors were excised and measured. The short and long diameters of the tumors were measured using a caliper and tumor volumes (cm^3) were calculated by using the following standard formula: tumor volumes (cm^3) = (the longest diameter) × (the shortest diameter)² × 0.5. All procedures for animal experimentation were

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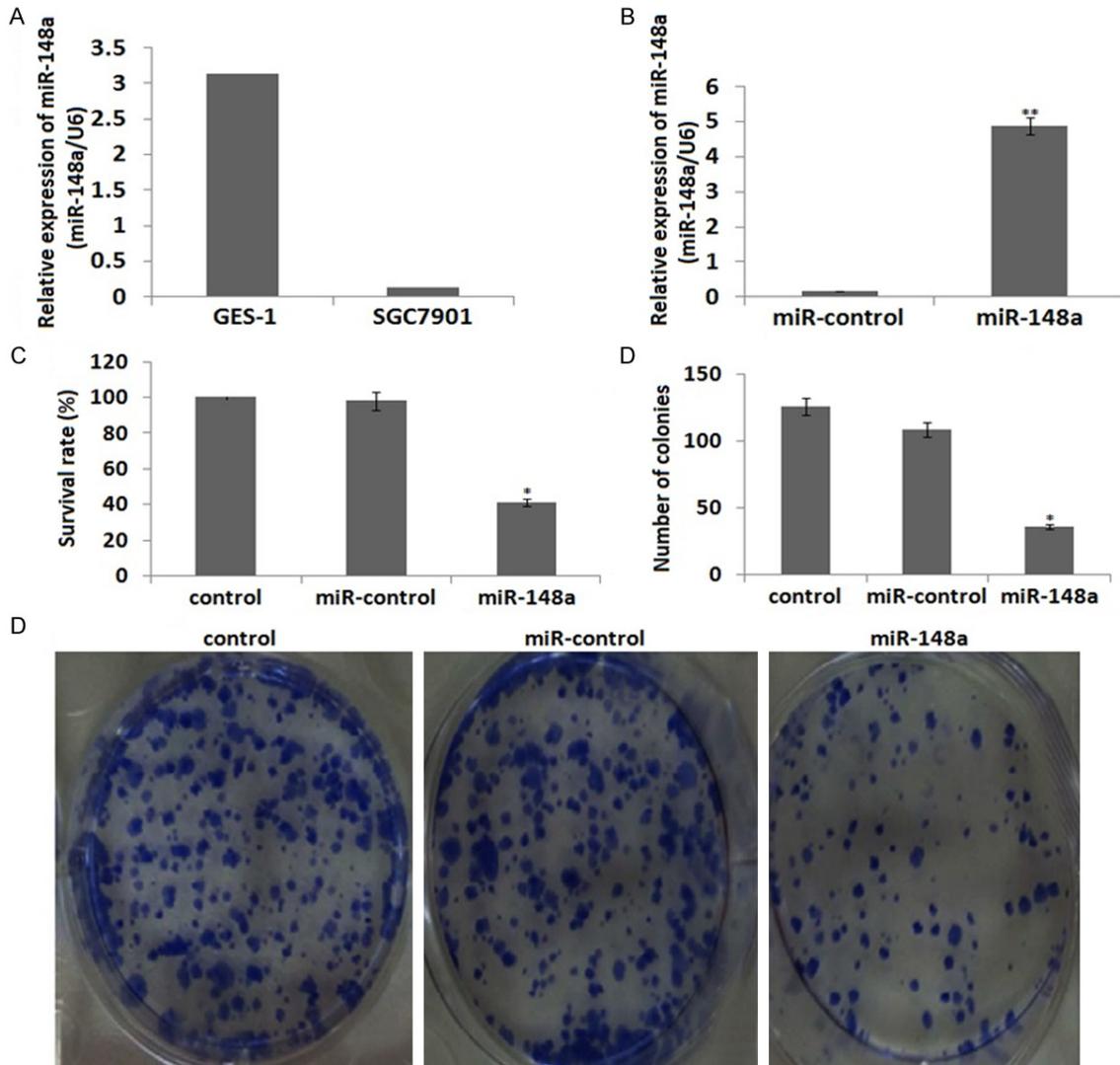


Figure 1. Effect of miR-148a on SGC-7901 cells. A. Relative expression of miR-148a in SGC-7901 cells is shown as compared with that in human normal gastric mucosal epithelial cell GES-1. B. Relative expression of miR-148a in SGC-7901 cells transfected with miR-148a is shown as compared with the SGC-7901 cells transfected with miR-control. C. Relative cell viability of SGC-7901 cells 48 h after transfection of miR-148a for 48 h. D. Colony formation of the stable miR-148a or miR-control transfected SGC-7901 cells for 2 weeks. *, $P < 0.05$; **, $P < 0.01$ using a two-tailed *t*-test.

performed in accordance with the Institutional Animal Care and Use Committee guidelines of the Experiment Animal center of the affiliated hospital of Qingdao University.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin blocks. Sections (4 μ m) were used for immunohistochemical examination. After deparaffinization and rehydration, antigen retrieval was performed by boiling in 10 mmol/l of citrate buffer (pH 6.0) for 10 min. After inhibition of endogenous peroxidase activ-

ity for 30 min with methanol containing 0.3% H_2O_2 , the sections were blocked with 2% bovine serum albumin in PBS for 30 min and incubated with mouse anti-BIM antibody. The immune complex was visualized by the Dako REAL™-EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse (Dako, Denmark), according to the manufacturer's procedures.

TUNEL assay

The detection of nuclei with fragmented DNA by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was

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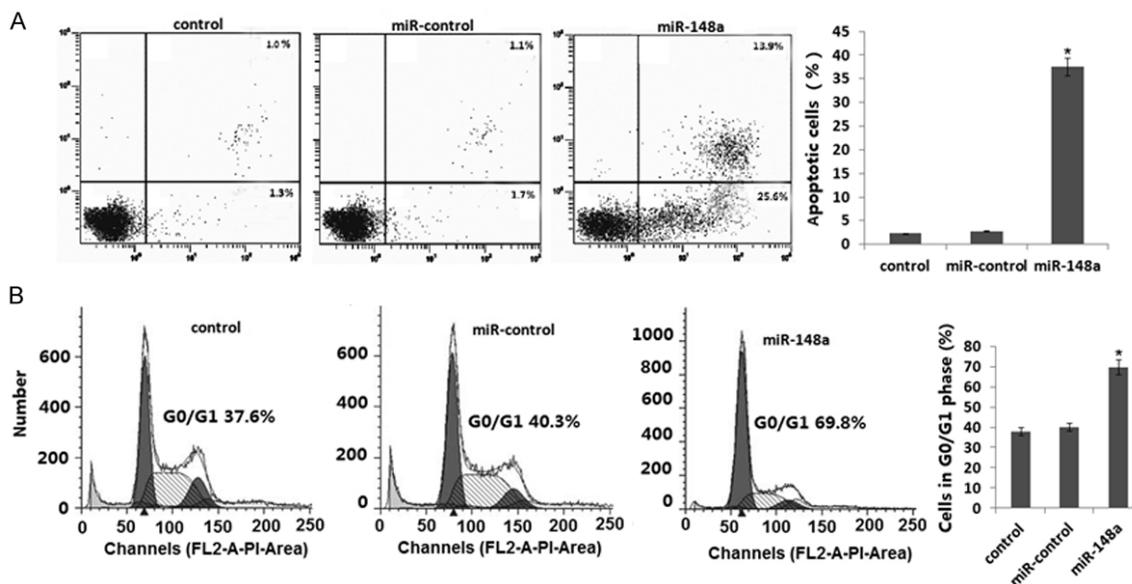


Figure 2. MiR-148a induced cell apoptosis and G0/G1 cell cycle arrest. A. Representative histograms depicting apoptosis of SGC-7901 cells transiently transfected with 100 nM miR-148a or miR-control. B. Representative histograms depicting cell cycle of SGC-7901 cells transiently transfected with 100 nM miR-148a or miR-control. The percentage of apoptotic and G0/G1 phase cells of three independent experiments, mean \pm S.D. (* $P < 0.01$).

accomplished using the ApopTag Peroxidase In situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. To quantify TUNEL-positive cells, the number of brown-positive cells was counted in 12 random fields at $\times 200$ magnification.

Statistical analysis

All values are shown as mean \pm SD. P values were calculated using student's two-tailed t test; $P < 0.05$ was considered significant.

Results

MiR-148a inhibits SGC-7901 cell proliferation and colony formation

To explore the role of miR-148a on cell proliferation, the expression of miR-148a in SGC7901 cells was detected by quantitative real-time RT-PCR (qRT-PCR). The results showed that expression of miR-148a was downregulated in SGC7901 cells compared with the immortalized normal gastric mucosal epithelial cell GES-1 (**Figure 1A**).

To investigate the role of miR-148a on cell proliferation of SGC7901 cells, we transfected SGC-7901 cells with miR-148a or miR-control for 48 h. Ectopic expression of miR-148a in SGC-7901 cells was confirmed by qRT-PCR.

Cell proliferation was detected by MTT assay. The results showed that miR-148a was enhanced 71 fold after miR-148a transfection (**Figure 1B**). Overexpression of miR-148a inhibited the growth rate of SGC-7901 cells compared with miR-control transfected cells ($P < 0.05$, **Figure 1C**).

The stable miR-148a or miR-control transfected SGC-7901 cells were collected and used for examining the effect of miR-148a on cell growth by colony formation assay. The number of colonies of miR-148a/SGC-7901 cells was smaller as compared to those of the miR-control/SGC-7901 cells ($P < 0.05$, **Figure 1D**). Together, these results provided strong evidence that miR-148a inhibits GC cell growth.

MiR-148a induces SGC-7901 cell apoptosis

Given that observed cellular growth may be affected by the rates of apoptosis and cell cycle analysis, we examined the effects of miR-148a on apoptosis *in vitro* by flow cytometry. Flow cytometry showed that the apoptotic rate was significantly increased in SGC-7901/miR-148a cells compared with miR-control cells (**Figure 2A**, $P < 0.01$). To further elucidate the mechanism of miR-148a-mediated growth inhibition of SGC-7901 cells, cell cycle analysis was performed (**Figure 2B**). Upon upregulation of miR-148a, the percentage of cells in G0/G1 phase

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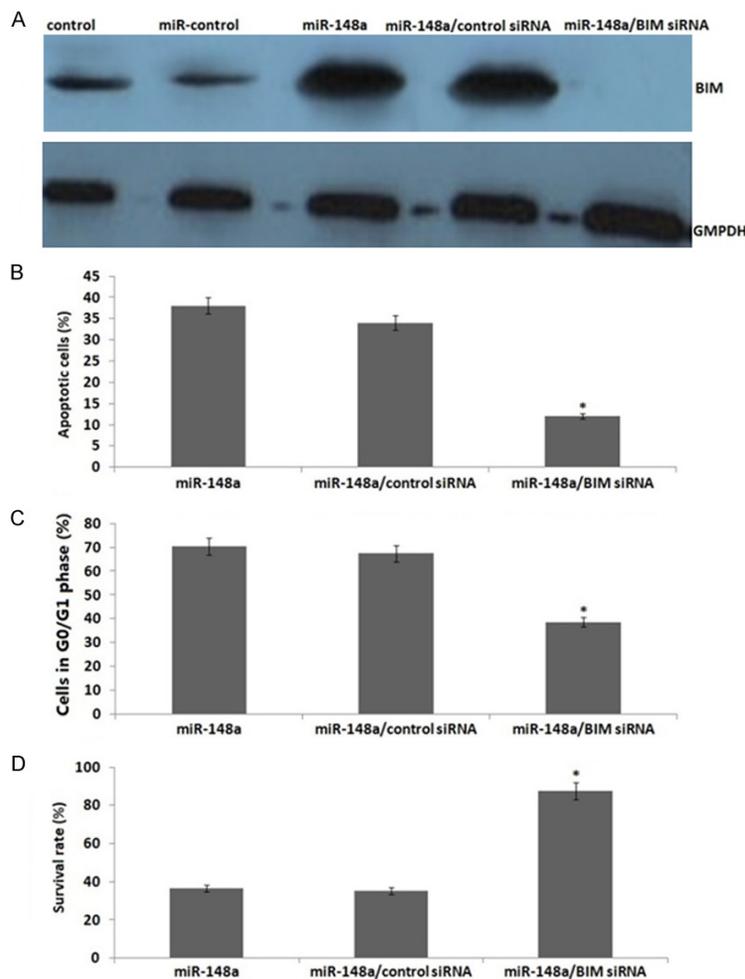


Figure 3. BIM-dependent apoptosis induced by miR-148a in SGC-7901 cells. A. BIM protein expression was detected by western blot assay in SGC-7901 cells; B. Cell apoptosis was detected by Flow cytometry assay; C. G0/G1 phase cells was detected by Flow cytometry assay; D. Relative cell viability of SGC-7901 cells 48 h after co-transfection of miR-148a and BIM siRNA for 48 h. *, $P < 0.01$ using a two-tailed *t*-test.

increased from $37.64\% \pm 3.27\%$ in controls to $69.8\% \pm 3.41\%$ in SGC-7901/miR-148a ($P < 0.01$).

BIM-dependent apoptosis induced by miR-148a in SGC-7901 cells

We first investigated the effects of miR-148a on *BIM* expression in SGC-7901 cells. Low *BIM* expression was found in the SGC-7901 cells by western blot assay. MiR-148a transiently transfected into SGC-7901 cells for 48 h significantly induced *BIM* protein expression (Figure 3A).

To detect the effect of *BIM* on miR-148a-induced apoptosis in SGC-7901 cells, miR-148a

and *BIM* siRNA or control siRNA was co-transfected into the SGC-7901 cells for 48 h. With *BIM* siRNA transfection, miR-148a-induced *BIM* upregulation was inhibited (Figure 3A). In addition, miR-148a-induced apoptosis was reversed by *BIM* siRNA transfection (Figure 3B). Cell cycle analysis showed after *BIM* siRNA transfection, the percentage of cells in G0/G1 phase decreased from $70.1\% \pm 4.36\%$ in miR-148a groups to $38.3 \pm 3.87\%$ in miR-148a/*BIM* siRNA groups ($P < 0.01$) (Figure 3C). MTT assay showed that miR-148a-induced cell proliferation was also restored by *BIM* siRNA transfection (Figure 3D).

MiR-148a inhibits tumor growth in vivo

We first investigated the efficacy of miR-148a against tumor growth *in vivo*. Stable transfection of miR-148a into SGC-7901 cells resulted in decreased growth of subcutaneous xenograft tumors in nude mice, when compared to those stably transfected with empty vector (NC) (Figure 4A). In addition, the miR-148a levels within tumors were increased (Figure 4B), and the intratumoral expression of *BIM* and E-cadherin was also increased by stable transfection of miR-148a by immunohistochemistry assay (Figure 4C, 4D). Moreover, stable transfection of miR-148a resulted in increase of apoptosis within tumors (Figure 4E). These results suggested that miR-148a could inhibit the growth of gastric cancer cells *in vivo*.

Discussion

Although deregulation of miRs has been observed in gastric cancer tissues and cell lines, the exact molecular mechanism by which miRNAs modulate the process of tumorigenesis is not yet fully elucidated. To date, a series

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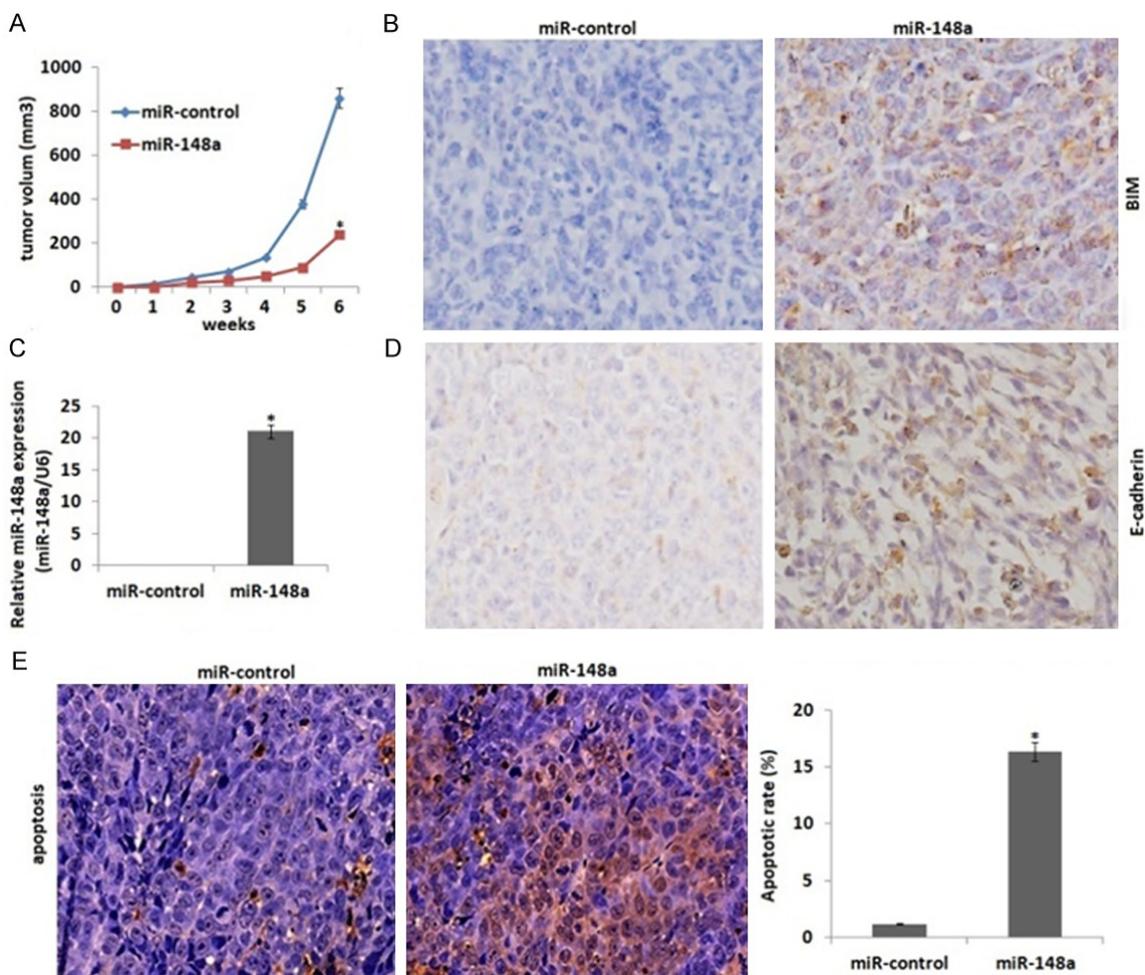


Figure 4. MiR-148a suppresses the growth of SGC-7901 xenograft tumors through apoptosis induction. A. Growth curve of SGC-7901 tumors (n = 6 per group); B. MiR-148a expression in tumors by qRT-PCR assay; C. BIM expression in tumors by immunohistochemistry assay ($\times 200$); D. E-cadherin expression in tumors by immunohistochemistry assay ($\times 200$); E. Apoptosis was analyzed by TUNEL staining ($\times 200$). *, $P < 0.01$ using a two-tailed *t*-test.

of miRNAs has been reported to function as oncogene in the development of gastric cancer [16-18]. However, miRs acting as tumor suppressor genes need to be further investigated.

Previous studies have suggested that miR-148a functioned in multiple cellular processes, including proliferation, differentiation, senescence and apoptosis, and their deregulation is a hallmark of human cancer [19]. MiR-148a was identified to be downregulated in diverse cancers, including oral Cancer [20], colorectal neoplasia [21], hepatocellular carcinoma [22, 23]. Here, we showed miR-148a was down-regulated in gastric cancer SGC-7901 cells. Our data also indicated that overexpression of miR-148a could improve the apoptosis of gastric cancer cells in vitro and in vivo, and miR-148a was able to suppress tumor growth of gastric

cancer in vivo. We further characterized BIM as a potential target of miR-148a. In addition, BIM is potentially involved in miR-148a-induced apoptosis. Above findings suggest miR-148a may act as a novel tumor suppressor by regulating the apoptosis of cancer cell in gastric cancer.

Apoptosis is an ordered cell death process that occurs in physiological and pathological conditions. A disruption of this delicate balance can lead to the development of cancer [24]. Now little is known about the effect of miR-148a on apoptosis in cancer. In current report, we found ectopic expression of miR-148a could induce the apoptosis of gastric cancer cells in vitro, and overexpression of miR-148a could significantly inhibit tumor growth in nude mouse xenograft model by inducing the apoptosis of

gastric cancer cells in vivo. Above findings suggest that miR-148a may play the potential role in gastric cancer development by promoting apoptosis of cancer.

To explore the molecular mechanism underlying miR-148a function, it is important to identify its target gene. Recently, several novel targets of miR-148a have been confirmed including TRAIL [9], PDIA3 [25], MIG6 and BIM [26], CCKBR and Bcl-2 [27, 28]. In our study, BIM was identified as a target gene of miR-148a in gastric cancer. Our study showed that BIM silencing could counteract the effect of promoting apoptosis by miR-148a. It suggests that the miR-148a-BIM axis may be involved in the development of gastric cancer. However, the downstream pathway is needed to be further investigated.

In summary, we report the down-regulation of miR-148a in gastric cancer cells, and investigate the potential role of miR-148a in tumorigenesis by regulating apoptosis. This study provides new insights into the role of miR-148a in gastric cancers. MiR-148a may function as a potential tumor suppressor gene in gastric cancer, and have the potential application as a biomarker or therapeutic target in gastric cancer therapy.

Disclosure of conflict of interest

None.

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