Int J Clin Exp Med 2017;10(7):10506-10513
www.ijcem.com /ISSN:1940-5901/IJCEM0053327

Original Article

MicroRNA-18b promotes cell proliferation and inhibits apoptosis of gastric cancer via targeting KIAA1324

Shengxi Wang, Wei Song, Zhaopei Li, Wanhua Ren

Department of Minimally Invasive Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong Province, China

Received March 20, 2017; Accepted April 17, 2017; Epub July 15, 2017; Published July 30, 2017

Abstract: Gastric cancer (GC) is the fourth most predominant malignancy worldwide and remains the second most common cause of cancer-related death globally. Recent studies have shown that miRNAs are involved in the tumorigenesis of GC. MicroRNA-18b (miR-18b) has been reported to be upregulated in primary gastric cancer tissues compared with adjacent non-tumorous tissues. However, the specific prognostic value and biological roles of miR-18b in gastric cancer still remains unknown. In this study, we firstly verified that miR-18b expression was really up-regulated in cancerous tissues and overexpressed miR-18b in tumor- and adjacent-tissue samples from patients with gastric cancer (GC). In addition, our results showed that low expression of miR-18b inhibited the growth and promoted the apoptosis in GC cells. From our further study, down-regulation of miR-18b also reduced tumor growth in xenograft models of gastric cancer. Moerover, we showed, by luciferase assay, a direct interaction between miR-18b and the KIAA1324 3'UTR, as overexpression of miR-18b was associated with suppression of luciferase activity. Furthermore, we demonstrated that low-expression of miR-18b caused a significant upregulation of both KIAA1324 mRNA and protein. Taken together, we concluded that miR-18b promotes cell proliferation and inhibits apoptosis of gastric cancer via targeting KIAA1324 and miR-18b may serve as a useful therapeutic agent for miRNA-based GC therapy.

Keywords: MiR-18b, proliferation, apoptosis, KIAA1324, gastric cancer

Introduction

Gastric cancer (GC) is the fourth most predominant malignancy worldwide, and is still the common cause of cancer-related death worldwide [1]. Although there has been great progress on traditional treatments, such as modus operandi, supplemented with adjuvant chemotherapy, a considerable number of patients with GC are diagnosed at the advanced stages and then attained poor prognosis. Thus, a better understanding of the molecular mechanisms of progression of gastric cancer may lead to important improvements in the development of new therapeutic agent [2]. Recently, studies on the effect of microRNAs on gastric cancer have shown great progress [3-5].

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs that regulate human gene expression [6]. Increasing evidences have showed that several miRNAs are aberrantly expressed in different cancer types, which can act as oncogenes or tumor suppressors during the development and progression of cancers through sequence-specific binding to their mRNA targets [7]. To date, the role of miR-18b in tumor remains controversial. On the one hand, Dar AA et al. reported that miR-18b as a tumor suppressor in melanoma [8]. On the other hand, Fonseca-Sanchéz MA et al. reported that miR-18b was upregulated in breast cancer and modulated genes involved in cell migration [9]. Although Guo et al. reported that miR-18b was highly expressed in gastric cancer (GC) tissues compared with normal gastric tissues [10], the biological roles of miR-18b and the underlying signal pathway in gastric cancer remains unknown.

It has been reported that KIAA1324 encodes a 1,013 amino acid transmembrane protein and is highly conserved among species [11]. The correlation between KIAA1324 expression and

prognosis in endometrial, ovarian, and pancreatic cancer patients was previously reported [11-13]. Although Kang et al. reported that KIAA1324 acted as tumor-suppressor in gastric cancer [14], the specific mechanism still needs to be further studied.

To further understand the regulatory mechanisms of miR-18b in GC progression, we firstly analyzed the expression of miR-18b in tumor- and adjacent-tissue samples from 64 patients with gastric cancer (GC). miR-18b expression was significantly up-regulated in cancerous tissues compared with noncancerous controls. From our further study, our results showed that the miR-18b down-expression inhibited the growth and promoted apoptosis in gastric cancer HGC-27 and MGC-803 cells. Furthermore, we demonstrated that KIAA1324 was the direct functional target of miR-18b in the progress of GC. Our study not only makes a contribution to the understanding of the roles and molecular mechanisms of miR-18b in GC progression, but also the data may be translated into new therapeutics and/or prognostic biomarkers for GC.

Materials and methods

Clinical tissues specimens and cell culture

Tissue specimens (tumor, adjacent samples) of 64 patients with gastric cancer were collected after informed consent and verification by a pathologist, and immediately frozen in liquid nitrogen. Fresh-frozen and/or formalin-fixed paraffin embedded samples were used for miR-18b and KIAA1324 expression analysis.

Human gastric cancer HGC-27 and MGC-803 cell lines were cultured in DMEM medium (Biological Industries) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) and antibiotics. All the cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. These cell lines were obtained from the American Type Culture Collection (USA).

Mice model

All animal work was conducted under the institutional guidelines of Shandong Province and approved by the Use Committee for Animal Care. Nude mice were purchased from the Vital River Laboratories (Beijing, China). 10×10^6 HGC-27 cells were transfected with 30 nM miR-18b inhibitor or control in replicate. Cells were cultured for 24 hours before harvest for transplantation into the animal. Before injection, cells were pooled and mixed with matrigel (BD Biosciences, USA). 5×10^6 cells were injected subcutaneously into the right flank of nude mice. Measurements were taken weekly and tumor volumes were calculated using the formula V = length × width ^ 2/2. The animals were sacrificed 4 weeks after injection. Pictures were recorded with a Nikon d800 digital camera (Nikon, Japan).

Cell transfection

Human gastric cancer HGC-27 and MGC-803 cell lines were transfected with miR-18b inhibitor or negative control. The miR-18b inhibitor were purchased from GenePharma (Shanghai, China). miR-18b mimics and inhibitors at a final concentration 50 nM were transfected using lipofectamine2000 (Invitrogen, Carlsbad, CA) into cells seeded onto 6 well plates. Twenty-four hours after transfection, cells were analysed as required.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated from tissues or cells using TRIzol (Invitrogen, Carlsbad, USA) and small RNA enrichment was conducted using a miRVana miRNA isolation kit (Ambion, Austin, USA), according to the manufacturer's instructions. The relative expression level of miR-18b (normalized to U6) was determined using Hairpin-itTM miRNA real-time PCR Detection Kit (GenePharma, Shanghai, China) according to the manufacturer's protocol in a StepOnePlus™ real-time PCR instruments (Applied Biosystems, San Diego, USA). Real-time PCR using SYBR Green (Takara, Japan) was performed to compare the relative expression levels of KIAA1324 mRNAs according to the manufacturer's instructions. The primers for mRNAs real-time PCR were shown as below. KIAA1324: Forward primer: 5'-TCCAGGGACCAAGAACAACAAGA-3'; Reverse primer: 5'-TCAGGAATCCGGAGGTCAGTG-3'. The relative expression levels of the mRNAs were determined using the $2^{-\Delta\Delta Ct}$ analysis method. All reactions were performed in triplicate.

Western blot

Total proteins were extracted from the cultured cells or tissues and quantified using a BCA

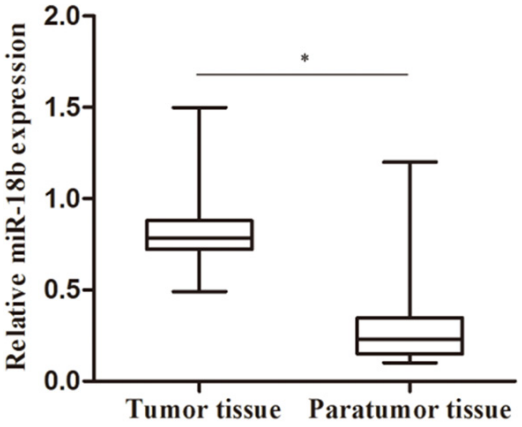

Figure 1. MiR-18b was highly expressed in clinical GC tumor tissues. MiR-18b mRNA level was examined by real-time PCR in 64 cases of clinical GC tumor tissues and paired adjacent tissues. Statistically significant differences are indicated: *$P < 0.05$; Student's t test.

Protein Assay Kit (Beyotime, Jiangsu, China) with BSA as a standard. Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% BSA (5% w/v in PBS + 0.1% Tween 20) and incubated with primary antibodies at room temperature. The antibodies which are against KIAA1324 and β-actin were used according to the manufacturer's instructions, and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After using the secondary antibodies (Santa Cruz, USA) at 1:2,000 (v/v) dilutions in PBS + 0.1% Tween 20, the signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA) according to manufacturer's instructions.

Cell proliferation assay

Cell proliferation was performed with Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan). According to the instructions, Cell Counting Kit-8 reagent was added at 0, 24, 48, and 72 h respectively after seeding 4×10^3 cells per well in into 96-well plates and transfected with miR-18b inhibitor or control, and incubated at 37°C for 2 h. The OD (optical density) 450 nm value was detected by using a microplate reader (Bio-Rad, Richmond, CA, USA).

Annexin V/PI analysis

Annexin V/PI staining was performed with BD Pharmingen. Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's protocol. Cells were trypsinized and washed twice with PBS. The cells were incubated in binding buffer containing Annexin V-FITC and propidium iodide (PI). Stained cells were analyzed by flow cytometry using CELLQUEST program (Becton Dickinson). Three independent experiments were performed.

In vitro luciferase assay

3'UTR sequence of KIAA1324 which was predicted to interact with miR-18b or a mutant sequence with the predicted target sites was inserted into pmirGLO vector (Promega, USA). HGC-27 and MGC-803 cells lines were transfected with miR-18b mimics or with pmirGLO-KIAA1324-wt and pmirGLO-KIAA1324-mut. Firefly and Renilla luciferase activities were assessed using the Dual-Glo luciferase assay system (Promega, USA) in accordance with the manufacturer's instructions. Luminescence readings were acquired using a TD 20/20 luminometer (Turner Design Inc., CA, USA). The Renilla luciferase signal was normalized to the firefly luciferase signal to adjust for variations in transfection efficiency. Sample values were compared to the reference value of cells expressing pmirGLO empty vector.

Statistical analysis

Data are presented as means ± SD. of three independent experiments. Differences between groups were analysed by GraphPad Prism 5 software (GraphPad Software, CA, USA) with Student's t-test. Differences were considered statistically significant at $P < 0.05$.

Results

MiR-18b was highly expressed in clinical GC tumor tissues

We examined the expression levels of miR-18b in 64 GC clinical samples via utilizing real-time PCR, with quantified values used to calculate miR-18b/U6 ratios. Our results exhibited that the expression of miR-18b was substantially higher in clinical GC tumor tissues (**Figure 1A**).

MiR-18b down-regulation inhibited the growth and promoted apoptosis in GC cells

Based on the expression pattern of miR-18b, the influences of low-expression of miR-18b on

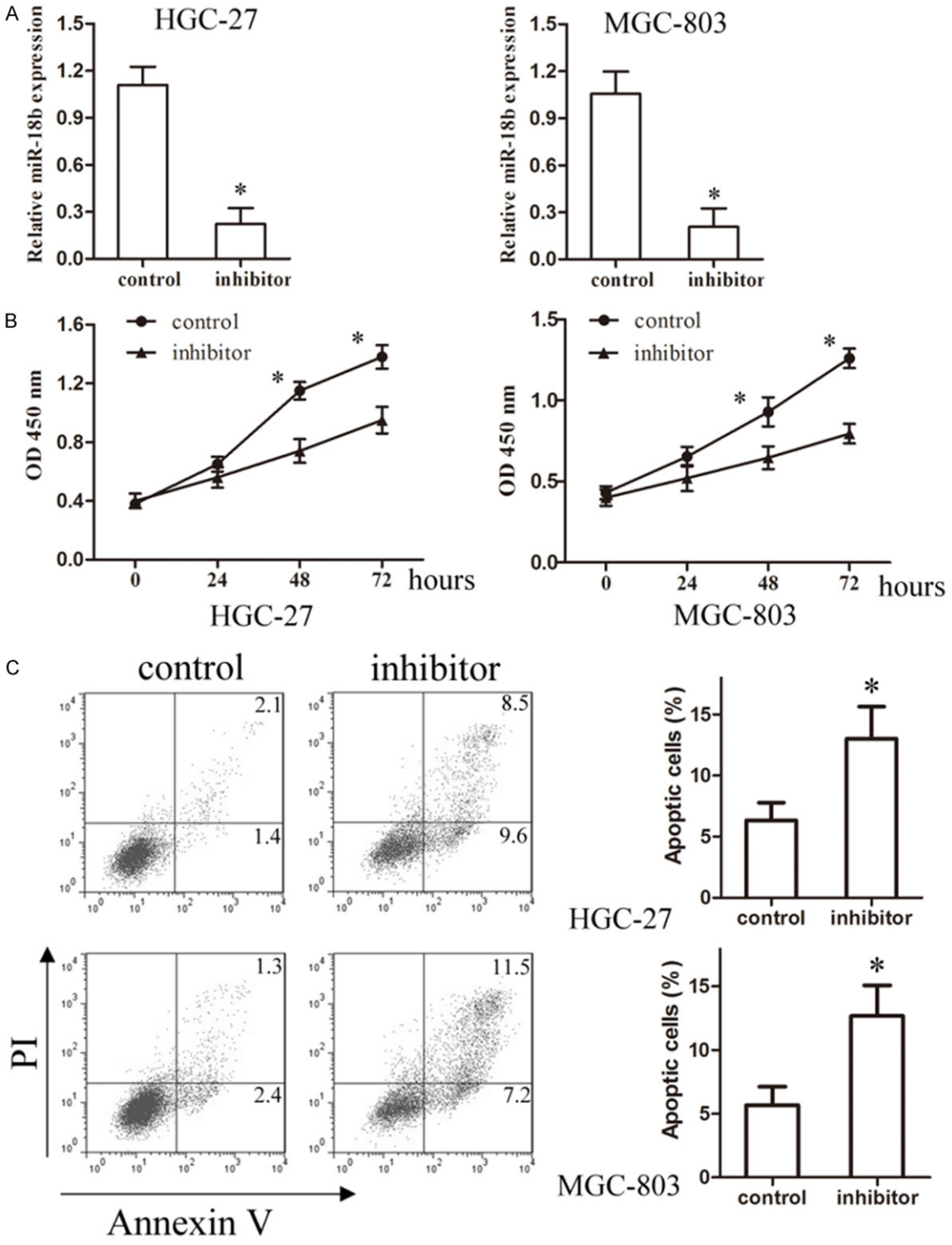

Figure 2. Down-regulation of miR-18b inhibited GC cells growth and promoted apoptosis. A. Transfection with miR-18b inhibitor for 24 hours, miR-18b mRNA levels was obviously decreased in HGC-27 and MGC-803 cells. B. The CCK-8 assay showed that low-expression of miR-18b inhibited the growth of HGC-27 and MGC-803 cells. Absorbance at 450 nm was measured. C. Flow cytometry analysis was performed using cells stained with Annexin V-FITC and propidium iodide (PI). The right panel shows quantification of apoptotic cell population from triplicate samples. Statistically significant differences are indicated: $*P < 0.05$; Student's t test. The experiment was repeated at least three times.

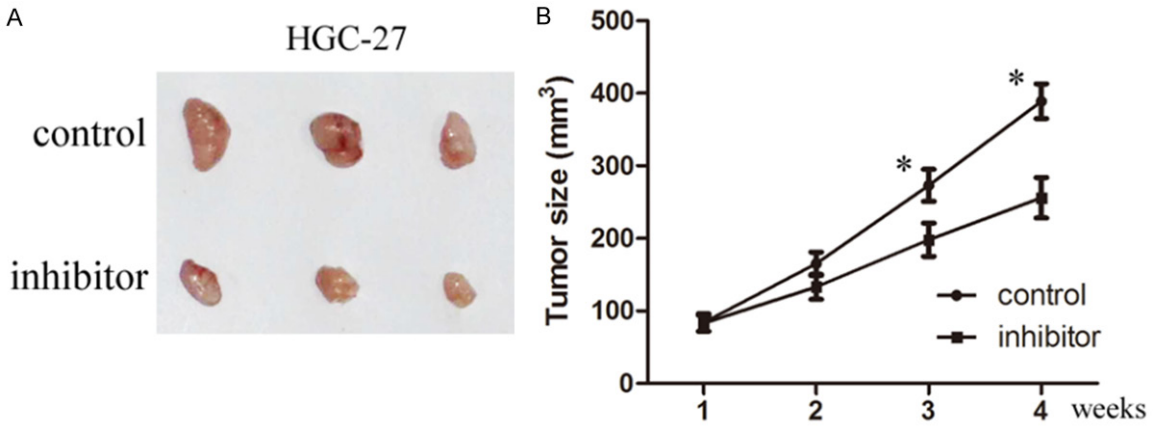

Figure 3. MiR-18b down-regulation suppressed tumor growth of gastric cancer xenografts. A. HGC-27 cells were transfected with control or miR-18b inhibitor and injected subcutaneously into 10 NOD/SCID mice using 5 × 10^6 cells per flank. Surgical resections of HGC-27 xenograft tumors on week 4 for animals were shown. B. Measurements of tumor volumes were taken weekly and tumor volumes were shown. Mean ± SD. *P < 0.05 (paired T-test).

cell proliferation and apoptosis were examined in human GC cell lines: HGC-27 and MGC-803. The cell lines were transfected with miR-18b inhibitor and decreased level of miR-18b was confirmed by real-time PCR assay (**Figure 2A**). Down-regulation of miR-18b obviously suppressed the cell growth compared with negative control in HGC-27 and MGC-803 cells, examined by the CCK-8 assay (**Figure 2B**). In addition, miR-18b low-expression increased the annexin V-positive cell population compared with negative control in HGC-27 and MGC-803 cells, examined by annexin V staining and flow cytometry analysis. Taken together, our findings verified that miR-18b low-expression inhibited the cell growth and promoted apoptosis in GC cells.

The down-regulation of miR-18b reduced tumor growth in xenograft models of gastric cancer

The in vitro data support a tumor suppressor role for miR-18b. Thus, we assessed tumor growth of xenografts derived from HGC-27 cells that were transfected with synthetic miR-18b inhibitor or negative control prior to subcutaneous injection into nude mice. As shown in **Figure 3A**, miR-18b inhibitor inhibited tumor growth of the HGC-27 xenograft. Moreover, tumor volumes of four time points were shown in **Figure 3B**. Together, the down-regulation of miR-18b inhibited tumor growth of HGC-27 cells in all animals tested.

KIAA1324 is a direct miR-18b target

miRNAs regulate their targets via binding to their 3'UTR. Based on three commonly used algorithms, Target Scan [15], Miranda [16] and PicTar [17], we found that the KIAA1324 was the candidate gene for miR-18b. We identified the binding site of miR-18b in the 3'UTR of KIAA1324 mRNA (**Figure 4A**) and cloned the 3'UTR of KIAA1324 mRNA and its mutants into downstream of pmirGLO-control luciferase reporter gene vector (named pmirGLO-KIAA1324-wt or pmirGLO-KIAA1324-mut), respectively. The luciferase reporter gene assays demonstrated that miR-18b significantly suppressed the firefly luciferase activities of pmirGLO-KIAA1324-wt, whereas it failed to work when the target site was mutated in HGC-27 and MGC-803 cells (**Figure 4B**). Moreover, the levels of KIAA1324 mRNA and protein were substantially increased when the expression of miR-18b of was inhibited in two kinds of GC cells (**Figure 4C**, **4D**). Thus, these data suggested that KIAA1324 was the target gene of miR-18b in GC cells.

Discussion

In this study, we have shown that miR-18b was obviously upregulated in human GC cancerous tissues compared with the corresponding non-cancerous tissues. We demonstrated, by the use of luciferase assay in vitro, that miR-18b acts as a regulator of KIAA1324 expression and activity. Furthermore, recent reports have

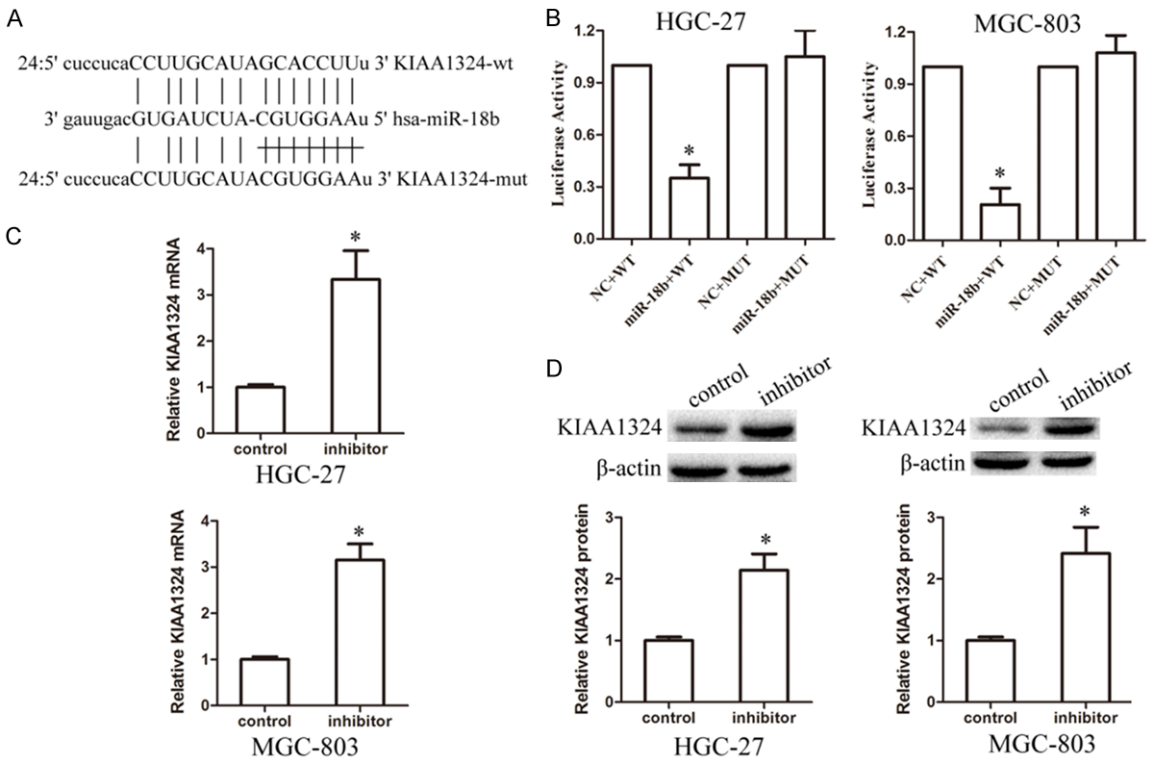

Figure 4. MiR-18b downregulated KIAA1324 expression by directly targeting its 3' UTR. A. The binding site of miR-18b in 3'UTR of KIAA1324 mRNA is shown in a model. Mutant was generated at the KIAA1324 3'UTR as indicated. A KIAA1324 3'UTR fragment containing wild type or mutant (wt or mut) of the miR-18b-binding sequence was cloned into the downstream of the pmirGLO-control luciferase reporter gene vector. B. The effect of miR-18b on reporters of pmirGLO-KIAA1324-wt and pmirGLO-KIAA1324-mut in HGC-27 and MGC-803 cells was measured by luciferase reporter gene assays, respectively. C, D. The mRNA and protein levels of KIAA1324 were examined in HGC-27 and MGC-803 cells transfected with anti-miR-18b by real-time PCR and Western blotting, respectively. Statistically significant differences are indicated: $*P < 0.05$; Student's t test. The experiment was repeated at least three times.

indicated that KIAA1324 was significantly associated with the prognosis of patients with GC [18-21]. These findings suggested that the overexpression of miR-18b correlates with the progression of GC patients, possibly because of repression of the function of the KIAA1324 protein.

The role of KIAA1324 in cancer has been evaluated in endometrial, pancreatic, ovarian cancer and gastric cancer to date [11-13]. In type I endometrial cancer, KIAA1324 expression is higher at early stage than that of benign tumors, but reduced in high grade and stage endometrial carcinoma. In addition, KIAA1324 is downregulated in type II endometrial cancer, which is more aggressive than type I. In pancreatic cancer, KIAA1324 is also highly expressed in early-stage tumor, but its expression is decreased in advanced cancer. High KIAA1324 expression in endometrial and pancreatic carcinoma is correlated with favorable prognosis in cancer patients. However, in high-grade serous carcinoma of the ovary/peritoneum, high expression of ERα and KIAA1324 is associated with poor survival in cancer patients. Furthermore, KIAA1324 acts as a tumor suppressor in gastric cancer through induction of apoptosis. This indicates that KIAA1324 may play different roles in various types of cancers.

As is well known, miRNAs modulate the expression of their target genes at post-transcriptional level [22]. They prevent their translation by directly binding to the corresponding complementary sequences of their target mRNAs, thereby downregulating protein expression. Many studies indicated that abnormal expression of miRNAs is associated with various human diseases, including malignancies. Dar AA et al. has reported that miR-18b expression is suppressed in melanoma through DNA meth-

ylation and is correlated with survival. miR-18b overexpression results in downregulation of MDM2, upregulation of p53, suppression of the proliferative and invasive ability of melanoma cells, induction of apoptosis, and reversal of EMT [8]. Fonseca-Sanchéz MA et al. has reported that the inhibition of miR-18b induces the modulation of genes with key roles in cancer. These genes have been implicated in tumor growth (CHRM2 and POSTN), cell proliferation (NLRP7 and CHMR2), anoikis (OLFM3), apoptosis (REG1B, SCN3B and POSTN), and angiogenesis (KLK3, CHRM2 and POSTN). Notably, eight genes (KIR3DL3, NLRP7, KLK3, OLFM3, SEMG1, CRX, POSTN and CEACAM5) are involved in apoptosis and metastasis of cancer cells [9]. However, whether KIAA1324 is posttranscriptionally regulated by miR-18b still remains unclear. Via using miRNA prediction programmes, we found that a fragment of the KIAA1324 3'UTR contained putative miR-18b binding site. To further confirm the prediction, the miR-18b mimics were co-transfected with a luciferase reporter construct containing wild-type KIAA1324 3'UTR. Our results indicated that transfection with miR-18b mimics downregulated luciferase activity compared with transfection with NC. Therefore, we focused on miR-18b that might have significant effects on the progression and development of GC. Furthermore, we performed a series of functional experiments to test the effect of miR-18b on GC cells. Our results suggested that low-expression of miR-18b resulted in inhibition of growth and promoted apoptosis in GC cells.

In conclusion, we identified and characterized the miR-18b-KIAA1324 axis, which is involved in proliferation and apoptosis of GC cells. Based on our findings, miR-18b may act as a therapeutic target for the treatment of human GC.

Acknowledgements

The authors thank Mingzhu Cui for technical support.

Disclosure of conflict of interest

None.

Address correspondence to: Wanhua Ren, Department of Minimally Invasive Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jingwuweiqi Road No. 324, Jinan 250021, Shandong Province, China. Tel: +86 0531 68777153; Fax: +86 0531 68776388; E-mail: wanhuaren@aliyun.com

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