Knockdown of MicroRNA-92a suppresses arcinogenic cellular activities of gastric cancer cells via targeting Bim

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Abstract Aim: This study aimed to explain the effects and mechanisms of microRNA-92a in gastric cancer. Methods: Thirty pairs of cancerous and adjacent normal tissues were collected from patients with gastric cancer. BCL2-interacting mediator of cell death (Bim) and miRNA-92a expressions were evaluated using immunohistochemistry and in situ hybridisation, respectively. SGC7901 cells were divided into three groups: control, miRNA inhibitor, and miRNA inhibitor + BIM inhibitor. Cell proliferation and apoptosis were measured using MTT assay and flow cytometry, respectively. Cell invasiveness and migration were evaluated using transwell and wound-healing assays, respectively. Relative BIM, BCL-2 and EGFR expressions were assessed using western blotting. Results: Bim and miRNA-92a expressions in tumor tissues significantly up-regulated compared with adjacent normal tissues ($P < 0.05$, *respectively*). Compared with control group, cell proliferation and apoptosis in miRNA inhibitor group was significantly suppressed and elevated ($P < 0.01$, *respectively*); Cell invasiveness and migration in the miRNA inhibitor group were significantly suppressed compared with those in the control group ($P < 0.01$, *respectively*). BCL-2 and EGFR expressions in the miRNA inhibitor group was significantly suppressed compared with those in the control group, whereas BIM expression was significantly elevated in the miRNA inhibitor group compared with that in the control group ($P < 0.01$). Conclusion: miRNA-92a knock-down thus suppresses in vitro SGC7901 cell activities in gastric cancer via BIM targeting.

Key words: miRNA-92a, Bim, Bcl-2, EGFR

Introduction

Globally, gastric cancer is the fourth most prevalent form of cancer, and the mortality rate associated with gastric cancer is the second highest in China (1). As patients with gastric cancer are mostly diagnosed in the late stage of the disease, lymph node metastasis rates range between 50% and 75% and distant metastasis is commonly observed; thus, such patients have a 5-year survival rate of <40% (2, 3). Therefore, it is clinically important to study molecular mechanisms underlying in the occurrence and development of gastric cancer. MicroRNAs (miRNAs) are non-coding RNA nucleotide sequences with a length of 22ntand play a role in inhibiting protein translation by generally binding to 3'UTR regions of target mRNAs (4). Reportedly, abnormal expression of several miRNAs such as miRNA-223, miRNA-21, miRNA-27a and miRNA-141 is closely related to gastric cancer cell proliferation, invasion and migration (5-9). The protein BCL-2 interacting mediator of cell death (BIM) is a member of the BH3-only subfamily of the BCL-2 family and is an important apoptosis-regulating protein. It exerts aninhibitory effect on tumour growth, and its deletion can cause many kinds of tumours (10, 11). Studies have reported that the *Bim* is a target gene of miRNA-92a (12). However, in gastric cancer, the correlation between miRNA-92a and BIM expression and the effects and mechanism of miRNA-92a in its development are still unclear. In the present study, we initially collected 30 pairs of cancerous and adjacent normal tissues from patients with gastric cancer patients and measured miRNA-92a and BIM expressions. We additionally evaluated the effects of miRNA-92a on gastric cancer cell proliferation, apoptosis, invasion and migration *in vitro.*

Material and methods

Clinical data

In total, 30 pairs of cancerous and adjacent normal tissues were obtained from patients with gastric cancer who were diagnosed and treated at the second people's hospital of Yinbin from April 2016 to November 2017. These specimens were

collected and stored in 4% polyoxymethylene for fixation, embedded in paraffin and sectioned at 4-μm thickness for measuring miRNA-92a and BIM expressions. Written informed consent from all patients and ethical approval from the Hospital Ethic Review Committee was obtained.

In Situ Hybridization (ISH)

ISH kit was performed using kits from Boster Biological Technology Co. Ltd. (Wuhan, China) according to the manufacturer's instructions. Every specimen was treated with digoxin-labelledmiRNA-92a(20 μl), and IOD values of difference tissues were measured.

Immunohistochemistry (IHC)

IHC was performed using rabbit anti-human BIM antibody (1:500; Neomarkers, Shanghai, China) with an IHC Envision staining kit (Beijing Zhongshan Biotechnology Co., Ltd., Beijing, China) following the manufacturer's instructions. Phosphate buffer saline (PBS) was used as the negative control. The differences in the IOD values between the tissue pairs were measured.

Cell and cell culture

SGC-7901 cells were purchased from ATCC (USA) and cultured in RPMI-1640 culture medium (Hyclone, USA) comprising 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2. Cells in logarithmic growth phase were used for flow cytometry experiments.

Grouping and treatment

SGC-7901 cell were divided into three groups: control group treated by negative control miRNA and cultivated byRPMI-1640 culture medium contained with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin; miRNA inhibitor group were transfected with miRNA-92a inhibitor (Sigma, USA) and miRNA inhibitor and Bim inhibitor group were transfected with miRNA-92a inhibitor and Bim inhibitor which was si-Bim as 1mg/mL (Sigma, USA).

miRNA-92a gene expression in difference groups by RT-PCR assay

The total RNA was extracted from SGC-7901 cells of difference groups by TRIzol (Thermo Fisher Scientific, Inc.) depending on manufacturer's protocol. The quality of the RNA samples was assessed by determining the optical density (OD)260/(OD)280 ratio. TaqMan miRNA assays (Applied Biosystems, Thermo Fisher Scientific, Inc.) were used to analyze miR-92a expression. U6 small nuclear RNA was used as a loading control and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

MTT assay

Treated SGC-7901 cells from thethree groups were then cultured for 72 h. Subsequently, 20μl of MTT solution (5g/l) was added to each well and cells were further cultured for 4 h. The supernatant was then removed, and 150-μl DMSO was added to each well. After10 min of incubation, absorbance (A) was measured at 490nm using an enzyme-linked immunosorbent analyser to determine proliferation rates of cells in the groups.

Cell apoptosis by flow cytometry

Treated SGC-7901 cells from the three groups were cultured for 72 h and harvested and centrifuged at 1500 rpm at 4℃ for 5 min. The supernatant was discarded and 400 μl of fresh 1×Binding Buffer was added. Cells were gently resuspended and incubated at room temperature for 20 min prior to the addition of 5 μl each of Annexin V-FITC and propidium iodide. After further incubation for 1 h, cell apoptosis was measured using flow cytometry.

Transwell assay

Treated SGC-7901 cells from the three groups were harvested, adjusted to the same cell concentration using serum-free 1640 medium and added to the upper chamber of the invasion kit plate (1×10^5 cell in every well). The bedroom were added 1640 medium contained 30% FBS, and cells cultured for 48 h. The bedroom was then removed, and the cells were fixed in 95% ethanol for 10 min. Using a cotton swab, cells that were retained on the microporous membrane in the upper chamber were carefully swiped off. After haematoxylin and eosin staining, cells that had

passed through microporous membrane were examined.

Wound healing assay

Cell migration was assessed using the wound-healing scratch test. Cells from the three groups were seeded into 6-well plates at a density of 1×10^5 cells/well and cultured to 90% confluence. After washing the cells thrice using serum-free medium, fresh serum-free medium was added, and each cell monolayer was scratched using a 10-μl Eppendorf pipette tip. After washing thrice with serum-free medium, cells were incubated in serum-free medium at 37℃ in a humidified atmosphere containing 5% CO2. The width of scratches in the three groups was measured at 0, 24 and 48 h. The experiment was repeated nine times.

Western blotting

Treated SGC-7901 cells from the three groups were collected and washed thrice in pro-ice PBS. Further, 1×10^5 cells were added 100 – 150 μl cell lysate on ice for 1 h and centrifuged at 12000 rpm for 10 min. The supernatant was removed and assayed for total protein concentration using the Bradford method. The proteins in the supernatant were denatured by boiling and separated using 10% sodium dodecyl -sulphate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. After blocking using TBST containing 5% bovine serum albumin, blots were probed overnight using BIM (1:500), BCL-2 (1:500), EGFR (1:500) and GAPDH (1:500) as primary antibodies. The membrane was washed thrice with TBST (5 min each), and secondary antibodies were added and incubated for 1 h. After washing thrice with TBST (5 min each), proteins were detected using chemiluminescence. GAPDH was used as the loading control.

Statistical analysis

Data were analysed using SPSS v23.0 software, and data was expressed as mean $\pm$ standard deviation. All statistical analyses were determined using one-way ANOVA with *boferroni*-test. Statistical significance was set at $P \leqslant 0.05$.

Results

Clinical data and analysis

Compared with adjacent normal tissues, ISH assay identified that the miRNA-92a expression in cancerous tissues was significantly elevated (*P ＜ 0.001*; Fig 1A); however, BIM expression, identified using ICH, was significantly supressed in cancerous tissues compared with that in adjacent normal tissues (*P ＜0.001*; Fig 1B). Based on these results, miRNA-92a can be a carcinogenic factor targeting BIM in gastric cancer.

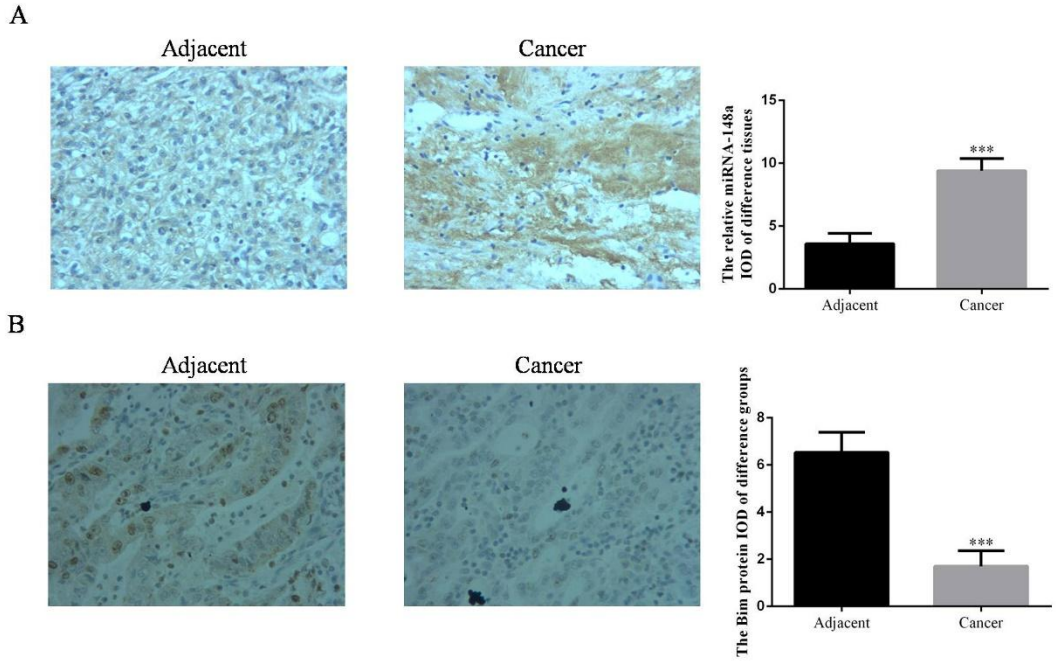

Fig 1. The Bim and miRNA-92a expression in difference tissues

A. The miRNA-92a expression of difference tissues by ISH (200×)

Adjacent: Adjacent normal tissues; Cancer: Cancer tissues

***:P＜0.001 compared with adjacent normal tissues

B. The Bim protein expression of difference tissues by IHC(200×)

Adjacent: Adjacent normal tissues; Cancer: Cancer tissues

***:P＜0.001 compared with adjacent normal tissues

miRNA-92a mRNA expression and Cell proliferation

By RT-PCR assay, the miRNA-92a mRNA expression of miRNA inhibitor and miRNA inhibitor +Bim inhibitor groups were significantly suppressed compared with that of Control group (P＜0.001, respectively, Fig 2A). This result was shown that

miRNA-92a inhibitor had effective suppressed miRNA-92a expression in cell experiment. Meanwhile, Cell proliferation in the miRNA inhibitor group was significantly suppressed compared with that in the control group ($P < 0.01$) whereas that in the miRNA inhibitor + BIM inhibitor group was significantly elevated compared with that in the miRNA inhibitor group ($P < 0.01$; Fig 2B). This suggests that inhibiting miRNA-92a may suppress SGC-7901 cell proliferation by regulating BIM expression.

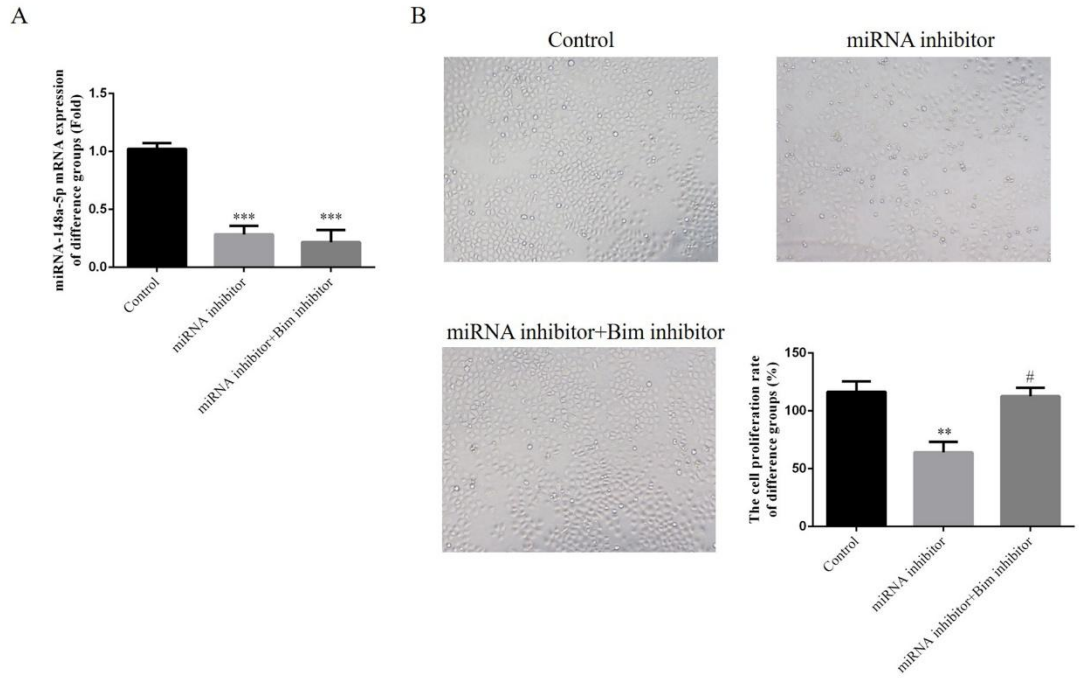

Fig 2. The cell proliferation rate of difference groups by MTT assay

Control: normal control group treated with normal treatment; miRNA inhibitor:miRNA-92a inhibitor group transfected with miRNA-92a inhibitor; miRNA inhibitor+Bim inhibitor group transfected with miRNA-92a inhibitor and Bim inhibitor. **: P＜0.01 compared with Control group; #:P＜0.01 compared with miRNA inhibitor group

Cell apoptosis

Cell apoptosis in the miRNA inhibitor group was significantly elevated compared with that of the control group ($P < 0.01$; Fig 3), whereas that in the miRNA inhibitor + BIM inhibitor group was significantly suppressed compared with that in the miRNA group (*$P < 0.01$*), indicating that apoptosis is regulated by

miRNA-92a via BIM targeting.

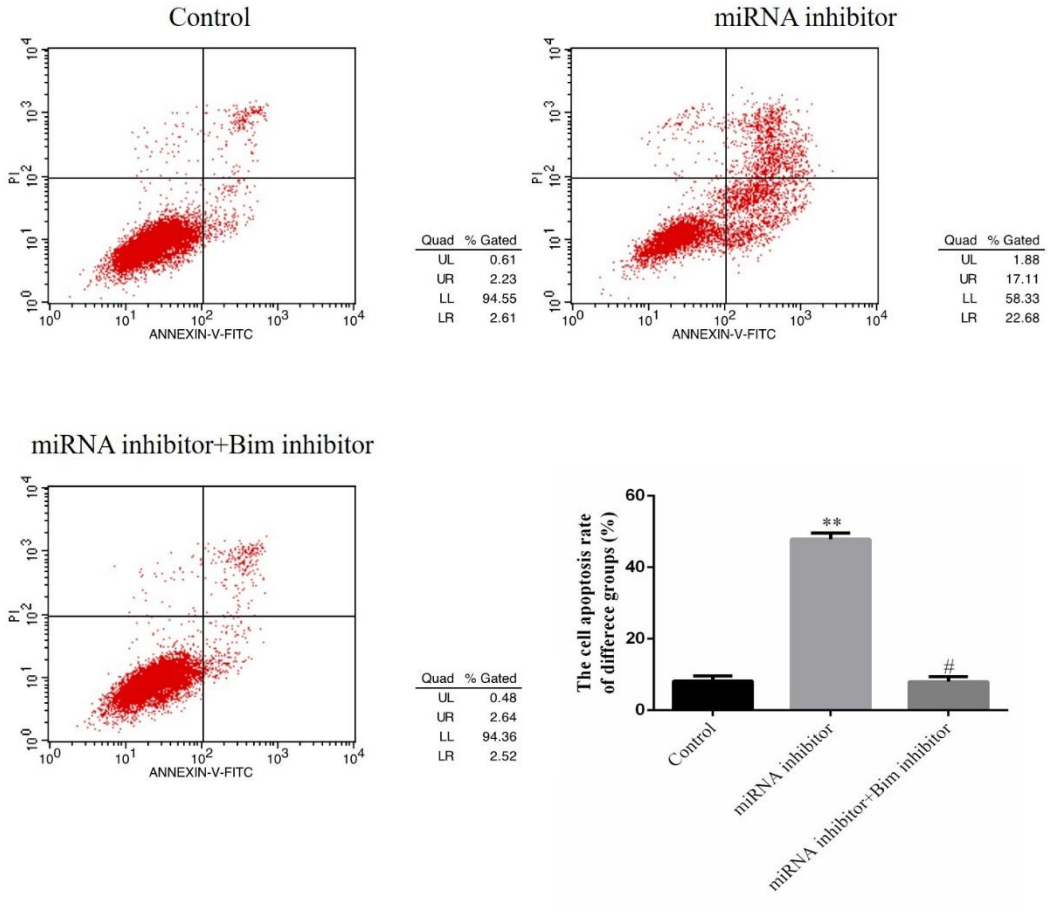

Fig 3. ThemiRNA-92a gene expression and cell apoptosis of difference groups

Control: normal control group treated with normal treatment; miRNA inhibitor:miRNA-92a inhibitor group transfected with miRNA-92a inhibitor; miRNA inhibitor+Bim inhibitor group transfected with miRNA-92a inhibitor and Bim inhibitor.

A. ***:P＜0.001 compared with Control group

B. **: P＜0.01 compared with Control group; #:P＜0.01 compared with miRNA inhibitor group

Cell invasiveness

SGC-7901 cell invasiveness in the miRNA inhibitor group was significantly suppressed compared with that of the control group ($P < 0.01$) whereas that in the miRNA inhibitor + BIM inhibitor groups was significantly increased compared with that in the miRNA group ($P < 0.01$; Fig. 4), indicating that cell invasiveness is regulated by miRNA-92a via BIM targeting.

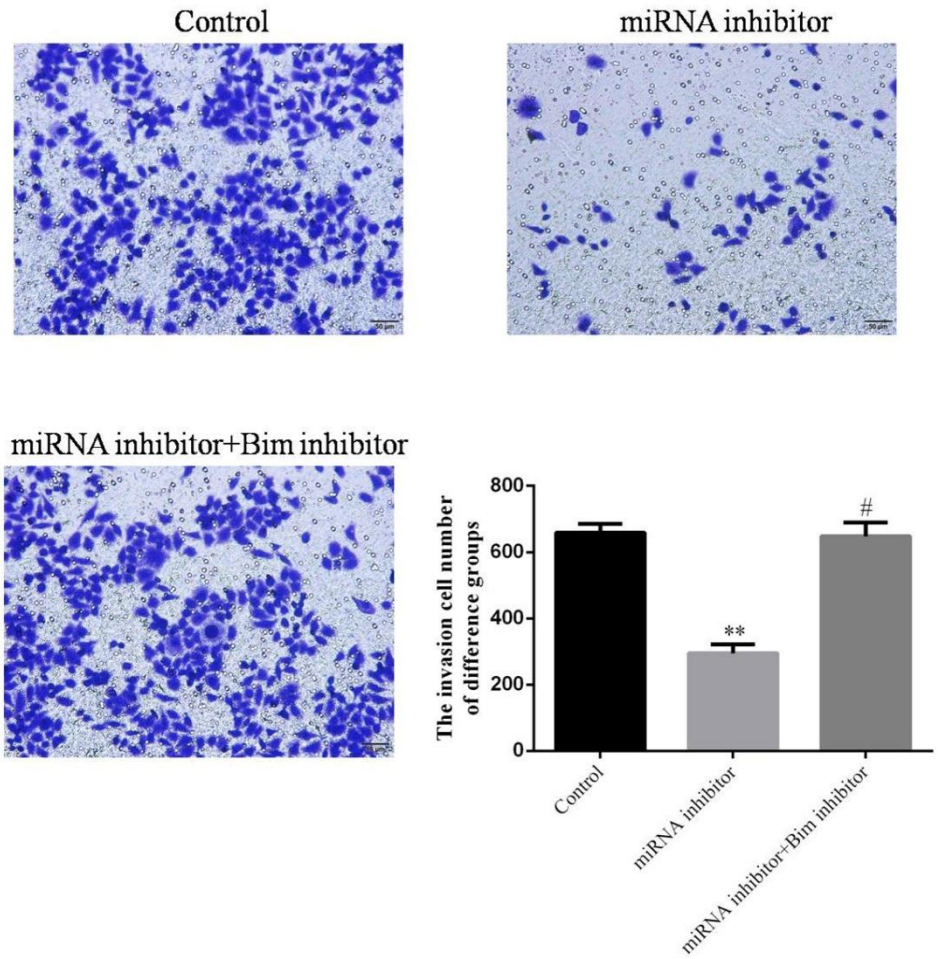

Fig 4. The invasion cell number of difference groups (×100)

Control: normal control group treated with normal treatment; miRNA inhibitor:miRNA-92a inhibitor group transfected with miRNA-92a inhibitor; miRNA inhibitor+Bim inhibitor group transfected with miRNA-92a inhibitor and Bim inhibitor. **: P＜0.01 compared with Control group; #:P＜0.01 compared with miRNA inhibitor group

Migration

Cell migration, as assessed using wound-healing scratch test, was significantly suppressed in the miRNA inhibitor group compared with that in the control group at both 24 and 48 h ($P < 0.01$; Fig 5). However, compared with that in the miRNA group, the miRNA inhibitor + BIM indicator group demonstrated significantly increased cell migration both time points ($P < 0.01$), indicating that cell migration is regulated by miRNA-92a via BIM targeting.

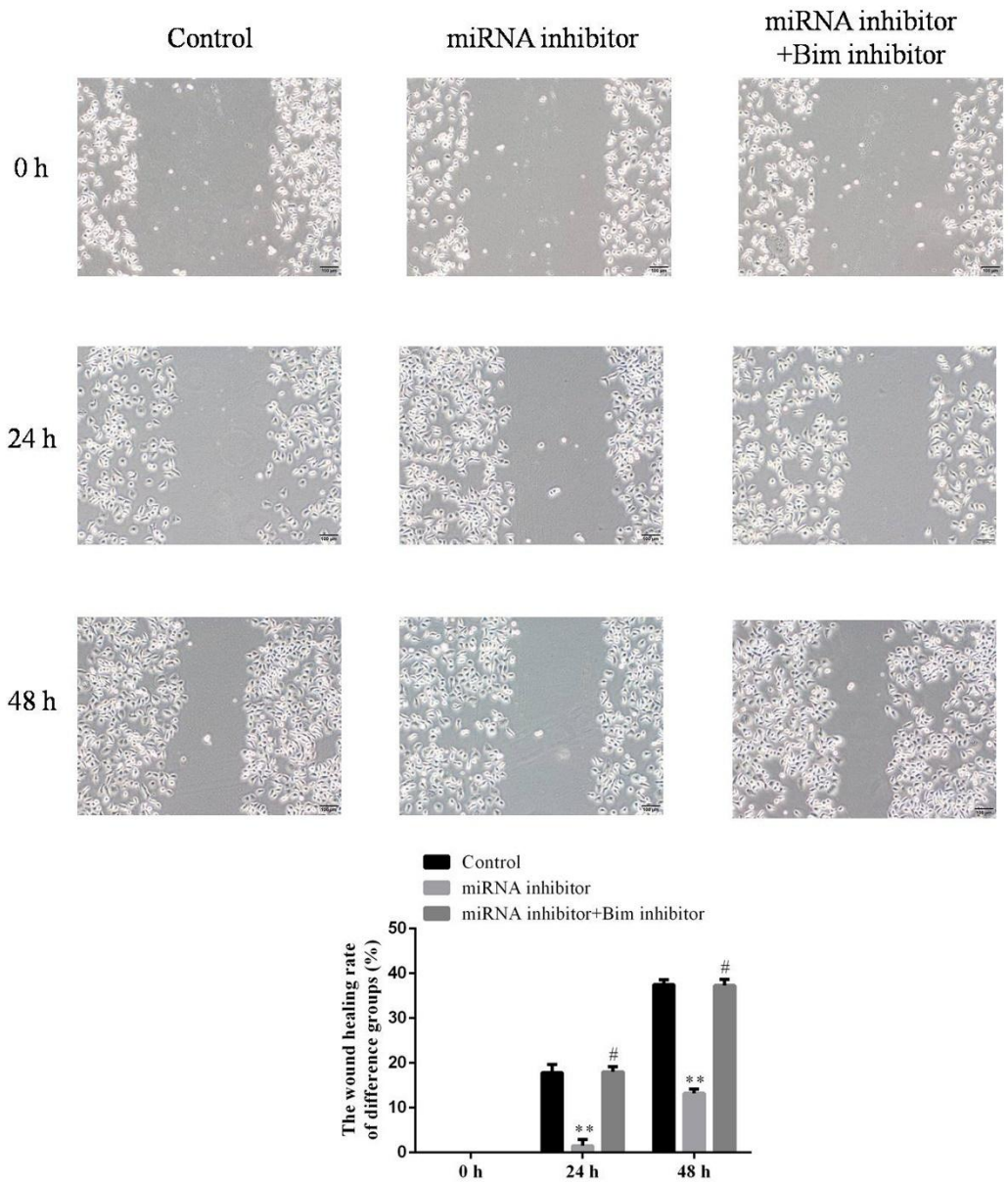

Fig 5. The wound healing rate of difference groups in 0h, 24h and 48 h(×100)

Control: normal control group treated with normal treatment; miRNA inhibitor:miRNA-92a inhibitor group transfected with miRNA-92a inhibitor; miRNA inhibitor+Bim inhibitor group transfected with miRNA-92a inhibitor and Bim inhibitor. **: P＜0.01 compared with Control group; #:P＜0.01 compared with miRNA inhibitor group

Relative protein expression

Bim expression was significantly upregulated but BCL-2 and EGFR expressions were significantly downregulated in the miRNA inhibitor + BIM inhibitor

group compared with those in the miRNA inhibitor group ((P ＜ 0.01; Fig. 6), suggesting that BIM may be a target gene of miRNA-92a.

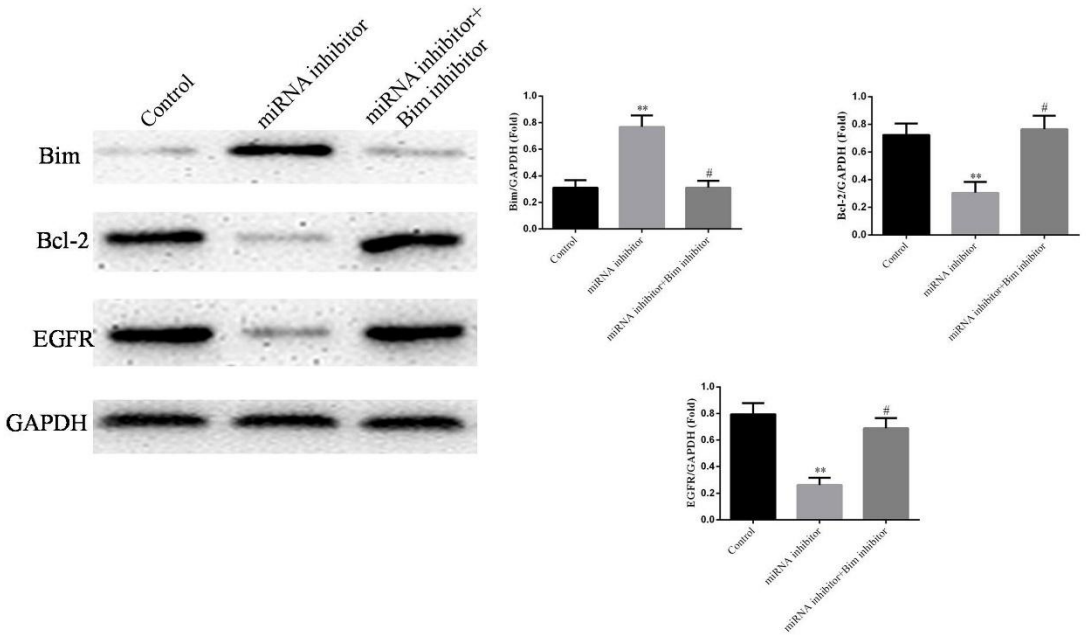

Fig 6. The relative protein expression of difference groups by WB assay

Control: normal control group treated with normal treatment; miRNA inhibitor:miRNA-92a inhibitor group transfected with miRNA-92a inhibitor; miRNA inhibitor+Bim inhibitor group transfected with miRNA-92a inhibitor and Bim inhibitor. **: P＜0.01 compared with Control group; #:P＜0.01 compared with miRNA inhibitor group

Discussion

Recent research has confirmed that miRNA-92a is abnormally expressed in multiple tumor tissues (13-15). BIM is an important member of the BCL-2 family and exerts apoptotic effects by suppressing BCL-2, which is an anti-apoptotic factor. Human BIM, located at 2q12 or 2q13, is expressed in haematopoietic, epithelial, germ, nerve and other normal tissue cells and plays an important role in maintaining a stable environment for haematopoietic cells, tumor occurrence and autoimmune diseases prevention (16). After the stimulation, BIM dissociates from the cell force protein complex and promotes apoptosis (17). Previous studies have reported that BIM is under-expressed or not expressed in cancers such as colon, breast and renal cancers

(18-21) and that changes in BIM expression are closely correlated with the occurrence and development of tumors. In the present study miRNA-92a expression was found to be significantly elevated in gastric cancer tissues compared with that in adjacent normal tissues. Additionally, BIM expression was significantly suppressed in gastric cancer tissues compared with that in adjacent normal tissues. These results suggest that miRNA-92a is negatively correlated with BIM expression in gastric cancer tissues. Additional in vitro cell studies using a miRNA-92a inhibitor in SGC-7901 cells showed that miRNA inhibitor inhibits cell proliferation, invasion and migration and stimulate cell apoptosis.

Previous studies have shown that abnormal BIM expression directly affects BCL-2 expression (22-24). BCL-2 is one of the anti-apoptotic genes discovered till date, and it can prolong cell lifespan without affecting cell cycling and differentiation. BCL-2 overexpression can inhibit apoptosis caused by various pro-apoptotic factors such as radiation and chemical drugs (25). In the present study, the results indicate that miRNA-92a knock-down suppresses cell proliferation induced by cell apoptosis up-regulated via BCL-2 expression, inducing BIM up-regulation. However, in the presence of a BIM inhibitor, cell proliferation normalized and apoptosis was suppressed.

EGFR overexpression is widespread in a variety of solid tumors, suggesting that abnormal EGFR expression is closely related to tumor cell proliferation, metastasis and invasiveness (26). Some studies have reported that BIM overexpression also suppresses EGFR expression and promotes tumor cell invasion and migration (27, 28). In present study, the increase in BIM expression induced by miRNA-92a knock-down suppressed SGC-7901gastric cancer cell invasiveness and migration in vitro via the regulation of EGFR expression. Both tumor cell invasiveness and migration were restored by the addition of BIM inhibitor.

In conclusion, miRNA-92a is a cancer promoting factor in gastric cancer as miRNA-92a knock-down in the gastric cancer cell line SGC-7901 suppresses cellular activities, such as proliferation, migration and invasion, in vitro.

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