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Original Research

MicroRNA-101-3p regulates gastric cancer cell proliferation, invasion and apoptosis by targeting PIM 1 expression

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Abstract: To investigate the effect of microRNA-101-3p (miRNA-101-3p) on proliferation, invasion and apoptosis of gastric cancer (GC) cells, and to explore its influence mechanism. Human GC cell line (AGS) and normal human gastric epithelial cell line (GES-1) were used in this study. The cells were transfected with proto-oncogene serine/threonine-protein kinase (PIM 1) overexpression plasmid, miRNA-101-3p mimics, or miRNA-101-3p non-homologous sequence using lipofectamine 2000. Real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting were used to determine the expressions of miRNA-101-3p and PIM 1 in GC cells. Cell counting kit-8 (CCK-8) and Transwell assays were used to assess the effect of miRNA-101-3p on proliferation and invasion of GC cells. The regulatory effect of miRNA-101-3p on PIM 1 was assessed using bioinformatics analysis and luciferase reporter gene assay. The expression of miRNA-101-3p was significantly down-regulated in GC cells, relative to normal human gastric epithelial cells ($p < 0.05$). However, the expression of PIM 1 mRNA was significantly upregulated in GC cells, when compared with normal gastric epithelial cells ($p < 0.05$). The expression level of miRNA-101-3p was significantly higher in miRNA-101-3p mimic group than in miRNA-101-3p control and normal control groups ($p < 0.05$). Cell proliferation in miRNA-101-3p mimic group was significantly and time-dependently reduced, when compared with miRNA-101-3p control and normal control groups ($p < 0.05$). Transfection with miRNA-101-3p mimics significantly increased the invasiveness of AGS cells, and significantly promoted their apoptosis ($p < 0.05$). Results of qRT-PCR and Western blotting showed that increased miRNA-101-3p expression significantly reduced the expression of PIM 1, while decreased miRNA-101-3p expression promoted the expression of PIM 1 ($p < 0.05$). Results of bioinformatics showed that miRNA-101-3p had specific binding sequence in the 3'UTR region of PIM 1. Cloning of miRNA-101-3p sequence into the luciferase reporter plasmid led to significant inhibition of the expression of PIM 1 ($p < 0.05$), but had no inhibitory effect on mutated PIM 1 3'UTR ($p > 0.05$). Overexpression of PIM 1 significantly reversed the inhibition of proliferation and invasion, and promotion of apoptosis by miRNA-101-3p ($p < 0.05$). These results indicate that miRNA-101-3p inhibits the proliferation and invasion of GC cells, and promotes their apoptosis by regulation of the expression of PIM-1.

Key words: Gastric cancer; MicroRNA-101-3p; Proliferation; Invasion; Apoptosis.

Introduction

Gastric cancer (GC) is a malignant tumor of the gastric mucosa epithelium. In the last few decades, the incidence of GC has increased due to a shift to Western-type diets, changing environment, *Helicobacter pylori (HP)* infection, and lifestyle modification. Although the pathogenesis of GC is not clear, it is speculated to be linked to heredity, diet, environmental influences and an physiological makeup (1). The involvement of microRNAs in GC progression has been reported (2). A microRNA is a small non-coding RNA molecule found in plants, animals and some viruses. It functions in RNA silencing and post-transcriptional regulation of gene expression. MicroRNAs function via base-pairing with complementary sequences within mRNA molecules (3). MiRNA-101-3p, a member of miRNA-101 family, is involved in the proliferation, differentiation and apoptosis of tumor cells, and studies have shown that it is significantly down-regulated in liver, lung, endometrial and glioma cancers (4-7). However, its role in GC progression has been scantly reported. MiRNA-101-3p-induced regulation of transcription of HOX antisense intergenic RNA (HOTAIR) has been shown to be mediated by serum response factor (SRF) (8). Proto-oncogene serine/threonine-protein kinase (PIM1) is highly expressed in cells of various tumors, where it participates in cell proliferation, apoptosis and metastasis. It has also been identified as a potential target for chemotherapy. MiRNA-144 inhibits the proliferation of GC cells via regulation of PIM 1 (9). The present study investigated the effect of miRNA-101-3p on proliferation, invasion and apoptosis of GC cells, and the mechanism involved.

Materials and Methods

Materials

Human GC cells (AGS) and normal human gastric

epithelial cells (GES-1) were obtained from Shanghai Huiying Biotechnology Co., Ltd. Fetal bovine serum (FBS), RPMI 1640 medium, Transwell chamber and primer sequences of MiRNA-101-3p, PIM 1 and GAPDH were purchased from Shanghai United Cell Bioengineering Co., Ltd. LipofectamineTM200 transfection reagent was a product of Thermo Fisher Scientific and Technology Co., Ltd. (USA). MiRNA-101-3p mimics, PcDNA-PIM 1, pcDNA, WT-3'UTR and mutant 3'UTR were obtained from Sigma-Aldrich (USA), and qRT-PCR kit was purchased from Biomics Biotechnology Co., Ltd. Cell counting kit-8 (CCK-8) was a product of Shanghai Xinrui Biotechnology Co., Ltd., and annexin V-FITC/PI apoptosis kit was purchased from Emjet Technology Co., Ltd. Microplate reader was purchased from Labsystems Diagnostics Co., (Finland). Flow cytometer was obtained from BD Biosciences (USA).

Cell culture, transfection and grouping

The AGS and GES-1 cells were cultured in RPMI-1640 medium supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. The culture medium was changed every 2 days, and subcultured with 0.25 % trypsin for 3 to 5 days. After attaining 50 - 60 % confluency, the cells were treated with serum-free medium and transfected. Cells in logarithmic growth phase were selected and used in this study. The cells (2 x 10^5 cells/well) were seeded in 6-well plates and incubated overnight, and thereafter, randomly assigned to three groups: normal control group, miRNA-101-3p control group, and miRNA-101-3p mimic group. The cells were transfected with PIM 1 over-expression plasmid, miRNA-101-3p mimics or miRNA-101-3p non-homologous sequence using lipofectamine 2000. After 24 h of transfection, the plasmid vector lipofectamine 2000 diluted in Opti-MEM medium was added and incubated for another 6 h. Thereafter, it was replaced with complete culture medium, and incubated for 24, 48 and 72 h.

In order to show whether miRNA-101-3p influenced the behavior of GC cells through PIM 1, miRNA-101-3p mimics and pcDNA-PIM 1 were simultaneously transfected into GC cells. The transfection procedure was the same as previously stated, and the cells were randomly assigned to three groups: miRNA-101-3p mimic group, miRNA-101-3p mimic + PIM1 control plasmid co-transfection group (miRNA-101-3p + pcDNA group), and miRNA-101-3p mimic + PIM 1 over-expression plasmid co-transfection group (miRNA-101-3p + pcDNA-PIM 1 group).

qRT-PCR

The levels of expression of miRNA-101-3p and PIM 1 mRNAs were determined using qRT-PCR. Trizol RNA extraction reagent was used to extract total RNA from cells of each group, while cDNA synthesis kit was used to perform the cDNA synthesis according to the instructions of the kit manufacturer. Light Cycler 1536 Real-time PCR Detection System was used for estimation of the expressions of mRNAs of miR-101-3p and PIM 1 with quantitative RT-PCR. Variation in the cDNA content was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR medium (20 µL) consisted of 6.4 µL of dH_2O, 1.6 µL of gene-specific primer (10 µM), 2 µL of synthesized cDNA and 10 µL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of miR-101-3p and PIM 1 (10).

Cell proliferation assay

The effect of miRNA-101-3p on proliferation of GC cells was determined using CCK-8 assay. The cells were seeded in 96-well plates at a density of 1 × 10^4 cells/mL, and cultured in Dulbecco's modified Eagle's medium (DMEM) for 24 h. After incubation for 24, 48 and 72 h, 10 µL of CCK-8 solution was added to the wells, followed by incubation at 37 ˚C for 2.5 h. The medium was finally replaced with 150 µL of 0.1 % dimethyl sulfoxide (DMSO), and the absorbance of each sample was read in a microplate reader at 450 nm. The assay was performed in triplicate. Cell proliferation was calculated as shown in Equation 1:

$$Cell\ proliferation\ (\%) = \frac{Absorbance\ of\ the\ experimental\ group \times 100}{Absorbance\ of\ the\ control\ group} \quad (1)$$

Cell invasion assay

The effect of miRNA-101-3p on invasive ability of GC cells was determined using Transwell chamber assay. The cells (2 × 10^5 cells/mL) were placed in Transwell chamber coated with substrate and cultured in serum-free medium. Medium containing 0.5 mL of 10 % FBS was added to the lower chamber. After 24 h, the cells that passed through the matrix gel membrane were stained with crystal violet after fixation, and were photographed and counted using an inverted microscope (11).

Apoptosis assay

The effect of miRNA-101-3p on apoptosis of GC cells was assessed using flow cytometric analysis. The AGS cells were seeded at a density of 2.5 x 10^6 cells/well in 6-well plates and cultured for 72 h. The cells were thereafter washed twice with phosphate-buffered saline (PBS), and thoroughly mixed with 500 µL binding buffer. The cells were then stained with 10 µL each of annexin V-fluorescein isothiocyanate and propidium iodide within 10 min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm. The level of cell apoptosis was analyzed using CellQuest software.

Luciferase reporter gene assay

The relationship between miRNA-101-3p and PIM 1 was assessed using luciferase reporter gene assay. Luciferase reporter gene was used to assess miRNA-101-3p localization sequence of PIM 1 gene in AGS cells. First, the target gene of miRNA-101-3p was predicted using Bioinformatics, and the target gene prediction database getScan, miRRB and miRanda were used to predict the target (PIM 1). The PIM 1 or mutated PIM 1 3' URT region was cloned into the psiCHECK2 vector, and the miRNA-101-3p mimics was simultaneously transfected with GC cells. After 48 h of transfection, luciferase activity was determined using luciferase reporter assay kit.

Western blotting

The cells were washed twice with PBS and lysed with 250 µL of ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitors. The resultant lysate was centrifuged at 15,000 rpm for 15 min at 4 °C, and the protein concentration of the supernatant was determined using BCA method. A portion of total cell protein (10 µg) from each sample was separated on 10 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of rabbit polyclonal anti-PIM 1, miRNA-101-3p and GAPDH, each at a dilution of 1 to 800. Then, the membrane was washed thrice with PBS and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Enhanced Chemiluminescence (ECL). The respective protein expression levels were normalized to that of GAPDH which was used as a standard.

Statistical analysis

Data are expressed as mean ± SEM, and statistical analysis was performed using SPSS (19.0). Groups were compared with Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Levels of expression of miRNA-101-3p and PIM 1 in AGS and GES-1 cells

As shown in Figure 1, the expression of miRNA-101-3p was significantly down-regulated in GC cells relative to normal human gastric epithelial cells ($p < 0.05$). However, the corresponding expression of PIM 1 mRNA was significantly upregulated in GC cells, when compared with normal gastric epithelial cells ($p < 0.05$).

Levels of expression of miRNA-101-3p in the different groups of AGS cells

The expression level of miRNA-101-3p was significantly higher in miRNA-101-3p mimic group than in miRNA-101-3p control and normal control groups ($p < 0.05$; Figure 2).

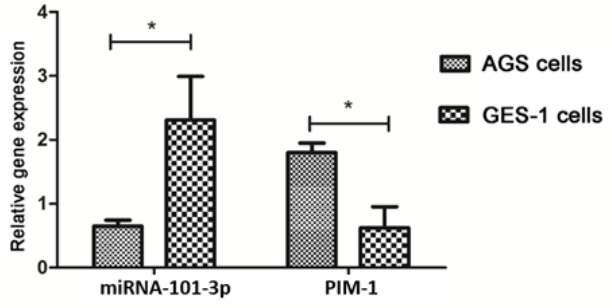

Figure 1. Expression levels of miRNA-101-3p and PIM-1 in AGS and GES-1 cells . *P<0.05, when compare with GES-1 cells.

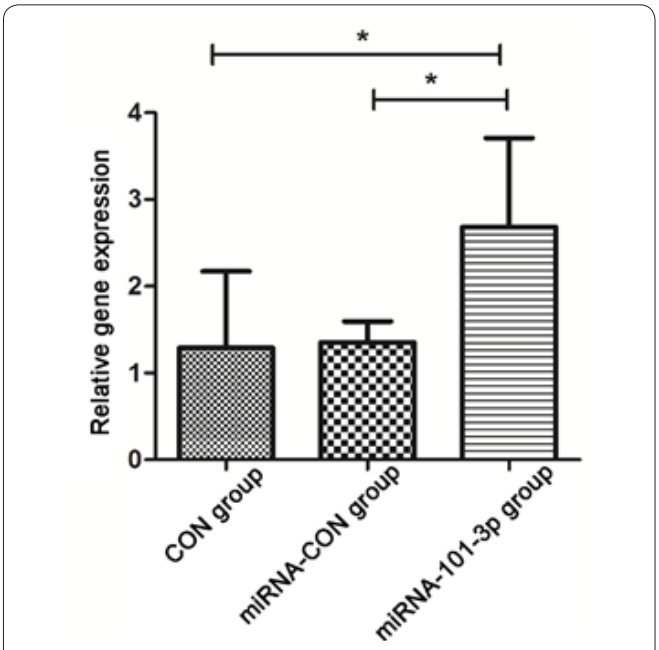

Figure 2. Effect of miRNA-101-3p mimic transfection on the expression levels of miRNA-101-3p (n = 6).

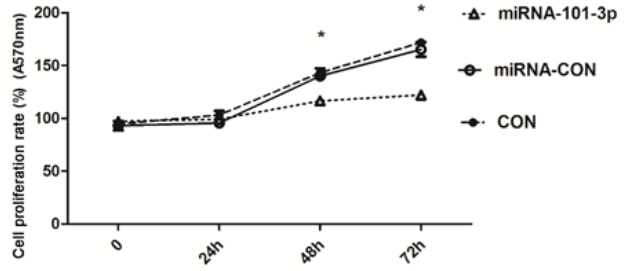

Figure 3. Effect of miRNA-101-3p mimic transfection on the proliferative activity of AGS cells (n = 6). $^{*}p < 0.05$, when compared with miRNA-101-3p control and normal control groups.

Effect of miRNA-101-3p mimic transfection on cell proliferation

Cell proliferation in miRNA-101-3p mimic group was significantly and time-dependently reduced, when compared with miRNA-101-3p control and normal control groups ($p < 0.05$). These results are shown in Figure 3.

Effect of miRNA-101-3p mimic transfection on invasiveness of AGS cells

Transfection with miRNA-101-3p mimics significantly increased the invasiveness of AGS cells ($p < 0.05$; Figure 4).

Effect of miRNA-101-3p mimic transfection on apoptosis

As shown in Figure 5, transfection with miRNA-101-3p mimics significantly promoted apoptosis in AGS cells ($p < 0.05$).

Results of bioinformatics analysis, qRT-PCR and Western blotting

Results of qRT-PCR and Western blotting showed that increased miRNA-101-3p expression significantly reduced the expression of PIM1, while decreased miRNA-101-3p expression promoted the expression of PIM 1 ($p < 0.05$; Figure 6).

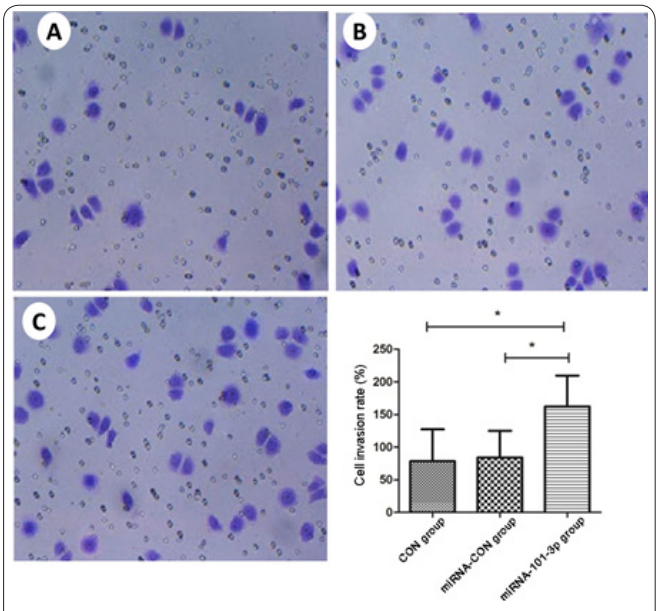

Figure 4. Effect of miRNA-101-3p mimic transfection on the invasion of AGS cells (n = 6). (A): Normal control group; (B): miRNA-101-3p control group; and (C): miRNA-101-3p mimic group. $^{*}p < 0.05$, when compared with normal and miRNA-101-3p control groups.

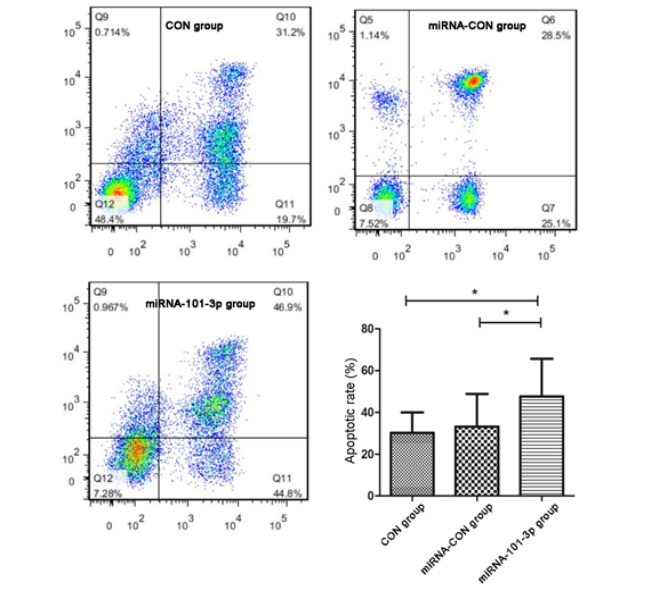

Figure 5. Effect of miRNA-101-3p mimic transfection on the apoptosis of AGS cells (n = 6). $^{*}p < 0.05$, when compared with normal and miRNA-101-3p control groups.

Results of Bioinformatics showed that miRNA-101-3p had specific binding sequence in the 3'UTR region of PIM 1 (Figure 7). After cloning the sequence into the luciferase reporter plasmid, miRNA-101-3p significantly inhibited the expression of PIM 1 ($p < 0.05$; Figure 8) , but had no inhibitory effect on mutated PIM 1 3'UTR ($p > 0.05$; Figure 8).

Effect of PIM 1 over-expression on miRNA-101-3p mRNA and protein expression in AGS cells

As shown in Figure 9, overexpression of PIM 1 significantly increased the RNA (A) and protein (B) expression levels of miRNA-101-3p.

Discussion

MicroRNA (miRNA) is an endophytic small RNA

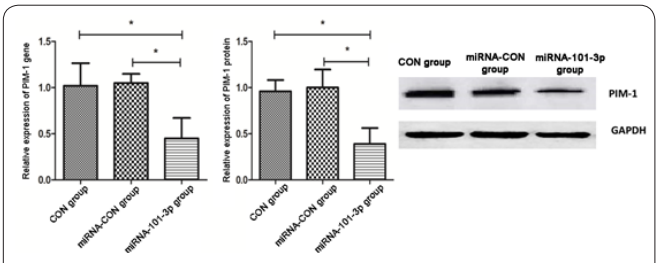

Figure 6. Effect of miRNA-101-3p on expression levels of PIM-1 gene and protein (n = 6). (A): Expression of PIM-1 mRNA as measured using qRT-PCR, and (B): Expression of PIM-1 protein as measured using Western blotting. $^{*}p < 0.05$, when compared with normal and miRNA-101-3p control groups.

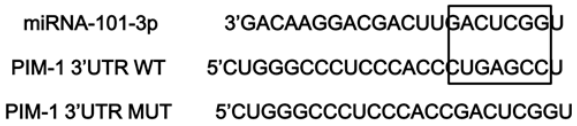

Figure 7. Results of Bioinformatics analysis.

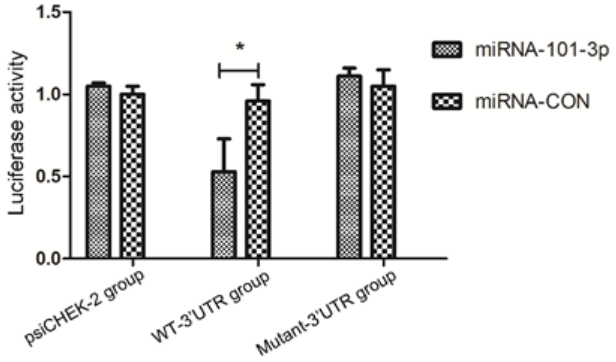

Figure 8. Results of luciferase reporter gene assay (n = 6). $^{*}p < 0.05$, when compared with normal and miRNA-101-3p control groups.

consisting of about 20 to 24 nucleotides, and plays a key regulatory role in cells. Each miRNA has multiple target genes, with several miRNAs regulating a single gene. This complex regulatory mechanism not only controls the expression of multiple genes through a single miRNA, but also fine-tunes gene expression via combination of several miRNAs (12). MiRNA-101-3p is a member of miRNA-101 family which is significantly down-regulated in liver, lung, endometrial and glioma cancers (4-7). It targets MCL1 and EZH2 genes, thereby mediating the proliferation, differentiation and invasion of tumor cells (5, 7). This study provides the first-time evidence that miRNA-101-3p regulates the proliferation, invasion and apoptosis of GC cells by targeting PIM 1 expression. The expression level of miRNA-101-3p was significantly lower in AGS cells than in GES-1 cells, an indication that miRNA-101-3p may be poorly expressed in GC cells. This suggests that miRNA-101-3p may inhibit GC progression. Cell proliferation in miRNA-101-3p mimic group was significantly and time-dependently reduced, when compared with miRNA-101-3p control and normal control groups. Transwell assay revealed that transfection with miRNA-101-3p mimics significantly increased the invasiveness of AGS cells. These results suggest that miRNA-101-3p may be involved in GC progression through enhancement of invasiveness of the cells.

Reports on the mechanism by which miRNA-

101-3p inhibits cancer cell proliferation, invasion, and apoptosis are scanty. Studies have shown that miRNA-101-3p targets adenylate-activated protein kinase (AMPK) in the regulation of glucose metabolism and proliferation in triple-negative breast cancer cells (13). It has also been reported that miRNA-101-3p acts on metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) by blocking phosphatidylinositol 3 kinase (PI3K)/akt murine thymoma viral oncogene homolog signaling pathway, thereby inhibiting the proliferation and migration of lung cancer cells (14). Proto-oncogene PIM 1 is located on the long arm of chromosome 6, and its protein product exhibits Akt kinase activity, which is closely linked to the development of tumors (15). It has been reported that PIM 1 is highly expressed in a variety of tumors, and participates in the proliferation, invasion, apoptosis and metastasis of the cells (16-18). This study shows for the first time the relationship between miRNA-101-3p and PIM 1 in GC.

Luciferase reporter gene assay showed that miRNA-101-3p has a specific binding sequence in the 3'UTR region of PIM 1. After cloning the sequence into the luciferase reporter plasmid, miRNA-101-3p significantly inhibited the expression of PIM 1, but had no inhibitory effect on mutated PIM 1 3'UTR. These results suggest that miRNA-101-3p mimics may inhibit the expression of PIM 1 via recognition of 3'UTR site on PIM 1 gene. It is likely that PIM 1 is the target gene of miRNA-101-3p. The results of this study also showed that co-transfection of miRNA-101-3p mimics and PIM 1 over-expression plasmid into AGS cells significantly reversed the inhibitory effect of miRNA-101-3p on proliferation, invasion and apoptosis of the cells. This suggests that miRNA-101-3p influences the proliferation, invasion and apoptosis of GC cells by targeting the expression of PIM 1.

The results obtained in this study indicate that miRNA-101-3p inhibits the proliferation and invasion of GC cells, and promotes their apoptosis by regulation of the expression of PIM-1.

Acknowledgements
None.

Conflict of Interest
There are no conflict of interest in this study.

Author's contribution
All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Fanggui Xu; Fubing Wu, Lili Yang, Wen Huang, Fanggui X collected and analysed the data; Fubing Wu and Lili Yang wrote the text and all authors have read and approved the text prior to publication.
Fubing Wu and Lili Yang contributed equally to this work and should be considered as co-first authors.

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