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Original Article

Down-regulation of miR-92a inhibits cell proliferation and invasion but induces apoptosis of gastric cancer by regulating FBXW7

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Abstract: This study aimed to investigate the potential roles of miR-92a expression in the development and invasion of gastric cancer, and to illustrate the potential mechanism. Expression of miR-92a in gastric cancer SGC7901 cells were analyzed using RT-PCR. MTT assay, Transwell assay, and Annexin V-FITC were used to analyze the influences of miR-92a expression on tumor cell viability, invasion, and apoptosis respectively. Western blot was used to detect the cell apoptosis-related protein expression in SGC7901 cells. Compared to the normal gastric mucosa epithelial 3T3 cells, miR-92a was highly expressed in SGC7901 cells. Accordingly, miR-92a suppression significantly decreased the cell viability, induced apoptosis, and suppressed cell invasion. Moreover, the suppressed miR-92a significantly increased the expression of FBXW7, but decreased the cycline E and c-myc expression in SGC7901 cells. Taken together, our study suggested that miR-92a suppression may play certain inhibit roles in the development and invasion of gastric cancer through involving in biological processes including proliferation, apoptosis, and invasion by negatively regulating FBXW7 and its downstream protein of cycline E and c-myc.

Keywords: Gastric cancer, miR-92a, cell proliferation, cell apoptosis, cell invasion

Introduction

Gastric cancer remains to be one of the most common malignancies, which ranks to the first place among the gastrointestinal cancers worldwide [1]. The incidence and mortality for gastric cancer are high among the deaths that brought from tumors [2]. Previous studies have demonstrated that various kinds of factors are involved in the pathogen and biology of gastric cancer, including inflammatory factors, EB virus, and other signal pathways [3-5]. Accordingly, a variety of treatment methods including the molecular targeted therapeutic drugs, surgery, and minimally invasive surgery have play certain roles in the clinical treatment of gastric cancer [6, 7]. However, the cures for gastric cancer still remain hard due to the complicate molecular pathogen mechanism of gastric cancer. Thus, to investigate the molecular mechanism of gastric cancer will be useful for the treatment of gastric cancer.

microRNAs (miRNAs) are some 20- to 23-nt noncoding RNAs in length that play pivotal roles in diversity of biological processes at the transcriptional or post-transcriptional level by targeting the 3'UTR of target genes [8]. Accumulating studies have reported that various miRNAs are involved in the biological processes and pathogen of gastric cancer, such as miR-133b, miR-10b, and miR-132 [9-11]. Recently, Zhu et al reported that a five-miRNA panel (miR-16, miR-92a, miR-451, miR-486-5p, and miR-25) in plasma was identified as the potential biomarker for the detection of gastric cancer [12]. miR-92a has been reported to play crucial roles in the biology of other tumors instead of gastric cancer through intricate mechanisms. For example, the overexpressed miR-92a promotes cervical cancer proliferation and invasion by targeting FBXW7 [13], and miR-92a functions as a diagnosis and prognosis biomarker for the colorectal cancer, even as that of colorectal metastasis [14, 15]. Besides, miR-

Table 1. Primers used for targets amplification in this study

Target	Primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
Cycline E	Sense	AGCCCAGCCAGACGGAATCC
	Antisense	GTCTAGAAGTATGGACCTCATC
c-myc	Sense	GAGCCCCTGGTGCTCCATGAG
	Antisense	AGGACTCTGACACTGTCCAACTTG
FBXW7	Sense	GTGATAGAGCCCCAGTTCCA
	Antisense	CCTCAGCCAAAATTCTCCAG

92a is associated to the invasion of lung cancer via involving in the abnormal expression of RECK [16]. Although increasing evidence has mentioned the pivotal roles of miR-92a in the tumor biology, few have reported its roles in the pathogen of gastric cancer.

In the current study, we measured the expression of miR-92a in the gastric SGC7901 cells and analyzed the roles of miR-92a expression in several biological processes of gastric cancer cells using the miRNA inhibitor transfection. Comprehensive experimental studies were used to assess the influences of miR-92a expression on cell proliferation, invasion, and apoptosis, as well as the cell biological process-related protein expression. This study was aimed to investigate the possible roles of miR-92a in the biological processes including proliferation and invasion of gastric cancer and to reveal its potential mechanism of action.

Materials and methods

Cell culture and cell transfection

Human gastric cancer SGC7901 cells and normal gastric mucosa epithelial 3T3 cells, which were obtained from the American Type Culture Collection (ATCC; MA, USA), were cultured in Dulbecco's modified Eagle medium (Gibco) with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma), and incubated at 37°C in 5% CO_2.

For cell transfection, miR-92a sponge and the miR-92a mimic (GenePharma, Shanghai, China) were transfected into the SGC7901 cells using the Lipofection 2000 regent (Invitrogen). Cells transfected without miR-92a sponge or with the co-transfection of miR-92a sponge and miR-92a mimic were considered as the controls.

Cell proliferation assay

Cell viability was assessed using the MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay according to previous described [17]. In brief, after 48 h of transfection, cells were adjusted to 5×10^3 cells for injection into the 96-well plates. Cells were centrifuged at 12,000 rpm, and then supernatant was removed. Followed with addition into 20 μL MTT and then cultured for 4 h. Finally, 150 μL dimethylsulfoxide (DMSO) was used to mix with the cells for 10 min. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan). All experiments were conducted independently for 3 times.

Cell apoptosis assay

Cell apoptosis was quantified using the flow cytometry with Annexin V-FITC cell apoptosis kit (Invitrogen, USA) according to manufacturer's protocol. Briefly, after 48 h of transfection, cells were harvested and then washed using PBS buffer (PH 7.4) for 3 times, and then resuspended in the staining buffer. After that, 5 μL of annexin-V-FITC and 5 μL of propidium iodide (PI) were mixed with the cells. After being cultivated at room temperature for 10 min, mixtures were analyzed using the FACScan flow cytometry. Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

Cell invasion assay

Cell invasion ability was analyzed using the Matrigel method as previously described [18]. Briefly, after 24 h of incubation, the upper chamber was enveloped with serum-free DMEM medium supplemented with 50 mg/L Matrigel and then air-dried at 4°C. After being sucked out the medium, 50 μL fresh serum-free medium containing 10 g/L BSA was added and then cultured for 30 min at 37°C. After that, Transwell was put into the 24-well plates and cultured with DMEM (Dulbecco's Modified Eagle Medium) medium mixed with 10% FBS. Then cells in Transwell were suspended with serum-free DMEM medium. After 48 h, Transwell in each group was washed with PBS buffer to remove the upper cells on microporous membrane, followed with fixed in ice-cold alcohol. Finally, Transwell from each group was stained with 0.1% crystal violet for 30 min, and

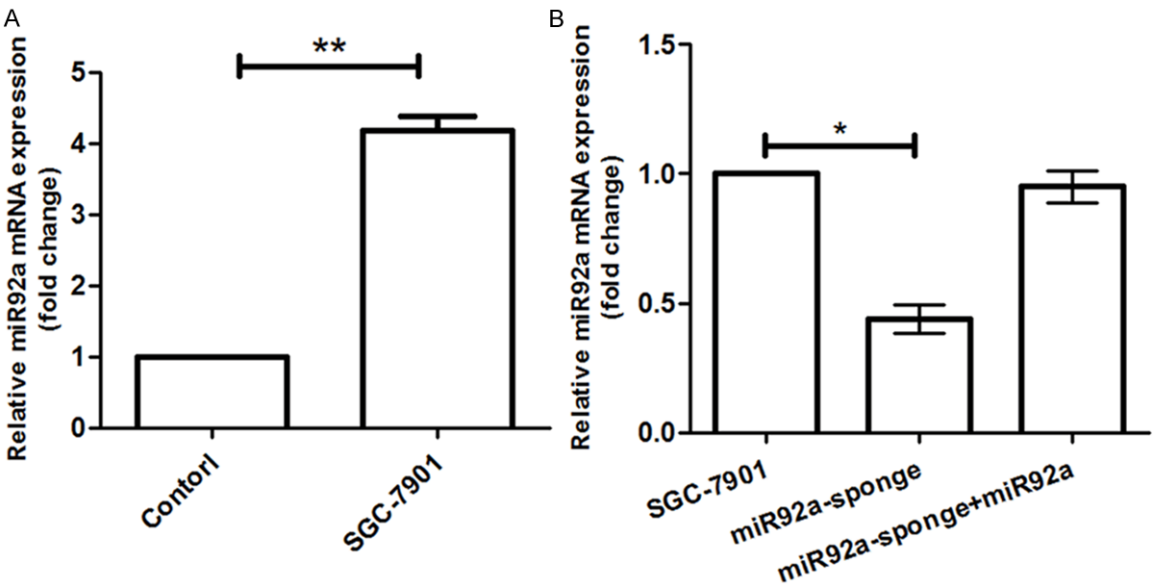

Figure 1. Expression of miR-92a in gastric cancer cells. A: Relative mRNA of miR-92a was highly expressed in SGC7901 cells, which was more than that in the normal gastric mucosa epithelial 3T3 cells. **: P<0.01, compared to the control cells (3T3); B: Relative RNA miR-92a expression was significantly decreased compared to the control (without miR-92a sponge transfection). *: P<0.05, compared to the control (SGC7901 cells without any transfection).

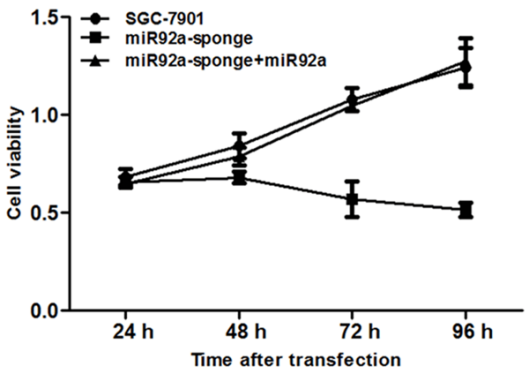

Figure 2. miR-92a suppression inhibited the gastric SGC7901 cell viability. After miR-92a sponge transfection, cell viability was significantly decreased with time increasing.

then decolorated with 33% acetic acid. The absorbance of eluents was observed at OD 570 nm using a microplate reader (Biotech, USA). Transwell in control group was treated without Matrigel.

Real time (RT)-PCR

Total RNA from the cells was isolated using TRIzol Reagent (Invitrogen) according to the manufacture's protocol. The extracted RNA were treated with RNase-free Dnase I to remove the mixed DNA, and concentration and purity for RNA were determined using SMA 400-UV-VIS (Merinton, Shanghai, China). The purified RNA (0.5 μg/μL) was used for the cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen). Expressions of targets were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). Primers used for targets amplification were shown in **Table 1**. GAPDH served as the internal control.

Western blot

Cells were lysed in the lysate buffer containing phenylmethanesufonyl fluoride (PMSF) and then were centrifuged at 12,000 rpm at 4°C for 5 min. The supernatant was collected to determine the concentration of isolated protein using bicinchoninic acid (BCA) protein assay kit (Pierce, Rochford, IL). For western blotting, a total of 50 μg protein per cell lysate was subjected into a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidencefluoride (PVDF) membrane (Mippore). Membrane was blocked in Tris Buffered Saline Tween (TBST) supplemented with 5% non-fat milk for 1 h, and subsequently incubated with monoclonal anti-

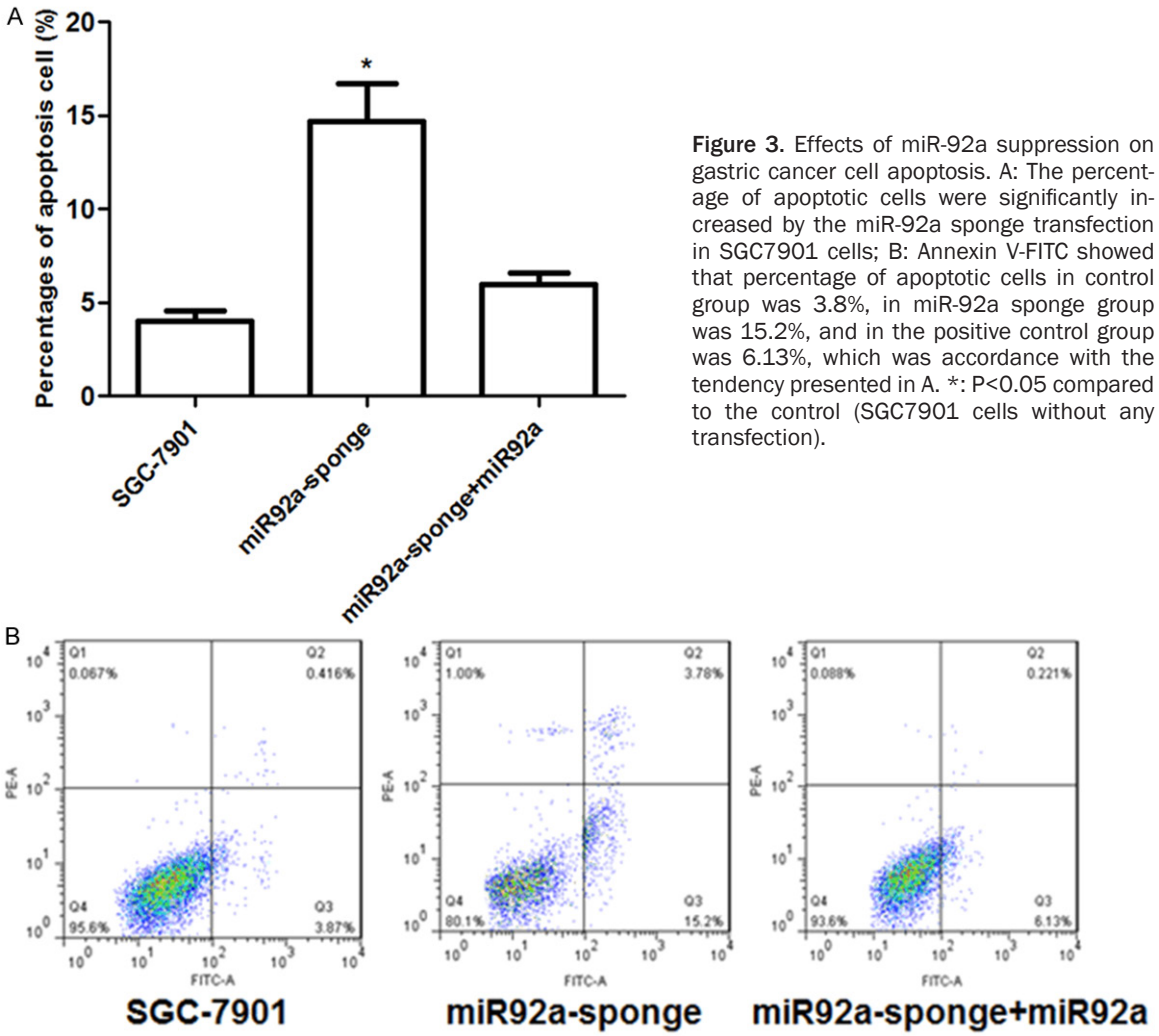

Figure 3. Effects of miR-92a suppression on gastric cancer cell apoptosis. A: The percentage of apoptotic cells were significantly increased by the miR-92a sponge transfection in SGC7901 cells; B: Annexin V-FITC showed that percentage of apoptotic cells in control group was 3.8%, in miR-92a sponge group was 15.2%, and in the positive control group was 6.13%, which was accordance with the tendency presented in A. *: P<0.05 compared to the control (SGC7901 cells without any transfection).

bodies (1:100 dilution, FBXW7, cycline E, and c-myc) overnight at 4°C, followed by incubation with horseradish-peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Then the membranes were washed with 1×TBST buffer for 10 min for 3 times. Finally, detection of PVDFs was performed using the development of X-ray after chromogenic substrate with an enhanced ECL (chemiluminescence) method. GAPDH was considered as the internal control.

Statistical analysis

All experiments were conducted 3 times independently in this study. The data are expressed as the mean ± SD. Statistical analysis was performed using SPSS 19.0 software (IBM, Armonk, NK). Differences for data among groups were calculated using a one-way analysis of variance (ANOVA) with post-hoc test. P<0.05 was considered as the statistically significant.

Results

Expression of miR-92a in gastric cancer cells

The relative mRNA expression for miR-92a in the gastric SGC7901 cells were highly expressed compared to that in normal cells (P<0.05; **Figure 1A**). Besides, when cells were transfected with miR-92a sponge, its relative expression was significantly decreased than that in the cells without any transfection (P< 0.05; **Figure 1B**). These results suggested the correlation between miR-92a expression and gastric cancer.

miR-92a suppression declined the gastric cancer cell viability

Accordingly, we analyzed the cell viability to assess the effects of miR-92a expression on gastric cancer proliferation. The cell viability was declined at 48 h and this effect was more

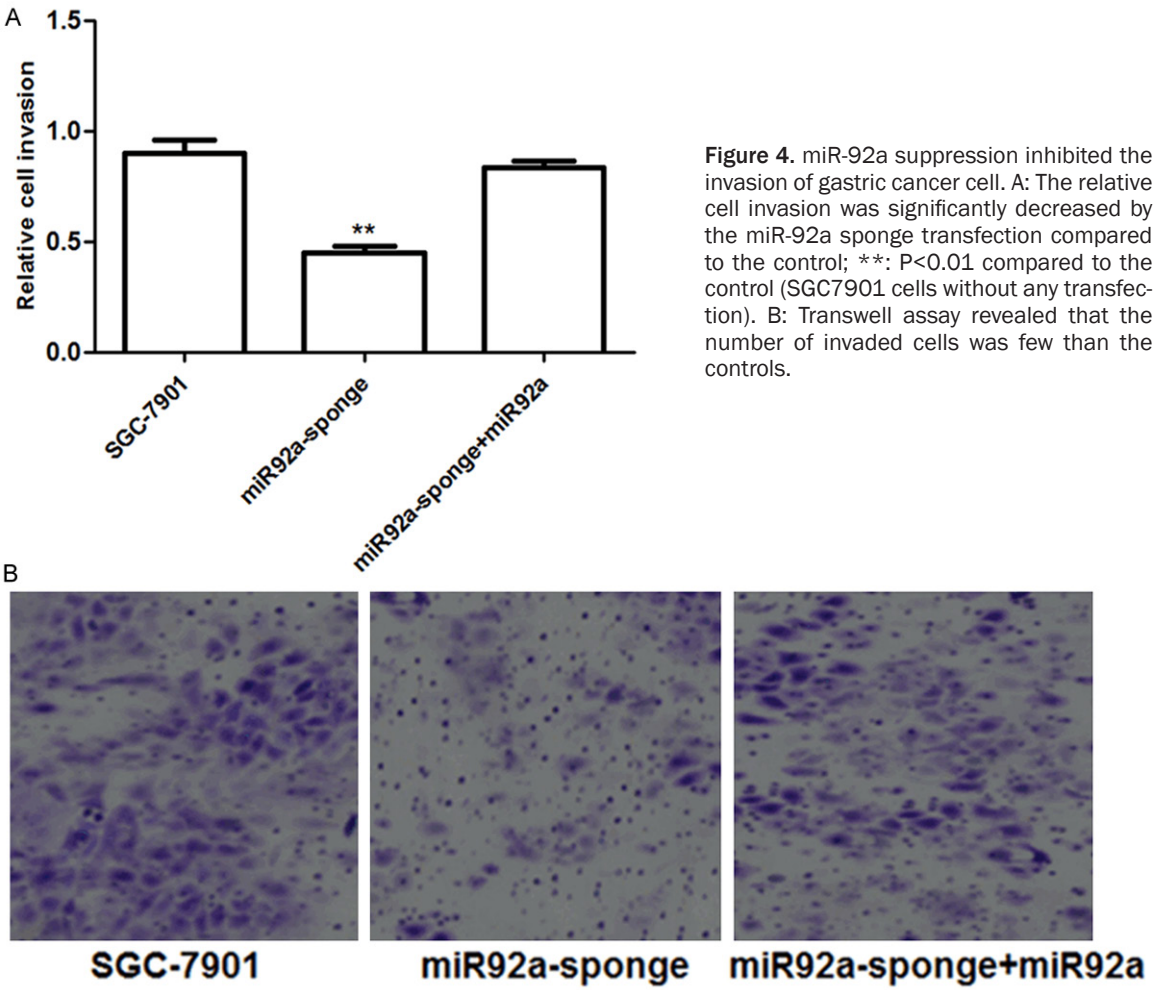

Figure 4. miR-92a suppression inhibited the invasion of gastric cancer cell. A: The relative cell invasion was significantly decreased by the miR-92a sponge transfection compared to the control; **: P<0.01 compared to the control (SGC7901 cells without any transfection). B: Transwell assay revealed that the number of invaded cells was few than the controls.

apparent with time increasing after miR-92a sponge transfection. However, when cells were transfected with the miR-92a and miR-92a sponge, cell viability was almost the same as that in control cells (**Figure 2**).

miR-92a suppression induced gastric cancer cell apoptosis

When cells were transfected with the miR-92a sponge, the percentage of apoptotic SGC7901 cells (about 15.2%) was significantly increased compared to the control (about 3.8%) (P<0.05; **Figure 3A**), which was accordance with the Annexin V-FITC results (**Figure 3B**).

miR-92a suppression was associated with gastric cancer invasion

We further analyzed the influence of miR-92a expression on the invasion ability of gastric cancer using Transwell assay (**Figure 4**). The relative invasion cells were significantly decreased by the miR-92a suppression than that in control cells (P<0.01; **Figure 4A**). In addition, Transwell assay revealed that the number of invaded cells was reduced compare to the two control group (**Figure 4B**).

Effects of miR-92a suppression on cell apoptosis-related protein expression

To analyze the molecular mechanism for miR-92a expression on the gastric cancer cell biological processes, we further measured the cell apoptosis-related protein expression (**Figure 5**). The relative mRNA and protein levels for FBXW7 were significantly increased by the suppressed miR-92a in SGC7901 cells compared to the control (**Figure 5A**). Besides, the relative mRNA level and protein expression for cycline E and c-myc were both drastically decreased by the suppressed miR-92a than that in controls (**Figure 5B**).

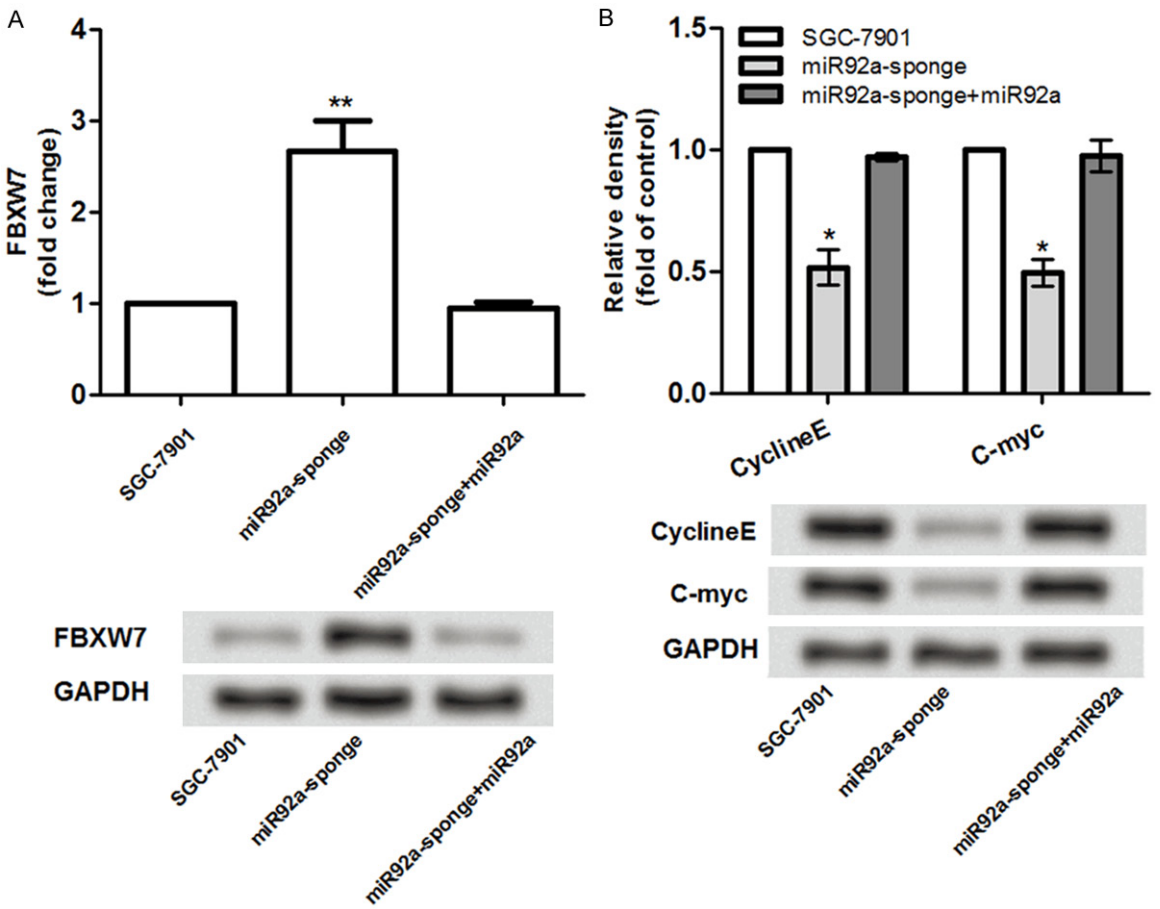

Figure 5. Influence of miR-92a expression on the expression of cell apoptosis-related protein. A: The relative mRNA and protein level for FBXW7 was significantly increased by the suppressed miR-92a in SGC7901 cells; B: The relative mRNA and protein expression for cycline E and c-myc was significantly decreased by the suppressed miR-92a. *: $P<0.05$ and **: $P<0.01$ compared to the control (SGC7901 cells without any transfection).

Discussion

Increasing evidence has reported the crucial roles of miRNAs in the biology and pathogen of gastric cancer [19, 20], and miR-92a has been demonstrated to play significant roles in the development, metastasis and diagnosis of lots of tumors [16, 21]. Recently, miR-92a has been reported functions as a biomarker in the detection of gastric cancer [12], but the molecular mechanism remains unknown. In this study, we analyzed the expression of miR-92a in gastric SGC7901 cells and investigated the effects of miR-92a expression on the biological processes of gastric cancer. miR-92a has been proved to be up-regulated in the serum from patients suffered with gastric cancer [22], and in agreement with previous data, our study showed that the relative mRNA expression of miR-92a in SGC7901 cells was highly expressed (**Figure 1**), indicating the correlation between miR-92a and gastric cancer.

Accordingly, we further analyzed the influences of miR-92a expression on SGC7901 cell biological processes using gene-mediated silencing, including proliferation, apoptosis and invasion. Our data revealed that the SGC7901 cell viability was significantly inhibited while cell apoptosis was induced by the miR-92a suppression (**Figures 2** and **3**). Qiong et al proved that miR-92a promotes the proliferation of gastric cancer stem cell [23]. The cervical cancer cell proliferation was significantly enhanced by the overexpressed miR-92a [13]. In addition, the cell viability was inhibited while apoptosis was induced by the anti-miR-92a oligonucleotide in gastric SGC7901 and MKN-45 cells [24]. Based on our data, we speculated that miR-92a suppression was closely correlated to

the proliferation and apoptosis of gastric cancer. From other point of view, our results presented that cell invasion was inhibited by the suppressed miR-92a in SGC7901 cells (**Figure 4**). The roles of miR-92a in the invasion of gastric cancer have not been fully discussed. But Zhao et al proved that the overexpressed miR-92a promotes cervical cancer invasion [13], and similar result for miR-92a in lung cancer was demonstrated by Huang et al [25]. Taken together, these results suggested that miR-92a suppression may play roles in gastric cancer development and progression via involving in biological processes including proliferation, apoptosis and invasion.

Furthermore, we further measured the cell apoptosis-related proteins to investigate the potential mechanism for miR-92a in gastric cancer. Our results showed that FBXW7 expression was highly expressed, whereas cycline E and c-myc expression was significantly decreased by the suppressed miR-92a (**Figure 5**). FBXW7is a F-box protein family which is characterized by an approximately 40 amino acid motif, and has potential roles in the pathogenesis of human cancers [26, 27]. Previous studies have demonstrated that FBXW7 regulates tumor apoptosis [28], and the overexpressed FBXW7 inhibits renal cancer proliferation and induces apoptosis [29]. Besides, several studies have proved that FBXW7 was targeted by several kinds of miRNAs and then play certain roles in the biology of gastric cancer [30]. On the other side, it has been said that the identified substrates were cycline E, JUN, MYC, and Notch1 released notch intracellular domain [31]. C-myc and cycline E abnormal expression was correlated to the apoptosis and proliferation of the development of gastric cancer [32]. FBXW7 induced cell apoptosis through depredating the c-myc and cycline E in gastric cancer [33]. Additionally, FBXW7 has been proved to be correlated to the invasion of gastric cancer [34]. Based in our data, we speculated that the cell invasion and apoptosis, and proliferation of gastric cancer may be affected by miR-92a via increasing FBXW7 and decreasing cycline E and c-myc.

In conclusion, the data presented in this study reveals that down-regulation of miR-92a may play certain inhibit roles in the development and invasion of gastric cancer through suppressing the cell viability and inducing apoptosis, and inhibiting invasion by negatively regulating FBXW7 and its downstream protein of cycline E and c-myc. Our study may provide theoretical basis for illustrating the possible pivotal roles of miR-92a in the development and progression of gastric cancer, and may provide basis for the potential roles of miR-92a in the clinical treatment of gastric cancer. Further experimental studies are still needed to explore the deep mechanism at transcriptional level.

Disclosure of conflict of interest

None.

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