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Original Research

MicroRNA-92a promotes proliferation and invasiveness of gastric cancer cell by targeting FOXO1 gene

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Abstract: The aim of this study is to investigate the effect of miRNA-92a on GC cell proliferation, migration and invasiveness, and the mechanism(s) involved. Four GC cell lines (SGC-7901, BGC-823, MKN45 and HGC-27) and normal human gastric epithelial cells (GES1) were used in this study. MicroRNA-92a mimics or inhibitor were transfected into the cells. The results of transfection were assessed using real-time quantitative polymerase chain reaction (qRT-PCR). Cell proliferation, migration, invasiveness and apoptosis were determined using cell counting kit 8 (CCK-8), scratch test, Transwell invasion assay, and flow cytometric analysis, respectively. The protein target of miRNA-92a was predicted using Bioinformatics. The expression of FOXO1 protein was measured using Western blotting. The expression of miRNA-92a was significantly upregulated in GC cells, relative to normal gastric epithelial cells ($p < 0.05$). Overexpression of miRNA-92a significantly promoted the proliferation, migration and invasiveness of GC cells, but significantly inhibited their apoptosis ($p < 0.05$). MicroRNA-92a directly targeted FOXO1 gene, and significantly reduced its protein expression. Overexpression of miRNA-92a promotes the proliferation, migration and invasiveness of GC cells, and plays a role similar to that of oncogene. It directly targets FOXO1 gene by inhibiting its protein expression.

Key words: Gastric cancer; MicroRNA-92a; Cell proliferation; FOXO1 gene; Expression.

Introduction

Gastric cancer (GC) is a frequently occurring digestive tract cancer characterized by high morbidity and mortality. Although it can be treated if diagnosed early, the 5-year survival and overall prognosis are unsatisfactory. The initiation of GC involves several signaling pathways and abnormal gene expression patterns which are often accompanied by inactivation of anti-oncogenes such as p53 and Rb, and activation of oncogenes such as transforming growth factor α (TGF-α), C-myc gene, and mitogen-activated protein kinase (MAPK) (1, 2). The abnormal expressions of some genes contribute significantly to the proliferation, differentiation, angiogenesis, metastasis and invasiveness of GC cells.

MicroRNAs (miRNAs) are a class of non-coding single-stranded small RNAs with a length of about 22 nucleotides. They function through base-pairing with complementary sequences within mRNA molecules. MicroRNAs influence the expression of target genes by pairing with their 3'UTR regions, thereby participating in life processes such as cell growth and differentiation, and apoptosis (3). MicroRNA expression is tissue-specific (i.e. the expression level of a particular miRNA varies from one tissue to another, and the level of expression of a different miRNA also varies within the same tissue). Studies have shown that miRNAs participate in tumorigenesis via the regulation of expression of target genes. They function as proto-oncogenes or anti-oncogenes.

MicroRNA-92a is a recently discovered miRNA, which plays a regulatory role in the pathogenesis of a variety of tumors. Its expression is upregulated in tumors and plays a role similar to that of oncogene. It has been reported that miRNA-92a promotes cell proliferation via negative regulation of cell cycle regulator p53 subtype. In addition, it promotes cell migration and invasive ability (4). Studies have shown that miRNA-92a promotes proliferation and inhibits apoptosis in CRC cell via regulation of PTEN gene. Clinical studies have provided supporting evidence that miRNA-92a can be used as an important biological marker for the diagnosis of CRC after surgical intervention, especially in patients with extra-colorectal lymph node metastasis. As a diagnostic tool, its sensitivity and specificity are approximately 85.7 and 90.9 %, respectively (5). It has been reported that miRNA-92a is highly expressed in hepatocellular carcinoma, and its expression level is closely correlated with those of other clinical and pathological indices (6). The present study investigated the effects of miRNA-92a on GC cell proliferation, migration and invasiveness, and the mechanism(s) involved.

Materials and Methods

Cell lines and culture

Four GC cell lines (SGC-7901, BGC-823, MKN45 and HGC-27) and normal human gastric epithelial cells (GES1) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences. The GC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/ streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. The GES1 cells were cultured in RPMI medium supplemented with 10 % FBS only.

Quantitative real time polymerase chain reaction (qRT-PCR)

The level of expression of miRNA-92a was determined using qRT-PCR.

Total RNA was extracted from the cells using Trizol total RNA extraction kit, and reverse transcribed to obtain the cDNA. Light Cycler 1536 RT-PCR detection system was used for the estimation of the mRNA expression of the gene. Variation in the cDNA content was normalized using glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene. The qRT-PCR reaction conditions were: pre-denaturation at 95 °C for 5 min; PCR reaction at 95 °C for 5 sec and 60 °C for 30 sec, and a total of 40 cycles. The PCR reaction mixture (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 level of the protein. The primer sequences used are shown in Table 1.

Cell transfection

Liposome transfection method was employed using lipofectamine 3000 transfection reagent. Cells in logarithmic growth phase were seeded in 6-well plates until they attained 60 – 80 % fusion. The cells were cultured in serum-free medium with equal volume of miRNA-92a mimics, control miRNA-92a mimics, inhibitor miRNA-92a, and miRNA-92a inhibitor control, each at 10 µmol/L. Incubation was carried out at room temperature for 5 min. Lipofectamine 3000 was dissolved in serum-free medium and incubated at room temperature for 10 min to form a mixture. The mixture was then added to cells in each group, and cultured at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air for 48 h. The transfection efficiency was determined using qRT-PCR.

Determination of levels of expression of miRNA-92a in GC cells

After transfection, the GC cells were assigned to four groups: miRNA-92a mimics group, miRNA-92a mimics control group, miRNA-92a inhibitor group, and miRNA-92a inhibitor control group. The levels of expression of miRNA-92a in the four groups were determined using qRT-PCR.

Cell proliferation assay

Cell proliferation was determined using CCK-8 assay kit. The cells were seeded in 96 well plates at a density of 6 x 10^3 cells/well, and cultured for 24 h at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Then, 10 µL of CCK-8 was added to each well, and cultured for72 h. The absorbance of the samples was read in a microplate reader at 450 nm. The assay was performed in triplicate.

Apoptotic assay

The cells were seeded at a density of 2.5 x 10^6 cells/well in 6-well plates and cultured at 37 °C for 72 h. They were thereafter washed 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 488 nm.

Cell migration assay

The migratory ability of GC cells was determined using scratch test. Cells in logarithmic growth phase were seeded in 6-well plates until they attained 90 - 100 % confluency, and scratches were made on the cell monolayers. After washing thrice with serum-free medium, the cells were further cultured for 72 h, and then observed and analyzed using Image Pro Plus (6.0). Cell motility was calculated as shown in Equation 1.

$$Cell\ mobility = \frac{1-scratch\ width\ during\ measurement \times 100\ \%}{initial\ scratch\ width} \quad (1)$$

Determination of cell invasiveness

The degree of invasiveness of GC cells was assessed using Transwell invasion assay. The cells (1×10^5 cells/mL) were placed in Transwell chamber coated with substrate and cultured in serum-free medium. Medium containing 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, photographed and counted using an inverted microscope.

Prediction of miRNA-92a target gene

TargetScan and miRanda were used to predict the target gene of miRNA-92a.

Luciferase reporter gene assay

The FOXO1 3'URT region or the mutated FOXO1 3' URT region was cloned into the psiCHECK2 vector,

Table 1. Primer sequences used for qRT-PCR.

Gene	Sequence
miRNA-92a	Forward: 5'-CGGATCAACTACATGAACCTCCAGC-3'
	Reverse: 5'-CCGGAATTGTG ATGTGCAGACAACAG-3'
GAPDH	Forward: 5'-ATTGGAACGTGATAAGATT-3'
	Reverse: 5'-GGAACGCATGATATATG-3'

and the miRNA-92 mimics was simultaneously transfected into GC cells. After 48 h of transfection, luciferase activity was determined using luciferase reporter assay kit.

Western blotting

The cells were washed with PBS and lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor. 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 bicinchoninic acid (BCA) protein kit. A portion of total cell protein (40 µg) from each sample was separated on 12 % 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 (3 %) 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 FOXO1 and GAPDH, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Bio-rad gel imaging system. The respective protein expression levels were normalized to that of standard GAPDH.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (19.0). Groups were compared using Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

Results

Level of expression of miRNA-92a in GC cells after transfection

The mRNA expression of miRNA-92a in GC cells and normal gastric epithelial cells was detected by qRT-PCR. The results showed that the mRNA expression of miRNA-92a in GC cells was significantly up-regulated compared with that in normal gastric epithelial cells ($p < 0.05$; Figure 1).

Expression levels of miRNA-92a in miRNA-92a mimics and inhibitor groups

As shown in Figure 2, after transfection, qRT-PCR was used to verify the transfection results. The results showed that the expression of miRNA-92a was significantly up-regulated in the mimics group and down-regulated in the inhibitor group, indicating successful transfection ($p < 0.05$).

Effect of miR-92a expression on GC cell proliferation

CCK-8 method was used to detect the effect of transfection of miRNA-92a on the proliferation of gastric cancer cells. The results showed that the overexpression of mirna-92a significantly promoted the proliferation of sgc-7901 cells compared with the control group ($p < 0.05$; Figure 3).

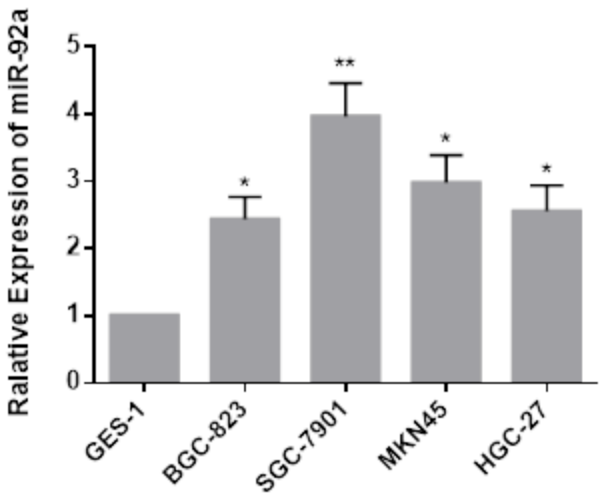

Figure 1. Expression of miRNA-92a in the four GC cell lines. $^{*}p < 0.05$; $^{*}p < 0.01$, when compared with GES 1 cells (control group).

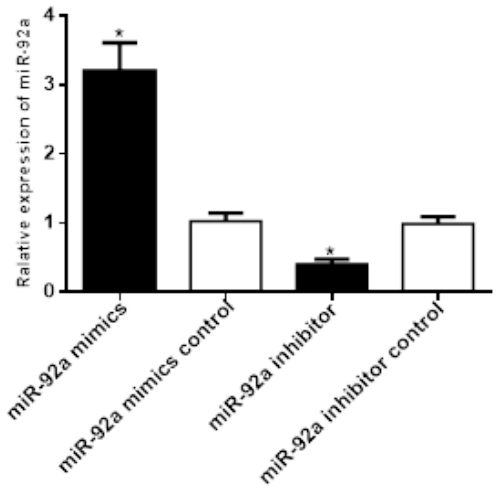

Figure 2. Levels of expression of miRNA-92a in the four transfection groups. $^{*}p < 0.05$, when compared with miRNA-92a inhibitor group.

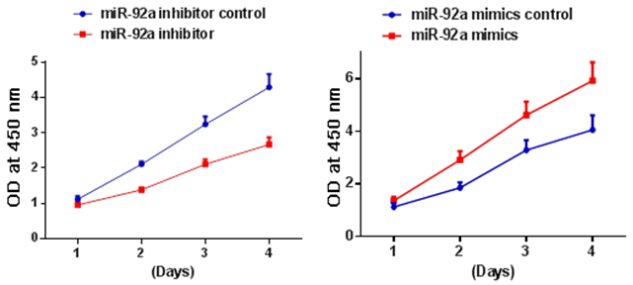

Figure 3. Growth curves of cells in the different transfection groups.

Effect of miRNA-92a expression on cell apoptosis

There were only few apoptotic cells in the miRNA-92a mimics group, relative to mimics control group, but the number of apoptotic cells was significantly increased in the miRNA-92a inhibitor group, when compared with the inhibitor control group ($p < 0.05$). These results are shown in Figure 4.

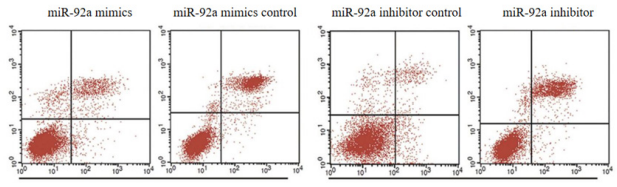

Figure 4. Flow cytometric analysis of cell apoptosis.

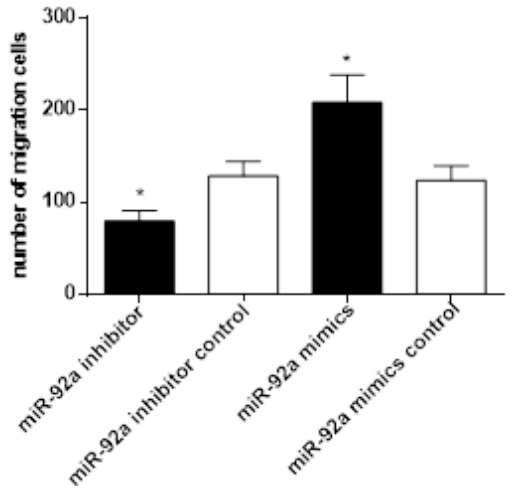

Figure 5. Effect of miRNA-92a overexpression on cell migration**.** *$p < 0.05$, when compared with mimics or inhibitor control group.

Effect of miRNA-92a on cell migration

The migratory ability of cells in miRNA-92a mimics group was significantly higher than that of mimics control group, and the mean number of cells migrating in the inhibitor group was significantly lower than that of the inhibitor control group ($p < 0.05$; Figure 5).

Effect of miRNA-92a mimics and inhibitor on GC cell invasiveness

As shown in Figure 6, cells in miRNA-92a mimics group were more invasive than those in the mimics control group, but cell invasiveness was significantly inhibited by miRNA-92a inhibitor ($p < 0.05$).

Gene target of miRNA-92a

The results of TargetScan and miRanda showed that miRNA-92a was able to bind to the 3'UTR end of transcription factor FOXO1. The results of luciferase reporter gene assay revealed that the activity of 3'UTR luciferase of FOXO1 mRNA was significantly reduced ($p < 0.05$), but there was no significant effect on the activity of the mutant luciferase ($p > 0.05$). These results are shown in Figure 7.

Effect of miRNA-92a on FOXO1 protein expression

Western-Blot assay was used to detect FOXO1 protein expression levels in different transfection groups, and the results showed that over-expression of miRNA-92a significantly down-regulated FOXO1 protein expression in GC cells. ($p < 0.05$; Figure 8).

Discussion

MicroRNAs (miRNAs) are a class of non-coding small RNAs containing about 22 nucleotides. They are widely distributed in animals, plants and some viruses.

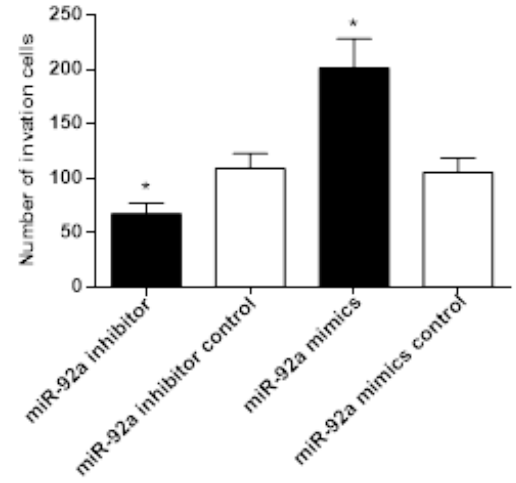

Figure 6. Invasiveness of GC cells after miRNA-92a overexpression. *$p < 0.05$, when compared with mimics or inhibitor control group.

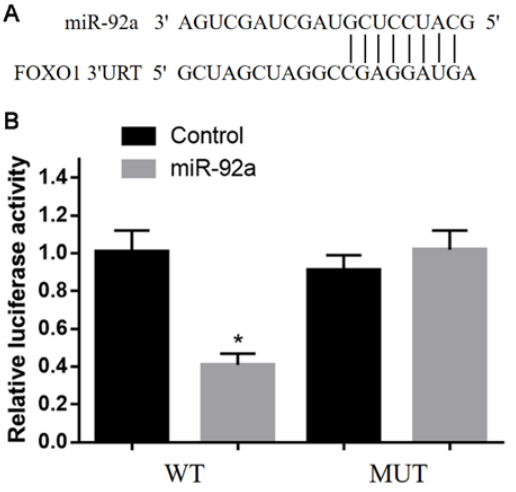

Figure 7. Results of luciferase reporter gene assay. (A): Results of TargetScan and miRanda analysis showing binding interaction between miRNA-92a and the 3'UTR end of FOXO1; and (B): Direct targeting of FOXO1 mRNA by microRNA-92a.

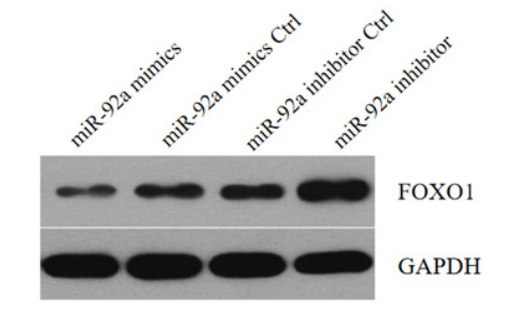

Figure 8. Effect of miRNA-92a on FOXO1 protein expression.

They were first discovered in larvae in 1993 (7). MicroRNAs regulate their target genes at the post-transcriptional level via complementary base pairing, thereby influencing their protein expressions. There are many types of miRNAs, many of which exist in cells, while others circulate freely in the blood and extracellular fluid. MicroRNAs participate in different physiological and pathological processes such as cell proliferation and differentiation. They also influence cell development, stress and apoptosis (8).

The pathogenesis of GC is a complex process involving several genes and regulatory pathways. MicroRNAs have been shown to participate in the pathogenesis of GC. However, the precise molecular mechanism involved remains unknown. MicroRNA-92a is a member of the miRNA-17-92 cluster, and has been shown to promote cell proliferation and angiogenesis, while inhibiting cell differentiation. It is a potential oncogene, whose expression in many tumors has been reported to be significantly upregulated (9-10). Studies have shown that the expression of miRNA-92a is significantly increased in patients with bladder cancer (11). Its expression is also significantly upregulated in CRC, and is closely related to the stage of the tumor, but decreases significantly after surgery (12). The expression of miRNA-92a is significantly upregulated in esophageal cancer cells, and its level is closely related to lymph node metastasis and TNM staging (13). Although miRNA-92a has been reported to be highly expressed in GC cells (14), the molecular mechanism involved has not been fully elucidated.

Forkhead (FOX) gene is a family of transcription factors with highly conserved sequences comprising 17 subfamilies, among which FOXO is closely related to animal cell growth and development, differentiation and proliferation, apoptosis, signal transduction, immunity and aging (15, 16). Studies have shown that FOXO1 is a protein product of an antioncogene. During tumorigenesis, FOXO1 activity decreases significantly, which in turns shortens the cell cycle and weakens repair of DNA damage, thereby resulting in genomic instability (17, 18). The involvement of FOXO1 protein in GC formation has been reported (19). The phosphorylation of FOXO1 protein inhibits the expressions of downstream genes such as P27 and Bim, thereby promoting the upregulation of anti-apoptotic genes, cell proliferation and tumor growth (20). However, the acetylation of FOXO1 weakens the ability of FOXO1 to bind homologous DNA sequences, thereby enhancing the phosphorylation of FOXO1, while reducing its transcriptional activity (21).

MiRNA-92a is one of the seven miRNAs encoded in the highly conserved miR-17-92 gene cluster. miR-17-92 clusters are potential oncogenes that are up-regulated in a variety of tumors, including hematopoietic malignancies and solid tumors from the breast, colon, lung, pancreas, prostate, and stomach. MiR-17-92 cluster is most likely involved in the regulation of gastric cancer, among which miRNA-92a may be an important regulatory factor in this process. Shapiro et al. (22) found that miRNA-92a was highly expressed in colon cancer, and the expression of miRNA-92a decreased significantly after surgical treatment. Volinia et al. (23) found that miRNA-92a was highly expressed in gastric cancer tissues. However, Ng et al. (24) found no statistically significant difference between the gastric cancer group and the normal group. Studies have shown that antisense oligonucleotides targeting miR-92a can effectively inhibit the proliferation of gastric cancer cells and promote their apoptosis. MiR-92a may be one of the key regulatory factors for the occurrence and development of gastric cancer (25). In this study, miRNA-92a was highly expressed in GC cells, an indication that it may play the role of an oncogene. The proliferation, migration and invasiveness of GC cells were also significantly enhanced by miRNA-92a overexpression. Inhibition of miRNA-92a overexpression significantly reduced the proliferation, migration and invasiveness of GC cells, while promoting their apoptosis. Overexpression of miRNA-92a also significantly reduced the protein expression of FOXO1. These results indicate that miRNA-92a may enhance the growth of GC cells. It is likely that miRNA-92a may promote the migration and invasive ability of GC cells, thereby playing an oncogenic role.

The results obtained in the present study suggest that overexpression of miRNA-92a promotes the proliferation, migration and invasiveness of GC cells, and plays a role similar to that of oncogene. It directly targets FOXO1 gene by inhibiting its protein expression.

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

Author's contribution
All work was done by the author 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 Shichun Feng; Jiawei Yu, Qingfeng Ni, Shu Zhang, Ruheng Hua, Ran Tao, Chong Tang, Shichun Feng collected and analysed the data; Jiawei Yu wrote the text and all authors have read and approved the text prior to publication.

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