MicroRNA-190 regulates FOXP2 genes in human gastric cancer

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Objective: To investigate how microRNA-190 (miR-190) regulates FOXP2 genes in gastric cancer (GC) cell line SGC7901.

Methods: We identified that miR-190 could target FOXP2 genes by using dual luciferase enzyme assay. Precursor fragment transfection of miR-190 was performed with GC cell line SGC7901 and human gastric mucosal cell line GES-1. miR-190 expression was detected by reverse transcription-polymerase chain reaction (RT-PCR) and FOXP2 protein expression was measured by Western blotting.

Results: FOXP2-3′-untranslated region (UTR) in miR-190 transfection group was significantly decreased as compared with other groups. There were no significant differences in fluorescence signals of FOXP2mut-3′-UTR in each group. Therefore, it was assumed that miR-190 can target FOXP2 genes. Through RT-PCR verification, it was observed that the expression level of miR-190 was significantly higher in GC cell line SGC7901 than in human gastric mucosa cell line GES-1 after transfection with miR-190 mimics. The expression level of miR-190 was significantly higher in GES-1 cells than in SGC7901 cells after transfection with miR-190 inhibitors. Western blotting results showed the expression level of FOXP2 was significantly lower in GC cell line SGC7901 than in GES-1 cells. Compared with blank, mimics control, and inhibitors control groups, the miR-190 mimics group showed significantly enhanced proliferation, migration, and invasion abilities, while miR-190 inhibitors group showed decreased abilities toward proliferation, migration, and invasion (P<0.05). The transcription level of miR-190 and the expression level of FOXP2 in tumor tissues and adjacent normal tissues in GC patients were verified to be consistent with those of cell line experiments.

Conclusion: Upregulation of miR-190 can lead to downregulation of FOXP2 protein expression. miR-190 may serve as a potential target for GC diagnosis.

Keywords: targeted regulation, SGC7901 cell line, RT-PCR, Western blotting, CCK-8, migration assay, transwell

Introduction

Gastric cancer (GC) remains the fourth most common cancer and the third main cause of cancer-related deaths around the world.¹ The incidence of GC is related to people’s diet habits, geographical environment, genetic factors, Helicobacter pylori infection, and some precancerous lesions. Targeted therapy, as a hot research topic in recent years, has played an important role in finding new types of GC-associated molecules and their working mechanisms in the occurrence or/and progression of GC, which is significant for GC diagnosis, precise biological classification, comprehensive treatment guidance, and prognosis evaluation. FOXP family members are involved in many important biological processes, including metabolism, development, differentiation, proliferation, apoptosis, migration, and invasion.² Function loss or functional change of
FOX has an important impact on cell migration and may lead to cell cancerization.3,4 FOXP2, as the first gene relevant to the human ability to develop language, is widely concerned in recent years.5 However, few literatures reported its relation with tumors and regulation of microRNAs (miRNAs).

miRNAs are small, regulatory, noncoding RNA molecules that govern the expression of their cognate target genes predominantly through binding to the 3′-untranslated region (UTR).7 miRNA has been shown to play important gene-regulatory roles in many physiological and pathological processes, including cell proliferation, differentiation, apoptosis, and hormone secretion.8,9 Multiple miRNAs have been found to be related to GC, such as let-7a and miR-21.10-12 Human miRNA-190 (miR-190) is derived from an intron region of the talin 2 gene on chromosome 15.13 Overexpression of miR-190 could lead to the inhibition of tumor growth and to prolonged dormancy periods in glioblastoma and osteosarcoma.14 Few studies explored the association of miR-190 and GC via targeting FOXP2 gene. In this study, we investigated the regulation of miR-190 to FOXP2 in GC cell line SGC7901, promising to offer a direction to follow-up studies as well as a new candidate for GC diagnosis.

Materials and methods
Reagents and materials
GC cell line SGC7901 was purchased from Nanjing KeyGEN Biotech. Co., Ltd (Nanjing, People’s Republic of China). The human gastric epithelial cell line GES-1 was purchased from Shanghai Bioleaf Biotech Co., Ltd (Shanghai, People’s Republic of China). RPMI-1640 culture medium was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The calf serum was purchased from Tianjin Hao Yang Biological Manufacture Co., Ltd (Tianjin, People’s Republic of China). Rabbit anti-human FOXP2 polyclonal antibody and mouse anti-human β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). IRDyeTM 700DX-labeled IgG and IRDyeTM 800DX-labeled sheep anti-rabbit IgG were purchased from LI-COR Biosciences (Lincoln, NE, USA). SiPORTTM NeoFXTM Transfection Agent, miR-190 mimics, inhibitors, and negative control segments were purchased from Ambion (Austin, TX, USA). Odyssey two-color infrared laser imaging system was purchased from LI-COR Biosciences.

Cell culture and cell transfection
GC cell line SGC7901 and normal human gastric mucosal cell line GES-1 were cultured in RPMI-1640 culture medium containing 10% fetal bovine serum (FBS), and were incubated at 37°C with 5% CO₂. SGC7901 and GES-1 cells in the logarithmic growth phase were transfected. The groups were designed as follows: 1) blank control group, 2) miR-190 mimics group, 3) miR-190 mimics control group, 4) miR-190 inhibitors group, and 5) miR-190 inhibitors control group. Twenty-four hours after the cell transfection, the total RNA of cells was extracted for real-time fluorescent quantitative polymerase chain reaction (PCR) to detect the expression changes of miR-190 in transfected cells. Seventy-two hours after the cell transfection, total protein was extracted and FOXP2 protein expression was detected by Western blotting assay.

Dual luciferase enzyme assay
miR-190 target gene prediction was carried out by using Target gene databases, namely TargetScan, PicTar, and miRanda. Dual luciferase reporter gene system was used to further verify whether FOXP2 was a direct target gene for miR-190. The full length of the 3′-UTR of FOXP2 gene was achieved by clonal expansion. PCR product was cloned into the downstream multiple cloning sites of pmiRGLO (Promega Corporation, Fitchburg, WI, USA) luciferase gene, and with bioinformatics tools, site-directed mutagenesis was performed by predicting the binding sites of miR-190 and target genes. Expression of Renilla luciferase PRL TK vector (TaKaRa, Dalian, People’s Republic of China) was used as an internal reference to adjust the number of cells and the transfection efficiency differences. miR-190 and negative control were cotransfected into SGC7901 cells with luciferase reporter vectors. As per the method provided by Promega Corporation, the double fluorescent luciferase activity was detected.

Reverse transcription-polymerase chain reaction
SGC7901 and GES-1 cells without transfection and with 24 hours’ transfection were collected. TRizol reagent was used to extract the total RNA which was converted into cDNA through reverse transcription. For the synthesis of cDNA, the TaqMan miRNA Reverse Transcription Kit (Thermo Fisher Scientific) was used. The reference U6 used specific primers for reverse transcription with the sequence 5′-CGCTTCAGAATTGGCGTGTCAT-3′. The reaction conditions were as follows: 16°C for 30 minutes, 42°C for 42 minutes, and 85°C for 5 minutes. With cDNA used as a template, PCR amplification was performed by using miR-190-specific primers and SYBR Green I dye molecules. miR-190 and U6 both used TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) for reverse transcription-PCR
(RT-PCR) amplification. The PCR reaction conditions were as follows: first step, 95°C for 5 minutes; second step, 95°C for 10 seconds, 60°C for 20 seconds, 72°C for 20 seconds with a total of 40 cycles. The data were analyzed by 2^-ΔΔCt method: ΔCt = Ct_target - Ct_β-actin. Ct refers to the number of cycles when amplification curve reached threshold value.

Western blotting for FOXP2 expression
After 72 hours of transfection, cells were collected and washed with phosphate-buffered saline (PBS) for two times. Thirty minutes after adding 100 µL cells lysis solution to split cells at 4°C, centrifugation was performed at 12,000× g for 10 minutes. Supernatant fluid was obtained and the concentration of protein was determined by the Bradford method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) was carried out with 50 µg total protein per lane. The protein was transferred to polyvinylidene fluoride membrane. Skim milk (5%) was added to block nonspecific binding. After 1 hour incubation in dark at room temperature, 1:200 diluted rabbit anti-human FOXP2 polyclonal antibody was added and was followed by the addition of mouse anti-human actin monoclonal antibody, and was kept overnight at 4°C. After washing the membrane, 1:10,000 diluted IRDyeTM 800DX-labeled sheep anti-rabbit IgG (for detecting FOXP2) and IRDyeTM 408DX-labeled sheep anti-rat IgG (for detecting β-actin) were added consecutively. After being cultured at room temperature in dark for 1 hour, the membrane was washed thoroughly and was placed in the Odyssey double color infrared laser imaging system for direct scanning/imaging. The integral optical density (OD) of each band was calculated, and the relative expression of the target protein was calculated as the ratio of the integral OD of the target band and the integral OD of the corresponding reference β-actin.

Cell counting kit-8 assay
After the transfected cells were collected, cells in the logarithmic growth phase were digested using trypsin. Then cells were subjected to centrifugation for 5 minutes at 559 g, followed by resuspension with complete medium. Cell suspension with 5×10^4 cells/mL was prepared and then cells were seeded into three 96-well plates. Each group was set for three wells. Blank control group was also set and cultured under normal culture conditions. After the cells were seeded for 24, 48, and 72 hours, a 96-well plate was selected for analysis. Subsequently, 10 µL of cell counting kit-8 (CCK-8) reagent was added to each well, followed by incubation for another 1.5 hours. Microplate spectrophotometer was used for the determination of OD at 450 nm.

Scratch test
Cells were first seeded in a six-well plate. After 80% of the cells were attached to the wall, the cells were rinsed in PBS twice to remove floating cells. Then sterile 10 µL Eppendorf tip was used to scratch the cell plate. Subsequently, the cells were washed twice and 2 mL RPMI-1640 culture medium containing 10% FBS was added to the culture plate. The scratch width after 0 and 48 hours was observed using an inverted microscope and photographed.

Transwell assay
The transwell assay was conducted according to the manufacturer’s instructions. Briefly, 60 µL of 5 mg/mL Matrigel (BD Biosciences, San Jose, CA, USA) was added to the upper chamber. Then 0.05% pancreatin/EDTA was added to digest the cells. Serum-free RPMI-1640 culture medium was added to the upper chamber to wash and resuspend the cells, and cell density was adjusted to 1×10^6 cells/mL. Subsequently, the upper chamber was added with 200 µL cell suspension medium while lower chamber was added with 600 µL RPMI-1640 culture medium containing 10% FBS, followed by incubation in a CO₂ incubator for 24 hours. Then a swab was used to wipe out the remaining Matrigel and cells in the upper chamber. Crystal violet staining was performed for 30 minutes in the lower chamber and then 10% acetic acid was used to wash out the crystal violet. Enzyme-labeled board was used to determine the OD at 570 nm with three replicates in each group. For performing migration assays, procedures similar to those followed for invasion assays were used except that the upper chamber was not coated with Matrigel.

GC tissue test
Fifty GC cases (32 males and 18 females) diagnosed and treated by surgeries from May 2014 to April 2015 were selected from Beijing Hospital. Tumor tissue and adjacent normal tissues were attained through surgical procedures treated by surgeries from May 2014 to April 2015 were selected from Beijing Hospital. Tumor tissue and adjacent normal tissues were attained through surgical procedures for subsequent RT-PCR and Western blotting verification. This study was approved by the Ethics Committee of Beijing Hospital, and written informed consent was obtained from the patients themselves or their families.

Statistical method
SPSS 20.0 software was used for data analysis. Count data were expressed as percentage or ratio and comparisons on count data were conducted using the chi-square test, while measurement data were presented with mean ± standard deviation (x̄±SD). Independent samples t-test was used to compare the mean differences between groups.
values of two independent samples and one-way analysis of variance (ANOVA) (after homogeneity-of-variance test) was used for multigroup comparisons. Fisher’s least significant difference (LSD) test was conducted for pairwise comparison between the mean values of multiple groups. P-value <0.05 was considered as statistically significant.

Results
Targeting relationship between miR-190 and FOXP2 gene
By searching databases TargetScan, PicTar, and miRanda, FOXP2 was found to be a potential target gene of miR-190. Results from the dual luciferase reporter gene system showed that compared with other groups, the luciferase signal of FOXP2-3′-UTR in the miR-190 mimics group decreased by ~80% (all P<0.05) (Figure 1). For mutant FOXP2mut-3′-UTR, the luciferase signals of different groups did not decrease significantly (all P>0.05). The result showed that the combination of miR-190 and FOXP2-3′-UTR binding sites could inhibit the transcription of FOXP2 and negatively regulate the expression of FOXP2. Therefore, FOXP2 may be the direct regulatory target gene of miR-190.

Expressions of miR-190 in SGC7901 and normal human gastric mucosa cells
RT-PCR results showed (Table 1, Figure 2) that miR-190 mimics group, miR-190 inhibitors control group, and blank control group showed no significant differences when compared in pairs (all P>0.05). The expression of miR-190 significantly increased (P<0.05) in the miR-190 mimics group when compared with the blank control group. The expression of miR-190 significantly decreased (P<0.05) in the miR-190 inhibitors group when compared with the blank control group. In all groups, SGC7901 cells showed a higher expression of miR-190 (P<0.05) than GES-1 cells. Therefore, the expression of miR-190 in GC tissues was higher than that of normal tissues.

Expression of FOXP2 in SGC7901 and GES-1 cells
The results of Western blotting showed that there were no significant differences when mimics control group, inhibitors control group, and blank control group were compared in pairs (all P>0.05) (Table 2, Figures 3 and 4). The expression of FOXP2 significantly decreased (P<0.05) in the miR-190 mimics group when compared with the blank control group.

The expression of FOXP2 significantly increased (P<0.05) in the miR-190 inhibitors group when compared with the blank control group. In all groups, the FOXP2 expression decreased in SGC7901 cells when compared to that in GES-1 cells (P<0.05). Therefore, upregulation of miR-190 could lead to downregulation of FOXP2 expression.

Expression of miR-190 in SGC7901 cells during proliferation
CCK-8 assays showed that in SGC7901 cell lines, miR-190 mimics group exhibited enhanced proliferation ability while
the miR-190 inhibitors group showed the opposite trend, as demonstrated in Figure 5. There was no significant difference in proliferation rate among different groups after transfection for 24 hours. After transfection for 48 and 72 hours, no significance was detected among blank group, mimics control group, and inhibitors control group, while the miR-190 mimics group showed elevated proliferation ability and miR-190 inhibitors group showed decreased proliferation ability compared with the other three groups (all P < 0.05).

Expression of miR-190 in SGC7901 cells during migration
The migration assay showed no significant difference on scratch width among the blank, mimics control, and inhibitors control groups after scratch test was performed for 48 hours (all P > 0.05). Compared with the blank, mimics control, and inhibitors control groups, the miR-190 mimics group showed a larger scratch width while the miR-190 inhibitors group showed a smaller scratch width (P < 0.05, Figure 6).

Expression of miR-190 in SGC7901 cells during invasion
As demonstrated in Figure 7, the comparison of expression of miR-190 in penetrating cells in blank, mimics control, and inhibitors control groups was not statistically significant (all P > 0.05). The miR-190 mimics group had more penetrating cells, while the miR-190 inhibitors group had less penetrating cells than the other three groups (P < 0.05).

Expression of miR-190 and FOXP2 protein in GC tissues
The expression of miR-190 in tumor tissues was significantly higher than that in normal tissues (P < 0.05), and the expression of FOXP2 protein in tumor tissues was significantly

Table 1
| Groups                | Relative expression in SGC7901 (x ± s) | Relative expression in GES-1 (x ± s) |
|-----------------------|----------------------------------------|-------------------------------------|
| Blank control         | 1.83 ± 0.51*                           | 1.00 ± 0.01                         |
| miR-190 mimics       | 13.01 ± 0.42**                         | 6.58 ± 0.62*                        |
| miR-190 inhibitors   | 1.36 ± 0.23**                          | 0.67 ± 0.21*                        |
| miR-190 mimics control | 1.85 ± 0.31*                          | 1.05 ± 0.42                         |
| miR-190 inhibitors control | 1.81 ± 0.40**                         | 0.99 ± 0.35                         |

Notes: The original GES-1 expression in normal gastric epithelial cells was used as a reference to attain relative expressions of each case; * compared with GES-1 group, P < 0.05; ** compared with blank group, P < 0.05; x ± s, mean ± standard deviation.

Table 2
| Groups                | SGC7901 (x ± s) | GES-1 (x ± s) |
|-----------------------|----------------|--------------|
| Blank control         | 0.65 ± 0.07*   | 1.00 ± 0.01  |
| miR-190 mimics       | 0.37 ± 0.04**  | 0.76 ± 0.07* |
| miR-190 inhibitors   | 1.05 ± 0.09**  | 1.71 ± 0.09* |
| miR-190 mimics control | 0.64 ± 0.04*   | 0.99 ± 0.06  |
| miR-190 inhibitors control | 0.62 ± 0.03*   | 1.05 ± 0.07  |

Notes: * compared with GES-1 group, P < 0.05; ** compared with blank group, P < 0.05; x ± s, mean ± standard deviation.

Abbreviation: miR-190, microRNA-190.
lower than that in normal tissues (Figure 8). Therefore, the upregulation of miR-190 in GC tissues can inhibit the expression of FOXP2.

Discussion

miRNA, a single-stranded and short noncoding RNA, is widely involved in the physiological and pathological regulations of the body. miRNA combines specifically with 3′-UTR of mRNA to degrade mRNA, inhibit mRNA translation, and negatively regulate the target gene. The expression dysregulation of specific miRNA can be involved in the initiation and progression of cancer. The relative ease of detection of miRNA also makes it a good choice for molecular diagnosis. Therefore, we designed experiments to look for a new type of miRNA related to GC to provide a reference for the future GC study and a possible molecular probe for the diagnosis and clinical monitoring of GC.

In this study, through a series of experiments performed, downregulation of FOXP2 expression and upregulation of miR-190 were observed in the GC cells and GC tissues, which indicated expression dysregulation of miR-190 and FOXP2 in GC cells, suggesting FOXP2 and miR-190 may have an impact on GC. It has been confirmed that FOXP2 is the direct target gene of miR-190 by using sequence matching retrieval and dual luciferase experiments. RT-PCR and Western blotting have confirmed that the overexpression of miR-190 decreases the expression of FOXP2 and the underexpression of miR-190 increases the expression of FOXP2 at RNA and protein levels. A series of experiments confirmed the negative regulation of miR-190 to FOXP2 and suggested that expression dysregulation of miR-190 in GC, at least partly, contributed to the pathological process of GC through FOXP2 pathway. While no information is available on the role of miR-190 in cell proliferation and tumorigenesis, our results obtained from transwell assays and scratch test showed that overexpression of miR-190 could enhance proliferation, migration, and invasion, and its downregulation can inhibit the ability of proliferation, migration, and invasion in SGC790 cells. Consistent with our results, miR-190 has been found to be upregulated in a variety of tumors, and its overexpression is able to enhance the cell proliferation and malignant transformation in the process of bronchial epithelial cell cancerization induced by arsenic. In the analysis of the expression of 95 kinds of miRNA in pancreatic cancer, miR-190 was one of the eight miRNAs that was significantly upregulated. The expression of miR-190 was confirmed to correlate with disease stage in human glioma specimens, and its expression was upregulated in other cancers such as bladder cancer, breast cancer, lung cancer, liver cancer, and colorectal cancer. It is worth noting that, in the direct target gene prediction of miR-190 made by Almog et al, FOXP2 was in the prediction. Our experimental results also confirmed the author’s prediction. However, in a study with the help of human glioblastoma and osteosarcoma transcriptome, researchers showed that miR-190 realized its function of miRNA related to tumor latency by affecting multiple transcription factors, tumor suppressor genes, and interferon reaction pathway and tended to inhibit tumor growth. This may suggest that miRNA plays a different role in different tumor environments and different stages of tumor development. More study should be carried out on the type of each tumor. There were no reports on the correlation between miR-190 and GC, so its biological function in the GC pathology has not been clear so far. As far as we know, this paper is the first to report on the expression of miR-190 in GC. Studying the regulation mechanism of miR-190 in GC不但 helps us to understand GC physiology, but also
**Figure 6** The comparisons on scratch width in SGC7901 cells.

**Notes:** (A) Comparison on scratch test among groups; 1, blank; 2, miR-190 mimics; 3, miR-190 inhibitors; 4, mimics control; 5, inhibitors control; (B) comparisons on scratch width (48 hours/0 hours); *compared with blank group, \( P < 0.05 \); #compared with mimics control group, \( P < 0.05 \); &compared with inhibitors control group, \( P < 0.05 \).

**Abbreviations:** miR-190, microRNA-190; h, hour(s).

**Figure 7** The comparisons on cell invasion in SGC7901 cells.

**Notes:** (A) Cell invasion in each group; blank; 2, miR-190 mimics; 3, miR-190 inhibitors; 4, mimics control; 5, inhibitors control (magnification, \( \times 400 \)); (B) penetrating cells in each group; *compared with blank group, \( P < 0.05 \); #compared with mimics control group, \( P < 0.05 \); &compared with inhibitors control group, \( P < 0.05 \).

**Abbreviation:** miR-190, microRNA-190.
provides a new choice for GC diagnosis, treatment, and prognosis.

Cell growth factor and its receptor pathway, cell cycle regulation, cell adhesion molecules and matrix degrading enzymes, inflammatory pathways, DNA methylation enzymes, histone modifying enzymes, DNA damage and repair, and other imbalances in biological processes are all involved in GC pathological process.\textsuperscript{24-26} We have confirmed that miR-190 has a direct regulatory effect on FOXP2, but follow-up researches are still needed to further understand the working mechanism of miR-190 in GC. The evaluation of cell growth and invasion of GC cells transfected with miR-190 mimics and inhibitors, and the reactivity of GC animal models to miR-190 mimics and inhibitors can help us to evaluate the functional significance of miR-190 in GC. Moreover, with a large number of miRNA target genes, the specific inhibition experiment of FOXP2 pathway is needed to verify the importance of FOXP2 pathway in the impact of miR-190 on GC cells. As a result, the sensitivity and accuracy of miR-190 as a diagnostic molecule are to be verified by clinical data.

**Conclusion**

We are the first to report that there is an increased expression of miR-190 and decreased expression of FOXP2 in GC cells and in GC tissues compared with normal gastric epithelial cells and neighboring tissues. We also confirmed the direct target regulation of miR-190 to FOXP2. It was suggested that miR-190 showed expression dysregulation in GC and might be partly involved in the GC pathological process via FOXP2 pathway. The report provides a reference for follow-up researches and suggests that miR-190 may be a candidate for GC diagnosis and monitoring. However, the sample size for the current study was rather limited, and a larger sample size was needed to validate our results.

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**Disclosure**

The authors report no conflicts of interest in this work.

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