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MicroRNA-936 promotes proliferation and invasion of gastric cancer cells by down-regulating FGF2 expression and activating P13K/Akt signaling pathway

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Abstract. – OBJECTIVE: This study was designed to investigate whether microRNA-936 can be involved in the development of gastric cancer (GCa) by down-regulating FGF2 expression and activating the phosphatidylinositol 3-kinase/protein kinase B (P13K/Akt) signaling pathway.

PATIENTS AND METHODS: Quantitative polymerase chain reaction (qPCR) was carried out to examine microRNA-936 and FGF2 levels in GCa tissue samples and adjacent normal ones, and further in GCa cell lines. After transfection of microRNA-936 inhibitor in GCa cell lines BGC and SGC, cell invasion, and proliferation abilities were evaluated by transwell and cell counting kit-8 (CCK-8) assays, respectively. In addition, the Dual-Luciferase reporting assay was conducted to verify the binding relationship between microRNA-936 and FGF2. After simultaneous transfection of microRNA-936 inhibitor and si-FGF2 in GCa cells, we detected the expression of FGF2/P13K/Akt by performing qPCR and Western blot experiments to further verify the regulation of microRNA-936 on FGF2 and PI3K/AKT pathway.

RESULTS: QPCR detection revealed that microRNA-936 was remarkably up-regulated while FGF2 was conversely down-regulated in GCa tissue samples, indicating a negative correlation between the two. In addition, compared with normal gastric mucosal cells GES, microRNA-936 showed a significant increased expression in GCa cell lines. Meanwhile, down-regulation of microRNA-936 caused a marked reduction in invasive and proliferation ability of GCa cells. Dual-Luciferase reporting assay demonstrated a direct binding of microRNA-936 to FGF2. QPCR and Western blot showed that microRNA-936 can inhibit FGF2 expression and activate the PI3K/AKT pathway at the same time. Further studies found that silencing FGF2 induced an enhancement in cell proliferation and invasiveness, which could be reversed by simultaneous downregulation of microRNA-936. The above observations suggested that microRNA-936 may accelerate the progression of GCa by inhibiting FGF2 expression and activating the PI3K/AKT pathway.

CONCLUSIONS: Overexpression of microRNA-936 can be conducive to the development of GCa, mainly through the down-regulation of FGF2 and activation of the P13K/Akt signaling pathway.

Key Words:
GCa, MicroRNA-936, FGF2, Cell invasion, Cell proliferation.

Introduction

As one of the most common malignancies of the digestive system, GCa was the most common cancer in the world less than a century ago. Although the incidence of GCa has decreased globally, it is still one of the major killers in the world[1]. GCa has always been the most common cancer in East Asia (including China)[2]. It is estimated that in 2018, among the new cancer cases in the world, GCa accounts for 5.7%, ranking sixth among all tumors, but GCa deaths account for 8.2% of all deaths, ranking second among all cancers[3]. Therefore, the treatment of GCa is still a great challenge to the whole world, especially East Asia. At present, surgery is considered to be the only radical treatment for GCa. With the continuous improvement of surgical techniques and the implementation of traditional radiotherapy, chemotherapy, and neoadjuvant therapy, the five-year survival rate of early GCa can reach over 95%[4]. Although there are a lot of treatment options available at present, for most patients with GCa, the therapeutic effect is still not optimistic since extensive invasion and lym-

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phatic metastasis may have occurred in the late diagnosis[5].

MicroRNA, a kind of small non-coding RNA with regulatory functions, can play a vital regulatory role in animals and plants through degradation of targeted mRNAs or inhibition of protein translation[6]. As an important component of the non-coding RNA family, miRNA can be engaged in a several cellular functions, and the imbalance of miRNA expression is not only related to tumorigenesis, but also to nerve, cardiovascular, developmental and other diseases[7]. MiRNAs function mainly by binding to the 3'-non-translation region (3'-UTR) of target mRNAs to regulate gene expression at post-transcription level. A single miRNA can target a variety of different mRNAs, and a single mRNA can also be regulated by various miRNAs[8]. In addition to the regulation of gene expression, miRNAs can serve as diagnostic markers for diseases[9]. Increasing data have confirmed that miRNA plays a pivotal role in many regulatory factors involved in tumor pathogenesis[10]. For example, microRNA-200 inhibits angiogenesis by targeting interleukin-8 (IL-8) and CXCL1 secreted by tumor endothelial cells and cancer cells, thereby significantly reducing tumor metastasis and angiogenesis and inducing vascular normalization in ovarian, lung, breast and kidney cancer tissues, confirming the potential therapeutic role of microRNA-200 in tumors[11].

MicroRNA-936 was highly expressed in endometrial carcinoma (EEC) and lingual squamous cell carcinoma compared to normal tissues[12,13]. Meanwhile, high expression of microRNA-936 has also been reported[14] in colorectal cancer metastatic tissues. MicroRNA-936 showed a high expression in blood cell microvesicles in patients with hepatocellular carcinoma[15,16]. However, another study showed that microRNA-936 was underexpressed in human non-small cell lung cancer cell lines (EPLC-32M1, A549, and 801D) and human pancreatic ductal adenocarcinoma cell lines (MiaPaCa-2, PANC-1 and Hs766T)[17-19]. Thus, microRNA-936 may play different roles in different tumors and tumor cell lines, but its specific role in the biological behavior of GCa cells is still unknown. In previous studies, our team has found that GCa tissue specimens contained a remarkably lower expression of microRNA-936 than adjacent normal tissues. Hence, in this study, we aimed to further discover the specific mechanism of microRNA-936 in the development of GCa.

Patients and Methods

Patients and Sample Collection

We collected 40 cases of GCa and adjacent tissue samples from our hospital and stored them in –80°C refrigerator. Patients and their families had signed informed consent and all samples were diagnosed by pathology. The investigation was approved by the Research Ethics Committee of our hospital.

Cell Culture and Transfection

Human normal gastric mucosal cells (GES) and GCa cell lines (BGC, SGC, MGC, MKN) were purchased from the Cell Culture Center of the Shanghai Research Institute (Shanghai, China). Cells were cultured with Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented by 10% fetal bovine serum (FBS) and placed in a humidified incubator at 37°C with 5% CO_2. The microRNA-936 inhibitor and FGF2 siRNA used and the corresponding negative controls were purchased from GenePharma (Shanghai, China). When the cell density reached 50%, transfection was performed according to the manufacturer's instructions.

RNA Extraction

After sufficient pre-treated tissue or cells were prepared in an Eppendorf (EP) tube, 500 μL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to directly lyse the tissue or cells, and let stand for 5 min. After RNA was extracted and dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China), the concentration was measured using a spectrophotometer. Then, RNA was stored in a refrigerator at –80°C until use.

RNA Reverse Transcription and Quantitative Polymerase Chain Reaction (qPCR) Detection

The RNA was subjected to a reverse transcription system using a PrimeScript RT reagent Kit to obtain a complementary deoxyribose nucleic acid (cDNA) under the following conditions: reverse transcription at 37°C for 15 min; inactivation of 85°C reverse transcriptase for 5 s. The reverse transcript template was added to the RNase-depleted water to a final concentration of 10 ng/μL. The quantitative PCR operation was carried out in accordance with the instruction of SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan). The total reaction system was 10 μL. The PCR reaction

conditions were: pre-denaturation at 94°C for 2 min, denaturation for 20 s, annealing at 50-65°C for 30 s, extension at 72°C for 60 s, extension at 72°C for 5 min, steps 2-4 for 35 cycles, annealing temperature at 55°C. Primer sequences were as follows: microRNA-936 (F: 5'- AACGAGACGACGACAGAC-3'; R: 5'-ACAGTAGAGGGAGGAATCGCAG-3'); FGF2 (F: 5'-CCGTTACCTGGCTATGAAGG-3'; R: 5'-ACTGCCCAGTTCGTTTCAGT-3'); P13K (F: 5'-AACGAGAACGTGTGCCATTTG-3'; R: 5'-AGAGATTGGCATGCTGTCGAA-3'); Akt (F: 5'-TGAGCGACGTGGCTATTG-3'; R: 5'-CAGTCTGGATGGCGGTT-3'); β-actin (F: 5'-CACCCGCGAGTACAACCTTC-3'; R: 5'-CCCATACCCACCATCACACC-3'); U6 (F: 5'-AGAGAAGATTAGCATGGCCCCTG-3'; R: 5'-ATCCAGTGCGGGTCCGAGG-3').

Dual-Luciferase Reporting Assay

Bioinformatics methods were used to predict potential targets for microRNA-936 and FGF2. A FGF2 3'-UTR or a mutant FGF2 3'-UTR having a predicted target site was inserted into the pGL3 promoter vector. The cells in the logarithmic growth phase were seeded in 96-well plates at 1.5×104 cells per well, and cultured in an incubator for 24 h. After co-transfection of 50 nmol/L miRNA mimics or negative control (NC) and 100 ng of dual reporter vector or mutant vector for 48 h, the medium was aspirated and added to fresh medium at 35 μL/well. The substrate was added to Luciferase (Promega, Madison, WI, USA) at 35 μL/well, shaken for 10 min, and the fluorescence value was measured.

Transwell Assay

Cells were seeded 1 x 10^4 cells per chamber into serum-free medium and aspirated to the upper chamber coated with 200 mg/mL Matrigel. After 24 h of culture, the cells invading the lower surface of the filter were fixed in 70% ethanol for 30 min and stained with crystal violet. The invading cells were counted by taking five random fields from each chamber under an optical microscope (magnification 200×).

Cell Counting Kit-8 (CCK-8) Assay

The pretreated cells were seeded in a 96-well plate at a concentration of 5×10^4 /mL, and the total liquid volume per well was 200 μL. At the 6, 24, 48, and 72 h after transfection, 20 μL/well CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added. After incubation for 1-4 h in a cell culture incubator, the optical density (OD) value was measured at 450 nm.

Western Blot

100 μL of radioimmunoprecipitation assay (RIPA) lysate containing phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China) was added to lyse cells on ice for 30 min. After the protein concentration was determined by the Bradford method, the protein sample was denatured in a water bath at 100°C for 5 min, and an appropriate amount of the loading buffer was added in. The denatured protein sample was pipetted to the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), and blocked with 5% skim milk powder for 1 h at room temperature. Primary antibodies (1:l000) were added for incubation overnight at 4°C shaker. The next day, the membrane was rinsed 3 times with Tris Buffered Saline and Tween-20 (TBST) and incubated with second antibody (concentration: 1:3000) for 1 h at room temperature. After that, the protein samples on the membrane were finally semi-quantitatively analyzed by alpha SP image analysis software.

Statistical Analysis

The *t*-test was used for comparison of measurement data between groups. Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA), and $p<0.05$ was considered statistically significant. Correlation analysis was performed using GraphPad Prism7 (La Jolla, CA, USA).

Results

MicroRNA-936 Enhances Invasion Ability and Proliferation Rate of GCa Cells

QPCR detected a significant reduced expression of microRNA-936 in GCa tissue samples when compared to the corresponding paracancerous tissues (Figure 1A). At the same time, *in vitro* experiments also revealed a reduction of microRNA-936 level in GCa cell lines (BGC, SGC, MGC, MKN) compared to GES, the normal gastric mucosal cell line (Figure 1B). BGC and SGC were selected for subsequent studies. We then transfected

microRNA-936 inhibitor in the gastric cells and confirmed the transfection efficiency through qPCR assay (Figure 1C). Subsequently, a significant reduction in cell invasion capacity was found by transwell assay after microRNA-936 expression was inhibited (Figure 1D). Meanwhile, the results of CCK-8 assay indicated that down-regulation of microRNA-936 also markedly suppressed the cell proliferation rate (Figure 1E-F).

MicroRNA-936 Can Directly Target FGF2

Through qPCR, we found that, in GCa tissues, FGF2 was remarkably lower than that in the adjacent ones (Figure 2A), and was inversely correlated with microRNA-936 expression (Figure 2B). Next, bioinformatics predicted a potential binding target for microRNA-936 and FGF2 (Figure 2C). Afterwards, we constructed FGF2 wild and mutant sequences, and found through the Dual-Luciferase reporter gene assay that the Luciferase in the FGF2-WT 3'UTR group decreased after transfection of microRNA-936 in both BGC and SGC cells , while no significant difference was found in FGF2-MUT 3'UTR group (Figure 2D-2E). From this we found that microRNA-936 can directly bind to FGF2.

MicroRNA-936 Activates P13K/Akt Signaling Pathway Via Down-Regulating FGF2

To further investigate the regulation of microRNA-936 on FGF2 and P13K/Akt signaling

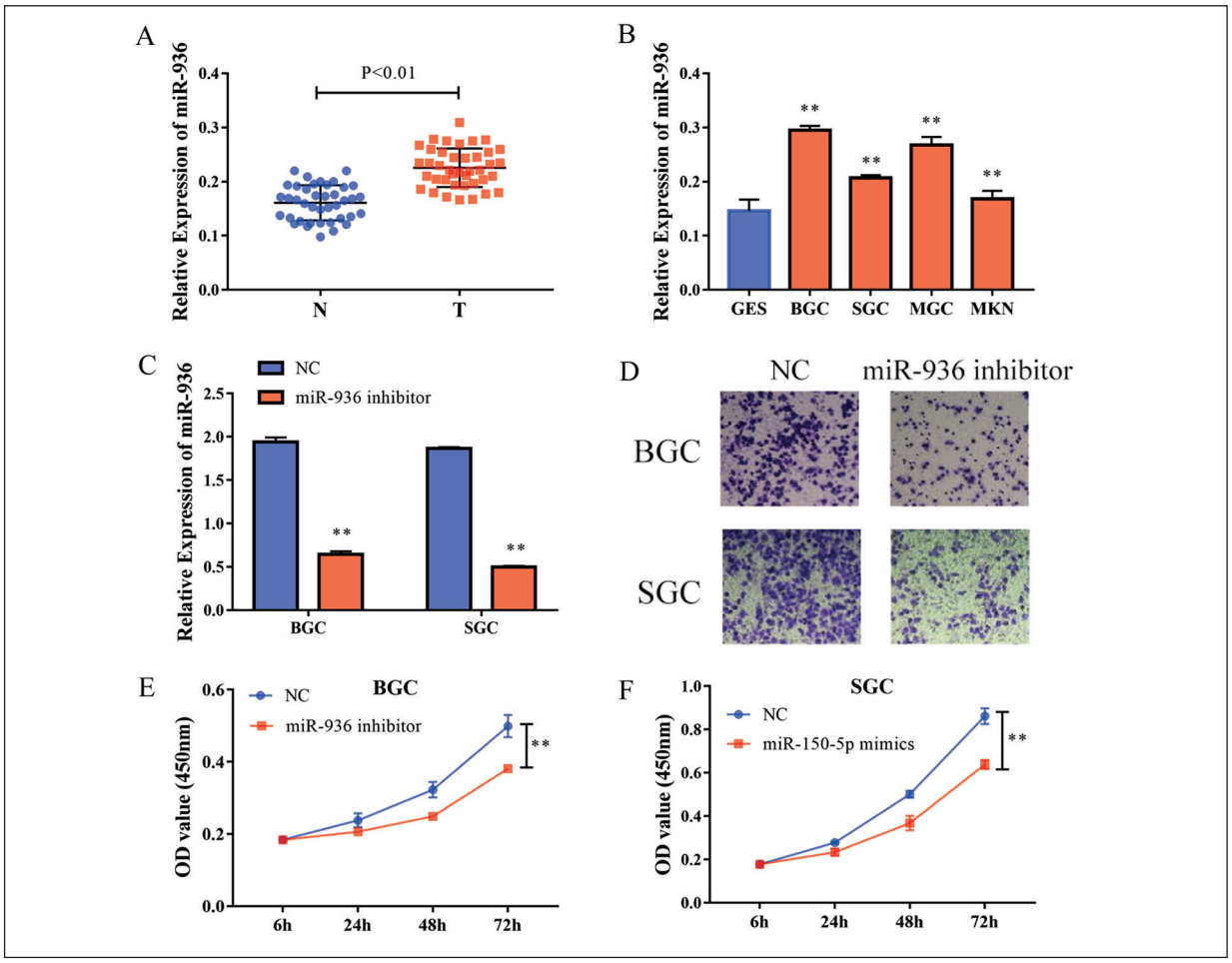

Figure 1. High expression of miR-936 in gastric cancer tissues. **A,** MiR-936 is highly expressed in gastric cancer tissues; **B,** Compared with normal gastric mucosal cell GES, the expression levels of miR-936 in gastric cancer cells BGC, SGC, MGC and MKN increased; **C,** Transfection efficiency of miR-936 inhibitor in BGC and SGC cells; **D,** Transwell invasion assay showed that down-regulation of miR-936 decreased cell invasion; (magnification: 40×) **E,** CCK-8 experiments showed that down-regulation of miR-936 expression decreased BGC cell proliferation ability; **F,** CCK-8 experiments revealed that down-regulation of miR-936 expression weakened SGC cell proliferation ability. (*p<0.05, **p<0.01).

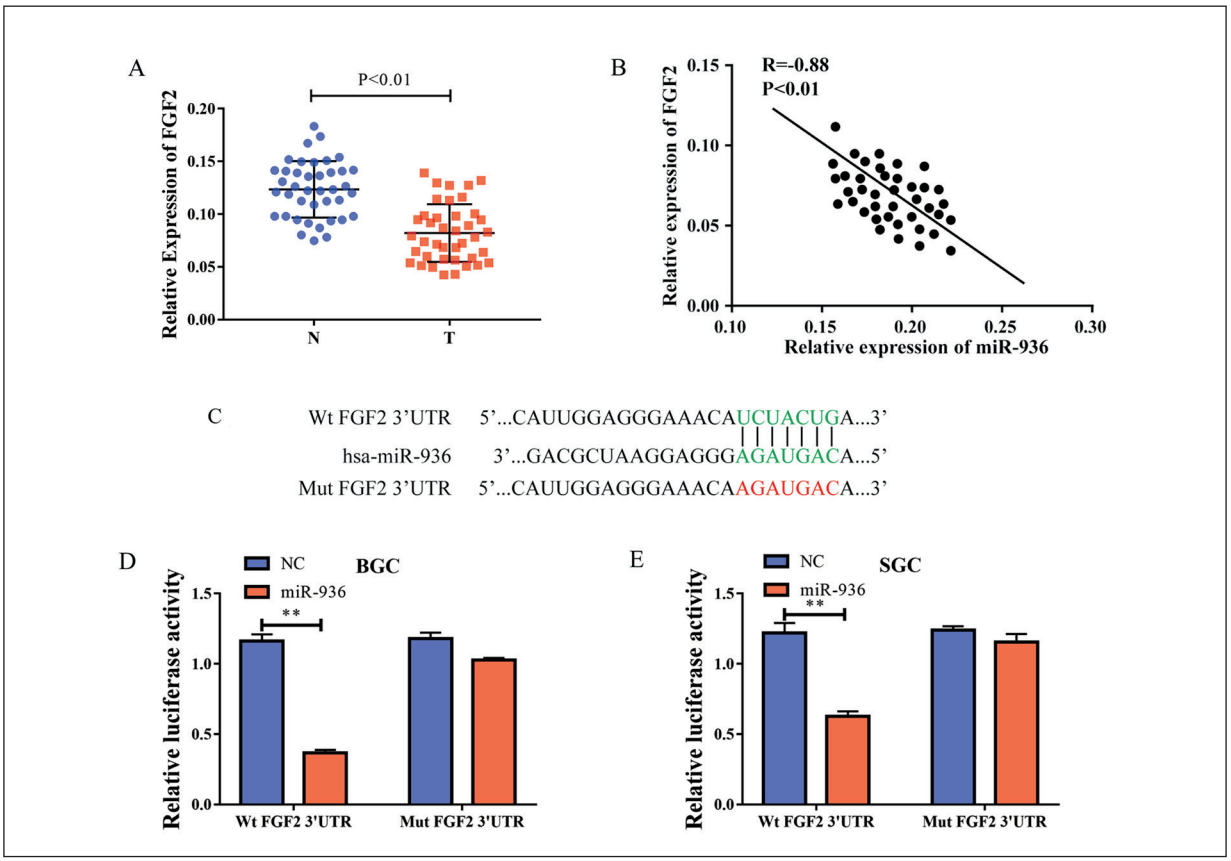

Figure 2. miR-936 can targeted binding to FGF2. **A,** FGF2 was lowly expressed in gastric cancer tissues; **B,** Expression level of FGF2 was negatively correlated with the expression level of miR-936; **C,** Bioinformatics tools predicted the potential binding between miR-936 and FGF2; **D,** Dual-Luciferase reporter gene experiments in BGC cells showed that miR-936 can targeted binding to FGF2; **E,** Dual-Luciferase reporter gene experiments in SGC cells indicated that miR-936can targeted binding to FGF2. (*p<0.05, **p<0.01).

pathways, we transfected microRNA-936 inhibitor and found by qPCR that FGF2 expression was remarkably enhanced in gastric cell lines (Figure 3A). Further, after transfection of si-FGF2, we found that FGF2 was remarkably down-regulated (Figure 3B), while microRNA-936 was conversely up-regulated (Figure 3C). In addition, it was found that P13K and Akt mRNA expression levels were remarkably decreased after down-regulating of microRNA-936 and were oppositely increased after down-regulation of FGF2, which, however, could be reversed by simultaneous down-regulation of this two (Figure 3D-3E); in addition, the same results were observed at the protein level (Figure 3F). We concluded that microRNA-936 may act by activating the PI3K/AKT pathway by inhibiting FGF2 expression.

Down-Regulation of FGF2 Enhances Invasiveness and Proliferation Ability of GCa Cells

To further investigate the mechanism by which microRNA-936 accelerates the progression of GCa, we evaluated the proliferative capacity and invasiveness of cells after transfection of si-FGF2. The results of transwell assay showed that down-regulation of FGF2 expression enhanced the cell invasion ability, which, however, could be attenuated by simultaneous silencing microRNA-936, as well as FGF2 (Figure 4A). Similarly, the same results in cell proliferation were observed in CCK-8 assay (Figure 4B). Therefore, the above results provided a clue that microRNA-936 may promote the proliferation and invasion abilities of GCa cells *via* inhibiting FGF2 expression.

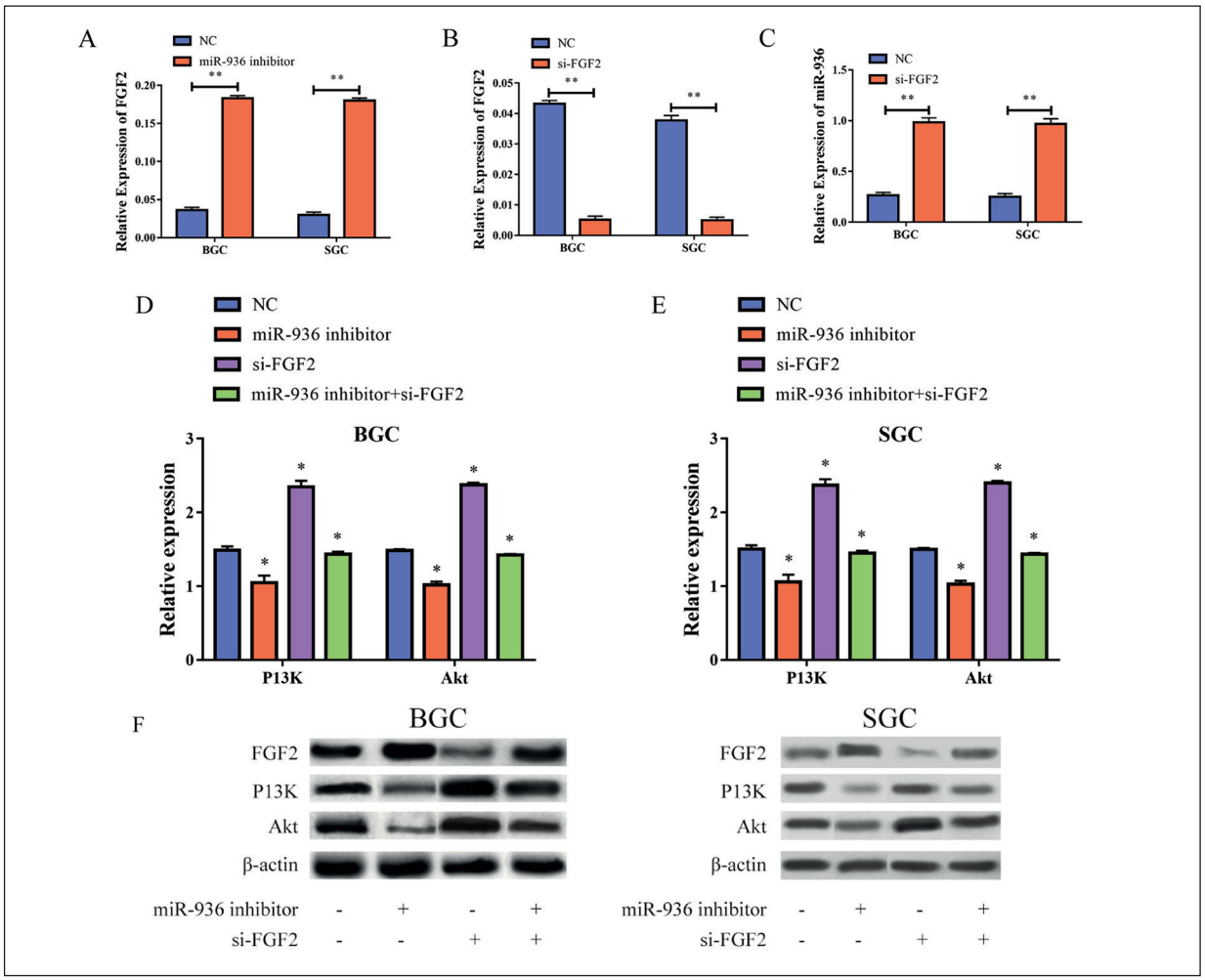

Figure 3. MiR-936 activates the P13K/Akt signaling pathway by down-regulating FGF2 expression. **A,** After down-regulation of miR-936 expression, FGF2 expression levels increased; **B,** Transfection efficiency of si-FGF2 in BGC and SGC cells; **C,** After down-regulation of FGF2 expression, miR-936 expression levels increased; **D,** After down-regulating the expression of miR-936 in BGC cells, the expression level of P13K/Akt decreased. After down-regulating the expression of FGF2, the expression level of P13K/Akt increased. Simultaneous downregulation of miR-936 reversed the expression level of P13K/Akt. **E,** Down-regulation of miR-936 in SGC cells reduced the expression level of P13K/Akt. Down-regulation of FGF2 increased the expression level of P13K/Akt, while simultaneous downregulation of miR-936 reversed P13K/Akt levels. **F,** Western blot analysis showed that FGF2 protein level increased and P13K/Akt protein level decreased after down-regulation of miR-936 expression. FGF2 protein level decreased and P13K/Akt protein level increased after down-regulation of FGF2 expression. Simultaneous down-regulation of miR-936 and FGF2 reversed P13K/Akt protein levels. (*p<0.05, **p<0.01).

Discussion

As a multi-factor disease, GCa can be induced by both environmental factors and genetic factors[20]. The incidence of this cancer can be effectively reduced by avoiding some predisposing factors. Although gender, age, and race cannot be changed[21], some other factors, such as Helicobacter pylori infection, high salt diet, smoking, and other pathogenic factors, can be completely controlled[7,22]. Zhou et al[23] have shown that eating fresh fruits and vegetables can effectively reduce the risk of gastric cancer. In addition, genetic factors are also important and cannot be ignored[24]. Due to environmental or genetic reasons, the occurrence of GCa tends to be a familial aggregation in many cases[25]. Many miRNAs have been observed to be involved in GCa progression. Mir-638 acts as a tumor suppressor gene in GCa; miR-206 inhibits tumor-initiating cells by targeting EHF in GCa; miR-27b inhibits GCa metastasis by targeting NR2F2; miR-532 promotes GCa mi-

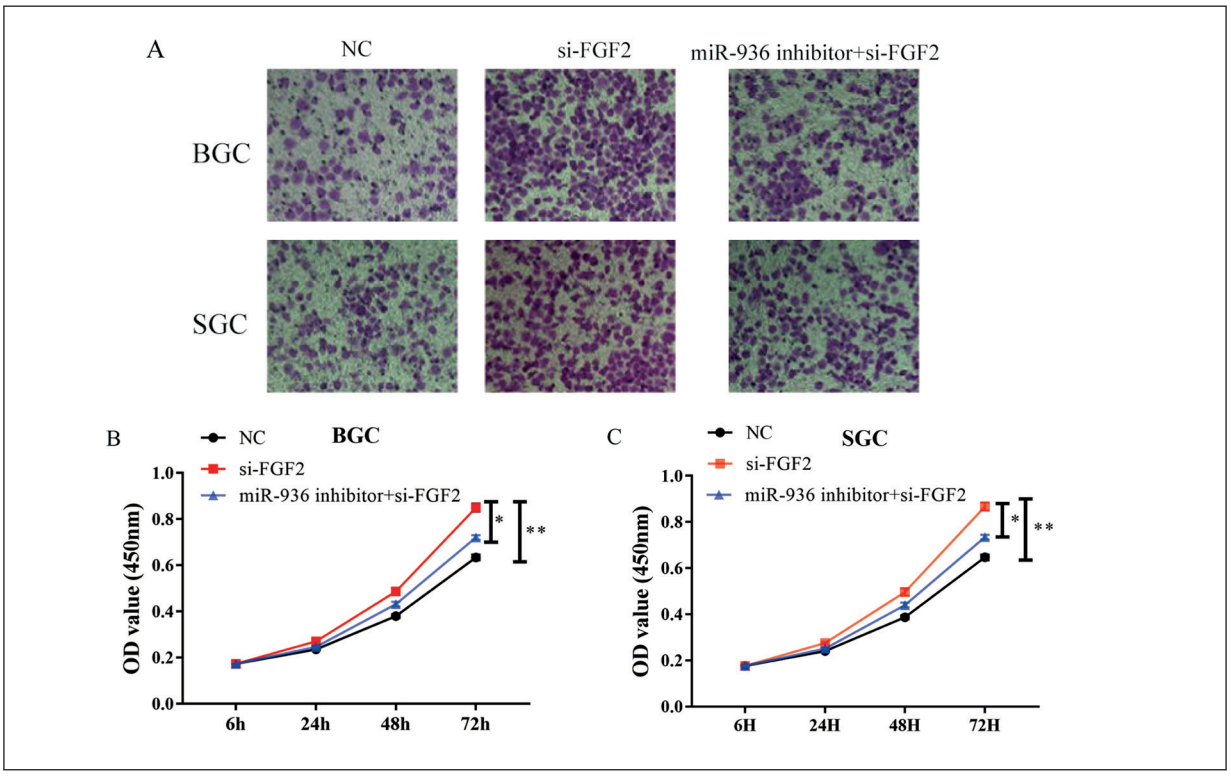

Figure 4. Down-regulation of FGF2 promotes invasion and proliferation of gastric cancer cells. **A,** Transwell invasion assay showed that the down-regulation of FGF2 expression increased cell invasive ability; while simultaneous down-regulating miR-936 expression level reduced cell invasive ability; (magnification: 40×) **B,** CCK-8 experiments showed that after down-regulating the expression of FGF2, the proliferation of BGC cells was enhanced; while simultaneous down-regulating miR-936 expression level reduced proliferation of BGC cells; CCK-8 experiments showed that the down-regulation of FGF2 expression increased the proliferation of SGC cells; while simultaneous down-regulation of miR-936 reduced the proliferation of SGC cells. (*p<0.05, **p<0.01).

gration and invasion by targeting NKD1; low expression of miR-144 may affect the prognosis of GCa[26-28]. However, there are still many miRNAs that play an important role in GCa development to explore.

Fibroblast growth factor 2 (FGF2) is an important growth factor that plays a role in promoting vascular growth[29]. FGF2 not only plays an essential role in tumor angiogenesis, but indirectly participates in the regulation of tumor cell migration and invasion, inflammatory response, and characteristics of tumor stem cells[30,31]. Moreover, FGF2 plays a vital part in regulating the sensitivity of chemotherapy drugs in tumor cells. The enhanced activity of FGF2 and its receptor FGFR1 in NSCLC have been verified[32] as one of the important mechanisms leading to gefitinib resistance in tumor cells. Meanwhile, in chronic myeloid leukemia, it has been shown[33] that elevated expression of FGF2 induced the insensitivity of CML cells to imatinib chemotherapy. Besides, Turner and Grose[34] have showed that FGF2 is highly expressed in a variety of human tumors and exerts vital functions in the proliferation and invasion of cancer cells. Soriano et al[35] suggested that FGF2 expression is remarkably higher in lung cancer patients and is associated with tumor progression and poor prognosis. FGF2 is remarkably increased in prostate cancer[36-38] and plays a pivotal role in prostate cancer malignant progression, while the knockout of FGF2 in mice remarkably inhibits the occurrence and progression. Additionally, knockdown of FGF2 was confirmed to be able to effectively inhibit tumor cell proliferation and migration of human umbilical vein endothelial cells, and suppress the growth and angiogenesis of Caki-1 and A549 xenograft animal models[36-38].

In this study, we found that microRNA-936 increased markedly in both GCa tissues and

cell lines. Down-regulation of microRNA-936 in GCa cells can attenuate the invasion and proliferation of cells. Further studies revealed that FGF2 was down-regulated in GCa tissues, which was negatively correlated with microRNA-936. In addition, microRNA-936 can inhibit FGF2 expression by directly binding to it and thus activate the P13K/Akt signaling pathway. Moreover, down-regulation of FGF2 enhanced cell invasiveness and proliferation rate, while down-regulation of these two can reverse this enhancement. Thus, we consider that microRNA-936 may promote the development of GCa by down-regulating FGF2 to activate the P13K/Akt signaling pathway.

Conclusions

Altogether the above data detected that microRNA-936 is able to prompt the progression of GCa by activating the P13K/Akt signaling pathway by down-regulating FGF2, and thus serves as a potential therapeutic target for GCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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