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MicroRNA-135 inhibits gastric cancer metastasis by targeting SMAD2

Y. HE, L. WU, Y. DAI, J. LI, S. LIU

Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Yun He and Li Wu contributed equally to this work

Abstract. – OBJECTIVE: The purpose of this study was to investigate whether microRNA-135 plays a role in the malignant progression of gastric cancer (GC) by regulating SMAD2 and its underlying mechanism.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine microRNA-135 expression in tumor tissue specimens and paracancerous ones of 28 patients with GC, and the interplay between microRNA-135 level and clinical indexes and prognosis of GC patients was also analyzed. Subsequently, when negative control (NC) sequence or microRNA-135 mimics were transfected into GC cell lines, Cell Counting Kit-8 (CCK-8), transwell and wound healing assays were used to analyze the impact of microRNA-135 on proliferation and apoptosis of GC cells. Finally, the mechanism of microRNA-135 on the downstream gene SMAD2 was explored by a cell recovery experiment.

RESULTS: QRT-PCR results revealed that in tumor tissues of GC patients, the microRNA-135 level was conspicuously lower than that in the adjacent tissues, and the difference was statistically significant. The overall survival rate was lower in patients with low expression of microRNA-135 compared with patients with a high one. In addition, in the GC cell lines including BGC-823 and SGC-7901, the cell proliferation, as well as invasion and migration ability of microRNA-135 mimics group, was significantly decreased compared with the NC group. Moreover, the research verified that SMAD2 expression in GC cell lines and tissues was markedly increased and negatively correlated with microRNA-135 level. The cell recovery study found that overexpression of SMAD2 can offset the influence of microRNA-135 mimics on proliferation and metastasis of GC cells, thus affecting the malignant progression of GC.

CONCLUSIONS: In this work, microRNA-135 was found conspicuously associated with lymph node or distant metastasis and poor prognosis of GC patients. Additionally, microRNA-135 may inhibit the malignant progression of GC by targeted regulating SMAD2 expression.

Key Words:
MicroRNA-135, SMAD2, Gastric cancer, Metastasis.

Introduction

Gastric cancer (GC) is one of the most aggressive malignancies, with its morbidity and mortality increasing year by year[1-3]. In the past few decades, comprehensive treatment schemes for gastric cancer have been greatly improved, but the prognosis of GC patients is still not optimistic[4,5], the main cause of which is that the primary tumor has very strong invasiveness to affect other tissues and organs[6]. Unfortunately, GC is easy to metastasize in the early stage but difficult to treat in the late stage and poor prognosis[7]. According to statistics, the mortality of GC in China is still on the rise as a whole. Therefore, it is urgent to find efficient treatment methods and tumor markers for early diagnosis of GC[8,9].

MicroRNA (miRNA) can play a biological role by binding to target genes to inhibit their transcription and translation[10,11]. Researchers[12,13] have shown that miRNA can be involved in cell proliferation, apoptosis and other processes, and is abnormally expressed in GC and other tumor cells. MiRNAs are a series of endogenous small non-coding RNAs (19-22 nucleotides) that are involved in a variety of regulatory functions in cell processes[14,15]. The miRNA gene is transcribed by RNA polymerase II (pol II) into the initial miRNA (pri-miRNAs), which is then processed by the DGCR8-formed micro-processing complex into a hairpin sequence of about 70 nucleotides known

Corresponding Author: Shuang Liu, BM; e-mail: xhfkzlls@163.com

as the precursor miRNAs (pre-miRNAs)[15]. The precursor miRNAs were further transported from the nucleus to the cytoplasm by the nuclear transposable protein exportin-5, and then were cut by RNase III Dicer to form double-stranded RNA[16]. Typically, one of these chains is selectively bound to the Argonaut protein and enters the RNA-induced silencing complex (RISC), where the complementary chains are degraded[16,17]. MiRNAs in RISC target the 3'-UTR of the target mRNAs based on the principle of sequence complementarity, thereby degrading the target mRNAs and regulating gene expression[17].

In this work, bioinformatics and molecular biology methods were used to clarify the expression of microRNA-135 and SMAD2 in GC tissues and their relationship with clinical phenotype. Meanwhile, the role of SMAD2 in GC invasion and metastasis was preliminarily explored through the application of molecular biology methods[18,19]. By using multiple bioinformatics databases, microRNA-135 was further identified as a specific microRNA capable of regulating the expression of gastric cancer, and its ability to proliferate and populate in gastric cancer cells was further confirmed by molecular biological means.

Patients and Methods

Patients and GC Samples

In this study, 28 pairs of GC tissues and the adjacent normal ones were selected from surgically treated GC cases and collected at -80°C. The collection of clinical specimens was approved by the Ethics Oversight Committee, and patients and their families had been fully informed that their specimens would be used for scientific research. All participating patients signed informed consent.

Cell Lines and Reagents

Human GC cell lines (AGS, BGC-823, SGC-7901) and immortalized normal gastric mucosal epithelial cell (GES-1) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured with Dulbecco's Modified Eagle's Medium high glucose medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; Gibco, Grand Island, NY, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in an incubator with 5% CO_2. Cells were passaged with 1% trypsin + EDTA (ethylenediaminetetraacetic acid) for digestion when grown to 80%-90% confluence.

Transfection

Negative control (NC) and microRNA-135 overexpression sequence (microRNA-135 mimics) were purchased from Shanghai Jima Company (Shanghai, China). After cells were plated in 6-well plates and grown to a cell density of 70%, transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell functional experiment after 48 hours.

Cell Counting Kit-8 (CCK-8) Test

The cells after 48 h of transfection were collected and plated into 96-well plates at 2000 cells per well. After culturing for 24 h, 48 h, 72 h and 96 h, the cells were added with CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan). After incubation for 2 hours, the optical density (OD) value of each well was measured in a microplate reader at 490 nm absorption wavelength.

Transwell Assay

The cells after transfection for 48 hours were digested, centrifuged and resuspended in medium without fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) to adjust the density to 5 x 10^5 cells/mL. 200 μL of cell suspension (1 x 105 cells) was added to the upper chamber, and 700 μL of medium containing 20% FBS was added to the lower chamber. According to the different migration abilities of each cell line, cells were put back into the incubator and continued to culture for a specific time. Then, the transwell chamber was taken out, washed 3 times with 1 x Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), and placed in methanol for cell fixation for 15 min. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with a cotton swab. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

Cell Wound Healing Assay

The cells after transfection for 48 hours were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5 x 105 cells/mL. The density of the cells was determined ac-

cording to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluence of the cells reached 90% or more the next day. After the stroke, cells were rinsed gently with PBS 2-3 times and observed again after incubated with low-concentration serum medium (such as 1% FBS) for 24 hours. The difference in cell healing ability was judged according to the migration area.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, and Real Time-Polymerase Chain Reaction (RT-PCR) was performed according to the SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) kit instructions. The PCR reaction was performed using the StepOne Plus Real Time-PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The following primers were used for qRT-PCR reaction: microRNA-135: F: 5'-AGCATAATACAGCAGGCACAGAC-3'; R: 5'-AAAGGTTGTTCTCCACTCTCTCAC-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; SMAD2: F: 5'-TACCATCAACTCCAACGG-3', R: 5'-GAACCCAAGGCATCTCCA-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'. Each sample was subjected to a three-well repeated experiment and repeated twice. Bio-Rad PCR instrument was used to analyze and process the data with software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The β-actin and U6 genes were used as internal parameters, and the gene level was calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22. 0 software (IBM, Armonk, NY, USA). Univariate analysis was performed using the χ^2 test and the exact probability Fisher test, while multivariate analysis was performed using COX regression analysis. Patient survival was analyzed using the Kaplan-Meier method, and intergroup curves were compared using the Log-rank test. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). $p<0.05$ was considered to be statistically significant.

Results

Downregulated MicroRNA-135 in Primary GC Tissues, and Reduced Level of MicroRNA-135 Connected with Advanced Clinical Procedure and Poor Patient Prognosis

MicroRNA-135 expression in GC tissues and cell lines were verified by qRT-PCR. The results showed that the microRNA-135 level in tumor tissues was conspicuously lower than that in adjacent tissues, and the difference was statistically significant (Figure 1A and 1B). Similarly, microRNA-135 was found lower in GC cell lines, especially in BGC-823 and SGC-7901, than in GES-1, the normal gastric cell line (Figure 1C). According to the level of microRNA-135 in above tissue specimens, they were divided into high and low expression group to explore the relationship between microRNA-135 expression and the prognosis of GC patients. Kaplan-Meier survival curves demonstrated that low level of microRNA-135 was markedly associated with the poor prognosis of GC patients ($p<0.05$; Figure 1D).

Subsequently, we further analyzed the association between microRNA-135 and age, pathological stage, lymph node metastasis and distant metastasis of GC patients. As shown in Table I, lowly-expressed microRNA-135 was positively correlated with lymph node or distant metastasis of GC, but not with age and pathological stage. Therefore, the above results suggested that microRNA-135 might be a new biological indicator for predicting the malignant progression of GC.

Upregulation of MicroRNA-135 Inhibited Cell Proliferation and Promoted Cell Apoptosis

To explore the effects of microRNA-135 on the function of GC cells, we first constructed the microRNA-135 overexpression model and verified the transfection efficiency by qRT-PCR (Figure 2A). Then, CCK-8, transwell and wound healing assays were performed in the BGC-823 and SGC-7901 cell lines, respectively. It was found that both cell proliferation and migration, as well as invasion ability of GC cells in microRNA-135 mimics group, were conspicuously reduced compared with the NC group (Figure 2B, 2C, 2D).

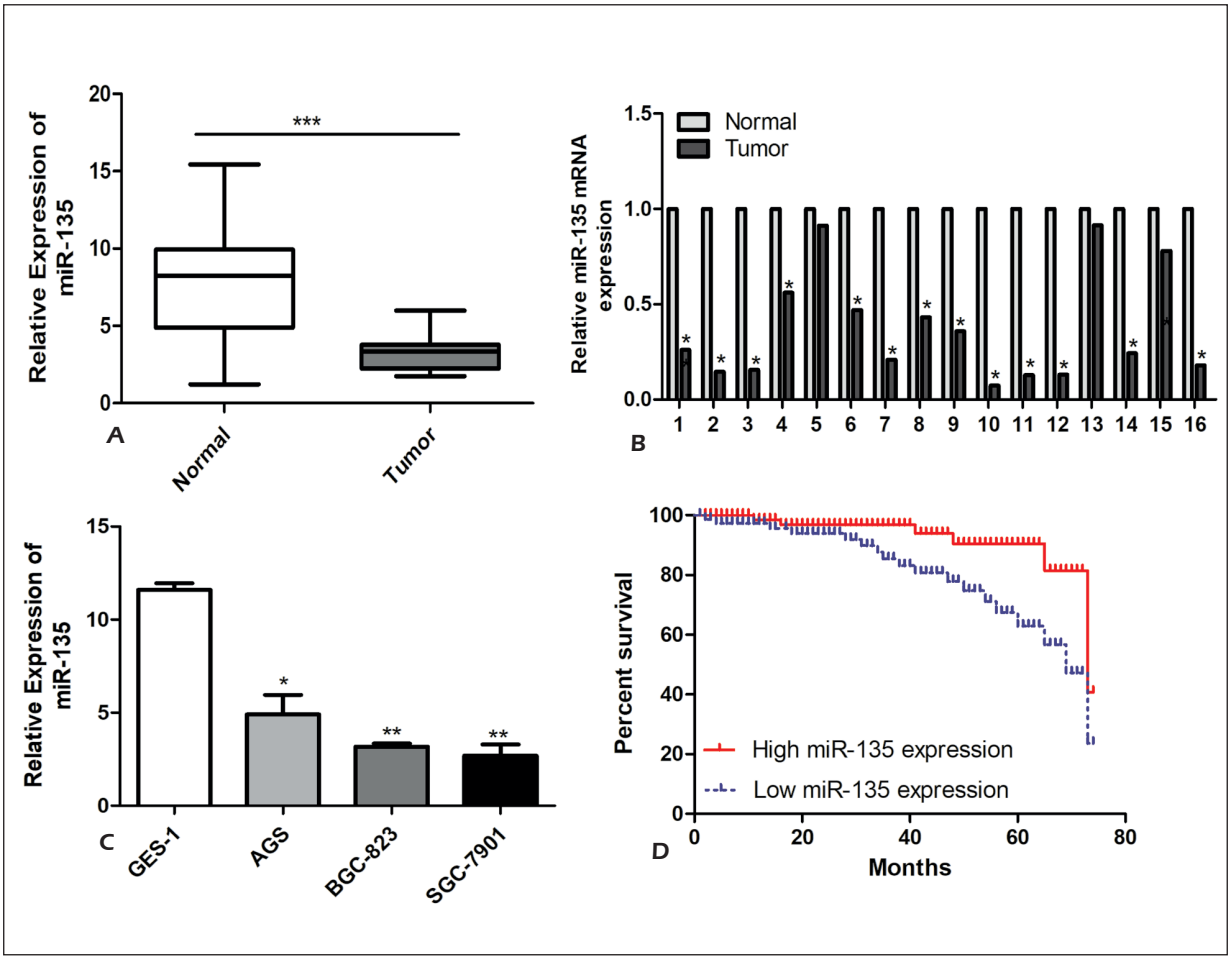

Figure 1. MiR-135 was underexpressed in gastric cancer tissues and cell lines. **A-B**, qRT-PCR was used to detect the differential expression of miR-135 in tumor tissues and adjacent tissues of gastric cancer. **C**, qRT-PCR was used to detect the expression level of miR-135 in gastric cancer cell lines. **D**, Kaplan-Meier survival curve of gastric cancer patients based on miR-135 expression. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Table I. Association of miR-135 expression with clinicopathologic characteristics of gastric cancer.

Parameters	Number of cases	miR-135 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.483
<60	9	6	3	
≥60	19	10	9	
T stage				0.907
T1-T2	19	11	8	
T3-T4	9	5	4	
Lymph node metastasis				0.031
No	18	13	5	
Yes	10	3	7	
Distance metastasis				0.030
No	20	14	6	
Yes	8	2	6	

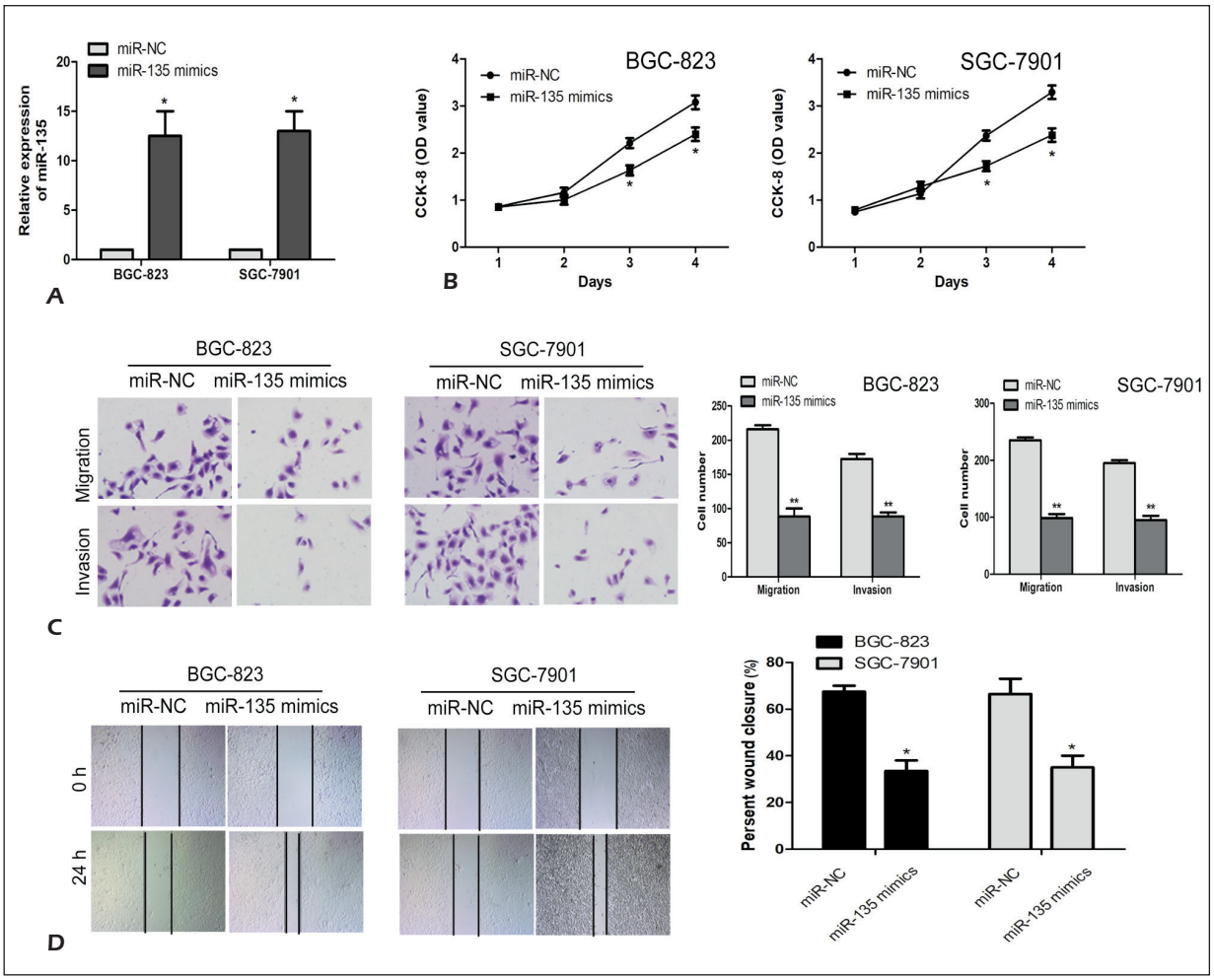

Figure 2. MiR-135 mimics inhibited proliferation, invasion and migration of gastric cancer cells. **A**, qRT-PCR verified the transfection efficiency of miR-135 after transfection of NC and miR-135 mimics in BGC-823 and SGC-7901 cell lines. **B**, The CCK-8 assay detected the effects of BGC-823 and SGC-7901 cell lines on the proliferation of gastric cancer cells. **C**, Transwell assay detected the ability of BGC-823 and SGC-7901 cell lines to invade and migrate (Magnification: 20X). **D**, The cell wound healing assay detected the ability of the BGC-823 and SGC-7901 cell lines to crawl (Magnification: 20X). Data are mean ± SD, *p<0.05.

SMAD2 Was Highly Expressed in GC Tissues and Cell Lines

SMAD2 expression was found remarkably inhibited in the GC cell line after overexpression of microRNA-135 (Figure 3A). Subsequently, qRT-PCR was used to verify SMAD2 expression in GC tissues and cell lines. The results revealed that SMAD2 expression was significantly increased in GC tumor tissues compared with the adjacent tissues (Figure 3B). Similarly, SMAD2 was conspicuously higher in GC cell lines than in normal gastric cell line (GES-1; Figure 3C). We then detected the level of microRNA-135 and SMAD2 by qRT-PCR in GC tissues, and the results showed that these two were negatively correlated in tumor tissues of GC patients (Figure 3D).

MicroRNA-135 Exactly Inhibited SMAD2 Gene Expression

To further explore the ways in which microRNA-135 inhibits the malignant progression of GC, we overexpressed SMAD2 in GC cells transfected with microRNA-135 mimics to investigate their role in GC, and confirmed the overexpressing transfection efficiency by qRT-PCR (Figure 4A). Subsequently, results of CCK-8, transwell and wound healing assays demonstrated that overexpression of SMAD2 could offset the impact of microRNA-135 mimics on GC cell proliferation, invasion and migration capacities (Figure 4B, 4C, 4D).

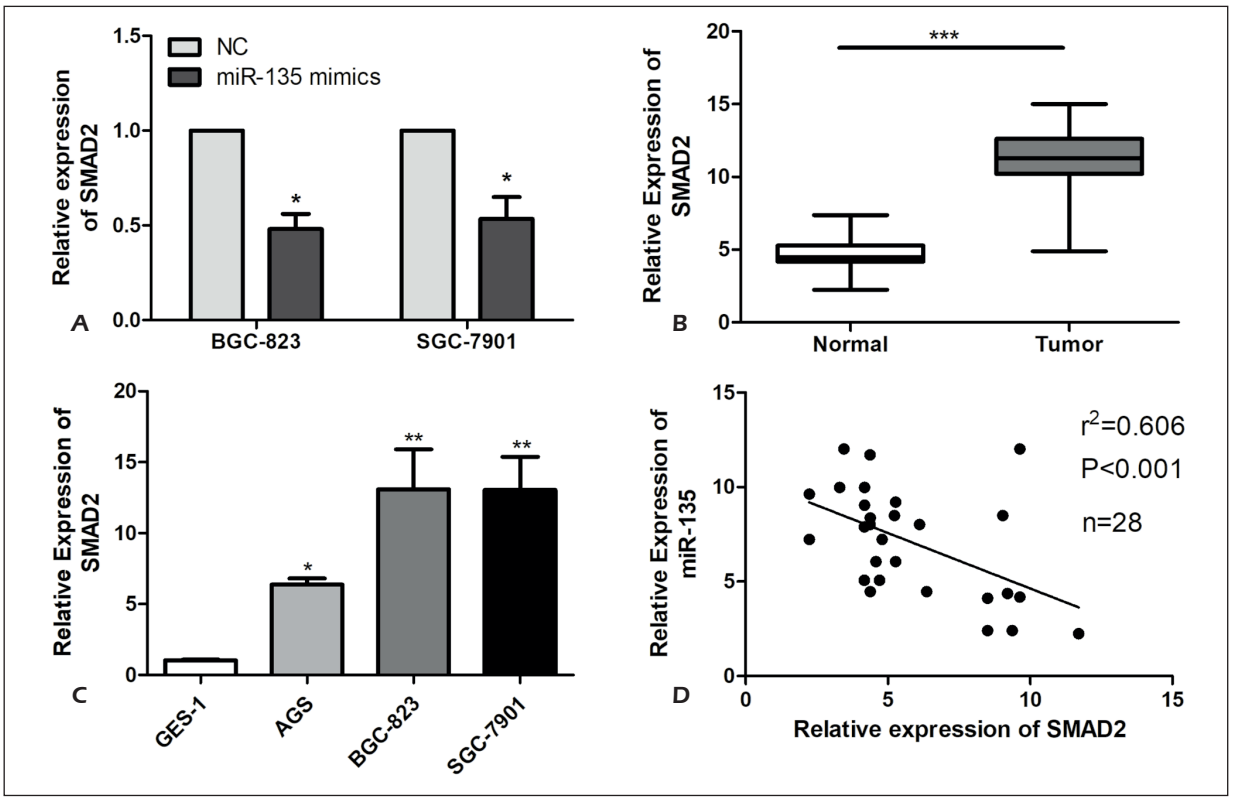

Figure 3. SMAD2 was highly expressed in gastric cancer tissues and cell lines. **A**, qRT-PCR verified the expression level of SMAD2 after transfection of miR-135 mimics in BGC-823 and SGC-7901 cell lines. **B**, qRT-PCR was used to detect the difference in expression of SMAD2 in gastric cancer tissues and adjacent tissues. **C**, qRT-PCR was used to detect the expression level of SMAD2 in gastric cancer cell lines. **D**, There was a significant negative correlation between miR-135 and SMAD2 expression in gastric cancer. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Discussion

GC is one of the most common malignant tumors of the digestive tract, with high incidence, low early diagnosis rate, high mortality and other malignant characteristics. Its early diagnosis rate is only about 10%, and the 5-year overall survival rate is only about 30%, which seriously affects the quality of life and health of human beings[1-3]. According to the results of the epidemiological investigation, China is a country with a high incidence of GC, and its incidence rate ranks first among all malignant tumors. The number of new cases accounts for about 28% of the global number of new cases, and the mortality rate is much higher than in other countries, with about 170,000 deaths every year[8,9]. Therefore, finding a solution that can improve the early diagnosis rate and therapeutic effect of gastric cancer is still a hot spot in the field of cancer research.

MiRNA (microRNA) is a group of endogenous, conserved single-stranded, non-coding RNA molecules of approximately 18-22 bases in length that have been shown to exert regulatory effects by binding to non-coding regions of mRNA. A large number of researches have confirmed that miRNA can regulate more than 30% of the genes in the human genome. Moreover, it can regulate the expression of individual mRNAs or simultaneously regulate the expression of multiple mRNA molecules, inhibit the translation of mRNA or degrade mRNA, and thus affect the functions related to cellular metabolism[10-12]. The discovery of miRNA functional diversity is important for studying its impact on the development of cancer[13]. A large number of studies[20] have found that miRNA expression in cancer tissues and their corresponding normal tissues are conspicuously different. Because of the distinct differences in miRNAs expression, they can serve as targets for clinical diagnosis and treatment[18-20]. Scholars[16,18] have shown that miRNA regulates the proliferation, cell cycle, angiogenesis, migration and invasion and metastasis of GC cells by regulating

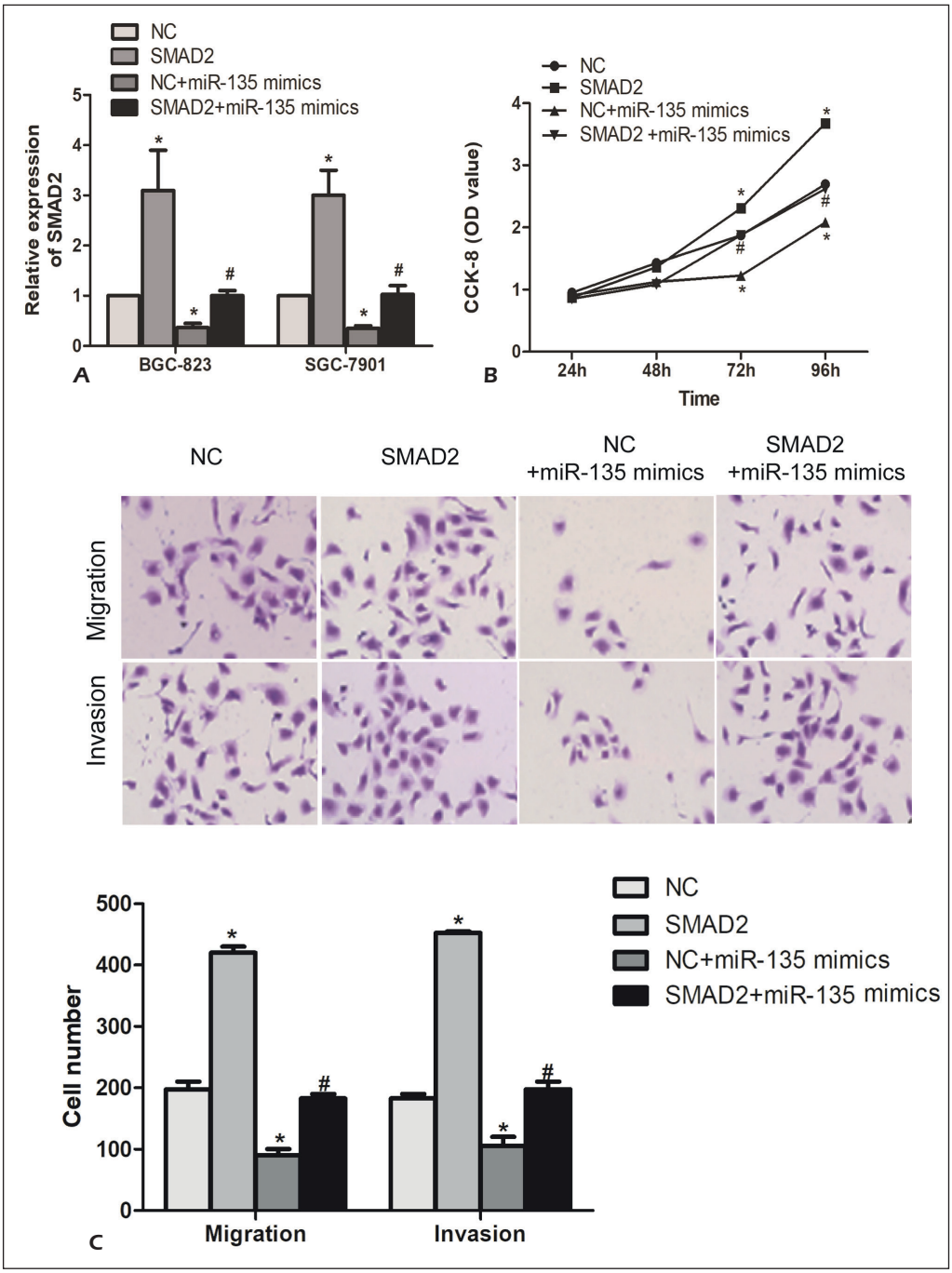

Figure 4. MiR-135 could inhibit gastric carcinogenesis via regulating SMAD2 expression. **A**, The expression level of SMAD2 in the cell line co-transfected with miR-135 and SMAD2 was detected by qRT-PCR. **B**, CCK-8 assay detected the proliferation of gastric cancer cells after co-transfection of miR-135 and SMAD2. **C**, The transwell assay detected the invasion and migration ability of gastric cancer cells after co-transfection of miR-135 and SMAD2 (Magnification: 20X). **D**, Cell wound healing assay detected the invasion ability of gastric cancer cells after co-transfection of miR-135 and SMAD2. Data are mean ± SD, $*^{\#}p<0.05$.

the expression of targeted genes. Studies have shown that cancer-promoting miRNAs can inhibit the expression of tumor suppressor genes when overexpressed in GC; on the contrary, tumor-suppressing miRNAs down-regulate the expression of oncogenes in GC[19]. MicroRNA-135 has been proved to be lowly expressed in various cancer tissues such as pancreatic cancer, and its expression level is closely related to clinicopathological features and clinical prognosis. It is suggested that

microRNA-135 plays a similar oncogene role in a variety of malignant tumors including GC[21]. To explore the role of microRNA-135 in the development and progression of GC, we conducted qRT-PCR to detect the expression of microRNA-135 in 28 pairs of GC tissues and adjacent normal tissues, and found microRNA-135 in GC tissues was conspicuously down-regulated and related to lymph node or distant metastasis. Therefore, we believe that microRNA-135 may play a role in GC suppression. Subsequently, to further explore the effect of microRNA-135 on the biological function of GC cells, we constructed a microRNA-135 mimics model in GC cell lines, and the results of CCK-8, transwell and wound healing assays revealed that microRNA-135 can inhibit the proliferation, invasion and migration abilities of GC cells.

In this work, the structural and functional verification of microRNA-135/SMAD2 interactions was performed. Subsequently, qRT-PCR confirmed that the levels of microRNA-135 and SMAD2 were negatively correlated in GC tissues, and the level of SMAD2 was conspicuously increased in GC tissues. In addition, overexpression of microRNA-135 in GC cells markedly decreased SMAD2 expression and attenuated the ability of GC cell malignant characteristics such as proliferation and invasion, reaching a similar effect to that of SMAD2 knockout, suggesting that microRNA-135/SMAD2 axis may exert a pivotal influence on the occurrence and development of GC.

Conclusions

As described in this work, microRNA-135 was conspicuously associated with lymph node or distant metastasis and poor prognosis of GC patients. Moreover, microRNA-135 may inhibit the malignant progression of GC by regulating SMAD2 expression.

Conflict of Interests

The authors declare that they have no conflict of interest.

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