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MiR-105 inhibits gastric cancer cells metastasis, epithelial-mesenchymal transition by targeting SOX9

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Abstract. – OBJECTIVE: Gastric cancer is one of the most common gastrointestinal malignancy, which is often diagnosed at an advanced stage. MicroRNA-105 (miR-105) was downregulated and acts as a tumor suppressor in various cancers. The purpose of this study was to explore the molecular mechanisms of miR-105 and sex-determining region Y-box 9 (SOX9) in gastric cancer.

PATIENTS AND METHODS: Western blot was performed to display the protein level of E-Cadherin, N-Cadherin, Vimentin and SOX9. Transwell assay was utilized to measure the capacity of migration and invasion. We employed the Luciferase reporter assay to determine miR-105 targeting to SOX9 in gastric cancer.

RESULTS: MiR-105 was downregulated in gastric cancer tissues and cells; it suppressed gastric cancer cell migration, invasion and epithelial-mesenchymal transition (EMT) in gastric cancer. SOX9 was upregulated in gastric cancer cells and had a negative correlation with miR-105. Moreover, the knockdown of SOX9 could inhibit gastric cancer cell migration, invasion and EMT. Furthermore, SOX9 was a target gene of miR-105 and mediated by miR-105. SOX9 could reverse the partial function of miR-105 on cell migration and invasion. In addition, miR-105 downregulation or SOX9 upregulation predicted a poor prognosis.

CONCLUSIONS: We showed that miR-105 was downregulated and inhibited cell migration, invasion and EMT in gastric cancer by binding to SOX9. In addition, we demonstrated that miR-105 downregulation or SOX9 upregulation predicted a poor prognosis. The newly discoverable miR-105/SOX9 axis provides novel insight into gastric cancer treatment.

Key Words
MiR-105, SOX9, Metastasis, EMT, Gastric cancer.

Introduction

Gastric cancer (GC) is one of the most common gastrointestinal malignancy and the leading cause of cancer death[1]. Gastric cancer is often diagnosed at the advanced stage, in which stage the cancer cells have fast proliferation and have a worse prognosis[2]. Almost no commonly-accepted biomarkers have been established to facilitate early diagnosis of GC, although many genes have been identified to participate in tumorigenesis. Therefore, improving GC diagnosis and identify new therapeutic targets and molecular mechanisms underlying gastric carcinogenesis is needed.

MicroRNAs (miRNAs) are non-coding small RNAs with 22-28 nucleotides, which could regulate targeted mRNA by binding to 3'-untranslated region (3'-UTR) at the post-transcriptional level[3,4]. MiRNAs may be detected in blood, which was secreted by solid tumors into circulation[5]. Studies[6,7] have demonstrated that miRNAs may be useful as a diagnostic or prognostic biomarker in many cancers. Among functional miRNAs, miR-105 was downregulated and acted as a tumor suppressor in various cancers, including triple negative breast cancer, non-small cell lung cancer, hepatocellular carcinoma and colorectal cancer[8-11]. In colorectal cancer, miR-105 was involved in tumor necrosis factor (TNF)-α-related tumor microenvironment and enhanced tumor progression[11]. Moreover, miR-105 suppressed cell proliferation, colony formation, migration and cell cycle, and inhibited xenograft growth in glioma[12]. Liu et al[13] discovered similar findings in glioma; miR-105 inhibited cell prolif-

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eration, invasion and xenograft growth and promoted cell apoptosis. However, almost no paper studied the impacts in gastric cancer and only Zhou et al[14] showed that miR-105 was downregulated in gastric cancer and mediated DNMT3A expression. Thus, we are interested to know the role and molecular mechanism of miR-105 in gastric cancer.

Sex-determining region Y-box 9 (SOX9), a member of the SOX family which served as a transcription factor, could play vital roles in the regulation of normal embryogenesis, neural crest development and differentiation[15-17]. SOX9 is up-regulated and improved cancer cell progress in different types of tumors, including glioma, cervical cancer, pancreatic cancer and renal cell carcinoma[18-22]. Huang et al[23] illustrated that knockdown of SOX9 could inhibit cell proliferation, invasion and epithelial-mesenchymal transition (EMT) in thyroid cancer. Furthermore, SOX9 upregulation indicated worse prognosis in solid tumors[24]. In gastrointestinal cancer, SOX9 was upregulated and promoted radiosensitivity or cisplatin sensitivity[25,26]. Considering these findings, we first proposed that miR-105 targeted to SOX9 and suppressed cell invasion, EMT and predicted patient's prognosis.

Patients and Methods

Patients and Clinical Samples

We collected 54 pairs of gastric cancer and corresponding paracancerous specimens from gastric cancer patients at the First Affiliated Hospital of Jiamusi University from 2014 to 2016, which included 25 early stage and 29 advanced stage tumors, based on the criteria of the World Health Organization (WHO). The complete clinicopathological characteristics of the patients were described in Table I. Before surgery, no patients received any therapeutic intervention. All the specimens of this study got informed consent from patients and were approved by the Ethics Committee of the First Affiliated Hospital of Jiamusi University.

Cell Lines and Cell Culture

Human gastric cancer cells lines (HGC-27 and MGC-803) and normal epithelial cell GES-1 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 μg/mL streptomycin, 100 IU/mL penicillin at 37°C containing 5% CO_2.

Table I. MiR-105 expression and clinicopathological features in 54 gastric cancer.

Clinicopathological features	Cases (No. =54)	miR-105 expression		*p*-value
		High (%)	Low (%)	
Gender				
Male	30	17 (56.7)	13 (43.3)	0.161
Female	24	9 (37.5)	15 (62.5)	
Age (years)				
≤60	22	13 (59.1)	9 (40.9)	0.182
>60	32	13 (40.6)	19 (59.4)	
Tumor size (mm)				
≤5.0	23	14 (60.9)	8 (39.1)	0.075
>5.0	31	12 (38.7)	19 (61.3)	
TNM stage				
I-II	27	17 (63.0)	10 (37.0)	0.029*
III-IV	27	9 (33.3)	18 (66.7)	
Differentiation				
poorly	26	16 (61.5)	10 (38.5)	0.058
Moderately/highly	28	10 (35.7)	18 (64.3)	
Lymph-node metastasis				
0-2	30	18 (60.0)	12 (40.0)	0.051
>2	24	8 (33.3)	16 (66.7)	
SOX9				
Negative	25	16 (64.0)	9 (36.0)	0.030*
Positive	29	10 (24.5)	19 (65.5)	

*p-values are calculated with Chi-square test.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs including miRNAs were extracted from tissues and cells using TRIzol Reagent (Invitrogen, Carlsbad, USA). Total RNAs were reverse transcribed to synthesize first complementary deoxyribose nucleic acid (cDNA) chain using One-Step PrimeScript® cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). Fast Start Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland) was employed to analyze quantitative Polymerase Chain Reaction (qPCR) using ABI PRISM7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). MiR-105 and SOX9 were normalized by U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Each reaction was performed in triplicate and the analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

Protein Extraction and Western Blotting

Radioimmunoprecipitation assay buffer (RIPA; Beyotime, Shanghai, China) and bicinchoninic acid (BCA) Reagent Kit (Solarbio, Beijing, China) were applied to extract total protein and measure the concentration of total proteins, respectively. The protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) through electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) with pore size of 0.45 μm; the members were blocked with 5% non-fat powdered milk in Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) at room temperature for 1 h. The blots were then incubated with mouse monoclonal against SOX9 (1:1000) and Vimentin (1:1000) antibodies, rabbit polyclonal antibody against N-Cadherin (1:1000), rabbit monoclonal antibody against E-Cadherin (1:1000; both from Abcam, Cambridge, MA, USA) and mouse monoclonal antibody against GAPDH (1:2000; Sigma-Aldrich, St. Louis, MO, USA). After washing extensively with Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA), the members were incubated rabbit (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse second antibody with Horseradish Peroxidase (HRP)-conjugated (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed by TBST and then developed with an enhanced chemiluminescence system (Pierce, Waltham, MA, USA).

Transwell Assay

Migration and invasion capacities were calculated by transwell assay using chamber (Corning Incorporated, Lowell, MA, USA) with a pore size of 8 mm with or without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). At first, cells were digested and resuspended in serum-free medium and put the chambers into 24-well plate; then the prepared cell suspension of 200 μL was added into the upper chamber. Simultaneously, 500 μL of medium containing 20% of FBS was added into the lower chamber acted as chemoattractant. After incubating for 24 h in 37°C incubator, we utilized cotton swab to remove the cells remained on the upper side of the membrane. The migrated or invaded cells were fixed with 4% paraformaldehyde and stained in 10% crystal violet; then, the cells were calculated microscopically.

Transfection

MiR-105 mimic and miR-105 inhibitor, pcDNA3.1-SOX9 and siRNA-SOX9 associated with their negative control were purchased from GenePharma (Shanghai, China). The sequences of siRNA-SOX9 and its negative control (siRNA-NC) were as follows: siRNA-SOX9: 5'-GCAGCGACGUCAUCUCCAA-3', siRNA-NC: 5'-UUCUCCGAACGUGUCACGU-3'.

MGC-803 cells were seeded in 6-well plates and cultured at 37°C for about 12 h. After the cells adhered to the plate, transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Plasmid Construction and Luciferase Reporter Assay

TargetScan (http://www.targetscan.org/vert_71/) was employed to forecast the target genes and binding sequences of miR-105, and SOX9 was one. The putative miR-105 binding site on SOX9 3'UTR was amplified and inserted into a pmirGlo vector, named WT. After that, the binding sequences mutated from GCAUUUG to CGUAAAC and sub-cloned into a pmirGlo vector (named WUT).

We seeded MGC-803 cells into 6-well plates, co-transfected WT or MUT and plasmid that with or without miR-105 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The normalization utilized the pmirGlo vector, carrying the Renilla Luciferase gene. Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) measured the Luciferase activity after 48 h of transfection.

Statistical Analysis

Data were shown as mean ± SD (Standard Deviation) and analyzed using Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) and GraphPad Prism 5 software (La Jolla, CA, USA). The correlation between miR-105 and SOX9 in gastric cancer tissues was analyzed by Spearman's correlation coefficient. The difference between the two groups was determined by Student's *t*-test. The comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Differences were considered statistically significant at $p<0.05$.

Results

The Correlation of MiR-105 and SOX9 in Gastric Cancer Tissues

To analyze the impacts of miR-105 in gastric cancer, we measured the miR-105 level using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). As expected, miR-105 was consistently downregulated ($p<0.0001$) in gastric cancer tissues *vs.* corresponding paracancerous tissues (Figure 1A). Moreover, SOX9 mRNA level in gastric cancer was higher ($p<0.0001$) than that in the corresponding paracancerous tissues (Figure 1B). Thus, we detected the association of miR-105 and SOX9 levels, and as expected, there was a negative correlation between miR-105 and SOX9 ($p<0.0001$, $r=-0.5814$; Figure 1C). In addition, we explored the expression of miR-105 in cells. Similar to the expression in tissues, miR-105 was downregulated in gastric cell lines HGC-27 ($p=0.0028$) and MGC-803 ($p=0.0004$) *vs.* normal gastric cell GES-1 (Figure 1D).

MiR-105 Inhibits Migration, Invasion and EMT of Gastric Cancer Cells

To verify the function of miR-105 on cell metastasis in gastric cancer, we used transwell and Western blot to measure the migration, invasion and EMT abilities. Before that, miR-105 mimic and inhibitor were employed to overexpress ($p=0.0003$) or knockdown ($p=0.0033$) miR-105, as shown in Figure 2A. As a result, the number of migration and invasion were reduced ($p=0.0010$ and 0.0042) with overexpression of miR-105, while increased ($p=0.0037$ and 0.0124) when inhibited miR-105 expression (Figure 2B). Furthermore, when transfected miR-105 mimic, the epithelial marker E-cadherin was increased, whereas mesenchymal markers N-cadherin and vimentin were rescued and showed opposite results when transfected with miR-105 inhibitor, suggesting that miR-105 played a great role in inducing EMT (Figure 2C).

SOX9 Was Upregulated in Gastric Cancer Cells and Knockdown SOX9 Inhibited Cell Migration, Invasion and EMT

To examined whether SOX9 is involved in regulating cell metastasis in gastric cancer cells, we measured SOX9 expression in gastric cells and discovered that SOX9 was overexpressed in gastric cancer cells HGC-27 ($p=0.0023$) and MGC-803 ($p=0.0007$) *vs.* normal gastric cell GES-1 (Figure 3A). Thus, siRNA-SOX9 was transfected into MGC-803 cells to suppress ($p=0.0024$) SOX9 expression, as shown in Figure 3B. Then, transwell assays illustrated that the abilities of migration ($p=0.0073$) and invasion ($p= 0.0081$) were decreased after transfection with siRNA-SOX9 (Figure 3C). Meanwhile, the results of Western blot explained that epithelial marker E-cadherin increased while mesenchymal markers N-cadherin and vimentin decreased (Figure 3D). Our results declared that SOX9 participated in cell migration, invasion and EMT in gastric cancer.

MiR-105 Targeting to SOX9 and Mediated its Expression

To explore the molecular mechanism between miR-105 and SOX9, TargetScan was employed to understand whether there were binding sites between them, and as expected, miR-105 bound to SOX9 at 3'-UTR. To verify miR-105 direct binding to SOX9, we mutated the binding sequences from GCAUUUG to CGUAAAC, and then measured the Luciferase activity (Figure 4A). SOX9 3'-UTR wild-type sequences (WT) or mutant sequences (MUT) were inserted into pmirGlo Luciferase vector. As expected, the wild-type of SOX9 3'-UTR could decrease ($p=0.0019$) the Luciferase activity, while mutant could not ($p=0.3401$; Figure 4B). In addition, we measured SOX9 mRNA levels in MGC-803 cells transfected with miR-105 mimic or miR-105 inhibitor. We discovered that miR-105 overexpression decreased ($p=0.0043$) SOX9 mRNA level, whereas the inhibition of miR-105 promoted ($p=0.0072$) SOX9 expression (Figure 4C).

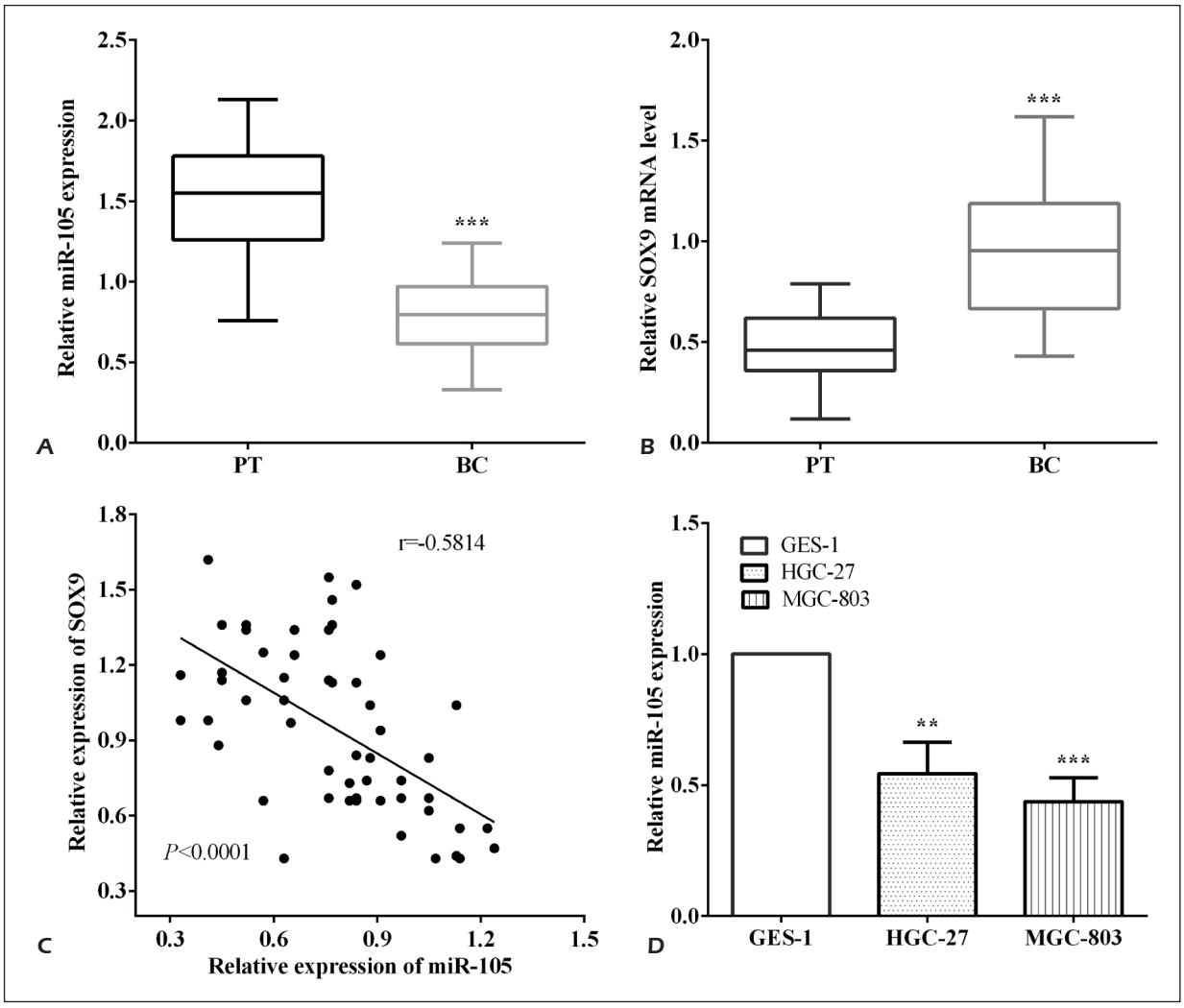

Figure 1. Correlation of miR-105 and SOX9 in gastric cancer tissues. ***A***, MiR-105 was downregulated in gastric cancer tissues vs. corresponding paracancerous tissues. ***B***, SOX9 mRNA level in gastric cancer was higher than that in the corresponding paracancerous tissues. ***C***, The association of miR-105 and SOX9 levels had a negative correlation between miR-105 and SOX9. ***D***, MiR-105 was downregulated in gastric cell lines HGC-27 and MGC-803 *vs.* normal gastric cell GES-1.

SOX9 Could Reverse the Partial Role of MiR-105 on Cell Migration and Invasion

As SOX9 was downregulated by miR-105, we next evaluated the functions of SOX9 in miR-105-mediated gastric cancer cell MGC-803. The mRNA and protein level of SOX9 were significantly restored (p=0.0384) in SOX9 re-expressed cells *vs.* cells only transfected with miR-105 mimic (Figure 5A). For transwell assay, SOX9 re-expression rescued the suppression of cell migration and invasion caused by miR-105 up-regulation in MGC-803 cells (Figure 5B), which illustrated that SOX9 could reverse the partial role of miR-105 on cell migration and invasion.

Identification of MiR-105 and SOX9 Associated With Poor Survival in GC

To identify whether miR-105 was associated with survival of gastric cancer patients, the Kaplan-Meier analysis was performed to measure the overall survival (OS) and disease-free survival (DFS). According to miR-105 expression level, we divided 54 gastric cancer patients into high expression group (miR-105(+)) and low expression group (miR-105(-)); there were 25 and 29 patients, respectively. We found that miR-105 had an association with TNM stage (p=0.029) and SOX9 (p=0.030). However, there was a tendency related with tumor size (p=0.075), differentiation (p=0.058) and lymph-node metastasis (p=0.051).

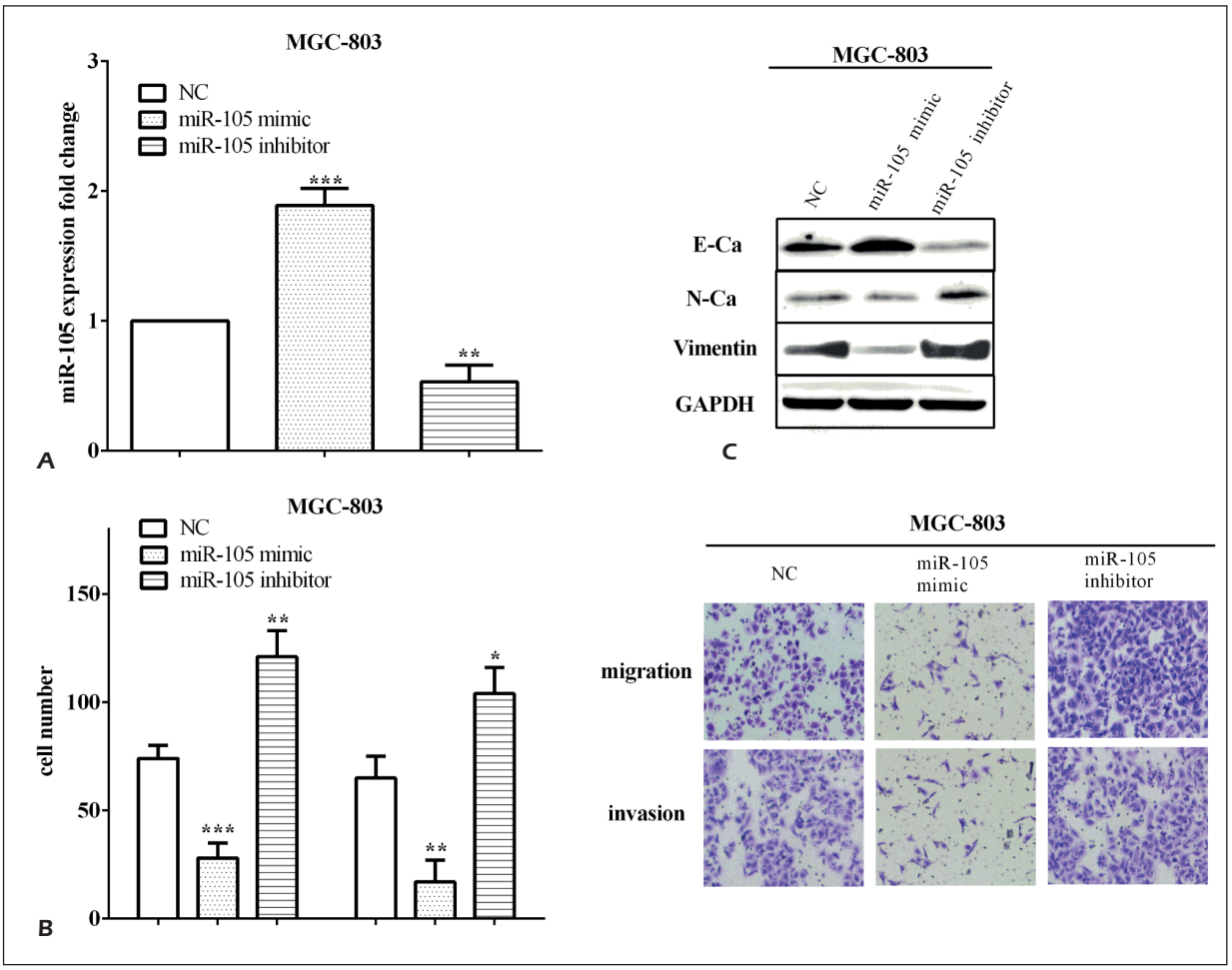

Figure 2. MiR-105 inhibits migration, invasion and EMT of gastric cancer cells. ***A***, MiR-105 mimic and inhibitor were employed to overexpress or knockdown miR-105. ***B***, Overexpression of miR-105 reduced migratory and invasive abilities, while increased when inhibited miR-105 expression. ***C***, MiR-105 overexpression inhibited gastric cancer EMT, while miR-105 inhibitor promoted cell EMT.

There was no correlation between miR-105 with gender (p=0.161) and age (p=0.182; Table I).

We measured OS and DFS based on miR-105 expression; as expected, the OS (Log-rank, p=0.0163) and DFS (p=0.0112) in the miR-105 (+) group was higher than miR-105 (-) group (Figure 6A and 6B). Meanwhile, according to SOX9 expression, we calculated OS and DFS, contrary to the results of miR-105 that the OS (p=0.0095) and DFS (p=0.0302) were lower with SOX9 over-expression *vs.* low expression (Figure 6C and 6D).

Discussion

Gastric cancer is one of the most common gastrointestinal malignancy, often diagnosed at the advanced stage[1,2]. Thus, it is necessary to discover new biomarkers to study the molecular mechanisms underlying gastric carcinogenesis. MicroRNAs may be useful as a diagnostic or prognostic biomarker, which could regulate gene expression by binding to 3'-untranslated region (3'-UTR) at the post-transcriptional level[3,4,6,7]. MiR-105 was downregulated and acted as a tumor suppressor in various cancers[8-11]. In glioma, miR-105 suppressed cell proliferation, colony formation, migration and cell cycle, inhibited xenograft growth and promoted cell apoptosis[12,13]. Similar findings were found by Honeywell et al[27], showing that miR-105 could inhibit cell proliferation and colony formation in prostate tumor. Considering these results, we evaluated that miR-105 was downregulated in gastric cancer tissues and cells. It was

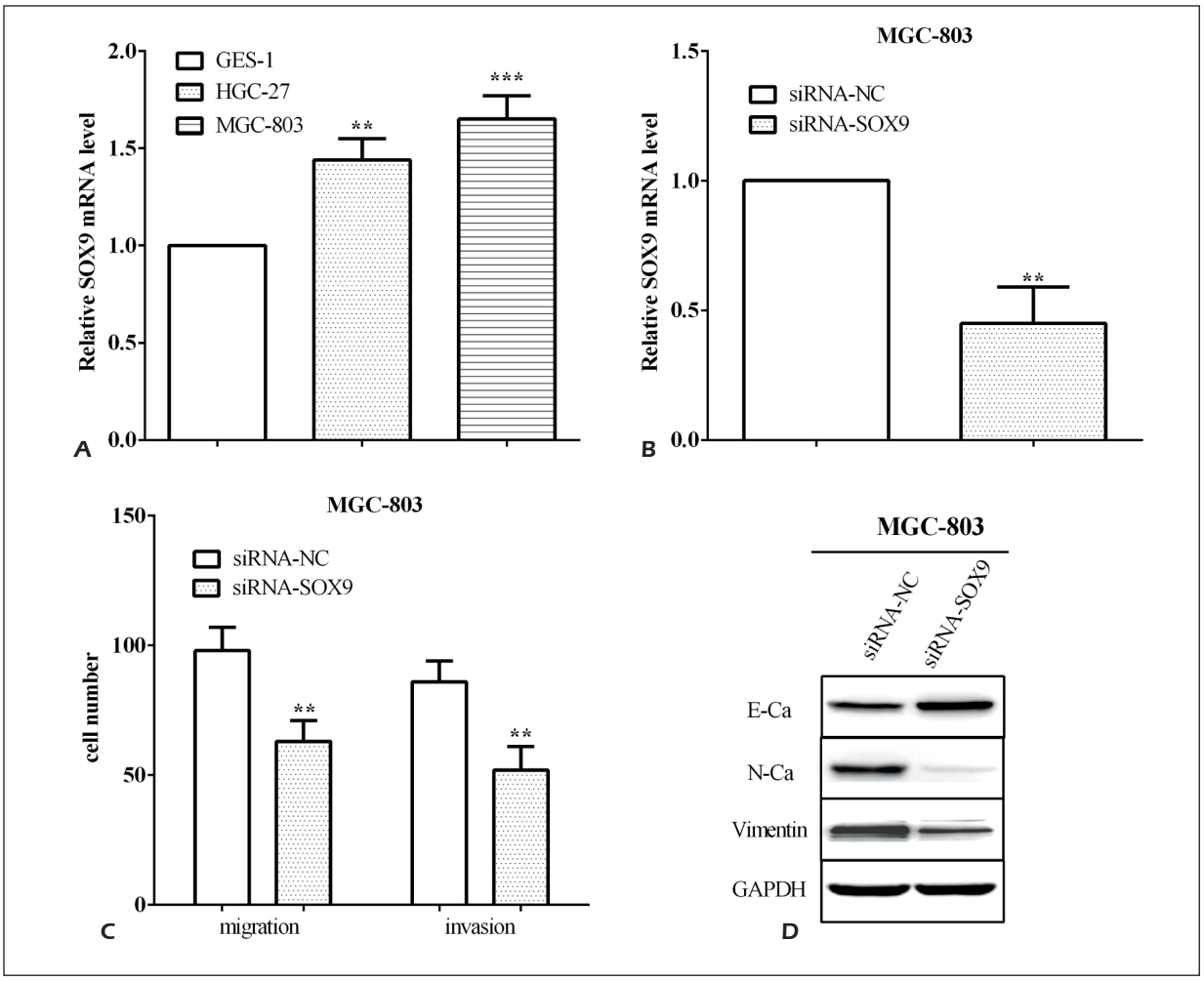

Figure 3. SOX9 was upregulated in gastric cancer cells and knockdown SOX9 inhibited cell migration, invasion and EMT. ***A***, SOX9 expression was calculated in gastric cells HGC-27 and MGC-803 versus normal gastric cell GES-1. ***B***, siRNA-SOX9 was transfected into MGC-803 cells to suppress SOX9 expression. ***C***, The abilities of migration and invasion were evaluated after transfected with siRNA-SOX9. ***D***, Knockdown SOX9 could inhibit gastric cancer EMT.

the first time to propose that miR-105 suppressed gastric cancer cell migration, invasion and EMT in gastric cancer. Moreover, miR-105 targeted to SOX9 and mediated SOX9, which was consistent with Liu et al[13] in glioma. In addition, we determined that miR-105 downregulation predict a poor prognosis, which is consistent with the findings of Lu et al[9] that reduced miR-105 expression in NSCLC tissues is associated with poor OS and DFS of NSCLC patients.

SOX9, a transcription factor, was up-regulated and improved cancer cell progress in different types of tumors[19-22]. Huang et al[23] illustrated that the knockdown of SOX9 could inhibit cell proliferation, invasion and EMT in thyroid cancer. Our results, consistent with all the findings, discovered that SOX9 was upregulated in gastric cancer cells and had negative correlation with miR-105. Knockdown of SOX9 could inhibit gastric cancer cell migration, invasion and EMT. Furthermore, SOX9 was a target gene of miR-105 and was mediated by miR-105. It is the first time for us to propose that miR-105 had a negative connection with SOX9 in gastric cancer. SOX9 could reverse partial function of miR-105 on cell migration and invasion, which were not presented in previous articles. Furthermore, SOX9 upregulation indicated worse prognosis in solid tumors[24]. Our results showed that miR-105 downregulation or SOX9 downregulation predicts a poor prognosis.

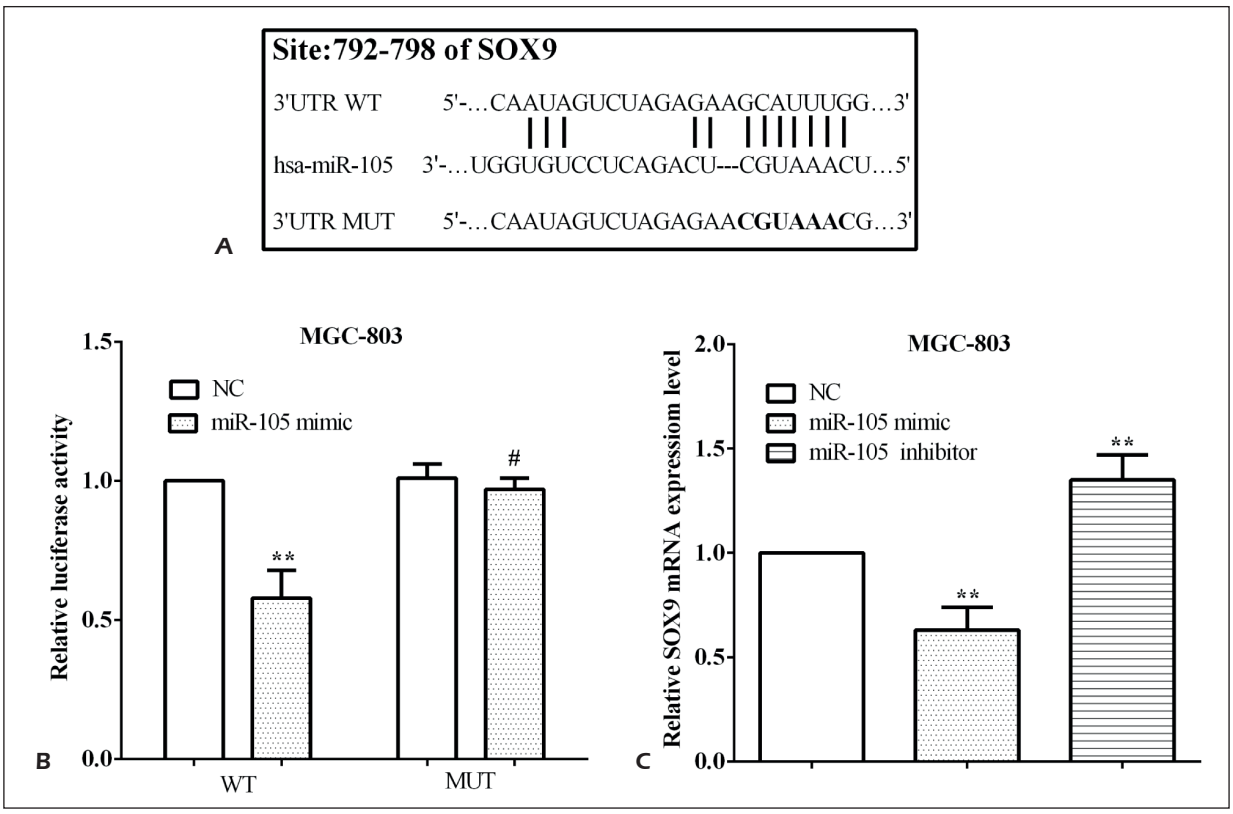

Figure 4. MiR-105 targeted to SOX9 and mediated its expression. ***A***, Wild-type and mutant of miR-381 binding sites on SOX9 3'-UTR and complementary sequences on miR-105. ***B***, The wild- type of SOX9 3'-UTR could decrease the Luciferase activity while mutant could not. ***C***, MiR-105 overexpression decreased SOX9 mRNA level, whereas the inhibition of miR-105 promoted it.

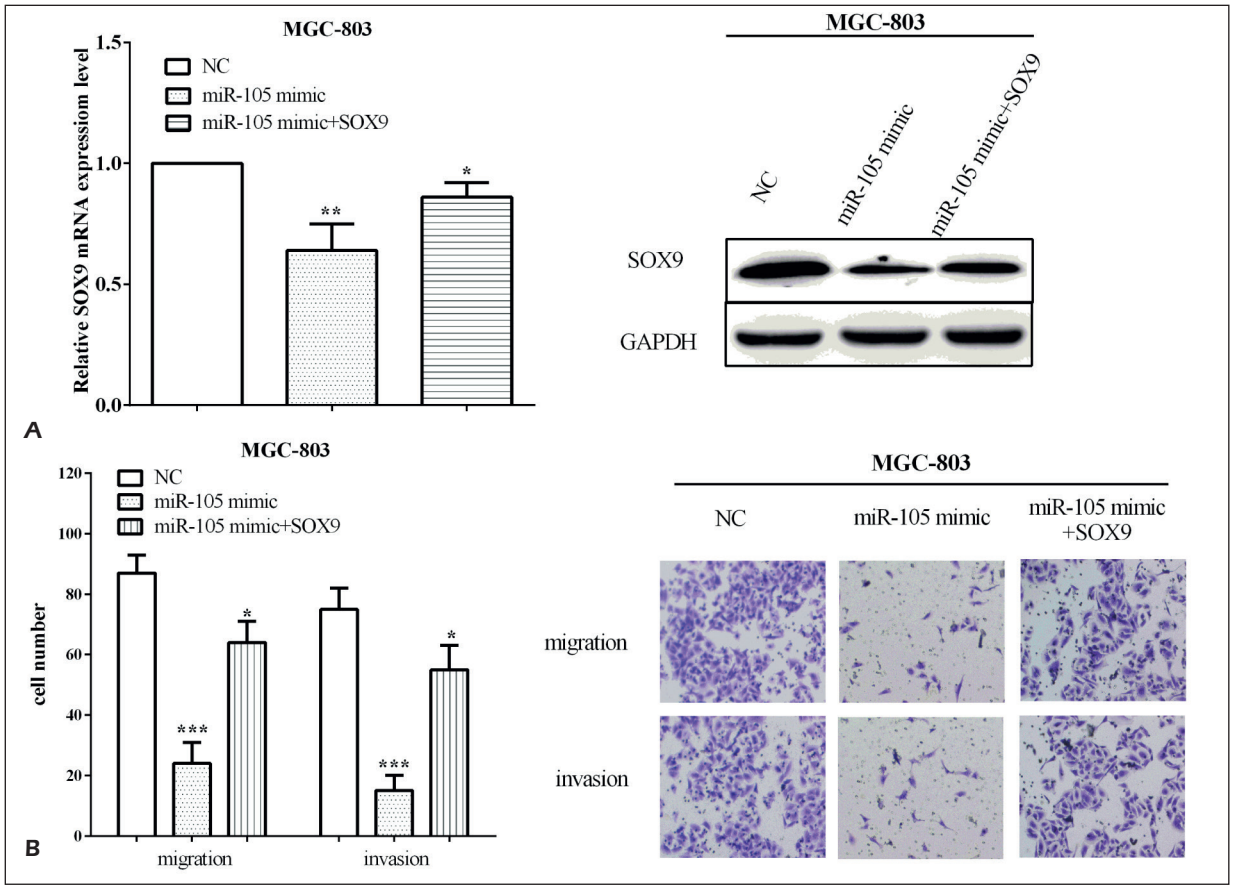

Figure 5. SOX9 could reverse the partial role of miR-105 on cell migration and invasion. ***A***, The mRNA and protein level of SOX9 was significantly restored in SOX9 re-expressed cells vs. cells only transfected with miR-105 mimic. ***B***, SOX9 re-expression rescued the suppression of cell migration and invasion caused by miR-105 up-regulation in MGC-803 cells.

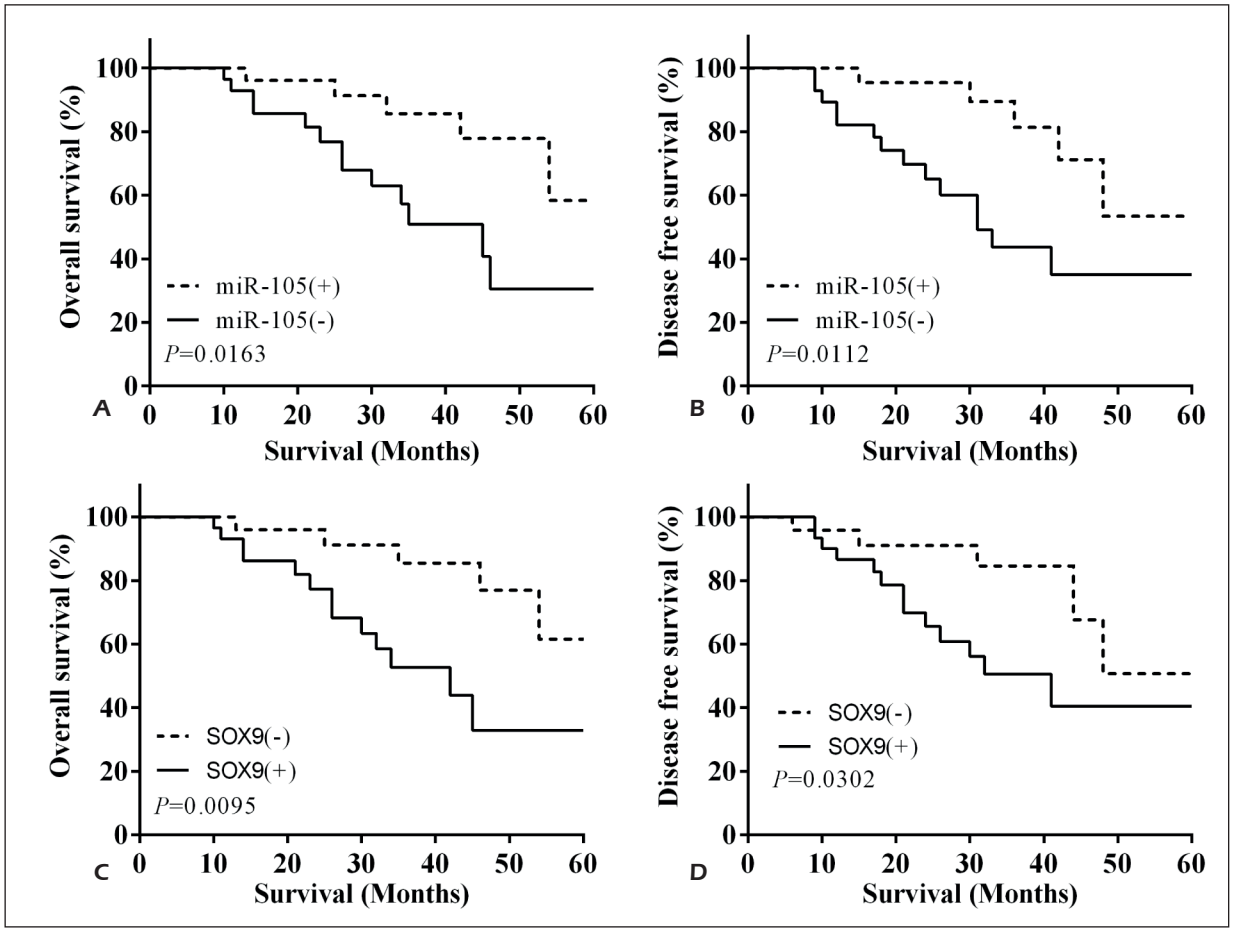

Figure 6. Identification of miR-105 and SOX9 associated with poor survival in GC. ***A***, and ***B***, The OS and DFS in the miR-105 (+) group was higher than miR-105 (-) group. ***C***, and ***D***, The OS and DFS were lower when SOX9 over-expression *vs.* low expression according to the SOX9 expression.

Conclusions

We have indicated that miR-105 was downregulated and inhibited cell migration, invasion and EMT in gastric cancer by binding to SOX9. In addition, we demonstrated that miR-105 downregulation or SOX9 upregulation predicted a poor prognosis. The newly discoverable miR-105/SOX9 axis provides novel insight into the gastric cancer treatment.

Conflict of Interests

The authors declared no conflict of interest.

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