

Downregulation of miR-95 in gastric cancer promotes EMT via regulation of Slug, thereby promoting migration and invasion

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Abstract. miR-95 has been revealed to be aberrantly expressed in multiple types of cancer and to regulate tumor development. Moreover, miR-95 has been revealed to be downregulated in gastric cancer. However, the detailed function of miR-95 in gastric cancer has remained largely unknown. In the present study, we found that miR-95 was downregulated in 63 pairs of gastric cancer tissue samples and adjacent normal tissue samples, as well as gastric cancer cell lines. Additionally, the expression of miR-95 was associated with tumor size, tumor-node-metastasis (TNM) stage and lymph node metastasis. Various functional experiments, including Cell Counting Kit-8 (CCK-8), colony formation, wound healing and Transwell assays were used to explore the effect of miR-95 on cell proliferation and migration as well as invasion, respectively. Overexpression of miR-95 significantly suppressed cell proliferation, migration and invasion. Moreover, miR-95 inhibited EMT by directly binding the 3'-untranslated region (3'-UTR) of Slug. Therefore, miR-95 may be used as a novel therapeutic target for suppressing gastric cancer growth and metastasis.

Introduction

Gastric cancer has become the second highest leading cause of cancer-related deaths worldwide (1). Since the development of new diagnostic techniques as well as improvements in radical lymphadenectomy surgical approaches, the prognosis of gastric cancer patients has improved, but the incidence and mortality rates remain high. The 5-year overall survival rate is still at ~20% (2-4), and the 5-year overall survival rate

of patients with late stage is nearly 4% (5). Therefore, it is urgent to identify potential predictive markers and effective molecular therapeutic targets of gastric cancer.

miRNAs (miRs), a class of small non-coding RNAs, have been reported to suppress the expression of multiple target genes by directly binding to a recognition sequence in the 3'-untranslated regions (3'-UTRs) of the mRNA of the target genes, causing mRNA degradation or translational repression (6-8). Increasing evidence has revealed that the expression of miRs is aberrant in various cancers, and the dysregulation of miRs plays crucial functions in the development and progression of cancers (9-12). Recently, miRs, including miR-93, miR-155 and miR-582 were revealed to promote or suppress gastric cancer proliferation and metastasis (13-15). Previous studies revealed that miR-95 was aberrantly expressed in multiple types of cancer and regulated tumor development (16-18). Chen *et al* revealed that miR-95 was downregulated in the GSRCC type of gastric cancer (19). However, the underlying mechanism of miR-95 has not yet been elucidated.

In the present study, it was demonstrated that miR-95 was downregulated in gastric cancer tissues and cell lines, consistent with a previously study (19). Moreover, the expression of miR-95 was significantly associated with tumor size, tumor-node-metastasis (TNM) stage and lymph node metastasis. Overexpression of miR-95 suppressed gastric cancer cell proliferation, migration and invasion. Additionally, miR-95 also regulated EMT in gastric cancer by directly inhibiting Slug. Collectively, our findings demonstrated that miR-95 is a tumor suppressor in gastric cancer.

Materials and methods

Patients and tissue samples. Patients admitted to the Affiliated Hospital of Jining Medical University between February 2012 and October 2016 were evaluated. These patients with gastric cancer included 41 males and 22 females aged between 32-86 years, with a mean age of 60.6 years. Clinical stages were classified according to the International Union against Cancer TNM classification system (20). The Research Ethics Committee of the Affiliated Hospital of Jining Medical University approved the present study (JN2017015), and all patients provided written informed consent. All tissue samples were stored at -80°C before use.

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Cell lines and cell culture. Human gastric cancer cell lines, such as CTC-141 (Laboratory of Stem Cell Biology of Sichuan University, Sichuan, China) and MKN45 [American Type Culture Collection (ATCC) Manassas, VA, USA], as well as normal human gastric epithelium cell line GES-1 (Bogu Biotechnology, Shanghai, China) were maintained in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin as well as 10% fetal bovine serum (FBS) at 37°C with 5% CO₂.

Cell transfection. Mimic control and miR-95 mimics (miR-95 mimic), as well as inhibitor control and miR-95 inhibitors (miR-95 inhibitor) were purchased from Qiagen (Duesseldorf, Germany). Cells were transfected using Lipofectamine 3000 according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). After transfection for 48 h, the transfected cells were used for further experiments.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from the gastric cancer tissue samples and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol and was quantified using NanoDrop 2000 (Thermo Fisher Scientific, Inc.). An RNA sample (2 µg) was used to synthesize cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). A SYBR-Green (Roche Molecular Diagnostics, Pleasanton, CA, USA) was used to determine relative mRNA expression with an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the instructions of the manufacturer. Primers for miR-95 were purchased from GeneCopoeia Co. (Guangzhou, China). The cycling parameters were as follows: 95°C for 5 min and then 40 cycles of 95°C for 15 sec and annealing/extension at 60°C for 1 min. E-cadherin: 5'-ACCTGGTTCAGATCAAATCC-3' (forward) and 5'-TCATTCTGATCGGTTACCGT-3' (reverse); N-cadherin: 5'-CAGAGTTTACTGCCATGACG-3' (forward) and 5'-AAAGTCGATTGGTTTGACCA-3' (reverse); vimentin: 5'-ATTGAGATTGCCACCTACAG-3' (forward) and 5'-ATCCAGATTAGTTTCCCTCAG-3' (reverse); Slug: 5'-AGATGCATATTCGGACCCACA-3' (forward) and 5'-CCTCATGTTTGTGCAGGAGAG-3' (reverse); GAPDH: 5'-GAGAAGTATGACAACAGCCTC-3' (forward) and 5'-ATGGACTGTGGTCATGAGTC-3' (reverse); miR-95: 5'-CTGGTGGAGGGATGGATG AA-3' (forward) and 5'-GGCCCGATCACAAACTCATC-3' (reverse); U6: 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse) GAPDH mRNA or small nuclear RNA U6 were used as internal controls. The relative mRNA expression was calculated via the 2^{-ΔΔC_q} method (21).

Western blot analysis. Whole protein was isolated from the transfected cells using RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China), and protein concentrations were assessed using Pierce BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Protein ~45 µg was separated via 10% SDS-PAGE. After being transferred to nitrocellulose

Table I. Clinicopathological variables in 63 gastric cancer patients.

Variables	No. (n=63)	miR-95 expression		P-value
		Low (n=42)	High (n=21)	
Age, years				0.285
<60	33	20	13	
≥60	30	22	8	
Sex				0.135
Male	41	30	11	
Female	22	12	10	
Tumor size (diameter) (cm)				0.031
Small (≤3)	27	14	13	
Large (≥3)	36	28	8	
TNM stage				0.020
I-II	29	15	14	
III-IV	34	27	7	
Lymph node metastasis				0.021
No	32	17	15	
Yes	31	25	6	

TNM, tumor node metastasis.

filter membranes (EMD Millipore, Bedford, MA, USA), the membranes were blocked with 5% skimmed milk at room temperature for 1 h. Subsequently, the membranes were incubated with indicated primary antibodies at 4°C overnight. After being washed with phosphate-buffered saline Tween-20 (PBST) three times at room temperature, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h and washed with PBST three times at room temperature. Finally, the blots were visualized by ECL kit (Pierce; Thermo Fisher Scientific, Inc.). Each independent experiment was performed three times. The antibodies as follow: EMT kit (1:1,000; cat. no. 9782; Cell Signaling Technology, Danvers, MA, USA), β-actin (1:2,000; cat. no. ab6276; Abcam, Cambridge, UK), goat anti-rabbit (HRP) (1:5,000; cat. no. ab205718; Abcam), goat anti-mouse (HRP) (1:3,000; cat. no. ab205719, Abcam). The densitometry of blots was determined using ImageJ software (version 4.1; National Institutes of Health, Bethesda, MD, USA).

Cell Counting Kit-8 (CCK-8) assay. CCK-8 assay was used to detect the effect of miR-95 on cell proliferation. In brief, 3,000 transfected CTC-141 or MKN45 cells with 200 µl media were seeded in 96-well plates. After transfection for 48 h, 20 µl CCK-8 solution (Beyotime Institute of Biotechnology) was added to the culture medium at 0, 24, 48 and 72 h, and incubated for 30 min at 37°C. The absorbance of 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each independent experiment was performed three times.

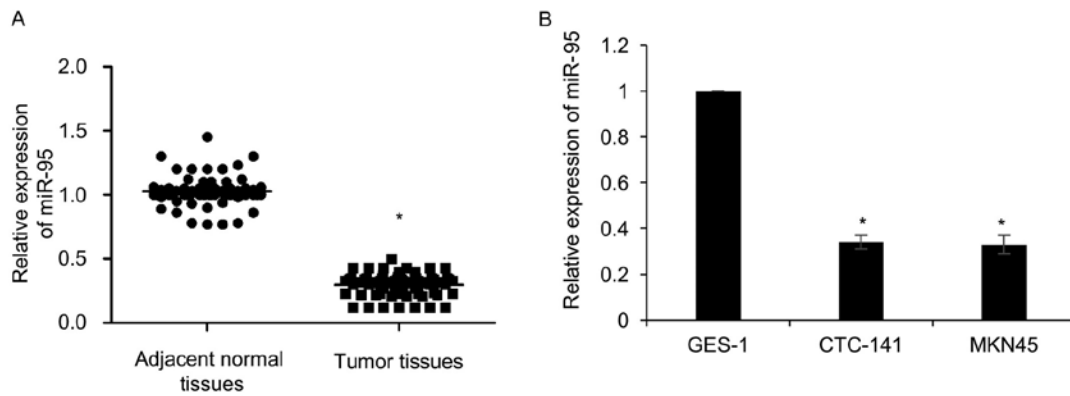


Figure 1. miR-95 is aberrantly downregulated in gastric cancer tissues as well as cell lines, and associated with poor tumor progression. (A) qRT-PCR result of the expression of miR-95 in 63 pairs of gastric cancer tissues and adjacent normal tissues. Tumor tissues vs. adjacent normal tissues, * $P < 0.05$. (B) qRT-PCR result of the expression of miR-95 in gastric cancer cell lines, such as CTC-141 and MKN45. GES-1 was used as a control. CTC-141 or MKN45 vs. GES-1, * $P < 0.05$.

Wound healing assay. A wound healing assay was used to determine the effect of miR-95 on cell migration. Briefly, 5×10^5 transfected CTC-141 or MKN45 cells were seeded in 6-well plates. When cell density was almost 90-100%, a linear wound was generated using a 20- μ l pipette tip, and the detached cells were washed with PBS three times. The distance of wound healing was assessed at 0 and 24 h with a light microscope. Each independent experiment was performed three times.

Transwell invasion assay. A Transwell assay was used to determine the effect of miR-95 on cell invasion. In brief, 80 μ l Matrigel was coated on the Transwell chambers (BD Biosciences, Bedford, MA, USA) and maintained at 37°C for 30 min. Approximately 4×10^4 transfected CTC-141 or MKN45 cells in 400 μ l serum-free medium were placed in the upper chamber in 24-well culture plates, and 500 μ l RPMI-1640 medium containing 10% FBS was added to the lower chamber. Cells were maintained at 37°C with 5% CO₂ for 16 h. Subsequently, the cells were stained with 0.5% crystal violet at room temperature for 10 min. The cells on the surface of the upper membranes were removed by cotton swab, and the number of invading cells was counted under a light microscope. Each independent experiment was performed three times.

EMT induction. When cell density was almost 60%, cells were cultured with serum-free media overnight. Recombinant human TGF- β 1 (10 ng/ml) was added into media for 72 h.

Luciferase reporter assay. TargetScan Human version 7.0 (www.targetscan.org) predicted that Slug was a potential target of miR-95. The 3'-UTR of Slug was cloned into the pGL3 luciferase vector (Invitrogen; Thermo Fisher Scientific, Inc.). For the luciferase assay, CTC-141 and MKN-45 cells were co-transfected with *Renilla*, pGL3-Slug 3'-UTR or and miR-95 mimics or miR-95 inhibitor. After transfection for 24 h, a luciferase reporter assay was performed using a Dual-Luciferase Reporter Assay kit according to the manufacturer's instructions (Promega Corp., Madison, Wisconsin, USA). The *Renilla* luciferase activity was used to normalize firefly luciferase activity. Each independent experiment was performed three times.

Statistical analysis. All data were analyzed using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA) and presented as the mean \pm SD. A Student's t-test or one-way ANOVA followed by Tukey's were used to analyze the differences between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-95 is aberrantly downregulated in gastric cancer tissues as well as cell lines, and associated with poor tumor progression. To investigate the functions of miR-95 in gastric cancer, we first detected the expression of miR-95 in gastric cancer tissues. We performed qRT-PCR to analyze the miR-95 expression levels in 63 gastric tumor tissue samples and adjacent normal tissues samples. As shown in Fig. 1A, our findings revealed that miR-95 expression was significantly downregulated in gastric cancer tissues compared with adjacent normal tissues (Fig. 1A). In addition, we detected the expression of miR-95 in gastric cancer cell lines, including CTC-141 and MKN45. GES-1 was used as a control. The expression of miR-95 was lower in CTC-141 and MKN45 cells compared to GES-1 (Fig. 1B). Subsequently, clinicopathological analyses of 63 gastric cancer patients demonstrated that the expression of miR-95 was closely associated with tumor size, lymph node metastasis as well as TNM stage (Table I).

miR-95 suppresses gastric cancer cell proliferation. To explore the function of miR-95 in gastric cancer, we first over-expressed or knocked down miR-95 in CTC-141 and MKN45 cells with miR-95 mimics or miR-95 inhibitor. The expression of miR-95 was determined by qRT-PCR (Fig. 2A). As shown in Table I, since the expression of miR-95 was closely associated with tumor size, we assumed that miR-95 may regulate cell proliferation. To verify our hypothesis, CCK-8 and colony formation assays were performed. The results of the CCK-8 assay revealed that compared with that of the mimic control or inhibitor control groups, the proliferation of CTC-141 and MKN45 cells in the miR-95-mimic group were significantly decreased and were clearly increased in the miR-95-inhibitor group (Fig. 2B). The colony formation assay also confirmed that

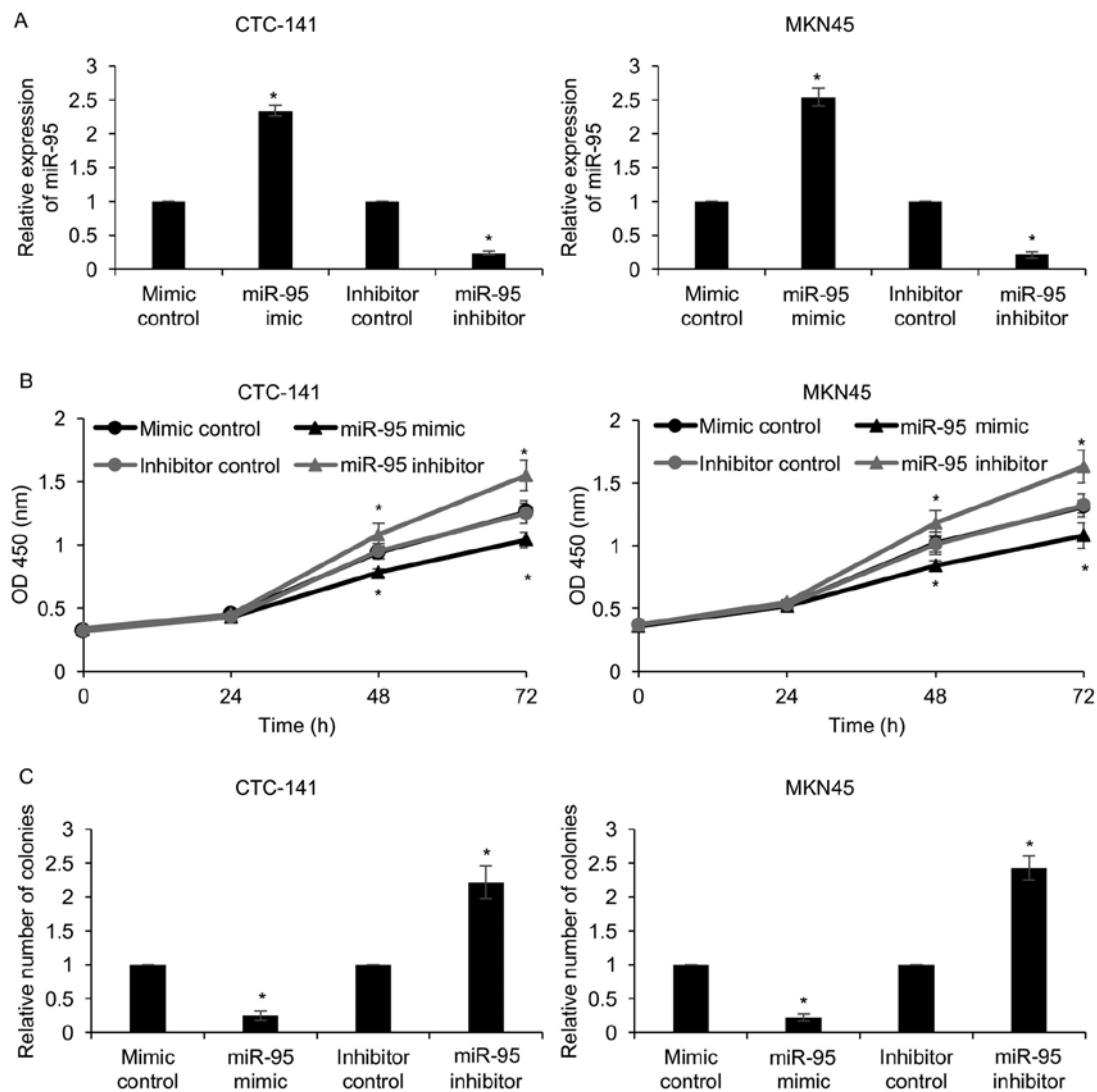


Figure 2. miR-95 suppresses gastric cancer cell proliferation. (A) miR-95 mimics or inhibitors significantly increased or decreased the expression of miR-95 in CTC-141 and MKN45 cells, respectively. miR-95 mimic vs. mimic control, miR-95 inhibitor vs. inhibitor control, * $P < 0.05$. (B) Representative graphs of the CCK-8 assay revealing CTC-141 and MKN45 cells transfected with miR-95 mimics or inhibitors. miR-95 mimic vs. mimic control, miR-95 inhibitor vs. inhibitor control, * $P < 0.05$. (C) Colony formation assay of CTC-141 and MKN45 cells transfected with miR-95 mimics or inhibitors. miR-95 mimic vs. mimic control, miR-95 inhibitor vs. inhibitor control, * $P < 0.05$. CCK-8, Cell Counting Kit-8.

ectopic expression of miR-95 led to a decrease in the number of colonies, and an inhibition of miR-95 led to an increase in the number of colonies (Fig. 2C). These results revealed that miR-95 suppressed gastric cancer cell proliferation.

Downregulation of miR-95 promotes gastric cancer cell migration and invasion. Our experiments revealed that miR-95 expression was negatively associated with lymph node metastasis. To determine whether miR-95 regulated migration and invasion in gastric cancer cells, we performed wound healing and Transwell assays. The results of the wound healing assays revealed that ectopic expression of miR-95 significantly decreased the distance of cell migration (Fig. 3A). In contrast, downregulation of miR-95 significantly increased the distance of cell migration (Fig. 3A). Similar results were observed in the Transwell assay, revealing that the ectopic expression of miR-95 resulted in less invading cells than that in the mimic control group (Fig. 3B). In contrast, the downregulation of miR-95 resulted in more invading cells than those in the

inhibitor control group (Fig. 3B). Since MMP9 is an invasion-related factor, we next determined whether miR-95 regulated MMP9 secretion using an ELISA assay, which revealed that the secretion of MMP9 was decreased when cells were transfected with miR-95 mimics, and increased when cells were transfected with the miR-95 inhibitor (Fig. 3C). Our experiments revealed that downregulation of miR-95 promoted gastric cancer cell migration and invasion.

miR-95 inhibits the TGF- β -induced EMT process of gastric cancer cells. To further decipher the detailed mechanisms of miR-95 in gastric cancer metastasis, we aimed to explore the effects of miR-95 on EMT, which was recognized as a main cause for cell migration and invasion. CTC-141 and MKN45 cells were transfected with miR-95 mimics and mimic control. After inducing 10 ng/ml of TGF- β 1, the expression of EMT-associated proteins was determined by RT-qPCR and western blotting, respectively. The results revealed that ectopic expression of miR-95 led to an increased expression

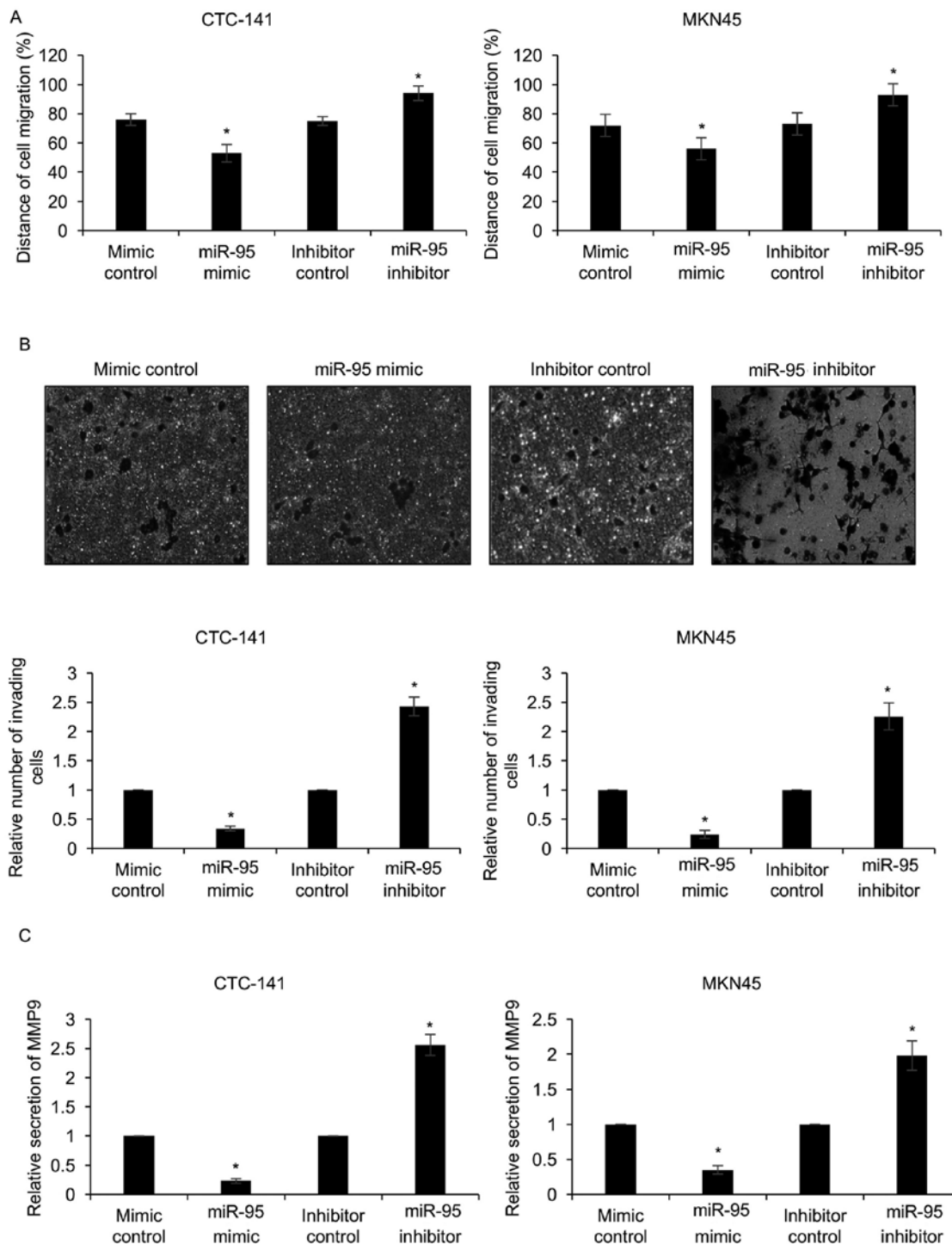


Figure 3. Downregulation of miR-95 promotes gastric cancer cell migration and invasion. (A) A wound healing assay of CTC-141 and MKN45 cells transfected with miR-95 mimics or inhibitors. miR-95 mimic vs. mimic control, miR-95 inhibitor vs. inhibitor control, * $P < 0.05$. (B) A Transwell assay of CTC-141 and MKN45 cells transfected with miR-95 mimics or inhibitors. miR-95 mimic vs. mimic control, miR-95 inhibitor vs. inhibitor control, * $P < 0.05$. (C) Levels of MMP9 proteins in gastric cancer cells assessed by ELISA analysis. miR-95 mimic vs. mimic control, miR-95 inhibitor vs. inhibitor control, * $P < 0.05$.

of E-cadherin both at the mRNA level and protein level, and a decreased expression of N-cadherin and vimentin both at the mRNA level and protein level (Fig. 4A and B). A suppression of miR-95 reversed these results (Fig. 4C and D). The mRNA and protein levels of E-cadherin were decreased, and the levels of N-cadherin as well as vimentin were increased (Fig. 4C and D). Overall, the results implied that miR-95 inhibits the TGF- β 1-induced EMT process of gastric cancer cells.

Slug is a target of miR-95 in gastric cancer cells. miRNAs have been found to participate in multiple physiological and pathological processes by regulating gene expression. To further decipher the detailed mechanism of miR-95 on EMT, we searched for potential targets of miR-95 by bioinformatics search using TargetScan (www.targetscan.org). We found that *Slug*, a key transcription factor of EMT, was a direct target of miR-95. To verify whether *slug* was regulated by miR-95,

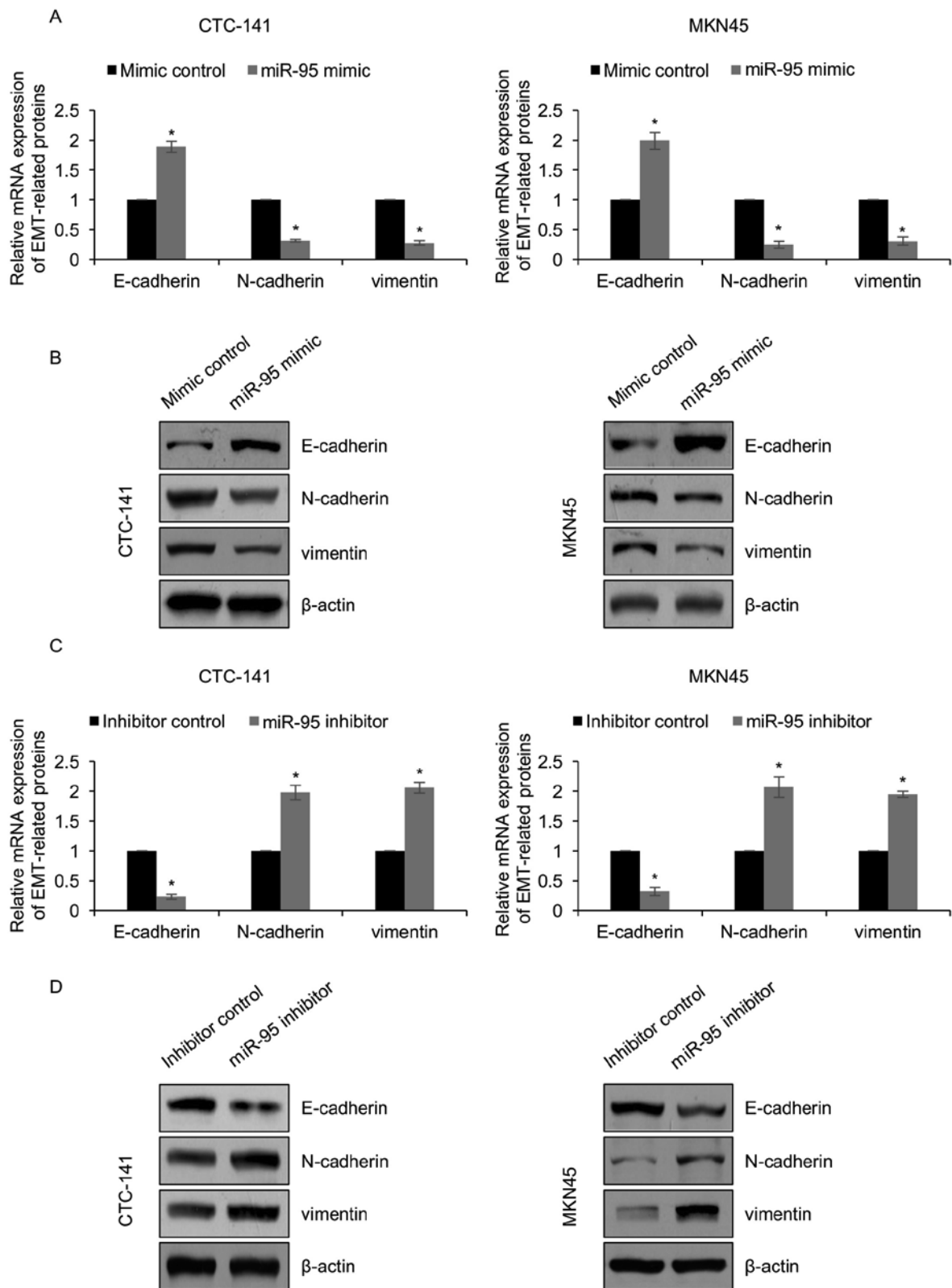


Figure 4. miR-95 inhibits the TGF- β 1-induced EMT process of gastric cancer cells. (A and B) miR-95 was overexpressed in CTC-141 and MKN45 cells. qRT-PCR and western blot analysis of E-cadherin, N-cadherin and vimentin in TGF- β 1-treated CTC-141 and MKN45 cells. miR-95 mimic vs. mimic control, * P <0.05. (C and D) miR-95 was knocked down in CTC-141 and MKN45 cells. qRT-PCR and western blot analysis of E-cadherin, N-cadherin and vimentin in TGF- β 1-treated CTC-141 and MKN45 cell lines. miR-95 inhibitor vs. inhibitor control, * P <0.05.

we overexpressed or knocked down miR-95 in CTC-141 and MKN45 and detected the expression of Slug. As shown in Fig. 5A, we found that Slug expression was decreased in

CTC-141 and MKN45 cells treated with miR-95 mimics, while Slug expression was increased in CTC-141 and MKN45 cells treated with miR-95 inhibitors (Fig. 5A and B). To

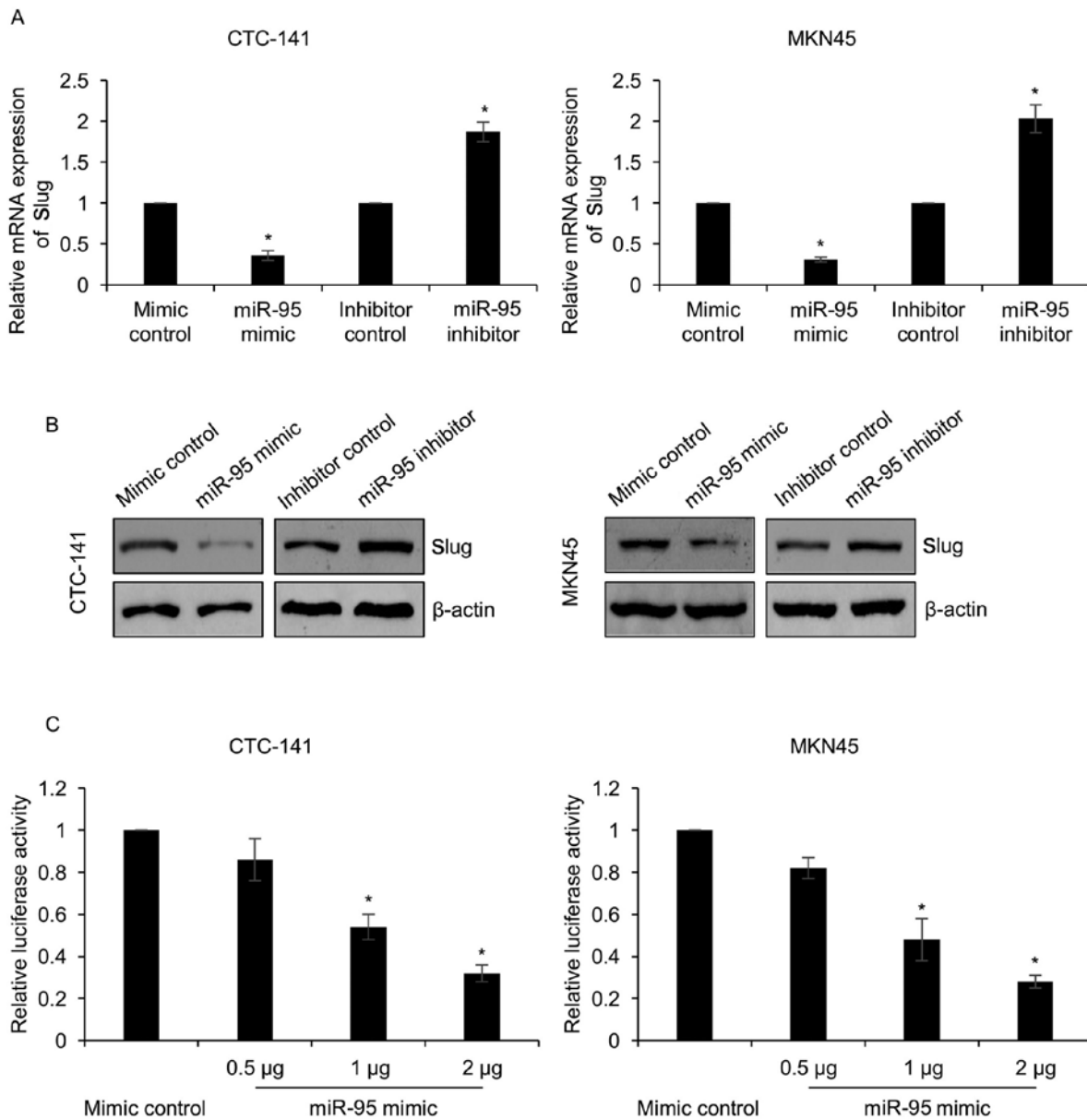


Figure 5. Slug is a target of miR-95 in gastric cancer cells. (A and B) miR-95 was overexpressed or knocked down in CTC-141 and MKN45 cells. qRT-PCR and western blot analysis of Slug. miR-95 mimic vs. mimic control, miR-95 inhibitor vs. inhibitor control, * $P < 0.05$. (C) Upregulation of miR-95 significantly inhibited the luciferase activity of the 3'-UTR of Slug. miR-95 mimic vs. mimic control, * $P < 0.05$.

examine whether miR-95 directly interacted with the 3'-UTR of Slug, we performed a luciferase reporter assay. The luciferase activity was significantly decreased when cells were co-transfected with Slug 3'-UTR and either miR-95 mimics or mimic control (Fig. 5C). These findings indicated that Slug is a target of miR-95 in gastric cancer.

Discussion

miR-95 has been reported to facilitate cell proliferation in non-small cell lung cancer (NSCLC) and colorectal carcinoma, suggesting that miR-95 acts as an oncogenic miRNA (17,22). Paradoxically, Chen *et al* revealed that miR-95 was downregulated in the GSRCC type of gastric cancer (19). Moreover, miR-95 was also revealed to regulate chemoresistance and radioresistance in NSCLC (22). A previous study also

revealed that miR-95 plays a key function in the anticancer activity of Brucein D which is in contrast to its proliferative effects (23). Several studies have indicated that miRNAs are new therapeutic targets for multiple diseases, such as cancers. In colorectal cancer (CRC), miR-95 was revealed to be overexpressed in the serum sample of CRC patients (24), suggesting that miR-95 may be a potential molecular target for cancer diagnosis.

In this study, we found miR-95 was downregulated in gastric cancer tissues and cell lines. Additionally, the expression of miR-95 was significantly associated with tumor size, TNM stage and lymph node metastasis. Furthermore, we found that miR-95 suppressed proliferation, migration and invasion of gastric cancer cells.

Metastasis is the main reason for treatment failure in cancer and results in >90% of cancer-related deaths (25-27). EMT is

a complex process which is associated with the progression of metastasis, and improves the migration as well as invasive abilities of cancer cells. Various studies have indicated that miRNAs are involved in this process (25,28). In the present study, we found that miR-95 suppressed EMT in gastric cancer cells. Notably, we found that Slug, a key transcription factor of EMT, was a target of miR-95. Therefore, we demonstrated that miR-95 suppressed cell migration and invasion through regulation of EMT in gastric cancer cells.

However, there are still some limitations in the present study. The upstream of miR-95 and detailed mechanism of miR-95 on cellular proliferation in gastric cancer is still unknown. Moreover, the function of miR-95 *in vivo* warrants further investigation.

Collectively, our findings revealed the important role of miR-95 in regulating EMT of gastric cancer. miR-95 suppressed Slug expression to regulate the progression of EMT, thereby inhibiting migration and invasion in gastric cancer. In addition, aberrant expression of miR-95 was closely associated with tumor size, lymph node metastasis as well as TNM stage, suggesting that miR-95 may play a key role in gastric cancer development. Our findings revealed miR-95 as a novel molecular therapeutic target of gastric cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WZ and LZ conceived and designed the study. WZ, JS, CX and JC performed the experiments. WZ and CX wrote the paper. WZ and LZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The Research Ethics Committee of the Affiliated Hospital of Jining Medical University, Jining, China approved the present study. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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