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Upregulation of circular SMAD7 inhibits tumorigenesis of gastric cancer by reversing epithelial-to-mesenchymal transition

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Abstract. – OBJECTIVE: Gastric cancer (GC) is one of the most ordinary malignant tumors. Recent studies have revealed that circular RNAs (circRNAs) play an important role in the progression of tumorigenesis. In this research, circ-SMAD7 was selected to identify how it functions in the progression of GC.

PATIENTS AND METHODS: Circ-SMAD7 expression in paired GC patients' tissue samples and cell lines was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). The role of circ-SMAD7 in the metastasis of GC was detected through wound healing assay and transwell assay. Western blot assay and RT-qPCR were used to discover the function of circ-SMAD7 in epithelial-to-mesenchymal transition (EMT) process. Furthermore, tumor metastasis assay was also performed *in vivo*.

RESULTS: In this study, RT-qPCR results showed that circ-SMAD7 expression was significantly lower in GC tissues compared to that in adjacent ones. Cell migration and invasion of GC were inhibited *via* upregulation of circ-SMAD7. Moreover, the results of further experiments revealed that the EMT-related proteins were regulated *via* upregulation of circ-SMAD7 in GC. Furthermore, tumor metastasis of GC was inhibited *via* upregulation of circ-SMAD7 in nude mice.

CONCLUSIONS: These results indicate that upregulation of circ-SMAD7 inhibits GC cell migration and invasion *via* reversing the EMT process.

Key Words:
Long noncoding RNA, Circ-SMAD7, Gastric cancer, Epithelial-to-mesenchymal transition.

Introduction

Gastric cancer (GC) remains a major threat to public health worldwide which is the most common gastrointestinal malignancy in East Asia[1,2]. It is estimated that approximately 28,000 cases were diagnosed with gastric neoplasms in 2017. Moreover, 10,960 deaths were expected to be related closely to GC which is one of the top leading causes of cancer-related death in the world[3]. Despite tremendous advances have been made in the development of detection techniques and interventions of GC in the past decades, the rate of local recurrence remains high, ranging from 2.8%-12.5%[4]. Also, most GC cases are diagnosed at late stages which are characterized by malignant invasion and distant metastasis. Therefore, it is very urgent to understand the underlying molecular mechanism of GC and improve the poor prognosis for the unfortunate patients.

Circular RNAs (circRNAs) have emerged as a new hot topic in the noncoding RNAs network which is formed by a covalently closed loop. Previous studies[5] have revealed that circRNAs were differentially expressed in various cancerous tissues or cells which are closely related to disease status and prognosis. For example, androgen-responsive circ-SMARCA5 is remarkably overexpressed in prostate cancer which enhances the proliferation of prostate cancer cells. By sponging miR-1271, circ-ABCB10 facilitates the proliferation and progression of breast cancer cell[6]. Through regulating the expression of miR-138, circ-0020397 promotes cell viability and invasion in colorectal cancer by targeting TERT and PD-L1[7].

Circ-SMAD7 is a novel circRNA which plays a vital role in malignant tumors. In the present study, circ-SMAD7 was remarkably downregulated in GC tissues and cell lines. Moreover,

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circ-SMAD7 inhibited migration and invasion of GC *in vitro* and *in vivo*. Our further experiments also showed that circ-SMAD7 inhibited epithelial-to-mesenchymal transition (EMT) process of GC.

Patients and Methods

Tissue Samples

In total, 46 patients were collected in this research. They underwent surgical resection at China-Japan Union Hospital of Jilin University. Human GC tissues and adjacent non-tumor tissues were obtained from patients during the surgery. After surgical resection, all the tissue samples were snap-frozen in liquid nitrogen immediately. Before the surgery, no radiotherapy and chemotherapy treatment were performed in any patient. This investigation was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University. Signed written informed consents were obtained from all participants before the study.

Cell Culture

The GC cell lines (BGC-823, SGC-7901, HGC-27, and MKN-45) and human gastric epithelial cell line (GES) were bought from American Type Culture Collection (ATCC; Manassas, VA, USA). 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) were used to culture the cells in a humidified incubator at 37°C with 5% CO_2.

Cell Transfection

According to the manufacturer's instructions, the circ-SMAD7 lentivirus or negative control (NC) was synthesized by GenePharma (GenePharma, Shanghai, China), which were then transfected into SGC-7901 cells through Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

According to the manufacturer's instructions, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to extract total RNA from tissue and cultured cells. By using a Reverse Transcription Kit (TaKaRa, Dalian, China), RNA was reverse transcribed to complementary deoxyribose nucleic acid (cDNA) for RT-qPCR. By using SYBR Green (TaKaRa, Dalian, China), RT-qPCR was performed to detect the expression of circ-SMAD7 in GC tissues and cells with a normalizing control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used for RT-qPCR were as follows: circ-SMAD7 forward: 5′-GGGGAGTGGCTGTGGATAA-3′ and reverse: 5′-TCTCAAGAGGGATTTACAAACG-3′; GAPDH, forward: 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse: 5′-TGGTGAAGACGCCAGTGGA-3′. The thermal cycle was as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression.

Wound Healing Assay

1.0×10^4 cells were seed into a 6-well plate. Three parallel lines were made on the back of each well. After growing to about 90% confluence, cells were scratched with a pipette tip and cultured in a medium. Cells were photographed under a light microscope after 0 h and 48 h. Each assay was independently repeated in triplicate.

Transwell Assay

After transfection, cells (5×10^4) in 200 μL serum-free RPMI-1640 were added to the top chamber of an 8 μm pore size insert (Corning, Corning, NY, USA) with or without 50 μg Matrigel (BD, Bedford, MA, USA). RPMI-1640 and FBS were then added to the lower chamber. 48 h later, the top surface of the chambers was treated with methanol for 30 min after being wiped by a cotton swab. Next, they were stained in crystal violet for 20 min. Three fields were used to count the data for migration and the invasion membrane.

Tumor Metastasis Assay

Transfected GC cells were injected into the tail vein of NOD/SCID mice (6 weeks old). The mice were sacrificed and the lung was extracted after 4 weeks. Then, the number of metastatic nodules in the lung was counted. The animal experiments were approved by the Animal Ethics Committee of Jilin University.

Western Blot Analysis

Reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was utilized to

extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China) was chosen to quantify protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were incubated with antibodies after being replaced to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-GAPDH and rabbit anti-E-cadherin, rabbit anti-N-cadherin, rabbit anti-Vimentin, as well as goat anti-rabbit secondary antibody. The Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of protein expression.

Statistical Analysis

Statistical analysis was conducted by Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA). Independent-sample *t*-test was used to compare the difference between the two groups. Moreover, $p<0.05$ was considered a statistically significant difference.

Results

The Circ-SMAD7 Expression Level in GC Tissues and Cells

RT-qPCR was used to monitor circ-SMAD7 expression in 46 GC patients' tissues and cells. Circ-SMAD7 was lower expressed in GC tissue samples than in adjacent tissues (Figure 1A). Meanwhile, the circ-SMAD7 expression level was lower in GC cells than in GES (Figure 1B).

Cell Migration and Invasion were Inhibited in GC Cells Via Upregulation of Circ-SMAD7

In our study, we chose the SGC-7901 cell line for the upregulation of circ-SMAD7. Then, RT-qPCR was utilized for detecting the circ-SMAD7 expression (Figure 2A). Moreover, wound healing assay showed that the migrated length of SGC-7901 cells was significantly decreased after circ-SMAD7 was upregulated (Figure 2B). Furthermore, the transwell assay showed that the number of migrated and invaded cells was significantly increased after circ-SMAD7 was upregulated (Figures 2C and 2D).

The Interaction Between EMT Process and Circ-SMAD7 in GC

To explore how circ-SMAD7 functioned in the EMT process of GC, RT-qPCR, and Western blot assay were conducted to detect the EMT-related proteins such as E-cadherin, N-cadherin, and Vimentin. The RT-qPCR assay showed that the expression of E-cadherin was higher in circ-SMAD7 lentivirus group than in NC group (Figure 3A), while the expression of N-cadherin and Vimentin was lower in circ-SMAD7 lentivirus group than in NC group (Figures 3B and 3C). Meanwhile, the Western blot assay also showed similar results (Figure 3D).

Tumor Metastasis of GC Was Inhibited In Vivo Via Upregulation of Circ-SMAD7

The number of metastatic nodules in the lung from the circ-SMAD7 lentivirus group was significantly reduced compared to NC group (Figure 4A). Moreover, the expression level of

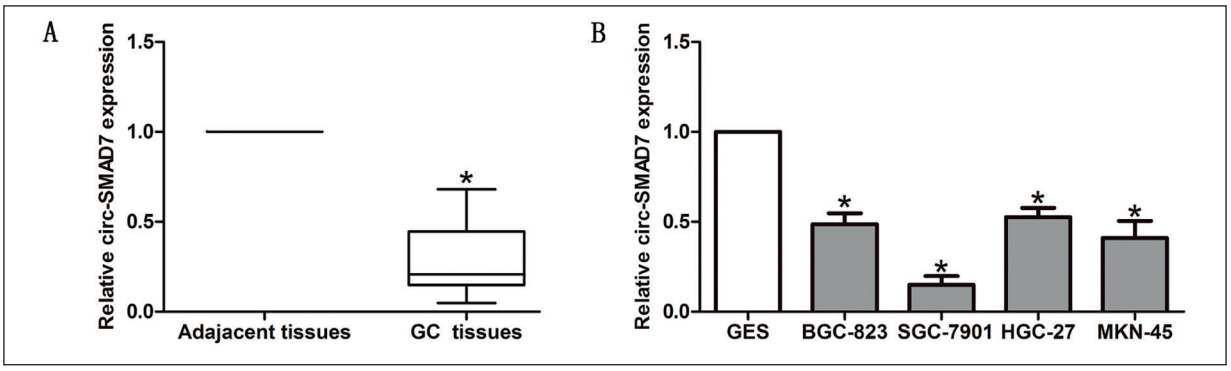

Figure 1. Expression level of circ-SMAD7 was decreased in GC tissues and cell lines. **A,** Circ-SMAD7 expression was significantly decreased in the GC tissues compared with adjacent tissues. **B,** Expression level of circ-SMAD7 was lower in the human GC cell lines than that in GES cells by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

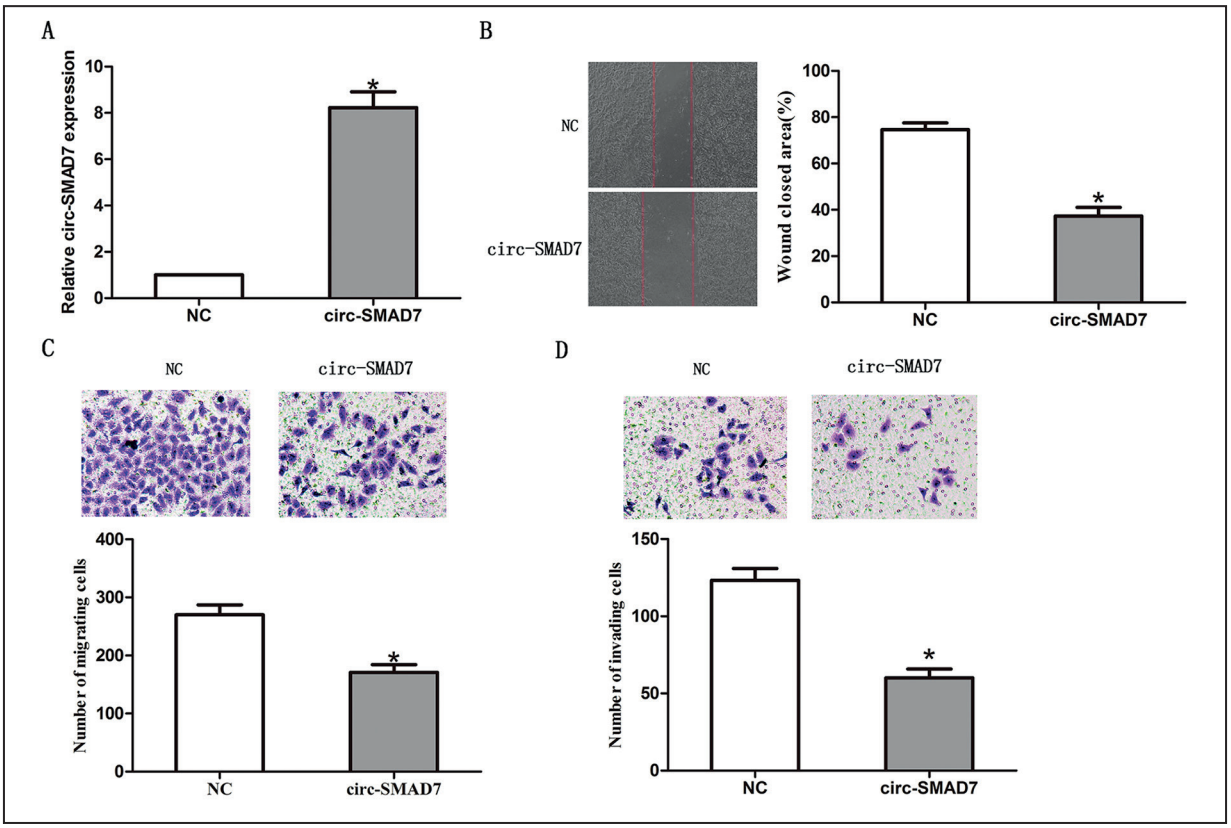

Figure 2. Upregulation of circ-SMAD7 inhibited GC cell migration and invasion. **A,** Circ-SMAD7 expression in GC cells transfected with negative control (NC) or circ-SMAD7 lentivirus (circ-SMAD7) was detected by RT-qPCR. **B,** Wound healing assay showed that the migrated length of cells in circ-SMAD7 group was significantly decreased compared with NC group in GC cells (magnification: 40×). **C,** Transwell assay showed that upregulation of circ-SMAD7 significantly repressed cell migration in GC cells (magnification: 40×). **D,** Transwell assay showed that upregulation of circ-SMAD7 markedly repressed cell invasion in GC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

circ-SMAD7 in extracted metastatic nodules was detected by RT-qPCR. The results showed that circ-SMAD7 was higher expressed in circ-SMAD7 lentivirus group compared with NC group (Figure 4B). Above results suggested that circ-SMAD7 could inhibit tumor metastasis *in vivo*.

Discussion

Recently, circRNAs have been reported to play an important role in the tumorigenesis of GC. For instance, the overexpression of circ-PVT1 promotes cell proliferation in gastric cancer by serving as a sponge for the miR-125 family which may be a potential factor and prognostic biomarker in GC[8]. By regulating expression of LATS1 and sponging miR-424-5p, circ-LARP4 suppresses the proliferation and invasion of GC cells[9]. CircRNA-100269 is downregulated in GC which inhibits cell growth in GC tumor via targeting miR-630[10]. The expression of circ-0003159 is remarkably negatively related to the progression of gastric cancer[11].

CircRNA SMAD7, generated from chromosomal 18, is reported to be lowly expressed in esophageal squamous cell carcinoma and inhibit tumor proliferation and migration[12]. In the current study, we conducted experiments to identify the role of circ-SMAD7 in GC. Results showed that circ-SMAD7 was downregulated in GC samples and cells. Besides, GC migration and invasion were found to be inhibited via the upregulation of circ-SMAD7. Experiments *in vivo* also showed that the inhibition of tumor metastasis was induced by circ-SMAD7. Above results indicated that circ-SMAD7 inhibited

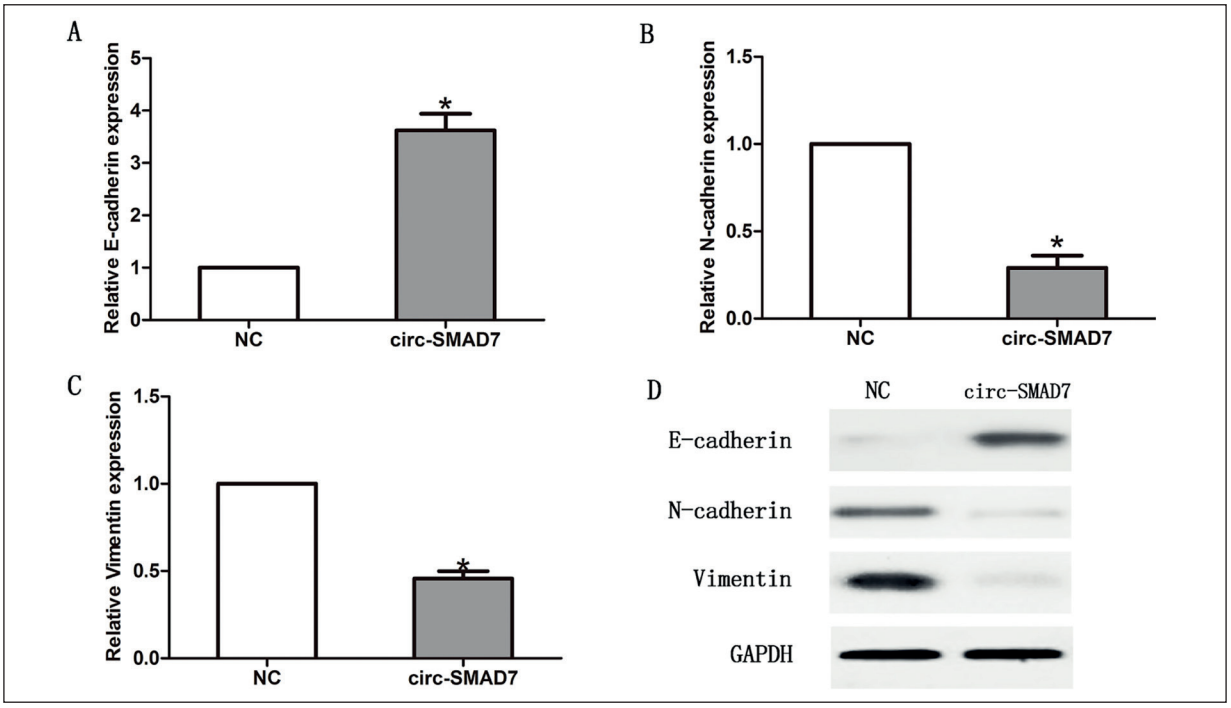

Figure 3. The association between circ-SMAD7 and EMT process of GC. **A,** RT-qPCR results revealed that the expression of E-cadherin in EMT process of GC was upregulated in circ-SMAD7 group compared with NC group. **B,** RT-qPCR results revealed that the expression of N-cadherin in EMT process of GC was downregulated in circ-SMAD7 group compared with NC group. **C,** RT-qPCR results revealed that the expression of Vimentin in EMT process of GC was downregulated in circ-SMAD7 group compared with NC group. **D,** Western blot assay was used to detect the protein level of E-cadherin, N-cadherin, and Vimentin in EMT process of GC after circ-SMAD7 was upregulated. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

EMT is a crucial biological process involved in a multitude of developmental and pathological events. EMT is characterized by the progressive loss of cell-to-cell contacts resulting in filopodia formation and mesenchymal gene expression which enables cell migration and invasion. For example, EMT is associated with poor tumor differentiation in pancreatic ductal adenocarcinoma which can be increased by gemcitabine[13]. URG11 promotes cell proliferation and EMT in benign prostatic hyperplasia cells via the RhoA/ROCK1 pathway[14]. Through the activation of ZEB1 and

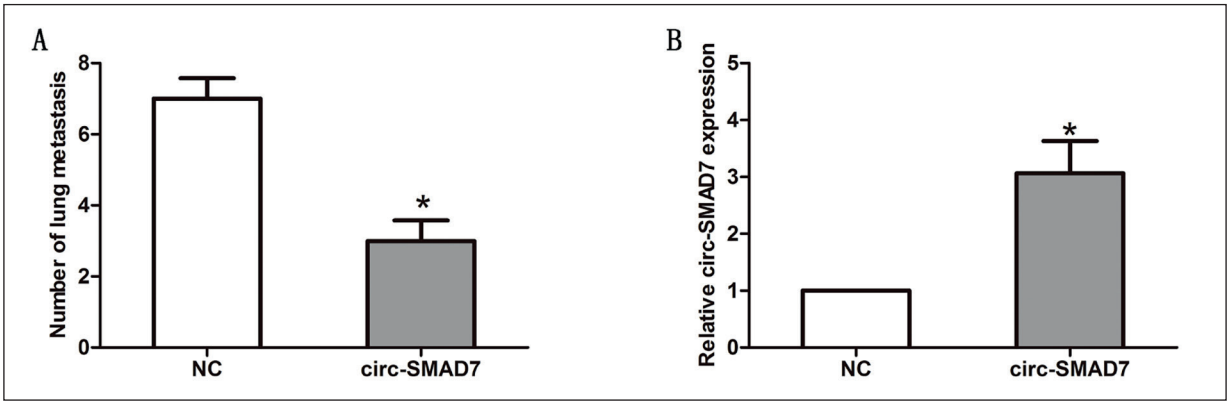

Figure 4. Upregulation of circ-SMAD7 inhibited tumor metastasis *in vivo*. **A,** Number of metastatic nodules in the lung from the circ-SMAD7 group compared with NC group. **B,** Relative expression of circ-SMAD7 in extracted nodules were examined by RT-qPCR. Data are presented as the mean ± SD of three independent experiments. *p< 0.05.

interaction with miR-139-5p, lncRNA HCP5 enhances epithelial-mesenchymal transition in colorectal cancer[15]. E-cadherin, N-cadherin, and Vimentin are vital proteins in the EMT process. To detect the effect of circ-SMAD7 during the EMT process of GC, we observed the changes of these proteins through the upregulation of circ-SMAD7 in GC cells. Results showed that the EMT process was reversed by the upregulation of circ-SMAD7.

Conclusions

We demonstrated that circ-SMAD7 could inhibit GC metastasis through suppressing the EMT process. These findings implied that circ-SMAD7 could serve as a prospective therapeutic target for GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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