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CircRNA_100782 promotes proliferation and metastasis of gastric cancer by downregulating tumor suppressor gene Rb by adsorbing miR-574-3p in a sponge form

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Abstract. – OBJECTIVE: The aim of this study is to investigate the expression levels of circRNA_100782 in gastric cancer tissues, and its function of regulating tumor suppressor gene Rb by absorbing miR-574-3p in a sponge form.

PATIENTS AND METHODS: qRT-PCR was performed to detect the expressions of circRNA_100782 at different stages during gastric cancer tissues. CCK-8 assay was performed to evaluate the osteoclast proliferation and differentiation. The correlation between miR-574-3p and circRNA_100782 was detected by statistical analysis. Bioinformatics and Luciferase assay were performed to explore the interaction and binding site of circRNA_100782 and miR-574-3p. The mice Rb 3′-UTR were cloned into the Luciferase reporter vector and miR-574-3p binding mutants were constructed to validate the inhibited regulation of miR-574-3p to the expression of Rb.

RESULTS: In the current study, compared with adjacent non-cancerous normal tissues, the expressions of circRNA_100782 and Rb were both downregulated in human gastric cancer cells. Through qRT-PCR and CCK-8 assay, we found that the expression of circRNA_100782 is related to the proliferation of gastric cancer cells. Besides, we also found that circRNA_100782 regulated the migration ability of gastric cancer cells through transwell assay. The bioinformatics prediction and luciferase assay demonstrated that circRNA_100782 can serve as a molecular sponge to further regulate the expression of Rb by sponging with miR-574-3p; moreover, circRNA_100782 can serve as a ceRNA for miR-574-3p to further regulate the expression of Rb.

CONCLUSIONS: In this research, we discovered that circRNA_100782 was downregulated in gastric cancer cells and is associated with cell proliferation and invasion by inhibiting tumor suppressor gene Rb by interacting with miR-574-3p.

Key Words:
CircRNA_100782, Gastric cancer, MiR-574-3p, Proliferation, Metastasis.

Introduction

Gastric cancer is a malignant tumor that originates in the gastric mucosa. Under the global cancer statistics released in 2018, gastric cancer (GC) is the fifth most commonly diagnosed cancer type and the third leading cause of cancer-related mortality worldwide, ranking first among various malignant tumors in China[1,2]. Although the survival rate of GC patients is increasing from popularization of gastroscopy, the prognosis of patients with advanced GC remains poor due to tumor metastasis and recurrence[3]. The identification of markers associated with tumorigenesis and cancer progression may enable the early detection of GC and finding potential targets for the treatment of gastric diseases[4].

The past decades an ever-growing list of diverse non-coding RNA species with functional capacity expressed has been observed in eukaryotic cells[5,6]. With the advent of next-generation sequencing, the catalog has grown more rapidly. Circular RNA (or circRNA) is a type of single-stranded RNA which is not the same as well-known linear RNA, forms a covalently closed continuous loop, i.e., in circular RNA the 3' and 5' ends normally present in an RNA molecule have been joined together. Moreover, circRNA has better stability and conservation compared with linear RNA. CircRNA molecules are rich in microRNA (miRNA) binding sites[7-12], and act as miRNA sponges in cells, thereby lifting the

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inhibitory effect of miRNA on its target genes to increase its target genes expression level. This mechanism of action is called the competitive endogenous RNA (ceRNA) mechanism[13-16]. There are many studies[17,18] showing that circRNAs participate in various pathological processes by interacting with miRNAs associated with diseases, such as proliferation, invasion, and metastasis of various malignancies including gastric cancer, breast cancer and pancreatic cancer.

In this study, we mainly explored the functional roles of circRNA_100782 in gastric cancer cells, as well as disclosed the molecular mechanisms. We detected the circRNA_100782 levels in colorectal cancer tissues and SGC-7901 cell line. Proliferation, migration, and invasion abilities of SGC-7901 cells were measured after transfection of lentiviral circRNA_100782. Finally, we assessed the regulatory relationship between circRNA_100782 and miR-574-3p, miR-574-3p and Rb respectively, and found that circRNA_100782 could bind with miR-574-3p to further regulate the expression of Rb. Our study uncovered circRNA_100782 serves as a molecular sponge by modulating the inhibitory effect of miR-574-3p on Rb gene. We found that circRNA_100782 plays a key role in the progression of gastric cancer by regulating the inhibitory effect of miR-574-3p on Rb as ceRNA.

Patients and Methods

Patients and Gastric Cancer Samples

In this work, 50 pairs of gastric tissue samples and adjacent normal ones were collected from surgically treated gastric cancer cases, and then, stored at -80°C. Patient information was included in Table I. No significant differences in the 50 pairs of samples in terms of diagnostic indicators and prognostic factors. This study was approved by the Ethics Committee of Nanchang University. Patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

Table I. Demographic data.

	Age < 45 years	Age > 45 years
Patients numbers	24	26
Sex ratio (M/F)	14/10	13/13
BMI (kg/m^2) ± SD	20.9 ± 5.3	22.2 ± 4.9

All the patients were selected randomly.

Cell Culture

The human gastric cancer cell line (SGC-7901) and normal gastric mucosal cell line, GES-1, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). The cells were cultured in DMEM medium containing 10% FBS at 37°C incubator with 5% CO_2.

Construction of Lentivirus and Cell Transfection

Lentiviral circRNA_100782 and circRNA_100782 shRNA were synthesized and constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For miR analysis, the miR-574-3p mimic, miR-574-3p inhibitor, and the negative control were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). To knockdown Rb, si-Rb plasma and negative control plasma were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For transfection, 1×10^4 cells were seeded in 6-well plates and cultured with RANKL (100 ng/mL) and M-CSF (100 ng/mL). Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I reduced serum medium were used for transfection. For analysis of circRNA_100782, the cells were transfected with circRNA_100782 shRNA (referred as to sh) and negative control shRNA (referred as to nc), respectively. For analysis of miR-574-3p, the cells were transfected with miR-574-3p inhibitor, and control cells were transfected with empty vector respectively. The cells without transfection were used as the control (referred as to control). After incubation for 30 min, the cultures were replaced with DMEM containing 10% FBS. Next, at indicated time point after transfection, cells were harvested for further study.

RNA Extraction and qRT-PCR

Taking out the culture plates, the cells were washed with PBS. After treatment, the total RNA of cells was extracted by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The samples were stored at room temperature for 30 min. The reverse transcription of cDNA was performed with a PrimeScript™ RT reagent

Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. For qRT-PCR, PCR primers were synthesized by GenePharma (ShangHai Gene Pharma, Shanghai, China) and sequences were listed in Table II. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression.

CCK-8 Assay

The CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) was used to measure the cells proliferation according to the manufacturers' instructions. In brief, 5×10^3 cells were seeded in 96-well plates uniformly. After being treated with regulated medium, the medium was removed, and the cells were washed with PBS solution for 3 times. Then, CCK-8 dilution was added to the 96-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 2 hours. After incubation, the plates were taken out, and cell proliferation was measured using multi-detection microplate reader. The absorbance (OD) value at 490 nm of each well was detected.

Luciferase Assay

After transfection for 48 h, the Luciferase activities were measured by using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Renilla Luciferase activities were normalized to the firefly Luciferase activities and the data were expressed as the fold change relative to the corresponding control groups which were defined as 1.0.

Statistical Analysis

Unless otherwise indicated, all data were processed by Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Each assay was applied at least three independent experiments or replicates. All data were presented as mean ± SD. Student's *t*-test, one-way analysis of variance (ANOVA) and multiple comparison between the groups were performed by using SNK method, in which $*p < 0.05$, $**p < 0.01$ represented as the difference significance.

Results

CircRNA_100782 and Rb Were Downregulated in Human Gastric Cancer Tissues and Cells

We first performed qRT-PCR to observe the expressions of circRNA_100782 and Rb in gastric cancer tissues and adjacent normal tissues to figure out the roles of circRNA_100782 and Rb in gastric cancer progression. The results showed that the expressions of both circRNA_100782 and Rb were significantly downregulated in gastric cancer tissues compared with adjacent normal tissues (Figure 1A and 1D). To further explain the biological function of circRNA_100782 in gastric cancer, we performed qRT-PCR to detect circRNA_100782 expression in human gastric cancer cell lines SGC-7901. The results showed that the expressions of both circRNA_100782 and Rb were significantly downregulated in gastric cancer cells compared with human epithelia cells GES-1 cells ($p < 0.05$) (Figure 1B and 1E). In addition, the Kaplan-Meier survival curves showed that low expression of both circRNA_100876 and Pb was conspicuously associated with poor prognosis of gastric cancer. The higher the expression level of both circRNA_100876 and Pb, the worse the prognosis ($p < 0.05$) (Figure 1C and 1F).

The Migration and Invasion of Gastric Cancer Cells Were Significantly Inhibited After Upregulated the CircRNA_100782 Expression

To explore the function of circRNA_100782 in the progression of gastric cancer, we constructed circRNA_100782 (Lnc-circRNA_100782) overexpressing lentivirus and transfected it into SGC-7901 cells. Besides, small interfering RNA (si-circRNA_100782) for circRNA_100782

Table II. Primer sequences for qRT-PCR.

Genes	Forward	Reverse	Tm (°C)
circRNA_100782	5'-ATGCTATGCTAGCTGATAGCTAG-3'	5'-ATCGTAGCTAGCTAGCATGCTCC-3'	60
miR-574-3p	5'-AGTCGATCGATGCTACACACC-3'	5'-CGCTAGCTGATGCATGCAGTCA-3'	61
Rb	5'-AGCTGACTGCATGCCCCCACAA-3'	5'-GCATGCATGACTCGACCCAGTCA-3'	61
GAPDH	5'-CGATCGTAGCTACGTGATCAC-3'	5'-GCTAGCTAGCTAGCTGATCGAT-3'	62
U6	5'-GACATAGCTAGCCTGCACCC-3'	5'-ACGGGATGAGGCGTACGCCAC-3'	62

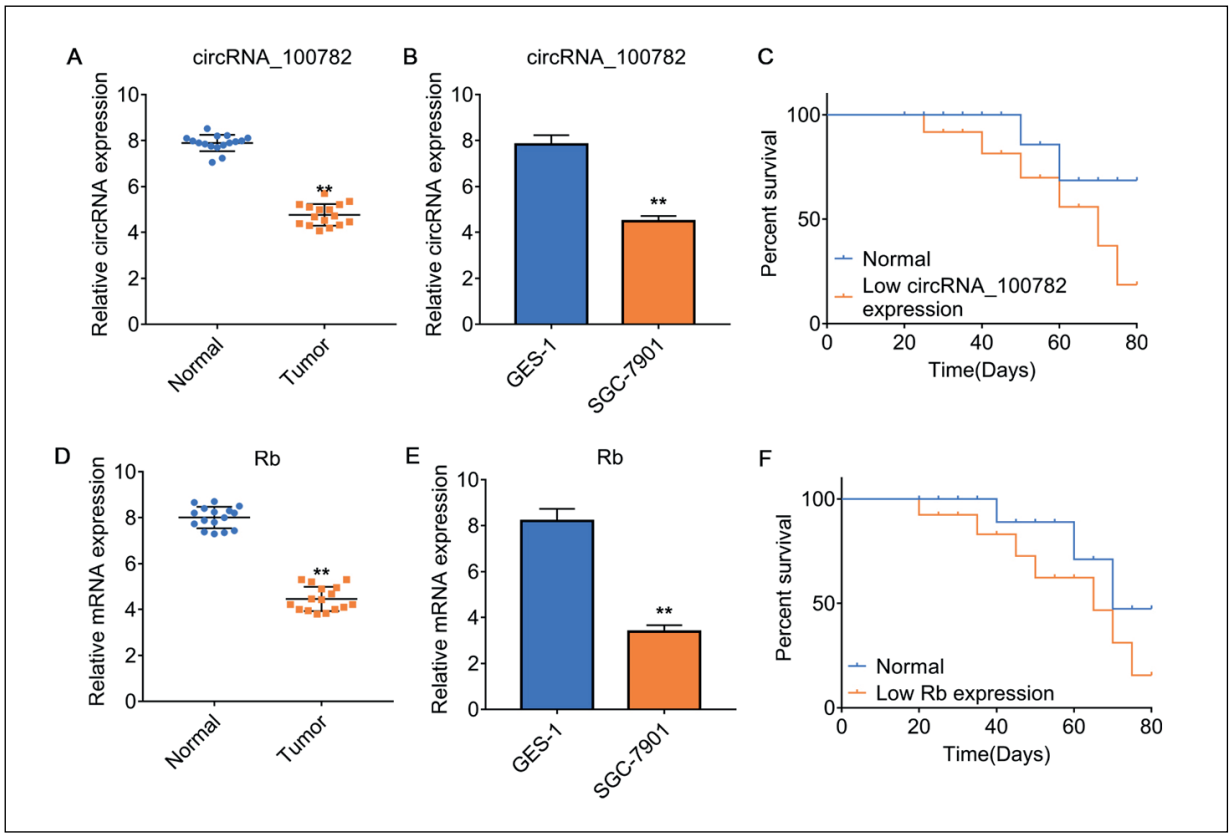

Figure 1. CircRNA_100782 and Rb were lowly expressed in human gastric cancer tissues and cells. **A,** Relative RNA expression levels of circRNA_100782 in human gastric cancer tissues and adjacent tissues. **B,** Relative mRNA expression levels of circRNA_100876 in human gastric cancer cell lines SGC-7901 and GES-1 cells. **C,** The Kaplan-Meier survival curve of gastric cancer patients based on circRNA_100782 expression showed that patients with low expression group had significantly worse prognosis than low expression group. **D,** Relative RNA expression of Rb in human gastric cancer tissues and adjacent tissues. **E,** Relative mRNA expression levels of Rb in human gastric cancer cell lines SGC-7901 and GES-1 cells. **F,** The Kaplan-Meier survival curve of gastric cancer patients based on Rb expression showed that patients with low expression group had significantly worse prognosis than low expression group. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

was synthesized and transfected into SGC-7901 cells. Subsequently, the expression of circRNA_100782 was detected by qRT-PCR. The results showed that the expression of circRNA_100782 was significantly increased in the Lnc-circRNA_100782 group compared with the vehicle (vector1) ($p < 0.05$), whereas the expression of circRNA_100782 was decreased in the si-circRNA_100782 group compared with the negative control vector2 ($p < 0.05$) (Figure 2A and 2B). To verify the role of circRNA_100782 in cell proliferation, CCK-8 analysis was performed on SGC-7901 cells after regulation of circRNA_100782 expression. The results showed that the overexpression of circRNA_100782 significantly reduced the proliferation of gastric cancer cells compared with the control group at 3 days, while inhibition of circRNA_100782 expression significantly increased the proliferation of gastric cancer cells (Figure 2C, 2D). The results suggest that changes in the expression of circRNA_100782 may affect the proliferation of gastric cancer cells. To further determine that circRNA_100782 affects migration and invasion of gastric cancer cells, we used transwell analysis to detect the ability of human gastric cancer cells to migrate after altering circRNA_100782 expression. Compared with the control group, when circRNA_100782 was upregulated, the response to fetal bovine serum and the migration of gastric cancer cells through the transwell chamber were significant-

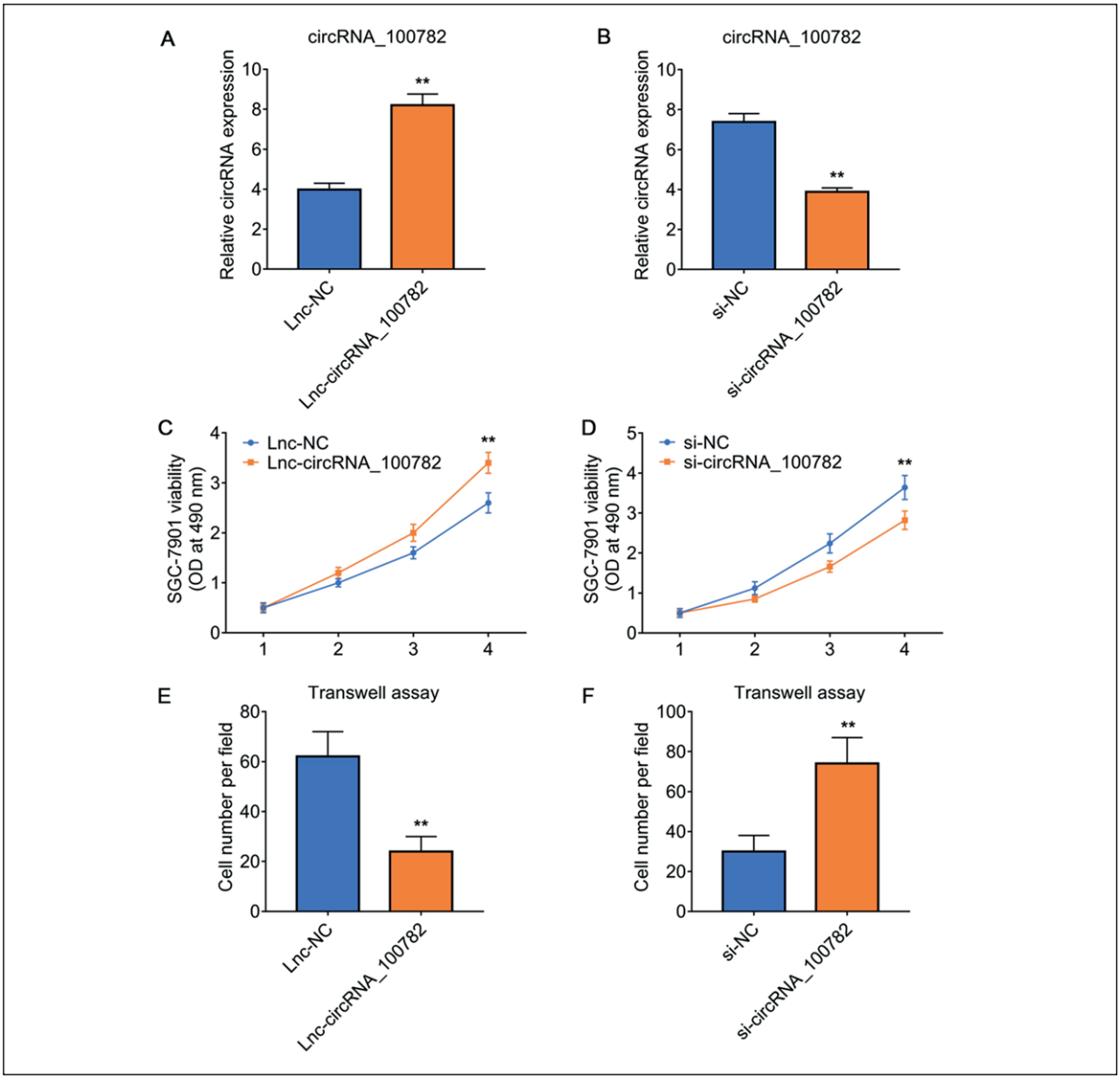

Figure 2. The migration and invasion of gastric cancer cells were significantly inhibited after upregulated the circRNA_100782 expression. **A,** Relative RNA expression levels of circRNA_100782 in SGC-7901 cells transfected with circRNA_100782 overexpressing lentiviral (Lnc-circRNA_100782) and Lnc-NC. **B,** Relative RNA expression levels of circRNA_100782 in SGC-7901 cells transfected with si-NC and si- circRNA_100782. **C,** Absorption at 490 nm of SGC-7901 cells treated with Lnc-circRNA_100782 and Lnc-NC detected by CCK-8 assay at 1 d, 2 d and 3 d. **D,** Absorption at 490 nm of SGC-7901 cells treated with si-circRNA_100782 and si-NC detected by CCK-8 assay at 1 d, 2 d, and 3 d. The data in the figures represent the averages ± SD. **E,** The number of migrated cells through transwell chambers was calculated after overexpression of circRNA_100782. **F,** The number of migrated cells through transwell chambers was calculated after knockdown of circRNA_100782. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

ly reduced (Figure 2E); when circRNA_100782 was downregulated, the response to fetal bovine serum, gastric cancer cells passed. The migration of transwell chambers has increased significantly (Figure 2F). In summary, the changes of circRNA_100782 regulate the migration and invasion of human gastric cancer cells. By upregulating circRNA_100782, the migration and invasion of gastric cancer cells can be effectively inhibited, and the migration and invasion of gastric cancer cells can be effectively promoted by downregulation.

MiR-574-3p Expression Was Upregulated in Gastric Cancer Cells and Negatively Correlated with CircRNA_100782

To investigate the relevance of circRNA_100782 to miRNA, we used StarBase 2.0 to predict the target miRNA of circRNA_100782 and found that miR-574-3p is one of the target miRNAs of circRNA_100782. We used qRT-PCR analysis to detect the miR-574-3p expressions of human gastric cancer tissues and SGC-7901 cells. The results showed that miR-574-3p was highly expressed in gastric cancer tissues compared to adjacent normal tissues, and was upregulated in SGC-7901 cells compared with GES-1 cells (Figure 3A and 3B); meanwhile, the results showed that the expression of U6 was no significantly different in SGC-7901 cells compared with GES-1 cells (Figure 3C). Then, we used a correlation analysis to further explore the relationship between circRNA_100782 and miR-574-3p. The results showed that miR-574-3p was significantly negatively correlated with circRNA_100782, suggesting that miR-574-3p may be regulated by circRNA_100782 (Figure 3D). From these results, we can see that miR-574-3p is highly expressed in gastric cancer tissues and SGC-7901 cell lines, and negatively correlated with circRNA_100782. It has been previously suggested that LncRNAs can act as a competing sponge in regulating the biological functions of miRNAs.

CircRNA_100782 can Sponge with MiR-574-3p and Inhibit its Expression in Gastric Cancer Cells

It has been previously suggested that LncRNAs can act as a competing sponge in regulating the biological functions of miRNAs. From the

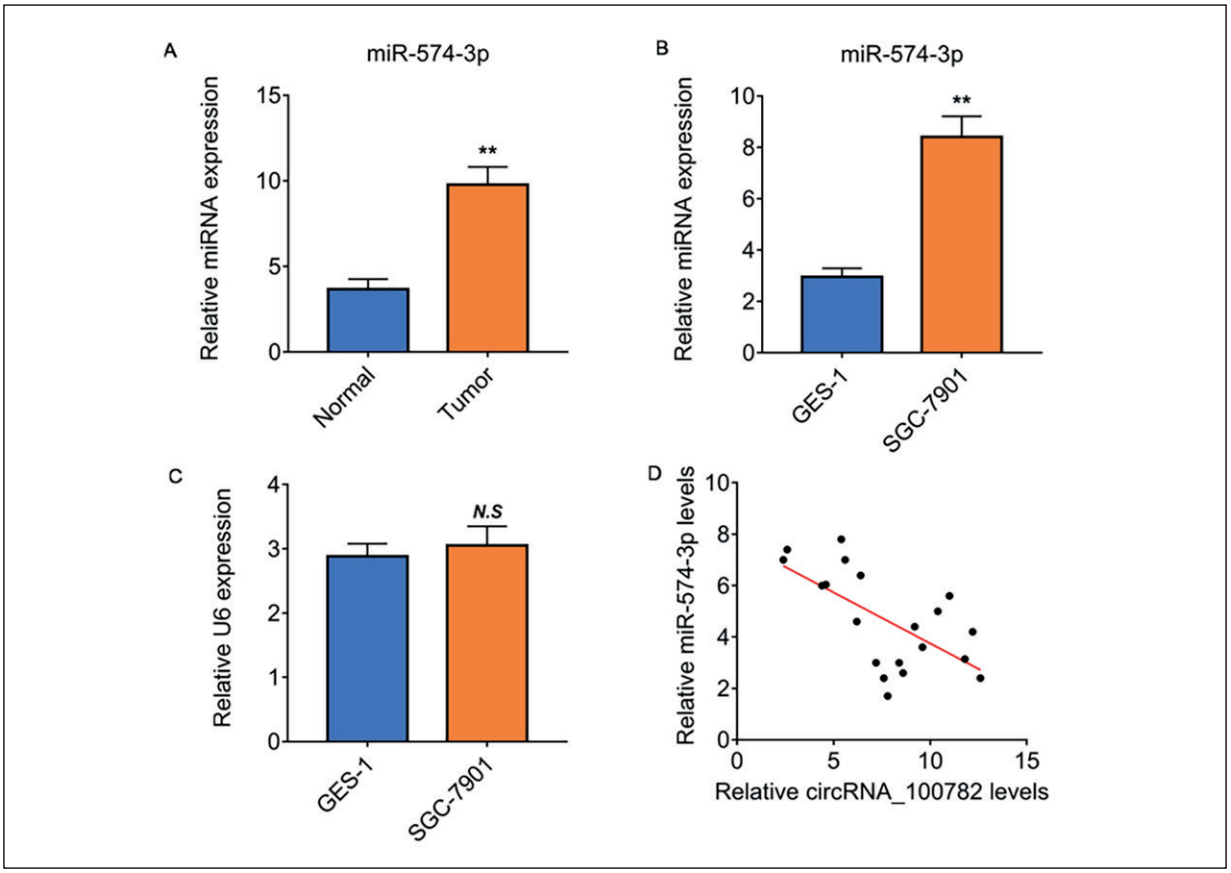

Figure 3. MiR-574-3p expression was upregulated in gastric cancer cells and negatively correlated with circRNA_100782. **A,** Relative miR-574-3p expression in gastric cancer tissues and adjacent normal tissues detected by qRT-PCR. **B,** Relative miR-574-3p expression in SGC-7901 cells and GES-1 cells detected by qRT-PCR. **C,** Relative U6 expression in SGC-7901 cells and GES-1 cells detected by qRT-PCR. **D,** Correlation analysis was performed to evaluate the relationship between miR-574-3p and circRNA_100782. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

previous conclusion, we know that miR-574-3p is negatively correlated with circRNA_100782. Therefore, we can assume that the interaction between circRNA_100782 and miR-574-3p is a way to regulate the migration and invasion of gastric cancer. To validate this hypothesis, we synthesized the circRNA_100782-wt Luciferase reporter vector and the circRNA_100782-mut 3'UTR Luciferase reporter vector and assayed for the Luciferase reporter gene (Figure 4A). Compared with the normal control, the Luciferase activity of SGC-7901 cells that co-transfected with wide type circRNA_100782 (circRNA_100782-wt) and miR-574-3p mimic was significantly decreased ($p < 0.01$), and it was reversely increased after mutation at the binding site of circRNA_100782 (circRNA_100782-mut) compared with circRNA_100782-wt ($p < 0.01$) (Figure 4B). The results indicate that circRNA_100782 can directly bind to miR-574-3p. In addition, when circRNA_100782 is overexpressed, the expression of miR-574-3p is inhibited, and when circRNA_100782 expression is inhibited, miR-574-3p expression is reversely promoted in SGC-7901 cells (Figure 4C, 4D). We also transfected miR-574-3p mimics and miR-574-3p inhibitors into SGC-7901 cells, which showed that miR-574-3p mimics inhibited circRNA_100782 expression, whereas miR-574-3p inhibitors increased circRNA_100782 expression (Figure 4E, 4F). All of these results indicate that miR-574-3p binds directly to circRNA_100782 at the recognition site.

CircRNA_100782 Served as CeRNA for MiR-574-3p to Further Modulate the Expression of Rb

Rb is a tumor suppressor gene whose mutation or deletion is closely related to the occurrence of various cancers. To investigate whether miR-574-3p interacts with Rb, we performed qRT-PCR analysis for Rb in the presence of miR-574-3p

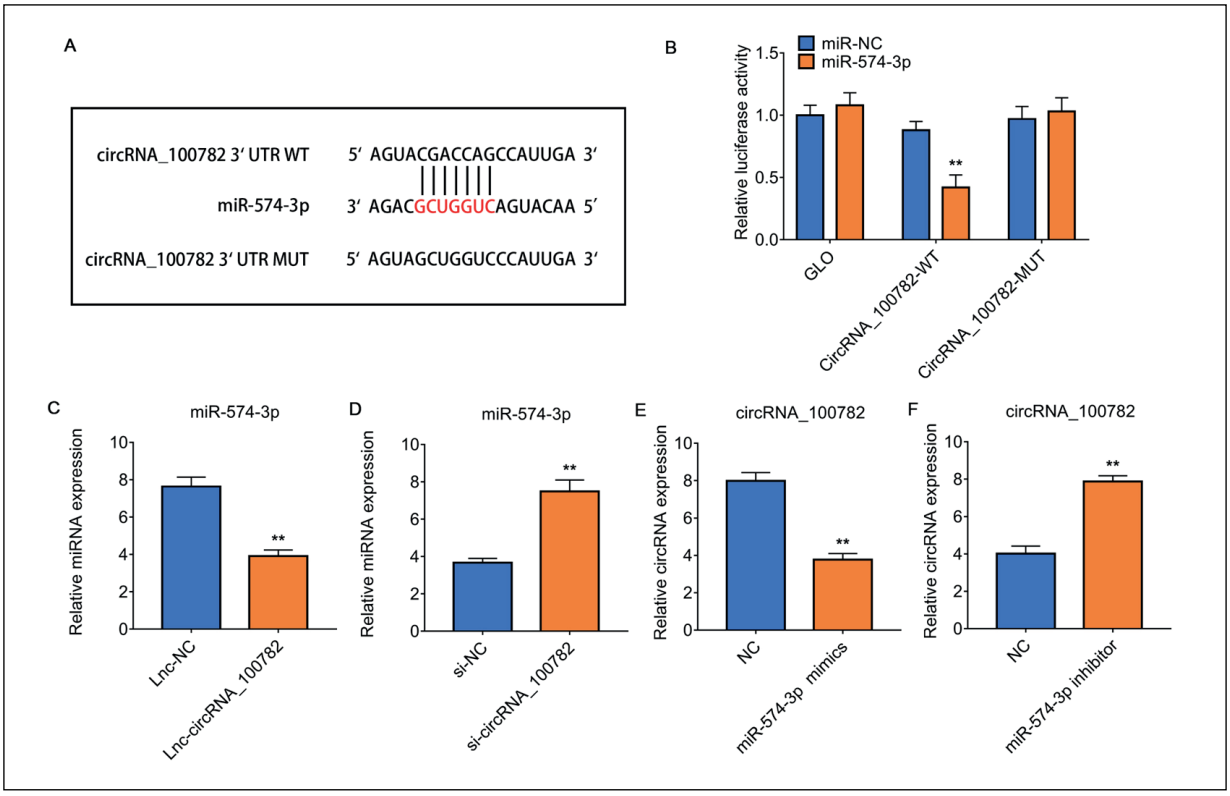

Figure 4. CircRNA_100782 can sponge with miR-574-3p and inhibit its expression in gastric cancer cells. **A,** Schematic illustration of the predicted miR-574-3p binding sites and mutant sites in circRNA_100782. **B,** Relative Luciferase activity of SGC-7901 cells. **C-D,** qRT-PCR analysis of miR-574-3p expression level in SGC-7901 cells transfected with lentiviral circRNA_100782 and si-circRNA_100782. **E-F,** Relative circRNA_100782 expression was detected in SGC-7901 cells after treated with miR-574-3p mimics and miR-574-3p inhibitor by RT-PCR. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

mimics or inhibitors. After treatment of SGC-7901 cells with the miR-574-3p mimics, we observed a decrease in Rb expression, indicating that miR-574-3p may interact with Rb (Figure 5A). To validate this mechanism, we cloned mice Rb 3'-UTR into a Luciferase reporter vector and constructed a miR-574-3p binding mutant in which the putative miR-21C binding site AUGC in Rb 3'-UTR. The mutation was UACG (Figure 5B). As expected, the Dual-Luciferase reporter results indicated that the miR-574-3p mimetic significantly downregulated Rb expression, while the point mutation in the Rb 3'-UTR abolished the inhibition of miR-574-3p (Figure 5C). Then, we further verified whether circRNA_100782 can regulate the expression of Rb by acting with the sponge of miR-574-3p. The results indicate that circRNA_100782 can significantly increase the expression of Rb, but the mutation of the binding site of miR-574-3p and circRNA_100782 is effectively eliminated (Figure 5D). On the contrary, inhibition of miR-574-3p overcomes the inhibitory effect of circRNA_100782 knockdown on Rb (Figure 5E). In summary, these data analysis showed that circRNA_100782 can be used as a ceRNA of miR-574-3p to further regulate Rb.

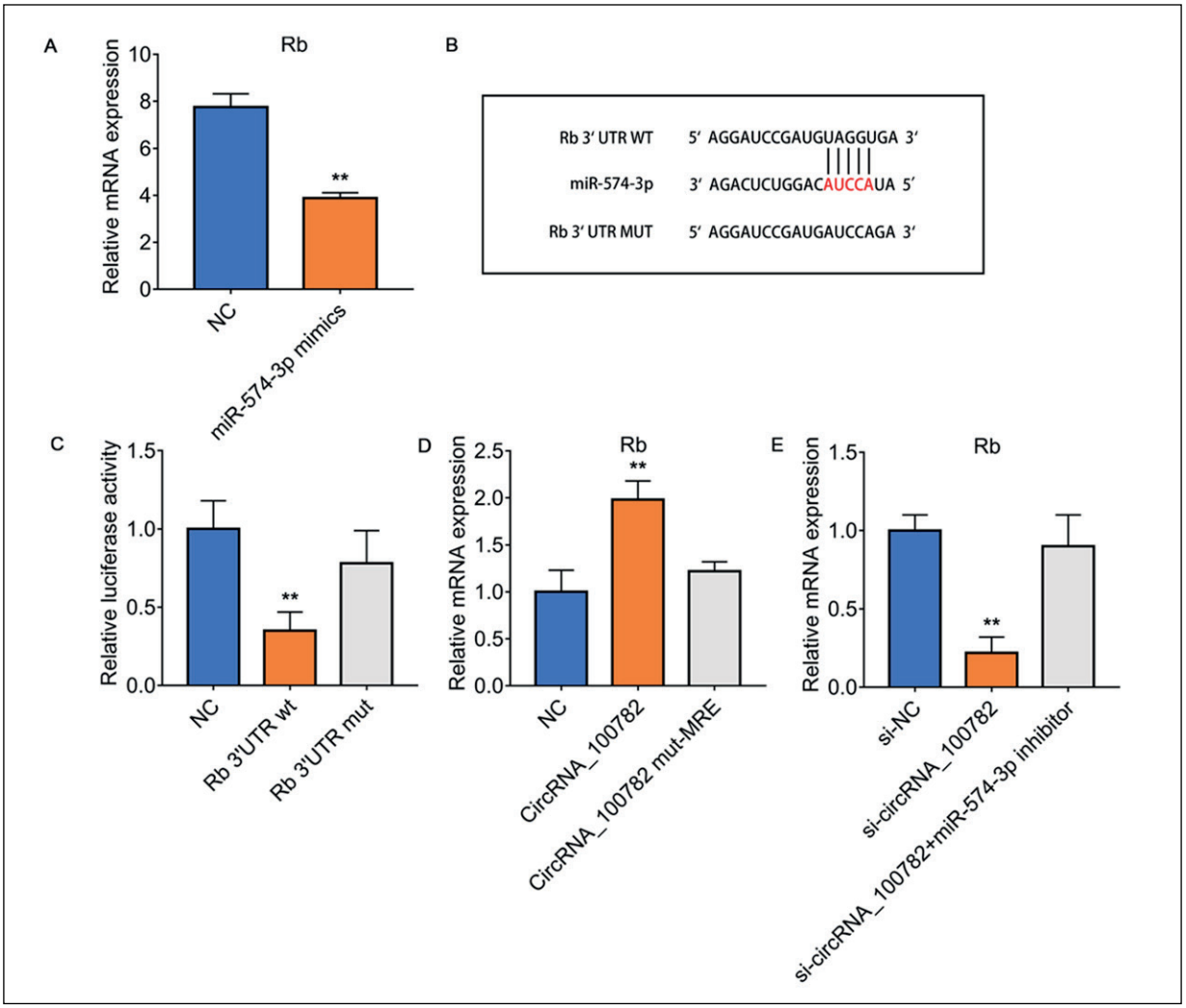

Figure 5. CircRNA_100782 served as a molecular sponge for miR-574-3p to further modulate the expression of Rb. **A,** qRT-PCR analysis of Rb mRNA expression level in SGC-7901 cells treated with the miR-574-3p mimics. **B,** Schematic illustration of the predicted Rb binding sites and mutant sites in miR-574-3p. **C,** Relative Luciferase activity of SGC-7901 cells. **D,** Relative mRNA expression levels of Rb in SGC-7901 cells transfected with circRNA_100782 and circRNA_100782 mut-MRE. **E,** Relative mRNA expression levels of Rb in SGC-7901 cells transfected with si-circRNA_100782, si-circRNA_100782 and miR-574-3p inhibitor by qRT-PCR analysis. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

Discussion

The number of gastric cancer patients increases yearly with the improvement of living standards and irregular diet, and it has become one of the common malignant tumors with high incidence[17,18]. Surgery combined with radiotherapy and chemotherapy are the most commonly used treatments, which can undoubtedly be able to conspicuously prolong the disease-free survival time of gastric cancer patients, but this advantage does not change gastric cancer as the most common cause of death in patients[19-21].

CircRNA is a closed circular RNA with stable expression, abundantly present in the eukaryotic transcriptome and it can also act as a miRNA sponge in different species, that is, competing endogenous RNA (ceRNA) can competitively bind to miRNA and regulate the expression of target genes[22-25]. MicroRNA (abbreviated as miRNA) is a ribonucleic acid (RNA) molecule with a length of about 21 to 23 nucleotides that is widely present in eukaryotes and can regulate other genes[26-28]. The expression of miRNA comes from some RNA (which is non-coding RNA) that is transcribed from DNA but cannot be further translated into protein[29]. MiRNAs bind to the target messenger ribonucleic acid (mRNA), thereby inhibiting post-transcriptional gene expression, and play an important role in regulating gene expression, cell cycle, and developmental timing of organisms[30-32]. In animals, one microRNA can usually regulate dozens of genes[33].

As a tumor suppressor protein, the retinoblastoma protein (protein name abbreviated pRb; gene name abbreviated RB or RB1) can prevent excessive cell growth by inhibiting cell cycle progression until a cell is ready to divide, which first reported on its role in the development of ocular malignancies and is dysfunctional in several major cancers[21,34,35].

In this study, we aimed to explore the functional roles of circRNA_100782 in gastric cancer, as well as to disclose the molecular mechanisms. Compared with gastric mucosal epithelial cells GES-1, the expression of circRNA_100782 in gastric cancer cells are lower; at the same time, the Kaplan-Meier survival curves showed that low expression of circRNA_100782 was conspicuously associated with poor prognosis of gastric cancer. Meanwhile, we found that the expression of Rb gene is similar to circRNA_100782. Based on the ceRNA hypothesis, we suggested that circRNA_100782 can act as an endogenous bait for miRNAs, thereby affecting the binding of miRNAs to their mRNA targets. The results indicate that circRNA_100782 binds directly to miR-574-3p and acts as a miR-574-3p sponge in colorectal cells (report by Dual-Luciferase). We can also draw the conclusions that miR-574-3p can regulate the expression of tumor suppressor gene Rb in this experiment.

Conclusions

We discovered that circRNA_100782 was downregulated in gastric cancer cells and is associated with cell proliferation and invasion by inhibiting tumor suppressor gene Rb by interacting with miR-574-3p.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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