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LncRNA DCST1-AS1 regulated cell proliferation, migration, invasion and apoptosis in gastric cancer by targeting miR-605-3p

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Abstract. – OBJECTIVE: Gastric cancer (GC) is one of the most common cancers in the world, with a high incidence and a poor prognosis. A large number of lncRNAs have been demonstrated to play multiple important roles in cancer development and progression. LncRNA is usually used as ceRNA and forms a regulatory network with miRNA in gastric cancer. However, the function and regulatory network of lncRNA in gastric cancer have not been fully elucidated.

MATERIALS AND METHODS: The qRT-PCR assay was used to detect DCST1-AS1 and miR-605-3p expression. Western blot was applied to measure the protein expression of CDK4, cyclin D1, MMP-2, MMP-9, cleaved caspase 3, Bcl-2, Bax and β-actin. MTT assay and flow cytometry were performed to assess cell proliferation and apoptosis, respectively. Transwell migration and invasion assay were used to determine cell migration capacity and invasion ability. Luciferase reporter assay was applied to determine the relationship of DCST-AS1 and miR-605-3p in GC.

RESULTS: In this study, we found that DCST1-AS1 was highly expressed while miR-605-3p was low expressed in GC tissues and cells. Moreover, DCST1-AS1 expression negatively regulated miR-605-3p expression in GC. Functionally test demonstrated that knockdown of DCST1 inhibited cell proliferation, migration and invasion as well as promoted cell apoptosis in GC cells. Interestingly, miR-605-3p has been verified to be a target miRNA of DCST1-AS1 with luciferase reporter assay. More than that, the reverse experiment determined that the inhibition of miR-605-3p could alleviate the suppressive effects of low DCST1-AS1 expression on cell growth in GC.

CONCLUSIONS: We proved the regulatory network of lncRNA DCST1-AS1 for the first time, and also explored and found that lncRNA DCST1-AS1 regulated cell proliferation, migration, invasion and apoptosis by regulation of miR-605-3p, providing a new therapeutic target for gastric cancer treatment.

Key Words:
GC, LncRNA DCST1-AS1, MiR-605-3p, Cell growth.

Abbreviations

GC = gastric cancer; si-DCST1-AS1 = siRNA targeting DCST1-AS1; RIPA = radioimmunoprecipitation; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF = polyvinylidene fluoride; HRP = horseradish peroxidase; DMSO = dimethyl sulfoxide; SD = standard deviation.

Introduction

Gastric cancer (GC) is one of the most common cancers with high mortality and high morbidity. Cell proliferation, metastasis, invasion and apoptosis are essential processes for cancer cell progression. Numerous studies have shown that cancer cells have strong proliferation, invasion, migration and anti-apoptosis ability. Therefore, research on them could contribute to understand the mechanism of cancer occurrence and development. At present, the regulation mechanism of gastric cancer cell growth and apoptosis has been studied, but the role of lncRNA as an important regulatory factor in gastric cancer has not been fully elucidated.

Recently, a large number of studies have shown that lncRNA is involved in the metabolism of a variety of cancers, including gastric cancer, non-small-cell lung cancer, prostate cancer and epithelial ovarian cancer[1-4]. Zhu et al[5] reported that lncRNA H19 repressed cell metastasis through targeting TGFB1 by sponging miR-675 in prostate cancer. More than that, the function of these

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lncRNAs is closely related to tumor formation, immune response, inflammatory response and cell growth[6-9]. In GC, the regulatory functions of many lncRNAs are involved in the proliferation, invasion, metastasis and apoptosis of GC cells[10-13]. In this way, the promotion of lncRNA BANCR was related to clinical progression and poor prognosis in GC[14,15]. In addition, lncRNAs can be used as biomarkers for the diagnosis of gastric cancer, including lncRNA MALAT1, Sox2ot and ABHD11-AS1[16-19]. Therefore, lncRNAs play an important role in the treatment and diagnosis of gastric cancer.

LncRNA DCST1-AS1 was an important regulator and was associated with prognosis prediction in hepatocellular carcinoma[20]. Here, we found that DCST1-AS1 expression was significantly increased in GC tissues and cells. However, the regulatory mechanism of DCST1-AS1 has not been explored in GC.

Materials and Methods

Patients and Tissues, Cell Culture and Transfection

Sixty GC tissues and adjacent non-tumor tissues were collected from patients at China-Japan Union Hospital of Jilin University. Informed consent was obtained from all patients in this experiment. All the protocols were approved by the Ethics Committee of China-Japan Union Hospital of Jilin University.

All GC cell lines (AGS, SGC-7901, BGC-823, HGC-27, MKN-45) and normal cell line (GES-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI 1640; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

siRNA targeting DCST1-AS1 (si-DCST1-AS1), pcDNA-DCST1-AS1, miR-605-3p inhibitor (anti-miR-605-3p) and their negative control (si-con, pcDNA and anti-miR-con) were purchased from GenePharma (GenePharma Co. Ltd, Shanghai, China), and transfected into the HGC-27 and MNK-45 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

qRT-PCR

Total RNA was extracted from GC tissues and cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations were detected using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For miR-605-3p, TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was applied to detect miR-605-3p expression according to the manufacturer's instructions. For DCST1-AS1, TaqMan® Reverse Transcription Reagents kit (Applied Biosystems) was used to detect according to the manufacturer's instructions. β-actin was employed as a normalizer. Then the qRT-PCR reactions were performed using Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Western Blot

Western blot was used to detect the protein expression of CDK4, cyclin D1, MMP-2, MMP-9, cleaved caspase 3, Bcl-2, Bax and β-actin in GC cells. Total proteins were extracted from cells with radioimmunoprecipitation (RIPA) buffer. The protein concentration was quantified by a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific). Then, the proteins were added into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After the membrane was blocked in 5% nonfat dried milk, it was incubated with primary antibodies CDK4 (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), cyclin D1 (1:1000; Santa Cruz Biotechnology), MMP-2 (1:1000; Santa Cruz Biotechnology), MMP-9 (1:1000; Santa Cruz Biotechnology), cleaved caspase 3 (1:1500; Santa Cruz Biotechnology), Bcl-2 (1:1000; Santa Cruz Biotechnology), Bax (1:1000; Santa Cruz Biotechnology) and β-actin (1:2000; Santa Cruz Biotechnology) at 4°C overnight. After washed in TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody anti-mouse IgG (1:1000 dilution; Abcam, Cambridge, MA, USA) for 1 h at 37°C. The blots were detected using the GelCapture version software (DNR Bio-Imaging systems, Jerusalem, Israel).

Cell Proliferation

MTT assay was used to detect cell proliferation ability. All transfected cells were seeded into 96-well plates with density of 2×10^3 cells/well and cultured in RPMI-1640 (Invitrogen,

Carlsbad, CA, USA) for 24, 48 and 72 h. Then, 20 µl MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and then incubated for 4 h at 37°C. Then, 150 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was also added into each well to dissolve formazan. The absorbance was measured at a wavelength of 490 nm using the SpectraMax 360 pc microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell Migration and Cell Invasion

The transwell migration and invasion assay were performed with transwell (Corning, Corning, NY, USA). For cell invasion, Matrigel (BD Biosciences, Franklin Lakes, NY, USA) was used according to the manufacturer's instructions. Cell migration was tested without Matrigel. Cells (1×10^6) were seeded into the upper chamber. RPMI 1640 medium was added into lower chamber. After incubation at 37°C for 24 h, the number of migrated and invasion cells in the lower chamber was calculated using Leica DM3000 microscope (Leica, Wetzlar, Germany).

Cell Apoptosis

Cell apoptosis was analyzed using a FACScan flow cytometer (BD Biosciences). The Annexin V-fluorescein isothiocyanate (FITC)/propidum iodide (PI) Apoptosis Detection Kit (Beyotime, Shanghai, China) was used to test according to the manufacturer's instructions. Briefly, cells (1×10^6) were harvested and stained with PI at 37°C for 15 min, then incubated with Annexin V at 37°C for 30 min.

Luciferase Reporter Assay

To determine the relationship between DCST1-AS1 and miR-605-3p, the fragment of DCST1-AS1-WT or DCST1-AS1-MUT was inserted into the pGL4.23 vector (Promega, Madison, WI, USA). Then these vectors were cotransfected with miR-NC or miR-605-3p into the HGC-27 and MNK-45 cells using Lipofectamine 2000 reagent (Invitrogen). And the Dual-Luciferase® Reporter Assay System (Promega) was used to analyze the luciferase activities.

Statistical Analysis

Data were shown as mean± standard deviation (SD). All data were analyzed and performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Student's *t*-test and ANOVA followed by Tukey's test were used to analyze the data of all groups. Pearson's correlation analysis was applied to determine the correlations between miR-605-3p and DCST1-AS1 in GC tissues. A p-value < 0.05 was considered statistically significant.

Results

DCST1-AS1 Was Highly Expressed While miR-605-3p Was Lowly Expressed in Tissues and Cells of GC

In GC tissues and cells, we found that DCST1-AS1 and miR-605-5p expression was obviously changed. Compared with adjacent tissues or normal cell lines (GES-1), the expression of DCST1-AS1 was improved in GC tissues or cells (Figure 1A and 1D), while the expression of miR-605-3p was inhibited in GC tissues and cells (Figure 1B and 1E). Moreover, Pearson's correlation analysis determined that DCST1-AS1 expression was negatively related to miR-605-3p expression in GC tissues (Figure 1C). Thus, the relationship between DCST1-AS1 and miR-605-3p play an important role in GC.

Knockdown of DCST1-AS1 Inhibited Cell Proliferation in GC

To further explore the function of DCST1-AS1, we performed a loss-of-function experiment in GC cells. Knocked down DCST1-AS1 (si-DCST1-AS1) was stably transfected into HGC-27 and MNK-45 cells (Figure 2A and 2B). As shown in Figure 2C and 2D, inhibition of DCST1-AS1 significantly decreased cell proliferation in HGC-27 and MNK-45 cells with MTT assay. Furthermore, si-DCST1-AS1 transfected remarkably reduced CDK4 and Cyclin D1 protein expression in HGC-27 and MNK-45 cells with Western blot (Figure 2E to 2G). Therefore, knockdown of DCST1-AS1 suppressed cell proliferation in GC.

Knockdown of DCST1-AS1 Inhibited Cell Migration and Invasion in GC

Next, transwell assay was used to explore cell migration and invasion in GC cells. Compared with NC and si-con groups, cell migration and invasion were sharply decreased in si-DCST-AS1 group in HGC-27 and MNK-45 cells (Figure 3A to 3D). Besides, the protein expression of MMP-2 and MMP-9 was significantly lower in si-DCST-AS1 group than that in NC and si-con groups (Figure 3E to 3G). Totally, silenced DCST-AS1 could inhibit GC cell migration and invasion.

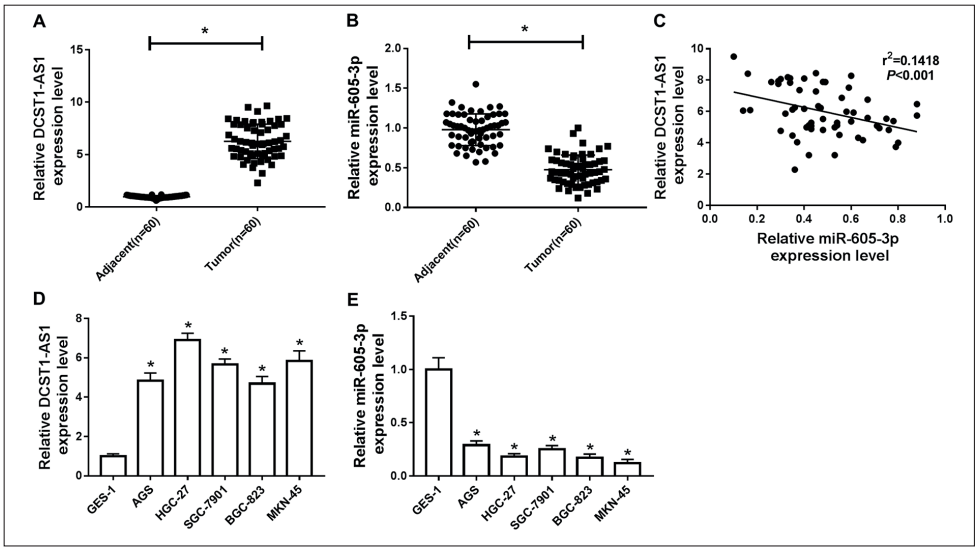

Figure 1. DCST1-AS1 was highly expressed while miR-605-3p was lowly expressed in tissues and cells of GC. **(A** and **B)** Compared with adjacent tissues, the expression of DCST1-AS1 **(A)** and miR-605-3p **(B)** was detected in tumor tissues with qRT-PCR. **(C)** Pearson's correlation analysis was used to analyze the correlation between DCST1-AS1 and miR-605-3p in tumor tissues. **(D** and **E)** Compared with normal cells (GES-1), the expression of DCST1-AS1 **(C)** and miR-605-3p **(D)** was detected in GC cells lines (AGS, HGC-27, SGC-7901, BGC-823 and MKN-45) with qRT-PCR. **p*<0.05.

Inhibition of DCST1-AS1 Promoted Cell Apoptosis in GC

Then, cell apoptosis was detected by flow cytometry and the results showed that inhibition of DCST1-AS1 contributed to cell apoptosis in HGC-27 and MNK-45 cells (Figure 4A to 4D). Meanwhile, the protein expression of Cleaved caspase-3 and Bax was remarkably increased

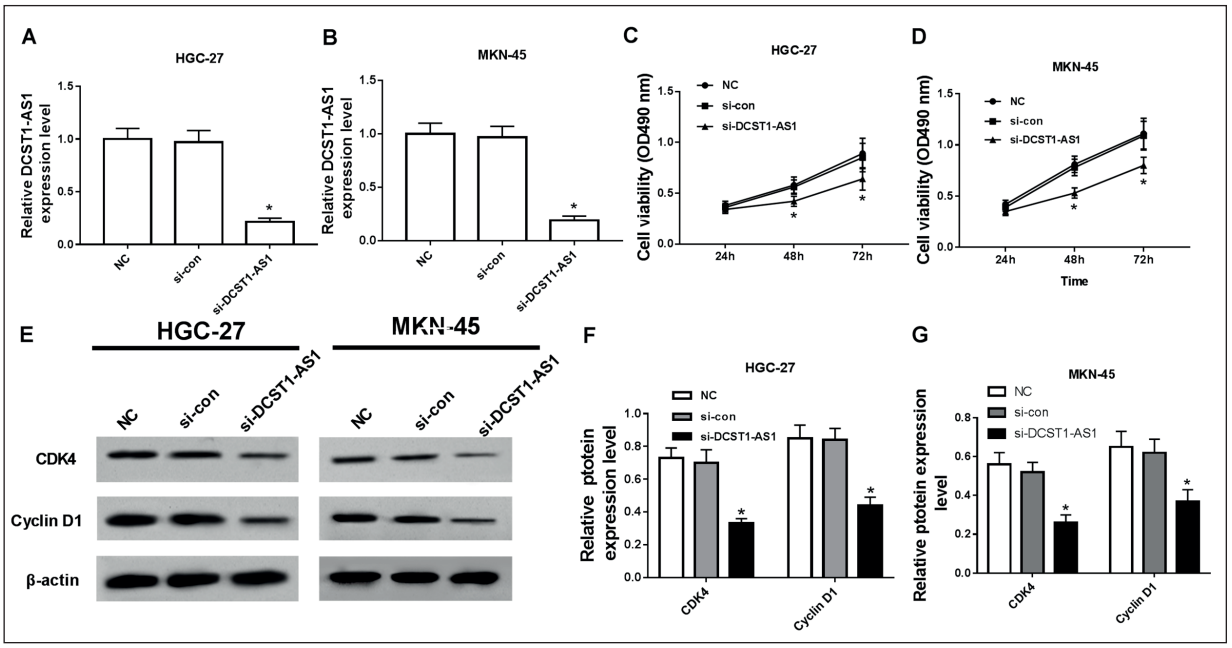

Figure 2. Knockdown of DCST1-AS1 inhibited cell proliferation in GC. **(A** and **B)** Compared with si-con and NC groups, the expression of DCST1-AS1 was detected in si-DCST1-AS1 group in HGC-27 **(A)** and MNK-45 **(B)** cell lines with qRT-PCR. **(C** and **D)** Compared with si-con and NC group, cell proliferation was measured in HGC-27 **(C)** and MNK-45 **(D)** cell lines with MTT assay. **(E** to **G)** Compared with si-con and NC groups, the protein expression of CDK4 and cyclin D1 was detected in si-DCST1-AS1 group in HGC-27 **(F)** and MNK-45 **(G)** cell lines with Western blot. E, the graph of Western blot. **p*<0.05.

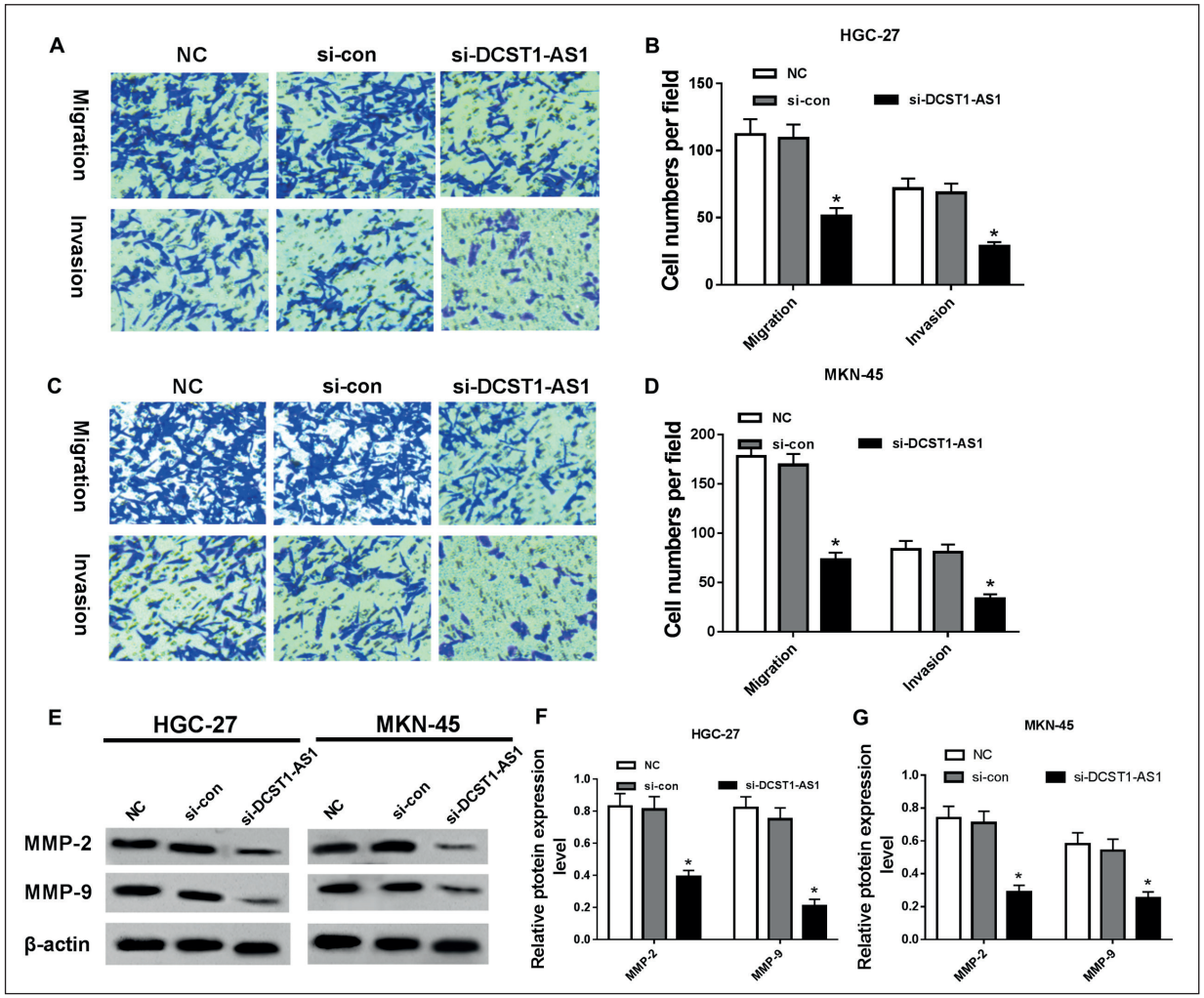

Figure 3. Knockdown of DCST1-AS1 inhibited cell migration and invasion in GC. **(A** and **B)** Compared with si-con and NC groups, cell migration and invasion were measured in si-DCST1-AS1 group in HGC-27 cell line with transwell. **(C** and **D)** Compared with si-con and NC group, cell migration and invasion were measured in MNK-45 cell line with transwell. **(E** to **G)** Compared with si-con and NC groups, the protein expression of MMP-2 and MMp-9 was detected in si-DCST1-AS1 group in HGC-27(F) and MNK-45 **(G)** cell lines with Western blot. **E,** the graph of Western blot. *$p<0.05$.

while Bcl-2 protein expression was suppressed by si-DCST-AS1 transfection in HGC-27 and MNK-45 cells (Figure 4E to 4F). Thus, knockdown of DCST1-AS1 dramatically induced cell apoptosis capacity in GC.

miR-605-3p was Identified as a Target miRNA of DCST1-AS1 in GC

In this study, we observed that miR-605-3p was a potential miRNA of DCST-AS1 using TargetScan (Figure 5A). Luciferase reporter assay was used to determine the relationship between DCST1-AS1 and miR-605-3p in HGC-27 and MNK-45 cells. As shown in Figure 5B and 5C, luciferase activities were significantly decreased when the DCST1-AS1-WT binds to miR-605-3p, but not DCST1-AS1-MUT in HGC-27 and MNK-45 cells. Moreover, the inhibition of DCST1-AS1 increased miR-605-3p expression while the induction of DCST1-AS1 decreased miR-605-3p expression in HGC-27 and MNK-45 cells (Figure 5D and 5E). Thus, these results proved that miR-605-3p was a target miRNA of DCST1-AS1 in GC cells.

Inhibition of miR-605-3p Reversed the Suppressive Effects of Low DCST1-AS1 on GC Cells

To further study the regulatory network of miR-605-3p and DCST1-AS1, reverse experiments were applied in GC. As shown in Figure

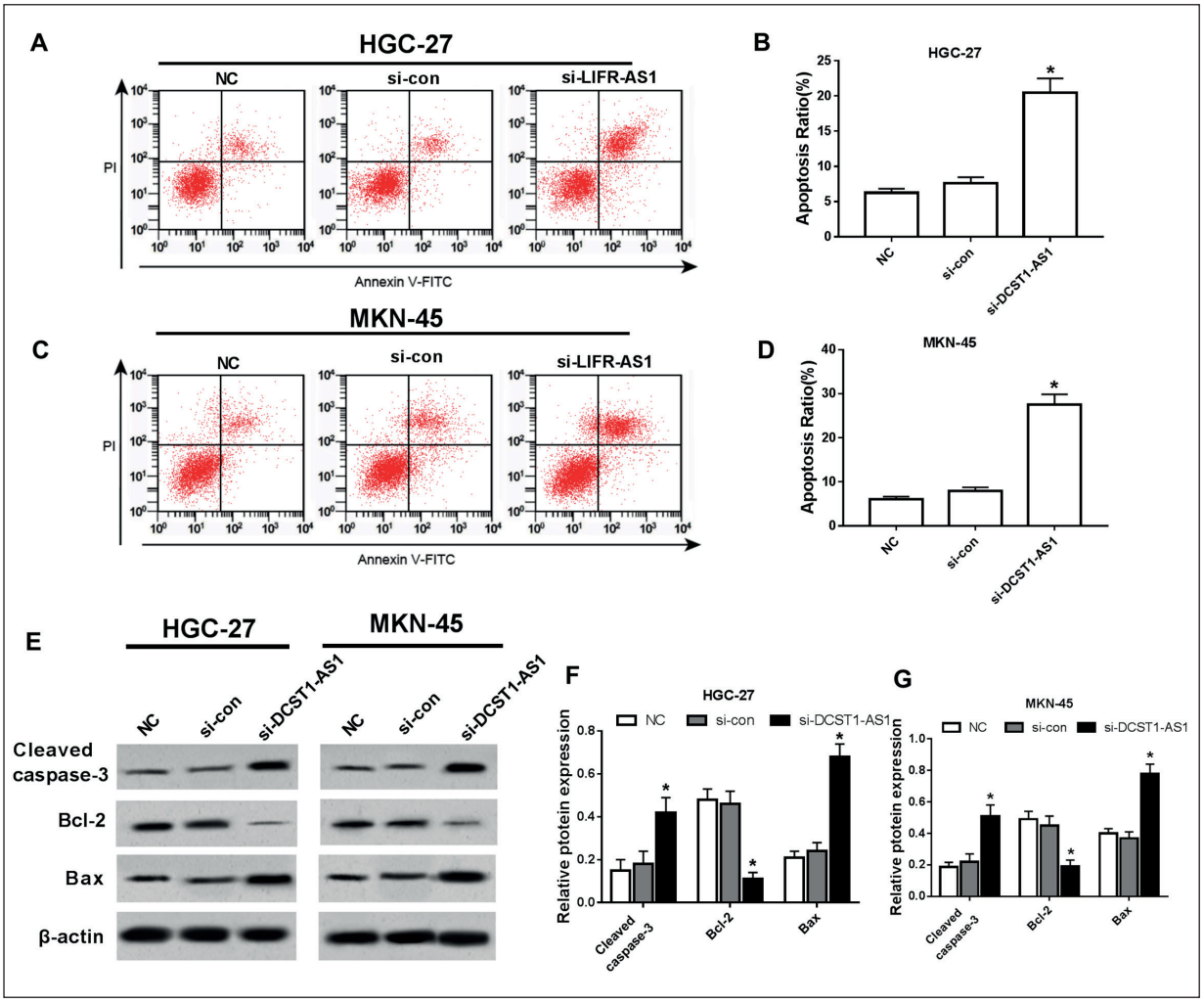

Figure 4. Inhibition of DCST1-AS1 suppressed cell apoptosis in GC. **(A** and **B)** Compared with si-con and NC groups, cell apoptosis was analyzed in si-DCST1-AS1 group in HGC-27 cell line with flow cytometry. **(C** and **D)** Compared with si-con and NC group, cell apoptosis was measured in MNK-45 cell line with flow cytometry. **(E** to **G)** Compared with si-con and NC groups, the protein expression of Cleaved caspase-3, Bcl-2 and Bax was detected in si-DCST1-AS1 group in HGC-27 **(F)** and MNK-45 **(G)** cell lines with Western blot. E, the graph of Western blot. *p<0.05.

6A to 6D, knockdown of DCST1-AS1 inhibited cell proliferation (Figure 6A and 6B), cell migration and invasion (Figure 6C and 6D) in HGC-27 and MNK-45 cells, which were weakened by downexpression of miR-605-3p. Moreover, the protein expression of CDK4, cyclin D1, MMP-2 and MMP-9 was also decreased by downregulating DCST1-AS1, which was increased by inhibition of miR-605-3p (Figure 6E to 6G). Additionally, si- DCST1-AS1 transfection dramatically promoted cell apoptosis, the protein expression of Cleaved caspase-3 and Bax and the inhibition of Bcl-2 protein, which was impaired by the reduction of miR-605-3p (Figure 6H to L). Therefore, these data suggested that downexpression of miR-605-3p alleviated the inhibition effects of si-DCST1-AS1 transfection on cell growth in GC, hinting DCST1-AS1 regulated cell growth through modulating miR-605-3p expression.

Discussion

In this study, we found and verified that miR-605-3p is the target miRNA of DCST1-AS1. We first studied the function and regulation of lncRNADCST1-AS1 in GC cells. The results showed that knockout of DCST1-AS1 significantly inhibited the proliferation, migration and

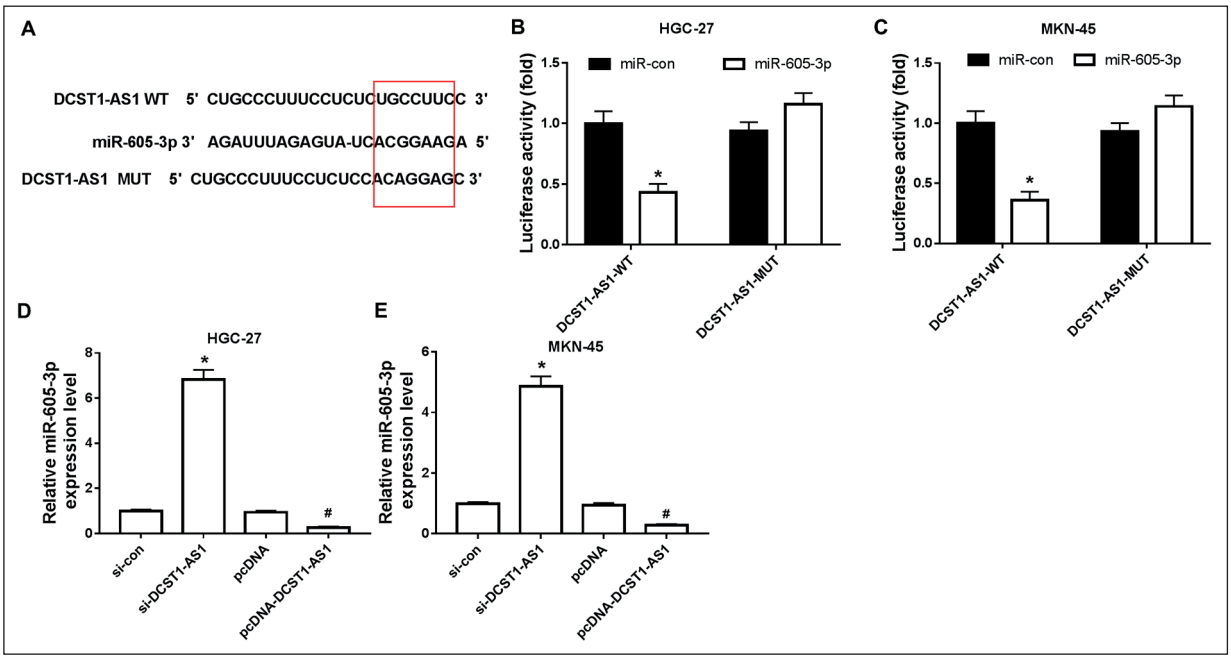

Figure 5. miR-605-3p was identified as a target miRNA of DCST1-AS1 in GC**. (A)** Predicted binding sites for miR-605-3p on the DCST1-AS1 3'UTR and displayed the mutate sites of DCST1-AS1 3'UTR binding sites. **(B** and **C)** luciferase activities were measured in HGC-27 **(B)** and MNK-45 **(C)** cells co-transfected with DCST1-AS1-WT or DCST1-AS1-MUT and miR-con or miR-605-3p. **(D** and **E)** compared with si-con or pcDNA, the expression of miR-605-3p was detected in si-DCST1-AS1 or pcDNA-DCST1-AS1 group in HGC-27 **(D)** and MNK-45 **(E)** cell lines. *p<0.05.

invasion of gastric cancer cells, and effectively promoted apoptosis in GC. The reverse experiments showed that low expression of miR-605-3p attenuated the inhibitory effect of knockout DCST1-AS1 on GC cells.

A variety of studies have shown that lncRNA has many functions in cancer, and it affects cell growth by binding to target miRNAs to regulate downstream mechanisms[21,22]. For example, lncRNA APF was associated with autophagy and myocardial infarction through the regulation of miR-188-3p[23]. He et al[24] determined that lncRNA H19 regulated cell proliferation and migration by modulating miR-141 in gastric cancer. In this paper, we found that the suppression of lncRNA DCST1-AS1 inhibited cell growth and was closely associated with GC occurrence.

More than that, miR-605-3p has been proved to be a target miRNA of lncRNA DCST1-AS1. Accumulating evidence suggested that miR-605-3p participated in cell progression in cancers, containing bladder cancer and prostate cancer[25,26]. In this study, the data determined that the inhibition of miR-605-3p could reverse the effects of low DCST1-AS1 expression on cell proliferation, invasion, migration and apoptosis in GC, suggesting DCST1-AS1 affected cell growth through modulating miR-605-3p in GC.

Here, we have only studied the regulatory network of lncRNADCST1-AS1 in cell growth, and its function in drug resistance and immune response has not been deeply observed. Therefore, we will further explore the more function of lncRNA in gastric cancer. In addition, lncRNA can target multiple miRNAs. MiR-675, miR-199a-5p and miR-22 have been verified to be a target miRNA of lncRNA H19[24,27-29]. Here, we only verified and explored the regulatory network between miR-605-3p. Next, we will further investigate and improve the regulatory network of lncRNA in gastric cancer, and supplement it with animal experiments.

Conclusions

These results proved the regulatory network of lncRNA DCST1-AS1 for the first time, and also explored and found that lncRNA DCST1-AS1 regulated cell proliferation, migration, invasion

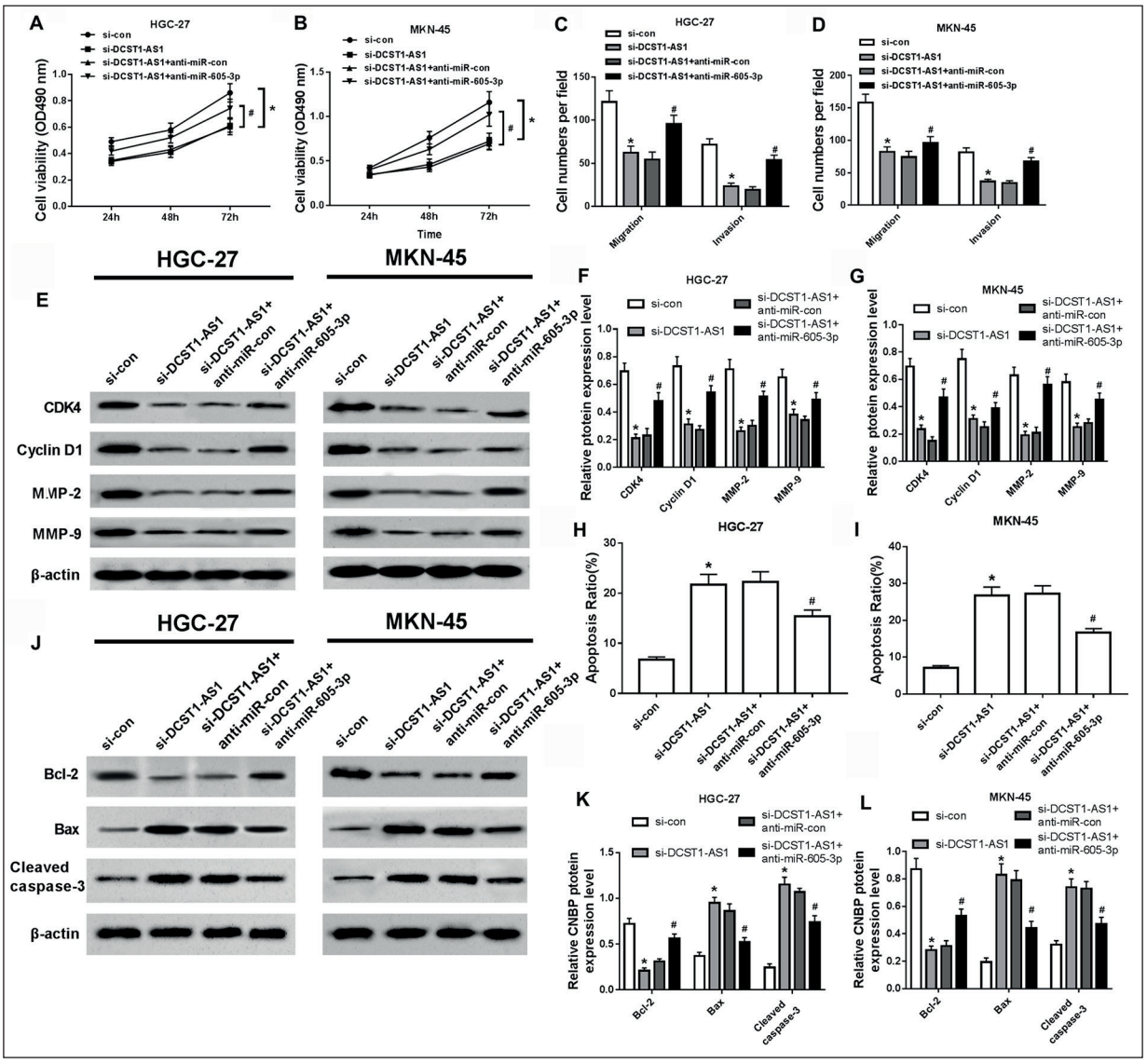

Figure 6. Inhibition of miR-605-3p reversed the suppressive effects of low DCST1-AS1 on GC cells. **(A** and **B)** MTT assay was used to measure cell proliferation in HGC-27 **(A)** and MNK-45 **(B)** cells transfected with si-con, si-DCST1-AS1, si-DCST1-AS1+anti-miR-con and si- DCST1-AS1+anti-miR-605-3p groups. **(C** and **D)** Transwell assay was used to detect cell migration and invasion in HGC-27 **(C)** and MNK-45 **(D)** cells transfected with si-con, si-DCST1-AS1, si-DCST1-AS1+anti-miR-con and si- DCST1-AS1+anti-miR-605-3p groups. **(E** to **G)** Western blot determined the protein expression of CDK4, cyclin D1, MMP-2 and MMP-9 in HGC-27 and MNK-45 cells transfected with si-con, si-DCST1-AS1, si-DCST1-AS1+anti-miR-con and si- DCST1-AS1+anti-miR-605-3p groups. **(H** and **I)** Flow cytometry was applied to measure cell apoptosis in HGC-27 **(H)** and MNK-45(I) cells transfected with si-con, si-DCST1-AS1, si-DCST1-AS1+anti-miR-con and si- DCST1-AS1+anti-miR-605-3p groups. **(J** to **L)** Western blot determined the protein expression of Cleaved caspase-3, Bcl-2 and Bax in HGC-27 and MNK-45 cells transfected with si-con, si-DCST1-AS1, si-DCST1-AS1+anti-miR-con and si- DCST1-AS1+anti-miR-605-3p groups. *p<0.05.

and apoptosis through the regulation of miR-605-3p, providing a new therapeutic target for gastric cancer treatment and improving our understanding of gastric cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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