Tumor suppressor role of miR-876-5p in gastric cancer

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Abstract. Gastric cancer (GC) is the second most common cancer cause of cancer-related mortality worldwide. Recent studies have demonstrated the function of microRNAs (miRNAs) in the pathogenesis of GC. miR-876-5p demonstrated an antitumor role in hepatocellular carcinoma and lung cancer; however, the function of miR-876-5p has not yet been fully identified in GC. Thus, the present study aimed to investigate the role of miR-876-5p in GC. The results of the present study demonstrated low expression levels of miR-876-5p in GC tumor tissues. Furthermore, overexpression of miR-876-5p inhibited GC cell proliferation and promoted apoptosis, whilst miR-876-5p knockdown promoted GC cell proliferation and decreased cisplatin sensitivity of GC cells. Transforming growth factor β-receptor 1 was demonstrated to be a potential target gene of miR-876-5p. Overall, the results of the present study suggest that miR-876-5p plays an antitumor role in GC.

Introduction

Gastric cancer (GC) is the second most common cause of cancer-associated mortality worldwide (1). In East Asia, based on GLOBOCAN 2018 data, the average incidence of gastric cancer is 32.1 per 100,000 among men and 13.2 among women (2). For the majority of cases, patients with GC are diagnosed at the advanced stage of disease and thus, successful therapeutic strategies are limited, and the prognosis is poor (3-5). Metastasis is a common trait of advanced GC progression (6); thus, in order to improve the survival rate of patients with GC, investigations that focus on the molecular mechanisms underlying pathogenesis of GC are required.

MicroRNAs (miRNAs) are small conserved non-coding RNAs, typically 20-25 nucleotides in length (7). miRNAs bind to complementary sequences that are frequently present in the 3'-untranslated region (UTR) of the target mRNA, thereby suppressing the translation of the target mRNAs (7). A number of studies have demonstrated that miRNAs play multiple roles in the pathogenesis of GC (8-14).

The function of miR-876-5p has been investigated in multiple types of cancer, including hepatocellular carcinoma (HCC) (15,16) and lung cancer (17). miR-876-5p has been demonstrated to inhibit cell proliferation of HCC and metastasis by targeting the DNMT3A gene (16), and also inhibit epithelial-to-mesenchymal transition (EMT) and metastasis by targeting the BCL6 corepressor-like 1 gene (15). With regard to lung cancer, miR-876-5p has been demonstrated to suppress EMT by targeting bone morphogenetic protein 4 (17). However, the role of miR-876-5p in GC is not yet fully understood. Thus, the present study investigated the functions of miR-876-5p in GC, with the aim of identifying potential, novel therapeutic targets for further development in the future.

Materials and methods

Patients and tissue samples. A total of 16 GC tissues and corresponding adjacent normal tissues were collected from 16 patients with GC between June 2015 and September 2016 who underwent surgery at the Department of Gastroenterology, West China Hospital (Chengdu, China). The age and sex distributions of the 16 patients with GC were listed as follows: Age, 46-72 years; mean age, 56; male/female, 11:5. The inclusion criteria were: i) Age ≥18 years; ii) the absence of radiotherapy, chemotherapy or any adjuvant therapy before hospitalization; iii) the absence of other malignant tumors; and iv) the absence of a family history of genetic diseases. Exclusion criteria were: i) The presence of antibiotics taking within three months before blood collection; ii) the presence of liver insufficiency and, iii) the presence of autoimmune system deficiency. The distance between the tumor tissues and adjacent non-tumor tissues was >5 cm. The histopathological diagnoses of all

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patients were confirmed by a senior pathologist at West China Hospital. The present study was approved by the Ethics Committee of Sichuan University (Chengdu, China) and all patients provided written informed consent.

Cell culture. The human GC cell line AGS-1 cell line and human gastric epithelial mucosa cell line GES-1 were purchased from the China Infrastructure of Cell Line Resources, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The two cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO_2. The cells were cultured until they reached 80% confluence. Cisplatin was purchased from Hansoh Pharma. A final concentration of 25 μM of cisplatin was added to the cultures as previously described (18).

miR-876-5p mimics and oligonucleotide transfection. miR-876-5p mimics, miR-876-5p antisense oligonucleotides (ASO) and negative controls (miR-NC mimics and miR-NC ASO) were constructed by Sangon Biotech Co., Ltd. The primer sequences were as follows: miR-876-5p mimics, sense 5'-UGGAUUUCUUUGUGAAUCACCA-3' and antisense 5'-GUGAUUCACAAAGAAAUCCAUU-3'; mimic control, sense 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sense 5'-ACGUGACACGUUCGGAGAATT-3'; miR-876-5p inhibitors 5'-UGGUGAUUCACAAAGAAAUCCA-3'; and inhibitor control 5'-CAGUACUUUUGUGUAGUACAA-3. A total of $2x10^5$ AGS-1 cells were seeded into 6-well plates overnight and transfected with 50 nM miR-876-5p mimics or miR-NC mimics using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Similarly, AGS-1 cells were seeded and transfected with miR-876-5p ASO (50 nM); miR-NC ASO (50 nM) was used as control. The cell growth and apoptosis assays were performed 24 h later.

Cell proliferation assay. Cell proliferation was assessed using an MTT assay. The AGS-1 cells were seeded into 96-well plates at a density of $5x10^5$ cells/plate. MTT was added to the medium at a final concentration of 0.1 mg/ml. The purple formazan crystals were dissolved using 100 μl of dimethyl sulfoxide and optical density was measured using a microplate reader (Multiskan Sky Thermo Fisher Scientific, Inc.) at a wavelength of 570 nm (19-21).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from GC tissues and AGS-1 cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The RNA was quantified by NanoDrop™ (ThermoFisher Scientific Inc.). Total RNA were reverse transcribed into cDNA using the All-in-One™ miRNA First-Strand cDNA Synthesis kit (Invitrogen, Thermo Fisher Scientific, Inc.). The PCR was performed by the SuperScript™ III Platinum™ SYBR™ Green One-Step qPCR kit (ThermoFisher Scientific Inc.). The primers were synthesized by Sangon Biotech Co., Ltd. The RNA sequences for the primers mentioned above were as follows: miR-876-5p sense 5'-AGGACUUCUCCCUCCUCCCAG-3' and anti-sense, 5'-UCCUCUUCUCCCUCCAGGGAG-3'; U6 snRNA sense

5'-CTCGCTTCGGCAGCACATATACT-3'; and anti-sense 5'-ACGCTTCACGAATTTGCGTGTC-3'. miR-876-5p expression was normalized to the internal reference gene U6 (18,22). PCR conditions were as follows: Denaturation for 10 min at 95°C, followed by 40 cycles of annealing for 1 min at 95°C and extension for 30 sec at 60°C. The dissolution procedure was designed as follows: 15 sec at 95°C, 30 sec at 60°C and 15 sec at 95°C. Quantification was performed using the $2^{-\Delta\Delta Cq}$ method (23).

Mutated site and target gene prediction. The mutated sites were generated by Gene Site-directed Mutagenesis System (Thermo Fisher Scientific, Inc.). The potential targets of miR-876-5p were predicted by TargetScan software (24-29).

Apoptosis analysis. The apoptotic ability of AGS-1 cells was assessed via flow cytometry using Annexin V-propidium iodide (PI) staining. AGS-1 cells were suspended in Annexin V-fluorescein isothiocyanate (FITC) (1 μg/ml) binding buffer (Abcam) at a density of $5x10^5$ cells/ml and incubated at room temperature for 20 min. Subsequently, PI (0.1 μg/ml) (Abcam) was added to the samples, incubated for 5 min at room temperature and analyzed using a BD FACSVerse™ flow cytometer (BD Biosciences) using the 488 nm excitation line (argon ion or solid-state laser), and emission was detected at 530 nm (green for Annexin V-FITC) and 575-610 nm (orange for PI). The data were analyzed using the BD FACSuite™ version 1.01 (BD Biosciences).

Western blot analysis. Total protein was extracted from AGS-1 cells using lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100 and 0.1% SDS) with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA). Proteins (10 mg) were separated via SDS-PAGE on a 10% gel and subsequently transferred onto a polyvinylidene difluoride membrane. The membranes were blocked in 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, prior to transforming growth factor β-receptor 1 (TGFBR1) analysis. The membranes were incubated with anti-TGFBR1 (1:1,000; cat. no. ab31013; Abcam) and β-actin (1:1,000; cat. no. ab8226; Abcam) overnight at 4°C. Membranes were washed three times with PBS and subsequently incubated with a horseradish peroxidase-conjugated rabbit IgG antibody (1:2,000; cat. no. ab218695; Abcam) for 2 h at room temperature. Protein bands were visualized using the ECL Western Blotting Detection reagents (GE Healthcare) and images were analyzed using ImageJ software (National Institutes of Health).

Luciferase reporter plasmid transfection. AGS-1 cells were seeded at $1x10^5$ per well and were serum-starved for 6 h pre-transfection. The 3' untranslated region (UTR) of TGFBR1 was cloned into the reporter plasmid (500 ng) and the pGL3-control (100 ng; Promega Corporation). miR-876-5p (30 nM) were transfected into AGS-1 cells containing the wild-type or mutant 3'-UTR plasmids using Lipofectamine® 2000 (Invitrogen, Thermo Fisher Scientific Inc.) for 5 min at room temperature. Mutants of the 3' UTR of TGFBR1 were generated using the Site-directed Mutagenesis kit (cat. no. A13282, Thermo Fisher Scientific Inc.). Cells were
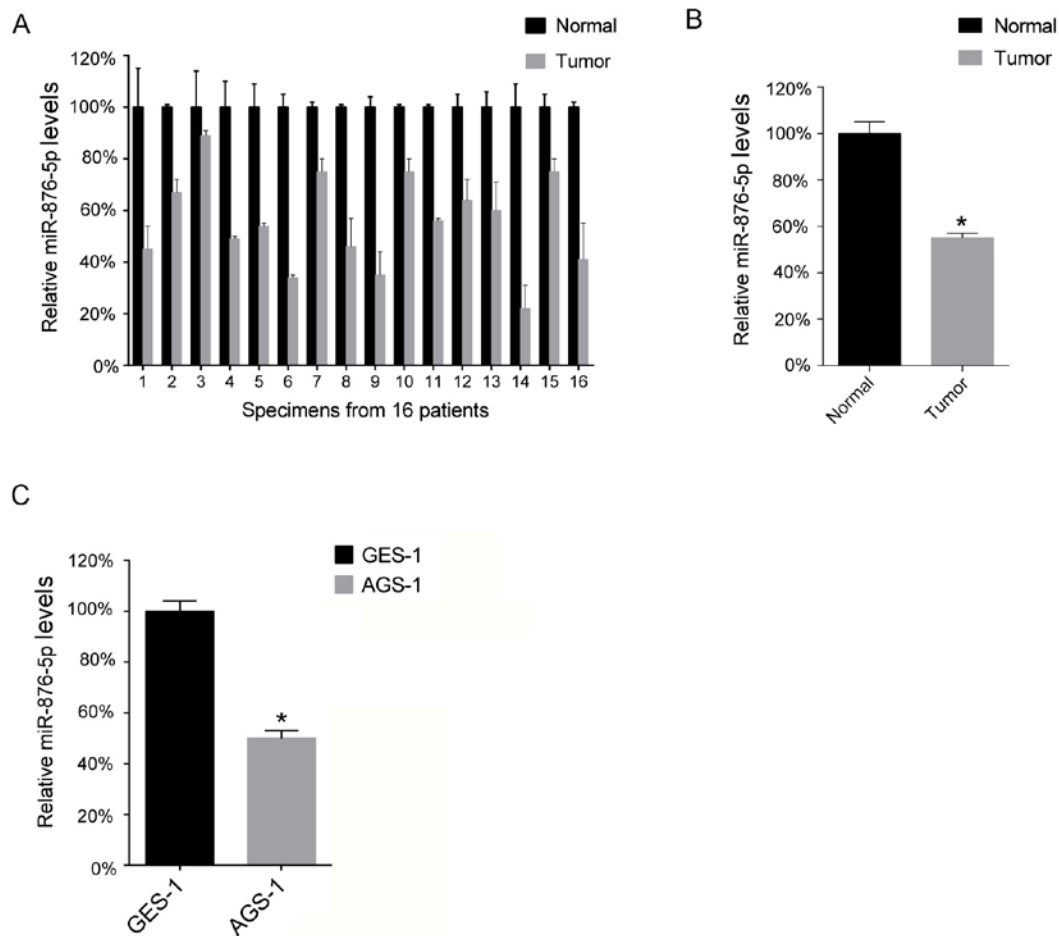
Figure 1. miR-876-5p is downregulated in GC tissue samples. (A) The miR-876-5p levels in 16 GC tissues and 16 corresponding matched normal adjacent tissues were analyzed via RT-qPCR. The miR-876-5p levels in the normal adjacent tissues were arbitrarily defined as 100%. (B) The mean values of miR-876-5p levels in the 16 GC tissues and 16 normal adjacent tissues were calculated and presented. (C) The miR-876-5p levels in AGS-1 cells were analyzed using RT-qPCR. *P<0.05; normal vs. tumor tissues and GES-1 vs. AGS-1 microRNA-876-5p. GC, gastric cancer; RT-qPCR, reverse transcription-quantitative PCR.

harvested 24 h later, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation). The firefly luciferase enzyme activity was normalized to the Renilla luciferase enzyme activity.

Statistical analysis. All data were analyzed using SPSS software version 16.0 (SPSS, Inc.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Two-tailed Student's t-test was used in order to analyze the mean values between two groups; one-way ANOVA was used in order to test the mean values among three groups or more, followed by Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-876-5p levels in GC tissues. The present study performed a RT-qPCR analysis in order to determine miR-876-5p levels in the 16 GC tissues and 16 corresponding normal adjacent tissues. The results of the present study demonstrated that miR-876-5p levels are downregulated in GC tissues compared with normal tissues (Fig. 1A), as the mean levels of miR-876-5p

in the 16 GC tissues was lower compared with that in the 16 normal adjacent tissues (Fig. 1B). Furthermore, the present study analyzed miR-876-5p levels in the GC cell lines and demonstrated that the AGS-1 cell line expressed lower levels of miR-876-5p compared with the GES-1 cell line (Fig. 1C).

Overexpression of miR-876-5p inhibits cellular proliferation and promotes apoptosis. The present study assessed the function of miR-876-5p *in vitro*. miR-876-5p levels in AGS-1 cells were overexpressed using miR-876-5p mimics. The results of the present study demonstrated that transfection with miR-876-5p mimics increased the miR-876-5p levels in AGS-1 cells (Fig. 2A). Subsequently, cell proliferation was assessed using an MTT assay. The results of the present study demonstrated that overexpression of miR-876-5p inhibits proliferation of AGS-1 cells (Fig. 2B). The present study assessed the apoptotic ability of the transfected cells and demonstrated that miR-876-5p increased the apoptotic rate of AGS-1 cells (Fig. 2C).

Suppression of miR-876-5p promotes cellular proliferation and inhibits apoptosis. miR-876-5p knockdown in AGS-1 cells was performed in the present study, via miR-876-5p
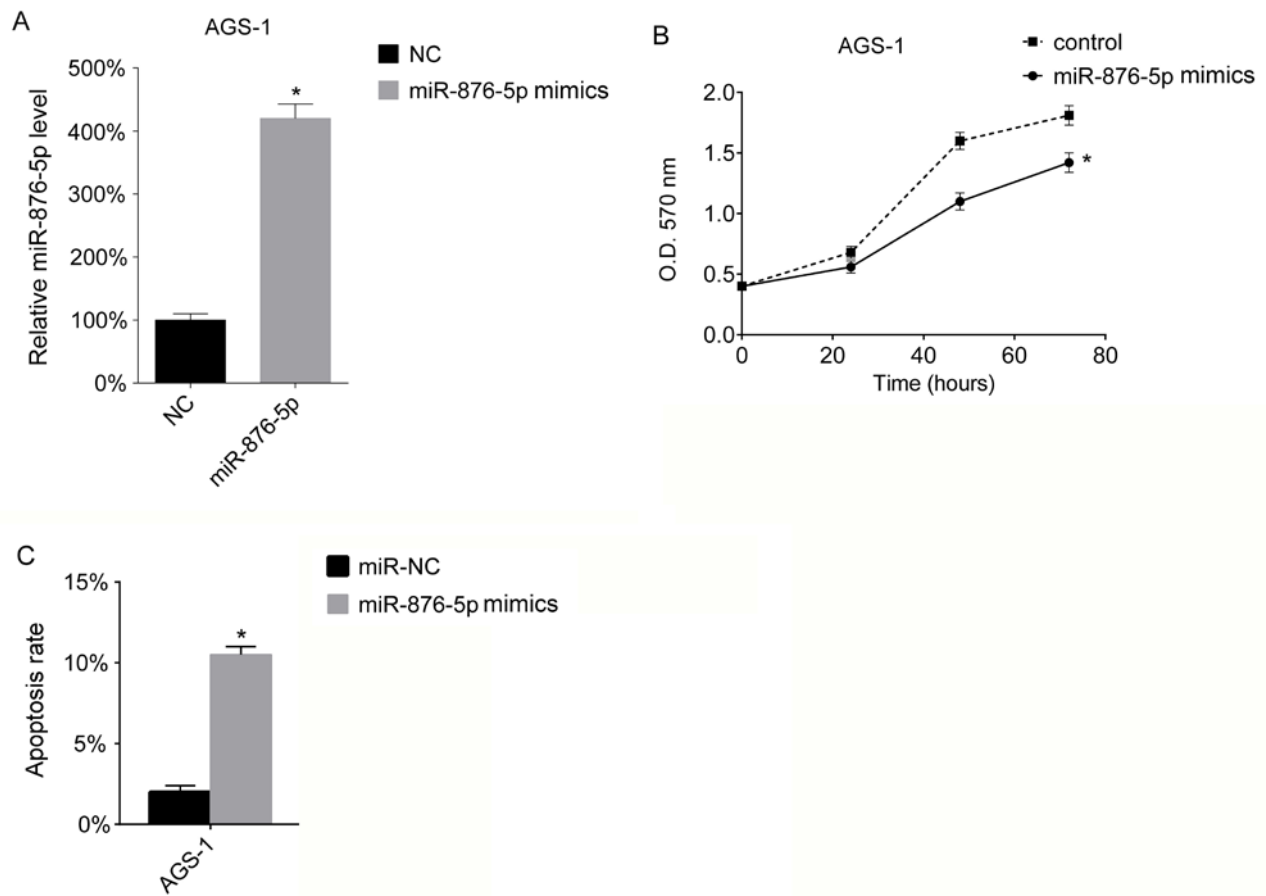
Figure 2. Overexpression of miR-876-5p inhibits proliferation and promotes apoptosis of AGS-1 cells. AGS-1 cells were seeded into 24-well plates at a density of 1x10^6 cells/well overnight. miR-876-5p mimics were transfected into AGS-1 cells. (A) After 24 h, miR-876-5p levels in AGS-1 cells were analyzed via reverse transcription-quantitative PCR. The miR-876-5p levels in miR-NC mimic-transfected cells. The miR-876-5p levels in the control group were arbitrarily defined as 100%. (B) Cellular proliferation was assessed using an MTT assay 24 h after transfection, at 24, 48 and 72 h. (C) Cell apoptosis rates were assessed by FACS 24 h after transfection with miR-876-5p mimics. *$P<0.05$, control vs. transfection group. miR-876-5p, microRNA-876-5p; miR-NC, microRNA-control; FACS, fluorescence-activated cell sorting; NC, control; OD, optical density.

ASO transfection. The miR-876-5p levels of AGS-1 cells were analyzed via RT-qPCR, and the results of the present study demonstrated that miR-876-5p ASO transfection downregulates miR-876-5p levels in AGS-1 (Fig. 3A). Furthermore, the results of the MTT assay in the present study demonstrated that miR-876-5p ASO transfection promotes the proliferation of AGS-1 cells (Fig. 3B). Subsequently, AGS-1 cells were treated with cisplatin following miR-876-5p ASO transfection. The results of the present study demonstrated that cisplatin increased the apoptotic rate of AGS-1 cells, whereas the downregulation of miR-876-5p decreased the apoptotic rate induced by cisplatin (Fig. 3C).

miR-876-5p targets TGFBR1. Physiologically, miRNAs play a role in targeting genes. The present study used the TargetScan software in order to predict the potential target genes of miR-876-5p. The results of the present study demonstrated that TGFBR1 may be targeted by miR-876-5p. The present study mutated the 2,151-2,158 position of TGFBR1 (Fig. 4A), in order to investigate the association between miR-876-5p and TGFBR1. The 3'-UTR TGFBR1 mutant was generated and subsequently transfected into a luciferase reporter plasmid. miR-876-5p and the 3'-UTR TGFBR1 mutant were

co-transfected into AGS-1 cells. At 24 h post-transfection, miR-876-5p was demonstrated to decrease the luciferase activity of the 3'-UTR of wild-type TGFBR1, but not that of the 3'-UTR of mutated TGFBR1 (Fig. 4B). After 48 h, TGFBR1 protein levels were determined using western blot analysis, and the results of the present study demonstrated that miR-876-5p mimics inhibited the expression of TGFBR1 in AGS-1 cells (Fig. 4D).

Discussion

The results of the present study demonstrated that miR-876-5p levels were downregulated in GC tumor tissues compared with normal adjacent tissues. Furthermore, overexpression of miR-876-5p inhibited cellular proliferation and promoted apoptosis, whereas miR-876-5p knockdown promoted cellular proliferation and inhibited apoptosis. The results of the present study suggest that TGFBR1 is targeted by miR-876-5p. To the best of our knowledge, the results of the present study are the first to report miR-876-5p involvement in human GC. As miR-876-5p may be associated with the survival time of patients with GC, future studies are required with larger patient sample sizes, in order to accurately assess survival
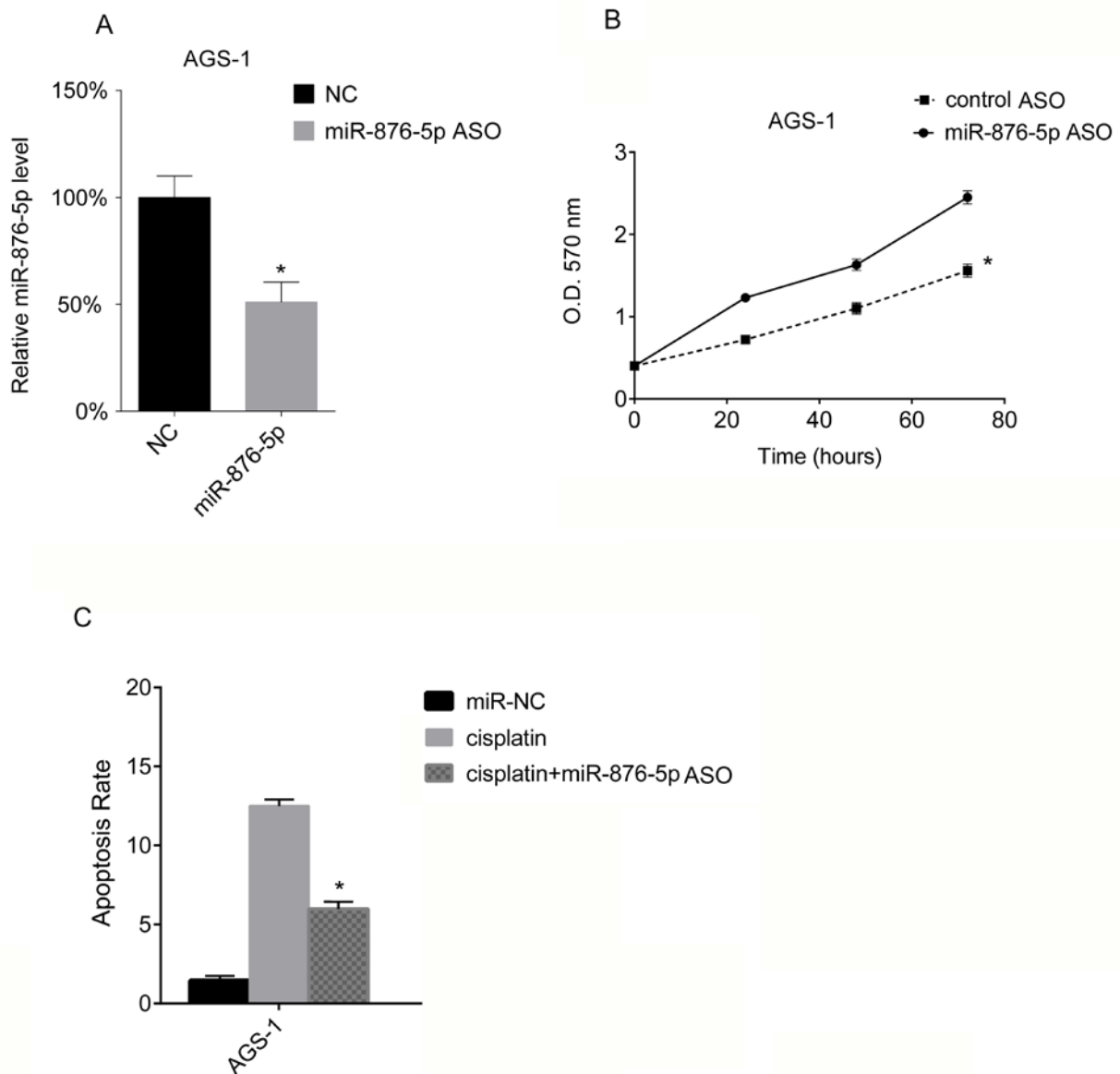
Figure 3. miR-876-5p knockdown promotes proliferation and inhibits apoptosis of AGS-1 cells. AGS-1 cells were seeded into 24-well plates at a density of 1x10^6 cells/well overnight. miR-876-5p mimics were transfected into AGS-1 cells. (A) After 24 h, miR-876-5p levels in AGS-1 cells were analyzed using reverse transcription-quantitative PCR. The miR-876-5p levels in miR-NC mimic-transfected cells. The miR-876-5p levels in the control group were arbitrarily defined as 100%. (B) Cellular proliferation was assessed using an MTT assay 24 h after transfection, at 24, 48 and 72 h. (C) Cell apoptosis rates were assessed by FACS 24 h after transfection with miR-876-5p ASO. *P<0.05, cisplatin vs. cisplatin + miR-876-5p ASO. miR-876-5p, microRNA-876-5p; miR-NC, microRNA-control; FACS, fluorescence-activated cell sorting; NC, control; miR-876-5p ASO, microRNA-876-5p antisense oligonucleotides; OD, optical density.

time analysis in mice models. Furthermore, future studies are critical in order to assess the association between miR-876-5p levels and the function in drug-treatment pressure.

A previous study demonstrated the involvement of miR-876-5p in viral infections. Wang *et al* (30) demonstrated that severe human enterovirus led to elevated expression of circulating miR-876-5p. Furthermore, miR-876-5p has been reported to participate in the miRNA-based antiviral defense mechanism against influenza virus in humans, by targeting the matrix protein of human influenza A H1N1 virus (31).

Previous studies have demonstrated that miR-876-5p inhibits cell proliferation, metastasis and EMT in hepatocellular carcinoma and lung cancer (15-17). The results of

the present study further confirmed the inhibitory role of miR-876-5p and proposed TGFBR1 as a target gene. TGFBR1 is the receptor of TGF-β, which is known to induce EMT (32). Thus, miR-876-5p may have the potential to regulate EMT through TGFBR1 targeting.

TGF-β has a dual role in cancer development, acting as a tumor suppressor and a tumor promoter; it promotes tumorigenesis indirectly by acting on the tumor microenvironment (33). Thus, there is potential that TGFBR1 may play a similar dual role in the pathogenesis of GC. It is predicted that the inhibition of TGFBR1 by miR-876-5p may result in inactivation of the TGF-β signaling pathway. The results of the present study demonstrated that miR-876-5p targeted
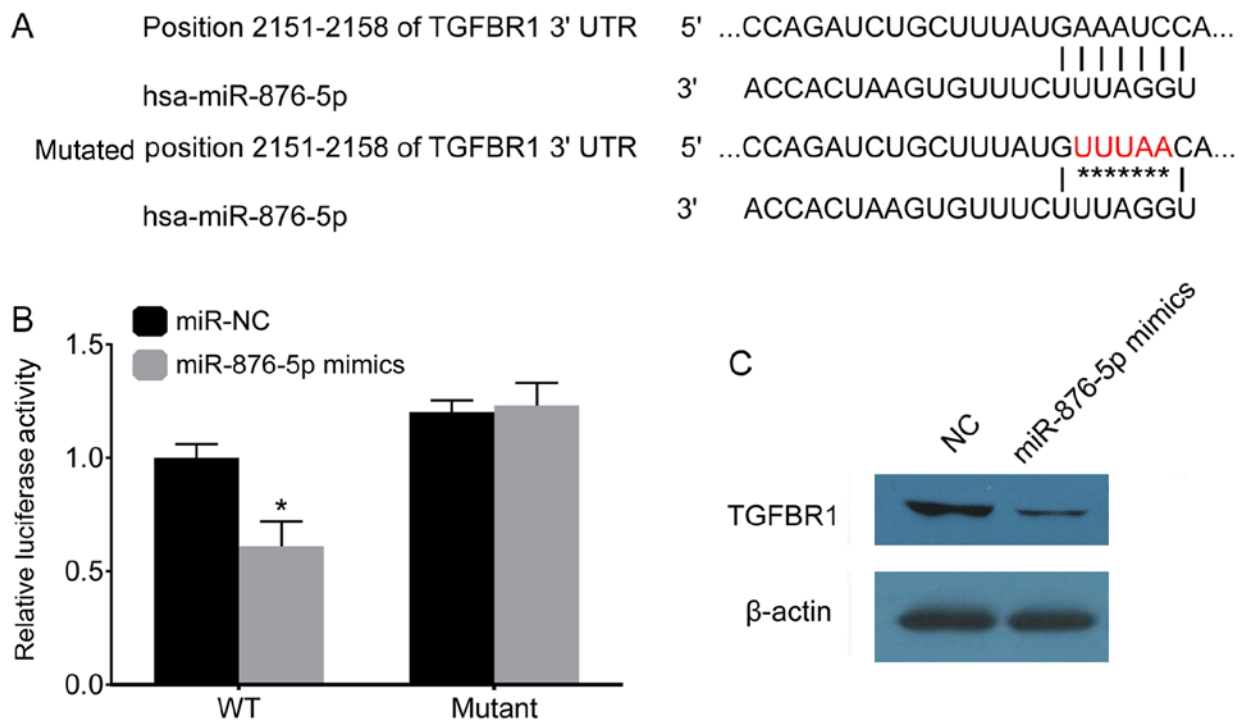
Figure 4. miR-876-5p targets TGFBR1. (A) The binding sites between miR-876-5p and TGFBR1 and the location of the mutant (highlighted in red). (B) miR-876-5p mimics and a plasmid containing either WT or the mutated 3'-UTR TGFBR1 sequence were transfected into AGS-1 cells and luciferase activity was measured after 48 h. (C) The amount of TGFBR1 protein in AGS-1 cells was determined post-transfection via western blot analysis. *P<0.05, WT miR-NC vs. miR-876-5p mimics. miR-876-5p, microRNA-876-5p; TGFBR1, transforming growth factor β-receptor 1; WT, wild-type; 3'-UTR, 3'-untranslated region; miR-NC, microRNA control; NC, control.

TGFBR1, but the role of TGFBR1 is not yet fully understood. Furthermore, the results of the present study suggest that miR-876-5p may be involved in the complex TGF-β signaling pathway by targeting TGFBR1. Another study demonstrated that variants exist in the whole TGFBR1 gene, represented by the single nucleotide polymorphisms, and that genetic variants in TGFBR1 may play a role in the development of GC (34). The results of the present study indicate that miR-876-5p could regulate TGFBR1 by binding to its 3'-UTR. Whether the variants of TGFBR1 have an effect on the regulation function of miR-876-5p warrants further investigations. Overall, the results of the present study confirm the inhibitory role of miR-876-5p in GC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HZ, YZ, JY and JX collected patient data and performed cell experiments. JX, SY and LX performed PCR, western blotting and other molecular experiments. YY and XS contributed to the study design and manuscript writing. ZJ and CZ contributed to funding support, data analysis and revising the manuscript for important intellectual content. All the authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Sichuan University (Chengdu, China) and the 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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