miR-135a-5p inhibits tumor invasion by targeting ANGPT2 in gallbladder cancer

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Abstract. Gallbladder cancer (GBC) is the most aggressive cancer type in the biliary tract, and our previous studies observed that microRNA (miR)-135a-5p expression was downregulated in GBC tissues. However, few studies have focused on the mechanism of action of the miR-135a-5p target genes in GBC. The present study aimed to investigate the regulatory role of miR-135a-5p signaling in GBC. The present study found that miR-135a-5p expression was downregulated in GBC tissue, as detected by immunohistochemistry and reverse transcription-quantitative PCR. In addition, overexpression of miR-135a-5p significantly inhibited the proliferation and migration of GBC-SD cells. Using a luciferase activity assay, it was identified that angiopoietin-2 (ANGPT2) was a potential target gene of miR-135a-5p in GBC. Knockdown of ANGPT2 expression significantly inhibited the proliferation and invasion of GBC-SD cells. In conclusion, the present results suggested that miR-135a-5p affected GBC cell proliferation and invasion by targeting ANGPT2. Moreover, miR-135a-5p may be a potential biomarker for GBC progression and a potential target for GBC therapeutic intervention.

Introduction

Gallbladder cancer (GBC) is the most aggressive cancer type in the biliary tract, accounting for 80-95% of all biliary tract malignancies (1). Despite significant efforts in clinical research on GBC, the prognosis of GBC remains poor, with a 5-year survival rate of ~5%, due to late diagnosis and easy metastasis (2,3). Therefore, it is important to elucidate the underlying molecular mechanisms of GBC that may serve a key role in developing GBC-targeted therapies.

MicroRNAs (miRNAs/miRs) are a type of small endogenous RNA that serve an important role in the regulation of gene expression by reducing the post-transcriptional translation of target mRNAs (4). Previous studies have reported that miRNAs are involved in the proliferation, migration and invasion of cancer cells (5,6). Moreover, miRNAs can be used as markers of cancer diagnosis and prognosis. For example, miR-135a-5p is significantly downregulated in several cancer types, such as gastric carcinoma, and low expression of miR-135a-5p is associated with a low overall survival in gastric cancer (7). Furthermore, miR-135a-5p induces apoptosis in prostate cancer cells by targeting the regulation of STAT6 expression (8). It has also been shown that miR-135a-5p inhibits glioma cell proliferation and invasion by targeting and regulating FOXO1 expression (9). Other previous studies have also identified miRNAs that affect GBC patient survival, such as miR-146b-5p, miR-335 and miR-101, amongst others (10-12).

Our previous studies have observed that miR-135a-5p was significantly downregulated in GBC tissues, as determined by analyzing a miRNA microarray (13). In addition, the relationship between miR-135a-5p and GBC was further examined through *in vivo* and *in vitro* experiments, and it was found that miR-135a-5p exerted strong therapeutic effects on GBC (14). However, few studies have focused on the mechanism of action of the miR-135a-5p target genes in GBC.

Angiopoietin-2 (ANGPT2) is a ligand for the tyrosine-protein kinase receptor Tie-2, which functions as a vascular stabilizing molecule and is an important regulator of vascular maturation (15,16). ANGPT2 is produced by endothelial cells and promotes angiogenesis. ANGPT2 is lowly expressed in normal tissues but tends to be highly upregulated in tumor blood vessels (17). It has been reported that elevated circulating ANGPT2 is associated with poor prognosis and tumor invasion in several cancer types, such as gastric carcinoma (18,19). However, no relevant studies reporting the function of ANGPT2 in GBC were identified in a preliminary search. Thus, the present study aimed to investigate the regulatory role of miR-135a-5p signaling and the function of ANGPT2 in GBC.

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Materials and methods

Tissues, cell lines and culture. GBC and matched adjacent non-tumorous gallbladder tissues (≥2 cm away from the tumor tissue) were obtained from 10 patients (age range, 45-63 years, mean age 53.8±8.6 years; seven female patients and five male patients) who underwent surgery in The Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine between January 2019 and December 2019. The study was approved by the Ethics Committee of The Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine. All enrolled patients signed an informed consent form before surgery. The Cancer Genome Atlas (TCGA) survival analysis data were evaluated using OncoLnc (oncolnc.org).

A human GBC cell line (GBC-SD) was purchased from The Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin (Corning, Inc.) with 5% CO_2 at 37˚C.

Immunohistochemistry (IHC). IHC staining was performed according to a method described in a previous study (20). GBC and matched adjacent non-tumorous gallbladder tissues (≥2 cm away from the tumor tissue) were fixed with 4% paraformaldehyde at room temperature for 30 min and then embedded in paraffin and sliced into 0.5-μm sections, followed by IHC. Briefly, the paraffin-embedded sections were dewaxed in xylene and rehydrated in a graded alcohol series (100, 95 and 80%). Subsequently, the sections were blocked with 5% BSA (cat. no. ST025; Beyotime Institute of Biotechnology) at 37˚C for 1 h, and heated in a microwave oven in sodium citrate buffer (0.1 mM; pH 6.0) for 5 min for antigen retrieval. The sections were then incubated overnight at 4˚C with primary antibodies against ANGPT2 (cat. no. ab56301; 1:200; Abcam). Following which, the sections were incubated with a secondary antibody (cat. no. ab6728; 1:1,000; Abcam) at 37˚C for 1 h. The sections were then stained with diaminobenzidine at room temperature for 3-15 min and counterstained with hematoxylin at room temperature for 5 min. Finally, the images were captured using an Olympus light microscope (Olympus Corporation) under x200 magnification (21). In total, two independent investigators performed the scoring of the respective expression profiles. The number of positive cells was graded as follows: 0 (<5%), 1 (6-20%), 1 (21-40%), 3 (41-60%), 4 (61-80%) or 5 (>80%) (22).

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA from gallbladder tissues and GBC-SD cells was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse-transcribed using a ReverTra Ace™ qPCR RT kit (Toyobo Life Science) according to the manufacturer's protocol. RT-qPCR was performed using a SYBR Supermix PCR kit (Kapa Biosystems; Roche Diagnostics) under the following conditions: 95˚C for 3 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 1 min, and melting curve analysis, according to the manufacturer's protocol. Primers were obtained from Sangon Biotech Co., Ltd., and are presented in Table I. GAPDH and U6 were used as endogenous controls. Data were analyzed using the $2^{-\Delta\Delta Cq}$ calculation (23).

Transfection of miRNAs and small interfering (si)RNAs. Synthesized RNA duplexes of miR-135a-5p mimics, miRNA mimics negative control (mi-NC), ANGPT2 siRNA and scrambled siRNA NC (si-NC) were designed and synthesized by Shanghai GenePharma Co., Ltd.. A total of $1x10^6$ cells/well were seeded into the 6-well plates and transfected with miR-135a-5p mimics and inhibitor using HilyMAX reagent (Dojindo Molecular Technologies, Inc.), according to the manufacturer's instructions. Transfection was performed using the following reagents: 120 μl Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.); 80 pmol (4 μl) mimics or inhibitor; 12 μl HilyMAX reagent, at room temperature for 15 min. GBC-SD cells were harvested for RT-qPCR at 48 h after transfection. The siRNA sequences and miRNA mimics are shown in Table II.

Luciferase activity assay. Wild-type (WT) and mutant (MUT) ANGPT2 3'-untranslated regions (UTRs) were cloned into pmiRGLO vectors (Shanghai GenePharma Co., Ltd.). For the luciferase activity assay, GBC-SD cells ($1x10^5$) were seeded in a 24-well plate. Cells were transfected with the miR-135a-5p mimics or mimic control and WT or MUT ANGPT2 3'-UTR, according to the manufacturer's instructions. Before transfection, the miR-135a-5p mimics or mimic control and WT or MUT ANGPT2 3'-UTR were incubated separately with HilyMAX reagent at room temperature for 15 min. Incubation was performed using the following reagents: 120 μl Opti-MEM; 4 μg WT or MUT ANGPT2 3'-UTR; 80 pM miR-135a-5p mimics or mimic control; 12 μl HilyMAX reagent. Then, cells were transfected with the incubated mixture at 37˚C for 4 h. After transfection, cells were cultured in DMEM (10% FBS) for 48 h. Subsequently, the relative luciferase activity was determined using a luciferase reporter system (Promega Corporation). Luciferase activity was normalized to *Renilla* luciferase activity.

Cell proliferation assays. Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. GBC-SD cells ($5x10^3$ cells/100 μl) were seeded in a 96-well plate. After 24, 48 and 72 h of culturing at 37˚C, proliferation was assessed using the CCK-8 solution. The cells in each well were incubated with 10 μl CCK-8 solution for 1 h on a shaker according to the manufacturer's instructions. Subsequently, the absorbance value was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). The experiments were performed in triplicate.

Wound healing assay. For the wound-healing assay, GBC-SD cells ($1x10^5$) were seeded in 24-well plates and cultured at 37˚C overnight. When they had reached 80% confluency, a 10-μl tip was used to create a 1-mm wide strip at the bottom of wells, and the floating cells were gently washed twice with DMEM. Cells were cultured at 37˚C in DMEM (1% FBS) for 24 h. The cells migrating into the wounded areas were captured using a light microscope at x100 magnification at 0 and 24 h. Wound healing was assessed using MShot Image Analysis System 1.3.10 (Guangzhou Mingmei Photoelectric Technology Co., Ltd.).

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
ANGPT2	AACTTTCGGAAGAGCATGGAC	CGAGTCATCGTATTCGAGCGG
miR-135a-5p	CCAGGCTTCCAGTACCATTAGG	GTTTCCGAGAGAGGCAGGTG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	GAGTCCACTGGCGTCTTCAC	TGCTGATGATCTTGAGGCTGTT

miR, microRNA; ANGPT2, angiopoietin-2.

Table II. ANGPT2 siRNA and miR-135a-5p mimics sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
ANGPT2 siRNA	GCATAGGAAAGAAGCAATATT	UAUUGCUUCUUUCCUAUGCTT
si-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
miR-135a-5p mimics	UAUGGCUUUUCAUUCCUAUGUGA	ACAUAGGAAUGAAAAGCCAUAUU
mi-NC	UAUAUCGUGUUAUUAGCGUUCCU	GAACGCUAAUAACACGAUAUAUU

si-NC, scrambled siRNA NC; mi-NC, miRNA mimics NC; siRNA, small interfering RNA; miR, microRNA; ANGPT2, angiopoietin-2.

Transwell assays. For the Transwell assay, Matrigel was diluted with serum-free medium (dilution concentration was no less than 1:3), and 50 μl Matrigel was added to the upper chamber (pore size, 8 μm) of each well in a 24-well plate, and incubated at 37˚C for 3-5 h, Subsequently, 70 μl serum-free medium was added to the upper chamber of each well and incubated at 37˚C for 30 min; residual liquid in the chamber was removed. GBC-SD cells were seeded into the upper chamber of Matrigel-plated with DMEM without serum ($5x10^4$ cells/well), and the lower wells contained 500 μl complete medium (DMEM and 10% FBS), following a routine procedure. After 48 h at 37˚C, cells invading into the lower chamber were collected, fixed with 5% glutaraldehyde for 10 min at room temperature. stained with 0.5% crystal violet and then counted under a light microscope at x100 magnification.

Western blotting. Western blot analysis was performed as previously described to determine migration- and invasion-related protein expression (24). The samples or cells were lysed in RIPA buffer (cat. no. P0013K; Beyotime Institute of Biotechnology). The obtained proteins were quantified using BCA Protein Assay kit (Sangon Biotech Co., Ltd.) and 30 μg proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gels for 2 h. Subsequently, the separated proteins were transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.). These membranes were then immersed in 5% skimmed milk (Sangon Biotech Co., Ltd.) diluted with PBS-0.1% Tween 20 at room temperature for 3 h to prevent nonspecific protein binding, followed by incubation with primary antibodies, including the endogenous reference, overnight at 4˚C and with HRP-conjugated anti-rabbit secondary antibodies (cat. no. ab205718; dilution, 1:5,000; Abcam) for 1 h at room temperature. The following primary antibodies were used: E-cadherin (E-cad; cat. no. ab40772; dilution, 1:5,000; Abcam) and Rho associated coiled-coil containing protein kinase 1 (Rock1; cat. no. ab97592; dilution, 1:500; Abcam). Anti-GAPDH (cat. no. ab9485; dilution, 1:2,500; Abcam) was used as the endogenous reference. Finally, bound HRP-conjugated antibodies were detected using Western Lightning Plus-ECL reagents (PerkinElmer, Inc.). The gray level of each band was obtained using ImageJ software (version 1.8.0; National Institutes of Health) (25).

Statistical analysis. The results are presented as the mean ± SD and all experiments were repeated at least three times. Statistical analyses were performed using GraphPad Prism (version 7.0; GraphPad Software, Inc.). The miRNA target gene was determined using prediction databases [TargetScan Release 7.2 (http://www.targetscan.org/vert_72/) and miRDB version 6.0 (http://mirdb.org/index.html)]. Survival analysis was conducted using the Kaplan-Meier method, and the Renyi test was used to compare the overall survival curves. An unpaired Student's t-test was used for comparison between two groups in cell assay, while a paired Student's t-test was used for comparison between two groups in Fig. 1, with the exception of IHC data. IHC data are presented as the median and range, and were analyzed used Wilcoxon test. One-way ANOVA followed by Tukey's post hoc test was used for comparisons between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-135a-5p and ANGPT2 in patients with GBC. To determine the expression levels of miR-135a-5p and ANGPT2 in GBC, RT-qPCR analysis was first performed for 10 GBC tissues and matched adjacent non-tumorous

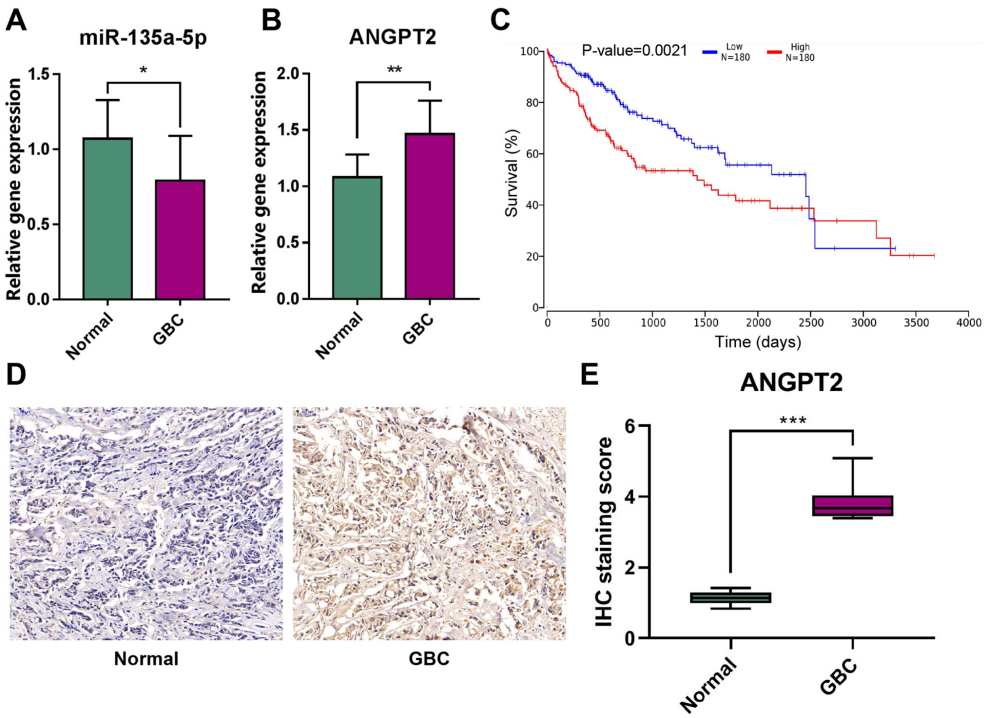

Figure 1. Expression levels of miR-135a-5p and ANGPT2 in patients with GBC. Expression levels of (A) miR-135a-5p and (B) ANGPT2 in GBC tissues. (C) The Cancer Genome Atlas survival analysis of ANGPT2 on the prognosis of patients with liver and biliary tumor. (D) IHC staining and (E) score of ANGPT2 in patients with GBC. *P<0.05, **P<0.01 and ***P<0.001. IHC, immunohistochemistry; miR, microRNA; ANGPT2, angiopoietin-2; GBC, gallbladder cancer.

gallbladder tissues. The results demonstrated that miR-135a-5p expression was significantly downregulated in GBC tissues (Fig. 1A). By contrast, the expression level of ANGPT2 in GBC tissues was significantly higher compared with that in normal tissues (Fig. 1B). Further TCGA survival (26) analysis identified that ANGPT2 expression had a significant impact on the prognosis of the patients with liver and biliary tumors, as patients with high ANGPT2 expression had a poorer prognosis (Fig. 1C). The IHC results demonstrated that the positive rate of ANGPT2 expression in GBC tissues was significantly higher compared with that in normal tissues (Fig. 1D and E). These results suggested that miR-135a-5p and ANGPT2 may be key biomarkers of GBC.

Overexpression of miR-135a-5p inhibits in vitro GBC cell proliferation, migration and invasion. To further investigate the effect of miR-135a-5p on GBC cell proliferation and migration, miR-135a-5p interference experiments were performed *in vitro*. The RT-qPCR results identified the overexpression of miR-135a-5p by miRNA mimics, which indicated that the transfection was successful (Fig. 2A). The CCK-8 assays demonstrated that the overexpression of miR-135a-5p significantly reduced the proliferation of GBC-SD cells (Fig. 2B). Moreover, the wound healing assay identified that the overexpression of miR-135a-5p effectively inhibited the migration of GBC cells. (Fig. 2C and D). Transwell assays results also revealed that GBC cell invasion was significantly inhibited after miR-135a-5p overexpression (Fig. 2E and F). Furthermore, the western blotting results demonstrated that Rock1 expression was decreased, while E-cad expression was notably increased in miR-135a-5p mimics group (Fig. 2G). These data indicated that the overexpression of miR-135a-5p could inhibit the proliferation, migration and invasion of GBC-SD cells.

ANGPT2 is the target gene of miR-135a-5p. Analysis of miRNA target gene prediction databases (TargetScan Release 7.2 and miRDB version 6.0) indicated that ANGPT2 may be a target gene of miR-135a-5p based on the putative target sequence ANGPT2 3'-UTR (Fig. 3A).

RT-qPCR analysis was performed to verify the regulatory relationship between miR-135a-5p and ANGPT2. The results demonstrated that ANGPT2 expression was significantly decreased after transfecting miR-135a-5p mimics in GBC-SD cells. Moreover, ANGPT2 was significantly inhibited by siRNA, indicating that transfection was successful (Fig. 3C). The luciferase reporter assay demonstrated that the signal from WT-ANGPT2 3'-UTR was significantly decreased after transfecting miR-135a-5p mimics, while the signal from MUT-ANGPT2 3'-UTR showed no significant difference (Fig. 3B and D). These results indicated that miR-135a-5p could directly target ANGPT2.

ANGPT2 inhibits the proliferation and invasion of GBC-SD cells. To investigate the effect of ANGPT2 on GBC cell proliferation and invasion, GBC-SD cell lines were transfected with siRNA targeting ANGPT2. The results demonstrated that ANGPT2 expression was significantly decreased after transfection (Fig. 2C). The CCK-8 and Transwell assay results indicated that the proliferation and invasion of GBC-SD cells were significantly inhibited by ANGPT2 knockdown (Fig. 4A-C). Western blot analysis results identified that Rock1 expression was inhibited, and E-cad expression was notably increased in ANGPT2-siRNA group (Fig. 4D). These

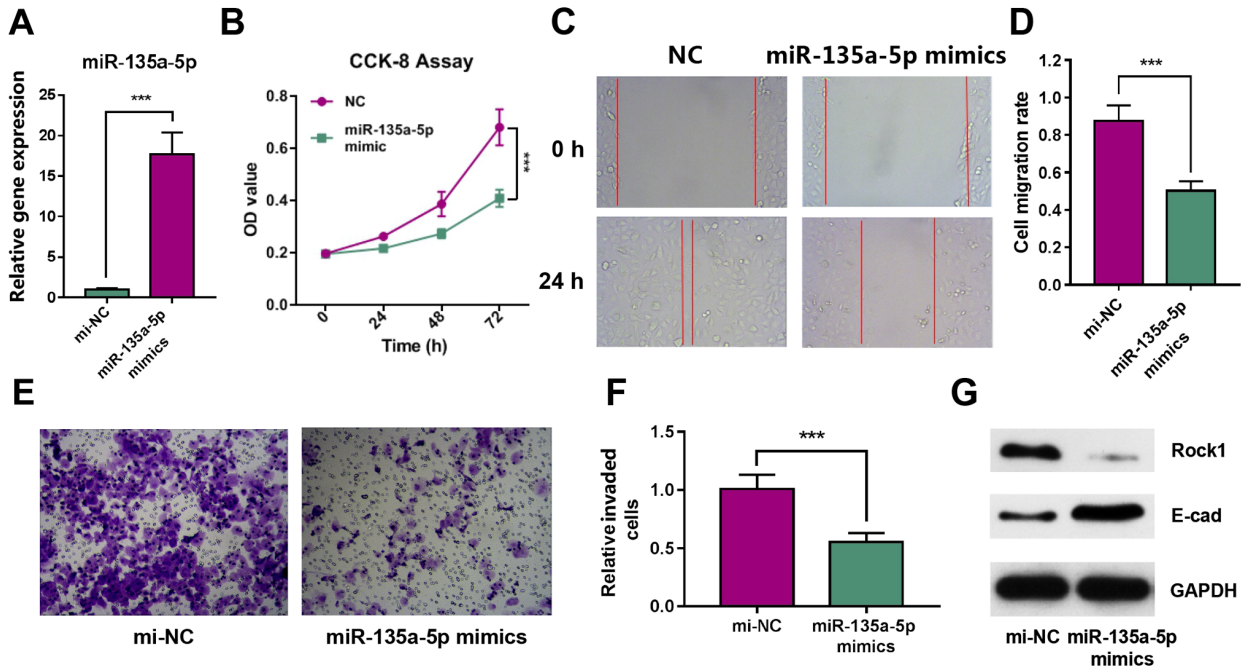

Figure 2. miR-135a-5p inhibits the proliferation, migration and invasion of GBC-SD cells. (A) miR-135a-5p expression in GBC-SD cells after transfection with miRNA mimics. (B) CCK-8 cell proliferation assay results. (C) Wound healing assay image and (D) quantification of results. Magnification, x200. (E) Cell invasion images and (F) quantification of results. Magnification, x200. (G) Expression levels of Rock1 and E-cad were determined using western blot analysis. ***P<0.001. miR/mi, microRNA; ANGPT2, angiopoietin-2; E-cad, E-cadherin; Rock1, Rho associated coiled-coil containing protein kinase 1; OD, optical density; NC, negative control; CCK, Cell Counting Kit.

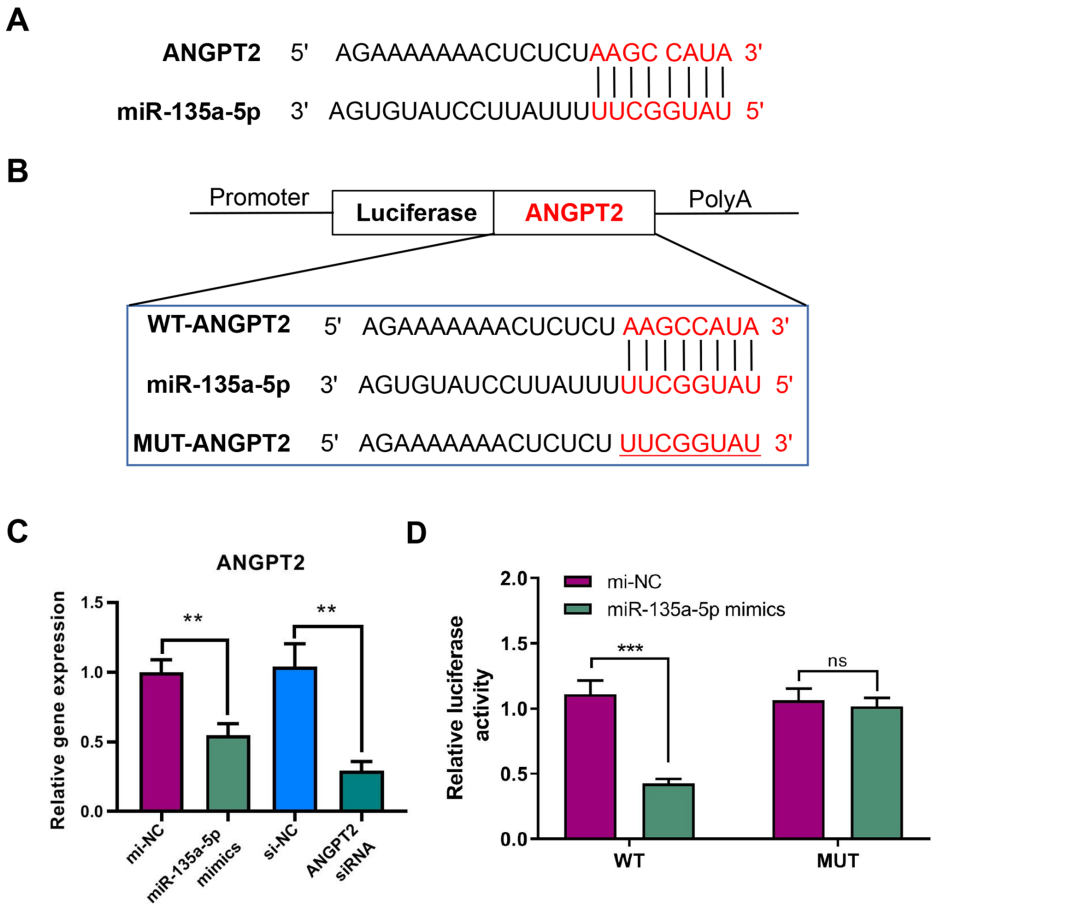

Figure 3. ANGPT2 is the target gene of miR-135a-5p. (A) Binding sites of miR-135a-5p on ANGPT2 3'-untranslated region. (B) WT-ANGPT2 and MUT-ANGPT2 sequences of luciferase activity assay. (C) Relative expression level of ANGPT2 in the miR-135a-5p mimics and ANGPT2 siRNA groups. (D) Relative luciferase activity results. **P<0.01 and ***P<0.001. miR/mi, microRNA; ANGPT2, angiopoietin-2; siRNA/si, small interfering RNA; NC, negative control; WT, wild-type; MUT, mutant; ns, not significant.

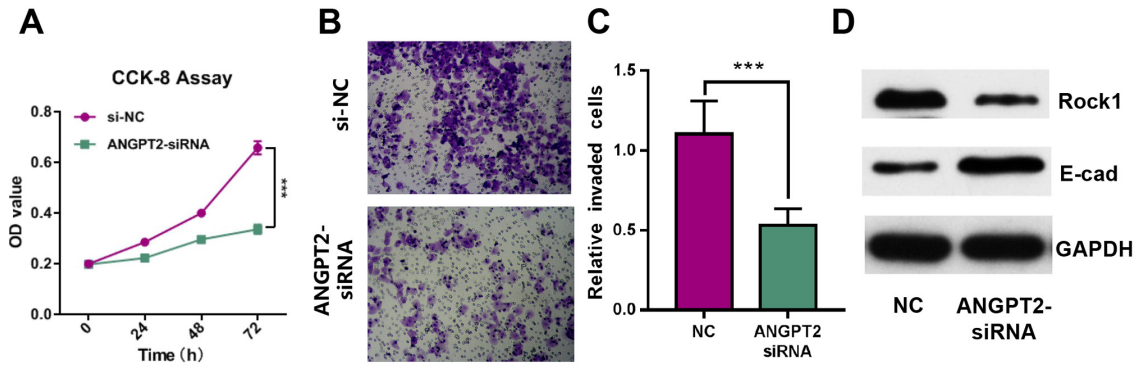

Figure 4. ANGPT2 inhibits the proliferation and invasion of GBC-SD cells. (A) CCK-8 cell proliferation assay results. (B) Cell invasion images and (C) quantification of results. Magnification, x200. (D) Expression levels of Rock1 and E-cad were determined using western blot analysis. ***P<0.001. ANGPT2, angiopoietin-2; E-cad, E-cadherin; Rock1, Rho associated coiled-coil containing protein kinase 1; OD, optical density; NC, negative control; CCK, Cell Counting Kit; siRNA/si, small interfering RNA.

data suggested that ANGPT2 inhibited the proliferation and invasion of GBC-SD cells.

Discussion

miRNAs are involved in the occurrence of a variety of tumors. The regulation of miRNAs on tumor suppressor genes and oncogenes is a new research approach. Each miRNA has hundreds of target genes that are involved in the translation or degradation of mRNAs in a base pairing (27). Recent studies have shown that miR-135a-5p served important but contradictory roles in different cancer progression (28,29). miR-135a-5p acts as a promoter or inhibitor of cancer cell proliferation and invasion by regulating specific signaling pathways and target genes. miR-135a-5p also regulates epithelial-mesenchymal transformation and chemoresistance in cancer cells (30). Previous studies reported that miR-135a-5p was upregulated in several cancer types, such as breast cancer, bladder cancer, melanoma and colorectal adenocarcinomas (31-34). By contrast, other studies have revealed that miR-135a-5p was downregulated, such as in lung cancer, prostate cancer and pancreatic cancer (30,35-37).

The present study demonstrated that miR-135a-5p expression was downregulated in GBC tissue, which was in line with previous studies (13,14). Furthermore, the overexpression of miR-135a-5p significantly inhibited the proliferation and migration of GBC-SD cells. The expression level of ANGPT2 was suppressed in the miR-135a-5p mimics group, suggesting that miR-135a-5p can specifically regulate the expression level of ANGPT2 in GBC-SD cells.

ANGPT2 is a ligand for the tyrosine-protein kinase receptor Tie-2, which functions as a vascular stabilizing molecule and is an important regulator of vascular maturation (16). It has previously been reported that angiogenesis serves a key role in the development and progression of various malignant tumors (16). Vascular formation and dilation are closely associated with VEGFs (38). It has been shown that VEGFs regulate ANGPT2, and the upregulation of ANGPT2 has been found clinically to be one of the mechanisms of acquired resistance during anti-VEGF treatment (39,40). Xu *et al* (41) revealed that ANGPT2 may be a serum marker for lung cancer prognosis. The present results suggested that ANGPT2 was a potential target gene of miR-135a-5p in GBC. Furthermore, knockdown of ANGPT2 expression significantly inhibited the proliferation and invasion of GBC-SD cells.

There are several potential limitations to the present study; one of which was the small number of patient cases enrolled. Moreover, there were few GBC-related data in the present study and TCGA dataset; therefore, there may be some sampling errors in the survival analysis. In future studies, additional clinical information of patients with GBC will be collected for relevant verification. Further verification may improve confidence in the present results, which indicated that the protein expression levels of ANGPT2 were reduced after miR-135a-5p mimics or siRNA treatment. Additional validation experiments in future animal model experiments will help to provide a more detailed understanding of the relationship between ANGPT2 and miR-135a-5p.

In conclusion, the present study demonstrated that miR-135a-5p was downregulated and was negatively associated with malignancies in GBC. It was identified that miR-135a-5p affected GBC cell proliferation and invasion by targeting ANGPT2. Moreover, miR-135a-5p may be a potential biomarker for GBC progression and a potential target for GBC therapeutic intervention.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GHY and HYD designed the work. GHY, HYD, XX and BZ contributed to the experiment, analyzed the data and drafted the manuscript. GHY and HYD confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine. All enrolled patients signed an informed consent form before surgery.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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