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MiR-335 promotes cell proliferation by inhibiting MEF2D and sensitizes cells to 5-Fu treatment in gallbladder carcinoma

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Abstract. – OBJECTIVE: Gallbladder carcinoma is a malignant tumor in the bile duct with poor prognosis. Although aberrant expression of miR-335 has been reported in the tumor tissues of gallbladder carcinoma, the biological role of miR-335 was still largely unknown. This study was intended to explore the role of miR-335 in the progression of gallbladder carcinoma.

PATIENTS AND METHODS: The gallbladder carcinoma cell lines GBC-SD and SGC-996 were used in our study. MiR-335 mimic, miR-335 inhibitor, and si-myocyte enhancer factor 2D (MEF2D) were transfected into gallbladder carcinoma cells, respectively. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay analysis was used to determine cell viability. The colony formation was also analyzed. Cell cycle progression was determined using flow cytometer. To verify the target gene of miR-335, the luciferase assay was used.

RESULTS: MiR-335 overexpression inhibited cell viability and colony formation of GBC-SD and SGC-996 cells. The percentage of cells in first gap phase (G1)/resting phase (G0) was significantly increased, and the expression of cell division cycle 2 (cdc2) and cell division cycle 25 (cdc25) was decreased after miR-335 was overexpressed, indicating its role in inducing the cell cycle arrest of GBC-SD and SGC-996 cells. MEF2D was up-regulated in gallbladder cancer and associated with tumor size and clinical stage. Down-regulation of MEF2D inhibited cell viability and colony formation, induced cell cycle arrest in G1/G0 phase, and decreased the expression of cdc2 and cdc25 in GBC-SD and SGC-996 cells. Bioinformatics analysis by TargetScan and luciferase assay verified that MEF2D could be targeted by miR-335. Importantly, the effects of miR-335 inhibitor on cell growth were rescued by small interfering RNA of MEF2D (siMEF2D) in GBC-SD and SGC-996 cells. Besides, miR-335 overexpression increased cell sensitivity to 5-Fluoracil (Fu) treatment and decreased the expression levels of ATP-binding cassette transporter B1 (ABCB1) and ATP-binding cassette G2 (ABCG2) in GBC-SD and SGC-996 cells.

CONCLUSIONS: MiR-335 participates in the progression of gallbladder carcinoma by targeting MEF2D. MiR-335 may be a potential therapeutic target for gallbladder carcinoma.

Key Words:
Gallbladder carcinoma, MiR-335, MEF2D, Cell viability, Cell cycle, 5-Fluoracil.

Abbreviations

MiRNAs: MicroRNAs; SP1: specificity protein 1; CRC: colorectal cancer; MEF2D: Myocyte enhancer factor 2D; NSCLC: non-small cell lung cancer.

Introduction

Gallbladder carcinoma is a common malignant tumor in the bile duct and is the seventh most common digestive tract cancer[1,2]. Patients with gallbladder carcinoma usually have a poor prognosis, and the median survival period is approximately 9 months[3]. The mortality rate of gallbladder carcinoma is high, causing the death of approximately 3300 patients each year[4]. At present, the major strategies for gallbladder carcinoma treatment were surgery, radiotherapy, and chemotherapy; however, the recurrence rate of gallbladder carcinoma remains high, and the 5-year survival rate is still low[5]. Therefore, it is essential to explore the mechanisms underlying the progression of gallbladder carcinoma and search new strategy for gallbladder carcinoma treatment.

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MicroRNAs (miRNAs) are members of small non-coding RNAs (~22 nucleotides) and exert vital roles in post-transcriptional regulation[6]. Scholars[7-9] have proved that miRNAs showed abnormal expression in many cancers, such as ovarian cancer, bladder cancer, and colon cancer, indicating the important role of miRNAs in the development and progression of cancers. Particularly, miR-335 was reported to be an important miRNA in digestive system cancers. For example, miR-335 showed low expression levels in gastric cancer, and miR-335 overexpression could inhibit gastric cancer cell proliferation and metastasis by targeting specificity protein 1 (SP1)[10]. In colorectal cancer (CRC), the expression level of miR-335 in primary CRC with metastasis ability and metastatic lesions was increased, revealing that miR-335 was associated with CRC progression[11]. In addition, Peng et al[12] showed that miR-335 level was decreased in primary gallbladder carcinoma and up-regulation of miR-335 indicated good prognosis of patients. However, the effect and regulatory mechanism of miR-335 on the development of gallbladder carcinoma were still unclear. Therefore, this study was performed to explore the role of miR-335 in gallbladder carcinoma *in vitro*.

Myocyte enhancer factor 2D (MEF2D) is a transcription factor that belongs to the member of the myocyte enhancer factor 2 (MEF2) family[13]. It could exert regulatory function in division, differentiation, and death of many kinds of cells in muscle, heart, and cancer cells[14]. For example, Song et al[15] found that the expression level of MEF2D in tumor tissues of pancreatic cancer was higher than that in normal tissues and MEF2D regulated pancreatic cancer cell proliferation and invasion. Previous studies[16,17] indicated that miR-1244 regulated cisplatin-treated non-small cell lung cancer (NSCLC) through MEF2D. In glioma[18], the expression level of MEF2D was increased and could inhibit cell invasion and angiogenesis which was finally identified as a target gene of miR-421[18]. However, the role of MEF2D in gallbladder carcinoma was still not elucidated.

In our study, gallbladder carcinoma cell lines GBC-SD and SGC-996 were used, and miR-335 mimic, miR-335 inhibitor, and small interfering RNA of MEF2D (siMEF2D) were transfected into gallbladder carcinoma cells, respectively. The results showed the important roles of miR-335 and MEF2D in cell viability, colony formation, and cell cycle arrest. Further analysis verified that MEF2D could be targeted and negatively regulated by miR-335. MiR-335 and MEF2D may be potential therapeutic targets for gallbladder carcinoma.

Patients and Methods

Tissues Collection and Cell Culture

Gallbladder carcinoma tissues and adjacent normal tissues were collected from a total of 60 patients with gallbladder carcinoma (stage I-IV) from January 2016 to June 2017 at the First Affiliated Hospital of Bengbu Medical College. The detail clinical-pathological characteristics were presented in Table I. The collected tissues were used for further analysis. Informed consents were obtained from every patient. This investigation is approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College.

GBC-SD and SGC-996 cell lines were obtained from Cell Bank of the Chinese Academy of Sciences (Xuhui, Shanghai, China). Cells were cultured at 37°C with 5% CO_2 using Dulbecco's Modified Eagle's Medium (DMEM) medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Grand Island, NY, USA).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The mRNA levels of miR-335 and MEF2D were detected using qRT-PCR. Briefly, total RNA was extracted using TRIzol reagent (Sigma-Aldrich, St Louis, MO, USA) from cells and tissues. Complementary deoxyribose nucleic acid (cDNA) for miRNA and mRNA was reverse transcribed from RNAs using Reverse Transcriptase Kit and PrimeScript RT Master Mix Kit, respectively (Sigma-Aldrich, St Louis, MO, USA). The qPCR was carried out on an Mx3000P Real-Time PCR system (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) using an SYBR-Green PCR Master mix (Sigma-Aldrich, St Louis, MO, USA). The primers of miR-335 and *MEF2D* were: miR-335: forward: 5'-ACACTCCAGCTGGGTCAAGAGCAATAACGAAA-3', reverse: 5'-CTCAACTGGTGTCGTGGA-3'[19]; *MEF2D*: forward: 5'-CGCGAATTCACCATGGGG AGGAAAAAGATT-3', reverse: 5'-TGGCTCGAGTCACTTTAATGTCCAGGT-3'[20]. The programs of PCR were: 95°C, 5 min; 92°C, 30 s, 60°C, 35 s,

Table I. Correlation between MEF2D expression and clinical-pathological characteristic in 60 cases of gallbladder carcinoma patients.

Characteristics	Case number	MEF2D expression		*p*-value
		Low	High	
Gender				0.693
Male	25	13	12	
Female	35	20	15	
Age				0.895
≤ 60	35	19	16	
> 60	25	14	11	
Tumor size				0.000***
< 5 cm	26	21	5	
> 5 cm	34	12	22	
Histological grade				
Well and moderately	31	18	13	0.622
Poorly and others	29	15	14	
N status				
N0	29	17	12	0.586
N1/2	31	16	15	
Clinical stage				0.014*
I-II	26	19	7	
III-IV	34	14	20	

***: $p < 0.001$.

72°C, 1 min of 42 cycles; 72°C, 10 min. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* was an internal control gene, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Plasmid Construction and Cell Transfection

The miR-335 mimic, miR-335inhibitor, and the corresponding controls were purchased from Shanghai GenePharma Co., Ltd (Pudong, Shanghai, China). Small interfering RNA of *MEF2D* (siMEF2D) and siRNA control (siNC) were synthesized by Thermo Fisher Scientific (Waltham, MA, USA). Cells (5×10^5 cells per well) were plated into 6-well plate and cultured for 24 h. The cells were transfected with miR-335 mimic, miR-335inhibitor, and the corresponding controls using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions.

(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

GBC-SD or SGC-996 cells (1×10^4 cells per well) were seeded into 96-well plates. At the indicated time, each well was added with 10 µl of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) and then incubated at 37°C with 5% CO_2 for another 4 h. Then, the absorbance was determined by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm.

Colony Formation Assay

The cells were seeded into 6-well plates and cultured for 14 days. Cells were fixed with 4% paraformaldehyde and stained using 0.1% crystal violet. Then, the cell numbers were counted, and the experiments were performed at least three times.

Cell Cycle Detection by Flow Cytometer

The cells (4×10^5 cells per well) were incubated, and were collected and permeabilized used 0.25% Triton X-100 for 15 min after overnight. Then, cells were added with 0.25% Triton X-100 propidium iodide and incubated for 30min. At last, the cell cycle was determined using flow cytometer (BD Biosciences, San Jose, CA, USA) after the phosphate-buffered saline (PBS) buffer was added.

Immunohistochemistry

All tissues were fixed and embedded in 10% formaldehyde and 4% paraffin respectively before cutting into 5 µm sections. The sections were incubated with *anti*-MEF2D antibody (dilution, 1:100) (Santa Cruz Biotechnology, Santa

Cruz, CA, USA) at 4°C for 12 h and subsequently treated with horseradish peroxidase (HRP)-conjugated goat *anti*-rabbit IgG antibody at room temperature for 1.5 h. The sections were observed under light microscopy (Olympus, Tokyo, Japan) at 200 × and 400 × amplification.

Luciferase Assay

The wild 3'-untranslated region (UTR) of *MEF2D* and the mutant 3'-UTR were constructed into the pmiRGLO vector (Promega, Madison, WI, USA). Cells were co-transfected with miR-335 mimic ormiR-335 inhibitor and pmiRGLO vector. After 48 h, the relative luciferase activities of cells were analyzed by normalizing to *Renilla* luciferase activity.

Western Blot

The proteins in tumor cells, tumor tissues, and adjacent tissues of gallbladder carcinoma were extracted with radioimmunoprecipitation assay (RIPA) buffer, and the concentration was detected by bicinchoninic acid (BCA) kit. RIPA buffer and BCA kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). 40 μg of total proteins were dissolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 4% non-fat milk for 1 h and probed with antibodies of MEF2D (dilution, 1:1000; Abcam, Cambridge, MA, USA), cell division cycle 2 (cdc2) (dilution, 1:500; Abcam, Cambridge, MA, USA), cell division cycle 25 (cdc25; dilution, 1:500; Abcam, Cambridge, MA, USA), ATP-binding cassette transporter B1 (ABCB1; dilution, 1:500; Abcam, Cambridge, MA, USA), ATP-binding cassette G2 (ABCG2; dilution, 1:1000; Abcam, Cambridge, MA, USA) and GAPDH (dilution, 1:1000; Abcam, Cambridge, MA, USA) at 4°C for overnight. The control protein was GAPDH, and the blots were imaged using ECL Plus reagents. The quantification of corresponding bands was conducted using Image J software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) software 22.0 (SPSS Corp., Armonk, NY, USA) was used for statistical analysis. All data were shown as mean ± standard deviation (SD). The differences between the two groups and multiple groups were analyzed by Student's *t*-test and two-way analysis of variance (ANOVA) with Bonferroni post-hoc test, respectively. The $p<0.05$, $p<0.01$, and $p<0.001$ were considered as statistically significant, highly statistically significant, and extremely statistically significant, respectively.

Results

MiR-335 Overexpression Inhibited Gallbladder Cancer Cell Growth

The expression of miR-335 was reported[12] to be significantly lower in gallbladder cancer tissues as compared to normal tissues. Our results also showed that the miR-335 levels in tumor tissues were significantly lower than that in normal tissues (Figure 1A, $p<0.01$). To confirm the influence of miR-335 on gallbladder carcinoma, miR-335 mimics were transfected into gallbladder carcinoma cell lines GBC-SD and SGC-996. QRT-PCR results verified the transfection efficiency (Figure 1A, all $p<0.001$). Then, we detected the influence of miR-335 mimics on gallbladder cancer cell growth by MTT assay, colony formation assay, and flow cytometry. MTT assay showed that miR-335 mimics significantly decreased cell viability of GBC-SD and SGC-996 cell lines as compared to mimic control group (Figure 1A, all $p<0.001$). Colony formation assay revealed that miR-335 mimics significantly inhibited colony rate of GBC-SD and SGC-996 cells as compared to mimic control (Figure 1B, $p<0.01$). Flow cytometry results showed that the gallbladder cancer cell percentage in the first gap phase (G1)/resting phase (G0) phase was increased in miR-335 mimics group as compared to mimic control (Figure 1C), indicating that miR-335 mimics induced cell cycle arrest. In addition, Western blot results revealed that miR-335 mimics significantly decreased the expression levels of cell cycle-related proteins cdc2 and cdc25 in gallbladder cancer cells (Figure 1D, all $p<0.01$). These results indicated that miR-335 overexpression inhibited cell viability and colony formation, and induced cell cycle arrest of GBC-SD and SGC-996 cells.

Down-Regulation of MEF2D Inhibited Gallbladder Cancer Cell Growth

To explore the effect of MEF2D on gallbladder cancer, the expression of MEF2D was determined in 60 gallbladder cancer patients. Results showed that the mRNA levels of MEF2D in tumor tissues were significantly higher than that

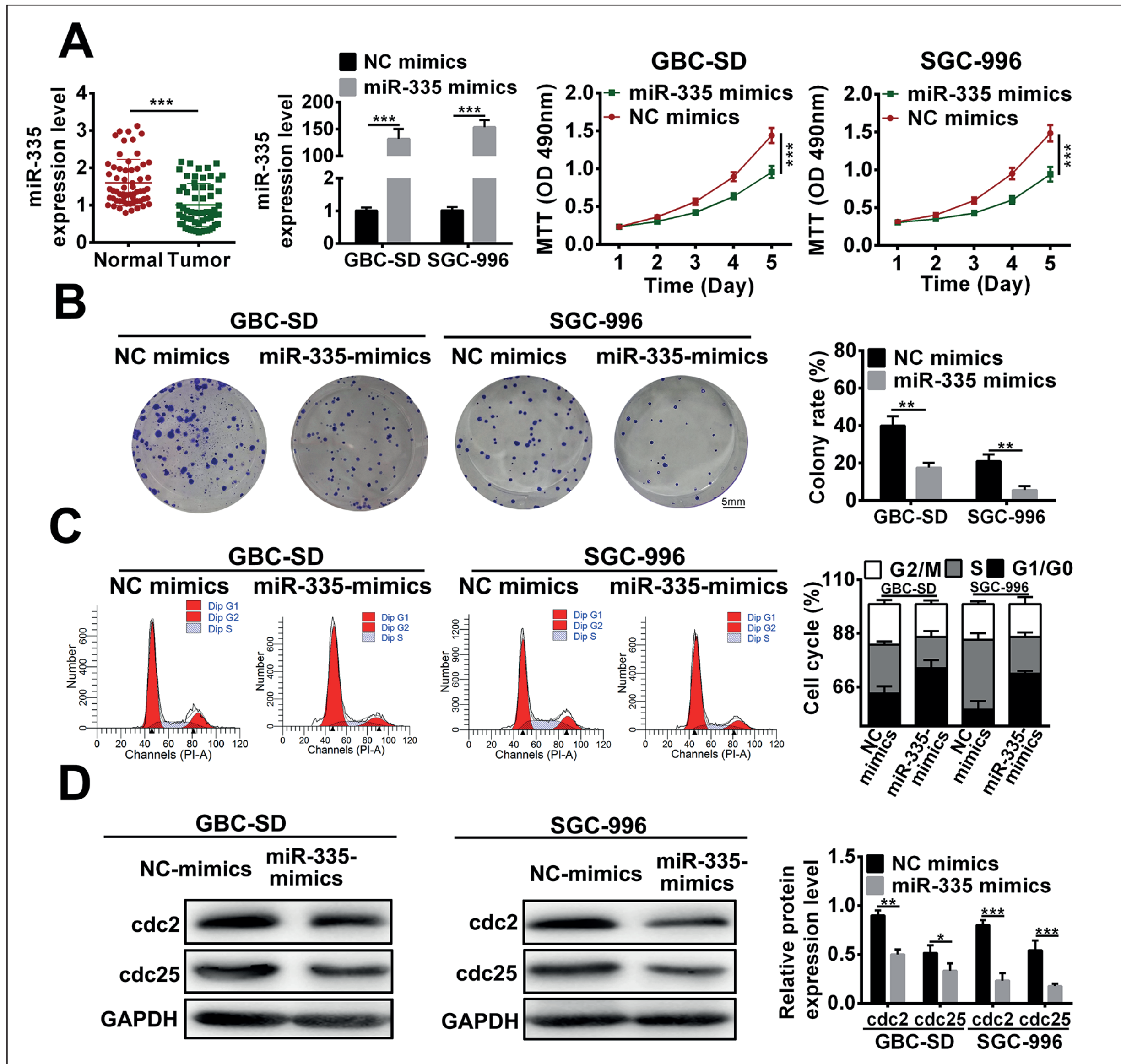

Figure 1. MiR-335 overexpression inhibited gallbladder cancer cell growth. **A,** QRT-PCR was performed to detect the mRNA level of miR-335 and MTT assay was performed to determine the viability of GBC-SD and SGC-996 cells after miR-335 mimics transfection. **B,** Colony formation of gallbladder carcinoma cells was determined after transfected with miR-335 mimics (Scale bar, 5 mm). **C,** Flow cytometer was used to examine cell cycle after transfected with miR-335 mimics. **D,** Western Blot was performed to determine the protein expression of cdc2 and cdc25 in cells transfected with miR-335 mimics. **: $p<0.01$. ***: $p<0.001$.

of normal tissues ($p<0.001$, Figure 2A). Immunohistochemistry results showed that MEF2D protein level was significantly up-regulated in tumor tissues as compared to normal tissues (Figure 2B). The correlation analysis showed that MEF2D expression was significantly correlated with tumor size ($p=0.000$) and clinical stage ($p=0.014$, Table I), indicating the potential role of MEF2D in the progression and development of gallbladder cancer.

SiMEF2D was used to knock down MEF2D in GBC-SD and SGC-996 cells, and the transfection efficiency was determined by analyzing the mRNA and protein levels of MEF2D (all $p<0.001$, Figures 2C and 2D). MTT assay results showed that siMEF2D significantly decreased the viable ability of GBC-SD and SGC-996 cells as compared to siNC control group (all $p<0.001$, Figure 2E). The colony formation assay revealed that the colony formation number in siMEF2D group was

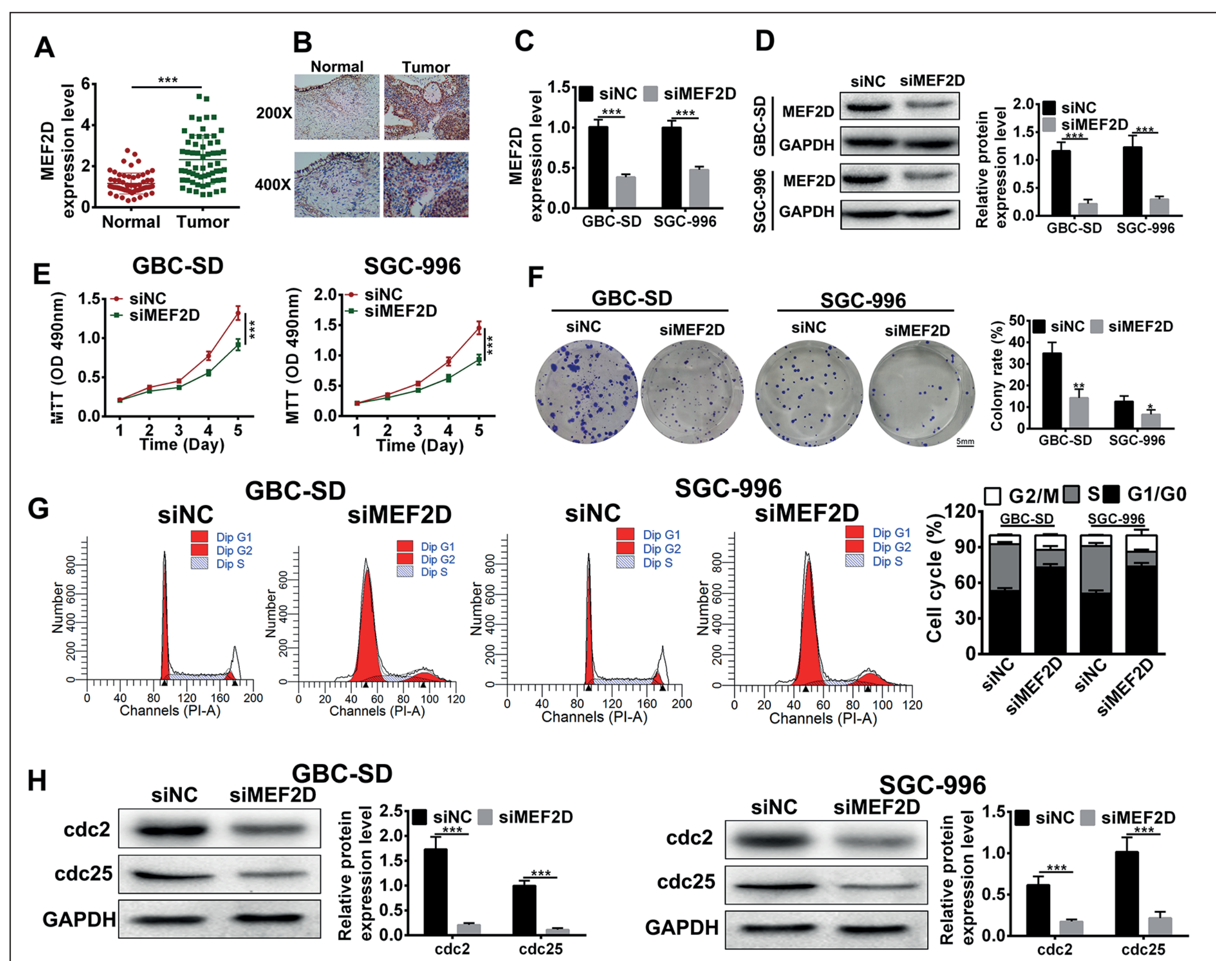

Figure 2. Down-regulation of MEF2D inhibited gallbladder cancer cell growth. **A,** QRT-PCR was used to determine the mRNA level of MEF2D in patients with gallbladder carcinoma. **B,** Immunohistochemistry was performed to detect the expression of MEF2D in patients with gallbladder carcinoma (under microscope of 200 and 400 magnifications). **C,** QRT-PCR was performed to determine the mRNA level of MEF2D in cells that transfected with siMEF2D. **D,** Western Blot was used to determine the protein level of MEF2D in cells that transfected with siMEF2D. **E,** MTT assay was used to detect the viability of cells that transfected with siMEF2D. **F,** Colony formation ability of cells that transfected with siMEF2D (Scale bar, 5 mm). **G,** Flow cytometer was performed to determine cell cycle of cells that transfected with siMEF2D. **H,** Western Blot was used to determine the protein expression levels of cdc2 and cdc25 in cells that transfected with siMEF2D. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$.

significantly decreased as compared to siNC control group (all $p<0.001$, Figure 2F). In addition, the cell percentage in G1/G0 phase was significantly increased in the siMEF2D group as compared with the siNC control group (all $p<0.001$, Figure 2G). Consistently, siMEF2D also significantly decreased the expression levels of cell cycle-related proteins cdc2 and cdc25 in gallbladder cancer cells (all $p<0.001$, Figure 2H). These results indicated that MEF2D was up-regulated in gallbladder cancer and associated with tumor size and clinical stage, and down-regulation of MEF2D inhibited gallbladder cancer cell viability and colony formation, and induced cell cycle arrest.

MEF2D Was Identified as a Target of MiR-335

Based on the completely opposite effects of miR-335 and MEF2D on gallbladder cancer cell growth, we inferred that MEF2D might be a target gene of miR-335. The relation between miR-335 and *MEF2D* was predicted by TargetScan (http//www.targetscan.org). The potential binding sites were shown in Figure 3A. The luciferase assay revealed that miR-335 mimic significantly decreased the luciferase activity of cells transfected with wild type of MEF2D 3'-UTR sequence ($p<0.001$), but had no significant effects on luciferase activity of cells transfected with

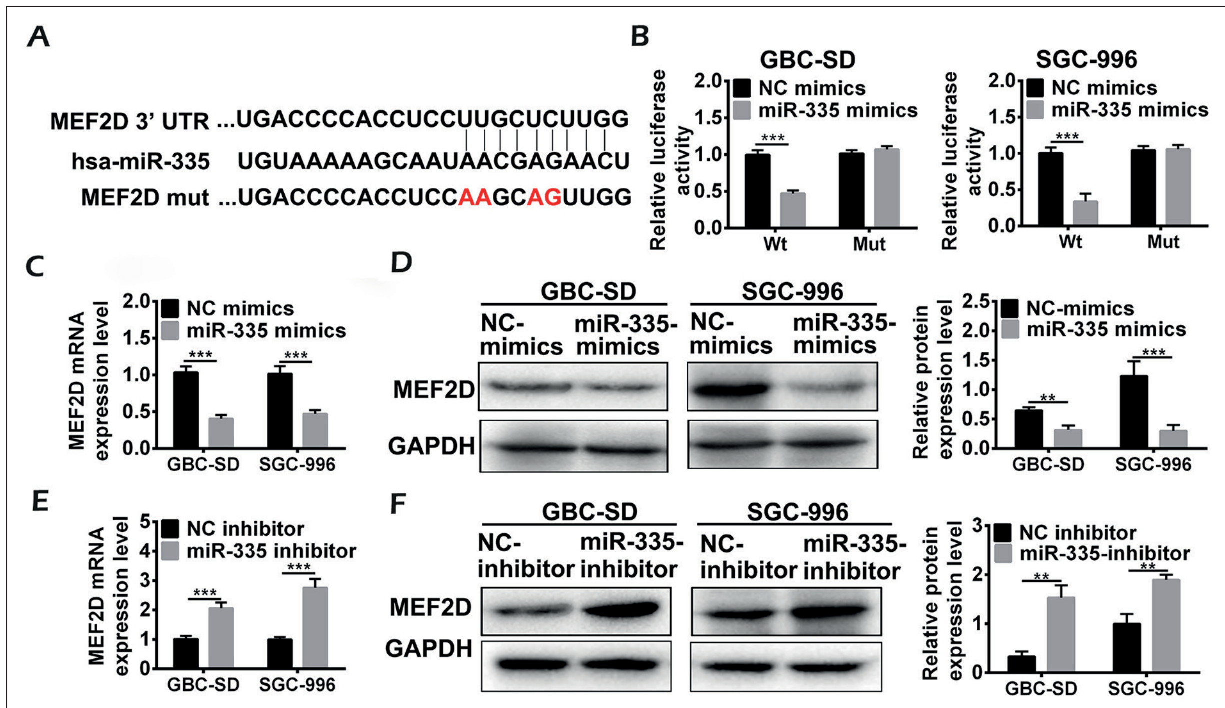

Figure 3. MEF2D was identified as a target of miR-335. **A,** Putative binding sequences between miR-335 and *MEF2D*. **B,** Luciferase assay was performed to explore the relationship between miR-335 and *MEF2D*. **C,** QRT-PCR was conducted to determine MEF2D mRNA levels in cells transfected with miR-335 mimics. **D,** Western Blot was performed to examine the protein level of MEF2D in cells transfected with miR-335 mimics. **E,** QRT-PCR was used to determine the mRNA level of MEF2D in cells transfected with miR-335 inhibitor. **F,** Western Blot was used to detect the protein level of MEF2D in cells with miR-335 inhibitor transfection. **: $p<0.01$. ***: $p<0.001$.

mutant type of MEF2D 3'-UTR ($p>0.05$, Figure 3B). In addition, miR-335 mimics significantly decreased the mRNA and protein expression levels of MEF2D in GBC-SD and SGC-996 cells (all $p<0.001$, Figures 3C and 3D). On the contrary, miR-335 inhibitor significantly increased the mRNA and protein level of MEF2D (all $p<0.001$, Figures 3E and 3F). Therefore, our results revealed that miR-335 could directly target and regulate MEF2D expression.

MiR-335 Inhibited Gallbladder Cancer Cell Growth by Inhibiting MEF2D Expression

We speculated that miR-355 might regulate the gallbladder cancer cell growth by regulating MEF2D expression, miR-335 inhibitor and siMEF2D were thus co-transfected into GBC-SD and SGC-996 cells, and MTT assay was performed. We found that the cell viability was significantly increased by miR-335 inhibitor ($p<0.001$), but was decreased after co-transfecting with miR-335 inhibitor and siMEF2D ($p<0.001$, Figure 4A). Colony formation assay results also proved that siMEF2D rescued the stimulating effects of miR-335 inhibitor on cell proliferation (Figure 4B). In addition, miR-335 inhibitor significantly decreased the cell numbers in the G1/G0 phase, while siMEF2D abolished these effects (all $p<0.001$, Figure 4C). MiR-335 inhibitor also significantly increased the expression of cdc2 and cdc25, and MEF2D knockdown showed reversed results (all $p<0.001$, Figure 4D). These investigations revealed that miR-335 inhibited gallbladder cancer cell viability and colony formation, and induced cell cycle arrest by inhibiting MEF2D expression.

Overexpression of MiR-335 Increased Sensitivity of Gallbladder Cancer Cells to 5-Fluoracil (5-Fu) Treatment

To explore the influence of miR-335 on the sensitivity of gallbladder cancer cells to 5- Fu treatment in GBC-SD and SGC-996 cells, miR-335 was overexpressed in GBC-SD and SGC-996 cells and treated with different concentrations of 5-Fu. As shown in Figure 5A, the vi-

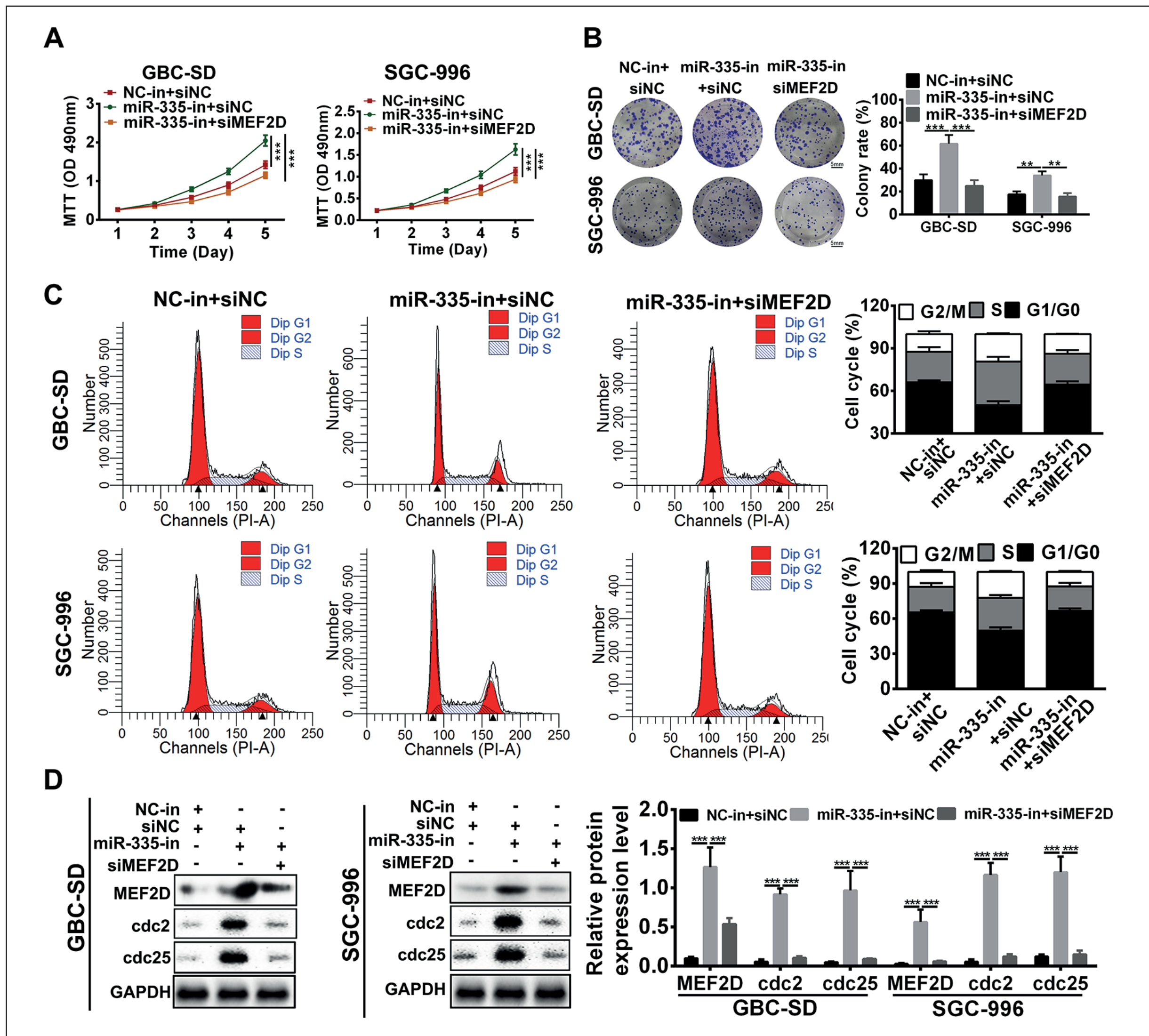

Figure 4. MiR-335 inhibited gallbladder cancer cell growth by inhibiting MEF2D expression. **A,** MTT was performed to detect the viability of GBC-SD and SGC-996 cells after co-transfected with miR-335 inhibitor and siMEF2D. **B,** Colony formation ability of GBC-SD and SGC-996 cells was determined after co-transfected with miR-335 inhibitor and siMEF2D (Scale bar, 5 mm). **C,** Flow cytometer was performed to detect cell cycle of GBC-SD and SGC-996 cells after s co-transfected withmiR-335 inhibitor and siMEF2D. **D,** Western Blot was used to examine the cdc2 and cdc25 expression in GBC-SD and SGC-996 cells that transfected with miR-335 inhibitor and siMEF2D. **: $p<0.01$. ***: $p<0.001$.

ability of 5-Fu-treated GBC-SD and SGC-996 cells in miR-335 mimics group was significantly lower than that in control group (all $p<0.001$, Figure 5A). In addition, miR-335 mimics decreased the protein expression levels of ABCB1 and ABCG2 (two drug resistance-related proteins) as compared to the control group (all $p<0.05$, Figure 5B). Therefore, these results demonstrated that overexpression of miR-335 increased the sensitivity of gallbladder cancer cells to 5-Fu.

Discussion

Although surgery, radiotherapy, and chemotherapy are developed for the treatment of gallbladder carcinoma, which is still a highly fatal disease with poor prognosis[5]. Therefore, explicating independent prognosticators and exploring potentially curative treatment options are necessary. Based on the fact that miR-335 was involved in the progression of cancers related to digestive system[10,11,21], we thus inferred that miR-335 might

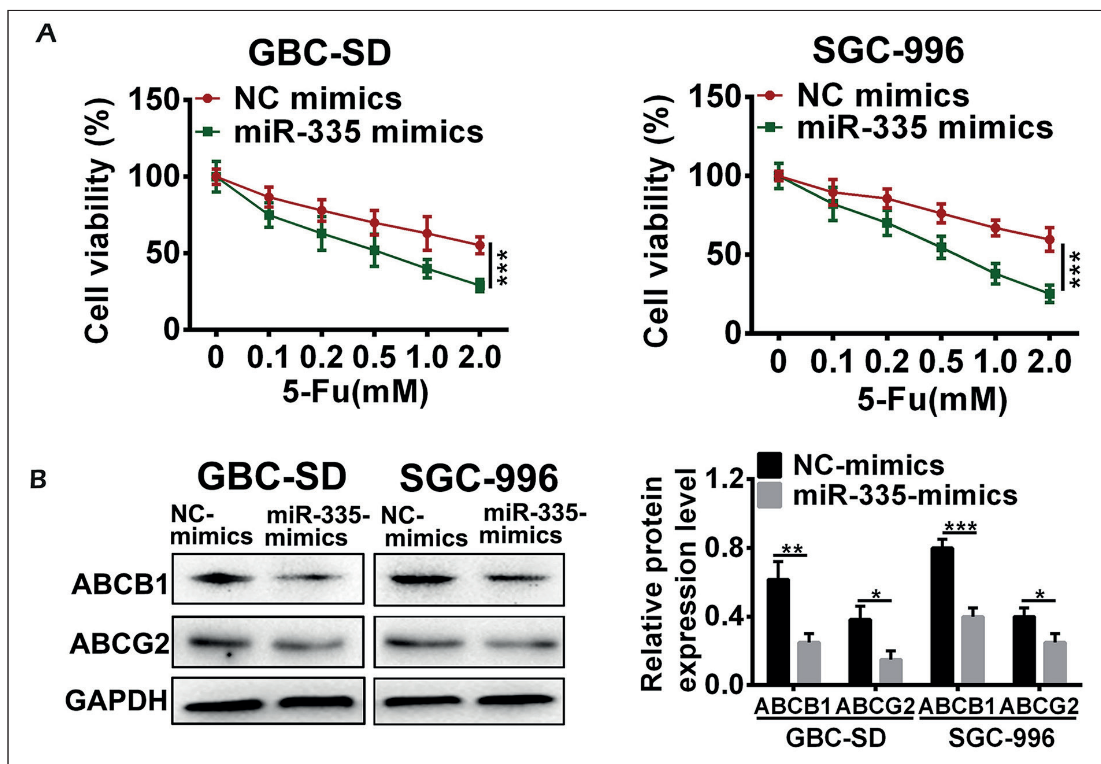

Figure 5. Overexpression of miR-335 increased the sensitivity of gallbladder cancer cells to 5-Fu treatment. **A,** MTT assay was performed to determine the viability of GBC-SD and SGC-996 cells that transfected with miR-335 mimics and treated with different concentrations of 5-Fu. **B,** Western Blot was performed to detect the protein expression levels of ABCB1 and ABCG2. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$.

also participate in the regulation of gallbladder carcinoma and the present experiment was performed.

In many cancers, scholars[22-24] proved that miRNAs showed abnormal expression. In digestive system cancers, such as gastric cancer[10] and CRC[11], the expression level of miR-335 was decreased. However, the up-regulation of miR-335 was also found in some cancers such as multiple myeloma[25] and acute leukemia[26]. These opposite results might be caused by tissue differences. In bladder cancer, miR-335 levels were decreased[27]. Particularly, Peng et al[12] showed that miR-335 levels were decreased in 96 primary gallbladder carcinoma patients among 166 cases. Therefore, we investigated the influence of miR-335 on cell growth of gallbladder carcinoma after miR-335 was overexpressed in GBC-SD and SGC-996 cell lines. The results revealed that miR-335 overexpression inhibited cell viability, colony formation, and induced cell cycle arrest in G1/G0 phase in GBC-SD and SGC-996 cells. These data indicated that miR-335 act as a tumor suppressor in gallbladder carcinoma.

Researches[17-20] showed that MEF2D was an important transcription factor in many cancers. Liu et al[18] proved that MEF2D expression was increased in glioma and could inhibit invasion and angiogenesis of glioma. Ma et al[28] showed that MEF2D was up-regulated in hepatocellular carcinoma (HCC) compared to normal tissues and silencing of MEF2D inhibited cell proliferation. We determined the role of MEF2D in GBC-SD and SGC-996 cells, and results demonstrated that the expression of MEF2D was increased in gallbladder carcinoma, and MEF2D level was significantly correlated with tumor size and clinical stage. The down-regulation of MEF2D inhibited cell viability, colony formation, and induced cell cycle arrest in GBC-SD and SGC-996 cells. These results indicated that MEF2D act as a tumor promoter in gallbladder carcinoma, which were in coincidence with previous studies[18,28].

Liu et al[18] showed that *MEF2D* was usually targeted by miRNA. For example, *MEF2D* was the target gene of miR-421 in glioma. MiR-218 could inhibit cell proliferation of lung carcinoma through decreasing MEF2D expression levels[29].

In our study, MEF2D and miR-335 had completely opposite effects on gallbladder carcinoma cell growth. The bioinformatics analysis and luciferase assay verified that miR-335 could target and negatively regulate *MEF2D* expression in gallbladder carcinoma cells. We also found that the suppressive role of miR-335 in cell growth was rescued by the down-regulation of MEF2D. Based on these results, we concluded that miR-335 inhibited gallbladder cancer cell growth by inhibiting MEF2D expression. In addition, we also found that miR-335 overexpression inhibited the viability of gallbladder carcinoma cells with different concentration of 5-Fu treatment and decreased the ABCB1 and ABCG2 levels, which indicated that overexpression of miR-335 increased the sensitivity of gallbladder cancer cells to 5-Fu.

Conclusions

This is the first evidence demonstrating the role of miR-335 and MEF2D in the progress and development of gallbladder carcinoma. MiR-335 participates in the progression of gallbladder carcinoma by targeting MEF2D. MiR-335 and MEF2D may be used as independent prognosticators and potential therapeutic targets for gallbladder carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

WW and QZ conceived and designed the experiments, LCC and JYQ performed the experiments.

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