circMTO1 sponges microRNA-219a-5p to enhance gallbladder cancer progression via the TGF-β/Smad and EGFR pathways

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Abstract. Circular mitochondrial translation optimization 1 homologue (circMTO1) has been reported to regulate the tumorigenesis of different types of cancer; however, the role of circMTO1 in gallbladder cancer (GBC) remains unknown. The present study aimed to identify the potential miRNAs and target genes of circMTO1 during GBC progression, and clarify the regulatory mechanism between circMTO1 and miRNAs or target genes. The present study performed MTT and Transwell assays, and Annexin V staining to assess cell viability, migration and apoptosis, respectively. In addition, a lymphatic vessel formation assay was performed to assess tube formation of human dermal lymphatic endothelial cells (HDLECs), and GBC-SD and NOZ cells. The results demonstrated that circMTO1 knockdown significantly attenuated the viability and migration of GBC cells and tube formation of HDLECs, and promoted apoptosis, indicating a tumor-promoting role of circMTO1. In addition, transfection with microRNA (miRNA/miR)-219a-5p inhibitor rescued short hairpin RNA-circMTO1-inhibited tumorigenesis of GBC cells, suggesting that miR-219a-5p acts as a downstream effector for circMTO1. Mechanistically, transfection with miR-219a-5p mimic suppressed the expression levels of Smad2/4 and epidermal growth factor receptor. Analysis of The Cancer Genome Atlas datasets revealed that circMTO1 expression was associated with overall survival and the stage of patients with GBC. Taken together, the results of the present study provide novel insight for the role of circMTO1-induced GBC tumorigenesis via regulation of miR-219a-5p expression.

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Introduction

Gallbladder cancer (GBC) is considered the most common bile tract cancer (1), and patients with advanced disease have a 5-year survival rate of <5% (2). Given that there are no obvious symptoms and relevant biomarkers for GBC, patients are commonly diagnosed at advanced stages (3,4). Chemotherapy, radiotherapy and surgical resection remain the most common treatment strategies for GBC (5). Despite advancements in treatment approaches, the overall survival rate of patients with GBC remains low (5). Thus, it is important to determine the molecular mechanisms underlying GBC tumorigenesis.

Circular RNAs (circRNAs) are derived from endogenous non-coding RNAs with a closed loop structure (6). circRNAs have been reported to play important roles in different types of cancer by binding to microRNAs (miRNAs/miRs) (7). Circular mitochondrial translation optimization 1 homologue (circMTO1) inhibits progression of gastric cancer (8), colorectal cancer (9), bladder cancer (10) and glioblastoma (11). However, the role of circMTO1 in GBC remains unknown. Recently, Wang *et al* (12) demonstrated that circMTO1 acts as a biomarker in GBC. Shi *et al* (13) reported that overexpression of circMTO1 suppresses cell apoptosis via flow cytometry. Thus, it is important to determine the molecular mechanism underlying circMTO1-induced GBC tumorigenesis.

miRNAs are small non-coding RNAs of 20-22 nucleotides in length, which regulate gene expression at the post-transcriptional level (14). Notably, miRNAs are involved in tumor progression (15,16). Previous studies have reported that miR-219a-5p inhibits cancer cell proliferation and migration (17,18). In addition, miR-219a-5p can sensitize non-small cell lung cancer cells to cisplatin by regulating fibroblast growth factor 9 expression (17). miR-219a-5p promotes irradiation-induced apoptosis of non-small cell lung cancer cells (19). Furthermore, miR-219a-5p can also induce neuronal apoptosis (20). Based on these findings, it was hypothesized that miR-219a-5p acts as a potential effector for circMTO1 in GBC.

The present study aimed to determine the underlying molecular mechanism during GBC progression. In addition, rationales for diagnosing and treating GBC via the circMTO1-targeted approach are discussed.

Materials and methods

Cell culture. Human GBC-SD cells were purchased from Kunming Cell Bank, while NOZ cells were purchased from the Japanese Collection of Research Bioresources Cell Bank. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all purchased from Gibco; Thermo Fisher Scientific, Inc.), at 37˚C with 5% CO_2, according to the manufacturer's instructions. Human dermal lymphatic endothelial cells (HDLECs; PromoCell GmbH) and maintained in endothelial cell growth medium (PromoCell GmbH), at 37˚C with 5% CO_2.

Generating stable cell lines. To generate stable cell lines, 1 μg pLKO.1-short hairpin (sh)RNAs (Invitrogen; Thermo Fisher Scientific, Inc.), packaging vectors 0.5 μg pCMV-VSVG (Addgene, Inc.) and 0.5 μg pCMV-PAX2 (Addgene, Inc.) were introduced into 293T cells to generate 2nd lentiviruses. Lentiviruses were collected from the medium after 36-48 h of transfection and maintained in fresh media. We used lentiviruses (MOI=5) to infect into GBC-SD and NOZ cells for 24 h. Puromycin (2 μg/ml) was used to obtain pooled resistant cells after 24 h and 1 μg/ml of puromycin were used for maintenance. After 1 week, subsequent experiments were performed. The following sequences were used: sh-negative control (NC), 5'-AUCAGCCAAUCGGUCAACCUUC-3'; sh-circMTO1-1, 5'-GUGGGGUUGUUUUGGGUCAGA-3'; and sh-circMTO1-2, 5'-GUUGUUUUGGGUCAGAUG UCA-3'.

Cell transfection. miRNAs (50 μM; Shanghai GenePharma Co., Ltd.) were transfected into GBC-SD and NOZ cells using Lipofectamine® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following incubation for 48 h at 37˚C, the cells were collected for subsequent analysis. The following sequences were used: NC mimic, 5'-CAUUCAUCCAUCAAUCGGGCAGGC CUUUAAGCUAACAUGGAA-3'; miR-219a-5p mimic, 5'-UGAUUGUCCAAACGCAAUUCUAAUUGCGUUUGG ACAAUCAUU-3'; and NC inhibitor, 5'-UUCAGGCAAUCC AAAUGCAGG-3'; miR-219a-5p inhibitor, 5'-AGAAUU GCGUUUGGACAAUCA-3'.

StarBase and TargetScan prediction. The StarBase 2.0 online software (http://starbase.sysu.edu.cn/starbase2) created by Sun Yat-sen University was used to predict the potential interacting miRNAs. In addition, TargetScan v7.2 (http://www.targetscan.org) created by MIT was used to predict protein-coding genes as potential targets.

MTT assay. GBC-SD and NOZ cells were seeded into 96-well plates at a density of ~5,000 cells/well. MTT reagent was added to each well and incubated for 4 h at 37˚C with 5% CO_2. Following the MTT incubation, the purple formazan crystals were dissolved using 150 μl DMSO for 10 min and viability was subsequently analyzed at a wavelength of 490 nm.

Migration assay. GBC cells ($1x10^5$ cells) were suspended in 200 μl of DMEM media (Gibco; Thermo Fisher Scientific, Inc.). In this assay, the upper chambers had 8-μm-pore membranes and incubated for 4-6 h at 37˚C. DMEM media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS was added to the lower chambers and incubated for 24 h at 37˚C. Migratory cells were stained with 0.005% crystal violet dye for 2 h at room temperature and observed under an invert light microscope (magnification, x200).

Immunofluorescence. GBC cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Triton was subsequently used to permeabilize the cell membranes. Annexin V-FITC (eBioscience; Thermo Fisher Scientific, Inc.) was used to probe the signal, and cell nuclei were stained with DAPI at room temperature for 30 min and observed under a fluorescence microscope (magnification, x200).

Western blotting. GBC cells were lysed using RIPA buffer and total proteins were extracted. The Rapid Gold BCA method (Thermo Fisher Scientific, Inc.) was used to determine protein concentrations, according to the manufacturer's protocol and ~50 μg total protein/lane was separated via SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto PVDF membranes and blocked with 5% non-fat milk at room temperature for 1 h. The membranes were incubated with primary antibodies against Smad2 (cat. no. 5339), Smad4 (cat. no. 46535), epidermal growth factor receptor (EGFR; cat. no. 2085), cleaved caspase-3 (cat. no. 25546-1-AP) and GAPDH (cat. no. 8884) overnight at 4˚C (all 1:1,000 and purchased from Cell Signaling Technology, Inc.). Following the primary incubation, membranes were incubated with anti-rabbit HRP-conjugated IgG (cat. no. 7074) and anti-mouse HRP-conjugated IgG (cat. no. 7076) secondary antibodies at room temperature for 1 h (both 1:2,000 and purchased from Cell Signaling Technology, Inc.). Protein bands were visualized using ECL reagents (Thermo Fisher Scientific, Inc.) and Bio-Rad gel imaging machine (Bio-Rad Laboratories, Inc.).

The Cancer Genome Atlas (TCGA) analysis. The gene expression data (RNAseq, NGS) of GBC were downloaded from TCGA dataset (36 tumor tissues and nine normal tissues, https://tcga.xenahubs.net). The present study did not have any inclusion or exclusion criteria. circMTO1 expression was analyzed using a two-tailed unpaired Student's t-test. Clinical characteristics of patients with gallbladder cancer were presented in Table I.

Patient sorting criterion. The patients were sorted into high and low expression groups according to the median circMTO1 RNA expression (cut-off value=2.8) in patients with GBC tumors. A total of 18 patients were classified into the high circMTO1 expression group and 18 into the low circMTO1 expression group.

Tube formation assay. GFR Matrigel was mixed with PBS (1:5) and coated in 24-well plates at 37˚C for 4-5 h. HDLECs were subsequently seeded into the plates (12 wells) at a density of ~5,000 cells/well, and the tubes were observed under an inverted light microscope (magnification, x200).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from GBC cells using TRIzol® reagent

Table I. Clinical characteristics of patients with gallbladder cancer (n=36).

Characteristic	Patients, n
Age, years	
≤60	17
>60	19
Sex	
Male	20
Female	16
Cholecystolithiasis	
Absent	28
Present	8
Diabetes	
Absent	12
Present	24
Jaundice	
Absent	21
Present	15
Pathological type	
Adenocarcinoma	36
Adenosquamous carcinoma	0
Papillocarcinoma	0
Degree of differentiation	
Poor	22
Moderate-well	14
T stage	
T1	10
T2	9
T4	17

(Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) at 37˚C for 15 min and 85˚C for 1 min. qPCR was subsequently performed to measure relative gene expression levels, using the $2^{-\Delta\Delta Cq}$ method (21). SYBR Green dye (Roche Applied Science) was used for qPCR. The following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min (step 1), 95˚C for 30 sec (step 2), 60˚C for 30 sec (step 3), 72˚C for 30 sec (step 4), 40 cycles (step 2-4). β-actin was used as the reference gene for circMTO1, Smad2, Smad4 and EGFR. U6 was used as the reference gene for miR-219a-5p. The following primer sequences were used for qPCR: circMTO1 forward, 5'-GCC TGAACACACTGGGAAAT-3' and reverse, 5'-CACAGATGC GAGAACACAGG-3'; Smad2 forward, 5'-CTTTGTGCAGAG CCCCAATT-3' and reverse, 5'-CTTGTTACCGTCTGCCTT CG-3'; Smad4 forward, 5'-TCCAGCCTCCCATTTCCAAT-3' and reverse, 5'-ACCTTGCTCTCTCAATGGCT-3'; EGFR forward, 5'-AGGTGAAAACAGCTGCAAGG-3' and reverse, 5'-AGGTGATGTTCATGGCCTGA-3'; miR-219a-5p forward, 5'-CTCCTGATTGTCCAAAC-3' and reverse, 5'-CGCTCG AGGTTTGGGG-3'; β-actin forward, 5'-TGGCATCCACGA AACTACCT-3' and reverse, 5'-TCTCCTTCTGCATCCTG TCG-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test was used to compare differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

circMTO1 knockdown impairs GBC tumorigenesis. To determine the role of circMTO1 in GBC progression, circMTO1 expression was suppressed using shRNAs (sh-circMTO1-1 and sh-circMTO1-2). RT-qPCR analysis was performed to detect circMTO1 expression. The results demonstrated that circMTO1 expression significantly decreased (~70-80%) in circMTO1 knockdown cells compared with sh-NC cells (Fig. 1A) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0002; sh-circMTO1-2 vs. sh-NC, P=0.0003; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0005; sh-circMTO1-2 vs. sh-NC, P=0.0006).

circMTO1 has been reported to promote tumorigenesis of cervical cancer (22). The MTT assay was performed to assess the viability of GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 and sh-circMTO1-2. The results demonstrated that cells transfected with sh-circMTO1-1 or sh-circMTO1-2 exhibited evidently lower viability compared with sh-NC cells (Fig. 1B) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0003; sh-circMTO1-2 vs. sh-NC, P=0.0004; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0002; sh-circMTO1-2 vs. sh-NC, P=0.0007). Annexin V staining was performed to assess apoptosis of GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2. The results indicated that circMTO1 knockdown increased the apoptotic rate of cells (Fig. 1C and D) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0005; sh-circMTO1-2 vs. sh-NC, P=0.0063; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0008; sh-circMTO1-2 vs. sh-NC, P=0.036). To confirm, western blot analysis was performed to detect cleaved caspase-3 protein expression in GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2. The results demonstrated that cleaved caspase-3 protein expression was upregulated in circMTO1 knockdown cells (Fig. 1E). In addition, the effect of circMTO1 on tube formation of HDLECs was investigated. The results indicated that circMTO1 knockdown attenuated tube formation of HDLECs (Fig. 1F and G) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0004; sh-circMTO1-2 vs. sh-NC, P=0.0003; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0007; sh-circMTO1-2 vs. sh-NC, P=0.0005). The results of the Transwell assay demonstrated that circMTO1 knockdown decreased the number of migratory GBC-SD cells (Fig. 1H). Taken together, these results suggest that circMTO1 acts as an oncogene in GBC progression.

miR-219a-5p inhibitor partially restores sh-circMTO1-attenuated GBC tumorigenesis. The StarBase database was used to determine the potential molecular mechanism underlying circMTO1-induced tumorigenesis

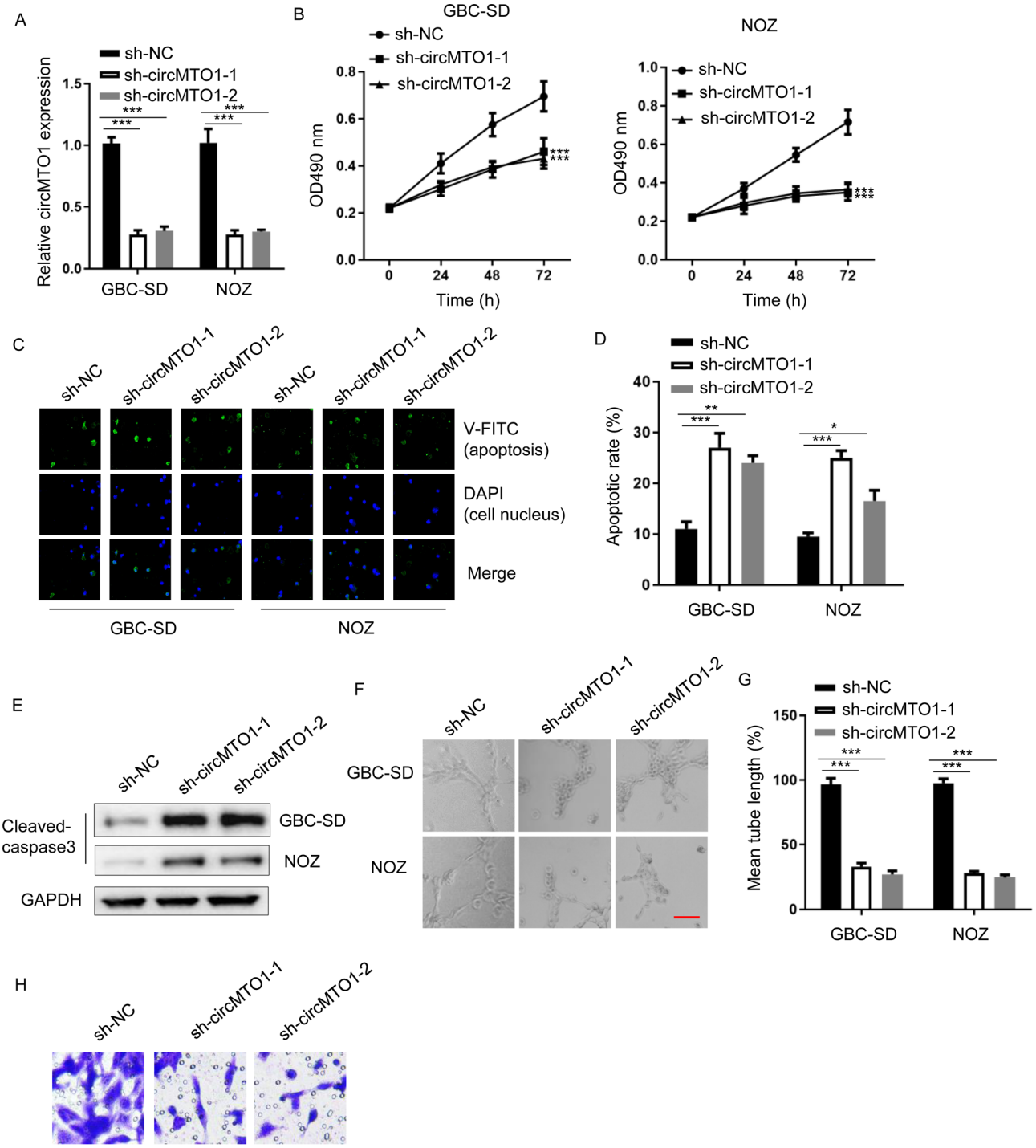

Figure 1. circMTO1 knockdown impairs GBC tumorigenesis. (A) Reverse transcription-quantitative PCR analysis was performed to detect circMTO1 expression in GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2. (B) The MTT assay was performed to assess the viability of GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2. (C and D) Annexin V staining was performed to assess the apoptosis of GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2. (E) Western blot analysis was performed to assess cleaved caspase-3 protein expression in GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2. GAPDH was used as the internal control. (F and G) The lymphatic vessel formation assay was performed to assess tube formation of human dermal lymphatic endothelial, GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2 (scale bar, 2 μm). (H) The Transwell assay was performed to assess the migratory ability of GBC-SD cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-2. *P<0.05; **P<0.01; ***P<0.001. circMTO1, circular mitochondrial translation optimization 1 homologue; GBC, gallbladder cancer; sh, short hairpin; NC, negative control; OD, optical density.

of GBC. Analysis revealed that miR-219a-5p is an interacting molecule for circMTO1 in GBC (Fig. 2A). RT-qPCR analysis demonstrated that miR-219a-5p expression was substantially regulated in GBC-SD or NOZ cells transfected with NC mimic, miR-219a-5p mimic, NC inhibitor or miR-219a-5p inhibitor (Fig. 2B) (GBC-SD, miR-219a-5p mimic vs. NC mimic, P=0.0004; miR-219a-5p inhibitor vs. NC inhibitor, P=0.0005; NOZ, miR-219a-5p mimic vs. NC mimic, P=0.0003; miR-219a-5p inhibitor vs. NC inhibitor, P=0.0003).

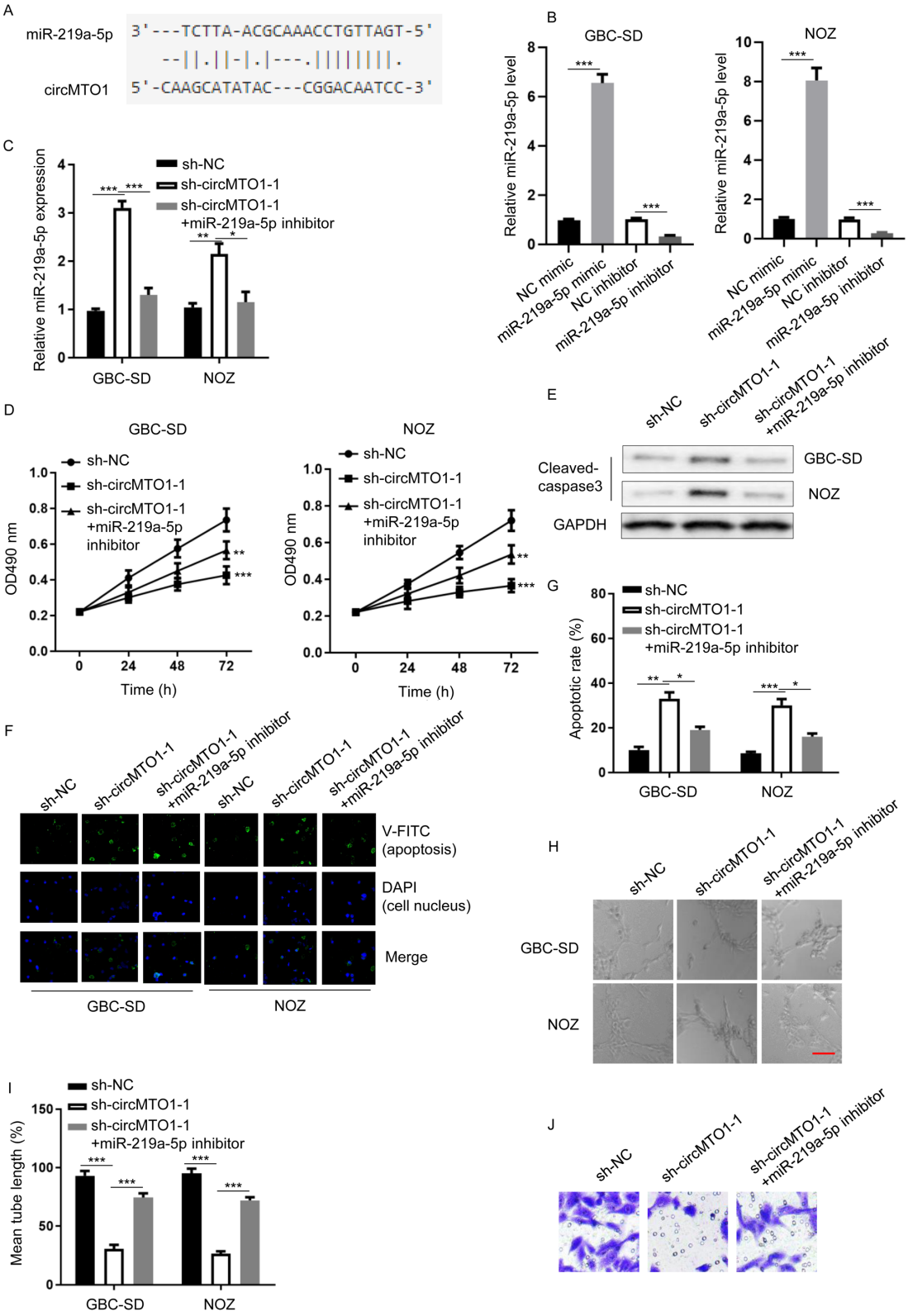

Figure 2. miR-219a-5p inhibitor partially restores sh-circMTO1-attenuated GBC tumorigenesis. (A) Bioinformatics analysis revealed that miR-219-5p is a potential target of circMTO1. (B) RT-qPCR analysis was performed to detect miR-219a-5p expression in GBC-SD and NOZ cells transfected with NC mimic, miR-219a-5p mimic, NC inhibitor or miR-219a-5p inhibitor. (C) RT-qPCR analysis was performed to detect miR-219a-5p expression in GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-1 + miR-219a-5p inhibitor. (D) The MTT assay was performed to assess the viability of GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-1 + miR-219a-5p inhibitor. (E) Western blot analysis was performed to detect cleaved caspase-3 protein expression in GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-1 + miR-219a-5p inhibitor. GAPDH was used as the internal control. (F and G) Annexin V staining was performed to assess the apoptosis of GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-1 + miR-219a-5p inhibitor. (H and I) The lymphatic vessel formation assay was performed to assess tube formation of human dermal lymphatic endothelial, GBC-SD and NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-1 + miR-219a-5p inhibitor (scale bar, 2 μm). (J) The Transwell assay was performed to assess the migratory ability of NOZ cells transfected with sh-NC, sh-circMTO1-1 or sh-circMTO1-1 + miR-219a-5p inhibitor. *P<0.05; **P<0.01; ***P<0.001. miR, microRNA; sh, short hairpin; circMTO1, circular mitochondrial translation optimization 1 homologue; GBC, gallbladder cancer; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

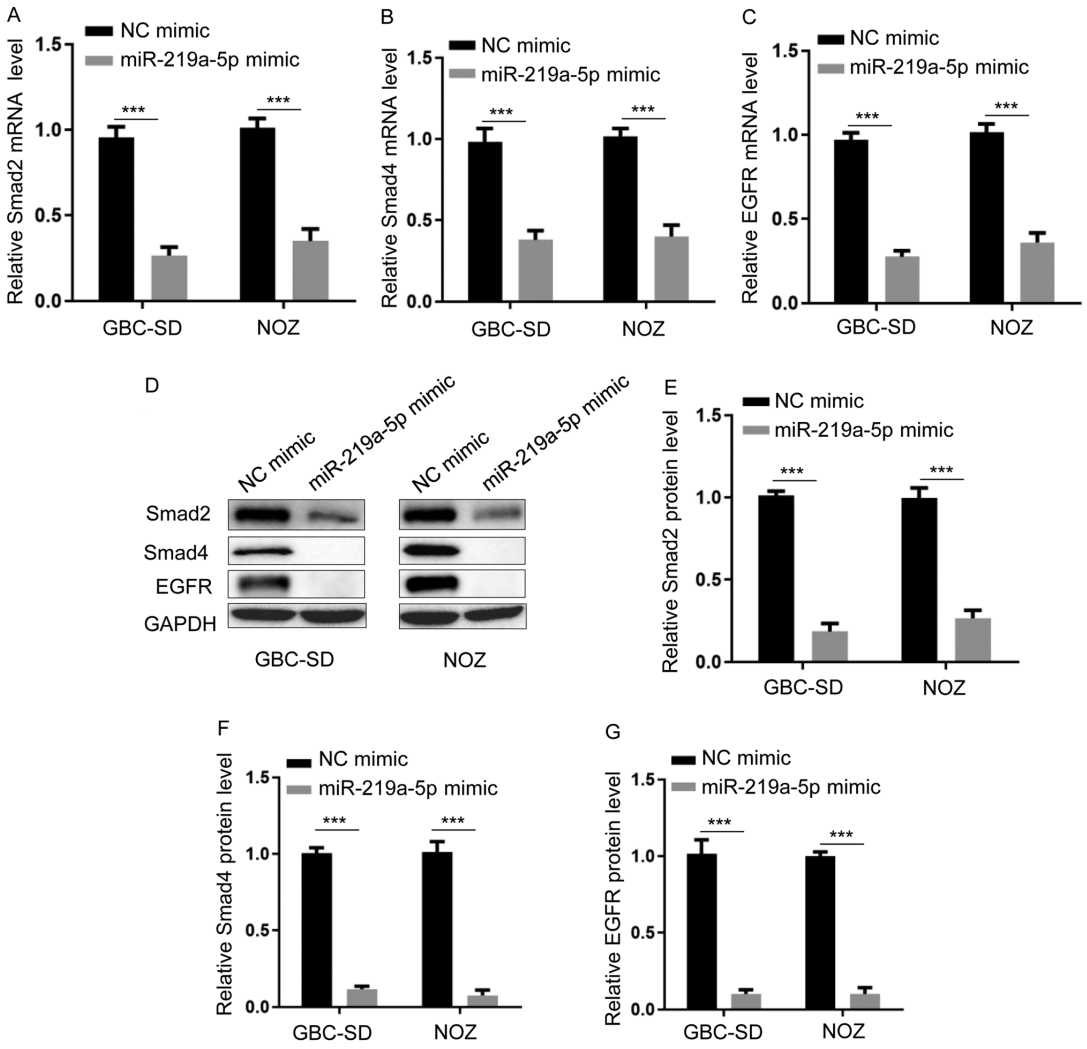

Figure 3. miR-219a-5p regulates the TGF-β/Smad pathway and EGFR expression. (A-C) Reverse transcription-quantitative PCR analysis was performed to detect the mRNA expression levels of Smad2, Smad4 and EGFR in GBC-SD and NOZ cells transfected with NC mimic or miR-219a-5p mimic. (D-G) Western blot analysis was performed to detect the protein expression levels of Smad2, Smad4 and EGFR in GBC-SD and NOZ cells transfected with NC mimic or miR-219a-5p mimic. ***P<0.001. miR, microRNA; NC, negative control. miR, microRNA; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; NC, negative control.

The function of miR-219a-5p in circMTO1-regulated GBC phenotypes was investigated. RT-qPCR analysis demonstrated that miR-219a-5p expression was upregulated following circMTO1 knockdown (Fig. 2C) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0002; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.0006; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0067; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.028). The results of the MTT assay demonstrated that transfection with miR-219a-5p inhibitor rescued the viability of GBC-SD or NOZ cells attenuated by circMTO1 knockdown (Fig. 2D) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0005; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.0036; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0007; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.0083). Western blot analysis revealed that cleaved caspase-3 protein expression was upregulated in circMTO1 knockdown cells, the effects of which were reversed following transfection with miR-219a-5p inhibitor (Fig. 2E). In addition, Annexin V staining demonstrated that transfection with miR-219a-5p inhibitor decreased the apoptotic rate of GBC-SD or NOZ cells enhanced by circMTO1 knockdown (Fig. 2F and G) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0072; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.032; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0007; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.014). Notably, transfection with miR-219a-5p inhibitor enhanced sh-circMTO1-modulated tube formation of HDLECs (Fig. 2H and I) (GBC-SD, sh-circMTO1-1 vs. sh-NC, P=0.0002; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.0003; NOZ, sh-circMTO1-1 vs. sh-NC, P=0.0006; sh-circMTO1-1 + miR-219a-5p inhibitor vs. sh-circMTO1-1, P=0.0003). The results of the Transwell assay indicated that circMTO1 knockdown decreased migration of NOZ cells, which was reversed following transfection with miR-219a-5p inhibitor (Fig. 2J). Collectively, these results suggest that miR-219a-5p serves as a downstream effector for circMTO1 in GBC cells.

miR-219a-5p regulates the TGF-β/Smad pathway and EGFR expression. To further investigate the detailed

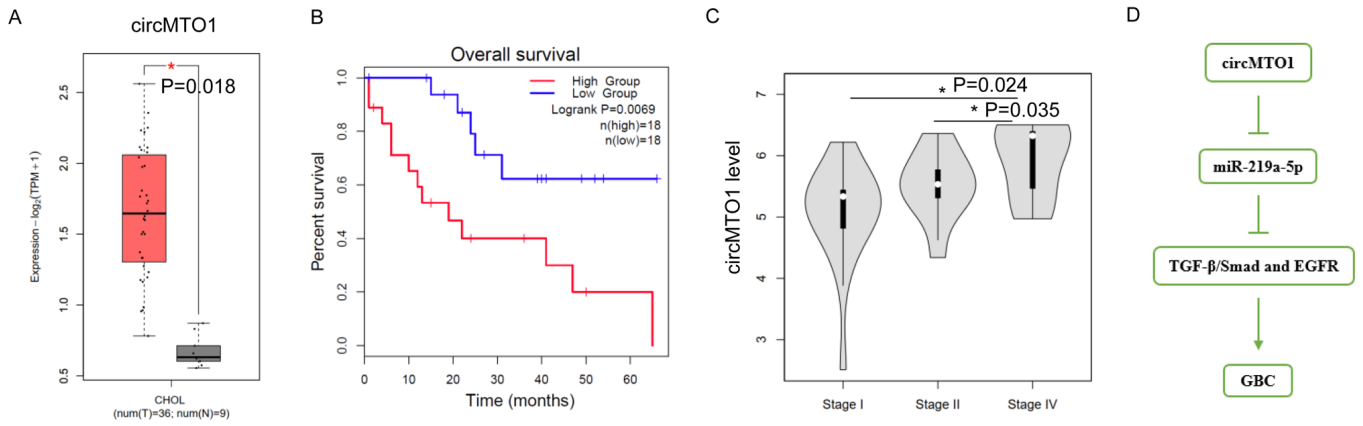

Figure 4. circMTO1 is closely associated with GBC progression. (A) circMTO1 expression in clinical normal tissues (n=9) and GBC tissues (n=36). (B) Overall survival analysis of patients with GBC, with high or low circMTO1 expression levels. (C) circMTO1 expression was measured at different stages of GBC. Stage I, the tumor has grown into the lamina propria or the muscle layer (muscularis); stage II, the cancer has grown through the muscle layer into the fibrous tissue on the side of the peritoneum or liver, but has not invaded the liver, and stage IV, the tumor has grown into one of the main blood vessels leading into the liver (portal vein or hepatic artery) or it has grown into 2 or more structures outside of the liver. (D) Mechanistic model of circMTO1-induced GBC. *P<0.05. circMTO1, circular mitochondrial translation optimization 1 homologue; GBC, gallbladder cancer; miR, microRNA; TGF, transforming growth factor; EGFR, epidermal growth factor receptor.

downstream pathway responsible for miR-219a-5p-associated GBC progression, TargetScan software was used to detect Smad2/4 and EGFR expression. RT-qPCR analysis demonstrated that transfection with miR-219a-5p mimic inhibited the expression levels of Smad2, Smad4 and EGFR (Fig. 3A-C) (Fig. 3A, GBC-SD, miR-219a-5p mimic vs. NC mimic, P=0.0003; NOZ, miR-219a-5p mimic vs. NC mimic, P=0.0005. Fig. 3B, GBC-SD, miR-219a-5p mimic vs. NC mimic, P=0.0004; NOZ, miR-219a-5p mimic vs. NC mimic, P=0.0008. Fig. 3C, GBC-SD, miR-219a-5p mimic vs. NC mimic, P=0.0005; NOZ, miR-219a-5p mimic vs. NC mimic, P=0.0004). Similarly, western blot analysis demonstrated that transfection with miR-219a-5p mimic decreased the protein expression levels of Smad2, Smad4 and EGFR (Fig. 3D-G) (Fig. 3E, GBC-SD, miR-219a-5p mimic vs. NC mimic, P=0.0002; NOZ, miR-219a-5p mimic vs. NC mimic, P=0.0003. Fig. 3F, GBC-SD, miR-219a-5p mimic vs. NC mimic, P=0.0004; NOZ, miR-219a-5p mimic vs. NC mimic, P=0.0007. Fig. 3G, GBC-SD, miR-219a-5p mimic vs. NC mimic, P=0.0006; NOZ, miR-219a-5p mimic vs. NC mimic, P=0.0005). Taken together, these results suggest that miR-219a-5p modulates the TGF-β/Smad pathway and EGFR expression.

circMTO1 is closely associated with GBC progression. To further determine whether circMTO1 plays a role in GBC, GBC datasets were downloaded from TCGA database. As expected, the results demonstrated that circMTO1 expression was higher in patients with GBC compared with healthy individuals (P=0.018; Fig. 4A). Notably, patients with low circMTO1 expression had prolonged overall survival times than those with high circMTO1 expression (P=0.0069; Fig. 4B). In addition, patients with high circMTO1 expression were associated with advanced disease (stage II vs. stage I; P=0.024; stage IV vs. stage II, P=0.035; Fig. 4C). Finally, we proposed the working model for circMTO1-induced GBC progression via miR-219a-5p (Fig. 4D). Collectively, these results suggest that circMTO1 is closely associated with GBC progression.

Discussion

The results of the present study confirmed that circMTO1 interacts with miR-219a-5p to promote the TGF-β/Smad signaling pathway and EGFR expression in GBC cells. To the best of our knowledge, the present study was the first to demonstrate that circMTO1 may serve as an oncogene in patients with GBC. The results demonstrated that circMTO1 expression was dysregulated in GBC cells and tumors. In addition, circMTO1 was involved in GBC progression. Based on these findings, circMTO1 may serve as a biomarker or target for diagnosis and treatment of GBC.

circRNAs are generated by back-splicing of 3' and 5' splice sites (23). As circRNAs are ubiquitously expressed in several tissues, they play important roles across various biological processes (24,25). Several circRNAs, including circERBB2, circFOXP1 and circHIPK3, have been reported to regulate GBC progression (26-28). Consistent with these findings, the results of the present study demonstrated that circMTO1 knockdown decreased cell viability and tube formation of HDLECs, while promoting the apoptosis of GBC cells. Notably, a few studies have reported that overexpression of circMTO1 enhances the apoptosis of gastric cancer cells and hepatoma cells (8,29). These findings oppose the results of the present study, thus it was hypothesized that circMTO1 may exert different roles in different types of cancer.

miRNAs play key roles in different types of human cancer by modulating gene expression levels (16). It has been reported that circRNAs sponge and decoy miRNAs in cancer cells (30). The results of the present study demonstrated that circMTO1 negatively regulated miR-219a-5p expression. Previous studies have verified that miR-92, miR-9 and miR-6893 are downstream effectors in different types of cancer (11,22,31). Thus, the results of the present study broaden the interacting spectrum of circMTO1. The results presented here demonstrated that miR-219a-5p inhibitor attenuated circMTO1 knockdown-induced apoptosis, which is consistent with previous studies (19,20). Long *et al* (32) reported that miR-219-5p targets

B-cell lymphoma 2 (Bcl-2) to inhibit melanoma growth and metastasis. Thus, it was hypothesized that circMTO1 interacts with miR-219-5p to regulate GBC progression by targeting anti-apoptotic genes, such as Bcl-2.

TGF-β signaling plays a key role in tumor progression, including epithelial-to-mesenchymal transition (EMT), via Smads and the MAPK pathway (33). A recent study suggested that TGF-β/Smad signaling promotes GBC metastasis by upregulating the miR-182/CADM1 axis (34). Smads exert their roles via phosphorylation (35). The results of the present study demonstrated that miR-219a-5p decreased Smad2/4 expression levels at the transcriptional level. Thus, detection of Smad 2/4 phosphorylation levels were not necessary and significant. In addition, miR-219a-5p downregulated EGFR expression in GBC cells, which was consistent with a previous study, suggesting that miR-219a-5p regulates EGFR to affect EMT of ovarian cancer cells (36). Notably, Shen *et al* (37) demonstrated that activation of the EGFR signaling pathway promotes gallbladder cancer invasion and metastasis. The authors reported that PLEK2 interacts with the kinase domain of EGFR and suppresses EGFR ubiquitination by c-CBL, which results in constitutive activation of EGFR signaling and elevated expression of the downstream effector, CCL2.

In conclusion, the results of the present study suggest that circMTO1 contributes to GBC progression via the miR-219a-5p/Smad/EGFR axis. This novel mechanism can be used to develop targeted therapeutic strategies for patients with GBC. However, the present study is not without limitations. First, further studies are required to determine the role of circMTO1 in different models (other GBC cell lines and animal models). Secondly, although the present study demonstrated that miR-219a-5p negatively regulated TGF-β signaling and EGFR, further studies are required to determine whether other downstream targets are involved in the circMTO1/miR-219a-5p axis-modulated GBC progression.

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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 upon reasonable request.

Authors' contributions

PW, CZ and YD designed the present study and performed the experiments. DL, LW and DZ analyzed the data and prepared the figures. PW and LW drafted the initial manuscript. DL, DZ and LW confirmed the authenticity of all the raw data. PW and YD reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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