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Abnormal expression and mechanism of miR-330-3p/BTG1 axis in hepatocellular carcinoma

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Abstract. – OBJECTIVE: Increasing evidence has suggested that microRNAs (miRNAs) played critical roles in cancer development by acting as a tumor suppressor or tumor-promoting genes. However, the role of microRNA-330-3p (miR-330-3p) in hepatocellular carcinoma (HCC) is still unknown. This study aimed to investigate the expression and role of miR-330-3p in hepatocarcinogenesis.

PATIENTS AND METHODS: A total of 30 human hepatocellular carcinoma tissues and adjacent normal tissues were obtained from 30 hepatocellular carcinoma patients. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was carried out to measure the expression of miR-330-3p in HCC tissues and cell lines. The relation between B-cell translocation gene 1 (BTG1) and miR-330-3p was predicted by TargetScan and confirmed by dual-luciferase reporter assay. Cell Counting Kit-8 (CCK-8), flow cytometry analysis, and transwell assay were used to determine cell viability, apoptosis, cell migration, and invasion, respectively. In addition, the mRNA and protein expression of Cyclin D1, Bcl-2, Bax, and matrix metalloproteinase (MMP)9 were detected using qRT-PCR and Western blotting.

RESULTS: We found that miR-330-3p expression was up-regulated in HCC tissues and cell lines. BTG1 was a direct target of miR-330-3p and it was down-regulated in HCC tissues and cell lines. Moreover, down-regulation of miR-330-3p suppressed HCCLM3 cell viability, migration, invasion, and enhanced cell apoptosis, while the tumor-suppressive effects were reversed by BTG1-siRNA. In addition, miR-330-3p inhibitor decreased the expression of Cyclin D1, Bcl-2, and MMP9 while enhanced the expression of Bax. Meanwhile, BTG1-siRNA led to the opposite effects.

CONCLUSIONS: The data suggested that miR-330-3p acted as a tumor gene in HCC by targeting BTG1 and it might be a potential therapeutic target for the HCC treatment.

Key Words:
MiR-330-3p, BTG1, Hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide[1]. Also, it is the second leading cause of cancer-related death with high morbidity and mortality[2-4]. In addition, the dysregulation of multiple genes results in the development and progression of HCC, making it become a complicated and intractable disease[5-7]. Although extensive research efforts have been made, the prognosis of HCC is still poor and the overall five-year survival rate worldwide is only about 3%[8]. Hence, understanding the pathogenesis of HCC development urgently needs to be defined. Therefore, exploring new biomarkers or therapeutic targets is essential for improving HCC diagnosis, treatment, and prevention.

MicroRNAs (miRNAs), endogenous and non-coding RNAs with 20-24 nucleotides in length, can regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of target genes[9-11]. Increasing evidence has indicated that miRNAs may act as oncogenes or tumor suppressors in various cancers[12,13]. As we all know, miRNAs play important roles in many cancers, including gallbladder cancer, cutaneous squamous cell carcinoma, osteosarcoma, bladder cancer, breast cancer, and ovarian carcinoma[14-18]. Moreover, evidence has demonstrated that miRNAs are involved in various biological processes such as cell development, proliferation, apoptosis, differentiation, migration, invasion, and differentiation[19-21]. Thus, concentrating on nuclear miRNAs can be a new insight into cancer therapy. Previous studies[22-26] have suggested that miR-330-3p is

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abnormally expressed in a variety of tumors, and it may act as a tumor-suppressor or a tumor-promotor in different types of cancers. However, as far as we know, the expression and regulatory function of miR-330-3p in HCC are still unclear. Therefore, in this study, we will investigate the expression and role of miR-330-3p in HCC.

B-cell translocation gene 1 (BTG1), belongs to the BTG anti-proliferative protein family, is initially identified as a transfer partner of the c-Myc gene in B-cell chronic lymphocytic leukemia[27,28]. Additionally, BTG1 is involved in the progression of several diseases by regulating various biological and cellular processes including cell proliferation, differentiation, apoptosis, angiogenesis, and survival[29-33]. However, the underlying regulatory mechanism of BTG1 in HCC remains poorly unknown.

Therefore, we aimed to investigate the effects and the potential mechanism of miR-330-3p and BTG1 in the progression of HCC. Our results showed vital roles for miR-330-3p in the development of HCC and provided new insights into the mechanisms by which miR-330-3p or BTG1 regulated the development of HCC.

Patients and Methods

Clinical Specimens Collection

A total of 30 human hepatocellular carcinoma tissues and adjacent normal tissues (>2 cm away from the edge of the tumors) were obtained from 30 hepatocellular carcinoma patients (age range: 28-69 years old; 20 male, 10 female; TNM stage II: 19, TNM stage III+IV: 11; all no alcoholism history) in Ming Zhou Hospital of Zhejiang University during surgical resection. None of these patients had received chemotherapy or radiotherapy before surgery. All specimens were rapidly frozen and stored in liquid nitrogen or at –80°C for subsequent experiments. Written consent was achieved from each patient approving the use of their specimens in this research. The investigation was approved by the Ethics Committee at Ming Zhou Hospital of Zhejiang University.

Cell Culture

HCC cell lines (MHCC-LM3, Huh7, MHCC97-L, HCCLM3) and the human normal liver cell line (LO2) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco, Rockville, MD, USA), supplemented with heat-inactivated 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1% penicillin/streptomycin and were incubated at 37°C in humidified atmosphere with 5% CO_2.

Cell Transfection and Reagents

The miR-330-3p inhibitor (5'-UCUCUGCAGGCCGUGUGCUUUGC-3'; GenePharma, Shanghai, China), inhibitor control (5'-CAGUACUUUUGUGUAGUACAA-3'; GenePharma, Shanghai, China), BTG1-siRNA (Cat No. sc-43644; Santa Cruz Biotechnology, Santa Cruz, CA, USA), control-siRNA (Cat No. sc-36869; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or miR-330-3p inhibitor+BTG1-siRNA were transfected into HCCLM3 cells for 48 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. QRT-PCR and/or Western blot assay was used to measure the transfection efficiency.

CCK-8 Assay

Cell Counting Kit-8 (CCK-8; Sigma-Aldrich, St. Louis, MO, USA) was used to detect the cell viability according to the manufacturer's instructions. Cells were seeded in 96-well plates ($1x10^4$ cells/well) and then incubated for 24 h at 37°C. Next, HCCLM3 cells were transfected with miR-330-3p inhibitor, inhibitor control, or miR-330-3p inhibitor+BTG1-siRNA for 48 h. Subsequently, the CCK-8 solution was added to each well and the cells were incubated for another 1 h. Finally, the micro-plate reader (Eon; BioTek, Winooski, VT, USA) was performed to measure the optical density (OD) at 450 nm.

Dual-Luciferase Reporter Assay

We used TargetScan Release7.2 (www.targetscan.org/vert_72) to predict the binding sites between miR-330-3p and BTG1. The BTG1 3'-UTR DNA segments were amplified by PCR and inserted into a pmirGLO vector (Promega, Madison, WI, USA) to construct the reporter vector BTG1-wild-type (BTG1-WT). Also, the mutant 3'-UTRs were cloned into a luciferase reporter vector (pmiR-REPORT; Promega, Madison, WI, USA) to form BTG1-mutated-type (BTG1-MUT) constructs. Then, HCCLM3 cells (5×10^4 cells per well) were co-transfected with mimic control (sence: 5'-UUCUCCGAACGUGUCACGUTT-3' and anti-sence: 5'-ACGUGACACGUUCGGAGAATT-3'; GenePharma, Shanghai, China) or miR-330-3p mimic (sence: 5'-GCAAAGCA-

CACGGCCUGCAGAGA-3' and anti-sence: 5'-UCUGCAGGCCGUGUGCUUUGCUU-3'; GenePharma, Shanghai, China) and BTG1-WT or BTG1-MUT using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, the dual-luciferase reporter assay system (Promega, Madison, WI, USA) was performed to measure the luciferase activity.

ORT-PCR

Total RNA was extracted from cells or HCC tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Then, 200 ng of total RNA was reversely transcribed to cDNA. Gene expressions were quantified by SYBR-Green quantitative real-time PCR using an ABI 7500 Real-Time PCR system (Applied Biosystems, Shanghai, China). GAPDH and U6 were performed as endogenous controls for mRNA and miR-330-3p respectively. The amplification conditions were as follows: 35 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min, chain extension at 72°C for 1 min, and final extension step at 72°C for 10 min. Primers were provided by Sangon Biotech (Shanghai, China) and listed as follows: U6, forward 5'-GCTTCGGCAGCACATATACTAAAAT-3';M reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'; GAPDH, forward 5'-CTTTGGTATCGTGGAAGGACTC-3'; reverse 5'-GTAGAGGCAGGGATGATGTTCT-3'; BTG1, forward 5′-CATCTCCAAGTTTCTCCGCACC-3′; reverse 5′-GCGAATACAACGGTAACCCGATC-3′; miR-330-3p, forward 5'-GCAGAGATTCCGTTGTCGT-3'; reverse 5'-GCGAGCACAGAATTAATACGAC-3'; Cyclin D1, forward 5′-GTCTTCCCGCTGGCCATGAACTAC-3′; reverse 5′-GGAAGCGTGTGAGGCGGTAGTAGG-3′; Bcl-2, forward 5′-TGGCGGTTTGCGGTGGAC-3′; reverse 5′-CCAGTGCAGGGTCCGAGGT-3′; Bax, forward 5′-ATCCAGAGACAAGACATGTAC-3′; reverse 5′-TTCAGATGTTCTAAGCCTACGG-3′; MMP9, forward 5′-GATCATTCCTCAGTGCCGGA-3′; reverse 5′-TTCAGGGCGAGGACCATAGA-3′. The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The results were repeated 3 times.

Western Blot Analysis

Total protein was extracted from cells using Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China). Bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to determine the protein concentrations. Then, the extracted protein samples were mixed with 5× loading buffer, boiled at 100°C for 5 min, loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in Phosphate Buffered Solution and Tween-20 (PBST) containing 5% non-fat dry milk for 1 h at room temperature and incubated with the primary antibody: BTG1 (1:1,000; cat. no. ab151740; Abcam, Cambridge, MA, USA), Cyclin D1 (1:1,000; Cat. No. 2978; Cell Signaling Technology Inc., Danvers, MA, USA), Bcl-2 (1:1,000; cat. no. 4223; Cell Signaling Technology Inc., Danvers, MA, USA), Bax (1:1,000; cat. no. 5023; Cell Signaling Technology Inc., Danvers, MA, USA), MMP9 (1:1,000; cat. no. 13667; Cell Signaling Technology Inc., Danvers, MA, USA) and β-actin (1:1,000; cat. no. 4970; Cell Signaling Technology Inc., Danvers, MA, USA), at 4°C overnight respectively. After that, the membranes were washed with PBST and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology Inc., Danvers, MA, USA) for 1 h at room temperature. Finally, the target proteins were visualized using SignalFire™ enhanced chemiluminescence reagent (Cat. No. 6883; Cell Signaling Technology Inc., Danvers, MA, USA) according to the manufacturer's instructions. The results were repeated 3 times.

Flow Cytometry

HCCLM3 cells were transfected with miR-330-3p inhibitor, inhibitor control, or miR-330-3p inhibitor+BTG1-siRNA for 48 h in 24-well plates ($1x10^6$ cells/well). After 48 h incubation, the cells double-stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) (cat. no. 70-AP101-100; MultiSciences Biotech, Co., Ltd., Hangzhou, China). Cell apoptosis was analyzed using a FACScan flow cytometer (Becton Dickinson; Billerica, MA, USA) according to the manufacturer's instructions. Data were analyzed with the ModFitLT V2.0 software (Becton Dickinson, Billerica, MA, USA). The results were repeated for 3 times. Cell apoptosis rate was calculated as early apoptosis + late apoptosis (the right quadrant).

Cell Migration and Invasion Assay

The un-coated transwell chambers (pore size, 8 μm; Costar; Corning, Corning, NY, USA) was used for cell migration detection, and Matrigel-coated (Sigma-Aldrich, St. Louis, MO, USA) transwell chambers were used for cell invasion analysis. HCCLM3 cells were transfected with miR-330-3p inhibitor, inhibitor control, or miR-330-3p inhibitor+BTG1-siRNA for 48 h. After transfection, $1x10^5$ HCCLM3 cells were incubated in serum-free medium for starvation and seeded into the upper chamber of transwell chambers. Then, 600μl RPMI-1640 culture medium with 20% FBS were added to the lower chambers. 48 h after incubation (37°C, 5% CO_2), the remaining cells on the upper chamber were removed with a cotton swab. Then, cells adhering to the under the surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 10 min at room temperature. The migratory and invasive cells were counted from 5 random fields using an inverted microscope (Olympus IX51; magnification, 40x).

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis was measured using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The differences between the groups were estimated by Student's *t*-test or one-way ANOVA followed by Tukey's test. All experiments were performed at least three times. *$p<0.05$ indicated a significant difference.

Results

MR-330-3p Expression Was Up-Regulated in Hepatocellular Carcinoma Tissues

The qRT-PCR assay was adopted to evaluate the expression level of miR-330-3p in 30 human hepatocellular carcinoma tissues and adjacent normal tissues from 30 hepatocellular carcinoma patients. Our results indicated that miR-330-3p expression was much higher in HCC tissues compared with adjacent normal tissues (Figure 1A). Compared with the human normal hepatocellular cells LO2, the miR-330-3p expression was also remarkably increased in hepatocellular carcinoma cell lines (MHCC-LM3, Huh7, MHCC97-L, and HCCLM3) (Figure 1B). HCCLM3 was chosen for the following experiments. Taken together, these results demonstrated that miR-330-3p was up-regulated in hepatocellular carcinoma tissues and cell lines.

BTG1 Was a Target of MiR-330-3p and It Was Down-Regulated in HCC Tissues and Cells

To identify the potential miR-330-3p targets, Targetscan 7.2 (http://www.targetscan.org/vert_72/) was performed. We found that miR-330-3p has hundreds of potential targets, including BTG1 (Figure 2A). BTG1 belongs to the BTG/Tob anti-proliferation gene family[29,30], and it plays critical roles in regulating various biological and cellular processes including cell cycle,

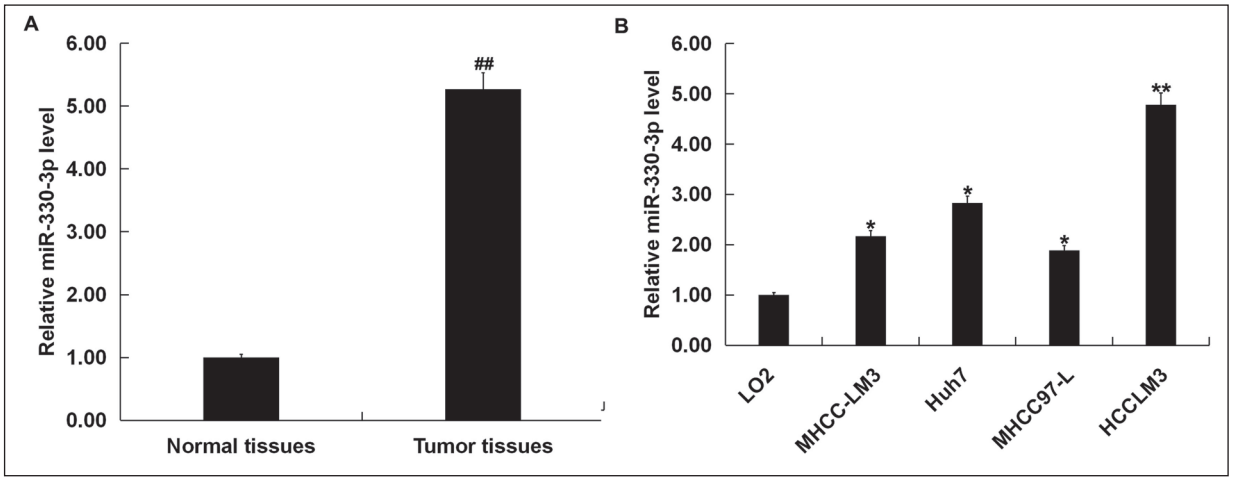

Figure 1. Expression of miR-330-3p in HCC tissues and cells. ***A,*** Expression of miR-330-3p in 30 hepatocellular carcinoma tissues and 30 adjacent normal tissues was determined by qRT-PCR. ***B,*** QRT-PCR assay was adopted to examine the miR-330-3p expression in human normal hepatocellular cells LO2 and hepatocellular carcinoma cells (MHCC-LM3, Huh7, MHCC97-L, and HCCLM3). The data were presented as the mean ± SD; $^{\#\#}p<0.01$ *vs.* Normal tissues; *$p<0.05$ *vs.* LO2 cells; **$p<0.01$ *vs.* LO2 cells.

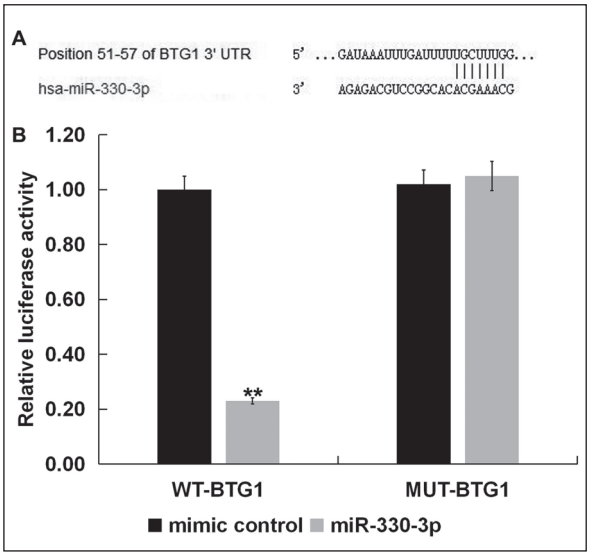

Figure 2. Relationship between miR-330-3p and BTG1. ***A*,** Binding sites between miR-330-3p and the 3′-UTR of BTG1. ***B*,** Luciferase activities were detected by Dual-Luciferase Reporter Assay System in HCCLM3 cells. The data were shown as the mean ± SD; $^{**}p<0.01$ *vs.* mimic control.

cell proliferation, and cell apoptosis[31-34]. Besides, the study has revealed that BTG1 plays an important role in apoptosis and negatively regulates cell proliferation, and it was down-regulated in HCC[35]. However, until now, the relationship between miR-330-3p and BTG1 in HCC remains unknown. Therefore, we choose BTG1 for further study, and we speculated that BTG1 was the direct target gene of miR-330-3p. Then, luciferase reporter assay was performed to confirm our prediction, and the data indicated that miR-330-3p mimic markedly decreased the luciferase activity of cells co-transfected with wild type BTG1 3'UTR construct and miR-330-3p mimic, but luciferase activity had no obvious decrease in cells co-transfected with BTG1-MUT and miR-330-3p mimic (Figure 2B).

Moreover, the mRNA and protein levels of BTG1 were detected in HCC tissues and cell lines by qRT-PCR and Western blot analysis. We found that BTG1 mRNA and protein expression was down-regulated in the HCC tissues and cell lines compared to adjacent normal tissues (Figure 3A and B) or normal hepatocellular cells LO2 (Figure 3C and D). Therefore, we concluded that BTG1 was a target of miR-330-3p and the expression level of BTG1 was down-regulated in the HCC tissues and cell lines.

BTG1-siRNA Reversed the Effects of MiR-330-3p Inhibitor in HCC Cells

To investigate the functional relevance of BTG1 in miR-330-3p-mediated effects in HCC cells, the miR-330-3p inhibitor, inhibitor control, BTG1-siRNA, control-siRNA, or miR-330-3p inhibitor+BTG1-siRNA was transfected into HCCLM3 cells. As shown in Figure 4A, we found that the level of miR-330-3p was reduced in HCCLM3 cells transfected with the miR-330-3p inhibitor. Meanwhile, BTG1-siRNA significantly decreased the mRNA level of BTG1 in HCCLM3 cells (Figure 4B). While down-regulation of miR-330-3p up-regulated BTG1 at both the mRNA and protein levels, on the contrary, BTG1-siRNA could partially restore the effects of miR-330-3p inhibitor on BTG1 expression in HCCLM3 cells (Figure 4C and D). Taken together, we concluded that BTG1 was negatively regulated by miR-330-3p in hepatocellular carcinoma cells.

MiR-330-3p Inhibitor Efficiently Inhibited Cell Viability, Migration, Invasion, and Promoted Apoptosis in HCCLM3 Cells by Directly Targeting BTG1

To investigate the effect of miR-330-3p inhibitor in HCCLM3 cells, HCCLM3 cells were transfected with miR-330-3p inhibitor, inhibitor control or miR-330-3p inhibitor+BTG1-siRNA for 48 h. CCK-8 assay demonstrated that cell viability was decreased when HCCLM3 cells were transfected with miR-330-3p inhibitor, but BTG1-siRNA could efficiently block the effects mediated by miR-330-3p inhibitor in the cells (Figure 5A). We also examined the influence of miR-330-3p inhibitor on the migration and invasion ability of HCCLM3 cells by transwell assay. As presented in Figure 5B and C, remarkably decreases of migratory and invasive cells were observed in HCCLM3 cells transfected with miR-330-3p inhibitor compared with control cells. Moreover, BTG1-siRNA enhanced HCCLM3 cell migration and invasion. In addition, flow cytometry assay was performed to evaluate HCCLM3 cell apoptosis, and the results demonstrated that miR-330-3p inhibitor-induced HCCLM3 cell apoptosis, while inhibition of BTG1 by siRNA-BTG1 clearly abolished the effects (Figure 5D and E). These data indicated that miR-330-3p inhibitor could inhibit the cell viability, migration, invasion, and induce cell apoptosis of HCCLM3 cells while BTG1-siRNA reversed the effects.

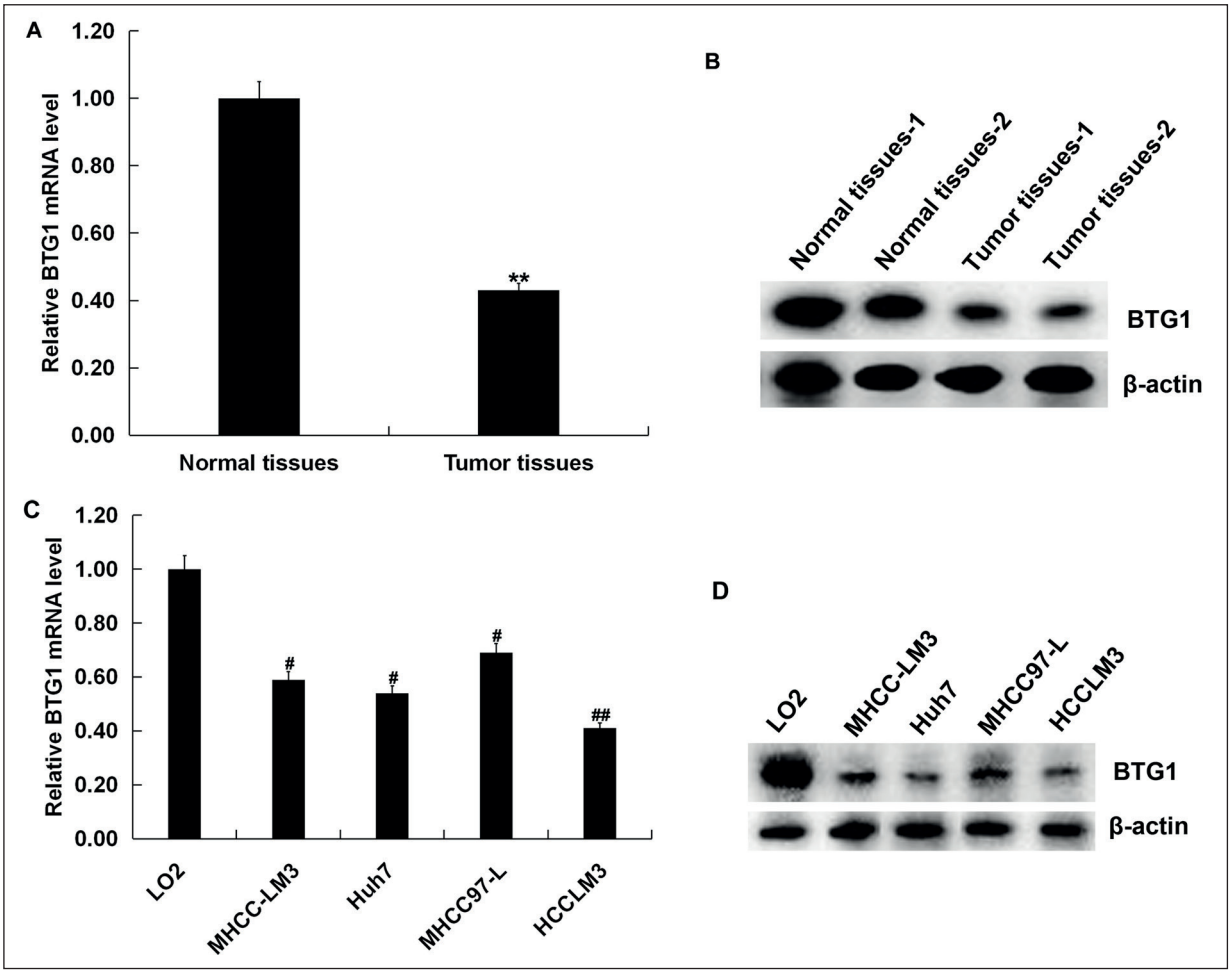

Figure 3. BTG1 expression in HCC tissues and cells. ***A*,** MRNA expression of BTG1 in 30 hepatocellular carcinoma tissues and 30 adjacent normal tissues was determined by qRT-PCR. ***B*,** Protein expression of BTG1 in the hepatocellular carcinoma tissues and adjacent normal tissues was determined by Western blot assay. ***C*,** QRT-PCR assay was adopted to examine the mRNA expression of BTG1 in human normal hepatocellular cells LO2 and hepatocellular carcinoma cells (MHCC-LM3, Huh7, MHCC97-L and HCCLM3). ***D*,** Western blot assay was used to examine the protein expression of BTG1 in human normal hepatocellular cells LO2 and hepatocellular carcinoma cells (MHCC-LM3, Huh7, MHCC97-L, and HCCLM3). The data were presented as the mean ± SD; $^{**}p<0.01$ *vs.* Normal tissues; $^{\#}p<0.05$ *vs.* LO2 cells; $^{\#\#}p<0.01$ *vs.* LO2 cells.

Down-Regulation of MiR-330-3p Regulated Cyclin D1/MMP9/Bcl-2/Bax Expression in HCCLM3 Cells

To further explore the molecular mechanism of the role of miR-330-3p in regulating HCC, the expressions of Cyclin D1, MMP9, Bcl-2, and Bax were detected by Western blot assay (Figure 6A) and qRT-PCR (Figure 6B-E). Our results demonstrated that miR-330-3p down-regulation decreased the protein expression of Cyclin D1, MMP9, and Bcl-2, and enhanced Bax protein level in HCCLM3 cells. Besides, miR-330-3p down-regulation significantly decreased the mRNA expression of Cyclin D1, MMP9, and Bcl-2, while the mRNA level of Bax was increased. These changes were eliminated by BTG1-siRNA. Our results clearly indicated that down-regulated of BTG1 successfully abolished the tumor-suppressive effects of miR-330-3p inhibitor in HCC cells.

Discussion

Dysregulation of oncogenes or suppressor genes is involved in HCC progression[34,35]. Moreover, many reports[36] have indicated that miRNAs serve as oncogenes or tumor suppressors in various types of cancers. It is necessary to investigate the roles of cancer-specific miRNAs

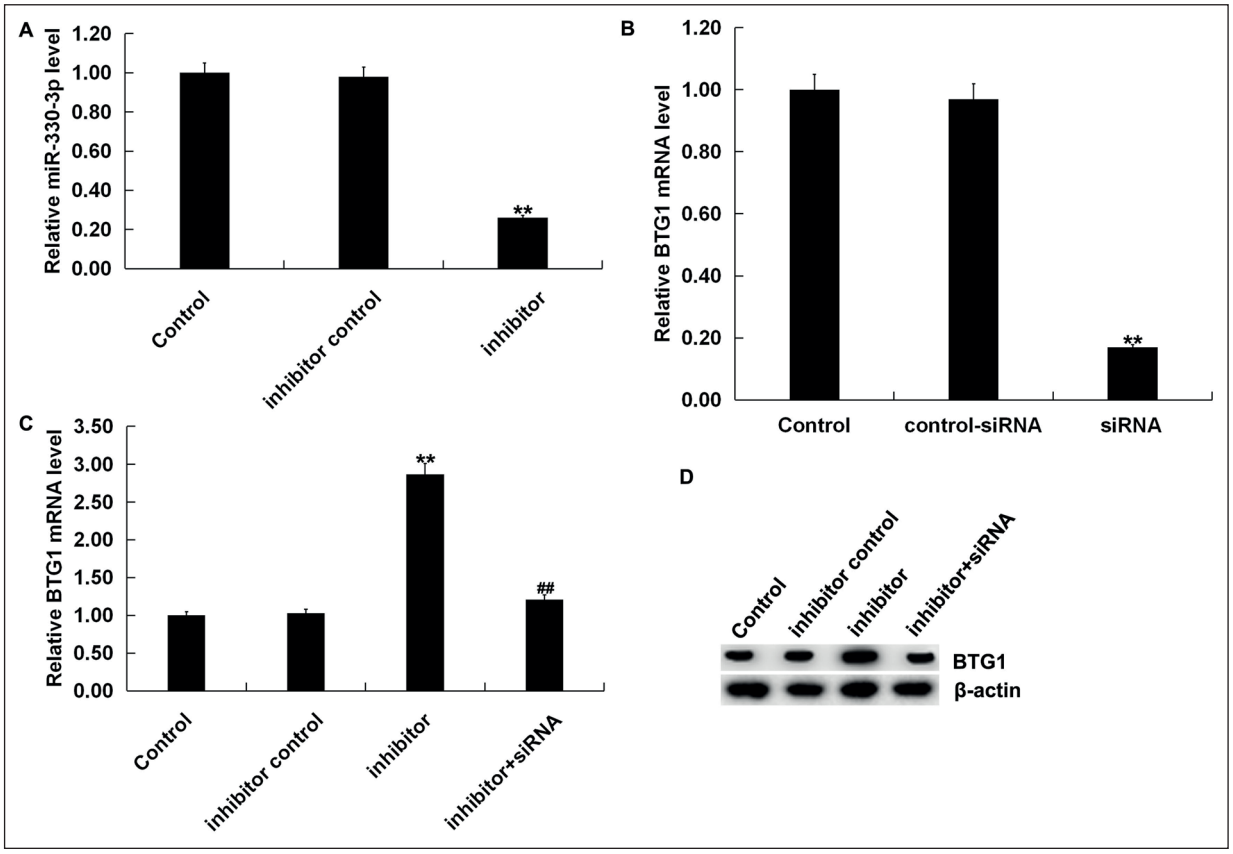

Figure 4. Effect of miR-330-3p inhibitor on BTG1 expression in HCCLM3 cells. HCCLM3 cell was co-transfected with the miR-330-3p inhibitor or BTG1-siRNA or corresponding control for 48 h. QRT-PCR and Western blotting assays were performed to evaluate the transfection efficiency. ***A,*** Expression of miR-330-3p in HCCLM3 cells was detected using qRT-PCR; ***B,*** QRT-PCR assay was performed to detect the BTG1 mRNA expression. ***C, D,*** QRT-PCR and Western blot assays were used to detect BTG1 mRNA and protein expression in HCCLM3 cells transfected with inhibitor control, miR-330-3p inhibitor or miR-330-3p inhibitor+BTG1-siRNA. Control: cells without any treatment; inhibitor control: cells transfected with inhibitor control; inhibitor: cells transfected with miR-330-3p inhibitor; control-siRNA: cells transfected with control-siRNA; siRNA: cells transfected with BTG1-siRNA; inhibitor+siRNA: cells co-transfected with miR-330-3p inhibitor and BTG1-siRNA. All experiments were performed three times, and representative images are presented. Data were shown as mean ± SD. $^{**}p<0.01$ *vs.* Control; $^{\#}p<0.05$ *vs.* Inhibitor; $^{\#\#}p<0.01$ *vs.* inhibitor.

or their target genes in oncogenesis[37-39]. Previous studies[40-42] demonstrated that miR-330-3p exerted important roles in the development of multiple tumors. In this study, we focused on investigating the mechanism of miR-330-3p in hepatocarcinogenesis.

First, we measured the expression of miR-330-3p in 30 HCC tissues and their adjacent tumor tissues by qRT-PCR assay. Notably, we found that miR-330-3p was significantly increased in HCC tissues compared to the adjacent tumor tissues. This result was further confirmed in HCC cell lines by qRT-PCR. Our results were in line with other researches which have indicated that miR-330-3p was also evidently up-regulated in various cancers. For example, Liu et al[40] found that the miR-330-3p expression was up-regulated in non-small cell lung cancer (NSCLC). Meng et al[41] demonstrated that the expression of miR-330-3p was increased in esophageal squamous cell carcinoma (ESCC) tissues. Therefore, aberrant up-regulation of miR-330-3p in multiple cancer cells and tissues, demonstrating that down-regulated miR-330-3p might could block tumorigenesis. As we all know, miRNAs could regulate gene expression by targeting the 3'UTR of targeted mRNAs. Qu et al[43] showed that miR-330-3p acted as an oncogenic in glioblastoma cells by regulating the SH3 domain-containing GRB2 like 2 (SH3GL2). Also, it has been reported that miR-330-3p exerted tumor suppressor roles by targeting E2F transcription factor 1

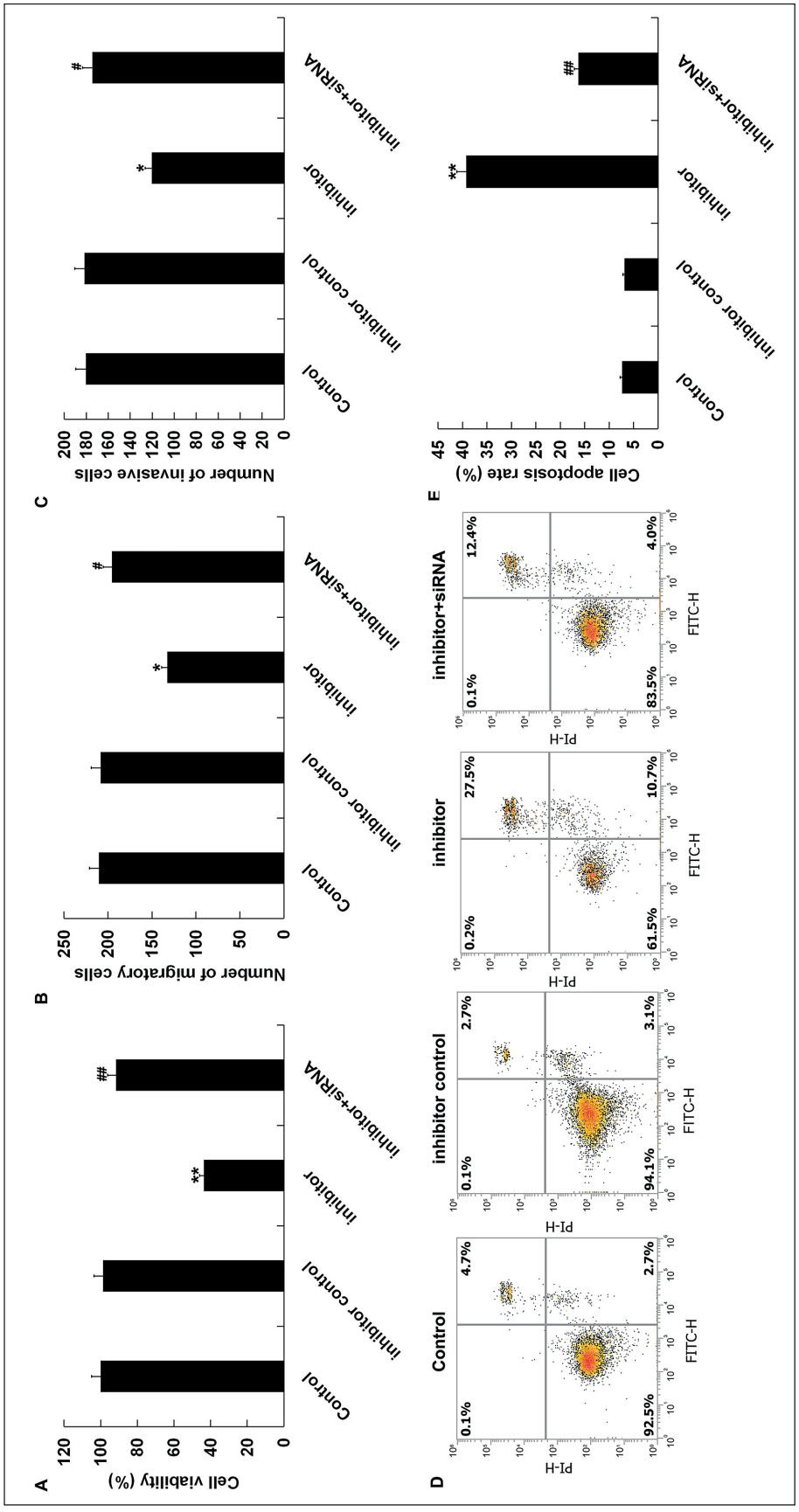

Figure 5. Effects of miR-330-3p inhibitor on cell proliferation, migration, invasion, and apoptosis in HCCLM3 cells. HCCLM3 cells were transfected with the inhibitor control, miR-330-3p inhibitor or BTG1-siRNA+miR-330-3p inhibitor for 48 h. Then, cell viability in HCCLM3 cells was determined by CCK-8 assay (***A***); Transwell assays were performed to detected cell migration (***B***) and invasion ability (***C***) in HCCLM3 cells; Flow cytometry was carried out to evaluate the percentages of apoptosis in HCCLM3 cells (***D*** and ***E***). All experiments were carried out in triplicate. Control: cells without any treatment; inhibitor control: cells transfected with inhibitor control; inhibitor: cells transfected with miR-330-3p inhibitor; inhibitor+siRNA: cells co-transfected with miR-330-3p inhibitor and BTG1-siRNA. Data were shown as mean ± SD. *$p<0.05$ *vs.* Control; **$p<0.01$ *vs.* Control; $^{\#}p<0.05$ *vs.* inhibitor; $^{\#\#}p< 0.01$ *vs.* inhibitor.

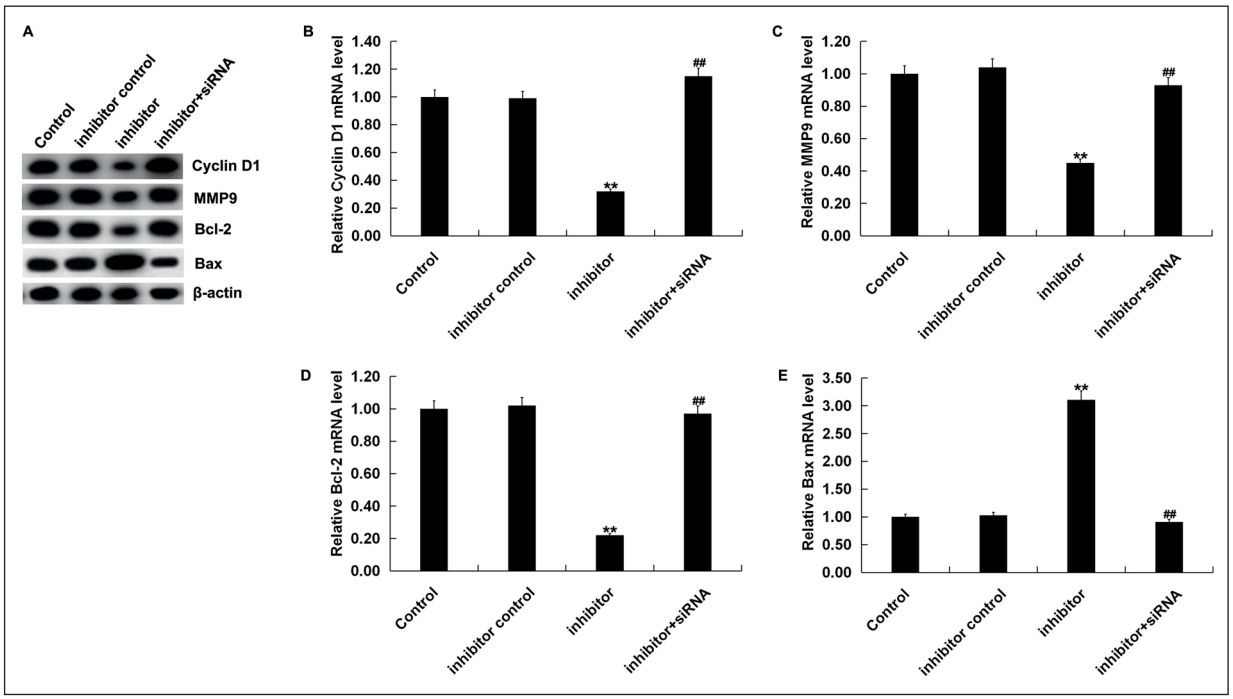

Figure 6. The expression of Cyclin D1, MMP9, Bcl-2, and Bax in response to miR-330-3p down-regulation. ***A,*** Cyclin D1, MMP9, Bcl-2, and Bax protein levels were detected by Western blotting in HCCLM3 cells. ***B-E,*** QRT-PCR was carried out to evaluate Cyclin D1, MMP9, Bcl-2, and Bax mRNA expression levels. Control: cells without any treatment; inhibitor control: cells transfected with inhibitor control; inhibitor: cells transfected with miR-330-3p inhibitor; inhibitor+siRNA: cells co-transfected with miR-330-3p inhibitor and BTG1-siRNA.The data were presented as mean±SD. $^{**}p<0.01$ *vs.* Control; $^{\#\#}p<0.01$ *vs.* inhibitor.

(VE2F1) or specificity protein 1 (Sp1)[44,45]. Then, we investigated the potential targets of miR-330-3p in HCC. Data from luciferase assay suggested that BTG1 was a direct target of miR-330-3p, revealing a possible mechanism of BTG1 with HCC oncogenesis. Previous studies have shown that BTG1 belongs to an anti-proliferative gene family and plays important roles in many types of cancers. Then, we focused on investigating whether BTG1 knock-down could reverse the effects of the miR-330-3p inhibitor on HCCLM3 cells. Our results suggested that silencing BTG1 reversed the effects of the miR-330-3p inhibitor on HCC cell viability, apoptosis, migration, and invasion. Besides, we also detected the relative genes of cell apoptosis, migration or growth including BTG1, Bcl-2, Bax, Cyclin D1, and MMP9. We found that the expression of BTG1, Bcl-2, Bax, Cyclin D1, and MMP9 was changed by miR-330-3p inhibitor and the changes were reversed by knockdown of BTG1. However, we did not set up the BTG1-siRNA alone group in this investigation and this might be a limitation of our study.

Conclusions

We indicated that miR-330-3p was up-regulated in both HCC cell lines and HCC tissues. MiR-330-3p down-regulation could inhibit HCC cell viability, migration, invasion, and induce cell apoptosis by targeting BTG1. These findings may help to provide new insights into the strategy for targeting HCC and identify novel molecular of miR-330-3p/ BTG1 in hepatocarcinogenesis. MiR-330-3p may be a potential diagnostic marker and therapeutic target for HCC. However, this is only a preliminary study of the role of miR-330-3p in hepatocellular carcinoma. To make the role of miR-330-3p in hepatocellular carcinoma more convincing, a lot of in-depth experimental research is still needed. For example, the relation between miR-330-5p and BTG1 expression and the clinical features (including tumor size, tumor nodule number, capsule formation, and Tumor Node Metastasis (TNM) stage, and survival rate) HCC patients should be investigated. The role miR-330-5p in HCC should also be investigated in other HCC

cell lines and *in vivo* experimental studies are necessary. Meanwhile, further functions of miR-330-3p in HCC, such as functions of miR-330-3p in HCC cell drug resistance and epithelial-mesenchymal transition, need to be explored. We will perform these issues in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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