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Long non-coding RNA MEG3 represses cholangiocarcinoma by regulating miR-361-5p/TRAF3 axis

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding RNA MEG3 (MEG3) and microRNA-361-5p (miR-361-5p) on cholangiocarcinoma cells and to explore the molecular mechanisms.

PATIENTS AND METHODS: The level of MEG3 and miR-361-5p was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between miR-361-5p and MEG3/TNF receptor-associated factor 3 (TRAF3) was confirmed by the Dual-Luciferase Reporter Assay. 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry analysis were used to determine cell viability and cell apoptosis. Moreover, the protein level of TRAF3, p65, and p-p65 was measured by Western blot assay.

RESULTS: We found that MEG3 was downregulated in CCA tissues and cell lines, especially in TFK1 and QBC939 cells. MEG3 directly bind to miR-361-5p, which was highly expressed in CCA tissues and cell lines. Further analysis indicated that MEG3-plasmid could inhibit cell viability and induce cell apoptosis in CCA cells, but these effects were significantly reversed by miR-361-5p mimic. Moreover, we proved that TRAF3 was a direct target of miR-361-5p and it was downregulated in CCA tissues and cell lines. In addition, we found that miR-361-5p downregulation significantly inhibited CCA cell viability and induced cell apoptosis, and these effects were eliminated by the knockdown of TRAF3. Further functional analysis showed that the knockdown of TRAF3 upregulated the expression of p-p65 decreased by miR-361-5p inhibitor in CCA cells.

CONCLUSIONS: Our results suggested that MEG3 repressed cholangiocarcinoma by downregulating miR-361-5p expression. Meanwhile, the suppression of miR-361-5p might improve CCA survival by targeting TRAF3 and inhibiting the NF-κB pathway, which might help to develop new strategies for CCA therapy.

Key Words:
Cholangiocarcinoma, MiR-361-5p, LncRNA-MEG3, TRAF3.

Introduction

Cholangiocarcinoma (CCA), known as bile duct cancer, is a hepatocellular carcinoma that originates in the bile ducts epithelial cells[1,2]. CCA could easily infiltrate into adjacent organs including liver and portal vein due to its high aggressive ability[3]. Although surgery, chemotherapy, and curative liver transplantation are methods for the treatment of CCA[4-6], the incidence of CCA has significantly increased[7,8], and the 5-year survival rate of CCA patients is still very low[9-12]. Therefore, it is important and urgent to develop new diagnostic or treatment strategies for CCA. In this work, we investigated the molecular mechanism correlated with the tumorigenesis and progression of CCA and hoped to seek more novel therapeutic targets for CCA.

MicroRNAs (miRNAs), a group of small non-coding RNAs with 20-22 nucleotides in length, can regulate the target gene expression by binding with the 3'-untranslated region (3'-UTR) of mRNAs[13-15]. MiR-361-5p, one of the miRNAs, was found to function as a tumor suppressor in various tumors. Sun et al[16] have indicated that the downregulation of miR-361-5p significantly inhibited tumor growth, and miR-361-5p played essential roles in the development and progression of cancers via NF-κB signaling pathway. However, whether miR-361-5p can regulate CCA cells and its regulatory functions is still unclear.

Long non-coding RNAs (lncRNAs), a group of RNAs, have more than 200 nucleotides seldom encoding proteins[17]. Previous reports[18,19] have suggested that lncRNAs act as promoter or inhibitor in cancer development by targeting oncogenes or tumor suppressors. LncRNA-MEG3 (MEG3) is located on chromosome 14q32[20,21]. Various researches have indicated that MEG3 suppressed cancer cell proliferation by activating the relative

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signaling pathway, such as NF-κB signaling pathway[22]. Also, MEG3 upregulation has been identified to inhibit cell proliferation in cancer cells[23-25]. However, the underlying mechanism of MEG3 in cholangiocarcinoma remains unexplored.

Therefore, the aim of the study was to investigate the effect of MEG3 and miR-361-5p on the progression of CCA and to explore the potential mechanism. In the present investigation, we detected the expression of MEG3 and miR-361-5p in CCA tissues and cell lines, investigated the effect of MEG3 and miR-361-5p on CCA cells, and further explored the molecular mechanism. We hope to provide therapeutic targets and more theoretical basis for the treatment of CCA.

Patients and Methods

Clinical Specimens Collection

A total of 20 cholangiocarcinoma tissues and 20 corresponding adjacent normal tissues were obtained from 20 cholangiocarcinoma patients (age range: 32-61 years old; 12 males, 8 female) who underwent surgical treatment at the Jiangsu Province Hospital. No patient received chemotherapy or radiotherapy before surgery. These specimens were immediately snap-frozen in liquid nitrogen and preserved at -80°C until need. All the patients enrolled in the present report were ≥18 years old, had no other cancer, and were not taking nonsteroidal anti-inflammatory drugs or proton pump inhibitors. The study was approved by the Institutional Ethics Committee of the Jiangsu Province Hospital. All patients provided the informed consent and were informed about the use of their specimens in this research.

Cell Culture

Human CCA cell lines CCLP1, SG231, HUCCT1, TFK1, QBC939 and the human intrahepatic bile duct epithelial cell line HiBECs, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Carlsbad, CA, USA), 1% penicillin/streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Cell Transfection and Reagents

The MEG3 sequence was synthesized (based on the MEG3 sequence) and then sub-cloned into the pCDNA3.1 vector (GeneChem, Shanghai, China) (MEG3-plasmid). The empty pCDNA vector was used as a control (control-plasmid). The TFK1 and QBC939 cells were seeded into the 6-well plates, miR-361-5p mimic (5′-ACGCCUGGAGAUUCUGAUAAUU-3′), mimic control (5′-UUCUCCGAACGUGUCACGUTT-3′), control-plasmid, MEG3-plasmid, MEG3-plasmid+miR-361-5p mimic, inhibitor control (Cat No. CS8005; Biomics Biotechnology, Nantong, China), miR-361-5p inhibitor (Cat No. CIH0153; Cohesion, Guangzhou, China), TRAF3-shRNA (Cat No. sc-29510-V; Santa Cruz Biotechnology, Santa Cruz, CA, USA), control-shRNA (Cat No. sc-108080; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or miR-361-5p inhibitor+TRAF3-shRNA were transfected into TFK1 and QBC939 cells respectively using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h of transfection, the cells were harvested for further experiments. The transfection efficiency was detected by qRT-PCR and/or Western blot analysis.

Dual-Luciferase Reporter Assay

StarBase (http://starbase.sysu.edu.cn/) and TargetScan Release 7.1 (www.targetscan.org/vert_71) were used to predict the binding sites between MEG3 and miR-361-5p, and the binding sites between miR-361-5p and TRAF3, respectively. The results indicated the binding sites between miR-361-5p and MEG3/TRAF3. As miR-361-5p and MEG3 for example, the fragment of the MEG3 containing the target sequence of miR-361-5p was amplified by qRT-PCR and then inserted into a pmirGLO vector (Promega, Madison, WI, USA) to form the reporter vector lncMEG3-wild-type (MEG3-WT). Another expressing vector was also constructed by inserting mutated binding site and was named as lncMEG3-mutated-type (MEG3-MUT). MEG3-WT or MEG3-MUT and miR-361-5p mimic or mimic control were co-transfected into 293T cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) respectively and incubated for 48 h. Then, the relative Luciferase activity was detected by Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. We used the consistent assay to investigate whether TRAF3 was a direct target of miR-361-5p. The experiment was performed at least three times.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

The total cellular RNA was extracted from CCA tissues and cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA) following the manufacturer's instructions. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Waltham, MA, USA) was used to measure the RNA concentrations at 260 and 280 nm (A260/280). PrimeScript™ RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan) was used to perform the reverse transcription experiment and qPCR was performed using a Prism 7000 Real Time-PCR system with SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) to detect the level of miRNA/mRNA. The amplification conditions were as follows: 35 cycles of denaturing at 94°C for 60 sec, annealing at 60°C for 60 sec, and chain extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. GAPDH and U6 were used as internal loading controls for mRNA and miRNA respectively. The primers were provided by Sangon Biotechnology (Shanghai, China) and the primer sequences were listed as follows: GAPDH, forward 5′-TGTTGCCATCAATGACCCCTT-3′; reverse 5′-CTCCACGACGTACTCAGCG-3′; U6, forward 5′-GCTTCGGCAGCACATATACTAAAAT-3′; reverse 5′-CGCTTCACGAATTTGCGTGTCAT-3'; lncMEG3, forward 5′-CTGCCCATCTACACCTCACG-3′; reverse5′-CTCTCCGCCGTCTGCGCTAGGGGCT-3′; miR-361-5p, forward 5′-ATAAAGRGCRGACAGTGCAGATAGTG-3′; reverse 5′-TCAAGTACCCACAGTGCGGT-3′; TRAF3, forward 5′-GAGCAAGGAGGCTACAAGGAG-3′; reverse 5′-CATGCAGCTCTCGCAGAAC-3′. The relative level of miR-361-5p, MEG3 and TRAF3 mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method. All the experiments were repeated at least 3 times.

Western Blot Analysis

CCA cells were washed with ice-cold PBS and then lysed with Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China), centrifuged at 12,000 rpm for 30 min at 4°C to get the total proteins. The protein concentration was determined with a bicinchoninic acid (BCA) protein kit (Beyotime Biotechnology, Shanghai, China). The equal amount of proteins was separated by 10% Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% nonfat milk for 1 h at room temperature, and incubated at 4° C overnight respectively with the primary antibody: TRAF3 (Cat No. 61095; dilution ratio: 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), p-p65 (Cat No. 3033; dilution ratio: 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), p-65 (Cat No. 8242; dilution ratio: 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (Cat No. 4970; dilution ratio: 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA). After that, the membranes were washed four times in PBST and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (cat No. 7074; dilution ratio: 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at room temperature. Finally, the protein bands were visualized using an enhanced chemiluminescence Western blotting substrate (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

MTT Assay

QBC939 cells and TFK-1 cells were seeded into 96-well plates in triplicate and incubated overnight. Then, the culture medium was removed and control-plasmid, MEG3-plasmid, MEG3-plasmid+miR-361-5p mimic, inhibitor control, miR-361-5p inhibitor, or miR-361-5p inhibitor+TRAF3-shRNA with 100 µl of fresh medium were added into 96-well plates and cultured for 48 h at 37°C. Then, MTT solution (10 µl) was added to each well and incubated for further 4 h. After that, the solution was removed and 100 µl DMSO was added to each well for 20 min to solubilize the formazan products. At last, the optical density (OD) was measured at 490 nm by a micro-plate reader (Bio-Rad, Hercules, CA, USA) after 15 min of vibration mixing. The relative cell viability was normalized with the control group using optical density values.

Flow Cytometry

In order to determine cell apoptosis, TFK1 and QBC939 cells were transfected with MEG3-plasmid, control-plasmid, MEG3-plasmid+miR-361-5p mimic, inhibitor control, miR-361-5p inhibitor, or miR-361-5p inhibitor+TRAF3-shRNA and cultured in triplicate in 24-well plates. After 48 h, the cells were trypsinized and double stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) according to the manufacturer's instructions (Cat No. 70-AP101-100;

MultiSciences, Hangzhou, China). Cell apoptosis was analyzed by flow cytometer (BD Biosciences, Bedford, MA, USA). The data were analyzed by applying the FlowJo7.6 analysis software.

Statistical Analysis

The data were expressed as the mean±standard deviation (SD) of at least three independent experiments performed in triplicate. The statistical analyses were carried out using the Statistical Product and Service Solution 18.0 software package (SPSS, Inc., Chicago, IL, USA). The significance of differences between the groups were estimated by the Student's *t*-test or One-way analysis of variance (ANOVA) followed by Tukey's test. All the *p*-values <0.05 were considered as statistically significant.

Results

MEG3 Was Downregulated in CCA Cells and Tumor Tissues

Firstly, the expression level of MEG3 in 20 CCA tissues and 20 normal tissues was detected by qRT-PCR. Our results showed that MEG3 expression was remarkably downregulated in CCA tissues compared with the normal tissues (Figure 1A). Compared with human normal intrahepatic bile duct epithelial cell line HiBECs, MEG3 was significantly downregulated in CCA cell lines (TFK1; QBC939; CCLP1; SG231; HUCCT1) (Figure 1B). As the downregulation of MEG3 was more evidently observed in TFK1 and QBC939 cells, these two cell lines were used for our following experiments. These data indicated that MEG3 was downregulated in CCA tissues and cell lines.

MiR-361-5p Interacted Directly with MEG3

To verify whether MEG3 could function as a competing endogenous RNA for a certain miRNA, we used the bioinformatics tool to hunt for candidate miRNAs. Therefore, putative MEG3 targets were predicted by bioinformatics analysis Starbase (http://starbase.sysu.edu.cn/). The predicted results showed the binding sites between miR-361-5p and MEG3 (Figure 2A). Moreover, we conducted a Luciferase Reporter Assay to prove the predicted binding sites between MEG3 and miR-361-5p, and the results confirmed the direct targeting relationship between miR-361-5p and MEG3 (Figure 2B).

MiR-361-5p Was Up-Expressed in CCA Cells and Tumor Tissues

Having learned the relationship between miR-361-5p and MEG3, we then explored the expression of miR-361-5p in cholangiocarcinoma cells and tumor tissues by qRT-PCR analysis. The results demonstrated that the expression level of miR-361-5p was significantly upregulated in CCA cancer tissue samples. (Figure 3A) Moreover,

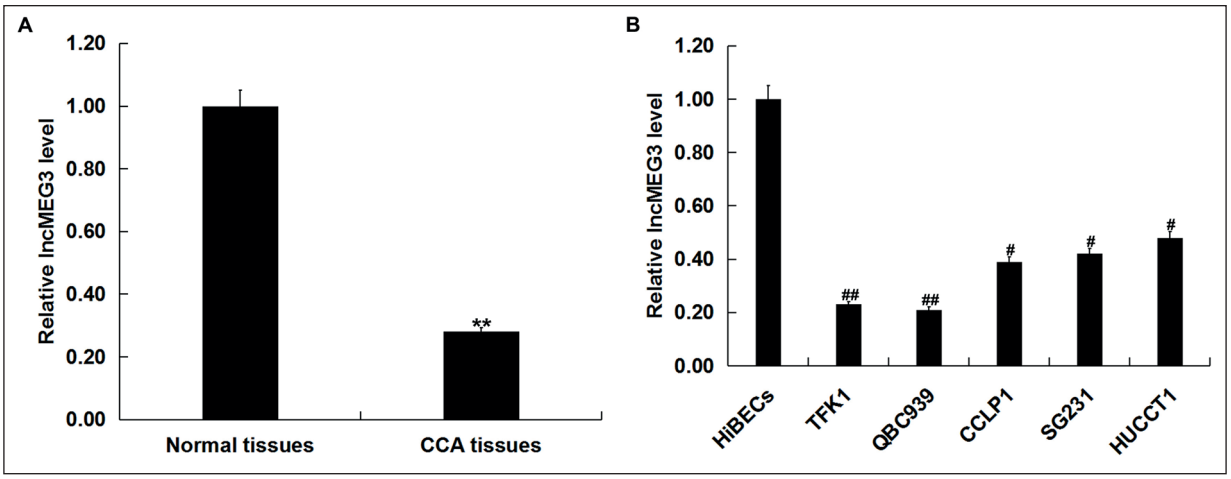

Figure 1. The low expression of MEG3 in cholangiocarcinoma tissues and cells. ***A,*** The expression of MEG3 in 20 cholangiocarcinoma tissues and 20 corresponding adjacent normal tissues of 20 cholangiocarcinoma patients was detected by qRT-PCR assay. ***B,*** MEG3 expression in cholangiocarcinoma cell lines (CCLP1; SG231; HUCCT1; TFK1; QBC939) and in the intrahepatic bile duct epithelial cell line (HiBECs) was detected by qRT-PCR assay. The data were expressed as the mean ± SD; ***p*<0.01 *vs.* normal tissues; #, ##*p*<0.05, 0.01 *vs.* HiBECs.

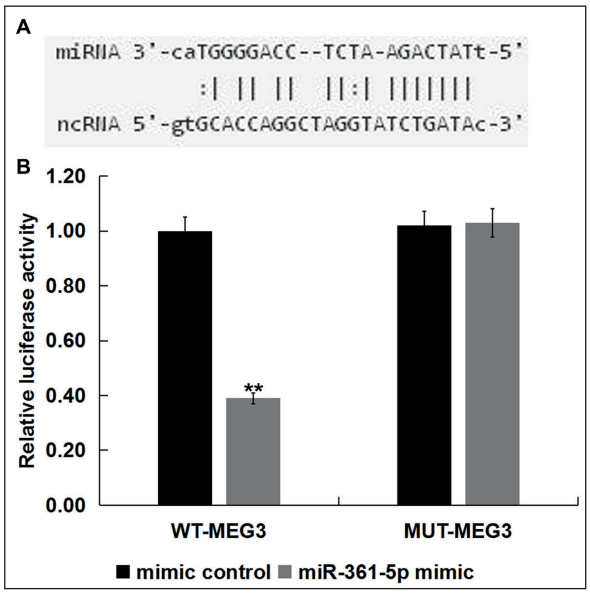

Figure 2. MEG3 binds to miR-361-5p. ***A,*** The binding sites between MEG3 and miR-361-5p. ***B,*** Dual-Luciferase Reporter Assay was used to confirm the binding sites between MEG3 and miR-361-5p. The data were expressed as the mean±SD; ***p*<0.01 *vs.* mimic control.

miR-361-5p was also increased in CCA cell lines (TFK1; QBC939; CCLP1; SG231; HUCCT1) compared with that in human normal intrahepatic bile duct epithelial cell line HiBECs, notably higher in TFK1 and QBC939 cells (Figure 3B). In summary, our data elucidated that MEG3 and miR-361-5p might play an important role in CCA cells.

MEG3-Plasmid Inhibited the MiR-361-5p Expression

To investigate the effect of MEG3 on CCA cells, control-plasmid, MEG3-plasmid, mimic control, miR-361-5p mimic, mimic control, or MEG3-plasmid+miR-361-5p mimic was transfected into TFK1 and QBC939 cells for 48 h. The transfection efficiency was measured by qRT-PCR assay. As presented in Figure 4A and 4B, MEG3 level was significantly increased in TFK1 and QBC939 cells transfected with MEG3-plasmid compared with the control group. In addition, the level of miR-361-5p was significantly higher in the miR-361-5p mimic group compared with the control group (Figures 4C and D). Meanwhile, MEG3-plasmid transfection significantly reduced the level of miR-361-5p in CCA cells, and this effect was significantly reversed by miR-361-5p mimic (Figures 4E and 4F). We found that MEG3 could negatively regulate miR-361-5p expression in CCA cells.

MEG3 Influenced Cell Viability and Apoptosis in CCA Cells Through Down-Regulating MiR-361-5p Expression

MEG3-plasmid was used to examine the specific influences of MEG3 upregulation on CCA cell viability. Our results indicated that the cell viability was significantly decreased by MEG3-plasmid in CCA cells. However, the effect of MEG3-plasmid on CCA cell viability was eliminated by miR-361-5p mimic (Figures 5A and B). Next, the increased apoptosis rates

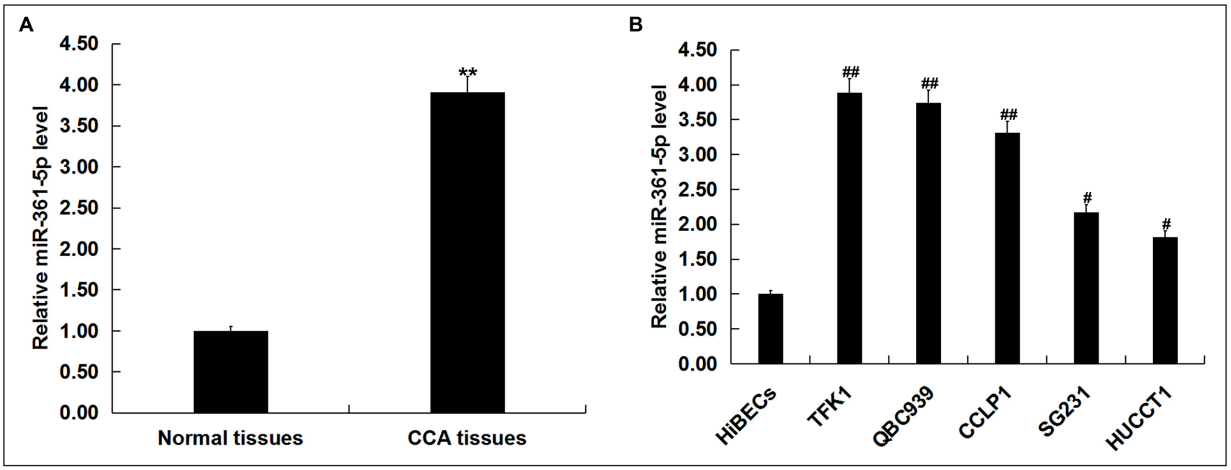

Figure 3. The high expression of miR-361-5p in cholangiocarcinoma tissues and cells. ***A,*** The expression of miR-361-5p in 20 cholangiocarcinoma tissues and 20 corresponding adjacent normal tissues of 20 cholangiocarcinoma patients was detected by qRT-PCR assay. ***B,*** MiR-361-5p expression in cholangiocarcinoma cell lines (CCLP1; SG231; HUCCT1; TFK1; QBC939) and in the intrahepatic bile duct epithelial cell line (HiBECs) was detected by qRT-PCR assay. The data were expressed as the mean ± SD; ***p*<0.01 *vs.* normal tissues; #, ##*p*<0.05, 0.01 *vs.* HiBECs.

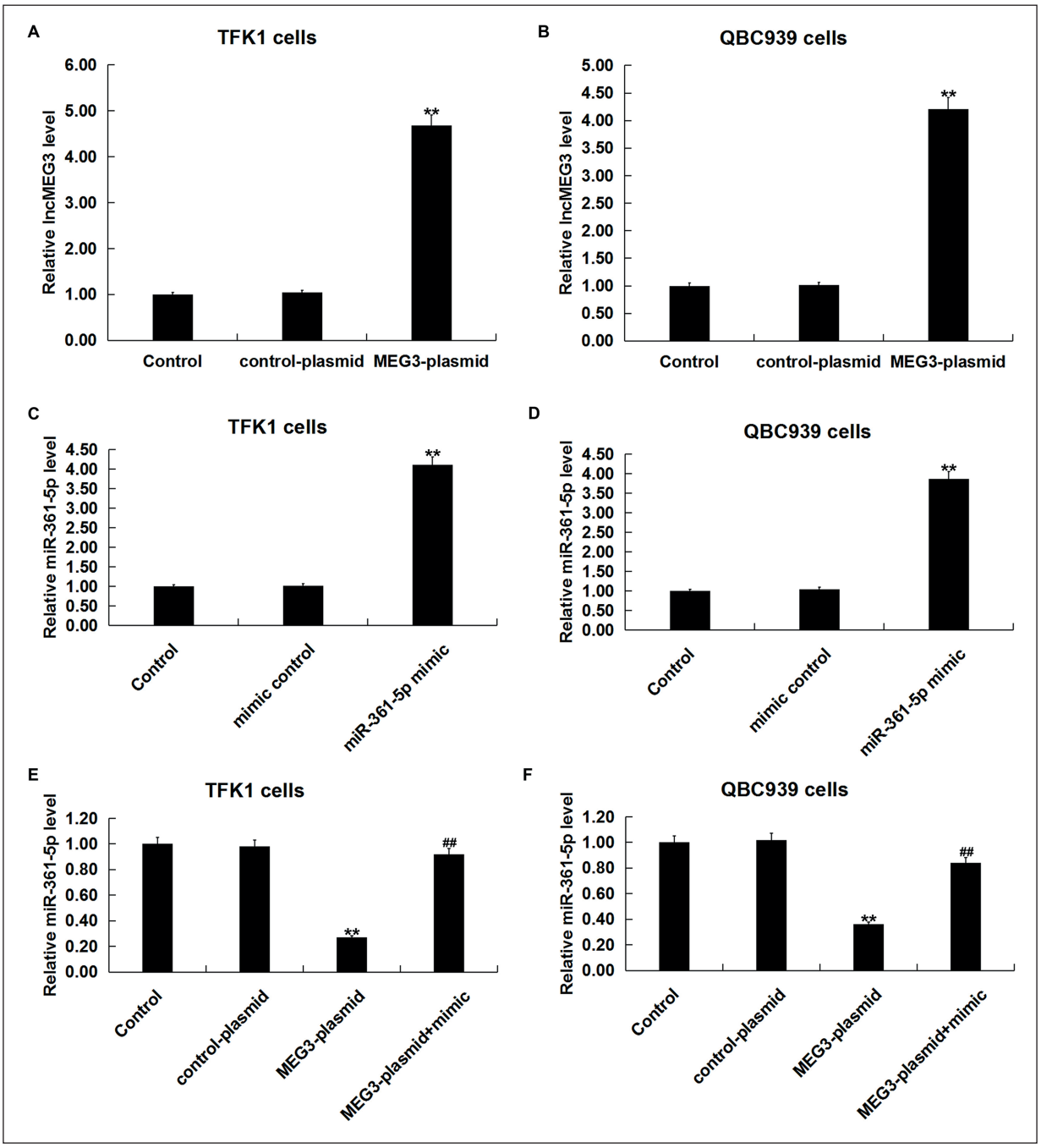

Figure 4. MEG3 negatively regulates miR-361-5p expression in CCA cells. ***A,*** and ***B,*** TFK1 and QBC939 cells were transfected with MEG3-plasmid for 48 h, then, the expression of MEG3 in ***(A)*** TFK1 and ***(B)*** QBC939 cells was detected by qRT-PCR analysis. ***C,*** and ***D,*** TFK1 and QBC939 cells were transfected with miR-361-5p mimic for 48 h, then, the expression of miR-361-5p in ***(C)*** TFK1 and ***(D)*** QBC939 cells was detected by qRT-PCR analysis. ***E,*** and ***F,*** The relative expression of miR-361-5p was measured by qRT-PCR in ***(E)*** TFK1 and ***(F)*** QBC939 cells transfected with MEG3-plasmid or MEG3-plasmid+miR-361-5p mimic. The results were presented as mean±SD. **$p<0.01$ *vs.* control; ##$p<0.01$ *vs.* MEG3-plasmid.

of CCA cells were measured by flow cytometry analysis after MEG3-plasmid transfection (Figures 5C and D), and the effect was reverse by miR-361-5p mimic co-transfection in TFK1 and QBC939 cells. According to all above findings, we confirmed that MEG3 inhibited cell viability and induced cell apoptosis by reducing the level of miR-361-5p.

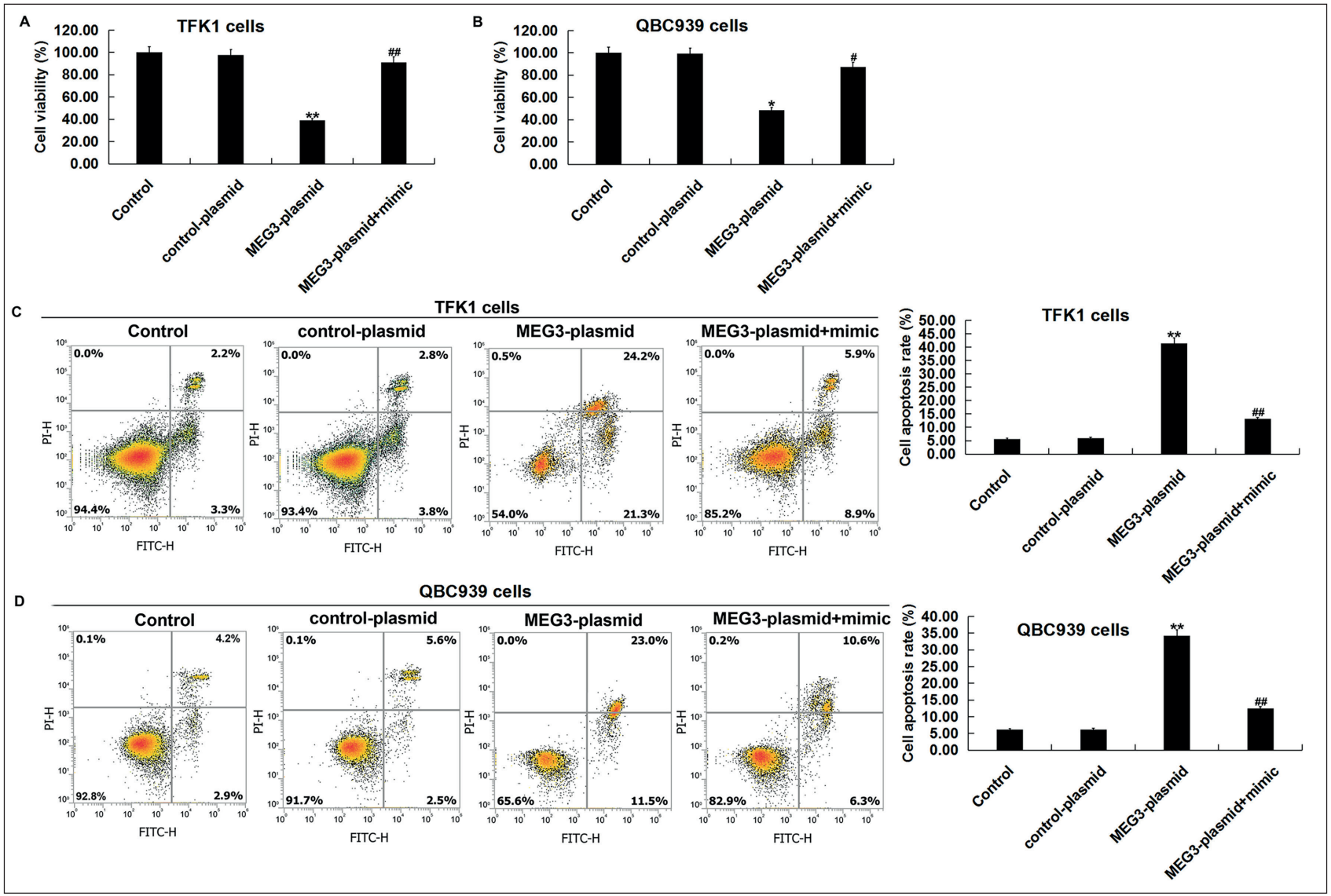

Figure 5. Effect of MEG3 on CCA cell viability and apoptosis. ***A,*** and ***B,*** The cell viability of TFK1 cells and QBC939 cells was measured to evaluate the roles of MEG3-plasmid in CCA cells through MTT assay. ***C,*** and ***D,*** Flow cytometry was performed to determine the percentages of apoptosis in TFK1 cells and QBC939 cells. Each bar in the histogram represented the mean±SD; *, **p<0.05, 0.01 *vs.* control; #, ##p<0.05, 0.01 *vs.* MEG3-plasmid.

TRAF3 Was a Target of MiR-361-5p

TRAF3 is found effective in various cancers, but few reports clarified how it works in cholangiocarcinoma. TargetScan Release 7.1 (www.targetscan.org/vert_71) was used to predict the targets of miR-361-5p, and the complementary sites of TRAF3 in miR-361-5p were firstly predicted. The results showed the binding sites between TRAF3 and miR-361-5p (Figure 6A). Then, the Luciferase Reporter Assay was used to confirm this prediction. As shown in Figure 6B, miR-361-5p mimic led to a significant decrease in the Luciferase activity in Wild-type 3'-UTR of TRAF3 reporter (WT-TRAF3) compared with the control group, while we detected no evident influence on TRAF3 3'UTR-mut reporter (MUT-TRAF3) (Figure 6B). All the results above illustrated the fact that TRAF3 was a direct target of miR-361-5p.

The Expression of TRAF3 Was Reduced in CCA Cells and Tumor Tissues

The expression of the TRAF3 in CCA cells and tumor tissues were further detected using qRT-PCR and Western blot assay. The findings indicated that TRAF3 significantly decreased in CCA tumor tissues compared with the normal tissues (Figures 7A and 7B). Besides, the expression of TRAF3 was found to be downregulated in CCA cell lines (TFK1; QBC939; CCLP1; SG231; HUCCT1) compared with the human normal intrahepatic bile duct epithelial cell line HiBECs (Figure 7C and D), and the downregulation of TRAF3 was more evidently observed in TFK1 and QBC939 cells.

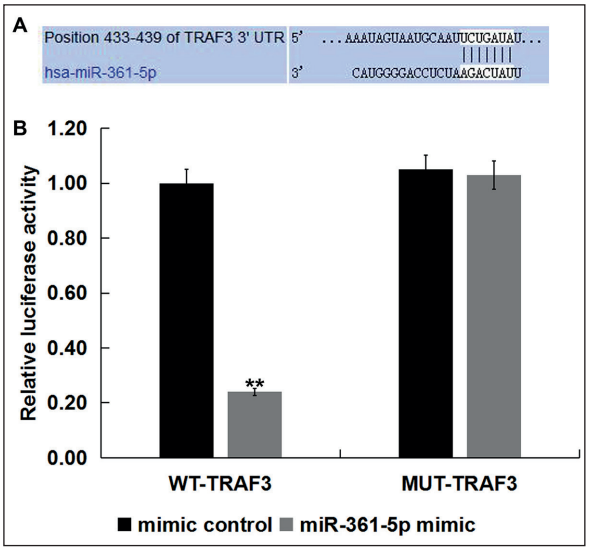

Figure 6. TRAF3 is a target of miR-361-5p. ***A,*** The binding sites between miR-361-5p and TRAF3 was predicted. ***B,*** Dual-Luciferase Reporter Assay was used to confirm the binding sites between miR-361-5p and TRAF3. The data were expressed as the mean±SD; $^{**}p<0.01$ *vs.* mimic control.

MiR-361-5p Downregulation Enhanced the Expression of TRAF3 in CCA Cells

To determine the role of miR-361-5p and TRAF3 in CCA cells, we transfected TFK and QBC939 cells with inhibitor control, miR-361-5p inhibitor, TRAF3-shRNA, control-shRNA, or miR-361-5p inhibitor and TRAF3-shRNA for 48 h. qRT-RCR assay showed that miR-361-5p level was reduced in miR-361-5p inhibitor transfected with CCA cells (Figures 8A and 8B). Furthermore, TRAF3-shRNA could significantly reduce the expression of TRAF3 in TFK1 and QBC939 cells (Figures 8C and 8D). Additionally, miR-361-5p inhibitor significantly promoted the mRNA level and the protein expression of TRAF3 in TFK1 and QBC939 cells, and this effect was reversed byTRAF3-shRNA (Figures 8E-8H). Based on these results, we confirmed that TRAF3-shRNA might reverse the tumor-repressive effects of miR-361-5p inhibitor on CCA cell lines.

Knockout TRAF3 Reversed the Tumor-Repressive Effects of MiR-361-5p Inhibitor in CCA Cells

To investigate the effect of miR-361-5p and TRAF3 on the biological behaviors of CCA cells, we performed some experiments on cell apoptosis and cell viability. The results demonstrated that the downregulation of miR-361-5p significantly reduced the cell viability of TFK1 and QBC939 cells (Figures 9A and B). In addition, the apoptosis assay results revealed that miR-361-5p inhibitor induced the apoptosis of CCA cells (Figures 9C and 9D). While all the effects of miR-361-5p inhibitor on CCA cells were eliminated by TRAF3-shRNA co-transfection.

Knockout TRAF3 Reversed the Inhibitory Effect MiR-361-5p Inhibitor on the Activation of NF-κB Signal Pathway

To further explore the signal pathway underlying the disincentive role in CCA cells, the protein levels of p65 and p-p65 on the NF-κB pathway were measured by Western blotting after transfection. Our results demonstrated that miR-361-5p inhibitor significantly depressed the p-p65 protein expression compared with the control group, and this effect was significantly elimi-

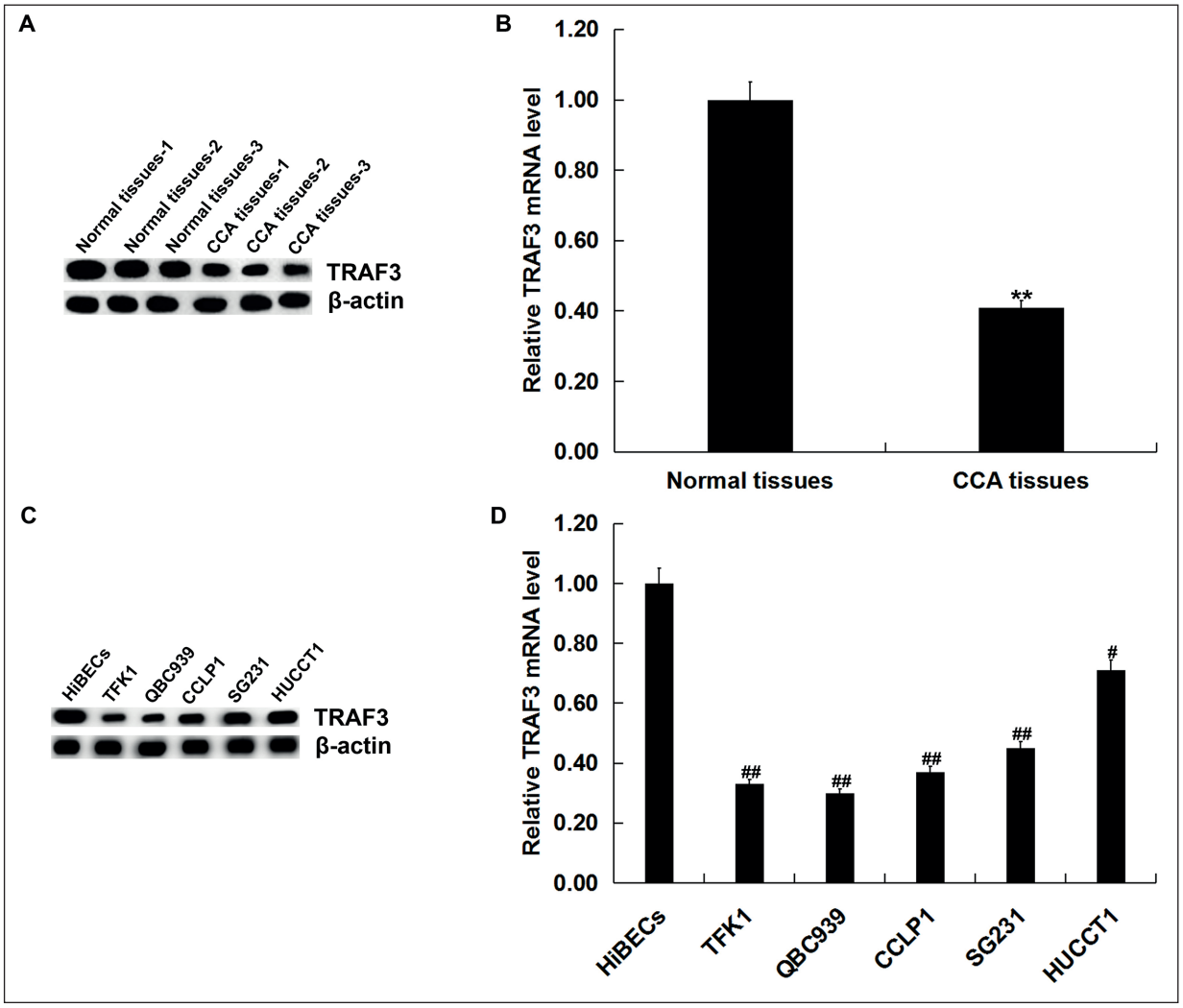

Figure 7. The low expression of TRAF3 in cholangiocarcinoma tissues and cells. ***A,*** and ***B,*** The protein (in three representatives of CCA tissues and human normal tissues), and the mRNA expression of TRAF3 in 20 cholangiocarcinoma tissues and 20 corresponding adjacent normal tissues of 20 cholangiocarcinoma patients was detected by Western blotting and qRT-PCR assay. ***C,*** and ***D,*** The protein and mRNA expression of TRAF3 in cholangiocarcinoma cell lines (CCLP1; SG231; HUCCT1; TFK1; QBC939) and in the intrahepatic bile duct epithelial cell line (HiBECs) was detected by Western blotting and qRT-PCR assay. The data were expressed as the mean ± SD; **$p<0.01$ *vs.* normal tissues; #, ##$p<0.05$, 0.01 *vs.* HiBECs.

nated by TRAF3-shRNA (Figures 9E and 9F). These results indicated that MEG3 repressed CCA by inhibiting the NF-κB signaling pathway by downregulating miR-361-5p expression in CCA cells.

Discussion

LncRNAs, emerging as vital regulatory molecules in tumor suppressing and oncogenic pathways, have been reported to be correlated with multiple physiological processes, such as cell proliferation and apoptosis[26]. Previous studies have shown that MEG3 acted as a tumor suppressor in various tumors. However, how MEG3 functions in CCA nosogenesis remains unknown. MiRNAs have obtained increasing attention as they are involved in tumor progression. Many reports[27] suggested that miRNAs acted as tumor suppressors or oncogenes and are involved in post-translational regulation of gene expression.

In the present work, we demonstrated that MEG3 functioned as a tumor suppressor in CCA. We firstly found that MEG3 was downregulated in CCA tissues and cell lines, indicating the critical roles in CCA development. The Dual-Luciferase Reporter Assay combined with RNA binding as-

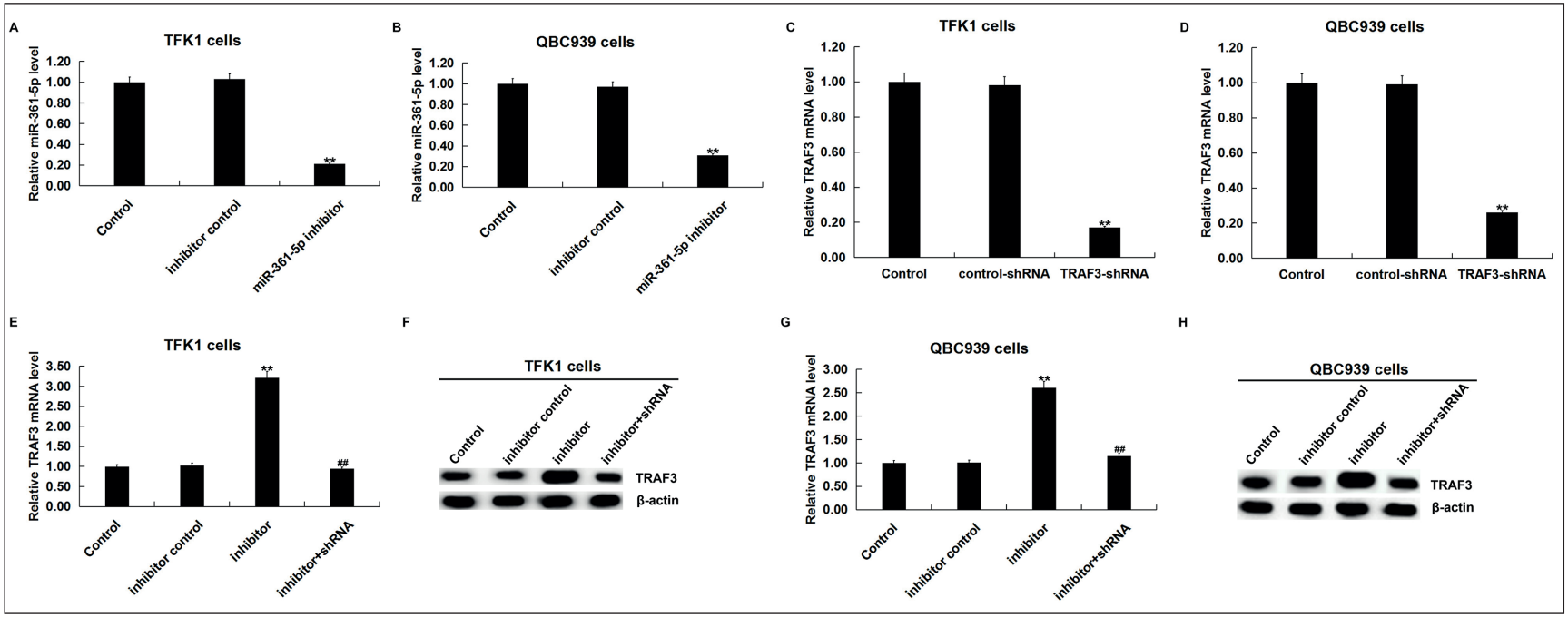

Figure 8. MiR-361-5p negatively regulates TRAF3 expression in CCA cells. ***A,*** and ***B,*** TFK1 and QBC939 cells were transfected with miR-361-5p inhibitor for 48 h, then, the expression of miR-361-5p in ***(A)*** TFK1 and ***(B)*** QBC939 cells was detected by qRT-PCR analysis. ***C,*** and ***D,*** TFK1 and QBC939 cells were transfected with TRAF3-shRNA for 48 h, then, the mRNA expression of TRAF3 in ***(C)*** TFK1 and ***(D)*** QBC939 cells was detected by qRT-PCR analysis. ***E,-H,*** Relative mRNA (***E*** and ***G***) expression of TRAF3 and TRAF3 protein (***F*** and ***H***) expression in TFK1 and QBC939 cells transfected with miR-361-5p inhibitor or miR-361-5p inhibitor+TRAF3-shRNA were measured by qRT-PCR and Western blotting. The results were presented as mean±SD. ***p*<0.01 *vs.* Control; ##*p*<0.01 *vs.* inhibitor.

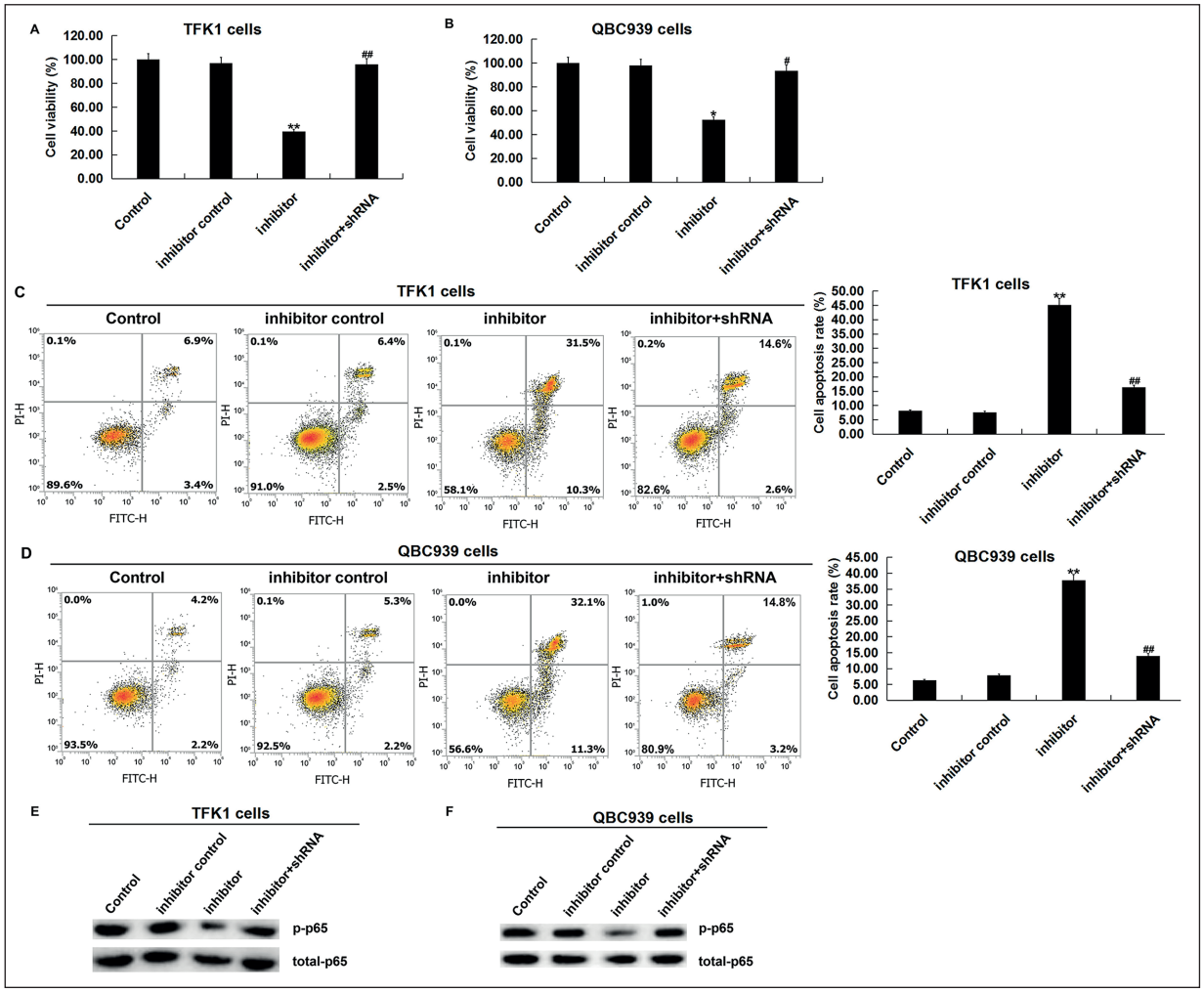

Figure 9. TRAF3 knockdown reversed the inhibitory effects of miR-361-5p inhibitor on CCA cells. ***A,*** and ***B,*** The cell viability of TFK1 cells and QBC939 cells transfected with miR-361-5p inhibitor or miR-361-5p inhibitor+TRAF3-shRNA was measured through MTT assay. ***C,*** and ***D,*** Flow cytometry was performed to determine the percentages of apoptosis in TFK1 cells and QBC939 cells transfected with miR-361-5p inhibitor or miR-361-5p inhibitor+TRAF3-shRNA. ***E,*** and ***F,*** Western blotting was performed to determine the expression of p65 and p-p65 in TFK1 cells and QBC939 cells transfected with miR-361-5p inhibitor or miR-361-5p inhibitor+TRAF3-shRNA. Each bar in the histogram represents the mean±SD; *, **p<0.05, 0.01 *vs.* control; #, ##p<0.05, 0.01 *vs.* inhibitor.

say provided further support for the interaction of MEG3-miR-361-5p activity. Meanwhile, we found that miR-361-5p was upregulated in CCA and it was negatively correlated with MEG3 expression in CCA cells. The upregulation of MEG3 inhibited cell viability and promoted cell apoptosis in TFK1 and QBC939 cells, which proved its tumor repressive effect and further revealed that MEG3 was able to lead to positively downregulate the expression of miR-361-5p. Another very important finding was that miR-361-5p mimic could reverse the effects induced by MEG3-plasmid in CCA cells.

Then, we speculated that miR-361-5p might be an oncogene in CCA progression. To confirm this inference, a bioinformatics tool was used to predict the potential targets of miR-361-5p. Our results demonstrated that TRAF3 was a direct target of miR-361-5p in CCA. Next, we used qRT-PCR and Western blotting assay to measure the TRAF3 mRNA level and protein expression in CCA tissues and cell lines. We showed that TRAF3 was decreased in CCA tissues and CCA cell lines. Then, we especially focused on the effect of miR-361-5p inhibitor treatment on CCA cell lines. Here, our results indicated that TRAF3 expression was enhanced by miR-361-5p inhibitor treatment in CCA cells. We then further confirmed whether MEG3 regulated CCA cell

growth via regulating miR-361-5p by targeting TRAF3, and we found that miR-361-5p inhibitor significantly inhibited CCA cell viability, induced cell apoptosis, and repressed NF-κB pathway. It was worth mentioning that all the effects of miR-361-5p inhibitor on CCA cells were eliminated by TRAF3 silencing.

Conclusions

LncRNA MEG3 served as a tumor suppressor in CCA development by regulating miR-361-5p/TRAF3/NF-κB pathway. This research better illuminated the pathogenesis and development of CCA and might provide novel clinical therapies for CCA treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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