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MicroRNA-361-5p suppresses the tumorigenesis of hepatocellular carcinoma through targeting WT1 and suppressing WNT/β-cadherin pathway

Y. CHENG[1], L. QIU[1,2], G.-L. HE[1], L. CAI[1], B.-J. PENG[1], Y.-L. CAO[1], M.-X. PAN[1]

[1]Second Department of Hepatobiliary Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong Province, China
[2]Department of General Surgery, Dongfeng Zhongshan People's Hospital, Zhongshan, Guangdong Province, China

Yuan Cheng and *Ling Qiu* contributed to the work equally and should be regarded as co-first authors

Abstract. – OBJECTIVE: MicroRNA-361-5p (miR-361-5p) has been found to be involved in the pathogenesis of several human cancers. However, the specific role of miR-361-5p is still unclear in hepatocellular carcinoma (HCC). Therefore, this study was designed to elucidate the function of miR-361-5p in HCC.

PATIENTS AND METHODS: The expression levels of miR-361-5p and Wilms' tumor-1 (WT1) were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. Moreover, the function of miR-361-5p was examined through Cell Counting Kit-8 (CCK-8) and transwell assays. The protein expressions were examined via Western blot analysis and immunocytochemical assay. Tumor growth of HCC was observed via xenograft tumor formation assay. The relationship between miR-361-5p and WT1 was verified by the Dual-Luciferase assay.

RESULTS: Downregulation of miR-361-5p was identified in HCC, which predicted a worse prognosis in HCC patients. Furthermore, it was found that miR-361-5p suppressed cell proliferation, migration, and invasion in HCC by inhibiting WT1. MiR-361-5p also inhibited tumor growth of HCC. Besides that, miR-361-5p suppressed EMT and negatively activated the WNT/β-cadherin pathway in HCC.

CONCLUSIONS: MiR-361-5p suppressed tumorigenesis of HCC by targeting WT1 and inactivating the WNT/β-cadherin pathway.

Key Words:
Hepatocellular carcinoma, MiR-361-5p, WT1, WNT/β-cadherin.

Introduction

Hepatocellular carcinoma (HCC) is the third most common malignant tumor after gastric cancer and esophageal malignant tumor[1]. Moreover, the early symptoms of HCC are not evident. The advanced HCC is mainly characterized by liver pain, angular, jaundice, ascites, and other symptoms[2]. However, the etiology and exact molecular mechanisms of HCC are not fully understood until now. It is currently believed that the pathogenesis of HCC is a complex process of multiple factors and steps, affected by its environment and genetics[3]. Although the tumorigenesis of HCC can be seen in all age groups, it is relatively rare in children. Most Chinese HCC patients are between 45 and 65 years old. However, clinical studies have found that the incidence of young people is significantly increased in recent years[4]. The incidence of HCC in men is higher than that in women, and the ratio of male to female is about 3:1[5]. Surgery, radiotherapy, and chemotherapy are widely used in clinical practice. However, the cure rate of patients with advanced HCC is still low due to cell proliferation[6]. The one-year survival rate after HCC resection is higher than 80%, and the five-year survival rate is over 50%[7]. In addition, the prognosis of HCC patients was found to be correlated with clinical and pathological type[8]. Therefore, the biomarkers for early detection, early diagnosis, and early treatment of HCC should be developed.

Zhang et al[9] have shown that microRNAs (miRNAs) play important roles in the pathogenesis of human cancers by affecting the expressions of the target genes. Moreover, many miRNAs that are oncogenes and tumor suppressors have been found to be involved in the tumorigenesis of HCC. For example, miR-199 functioned as a suppressive miRNA by downregulating the regulators of G-protein signaling 17 in HCC[10]. In contrast, miR-219-5p promoted tumor growth and

Corresponding Author: Mingxin Pan, MD; e-mail: pmxwxy@sohu.com

cell metastasis in HCC by regulating Cadherin 1[11]. In recent years, miR-361-5p has been found to exert different effects in some human cancers. Wu et al[12] proposed that miR-361-5p facilitated the development of cervical cancer by mediating epithelial-mesenchymal transition (EMT). By contrast, the inhibitory effect of miR-361-5p was observed in prostate cancer[13]. Moreover, miR-361-5p suppressed chemoresistance by modulating PI3K/AKT/mTOR pathway in gastric cancer[14]. However, the specific role of miR-361-5p is still unclear in HCC by now. Therefore, we designed this research to explore its function in HCC.

Wilms' tumor-1 (WT1) has been found to play a diagnostic and prognostic role in high-grade serous ovarian carcinoma[15]. Moreover, abnormal expression of WT1 has been identified in human breast cancer[16], and WT1 was found to be upregulated in desmoids tumors[17]. Liu et al[18] reported that high WT1 expression was associated with aggressive clinical features of ovarian cancer. Besides that, the WNT/β-cadherin pathway has been demonstrated to regulate the development of HCC. For example, the carcinogenic effect of miR-324-3p was found in HCC by activating the WNT/β-catenin pathway[19]. Cui et al[20] proposed that miR-337 regulated PI3K/AKT and WNT/β-catenin signaling pathways to inhibit HCC progression. However, the interaction between miR-361-5p and WT1 or WNT/β-catenin signaling pathway has not been investigated in HCC.

In the current study, the potential function of miR-361-5p was explored in HCC. Moreover, the downstream regulatory mechanism of miR-361-5p was also investigated in HCC. The interaction between miR-361-5p and WT1 or WNT/β-catenin signaling pathway was verified in this study.

Patients and Methods

Clinical Tissues

The experimental tissues were acquired from 72 HCC patients in the Second Department of Hepatobiliary Surgery of Zhujiang Hospital. All patients who participated in this study provided written informed consent. The study was approved by the Institutional Ethics Committee of Zhujiang Hospital, Southern Medical University. All HCC patients did not receive any treatment before surgery. Finally, these tissues were frozen in liquid nitrogen and then stored in -80°C refrigerator for further experiments.

Cells Culture

The normal human hepatic cell LO2 and MHCC97H, HCCLM3, Huh7 HCC cell lines were used for this experiment. These cell lines were purchased from Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China). These cell lines were then inoculated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). Finally, the cells were cultured at 37°C in an atmosphere with 5% CO_2.

Cell Transfection

MiR-361-5p mimic or inhibitor, miR-361-5p plasmid, and negative control (NC) were obtained from RiboBio (Guangzhou, China). They were separately transferred into HCCLM3 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturers' protocols.

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

The total RNA was extracted using TRIzol reagent (Promega, Madison, WI, USA) according to the standard method. Based on the instructions of the manufacturer, the First Stand cDNA Synthesis Kit (TIANGEN Biotechnology, Beijing, China) was used for the synthesis of cDNA. Quantitative RT-PCR was performed by SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on ABI 7300 Sequence Detection System (Thermo Fisher Scientific, Inc., Waltham, MA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-361-5p and WT1. Their expressions were calculated using the $2^{-\Delta\Delta ct}$ method. The qRT-PCR primers were designed as follows: miR-361, Forward: 5'-UCC CCC AGG UGU GAU UCU GAU UU-3', Reverse: 5'-GCA AAT CAG AAT CAC ACC TG-3'; U6, Forward: 5'-CTC GCT TCG GCA GCA CA-3', Reverse: 5'-AAC GCT TCA CGA ATT TGC GT-3'; Human WT1, Forward: 5'-CAG GCT GCA ATA AGA GAT ATT TTA AG CT-3', Reverse: 5'-GAA GTC ACA CTG GTA TGG TTT CTC A-3'; Human GAPDH, Forward: 5'-TGC ACC ACC AAC TGC TTA GC-3', Reverse: 5'-GGC ATG GAC TGT GGT CAT GAG-3'.

Western Blot Analysis

The protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The proteins

were then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and incubated with 5% non-fat milk in polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at room temperature. Next, we incubated the membranes overnight at 4°C with WT1, EMT markers (E-cadherin, N-cadherin, Vimentin), WNT/β-cadherin markers (β-cadherin, p-β-cadherin), and GAPDH primary antibodies (1:1000; Abcam, Cambridge, MA, USA). After washing, they were incubated with the corresponding secondary antibodies. Finally, the protein expression levels were measured by electrochemiluminescence (ECL; Pierce Biotechnology, Waltham, MA, USA).

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 assay was performed to examine cell proliferation. First, 3×10^4 HCCLM3 cells were plated and incubated in 96-well plates for 0, 24, 48, and 72 h. Next, the cells were placed in an incubator with 5% CO_2 at 37°C. After that, 10 μl of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well for 2 h. Finally, they were detected using a microplate reader (Molecular Devices, Eugene, OR, USA) at an absorbance of 450 nm.

Transwell Assays

Cell migration and invasion assays were performed using the transwell chambers (8-μm pore size; Millipore, Billerica, MA, USA). The lower chamber was added with 10% FBS. The experiment was performed in an atmosphere with 5% CO_2 at 37°C. The upper chamber with matrigel (BD Biosciences, Billerica, MA, USA) was used for cell invasion. But cell migration assay was conducted without matrigel. After that, 3×10^4 HCCLM3 cells were seeded in the upper chamber with a serum-free medium. After 24 hours, the migrated or invaded cells were fixed with methanol and stained with crystal violet. Finally, we counted the number of removed cells using a microscope (Olympus, Tokyo, Japan).

Xenograft Tumor Formation Assay

The nude mice (6 weeks old) were purchased from the Model Animal Research Center at Nanjing University (Nanjing, China). All animal experiments were approved by the Animal Care and Use Committee of Zhujiang Hospital and performed based on the Guide for the Care and Use of Laboratory Animals. First, 4×10^6 HCCLM3 cells with miR-361-5p plasmid or negative control were injected into the upper left flank of nude mice. The tumor volume was observed every 4 days. After 4 weeks, the mice were sacrificed, and the tumors were used for further study.

Dual-Luciferase Reporter Assay

The 3'-UTR of wild or mutant WT1 was inserted into the pmirGLO Luciferase vector (Promega, Madison, WI, USA) for Luciferase reporter assay. After that, the vector with 3'-UTR of WT1 and miR-361-5p mimics were transfected into HCCLM3 cells. Finally, the Dual-Luciferase assay system (Promega, Madison, WI, USA) was applied to analyze the Luciferase activity.

Immunocytochemical Assay

The sections of HCC tissues were dewaxed, hydrated, and washed twice with phosphate-buffered saline (PBS) for 5 min. After blocking with 5% goat serum (diluted in PBS), we incubated the cells with anti-WT1 (Nucleus) antibody at 37°C for 1-2 h. Next, we washed them three times with PBS for 5 min. We then incubated them with the corresponding secondary antibodies for 1 h at 37°C. After washing 3 times with PBS, a mixture of diaminobenzidine (DAB) was used for color development of this section. The section was washed, counterstained, dehydrated, transparentized, and fixed. The images were captured using a microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Data were calculated by Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). Data were shown as mean ± SD (Standard Deviation). The differences between groups were calculated through χ^2-test or One-way ANOVA with Bonferroni post-hoc test. Kaplan-Meier analysis with log-rank test was applied to measure the survival of HCC patients. Significant differences were defined as $p<0.05$.

Results

MiR-361-5p Expression Was Declined in HCC Tissues

First, the expression of miR-361-5p was detected in HCC tissues by qRT-PCR assay. We found that miR-361-5p expression was reduced in HCC tissues compared with normal tissues (Figure 1A). Moreover, abnormal miR-361-5p expression

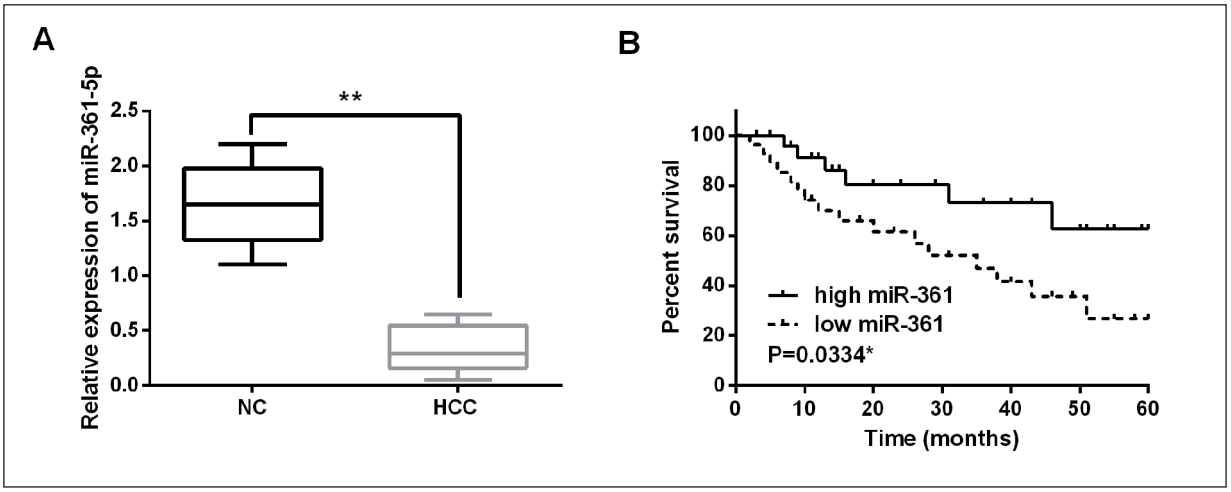

Figure 1. MiR-361-5p expression was declined in HCC tissues. **A,** The expression of miR-361-5p in HCC tissues. **B,** Low miR-361-5p expression was related to shorter overall survival in HCC patients. *$p < 0.05$, **$p < 0.01$.

was found to be associated with lymph node metastasis (p=0.015) and TNM stage (p=0.005, Table I). It indicated that the abnormal expression of miR-361-5p might be involved in the development of HCC. Besides that, the aberrant miR-361-5p expression also can predict prognosis in HCC patients. It was also found that high miR-361-5p expression had a longer overall survival in HCC patients (p=0.0334, Figure 1B). Therefore, miR-361-5p was considered to act as a biomarker for the diagnosis and prognosis of HCC.

MiR-361-5p Suppressed the Development of HCC

Next, miR-361-5p expression was examined in MHCC97H, HCCLM3, Huh7, and LO2 cell lines. We found that miR-361-5p was downregulated in MHCC97H, HCCLM3, and Huh7 cell lines compared with LO2 cells (Figure 2A). After that, miR-361-5p mimics or inhibitor was transfected into HCCLM3 cells. The results showed that miR-361-5p expression was enhanced by its mimics and reduced by its inhibitor (Figure 2B). After-

Table I. Relationship between miR-361-5p expression and their clinic-pathological characteristics of HCC patients.

		miR-361-5p		
Characteristics	**Cases**	**High**	**Low**	***p*-value**
Age (years)				0.073
≥ 50	40	15	25	
< 50	32	13	19	
Gender				0.137
Male	42	15	27	
Female	30	12	18	
Tumor size				0.395
< 5 cm	38	14	24	
≥ 5 cm	34	15	19	
TNM stage				0.005*
I-II	22	9	13	
III-IV	50	20	30	
Lymph node metastasis				0.015*
No	20	7	13	
Yes	52	19	33	
Differentiation				0.539
Well and Moderate	41	15	26	
Poor	31	14	17	

Statistical analyses were performed by the χ^2-test. *$p < 0.05$ was considered significant.

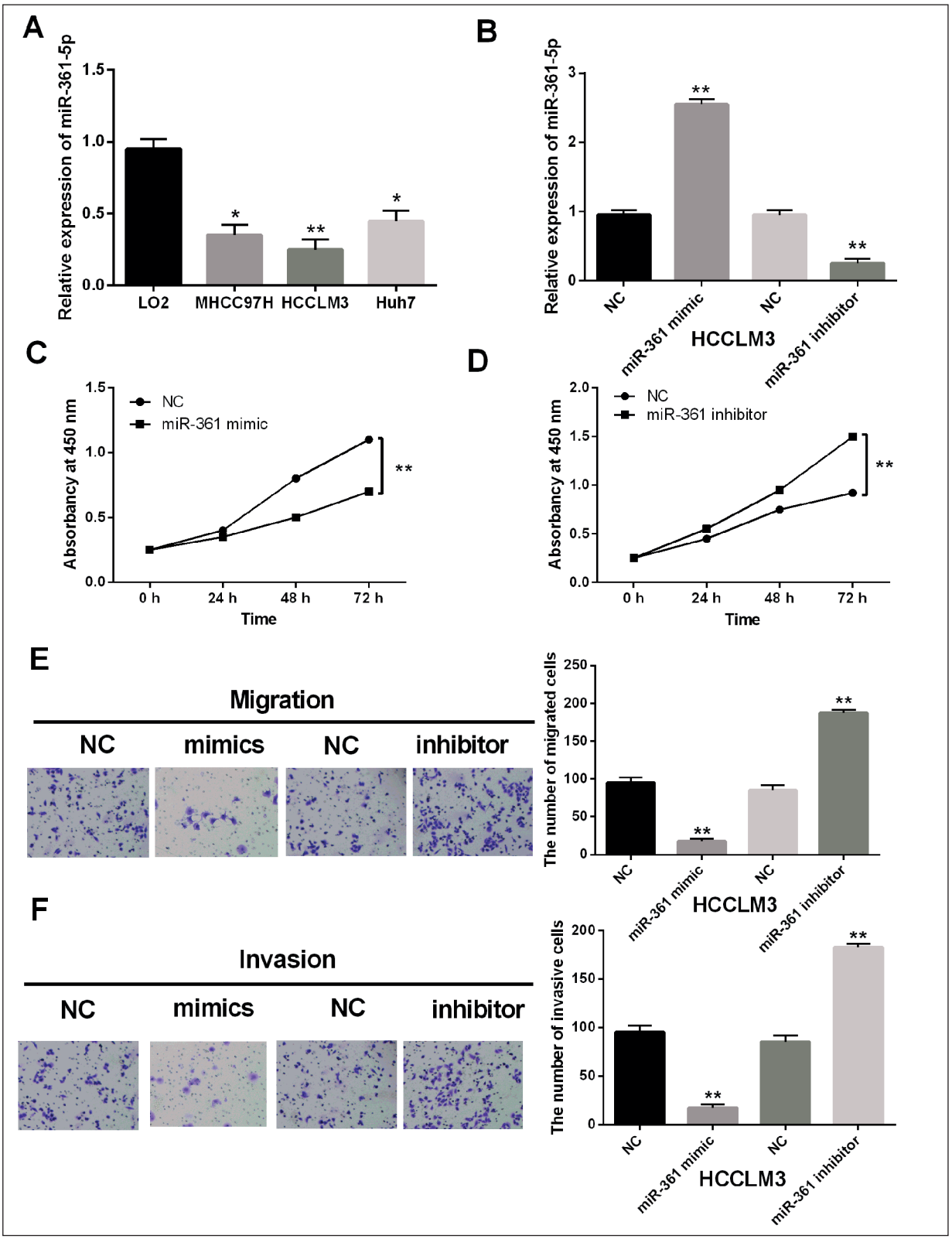

Figure 2. MiR-361-5p suppressed cell proliferation, migration, and invasion in HCC. **A,** The expression of miR-361-5p in MHCC97H, HCCLM3, Huh7, and LO2 cell lines. **B,** The expression of miR-361-5p in HCCLM3 cells containing miR-361-5p mimics or inhibitor. **C-D,** Cell proliferation in cells containing miR-361-5p mimics or inhibitor. **E-F,** Cell migration and invasion in cells with miR-361-5p mimics or inhibitor was detected. $^{**}p < 0.01$.

ward, we found that the overexpression of miR-361-5p repressed cell proliferation in HCCLM3 cells (Figure 2C). By contrast, the downregulation of miR-361-5p promoted cell proliferation in HCCLM3 cells (Figure 2D). In addition, the same effect of miR-361-5p on cell migration and invasion was also found in HCC (Figures 2E, 2F). Hence, we considered that miR-361-5p acted as a tumor suppressor in HCC. Besides that, we injected HCCLM3 cells with miR-361-5p stable transfection plasmid or miR-NC into nude mice. The tumor volume of HCC in nude mice with miR-361-5p plasmid was smaller than that of miR-NC (Figure 3A). Moreover, the growth rate of HCC tumors in nude mice with miR-361-5p plasmid was lower than that of miR-NC (Figure 3B). In conclusion, miR-361-5p showed an inhibitory effect in HCC.

MiR-361-5p Directly Targeted WT1 in HCC

Further, TargetScan (http://www.targetscan.org/) database indicated that miR-361-5p had binding sites with the 3'-UTR of WT1 (Figure 4A). Luciferase reporter assay was then performed to testify the prediction. We found that the Luciferase activity of wt-WT1 was reduced by miR-361-5p mimics. However, no decrease in the Luciferase activity was observed in HCCLM3 cells with mut-WT1 and miR-361-5p mimics (Figure 4B). Moreover, WT1 was found to be negatively correlated with miR-361-5p expression in HCC tissues ($p<0.01$, $R^2=0.2687$; Figure 4C). Furthermore, we detected the expression of WT1 in HCCLM3 cells with miR-361-5p mimics or inhibitor to further confirm the above results. The results showed that WT1 was downregulated in HCCLM3 cells with miR-361-5p mimics (Figure 4D) and upregulated in HCCLM3 cells with miR-361-5p inhibitor (Figure 4E). In conclusion, miR-361-5p directly targeted WT1 and negatively regulated its expression in HCC.

WT1 Was Upregulated in HCC Tissues

Next, the abnormal expression of WT1 was observed in HCC tissues by IHC analysis. The protein expression of WT1 was positive in the nucleus of HCC cells (Figure 5A). Moreover, WT1 was found to be upregulated in HCC tissues (Figure 5B). Furthermore, WT1 could predict the prognosis of HCC patients, and high WT1 expression showed a shorter overall survival in HCC patients ($p=0.0209$, Figure 5C). We considered that WT1 might be involved in the pathogenesis and prognosis of HCC.

MiR-361-5p Suppressed EMT and WNT/Ð-Cadherin Pathway in HCC

Finally, the effect of miR-361-5p on EMT and WNT/β-cadherin pathway was explored in HCC. The overexpression of miR-361-5p was found to block N-cadherin and Vimentin expressions and promoted the expression of E-cadherin in HCCLM3 cells (Figure 6A). In contrast, the knockdown of miR-361-5p showed an opposite effect on the three markers (Figure 6B). Thus, the overexpression of miR-361-5p was considered to inhibit cell metastasis *via* blocking EMT in

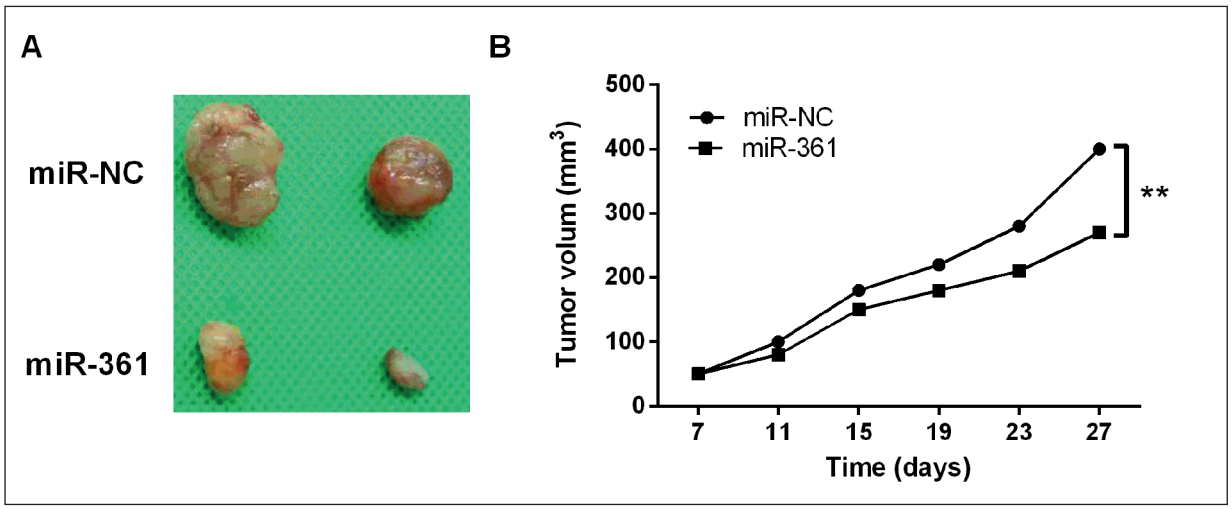

Figure 3. MiR-361-5p inhibited tumor growth of HCC. **A,** Photographs of HCC tumor tissue in miR-361-5p and miR-NC groups. **B,** Growth rate for tumor volumes of HCC was examined every 4 days. $^{**}p < 0.01$.

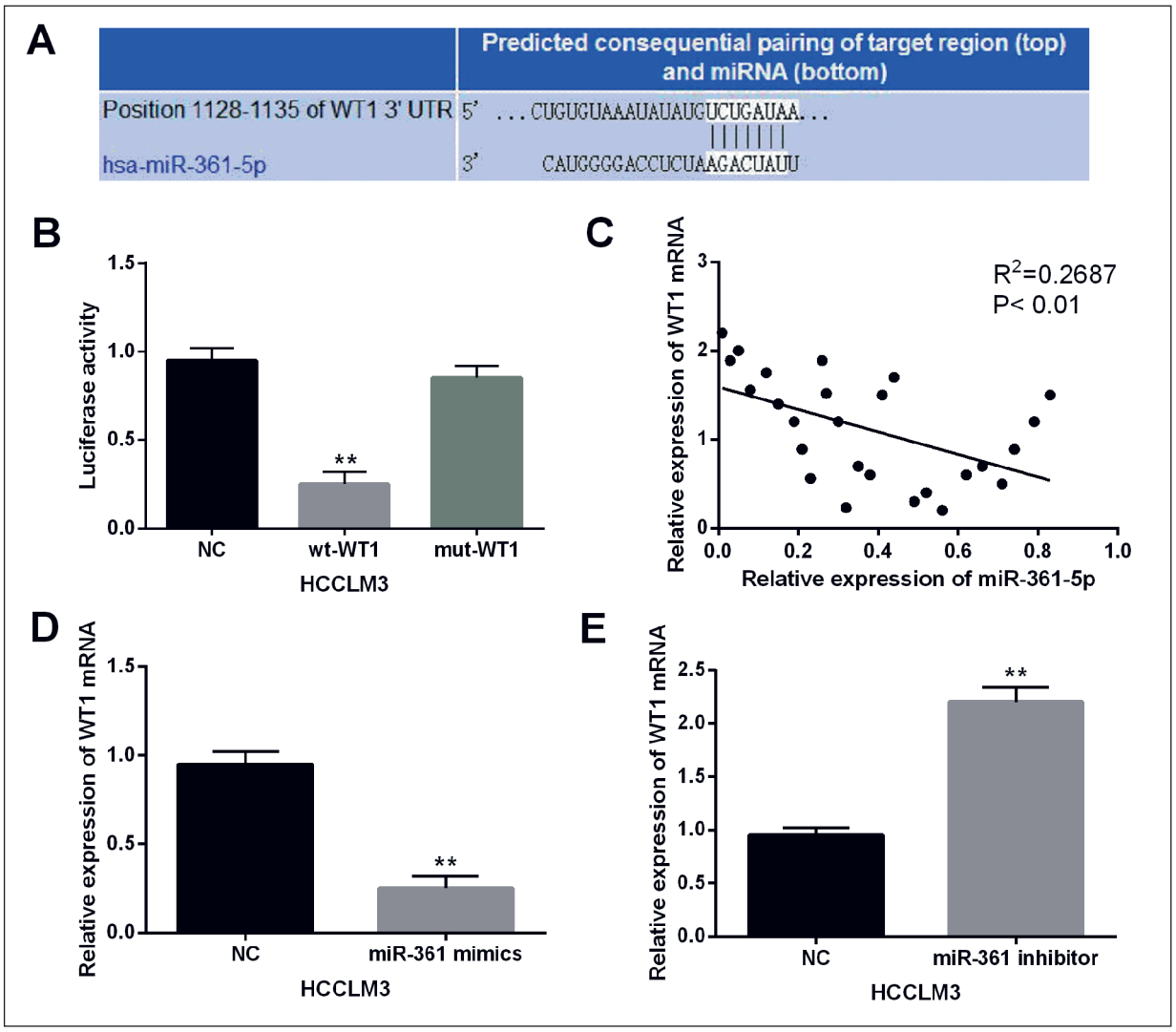

Figure 4. MiR-361-5p directly targeted WT1 in HCC. **A,** The binding sites between miR-361-5p and the 3'-UTR of WT1. **B,** Luciferase reporter assay. **C,** The correlation between miR-361-5p and WT1. **D-E,** The expression of WT1 in HCCLM3 cells containing miR-361-5p mimics or inhibitor $^{**}p < 0.01$.

HCC. In addition, the upregulation of miR-361-5p remarkably reduced the expression of p-β-cadherin. The downregulation of miR-361-5p promoted p-β-cadherin expression in HCCLM3 cells (Figures 6A, 6B). However, the expression of β-cadherin expression was not affected by miR-361-5p. Briefly, miR-361-5p suppressed EMT and WNT/β-cadherin pathway in HCC.

Discussion

Wong et al[21] have shown that miRNAs have molecular functions and pathological significance in HCC. In the present study, the relationship between miR-361-5p and HCC development was elucidated. The downregulation of miR-361-5p was identified in HCC, which was associated with lymph node metastasis and TNM stage. Furthermore, low expression of miR-361-5p predicted poor prognosis of HCC patients. Functionally, the overexpression of miR-361-5p showed an inhibitory effect in HCC.

It has been reported that miR-361-5p is abnormally expressed in human cancers. The downregulation of miR-361-5p was examined in lung cancer[22] and papillary thyroid carcinoma[23], which was similar to our results. Moreover, the downregulation of miR-361-5p was found to be associated with aggressive clinico-

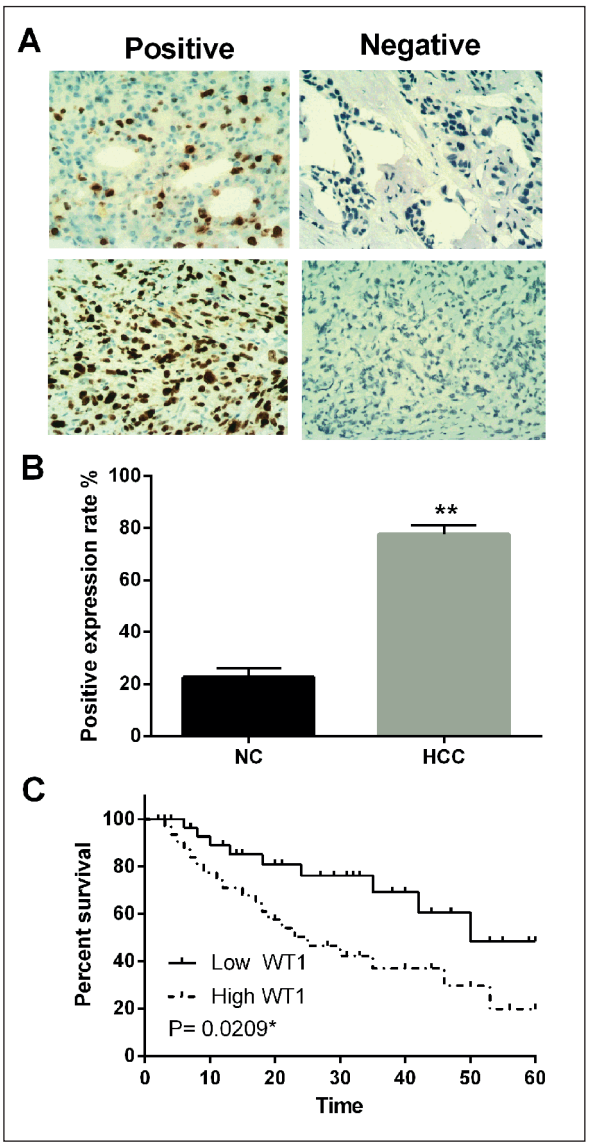

Figure 5. WT1 was upregulated in HCC tissues. **A-B,** The protein expression of WT1 in HCC tissues detected by immunohistochemistry. **C,** High WT1 expression was related to shorter overall survival in HCC patients. *$p < 0.05$.

pathological features and unfavorable prognosis of non-small cell lung cancer[24]. The same results of miR-361-5p were also found in HCC. Furthermore, miR-361-5p suppressed HCC cell proliferation and invasion by inhibiting VEGFA expression[25]. It was also reported[26] that miR-361-5p inhibited tumor growth in HCC by affecting CXCR6. These findings were consistent with our findings. In addition, miR-361-5p inhibited EMT in glioma[27]. In this study, the same effect of miR-361-5p was also observed. It was then confirmed that miR-361-5p directly targeted WT1 and negatively regulated its expression in HCC.

WT1 is a transcriptional activator of the erythropoietin gene, which affects cell growth[28]. The role of WT1 is different in some human cancers. In this study, WT1 was found to function as an oncogene. The upregulation of WT1 was identified in HCC, which predicted poor prognosis of HCC patients. Moreover, miR-361-5p was found to suppress tumorigenesis of HCC by targeting WT1. It has also been proposed that miR-193a inhibited proliferation and metastasis by down-regulating WT1 in breast cancer[29]. Recently, WT1 was identified as a direct downstream target of

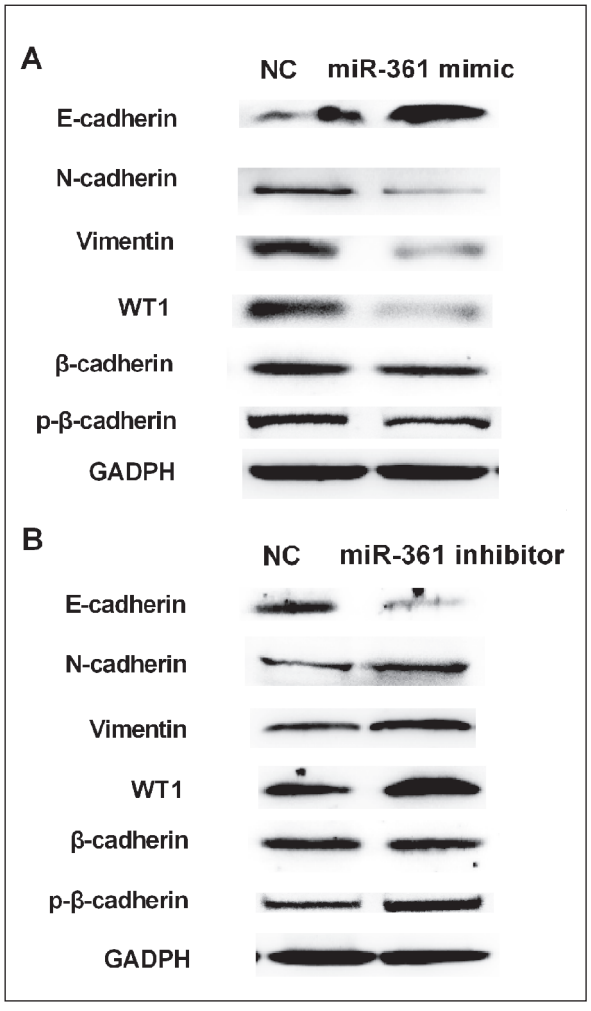

Figure 6. MiR-361-5p suppressed EMT and WNT/β-cadherin pathway in HCC. **A-B,** The protein expressions of E-cadherin, N-cadherin, Vimentin, β-cadherin, and p-β-cadherin in HCCLM3 cells with miR-361-5p mimics or inhibitor.

several miRNAs. For example, miR-15a and miR-16-1 were found to suppress cell proliferation by targeting WT1 in leukemic cells[30]. MiR-590 inhibited the proliferation of nephroblastoma cells by regulating WT1 expression[31]. In addition, we found that miR-361-5p inactivated WNT/β-cadherin pathway in this study to inhibit the proliferation of HCC cells.

It has been reported that the WNT/β-catenin signaling pathway can regulate the proliferation and cell cycle of HCC cells[32]. The interaction between miRNAs and the WNT/β-catenin signaling pathway has been reported in human cancers[33]. In HCC, miR-122 was found to inhibit EMT by suppressing Snail1, Snail2, and WNT/β-cadherin signaling pathway[34]. Same as our findings, it was reported that the overexpression of miR-212 inhibited migration and tumorigenicity by inactivating WNT/β-catenin pathway in HCC[35]. Therefore, the WNT/β-catenin signaling pathway was impeded by miR-361-5p to regulate the progression of HCC.

Conclusions

We showed that the expression of miR-361-5p was decreased in HCC, which was associated with poor prognosis in HCC patients. Furthermore, miR-361-5p inhibited tumorigenesis of HCC by targeting WT1 and inactivating the WNT/β-cadherin pathway. These findings will help to improve the diagnosis and treatment of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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