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MiR-16 inhibits hepatocellular carcinoma progression by targeting FEAT through NF-κB signaling pathway

X.-F. SU[1], N. LI[2], F.-L. MENG[3], Y.-L. CHU[1], T. LI[1], X.-Z. GAO[1]

[1]Department of Gastroenterology, Weihai Municipal Hospital, Weihai, China
[2]Department of Healthcare, Weihai Municipal Hospital, Weihai, China
[3]Department of Hepatology, Qilu Hospital of Shandong University and Institute of Hepatology, Shandong University, Jinan, China

Xiufeng Su and Na Li contributed equally to this work

Abstract. – OBJECTIVE: MicroRNA-16 (miR-16) expression has been proved to take part in the initiation and development of several cancers, including hepatocellular carcinoma (HCC). However, its role and its molecular mechanism in HCC cells remain unclear. Our study aimed to elucidate miR-16 probable role and potential mechanism in HCC cells.

PATIENTS AND METHODS: MiR-16 expression in HCC was measured by Real Time-Polymerase Chain Reaction (RT-PCR). MiR-16 mimic or inhibitor was applied to increase or decrease miR-16 expression in Huh7 cells separately. The cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The invaded cells and migrated cells were detected by the transwell assay. The epithelial-mesenchymal transition (EMT) and the nuclear factor-κB (NF-κB) were performed using Western blot. The tumor growth was measured via xenograft tumor formation assay. Moreover, bioinformatical methods and luciferase reporter assay were carried out to confirm the miR-16 target gene.

RESULTS: MiR-16 expression was downregulated in HCC tissues and cells. Furthermore, the increasing miR-16 expression was suppressed, whereas the decreasing miR-16 expression promoted cell proliferation, invasion, and migration in Huh7 cells. Moreover, miR-16 targeted FEAT in regulating HCC progression. FEAT was associated with a poor prognosis of HCC patients. Especially, miR-16 suppressed EMT and NF-κB pathway in HCC and inhibited the tumor growth *in vivo*.

CONCLUSIONS: We stated that miR-16 suppressed HCC cell progression by targeting FEAT and inhibiting EMT and NF-κB pathway. MiR-16 may be clinically utilized as a factor for the clinical diagnosis and prognosis of HCC.

Key Words:
MiR-16, Progression, Hepatocellular carcinoma (HCC), FEAT, Nuclear factor–κB (NF-κB).

Introduction

Hepatocellular carcinoma (HCC), as common and deadly cancer in the world, has few effective treatments to date[1,2]. Currently, surgical resection and gene therapy have been the main treatments for HCC[3]. Although there have been significant improvements, the curative ratio is still very low because of extrahepatic metastasis, frequent intrahepatic spread and tumor invasion. Hence, it is very urgent to reveal the treatment strategies and uncover the underlying mechanism of HCC progression.

MicroRNAs (miRNAs), which was well known to suppress the protein expression via binding to its target mRNA 3'-untranslated region (3'-UTR)[4]. Emerging evidence proved that the maladjustment of miRNAs was related to the physiological processes of prostate cancer, breast cancer, lung cancer, and HCC[5-7]. In HCC, miR-205 negatively regulated HCC cell progression via binding to the 3'-UTR of VEGFA[8]. Han et al[9] concluded that miR-9/St6gal1 axis played an inhibitory effect on HCC metastatic ability. MiR-298 and miR-98 negatively regulated HCC cell progression via EMT and Wnt/β-catenin signaling[10,11].

Previous studies reported that miR-16 regulated or negatively regulated the development of various cancers. It was proved that miR-16 was lower expressed in chordoma cells, and Smad3 acted as its target to suppress the cancer progression[12]. Moreover, You et al[13] provided evidence that down-regulation of miR-16 promoted colorectal cancer cell progression by targeting KRAS. In HCC, miRNA-16 expression was down-regulated and inhibited cell growth[14]. Furthermore, Liang et al[5] showed that FEAT was a target of miR-16 in promoting the apoptosis of a variety of human cancers. However, whether miR-16 targeting

Corresponding Author: Xiaozhong Gao, MD; e-mail: Vicky1029@163.com

FEAT in regulating the proliferation, invasion, and migration of HCC is not reported until now. It is well known that the role of the epithelial-mesenchymal transition (EMT) and nuclear factor-κB (NF-κB) signaling pathway in cancer cells metastasis is very important. Therefore, the discovery of molecular targets is very important for the research of pathogenesis and therapeutic strategies of HCC.

FEAT, an unrecognized protein, was identified as a promoter of tumorigenesis[16]. FEAT protein is aberrantly over-expressed in many human tumors, whereas faintly expressed in normal tissues, including breast, prostate, pancreatic cancer[17-19]. Furthermore, other authors[15] suggested that FEAT protein was reported to be highly expressed in liver cancer and lung cancer tissues. These findings indicate that FEAT is an oncogene in the different human diseases. There have been increasing reports in the last few years demonstrated that FEAT expression was increased in HCC cells and could promote HCC progression. Nevertheless, its precise molecular mechanism in HCC regulated by miR-16 was not fully elucidated.

In our research, we indicated that miR-16 negatively regulated HCC cell proliferation, invasion, and migration. We also verified FEAT as the direct target of miR-16 and there was a negative correlation between them. In a word, miR-16 inhibited HCC cell progression via EMT and NF-κB signaling pathway by targeting FEAT. Our results indicated that miR-16 played a critical tumor-suppressive role in the process of HCC progression and could be a potential candidate for the treatment of HCC.

Patients and Methods

Acquisition of Tumor Tissues

HCC tissues were provided by 101 patients who underwent surgical resection at Weihai Municipal Hospital from April 2014 to March 2018. All the patients have no treatment before surgical resection. The Ethics Committee of Weihai Municipal Hospital approved this study, and the patients signed the written informed consent before surgery.

Cell Lines Culture and Cell Transfection

The Huh7, MHCC-97L, HCC-LM3, MHCC-97H cell lines used for this experiment were taken from the American Type Culture Collection (ATCC; Manassas, VA, USA). Then, the cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) and then, they were cultured at 37°C with 5% CO_2 atmosphere.

MiR-16 mimic/inhibitor or negative control was provided by GenePharma (Suzhou, China). Huh7 cells were seeded in 24-well plates respectively, performed the transfection with the help of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and then, the cells were incubated for 48 h with 5% CO_2 at 37°C.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

MTT assay (Sigma-Aldrich, St. Louis, MO, USA) was applied for testing the cell viability of Huh-7 cells. A density of 5×10^3 cells in 96-well plates was cultured at 37°C with 5% CO_2. MTT (20 μL) was added into 96-well plates when cell viability was needed to measure, and then, incubated for another 4 h. The media were removed after the incubation; then, 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into the wells. The cell viability was calculated by measuring the optical density (OD) at 490 nm.

Transwell Assay

The transwell assay was applied for measuring cell migration and invasion. The transwell chamber (8 μm pore size polycarbonic membrane) was used for separating upper and lower chambers. For migration assay, Huh7 cells transfected with miR-16 mimic or inhibitor were seeded in the upper chamber, and the Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS was seeded in the lower chambers. The cotton swabs were carried out to wipe the cells in the upper chambers after they were cultured for 48 h at 37°C. 90% of ethanol was added in the lower chambers to fix the cells, and then 0.05% of crystal violet was added to stain the cells after washing with Phosphate-Buffered Saline (PBS) three times later. Finally, we photographed the migrated cells under a microscope. Invasion ability was also detected by using the same procedure mentioned before, but the upper chamber was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

Real Time-Polymerase Chain Reaction (RT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract the HCC tissues or cells. The purity and concentration of RNAs were

detected by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and the TaqMan Human miRNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) were carried out to synthesize the complementary deoxyribose nucleic acid (cDNA) and amplify PCR and quantify miR-16. U6 was used as a control to normalize miR-16 expression. SYBRR Premix Ex Taq™ II Kit (TaKaRa, Dalian, China) was applied for amplifying the PCR and quantifying FEAT. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control of FEAT. The relative expressional level was calculated by the $2^{-\Delta\Delta CT}$ method. The primer sequences were as follows: miR-16-F: TAGCAGCACGTAAATATTGGCG; miR-16-R: TGCGTGTCGT GGAGTC. FEAT-F: CTTCACCGAGGTCAGCAGTA; FEAT-R: CTCCATGACTCT AGCCGACA. GAPDH-F: AGAAGGCTGGGGCTCATTTG; GAPDH-R: AGGGGC CATCCACAGTCTTC. U6-F: CTCGCTTCGGCAGCACA, U6-R: AACGCTTCAC GAATTTGCGT.

Western Blotting

The radioimmunoprecipitation assay (RIPA) lysis containing proteinase inhibitors (Beyotime, Shanghai, China) and PMSF were used for extracting the proteins from the HCC cells. The bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was applied for testing the protein concentrations. The proteins (50 μg) were added in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to be separated. Then, they were transferred to nitrocellulose filter (NC) membranes. After blocking the membranes for 2 h at room temperature using skim milk (5%-10%), the membranes was incubated with the primary antibodies (E-cadherin, N-cadherin, vimentin, and NF-κB, GADPH) at 4°C overnight, subsequently, the secondary antibodies for 2 h at room temperature. The enhanced chemiluminescence kit (ECL, Millipore, Billerica, MA, USA) were used to detect the signals. GADPH served as a loading control.

Luciferase Reporter Assay

The recombinant pMIR-reporter luciferase vector was applied for the luciferase assays. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was carried out to co-transfected miR-16 mimic or control mimic with pMIR-reporter luciferase vector containing 3′-UTR of wild (Wt) or mutated (Mut) FEAT into Huh7 cells. After transfection for 48 h, the Dual Luciferase Reporter System (GeneCopoeia, Rockville, MD, USA) was applied to measure the luciferase activity of Huh7 cells treated with different transfection.

Immunohistochemistry Analysis

The HCC tissues were fixed with 4% paraformaldehyde in PBS for 12 h. The paraffin sections (8 μm) were obtained and then incubated with 3% H_2O_2 in PBS. After blocking with 5% goat serum, we incubated the sections with primary antibody anti-FEAT (Cell Signaling Technology, Danvers, MA, USA) for 24 h at 4°C and then, incubated with biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37°C. The diaminobenzidine (DAB) mixture (Solarbio, Beijing, China) was used to stain the sections. Then, we made the stain sections dehydrated using a graded alcohol series, cleared using xylene, and cover-slipped using neutral balsam. Finally, the protein density per section was determined by the Image Pros Plus 5.0 software (Silver Springs, MD, USA).

Xenograft Tumor Formation Assay

The Shanghai Lab Animal Research Center (Shanghai, China) provided us the nude mice (3-5 weeks old). All animal experiments were approved by the Animal Care and Use Committee of Weihai Municipal Hospital and conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. 5×10^6 transfected cells with pre-miR-16 plasmid or negative control were injected into the right flank of nude mice. The tumor volume was observed every 3 days. After 4 weeks, the mice were sacrificed by CO_2 asphyxiation, and the tumors were used for further study.

Statistical Analysis

The data are represented as mean ± standard deviation, and the experiments were repeated in triplicate. The differences between two groups were compared by Student's *t*-test and one-way ANOVA followed by the post-hoc test (Least Significant Difference) was used to compare differences among multiple groups. GraphPad Prism 5.02 Software (La Jolla, CA, USA) was applied for completing the graph presentations. Correlation analysis of two genes expression was performed using Pearson correlation analysis. The Kaplan-Meier method followed by the log-rank test was used to analyze the association between miR-16 expression levels and survival rate. $p<0.05$ indicated a statistical difference.

Results

Low Expression of MiR-16 Examined in HCC Tissues

For understanding the role of miR-16 played in HCC progression, we examined miR-16 expression level in the HCC tissues. In Figure 1A, we can see that miR-16 expression level was observably lower in HCC tissues than in normal tissues. Moreover, we also showed that miR-16 expression was correlated with TNM stage (p=0.017) and lymph node metastasis (p=0.007) as shown in Table I. Furthermore, the Kaplan-Meier survival curve stated that the lower miR-16 expression, the poorer prognosis of HCC patients, whereas the higher the miR-16 expression, the good prognosis of HCC patients (p=0.0339, Figure 1B). Thus, we concluded that miR-16 might predict the prognosis of HCC patient.

Inhibition Effect of MiR-16 Examined on HCC Cell Proliferation, Migration, and Invasion

To further explore the miR-16 role in HCC, its expression level was detected in Huh7, MHCC-97L, HCC-LM3, and MHCC-97H cell lines. As for the results of HCC tissues, miR-16 expression was declined in Huh7, MHCC-97L, HCC-LM3, and MHCC-97H cells in comparison with control (Figure 2A). Then, the Huh7 cell line was transfected with miR-16 mimic or inhibitor to increase or decrease miR-16 expression. The miR-16 expression we found in Figure 2B was as we expected. MTT assay was applied for the examination of cell proliferation. The results stated that the relative cell viability was significantly lower in miR-16 mimic group, whereas it was higher in miR-16 inhibitor group (Figures 2C, 2D). The transwell assay was carried out to measure the changes in the HCC cell invasion and migration. The results in Figure 2E stated that overexpression of miR-16 inhibited cell migration while silencing miR-16 enhanced cell migration. Consistently, the same tendency of cell invasion was also identified in Huh7 cells with miR-16 mimic or miR-16 inhibitor (Figure 2F). These results made clear that miR-16 downregulated the HCC development.

The Direct Target of MiR-16 Was FEAT in HCC

Previous studies have confirmed FEAT as a target of miR-16 in human cancers. We first predicted that FEAT was the potential target of miR-16 in regulating the HCC cells by using TargetScan Human 7.1. The

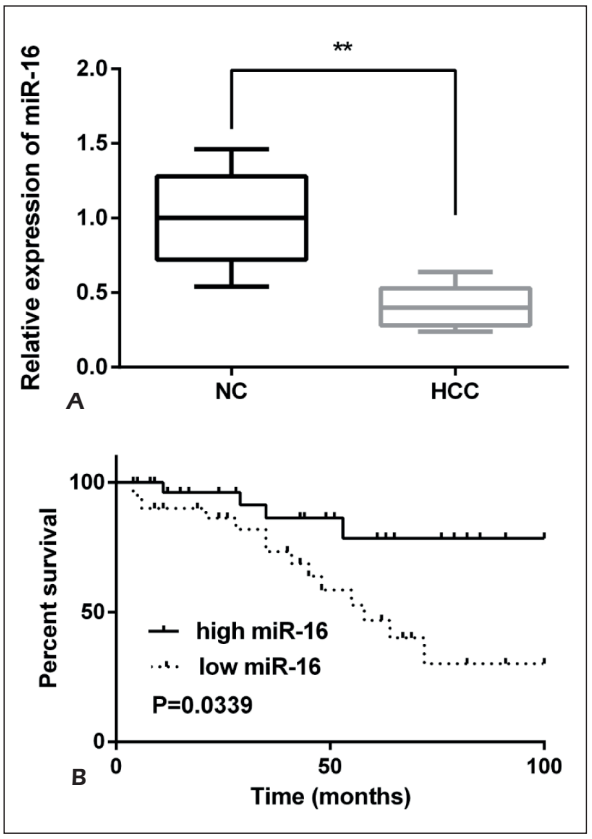

Figure 1. Low expression of miR-16 examined in HCC tissues. **A**, Examination of miR-16 expression in HCC tissues via RT-PCR. **B**, High expression of miR-16 was related to higher overall survival (OS) in HCC patients. **p<0.01.

FEAT 3'UTR and miR-16 binding site were shown in Figure 3A. Moreover, the Luciferase Reporter Assay was performed to further explore whether FEAT was the direct target of miR-16. As predicted, the reduction of the luciferase activity was observed in Huh7 cells co-transfected with miR-16 mimic and FEAT-Wt vector. But the luciferase activity has barely changed in FEAT-Mut (Figure 3B). The Pearson correlation showed a negative correlation between FEAT and miR-16 expression in HCC tissues (Figure 3C). Besides that, FEAT mRNA expression was identified to be reduced by the transfection of miR-16 mimic into Huh7 cells (Figure 3D) and increased by the transfection of miR-16 inhibitor (Figure 3E). Hence, we considered that miR-16 directly targeted FEAT and negatively regulated its expression.

High Expression of FEAT Examined in HCC Tissues

The alternation of FEAT expression in HCC was explored by Immunohistochemistry analysis. The results showed the positive detection of FEAT in the cytomembrane of the HCC cells

Table I. Relationship between miR-16 expression and their clinic-pathological characteristics of HCC patients.

Characteristics	Cases	miR-16		*p*-value
		High	Low	
Age (years)				0.572
≥ 60	61	24	37	
< 60	40	18	22	
Gender				0.719
Male	59	26	33	
Female	42	17	25	
Tumor size				0.116
< 5 cm	38	18	20	
≥ 5 cm	63	20	43	
Clinical stage				0.016*
I-II	32	15	17	
III-IV	69	16	53	
Lymph node metastasis				0.002*
No	75	10	65	
Yes	26	11	15	

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

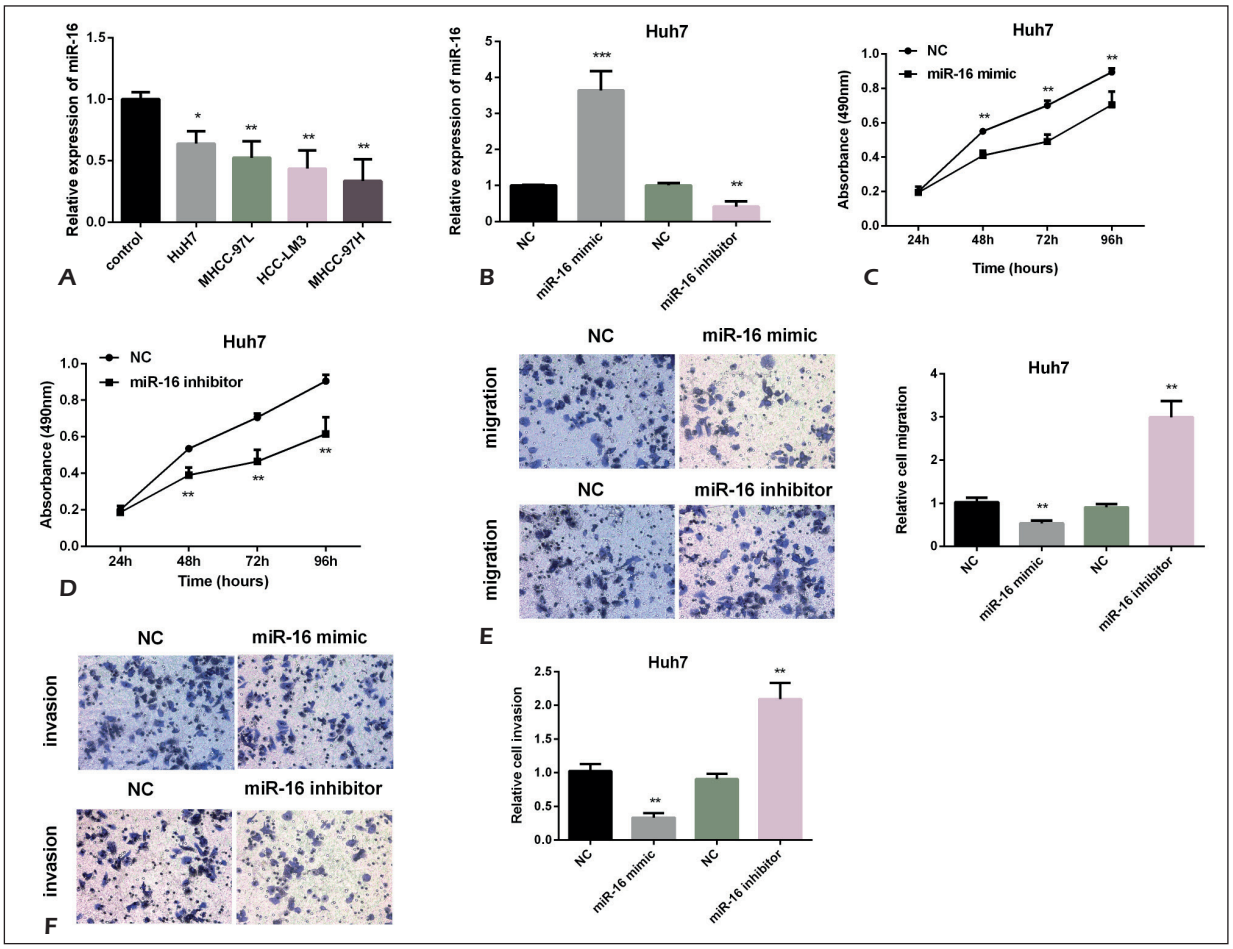

Figure 2. Inhibition effect of miR-16 examined on HCC cell proliferation, migration, and invasion. **A**, MiR-16 expression in HCC cell lines was detected by RT-PCR. **B**, MiR-16 expression examined in Huh7 cells after transfecting with miR-16 mimic or inhibitor by RT-PCR. **C-D**, HCC cells viability measured after treating with miR-16 mimic or inhibitor by MTT assay. E, F, HCC cell migration and invasion measured after treating with miR-16 mimic or inhibitor by transwell assay (magnification: 40×). **$p<0.01$.

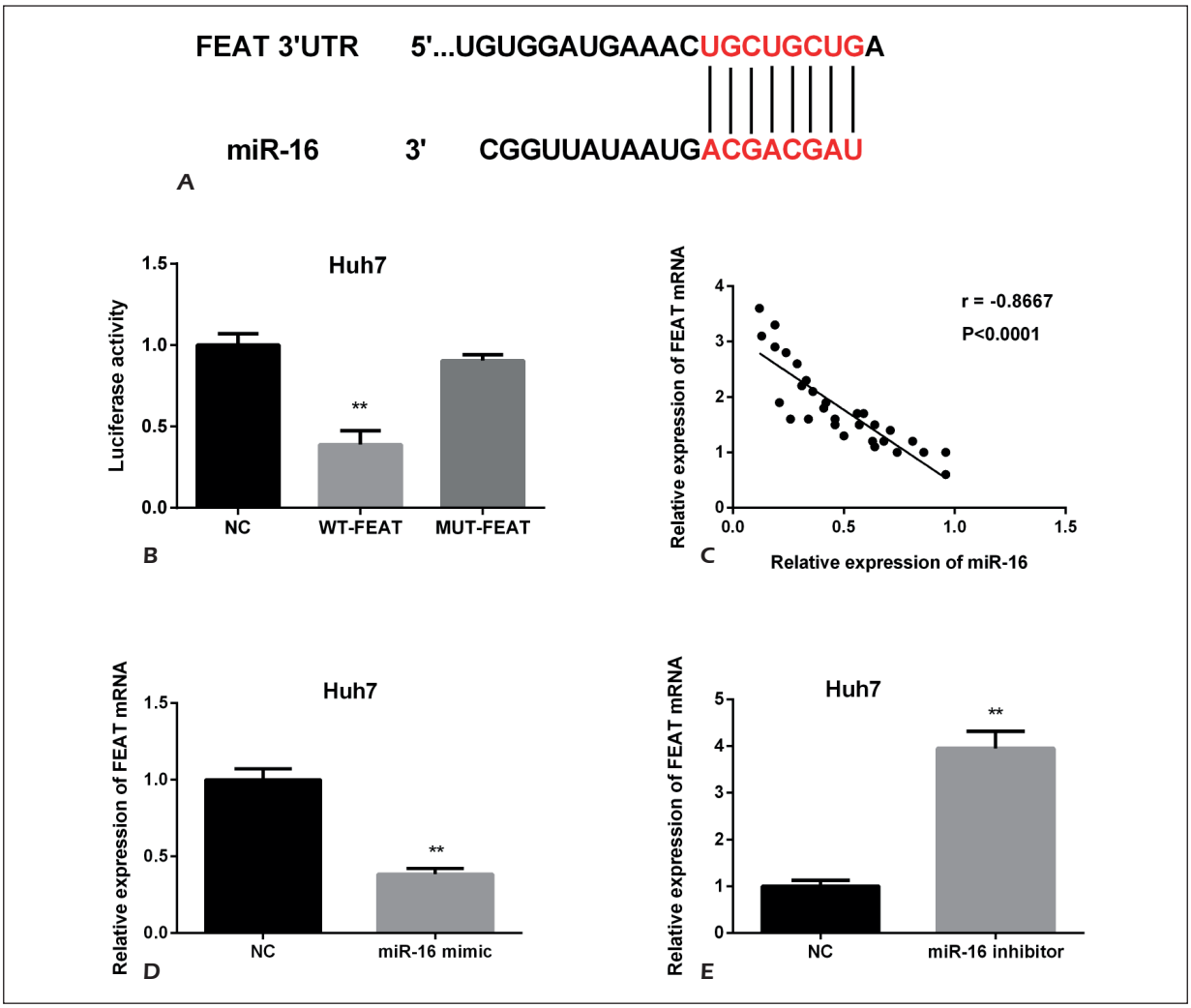

Figure 3. FEAT, the direct target of miR-16 in HCC cells. **A**, Prediction of the binding site of miR-16 with FEAT. **B**, Detection of luciferase activities in Huh7 cells after transfection with FEAT-3'-UTR-Wt (wild-type) or FEAT-3'-UTR-Mut (mutated-type). **C**, Relative FEAT mRNA expression tested using RT-PCR in HCC cells after transfected with miR-16 mimic or inhibitor. **D**, The negatively correlation between FEAT and miR-16 expression in HCC tissues (r=-0.7456, p<0.001) ***p*<0.01.

(Figure 4A). Moreover, the intensity of E2F7 was significantly increased in HCC tissues in comparison with the adjacent normal tissues (Figure 4B). In addition, the Kaplan-Meier survival curve stated that the higher the FEAT expression, the poorer prognosis of HCC patients, while the lower the FEAT expression, the good prognosis of HCC patients (p=0.0545, Figure 4C). Thus, FEAT might take part in the tumorigenesis of HCC.

NF-κB Signaling Pathway Involved in the Progression of HCC Regulated by MiR-16

MiR-16 was proved to suppress HCC cell proliferation, migration, and invasion via FEAT. Therefore, we speculated that miR-16 might regulate EMT in HCC. As we expected, the re-expression of miR-16 inhibited the N-cadherin and Vimentin expression, while increased the E-cadherin expression in HCC cells (Figure 5A). Inversely, the silence of miR-16 enhanced the E-cadherin expression and suppressed the N-cadherin and Vimentin expression level (Figure 5B). So, we predict that miR-16 regulated cell invasion and migration maybe by regulating EMT. In addition, we detected the NF-κB protein expression in Huh7 cells after being treated with miR-16 mimic or inhibitor to further explore the underlying mechanism of miR-16 in HCC cell proliferation. The results showed that the increased miR-16 expression remarkably repressed the phosphorylation of

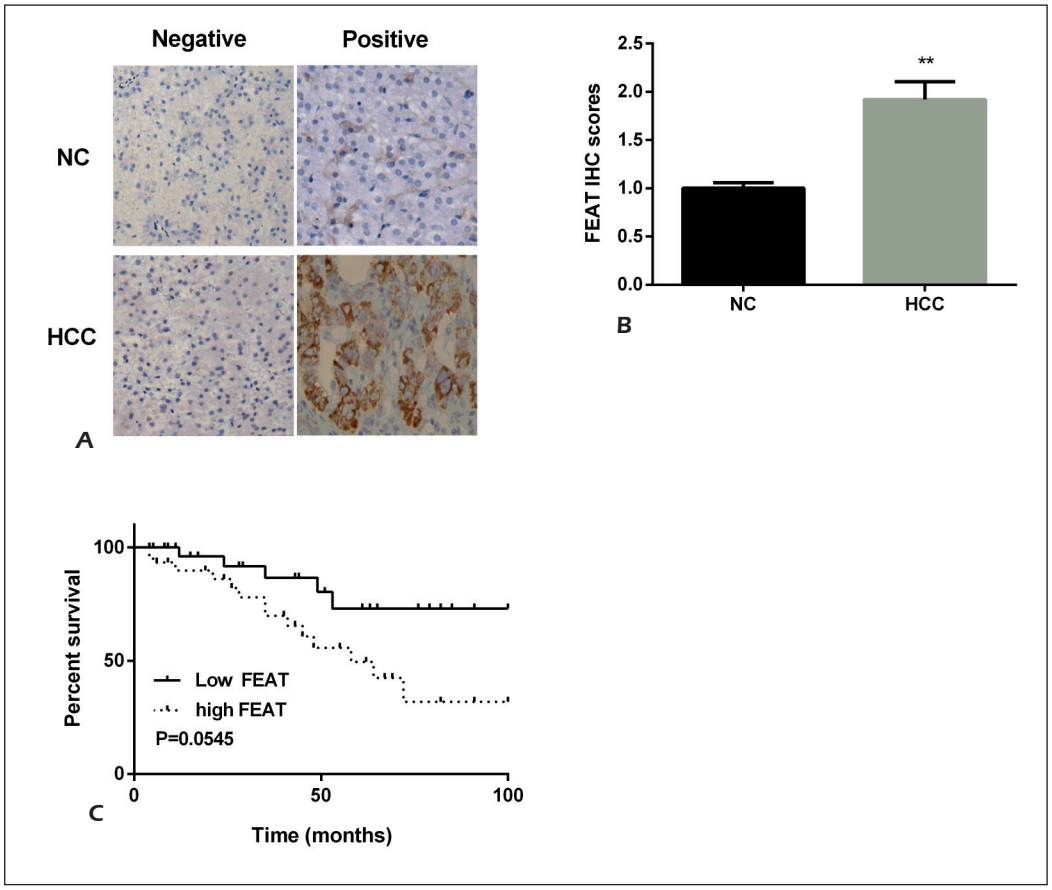

Figure 4. FEAT was upregulated in HCC tissues. **A-B**, FEAT protein expression measured in HCC tissues by immunohistochemistry (magnification: 100×). **C**, Lower FEAT expression was related to higher OS in HCC patients. $^{**}p<0.01$.

NF-κB expression (Figure 5C). On the contrary, the decreased miR-16 expression enhanced the phosphorylation of the NF-κB expression level (Figure 5D). Taken together, miR-16 was examined to regulate EMT and NF-κB pathway in HCC progression.

MiR-16 Inhibited the Tumor Growth In Vivo

The xenograft tumor formation assay was used to measure the tumor growth *in vivo*. The Huh7 cells with miR-16 stable transfection plasmid or miR-NC were injected subcutaneously into nude mice. The results showed that the increased miR-16 expression significantly declined the tumor volume compared to the control group (Figure 6A). Moreover, the tumors transfected with miR-16 stable plasmid grew more slowly than that with miR-NC (Figure 6B). These findings showed that miR-16 inhibited the tumor growth of HCC *in vivo*.

Discussion

Many miRNAs were reported by many researchers to be abnormally expressed in HCC. For example, miR-766 was low expressed in HCC[20], miR-504 acted as a tumor suppressor[21], while miR-106b and miR-223 were highly expressed in HCC[22,23]. Although miRNAs abnormally expressed were related to HCC, to further explore the mechanism of miRNAs in HCC progression was important. Qi et al[14] have proved that miRNA-16 expression was significantly decreased in HCC and that the up-regulation of miR-16 inhibited HCC cell growth, which was similar to our study demonstrating that miR-16 was low-expressed in HCC tissues and cells.

The tumor cells have the characteristics of proliferation, migration, and invasion, which were proved by evidence[24]. In HCC, the abnormal expression of miRNAs affected HCC cell development. Xie et al[25] reported that miR-765

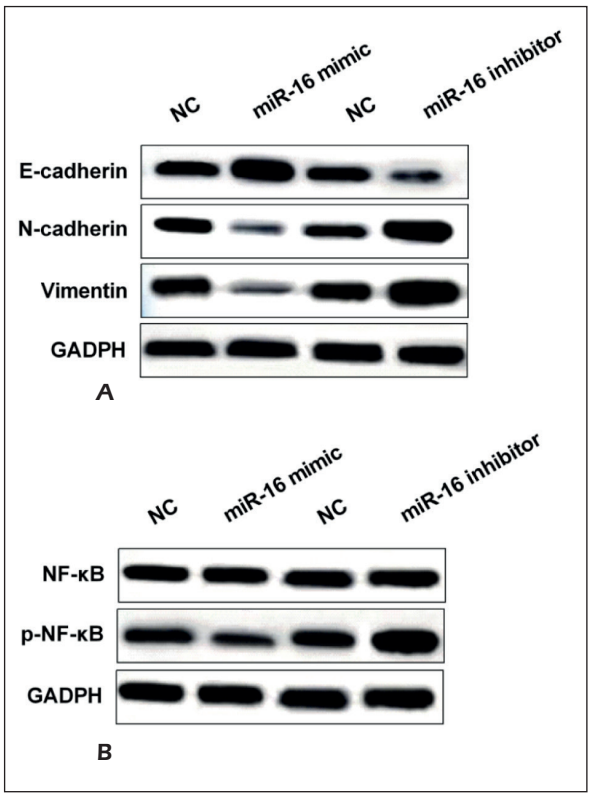

Figure 5. MiR-16 regulated EMT and NF-κB pathway in HCC progression. **A-B**, Western blot analysis of E-cadherin, N-cadherin, and Vimentin in Huh7 cells after treated with miR-16 mimic or inhibitor. **C-D**, Western blot analysis of NF-κB and the phosphorylation of p-NF-κB in Huh7 cells after treating with miR-16 mimic or inhibitor.

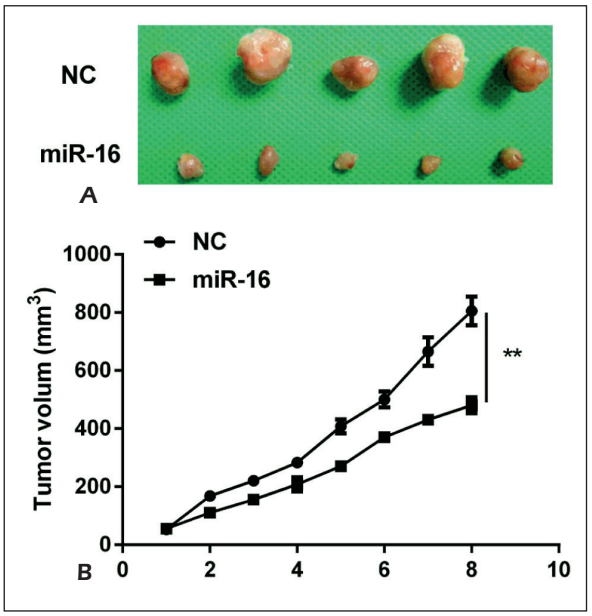

Figure 6. Inhibition effect of miR-16 on the tumor growth in vivo. **A**, The reduced tumorigenic ability of Huh7 cells treated with miR-16 stable transfection plasmid. **B**, The declined growth rate of tumors treated with miR-16 stable transfection plasmid. $^{**}p<0.01$.

mimic increased the HCC cells number by regulating INPP4B. However, increasing miR-375 suppressed HCC cell growth by targeting ErbB2[26]. Our study stated that the up-regulation of miR-16 suppressed HCC cell progression, whereas the downregulation of miR-16 enhanced HCC cell progression, which was consistent with Wu et al[27] reporting that the effect of miR-16 on HCC cell proliferation, invasion, and metastasis. However, the precise molecular mechanism of miR-16 in regulating the HCC progression was underestimated.

It is worth noting that FEAT was recently reported to be overexpressed in various tumors, but rarely in normal tissues[16], and this indicated that FEAT was involved in human cancer as a ubiquitous protein. Takahashi et al[16] reported that FEAT was an oncogene and attenuated apoptotic cell death. However, although great advances in the understanding of the important role of the cancer progression, it is not very clear the exact molecular mechanisms that FEAT was regulating during tumor progression. In HCC, the FEAT expression was higher detected, and the high FEAT protein promoted tumorigenesis *in vivo*[16]. Furthermore, FEAT inhibited the apoptosis of HCC cell regulated by miR-16[15]. The above data like our results demonstrated that FEAT expression was higher in HCC, and it was directly targeted by miR-16. However, it was the first time we found that FEAT inhibited HCC cell proliferation, invasion, and migration as a target of miR-16. Moreover, we also showed that miR-16 regulated the EMT and the NF-κB signaling pathway in HCC cells.

Conclusions

In this study miR-16 expression was lower while FEAT was higher in HCC tissues and cell lines, and their correlation was negative. It is the first time we proved that FEAT was the target of miR-16 in the development of HCC through the NF-κB signaling pathway, indicating that miR-16/ FEAT/ NF-κB signaling pathway maybe a potential application in HCC diagnosis and therapy.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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