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LncRNA MIR503HG regulated cell viability, metastasis and apoptosis of cervical cancer *via* miR-191/CEBPB axis

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Abstract. – OBJECTIVE: The long non-coding RNA MIR503 host gene (MIR503HG) plays a role in suppressing or promoting cancer in many types of human malignant tumors. The role of MIR503HG in cervical cancer is still unknown.

PATIENTS AND METHODS: The expression level of MIR503HG in cervical cancer tissues and cell lines was accessed using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. The Cell Counting Kit-8 (CCK-8) assay and flow cytometric analysis were performed to assess cell proliferation and apoptosis in cervical cancer. The nude mouse xenograft experiment was used to examine the ability of MIR503HG in tumor formation. In our study, we found that the expression of MIR503HG was significantly reduced in cervical cancer tissues and cell lines. In vitro studies have shown that MIR503HG inhibited cell proliferation and invasion, and enhanced cell apoptosis in cervical cancer through the miR-191/CEBPB axis. MIR503HG regulated the expression of miR-191 via directly binding to miR-191.

RESULTS: The expression of MIR503HG had a negative correlation with miR-191 expression in cervical cancer tissues. MiR-191 regulated the expression of CEBPB by directly targeting 3'-UTR of CEBPB mRNA. Overexpression of MIR503HG inhibited cell proliferation, invasion and apoptosis in vitro, and inhibited tumor growth *in vivo*.

CONCLUSIONS: MIR503HG plays a role in suppressing tumors in cervical cancer and is a long-term non-coding RNA.

Key Words:
MIR503HG, MiR-191, CEBPB, Cervical cancer, Apoptosis.

Introduction

Cervical cancer (CC) is considered to be a fatal malignant gynecological tumor, and is diagnosed as the leading cause of cancer-related mortality in women worldwide[1]. The morbidity and mortality of cervical cancer ranks as the 4th place among all types of malignancies[2]. In 2015, there were 98,900 new cases of cervical cancer and 30,500 deaths[3]. Most patients with early cervical cancer can be cured by surgery. However, for those patients with advanced cervical cancer, there is no effective therapy, so the survival rate of these patients is still very low[4]. Therefore, it is necessary to develop biomarkers that can predict the biological behavior of cervical cancer.

Long non-coding RNA (LncRNA) is more than 200 nucleotides in length, which is generally considered to be a potential key factor for gene regulation, affecting the phenotype impact of tumor cell[5]. LncRNA lacks the ability to encode proteins due to the lack of an open reading structure of the necessary length[6]. Many studies have found that lncRNAs were related to the molecular mechanism of cancer[7]. Lin et al[8] clarified the key roles of lncRNAs in regulating a series of complex biological behaviors. For example, MIR503HG, located on chromosome Xq26.3, emerged as a tumor suppressor that suppressed cell proliferation, metastasis and EMT of bladder cancer[9]. Similarly, MIR503HG serves as a tumor suppressor, impairing cell proliferation and inducing cell cycle arrest in NSCLC[10,11]. Moreover, MIR503HG was downregulated in multiple cancers, such as colorectal cancer, gastric cancer and ovarian cancer[12-14]. The roles of MIR503HG in cervical cancer remains unclear, thus, the purpose of this study was to investigate the effects of MIR503HG in cervical cancer.

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More importantly, the lncRNAs were involved in the occurrence and development of various cancers by competing for endogenous RNA (ceRNA)[15,16]. MiR-191 emerged as a diagnostic and prognostic marker of advanced gastric cancer[13]. Similarly, miR-191 served as a prognostic marker, which could enhance survival after radiotherapy, thereby improving radiotherapy response of prostate cancer[17,18]. Based on these reports, we hypothesized that MIR503HG may be a regulator of cervical cancer function, and studied its effects on cervical cancer cell proliferation, migration, and apoptosis.

Patients and Methods

Clinical Specimens

Forty-seven patients with cervical cancer were included in this study from People's Hospital of Rizhao during January 2015 to December 2017. Through operation, we obtained 47 pairs of cervical cancer tissues and adjacent normal tissues. Before the study, all patients did not receive radiotherapy and chemotherapy. All tissues were immediately frozen in liquid nitrogen and stored at -80°C. Each patient signed a written informed consent before the operation. This study was approved by the People's Hospital of Rizhao Ethics Committee. The inclusion criteria for patients were complete medical records and follow-up data, postoperative pathological diagnosis of CC, no preoperative chemotherapy, radiotherapy, endocrine therapy, and other anti-tumor treatments, and no hormones have been used recently. The exclusion criteria for patients were patients with other malignant tumors, systemic infectious diseases, severe liver and kidney dysfunction, and mental illnesses who cannot cooperate with treatment.

Cell Culture

Cervical cancer cell HeLa and a normal cell Ect1/E6E7 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). 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), 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were maintained at 37°C with 5% CO_2.

Cell Transfection

Sh-MIR503HG, miR-191 inhibitor and control oligos were purchased from GenePharma (Shanghai, China). pEX-MIR503HG and pcDNA-CEBPB were obtained from RiboBio (Guangzhou, China). HeLa cells were transfected with the vector using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA).

Quantitative Real-Time PCR Analysis

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract total RNA. PrimeScript RT reagent Kit (Invitrogen, Carlsbad, CA, USA) was then performed to reverse-transcribe the complementary DNA (cDNA) from RNA. Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) on the ABI 7900 System (Applied Biosystems, Foster City, CA, USA). The expression of mRNA and lncRNA was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH), while U6 was the internal control of miRNA-191. The primers for qRT-PCR were as follows: MIR503HG forward 5'-AAGGAATCCTCTCCCACCATTT-3' and reverse 5'-ACTCATTTGGCGGGAAAAC-3'; CEBPB forward 5'-AAGCTGAGCGACGAGTACAA-3' and reverse 5'-GACAGCTGCTCCACCTTCTT-3'; GAPDH forward 5'-GACATCAAGAAGGTGGTGAA-3' and reverse 5'-TGTCATACCAGGAAATGAGC-3'; miR-191 forward 5'-GTGCAGGGTCCGAGGT-3' and reverse 5'-CAACGGAATCCCAAAAGCAGC-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-ACGCTTCACGAATTTGCGT-3'.

Cell Viability Assay

HeLa cells at a density of 5×10^4 cells/well were seeded in a 96-well plate. The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) was employed to measure cell viability at 0, 24, 48 and 72 h. The optical density at 450 nm was measured using a microplate reader (Bio-Tek, Winooski, VT, USA). All the experiments were done in triplicate.

Cell Invasion Assay

Transwell inserts (8 μm in pore size, Millipore, Burlington, VT, USA) coated with Matrigel were carried out to measure cell invasive ability. The upper compartment was added in 200μl cell suspension, while 600 μl medium with 20% FBS was added in the lower chamber. After incubating at 37°C for 24 h, a cotton

swab was utilized to wipe up the non-invasive cells. However, 4% paraformaldehyde and 0.5% crystal violet were employed to fix and stain the invasive cells. The invading cells were counted and photographed using a light microscope (Leica, Heidelberg, Germany).

Cell Apoptosis Assay

Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) was conducted to assess the cell apoptosis ability. In brief, the cells were incubated with equal amounts of Annexin V and propidium iodide (PI) binding buffer in the dark for 15 minutes. Followed, binding buffer was added in the cells and resuspended the cells. Cell apoptosis was analyzed by FACS Calibur Flow Cytometry (BD Biosciences, San Jose, CA, USA).

Xenograft Model

We purchased 4-6 weeks old female BALB/c nude mice from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Cells (1×10^6 cells) were injected subcutaneously into the ventral side of nude mice. The tumor volumes were calculated every two days, and the mice were killed 26 days after inoculation. Then, the tumors were dissected and weighed. All procedures were approved by People's Hospital of Rizhao's Institutional Animal Care and Use Committee.

Statistical Analysis

All data recorded were exhibited as mean ± standard deviation (SD). The GraphPad Prism 5 (La Jolla, San Diego, CA, USA) was employed to analyze the data. The comparison between two groups were analyzed using Student *t*-test, whereas three or more groups were compared using one-way analysis of variance followed by Bonferroni's post hoc test. $p<0.05$ was considered statistically significant.

Results

MIR503HG was Downregulated in Cervical Cancer Tissues and Cell Lines

The level of MIR503HG in 47 pairs of cervical cancer and non-tumor tissues was analyzed by RT-qPCR. MIR503HG was low expressed in cervical cancer tissues versus adjacent tissues ($p<0.05$) (Figure 1A). In addition, the relationship between the expression of MIR503HG in cervical cancer tissues and clinical features of patients was analyzed. As expected, the downregulation of MIR503HG was related to tumor size, TNM stage and lymph node metastasis (Table I). In addition, MIR503HG was also low expressed in cervical cancer cells HeLa versus normal cells Ect1/E6E7 ($p<0.05$) (Figure 1B). These data suggested that MIR503HG may be a potential tumor suppressor in cervical cancer.

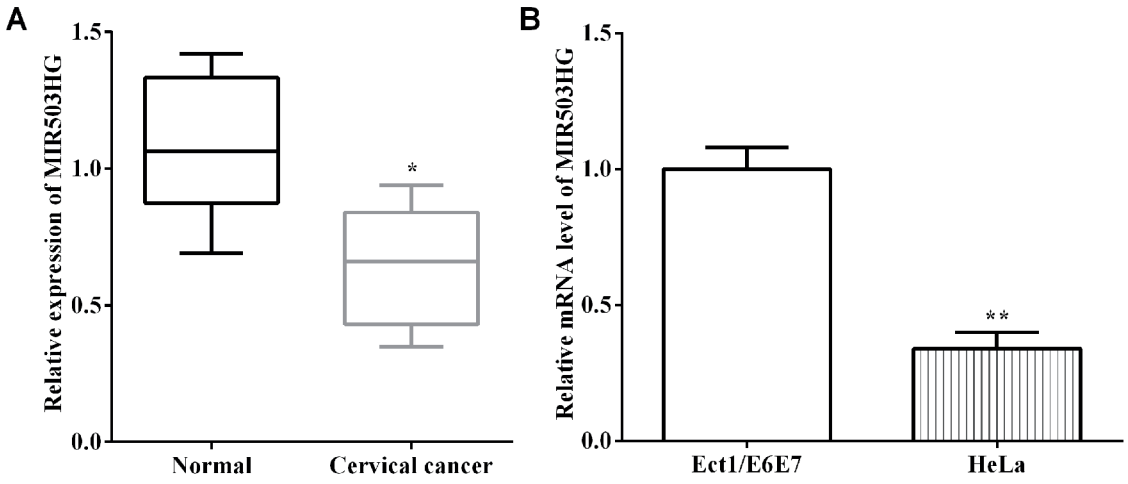

Figure 1. MIR503HG was downregulated in cervical cancer. **A**, MIR503HG was low expressed in cervical cancer tissues versus adjacent tissues. **B**, MIR503HG was also expressed in cervical cancer cells HeLa versus normal cells Ect1/E6E7.

The Biological Functions of overexpressing MIR503HG in Cervical Cancer Cells

Since MIR503HG was low expressed in cervical cancer cells, we then overexpressed MIR503HG in HeLa cells using pEX-MIR503HG, and the transfection efficiency was confirmed using the qRT-PCR ($p<0.05$) (Figure 2A). CCK-8 and transwell assays were utilized to calculate cell proliferation and invasion. As expected, the upregulation of MIR503HG inhibited cell proliferation ($p<0.05$) (Figure 2B). Cell invasion was suppressed after overexpressing MIR503HG

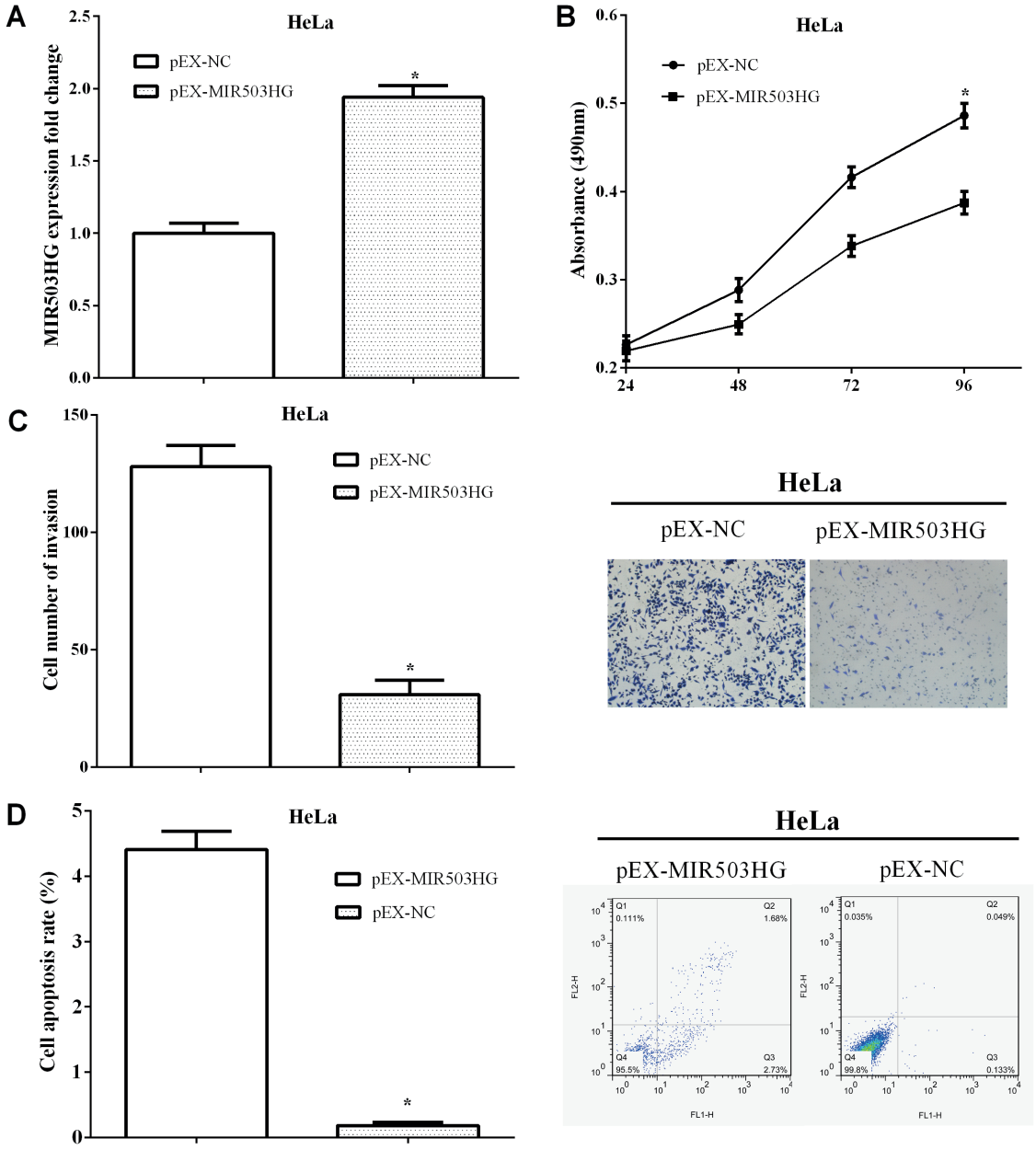

Figure 2. The biological functions of knocking down MIR503HG in cervical cancer cells. **A**, We overexpressed MIR503HG in HeLa cells using pEX-MIR503HG. **B**, Upregulation of MIR503HG inhibited cell proliferation. **C**, Cell invasion was suppressed after overexpressing MIR503HG (magnification 200×). **D**, The results indicated that MIR503HG overexpression promoted cell apoptosis.

Table I. The expression of MIR503HG and clinicopathological features in 47 cervical cancer.

Clinicopathological features	Cases (n=47)	MIR503HG expression		*p*-value
		27 Low (%)	20 High (%)	
Age (years)				
>50	24	13 (54.2)	11 (45.8)	0.642
≤50	23	14 (60.9)	9 (39.1)	
Tumor size (mm)				
>5.0	25	17 (68.0)	6 (32.0)	0.025*
≤5.0	22	10 (45.4)	14 (54.5)	
Histology				0.903
Squamous	42	24 (57.1)	18 (42.9)	
Adenocarcinoma	5	3 (60.0)	2 (40.0)	
FIGO stage				0.031*
III-IV	25	18 (72.0)	7 (28.0)	
0-II	22	9 (40.9)	13 (59.1)	
Lymph-node metastasis				0.037*
Yes	27	19 (70.4)	8 (29.6)	
No	20	8 (40.0)	12 (60.0)	
Metastasis				
Present	26	16 (61.5)	10 (38.5)	0.528
Absent	21	11 (52.4)	10 (47.6)	

**p*-values are calculated with Chi-square test.

($p<0.05$) (Figure 2C). In addition, cell apoptosis was measured using Flow cytometry. The results indicated that the overexpression of MIR503HG promoted cell apoptosis ($p<0.05$) (Figure 2D).

Downregulation of MIR503HG Promoted Cervical Cancer Progression

Moreover, cell proliferation, invasion, and apoptosis were assessed after knocking down MIR503HG in HeLa cells. At first, MIR503HG was silencing using sh-MIR503HG and the transfection efficiency was calculated using qRT-PCR ($p<0.05$) (Figure 3A). Not unfortunately, cell proliferation and invasion were enhanced after downregulating MIR503HG ($p<0.05$) (Figure 3B,C). By contrast, cell apoptosis was inhibited when silencing MIR503HG in HeLa cells ($p<0.05$) (Figure 3D). All the results indicated that MIR503HG may act as a tumor suppressor in cervical cancer.

MIR503HG Inhibited Tumor Growth In Vivo

Next, we constructed a mouse xenograft model and studied the antitumor effect of MIR503HG *in vivo*. Upregulation of MIR503HG inhibited xenograft growth in the nude mice ($p<0.05$) (Figure 4A). In addition, in the dissected tumor tissues, MIR503HG level in MIR503HG overexpression group was higher than the control group ($p<0.05$) (Figure 4B). The tumor volumes in MIR503HG overexpression group were smaller than those of the control group ($p<0.05$) (Figure 4C).

MIR503HG Emerged as CeRNA of MiR-191 in Cervical Cancer

An online prediction tool StarBase (http://starbase.sysu.edu.cn/) was used for performing the bioinformatics analysis, to search for miRNAs interact with MIR503HG. The results demonstrated that miR-191 has a putative binding site with MIR503HG (Figure 5A). The predicted binding sequences have been mutated from UUCCGUU to AAGGCAA. The Luciferase report gene assay was applied to verify the relationship between MIR503HG and miR-191. As we expected, miR-191 mimic reduced the Luciferase activity of wild type MIR503HG ($p<0.05$), but it had no effect on mutant MIR503HG ($p<0.05$) (Figure 5B). The mRNA

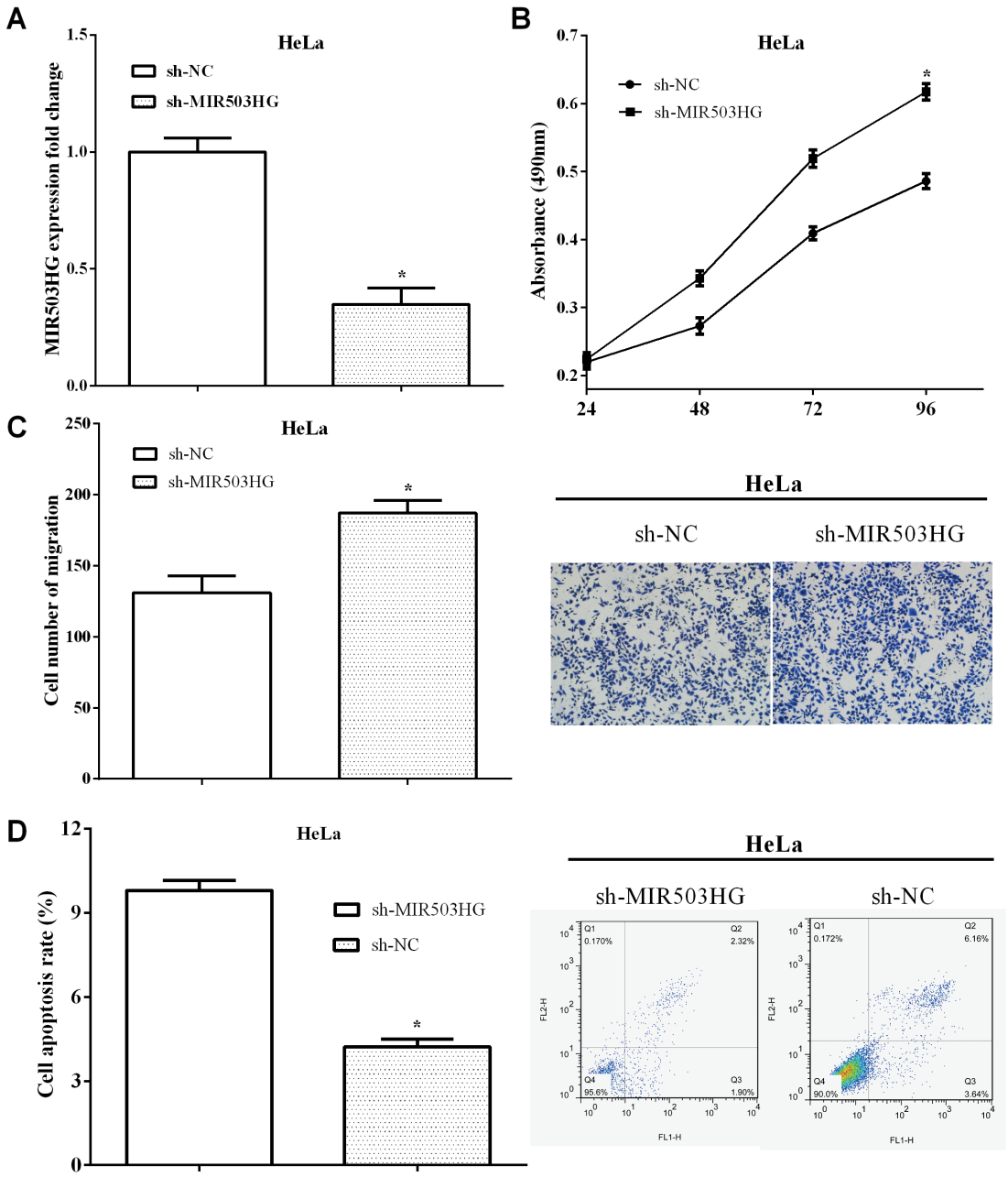

Figure 3. MIR503HG downregulation promoted cervical cancer progression. **A**, MIR503HG was silencing using sh-MIR503HG. **B**, Cell proliferation was enhanced after upregulating MIR503HG. **C**, Cell invasion was promoted when knocking down MIR503HG (magnification 200×). **D**, The apoptosis was inhibited when silencing MIR503HG in HeLa cells.

level of miR-191 was calculated after transfecting pEX-MIR503HG or sh-MIR503HG. MiR-191 was downregulated after overexpressing MIR503HG ($p<0.05$), while it was upregulated when transfecting sh-MIR503HG ($p<0.05$) (Figure 5C). The expression of MIR503HG had negative connection with miR-191 expression in cervical cancer tissues ($p<0.05$) (Figure 5D). The results indicated that MIR503HG emerged as ceRNA of miR-191 by directly binding to miR-191 in HeLa cells.

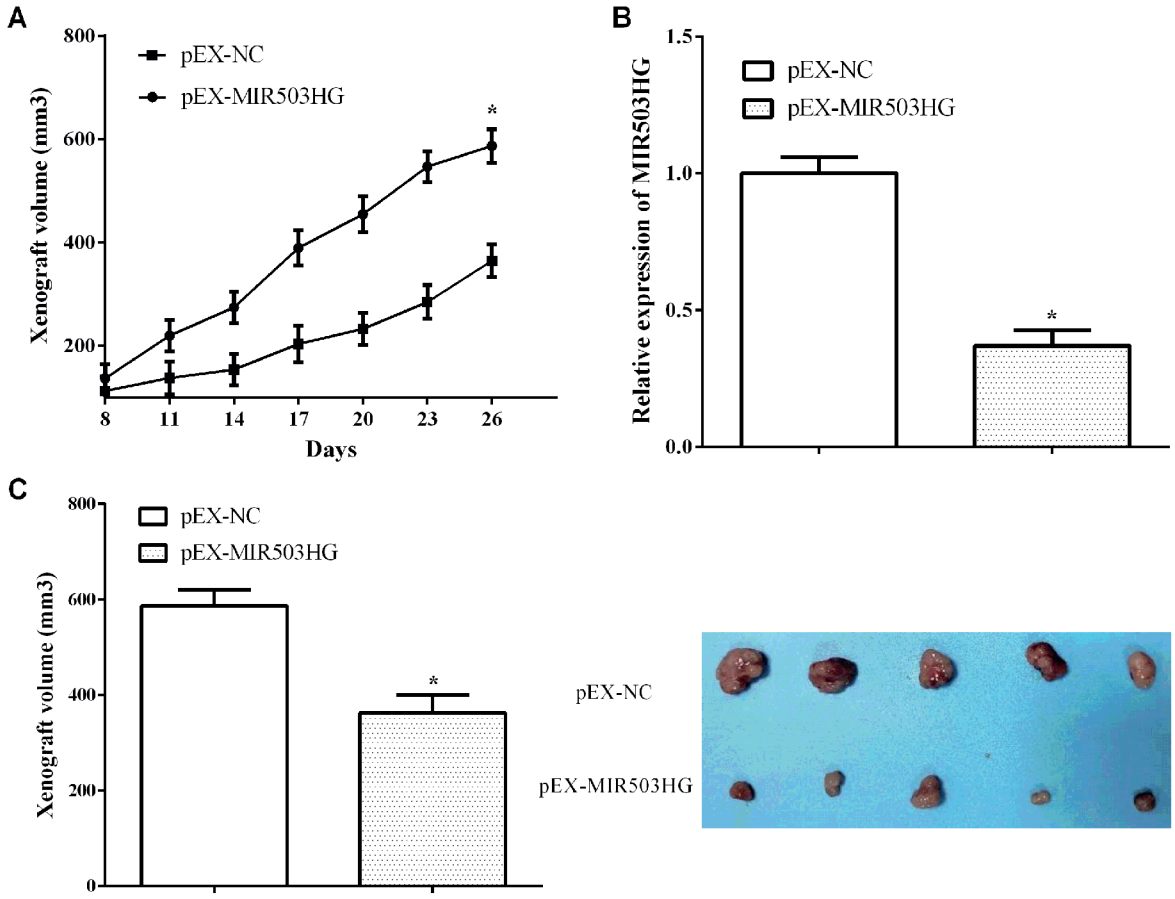

Figure 4. MIR503HG inhibited tumor growth *in vivo*. **A**, MIR503HG upregulation inhibited xenograft growth in the nude mice. **B**, MIR503HG level of the dissected tumor tissues in MIR503HG overexpression group was higher than the control group. **C**, The tumor volumes in MIR503HG overexpression group were smaller than the control group.

CEBPB as a Target Gene of MiR-191 is Modulated by MIR503HG in Cervical Cancer

CEBPB was predicted as a target gene of miR-191 by TargetScan, and the potential sequences were mutated from UUCCGUU to AAGGCAA (Figure 6A). The Luciferase activity of wild type CEBPB was inhibited by miR-191 mimic ($p<0.05$), and miR-191 mimic has no effect on the mutant CEBPB ($p>0.05$) (Figure 6B).

Then, we tried to investigate whether MIR503HG mediate cervical cancer progression through miR-191/CEBPB axis. qRT-PCR assay demonstrated that pEX-MIR503HG enhanced the expression of CEBPB ($p<0.05$), and siRNA-MIR503HG reduced CEBPB expression in HeLa cells ($p<0.05$) (Figure 6C). Additionally, miR-191 mimic suppressed the expression of CEBPB ($p<0.05$), whereas miR-191 inhibitor increased the CEBPB expression in HeLa cells ($p<0.05$) (Figure 6D). The expression of MIR503HG had positive relationship with CEBPB in cervical cancer tissues ($p<0.05$) (Figure 6E). On the contrary, the expression of MIR503HG had negative connection with miR-191 in cervical cancer tissues ($p<0.05$) (Figure 6F).

Discussion

In our study, we found that MIR503HG suppressed HeLa cell viability and invasion, and

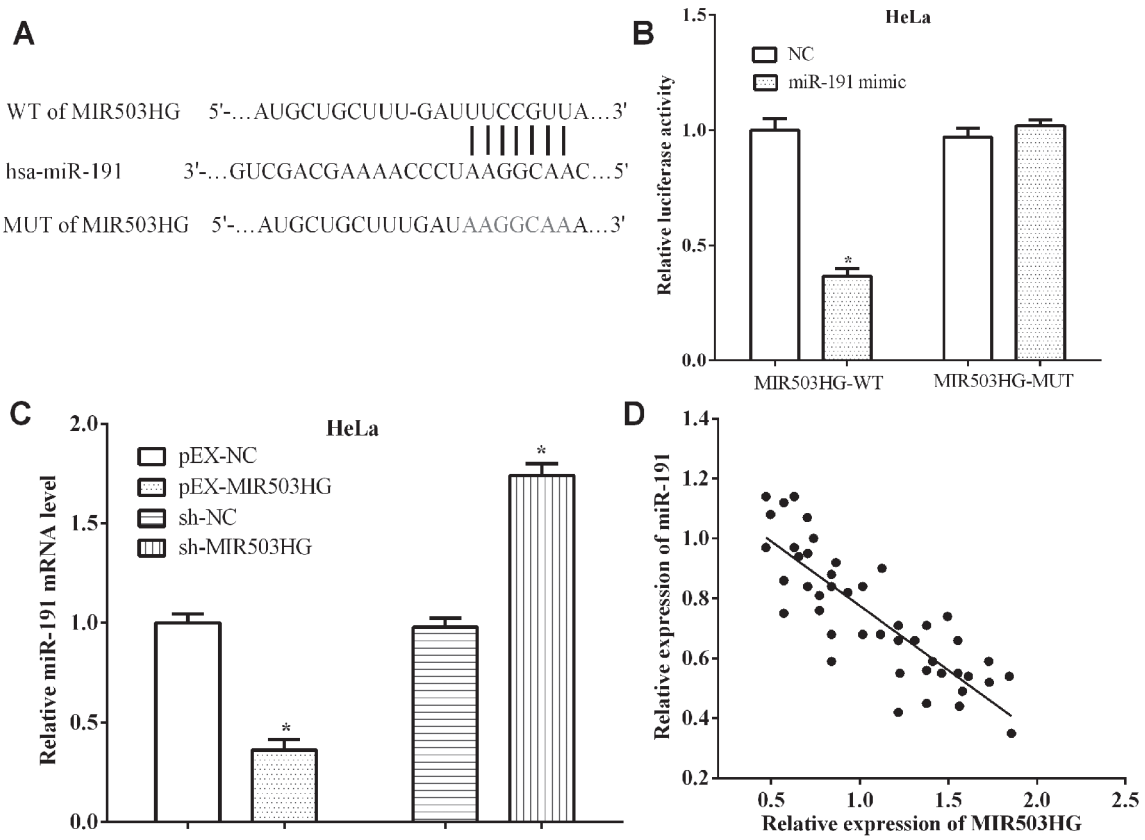

Figure 5. MIR503HG interacts with miR-191 in cervical cancer. **A**, The results demonstrated that miR-191 has a putative binding sites with MIR503HG. **B**, The Luciferase report gene assay was applied to verify the relationship between MIR503HG and miR-191. **C**, MiR-191 level was calculated after transfecting pEX-MIR503HG or sh-MIR503HG. **D**, The MIR503HG had negative connection with miR-191 in cervical cancer tissues.

enhanced cell apoptosis. MIR503HG regulated cervical cancer progression *via* miR-191/CEB-PB. Among all types of malignant tumors, the cervical cancer is ranked fourth in morbidity and mortality. The purpose of the study is to investigate the biomarkers in cervical cancer.

LncRNA MIR503HG plays suppressive roles in multiple tumors. In particular, MIR503HG silencing inhibited cell proliferation and enhanced cell apoptosis in NSCLC[19]. Also, it has been reported[20,21] that MIR503HG acted as a prognostic indicator and suppressed tumor metastasis in TNBC and HCC. Consistent with all the findings, we discovered that the upregulation of MIR503HG inhibited cell viability and invasion, but enhanced cell apoptosis. In contrast, the knockdown of MIR503HG promoted cell viability and invasion, while suppressed cell apoptosis. In addition, we also found that MIR503HG overexpression could inhibit xenograft growth. Therefore, MIR503HG is likely a tumor suppressor in cervical cancer. We first studied the role of MIR503HG in cervical cancer and its molecular mechanism. It is the first time we propose that MIR503HG acted as ceRNA of miR-191 in cervical cancer.

It has been reported that miR-191 exerted a tumor suppressive functions in cancers, including renal cell carcinoma, hepatocellular carcinoma, colon carcinoma and esophageal cancer[22-25]. MiR-191 enhanced cell proliferation and metastasis in breast cancer[26]. Consistent with the above findings, miR-191 binding to MIR503HG, and its expression was regulated by MIR503HG. MIR503HG had negative relationship with miR-191 in cervical cancer tissues.

CCAAT/enhancer-binding protein B (CEB-PB) enhanced flux through the hexosamine biosynthetic pathway *via* regulating key enzyme

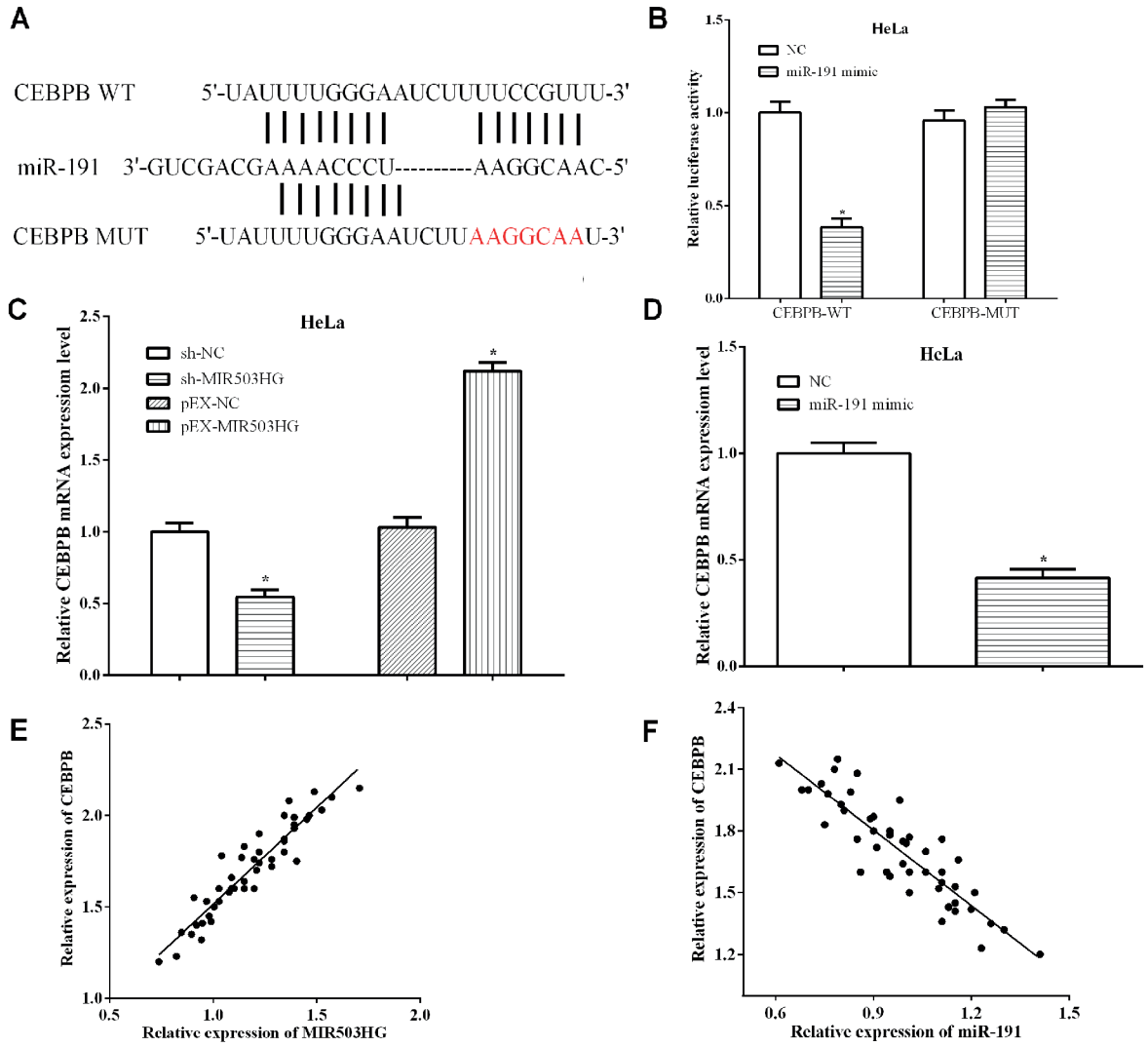

Figure 6. CEBPB as a target gene of miR-191 is modulated by MIR503HG in cervical cancer. **A**, CEBPB was predicted as a target gene of miR-191 by TargetScan. **B**, The Luciferase activity of wild type CEBPB was inhibited by miR-191 mimic. **C**, pEX-MIR503HG enhanced CEBPB expression in HeLa cell, and siRNA-MIR503HG reduced CEBPB expression. **D**, CEBPB expression was altered by changing miR-191 in HeLa cell. **E**, MIR503HG had positive relationship with CEBPB in cervical cancer tissues. **F**, The MIR503HG had negative connection with miR-191 in cervical cancer tissues.

glutamine expression[27]. CEBPB acted as a transcription factor to suppress cell proliferation of osteosarcoma[28]. In this study, we found that CEBPB was a target gene of miR-191, and CEBPB expression was altered by miR-191. It had negative connection between miR-191 and CEBPB in cervical cancer tissues. Moreover, MIR503HG regulated CEBPB expression and it had positive correlation with CEBPB in cervical cancer tissues.

Conclusions

MIR503HG suppressed cell proliferation, invasion and enhanced cell apoptosis *via* miR-191/CEBPB axis in cervical cancer. MIR503HG may acted as a tumor suppressor in cervical cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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