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Up-regulation of long non-coding RNA MFI2 functions as an oncogenic role in cervical cancer progression

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Abstract. – OBJECTIVE: Cervical cancer is one of the gynecologic tumors in the world. The main aim of this study was to elucidate the functional role of MFI2 in cervical cancer and provide novel insight into biomarkers and therapeutic strategies for cervical cancer.

PATIENTS AND METHODS: The relative expression level of MFI2 was examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell counting kit-8 (CCK-8) assay was involved to determine the ability of cell proliferation. Flow cytometric analysis was performed to detect cell apoptosis. Transwell assay and Matrigel assay were involved to determine cell migration and invasion. Expressions of protein kinase B (AKT), phosphorylated-AKT (p-AKT), B-cell lymphoma-2 (BCL2), and BCL2-Associated X (Bax) protein levels were detected in Western blotting. Transfected cells were used to perform tumor xenograft formation assay.

RESULTS: Our research validated that MFI2 was up-regulated in cervical cancer by qRT-PCR. Through CCK-8 assay, flow cytometric analysis, transwell assay, and Matrigel assay, we verified that MFI2 can promote cell proliferation, cell metastasis and inhibit cell apoptosis in cervical cancer. Subsequently, we used Western blotting assay to determine the alteration of protein expression of p-AKT, BCL2, and Bax. The results indicated that MFI2 may exert its function by regulating phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway. In tumor xenograft formation assay, up-regulated MFI2 accelerated tumor formation.

CONCLUSIONS: Current research elucidated that MFI2 promoted cell proliferation, cell metastasis and inhibited cell apoptosis in cervical cancer by regulating the PI3K/AKT signaling pathway. Our results may provide a novel insight into finding new therapeutic targets and biomarkers for cervical cancer.

Key Words:
MFI2, Cervical cancer, Proliferation, Apoptosis, Migration, Invasion, PI3K/AKT.

Introduction

Cervical cancer is the fourth most common gynecologic tumor and the fourth leading cause of cancer-related death among women worldwide. The incidence of cervical cancer is approximately 570,000 cases per year and nearly 310,000 deaths per year. The age of onset tends to be younger[1]. However, little is known about the molecular mechanisms involved in the development of cervical cancer.

Long noncoding RNA (LncRNA) is defined as a non-coding RNA transcript with a length greater than 200 nucleotides which has the function of gene expression regulation but does not encode proteins[2,3] . LncRNA can affect cell embryonic development, stem cell differentiation, protein-coding gene regulation, cell proliferation and apoptosis, tumor cell radiochemotherapy tolerance, and other physiological and pathological processes. These participate in epigenetic regulation, transcriptional regulation, and post-transcriptional regulation[3,4]. Moreover, increasing studies[5-8] have confirmed that lncRNA is involved in many different types of tumors, such as the biological processes of proliferation, metastasis, and invasion of liver cancer, gastric cancer, osteosarcoma, colorectal cancer, breast cancer, etc.

This work was aimed to investigate the expression of lncRNA MFI2 and its role in promoting

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the proliferation of cervical cancer. We recruited cell counting kit-8 (CCK-8) assay, flow cytometric assay, and transwell assay to determine the effect of MFI2 on cell proliferation, apoptosis, and cell metastasis. All the results indicated that MFI2 functioned as an oncogenic role in the cervical cancer progression.

Patients and Methods

Tissue Specimens

Here, 35 pairs of cervical tissues and para-tumor tissues were observed. All tissue specimens were obtained from patients who got surgery treatment in Gansu Provincial Hospital from 2016 to 2017. Tissue specimens were immediately put into liquid nitrogen. This investigation was approved by the Ethics Committee of Gansu Provincial Hospital. Written informed consents were signed from all participants before the study.

Cell Lines

Four cervical cell lines including SiHa, Caski, C33A, HeLa, and one human normal cervical epithelial cell line NCEC were obtained from the Shanghai Cell Bank (Shanghai, China). All cell lines were cultured by Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2.

Cell Transfection

To up-regulate MFI2, the selected cell lines were transfected with the lentiviral vector following standard instructions. For the knocking-down of MFI2, selected cell lines were transfected with shRNA following the standard protocol. All the plasmids were obtained from GenePharma (Shanghai, China).

Isolation of Total RNA and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Tissue specimens were taken from liquid nitrogen and cell lines were used to extract total RNA *via* TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Complementary deoxyribose nucleic acids (cDNAs) were synthesized *via* Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) according to the standard protocol. The MFI2 expression level was assessed through SYBR Green real-time PCR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as normalization. GAPDH forward 5'-3': TATCGGACGCCTGGTTAC, reverse 5'-3': TATCGGACGCCTGGTTAC; LINC00961 forward 5'-3': AACAGCCAGGAGCGGTATTACG, reverse 5'-3': GTCAAAGACGGTTGTGTGCCTG.

Cell-Counting Kit-8 Assay (CCK-8)

The CCK-8 assay (Dojindo, Kumamoto, Japan) was involved to examine cell proliferation in cervical cancer. Transfected cells were plated into 96-well plates (6×10^3/well) and the CCK-8 solution (10 μL/well) was used to stain cells for 2 h at 37°C.

Flow Cytometric Analysis

To detect the apoptotic cells, the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (Vazyme, Nanjing, China) was performed according to the instructions. The flow cytometric analysis took place at BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometry.

Transwell Assay and Matrigel Assay

We carried out the transwell assay to figure out the invasion ability of transfected cervical cancer cells. Transwell chambers and 24-wells plates were obtained from Corning (Corning, NY, USA). 1×10^5 cells were suspended with serum-free medium (100 μL) and transferred to the upper chamber. In the Matrigel assay, cells were transferred to the Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated upper chamber. After 36 h, the invasive cells were counted from images of five random fields using an inverted microscope (Olympus, Tokyo, Japan).

Western Blot

Total protein was isolated by radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) and phenylmethylsulfonyl fluoride (PMSF; Thermo Fisher Scientific, Waltham, MA, USA). Protein lysates isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membrane was immunostained at 4°C by primary antibodies overnight. Primary rabbit antibodies used in the current study including

anti-AKT (protein kinase B; CST, Danvers, MA, USA), anti-p-AKT (CST, Danvers, MA, USA), anti-BCL2 (B-cell lymphoma-2; CST, Danvers, MA, USA), anti-Bax (BCL2-Associated X; CST, Danvers, MA, USA). Rabbit anti-GAPDH (CST, Danvers, MA, USA) was taken as a loading control. The protein relative expression level was determined by the Image Lab software (Bio-Rad, Hercules, CA, USA).

Xenograft Model

The current study was approved by the Animal Ethics Committee of Lanzhou University Animal Center. Transfected SiHa or HeLa cells (6×10^5 / mL) were injected into two flanks of nude mice (6 weeks old) subcutaneously. Tumors growth were monitored and recorded every week. The formula (volume= length × width^2 × 1/2) was used to calculate tumor volume. Tumors were extracted after 4 weeks.

Statistics Analysis

All experiments in this work were performed three times independently at least. All data recorded were exhibited as mean ± standard deviation (SD). Student's unpaired *t*-test was used to undergo statistics analysis. $p<0.05$ was expected to be significant.

Results

MFI2 was Up-Regulated in Cervical Cancer Tissues and Cell Lines

To determine the relative expression level of MFI2 in cervical cancer, we used the qRT-PCR assay. As shown in Figure 1A, MFI2 was over-expressed in cervical cancer tissues. Consistently, the expression level of MFI2 was up-regulated in cervical cancer cell lines (Figure 1B). Subsequently, we transfected the selected

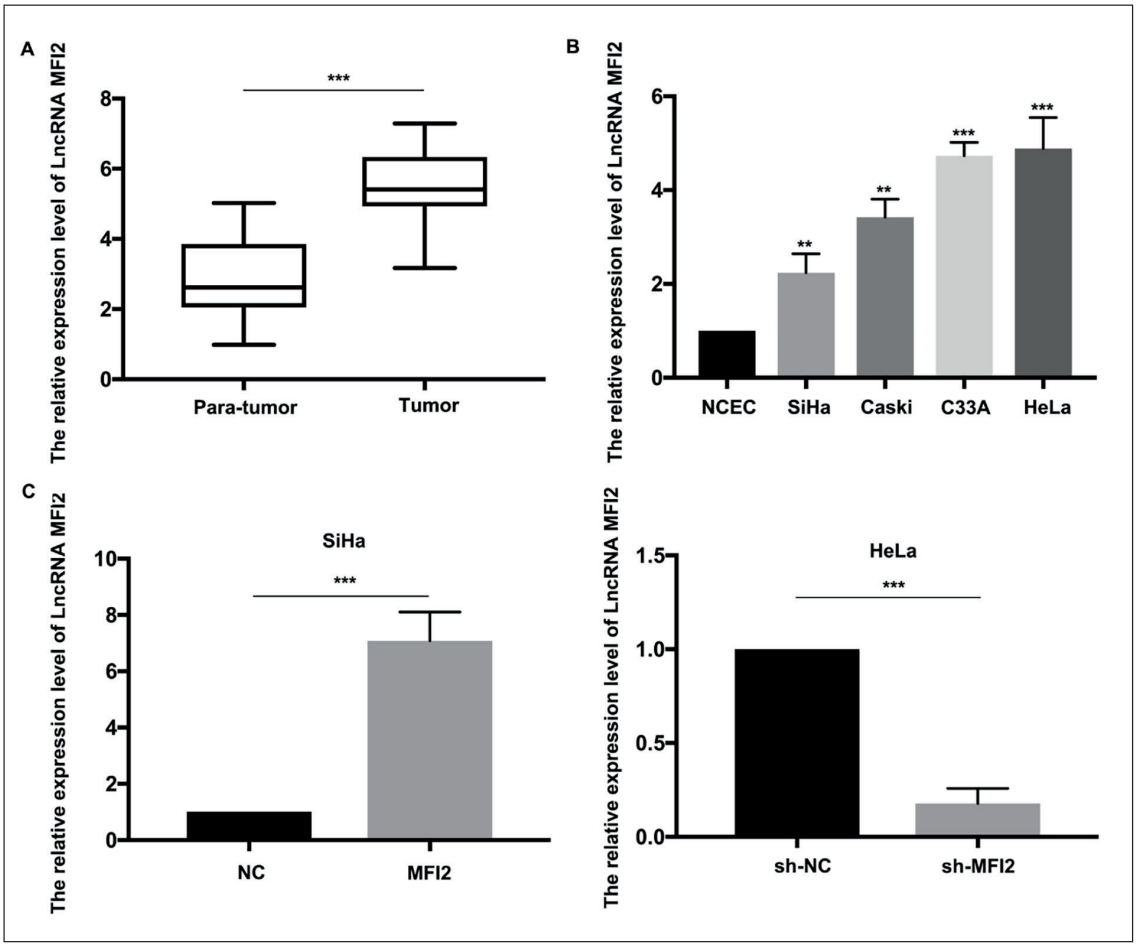

Figure 1. MFI2 had up-expression level in cervical cancer tissues and cell lines. ***A,*** Expression level of MFI2 in cervical cancer; ***B,*** Analysis of MFI2 expression level in cervical cancer cell lines. ***C,*** Transfection efficiency was evaluated by qRT-PCR. Data are presented as the mean ± SD of three independent experiments. **$p<0.01$, ***$p<0.001$.

cell lines with lentivirus for over-expressing MFI2 or down-expressing MFI2. The transfection efficiency was examined by qRT-PCR (Figure 1C).

MFI2 Functioned as an Oncogenic Role in Cervical Cancer

The CCK8 assay was recruited to examine the ability of MFI2 on cell proliferation. As shown in Figure 2A, MFI2 up-regulation group promoted cell proliferation in comparison with control group. To figure out the effect of MFI2 on cell apoptosis, flow cytometric analysis was involved in this study. As shown in Figure 2B, apoptotic cells were significantly decreased in MFI2 over-expression group while increased in MFI2 down-expression group. Besides, we employed transwell assay and Matrigel assay to detect the ability of cell migration and invasion. As shown in Figures 3A and 3B, over-expressed MFI2 significantly accelerated cell migration and invasion. Taken together, the results showed that MFI2 functioned as an oncogenic role in cervical cancer.

MFI2 Over-Expression Promoted Tumor Formation In Vivo

To examine the ability of MFI2 on tumor formation *in vivo*, we recruited tumor formation assay. Figures 4A and 4B showed that tumors generated from up-regulated MFI2 group were relatively larger than negative control group. Conversely, MFI2 down-expression group formed smaller tumors than control group.

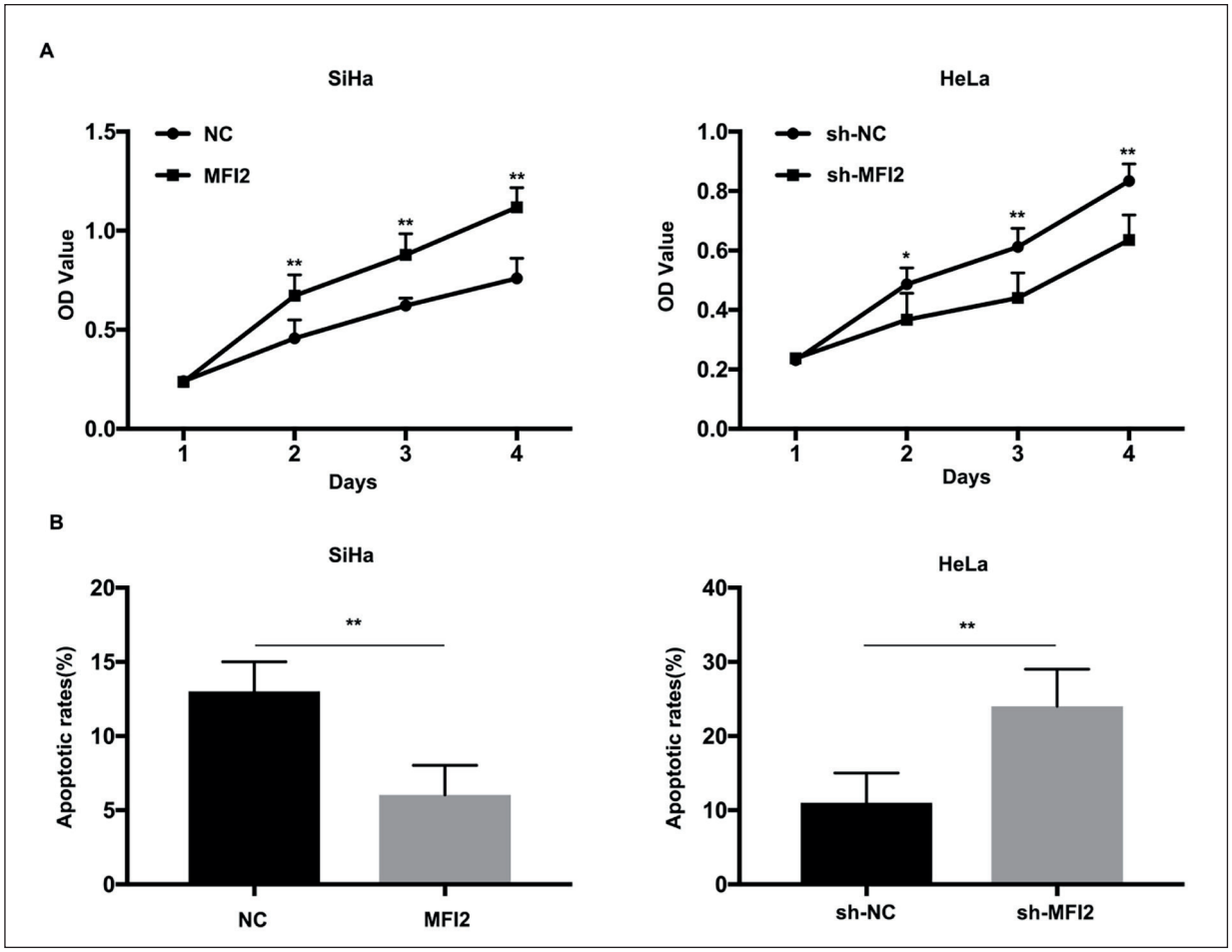

Figure 2. MFI2 promoted cell proliferation and inhibited cell apoptosis in cervical cancer. ***A,*** Cell proliferation ability was determined by CCK-8 assay. ***B,*** Flow cytometric analysis was performed to detect the apoptotic rates in transfected cells. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01.

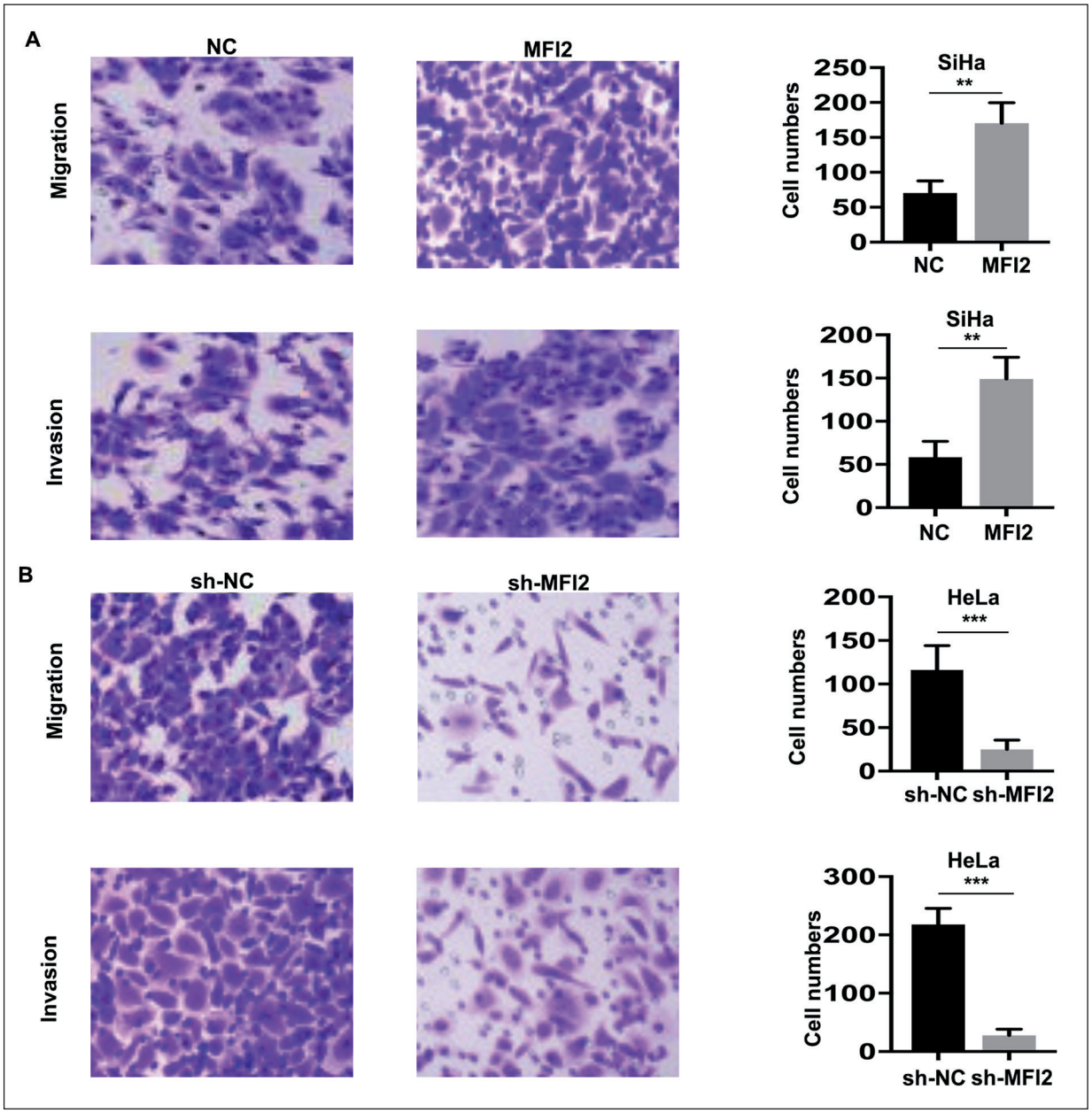

Figure 3. Up-regulated MFI2 can accelerate cell migration and invasion *in vitro*. ***A,*** Cell migration and cell invasion were detected by transwell assay and matrigel assay in MFI2 over-expression groups (magnification: 40×); ***B,*** Cell migration and cell invasion were detected by transwell assay and matrigel assay in MFI2 down-expression groups. Data are presented as the mean ± SD of three independent experiments (magnification: 40×). **p<0.01, ***p<0.001.

MFI2 Exerted Its Functions Through Activating the Phosphatidylinositol 3-Kinase (PI3K)/AKT Signaling Pathway

To explore the underlying mechanism of LNC00961 on the progression of OSCC, we used Western blotting assay to determine if there were alternations on some markers in the PI3K/AKT pathway. As shown in Figure 5, in MFI2 over-expression group, the expression level of phosphorylation-AKT was significantly decreased. Besides, the alternation of BCL2 and Bax indicated that MFI2 over-expression can induce cell apoptosis. Therefore, MFI2 functioned as an oncogenic role in cervical cancer by activating the PI3K/AKT signaling pathway.

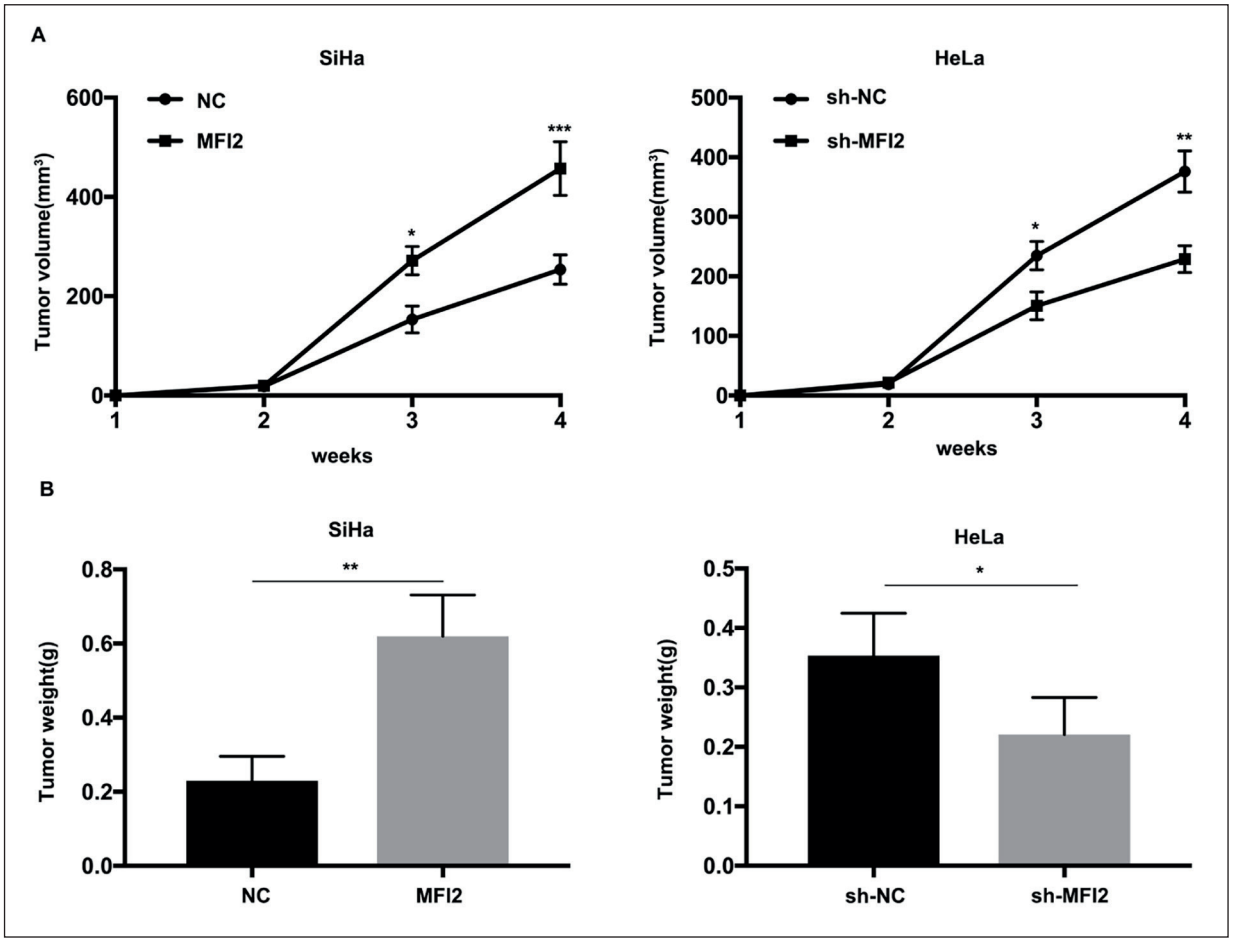

Figure 4. Up-regulated MFI2 can promote tumor formation *in vivo*. ***A,*** After tumor extraction, tumor volume was calculated respectively and made into a graph; ***B,*** Tumor weight were recorded. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Discussion

Cervical cancer is a common gynecological tumor in women and its incidence rate is the first among female genital tract tumors and tends to be younger, seriously threatening women's life and health[1]. The incidence of cervical cancer is a complex physiological process, both environmental and genetic factors can lead to cervical cancer, and its pathological mechanism has not been fully clarified. A large number of epidemiological studies have shown that HPV infection is a key factor in cervical cancer. Many molecules, including genes, proteins and RNA, are involved in the process of HPV causing cervical cancer[9]. It is an important clinical issue to search new targets for early diagnosis, treatment, and prognosis of cervical cancer.

LncRNA is a hot molecule in tumor research in recent years, with a length of more than 200 nucleotides and no or only weak protein-coding capacity, usually located in the nucleus and cytoplasm[10]. LncRNA has cis-transcriptional or trans-transcriptional regulation, regulating nuclear domain tissues, proteins, RNA and other different functions[11]. Studies[12,13] revealed that some LncRNA transcripts actually encoded small protein. Recent studies have shown that lncRNA was involved in X chromosome silencing, transcriptional activation and inhibition, epigenetics, gene expression, chromatin modification, genomic imprinting, and nuclear transport and other regulatory processes in development, differentiation, and metabolism. Abnormal expression of lncRNA was related to the diagnosis, recurrence, and metastasis of tumors. Abnormal expression of ln-

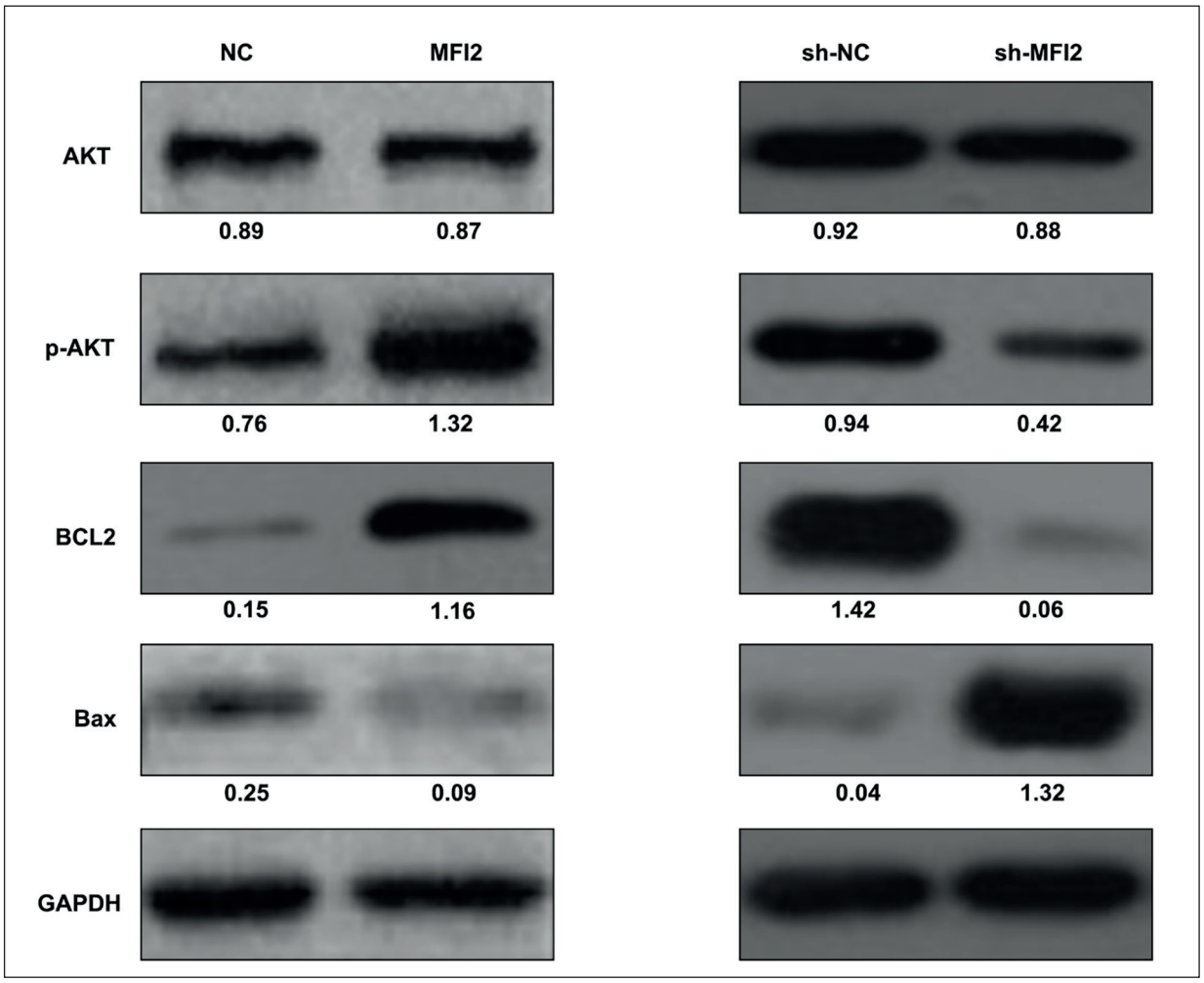

Figure 5. The influence of MFI2 on cervical cancer might be relied on the PI3K/AKT signaling pathway. AKT, p-AKT, BCL2, and Bax protein expression levels were examined in over-expression MFI2 group; AKT, p-AKT, BCL2, and Bax protein expression levels were examined in down-expression MFI2 group.

cRNA was found in all malignant tumors, including cervical cancer[5,14,15]. H19 is the first lncRNAs to be related to the tumor and previous studies have shown that the expression level of lncRNA H19 in cervical cancer tissues was significantly higher than that in normal cervical tissues[16]. HOTAIR is the first gene in lncRNAs that has been found to have a trans effect[17]. The expression level of HOTAIR in cervical cancer tissues was significantly higher than its corresponding paracancer tissues, and the expression level was related to the clinical stage and lymph node metastasis of patients[18]. Studies on lncRNAs were expected to find more effective biomarkers for the diagnosis of cervical cancer. LncRNA MFI2 was found to be associated with tumorigenesis. LncRNA MFI2 promoted the proliferation of osteosarcoma cells by accelerating FOXP4[19]. However, the role of lncRNA MFI2 in the development of cervical cancer has not been revealed.

In the current study, we found that MFI2 was up-regulated in cervical cancer *via* qRT-PCR. Through the CCK-8 assay, the results showed that MFI2 could promote cell proliferation. The flow cytometric assay indicated that MFI2 may inhibit cell apoptosis in cervical cancer cell lines. Besides, MFI2 accelerated cervical cancer cell migration and invasion through transwell assay and Matrigel assay. To explore the underlying mechanism of the role of MFI2 in cervical cancer, we examined the protein expression level of p-AKT, BCL2, and Bax. The data indicated that the influence of MFI2 could be by activating the PI3K/AKT signaling path-

way. Therefore, our research verified that MFI2 functioned as an oncogenic role in the cervical cancer progression.

Conclusions

We elucidated that MFI2 promoted cell proliferation, cell metastasis and inhibited cell apoptosis in cervical cancer by activating the PI3K/AKT signaling pathway. Our findings may provide a novel insight into finding new therapeutic targets and biomarkers for cervical cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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