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MicroRNA-101-5p inhibits the growth and metastasis of cervical cancer cell by inhibiting CXCL6

W. SHEN, X.-Y. XIE, M.-R. LIU, L.-L. WANG

Obstetrics Department, Maternal and Child Health Care Hospital of Laiwu City, Laiwu, Shandong, China

Abstract. – OBJECTIVE: The objective of this study is to explore the biological roles of microRNA-101-5p (miR-101-5p) in the growth and metastasis of cervical cancer.

PATIENTS AND METHODS: The levels of miR-101-5p and chemokine (C-X-C motif) ligand 6 (CXCL6) in cervical cancer tissues and cells were detected using the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. The proliferation, colony formation, migration, and invasion assays were conducted using miR-101-5p transfected cervical cancer cell. The expression of CXCL6 was measured by the immunoblotting assay. Xenograft model was constructed to reveal the precise roles of miR-101-5p in the growth of cervical cancer cell in vivo.

RESULTS: MiR-101-5p was down-regulated in cervical cancer tissues when compared to the normal controls. The levels of miR-101-5p were higher in cervical cancer cells (SiHa, Caski, C-4-I, C-33 A) than that in the human cervical surface epithelial cell line, HcerEpic. Over-regulation of miR-101-5p inhibited the aggressiveness phenotypes of a cervical cancer cell in vitro. Furthermore, over-regulation of miR-101-5p reduced the tumor growth of cervical cancer cell in vivo. CXCL6 was the target protein of miR-101-5p in cervical cancer as demonstrated by luciferase reporter assay. The mRNA level of CXCL6 was negatively associated with the miR-101-5p level in cervical cancer tissue. Finally, the rescue experiments suggested that the inhibitory role of miR-101-5p was mediated by regulating the expression of CXCL6 in cervical cancer.

CONCLUSIONS: These findings indicated that the over-regulation of miR-101-5p suppressed the progression of cervical cancer by targeting CXCL6 and might function as a potential therapeutic target for cervical cancer.

Key Words:
MiRNA, Cervical cancer, CXCL6, Migration, Invasion.

Introduction

Cervical cancer is one of the most common gynecological malignancies in women that affects female reproductive organs, including ovaries, fallopian tubes, uterus, cervix, vagina, and vulva[1,2]. The 5-year survival rate remains unsatisfactory despite the significant improvements in the therapeutic strategies for cervical cancer during the past decade[3,4]. The underlying mechanisms that promote the progression of cervical cancer are not fully known. Therefore, elucidating novel mechanisms of cervical cancer initiation and progression is critical for improving the diagnostics and therapeutics. MicroRNAs (miRNAs) are a kind of small non-coding RNAs, which regulate the expression of a target gene through promoting the degradation of target messenger RNA (mRNA) or inducing translational repression via incompletely binding with the 3'-untranslated region (3'-UTR) of target protein[5,6]. Substantial evidence demonstrates that dysregulation of miRNAs is associated with the development and metastasis of several cancers, and miRNAs might be the potential treatment targets in cancers[7,8]. For example, miR-661 serves as a suppressive miRNA in breast cancer and reduces the growth and invasion of cancer cell by regulating metastasis associated 1 (MTA1)[9]. MiR-490-3p regulates the proliferation and epithelial to mesenchymal transition (EMT) process of hepatocellular carcinoma (HCC) cell through regulating the expression of endoplasmic reticulum-Golgi intermediate compartment protein 3 (ERGIC3)[10]. Researches[11,12] also report that miR-101 is significantly up-regulated in non-small cell lung cancer (NSCLC). Nevertheless, the potential roles of miR-101-5p

Corresponding Author: Li Li Wang, MD; e-mail: wangliliwang20@foxmail.com

in regulating the growth and metastasis of cervical cancer cell remains not yet well investigated. Chemokines are a kind of small heparin binding cytokines, which regulate the migration of leukocytes through binding with G protein-coupled receptors (GPCR)[13,14]. More than 20 chemokine receptors and 50 chemokines have been found, and they are divided into four kinds (C, CC, CX3C and CXC)[15,16]. Chemokine receptor system, which extends to various types of neoplastic cell, is proved to be altered in neoplastic tissues[17]. C-X-C motif chemokine ligand 6 (CXCL6) is first identified in osteosarcoma cell line and CXCL6 participates into the progression of various types of cancers, including NSCLC, colon cancer and small cell lung cancer (SCLC)[18-21]. In a murine model of melanoma, down-regulation of CXCL6 inhibits tumor cell growth, invasion and metastasis[22]. In addition, the angiogenic role of CXCL6 is associated with the intratumoral expression of matrix metallopeptidase 9 (MMP-9) and the degradation of the extracellular matrix (ECM)[23]. Previous investigations have demonstrated the important role of CXCL6/C-X-C motif chemokine receptor 6 (CXCL6/CXCR6) in the metastasis of different cancers, including colorectal cancer and NSCLC[18,19]. These results indicate that CXCL6/CXCR6 interaction is important for the metastasis and progression of cancer. MiR-101-5p is a cancer-related miRNA, which plays an oncogenic or tumor suppressive role in different cancers. However, its role in cervical cancer has remained elusive so far. Therefore, in this study, we investigated the role of miR-101-5p in the progression of cervical cancer.

Patients and Methods

Clinical Samples

Fifty pairs of cancer and adjacent normal tissues were obtained from patients with cervical cancer that underwent surgical resection at Maternal and Child Health Care Hospital of Laiwu City. The tissues were snap-frozen and stored in liquid nitrogen for further investigation. We excluded patients that received immunotherapy, chemotherapy or radiotherapy before surgical treatment. Ethical approval was obtained from the Ethics Committee of the Maternal and Child Health Care Hospital of Laiwu City. The study conforms to the Code of Ethics of the World Medical Association (Declaration of Helsinki) printed in the British Medical Journal (18 July 1964). Informed consent forms were signed by all patients before surgery.

Cell Lines and Cell Culture

The four human cervical cancer cell lines (SiHa, Caski C-4-I, C-33 A) and the human cervical surface epithelial cell line, HcerEpic were obtained from the Chinese Academy of Sciences (Shanghai, China). 293T cell was purchased from the GuangZhou Jennio Biotech Co., Ltd (GuangZhou, GuangDong, China). Cervical cancer cell lines and HcerEpic were grown in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South-Logan, UT, USA) and 1% penicillin/streptomycin at 37°C and 5% CO_2. 293T cell was grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South-Logan, UT, USA) and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Transfection Studies

Lentiviral vector pGreenPuro (System Biosciences, Palo Alto, CA USA) that containing miR-101-5p (miR-101-5p) or miR-negative control (miR-NC) was packaged into 293T cells using the lentiviral packaging kit (GeneCopoeia, Shanghai, China) according to manufacturer's manual. Stable cell lines were established using the infecting SiHa cells with lentiviruses encoding miR-101-5p or miR-NC followed by 2 mg/ml puromycin selection. The stable cell line was cultured in RPMI-1640 medium containing 10% FBS and puromycin (0.5 mg/ml) for colony formation assay and in vivo experiments. The miR-101-5p mimic and the corresponding negative control, miR-NC was brought from GenePharma (Shanghai, China). The CXCL6 overexpression vector (pCDNA3.1-CXCL6) without 3'-UTR was constructed by GenePharma (Shanghai, China). MiR-101-5p mimic, miR-NC, or pCDNA3.1-CXCL6 plasmid were transfected into cell using the Lipofectamine™ 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol.

Quantitative Real-Time PCR

Total RNA was extracted from cervical cancer tissues and cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's manual. The relative level of miRNA-101-5p was detected using a SYBR PrimeScript miRNA RT PCR kit (TaKaRa, Dalian, Liaoning, China) in accordance with the manufacturer's

instructions. For detection of CXCL6 mRNA expression, the first-strand cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer′s instructions. Real time PCR was conducted using SYBR Premix Ex Taq (TaKaRa, Dalian, Liaoning, China) in the ABI 7900 Fast system (Applied Biosystems, Foster City, CA, USA). The relative levels of miR-101-5p and CXCL6 were normalized to U6 and GAPDH, respectively using the 2-ΔΔCT method. The primers were as follows: miR-101-5p (forward primer: 5'-GCCGGCAGCATTATGTCAAT-3'; reserve primer: 5'-GCCAGCAGCTTGATGTCAAT-3'), CXCL6 (forward primer: 5'-AGAGCTGCGTTGCACTTGTT-3'; reserve primer: 5'-GCAGTTTACCAATCGTTTTGGGG-3'), U6 (forward primer: 5'-AAAGCAAATCATCGGACGACC-3'; reverse primer: 5'-GTACAACACATTGTTTCCTCGGA-3'), GAPDH (forward primer: 5'-TGTGGGCATCAATGGATTTGG-3'; reverse primer: 5'-ACACCATGTATTCCGGGTCAAT-3'). U6 and GAPDH were the internal controls.

Cell Proliferation

We determined the proliferation of cervical cancer cell using the Cell Counting Kit-8 (CCK8) assay. Briefly, 2 × 103 transfected cells were seeded into 96-well plates and cultured at 5% CO2 and 37°C for 24 h, 48 h or 72 h, respectively. At each time point, 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added into 96 well plate and the cells were further incubated for 1 h. The absorbance was read at 450 nm was in a microplate reader (Bio-Tek Company, Winooski, VT, USA).

Colony Formation Assay

The miR-101-5p or miR-NC transfected cells (500 cells per well) were cultured in six-well plates for 14 days. Then, the colonies were fixed in 20% methanol and stained with 0.5 % crystal violet for 15 min. The number of colonies was counted using the ZEN 2011 imaging software on a Zeiss invert microscope (CarlZeiss, Hallbergnoos, Germany).

Migration Assay

To determine the migration ability of cervical cancer cell, a wound healing assay was conducted. MiR-101-5p or miR-NC transfected cells (1×105/well) were cultured in 6-well plates for 24 h. Subsequently, the cells were scratched with a sterile plastic micropipette tip to generate a wound in the monolayer. The cells were washed with PBS and cultured under standard conditions for 48 h. Then, the wound was imaged at 0 h and 48 h under an inverted Nikon Eclipse TS100 phase-contrast microscope (Nikon, Tokyo, Japan). Scratch healing rate = (0 h width of scratch-48 h width of scratch)/0 h width of scratch × 100%. 24.

Invasion Assay

Cell invasiveness was determined using Transwell assay. Briefly, 2×105 cells were seeded into the upper chamber that was pre-coated with Matrigel (Shanghai, China). In the lower chamber, 600 μl RPMI 1640 medium with 20% fetal bovine serum (FBS) was added. After 24 h, the non-invading cells were gently removed with a cotton swab, whereas the cells that invaded the lower surface of the filter were fixed with 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min. The cells were photographed with an inverted light microscope (CarlZeiss, Hallbergnoos, Germany) and the cell numbers were counted in five random fields of view25.

Dual-Luciferase Reporter Assay

The 3′-untranslated region (3'-UTR) of CXCL6 that containing the binding sites of miR-101-5p was PCR amplified and sub-cloned into psiCHECK2 vector (Promega, Madison, WI, USA). It is referred to as wt-CXCL6-3'UTR. The mutant 3'-UTR of CXCL6 was constructed with QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies; Santa Clara, CA, USA) and sub-cloned into psiCHECK2 vector. It is referred to as mut-CXCL6-3'UTR. In the luciferase reporter assay, SiHa cells were grown to 70-80% confluence and transfected with miR-101-5p mimic or miR-NC in combination with wt-CXCL6-3'-UTR or mut-CXCL6-3'-UTR. The firefly and renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blotting

Briefly, cervical cancer cells or tissues were lysed using RIPA buffer (Beyotime, Najing, Jiangsu, China. The protein concentration was detected using a BCA protein assay kit (Pierce, Bonn, Germany). Protein samples were fractionated by 10% SDS-PAGE, then the proteins were blotted onto PVDF membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were immunoblotted with antibodies against CXCL6 (Santa Cruz Biotechnology, Santa Cruz,

CA, USA) or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by HRP-linked secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA). GAPDH was used as a control.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded tumor tissues were cut into 4 μm sections. Then, paraffin sections were deparaffined in xylene and dehydrated in graded ethanol. Heat-induced antigen retrieval were performed using Citrate buffer (Invitrogen, Carlsbad, CA, USA). Then, sections were incubated with the primary anti-CXCL6 antibody (1:400, Santa Cruz Biotechnology, CA, USA) for overnight at 4°C. The following day, the slides were incubated with horseradish peroxidase-conjugated secondary antibody (ZSGB BIO, Beijing, China) for 90 min at room temperature, and a peroxidase-labeled polymer for 10 min at room temperature. The slides were developed with freshly prepared DAB solution for 5 min followed by hematoxylin counterstain. Then, after dehydration and mounting, they were photographed under a light microscope.

Immunofluorescence

Cells were fixed by pre-cold acetone and then rinsed three times with PBS. The cells were permeabilized in 0.1% Triton X-100 and incubated with 1% BSA/PBS to block nonspecific binding. Subsequently, the cells were immunostained by incubating with rabbit monoclonal antibody against CXCL6 (diluted 1:500, Epitomics, Burlingame, CA, USA) for overnight at 4°C. After being washed with PBS, cells were incubated with FITC-conjugated goat anti-rabbit secondary antibody (diluted 1:60, Boster Biotechnology, Wuhan, Hubei, China). Nuclei were counterstained with DAPI (Beyotime Biotech, Haimen, Jiangsu, China). Images were taken and analyzed using the ZEN 2011 imaging software on a Zeiss invert microscope (CarlZeiss, Hallbergnoos, Germany).

In vivo Xenograft Study

Animal study was approved by the Committee on the Use of Live Animals in Teaching and Research of the Animal Research Committee of Maternal and Child Health Care Hospital of Laiwu City. Tumor xenograft model was established using 6-week old female BALB/c nu/nu mice by subcutaneously injecting with 2×106 SiHa cells that expressing miR-101-5p or miR-NC. Tumor growth was monitored every 3 days with a fine digital caliper by measuring tumor length (L) and width (W). Tumor volume was calculated as (L × W2)/2. After 20 days, all mice were sacrificed, and the tumors were harvested and weighed. The animal experiment was conducted in accordance with Institutional Guidelines and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996).

Statistical Analysis

Statistical Package of the Social Sciences 17.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. The data shown are presented as the mean ± SD (standard deviation) at least from three independent experiments. Differences in the results of two groups were evaluated using either two-tailed Student's t-test or one-way ANOVA followed by post-hoc Dunnett's test. The relationship between miR-101-5p and CXCL6 expression was assessed by Spearman's correlation analysis. $p < 0.05$ was considered statistically significant.

Results

MiR-101-5p is Down-regulated in Cervical Cancer Tissues and Cell Lines

Gene expression dataset (GSE19611) that was used for statistical analysis was acquired from the GEO database (https://www.ncbi.nlm.nih.gov/gds/). The gene expression dataset (GSE19611) contains both the cervical cancer tissues and the matched normal tissues. As shown in Figure 1A, miR-101-5p was significantly down-expressed in cervical cancer tissues compared to normal tissues. We then determined the levels of miR-101-5p in 50 pairs of cervical cancer and adjacent normal tissues by qRT-PCR. As shown in Figure 1B, miR-101-5p was down-regulated in cervical cancer tissues compared to the adjacent normal tissues. Next, we observed that the levels of miR-101-5p were also significantly reduced in four cervical cancer cell lines than that in the human cervical surface epithelial cell line, HcerEpic (Figure 1C). Finally, the Kaplan Meier analyses indicated that the low level of miR-101-5p was associated with the poor overall survival of patients (Figure 1D). Thus, these findings demonstrate that miR-101-5p is down-regulated in cervical cancer and is a potential prognostic biomarker for patients with cervical cancer.

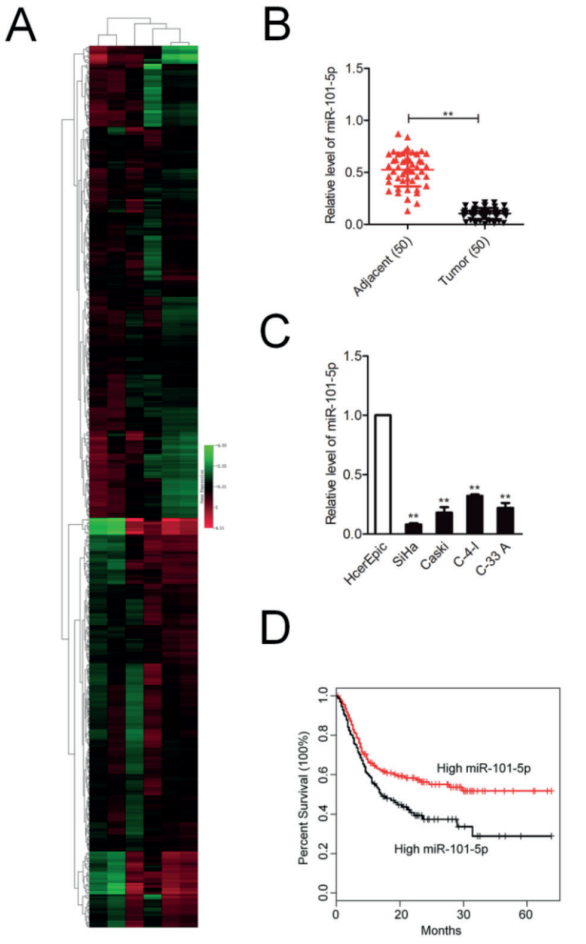

Figure 1. MiR-101-5p is under-regulated in cervical cancer. ***(A)*** Microarray analysis of miRNA in cervical cancer tissues and corresponding normal tissues; ***(B)*** The levels of miR-101-5p in cervical cancer and adjacent normal tissues were analyzed using qRT-PCR assay. $^{**}p < 0.01$ compared to adjacent; ***(C)*** qRT-PCR analysis of miR-101-5p in four human cervical cancer cell lines, SiHa, Caski C-4-I, C-33 A and the human cervical surface epithelial cell line, HcerEpic. $^{**}p < 0.01$ compared to HcerEpic; ***(D)*** Kaplan-Meier survival curve analysis demonstrated that the low level of miR-101-5p was associated with the poor prognosis of patients with cervical cancer.

Up-regulation of MiR-101-5p Suppresses Cervical Cancer Cell Proliferation and Xenograft Tumor Growth

To investigate the biological function of miR-101-5p in cervical cancer, we generated control or miR-101-5p overexpressing SiHa cells with lentiviruses that carrying miR-101-5p or miR-NC (Figure 2A). The CCK-8 assay demonstrated that overexpression of miR-101-5p inhibited the proliferation of SiHa and Caski cell (Figure 2B). Moreover, up-regulation of miR-101-5p decreased the colony formation of cervical cancer *in vitro* (Figure 2C). To determine the significance of these findings *in vivo*, SiHa cells that stably expressing miR-101-5p or miR-NC were subcutaneously injected into the flank region of nude mice. We observed that the tumor growth in the miR-101-5p group was inhibited than that in the miR-NC group (Figure 2D). Consistently, we also observed that the tumor size in miR-101-5p group was decreased than in the miR-NC group (Figure 2E). These data demonstrate that miR-101-5p inhibits the growth of cervical cancer cell *in vitro* and *in viv*o.

MiR-101-5p Suppresses Cervical Cancer Cell Migration and Invasion

Next, we investigated the role of miR-101-5p in the migration and invasion of cervical cancer cell using the wound healing and Transwell invasion assay in vitro. As shown in Figure 3A-3B, over-regulation of miR-101-5p significantly inhibited the migration and invasiveness of SiHa and Caski cell.

CXCL6 is a Target of MiR-101-5p in Cervical Cancer Cell

Next, the Targetscan (http://www.targetscan.org/vert_72/) and miRanda (http://www.microrna.org/microrna/) websites were applied to investigate the potential target gene of miR-101-5p. As shown in Figure 4A, we identified that CXCL6 was the potential target gene of miR-101-5p with a probable binding site at bases 317 to 324. We then performed dual luciferase reporter assays using miR-NC or miR-101-5p overexpressing SiHa and Caski cell that was transfected with wild type (wt) or mutant type (mut) 3'-UTR of CXCL6. As shown in Figure 4B, the luciferase activity of cell that was transfected with the wt 3'-UTR of CXCL6 was decreased by miR-101-5p transfection whereas the luciferase activity of cell that was transfected with mut 3'-UTR of CXCL6 was not significantly inhibited by miR-101-5p. Moreover, up-regulation of miR-101-5p decreased the mRNA level and protein expression CXCL6 in SiHa and Caski cell (Figure 4C-4D).

CXCL6 Mediates the Functional Effects of MiR-101-5p on Cervical Cancer Cell

To explore the role of miR-101-5p/CXCL6 in the progression of cervical cancer, SiHa cells were cotransfected with miR-101-5p mimics and CXCL6 overexpressing plasmid. As shown in Figure 5A-5B, the level of CXCL6 was significantly increased in SiHa cell that was cotransfected with v compared to the cell that was transfected with miR-101-5p mimics alone Then, we found

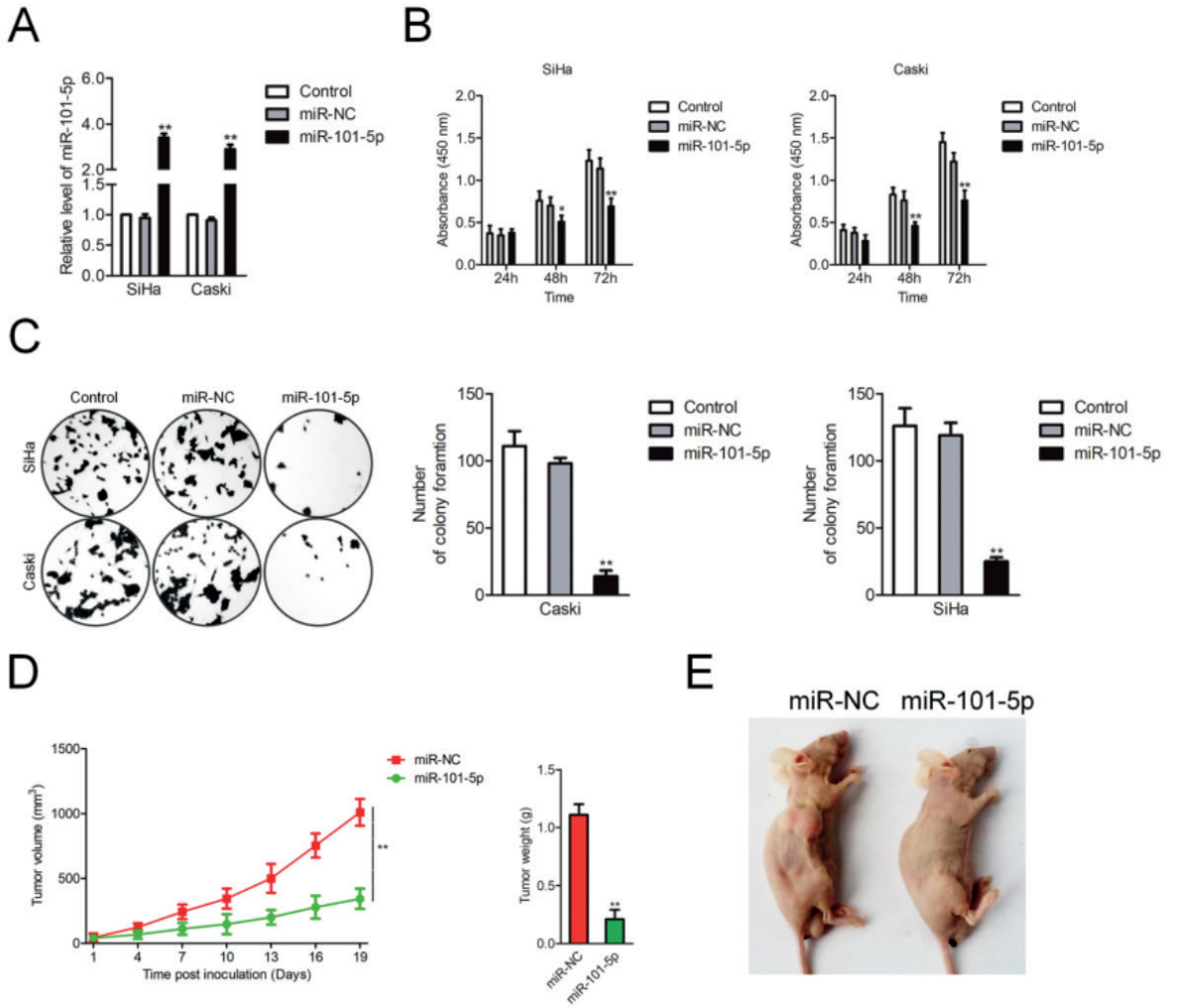

Figure 2. MiR-101-5p inhibits the growth of cervical cancer cell *in vitro* and *in vivo*. ***(A)*** qRT-PCR analysis of miR-101-5p expression in miR-101-5p and miR-NC transfected SiHa and Caski cell; ***(B)*** The proliferation of miR-101-5p and miR-NC transfected SiHa and Caski cell was assessed by CCK-8 assay; ***(C)*** Colony formation assay. *$p < 0.05$, **$p < 0.01$ compared to control; ***(D)*** Xenograft tumor volumes in nude mice injected with SiHa cells stable expressing miR-101-5p and miR-NC; ***(E)*** Representative images of xenograft tumors that was formed by miR-101-5p or miR-NC transfected SiHa cells. *$p < 0.05$, **$p < 0.01$ compared to miR-NC.

that up-regulation of CXCL6 level restored the proliferation, colony formation, migration and invasion of SiHa cell that was inhibited by miR-101-5p (Figure 5C-5F). These data demonstrate that miR-101-5p inhibits the growth and metastasis of cervical cancer cell by decreasing CXCL6 expression.

CXCL6 is Inversely Correlates with MiR-101-5p in Cervical Cancer Tissues

Finally, we investigated the relationship between CXCL6 mRNA and miR-101-5p in 50 pairs of cervical cancer and corresponding adjacent normal tissues by qRT-PCR. As shown in Figure 6A, the mRNA levels CXCL6 were higher in the cervical cancer tissues than those in the adjacent normal tissues. Consistently, the immunohistochemical analysis demonstrated the protein expression of CXCL6 was higher in cervical cancer tissues than that in the adjacent normal tissues (Figure 6B). Moreover, the Spearman's correlation analysis demonstrated that the levels CXCL6 were inversely correlated with miR-101-5p levels in cervical cancer tissues (Figure 6C).

Discussion

Human cervical cancer is the fourth leading cause of cancer death in women worldwide and is

one of the main causes of cancer-related death in the developing countries[26]. Therefore, it is important to investigate the molecular mechanisms underlying the progression of cervical cancer. MiRNAs are well known as a class of small regulatory RNA molecules that regulate gene expression in a sequence-specific manner[27]. Many researchers have reported that the expression and functions of miRNAs are cell and tissue specific[28]. Importantly, the dysfunctions of miRNAs are also

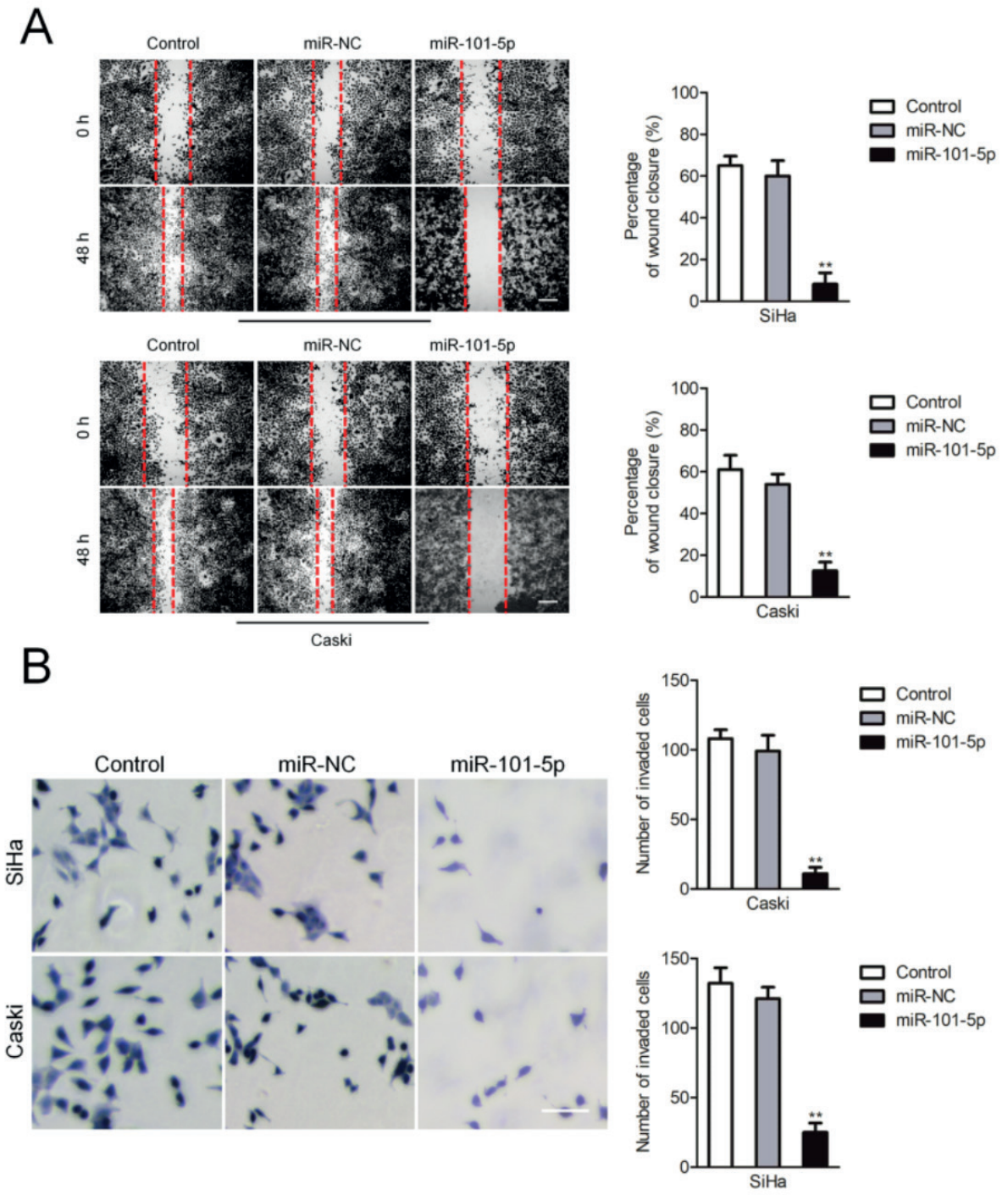

Figure 3. MiR-101-5p inhibits the migration and invasion of cervical cancer cell. ***(A)*** The migration of miR-NC or miR-101-5p transfected SiHa and Caski cell was determined using the wound healing assay; ***(B)*** The invasion ability of miR-NC or miR-101-5p transfected SiHa and Caski cell was analyzed by transwell invasion assay. $^{**}p < 0.01$ compared to control.

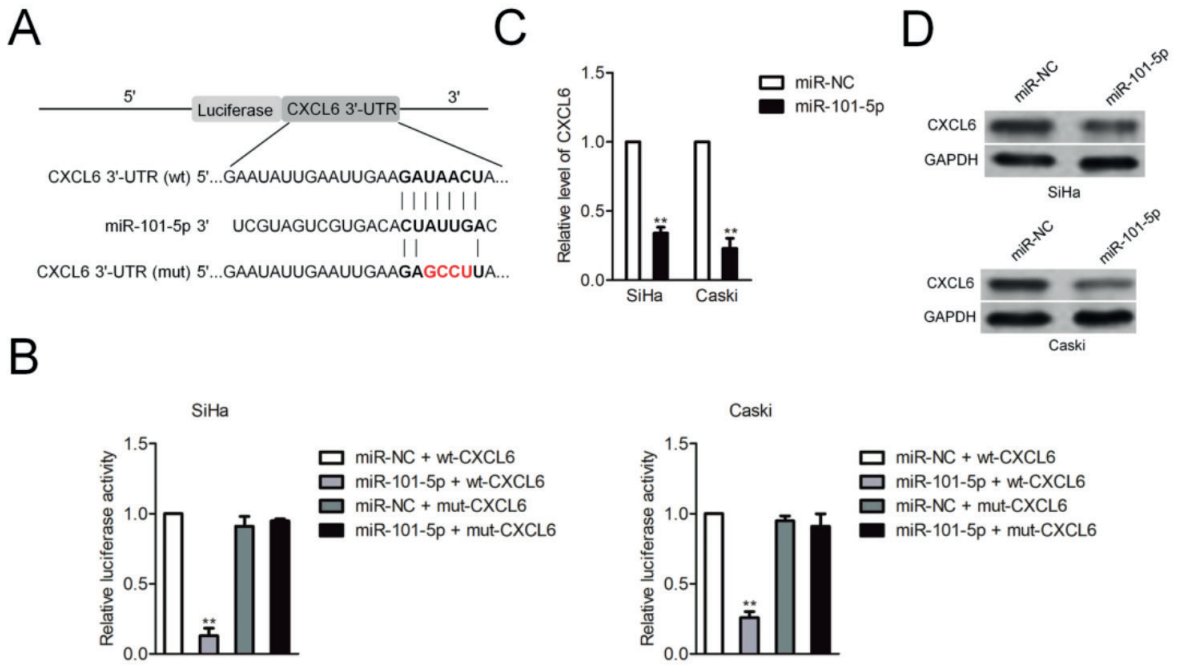

Figure 4. CXCL6 is the target of miR-101-5p in cervical cancer cell. ***(A)*** The binding sites between miR-101-5p and the 3'-UTR of CXCL6; ***(B)*** SiHa and Caski cell was cotransfected with miR-101-5p and wt 3'-UTR of CXCL6 or cotransfected with miR-101-5p and mut 3'-UTR of CXCL6. The luciferase activity was detected using the luciferase reporter assay. $^{**}p < 0.01$ compared to miR-NC + wt-CXCL6; ***(C)*** The mRNA level of CXCL6 in miR-NC or miR-101-5p transfected SiHa and Caski cell was detected using qRT-PCR assay. $^{**}p < 0.01$ compared to miR-NC; ***(D)*** The protein expression of CXCL6 in miR-NC or miR-101-5p transfected SiHa and Caski cell was determined by Western blotting assay.

commonly associated with the initial and developmental stages of human cancers, including cellular proliferation, oncogenesis, invasion, and metastasis[29-31].

In this study, we investigated the role of miR-101-5p in the growth, migration and invasion of cervical cancer cell. We found that miR-101-5p was significantly down-regulated in cervical cancer tissues and cell lines than normal cervical tissues and cell line, respectively. Moreover, the lower level of miR-101-5p was correlated with the poor prognosis of patient with cervical cancer. Moreover, overexpression of miR-101-5p inhibited the proliferation, colony formation, migration and invasion of cervical cancer cell *in vitro*. Meanwhile, up-regulation of miR-101-5p suppressed the growth of cervical cancer cell *in vivo*.

The previous study[11] proves that miR-101 exerts tumor-suppressive effects in multiple malignancies, including NSCLC. Down-regulation of miR-101 promotes the epithelial to mesenchymal transition (EMT) of cisplatin-resistant NSCLC cell. Moreover, miR-101 inhibits the proliferation, invasion of tumor cell and enhances the paclitaxel-induced apoptosis in NSCLC cell by directly targeting enhancer of zeste homolog 2 (EZH2)[32,33]. All these results demonstrate the important roles of miR-101 in cancers. However, the expression and role of miR-101-5p in the progression of cervical cancer is not known. Our study demonstrates the tumor suppressor function of miR-101-5p in cervical cancer. Bioinformatics analysis and luciferase reporter assay identified that CXCL6 was the directly target gene of miR-101-5p in cervical cancer. Our data also revealed that CXCL6 was up-regulated in human cervical cancer tissues, which was consistent with the previous findings, in which CXCL6 was found to be highly expressed in hepatocellular carcinoma (HCC) and NSCLC[18,34].

These results suggested the oncogenic role of CXCL6 in the progression of cervical cancer. In this study, overexpression of CXCL6 partially abrogated the inhibitory effect of miR-101-5p on the proliferation, colony formation, migration and invasion of cervical cancer cell. Moreover, the expression of CXCL6 was negatively associated

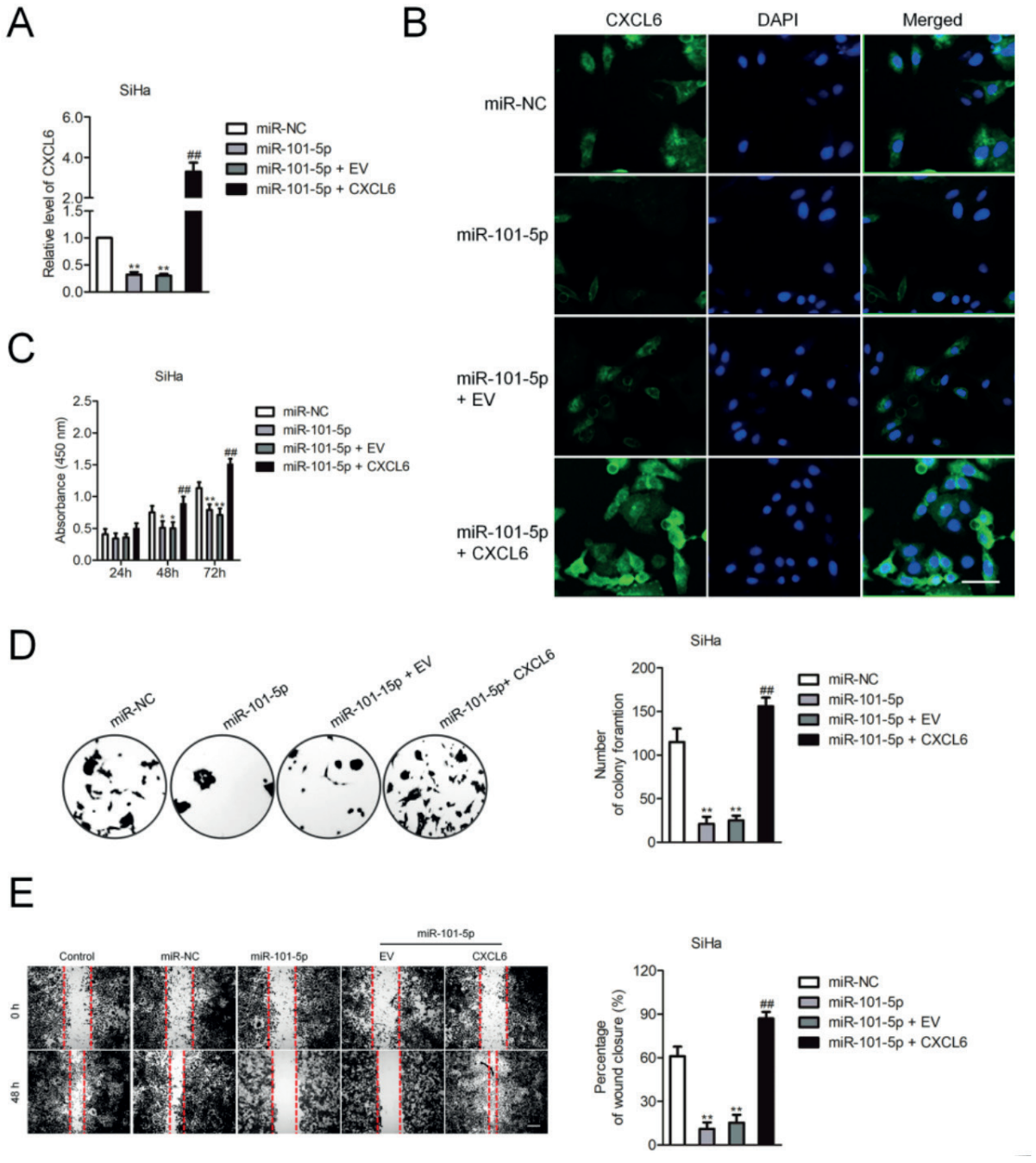

Figure 5. MiRMiR-101-5p inhibits the growth and metastasis of cervical cancer by targeting CXCL6. ***(A)*** SiHa cells were co-transfected with CXCL6 plasmid and miR-101-5p mimic or co-transfected with CXCL6 plasmid and miR-NC. The mRNA level of CXCL6 was detected using qRT-PCR. **$p < 0.01$ compared to miR-NC, ##p < 0.01 compared to miR-101-5p; ***(B)*** The expression of CXCL6 in SiHa cells that were co-transfected with CXCL6 plasmid and miR-101-5p mimic or co-transfected with CXCL6 plasmid and miR-NC was detected using western blotting assay; ***(C)*** The proliferation of SiHa cells that were co-transfected with CXCL6 plasmid and miR-101-5p mimic or co-transfected with CXCL6 plasmid and miR-NC was detected using CCK-8 assay. *$p < 0.05$ and **$p < 0.01$ compared to miR-NC, ##$p < 0.01$ compared to miR-101-5p; ***(D)*** Colony formation. ***(E)*** The migration of SiHa cell was determined using the wound healing assay.

Figure continued

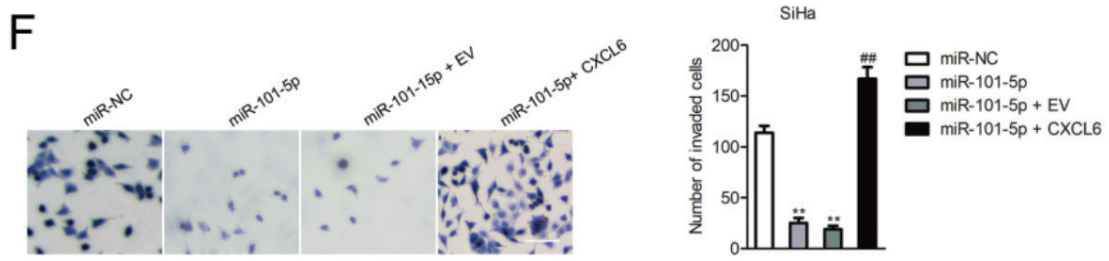

Figure 5. *(Continued).* ***(F)*** The invasion ability of SiHa cell was analyzed using the Transwell invasion assay. $^{**}p < 0.01$ compared to miR-NC, $^{\#\#}p < 0.01$ compared to miR-101-5p.

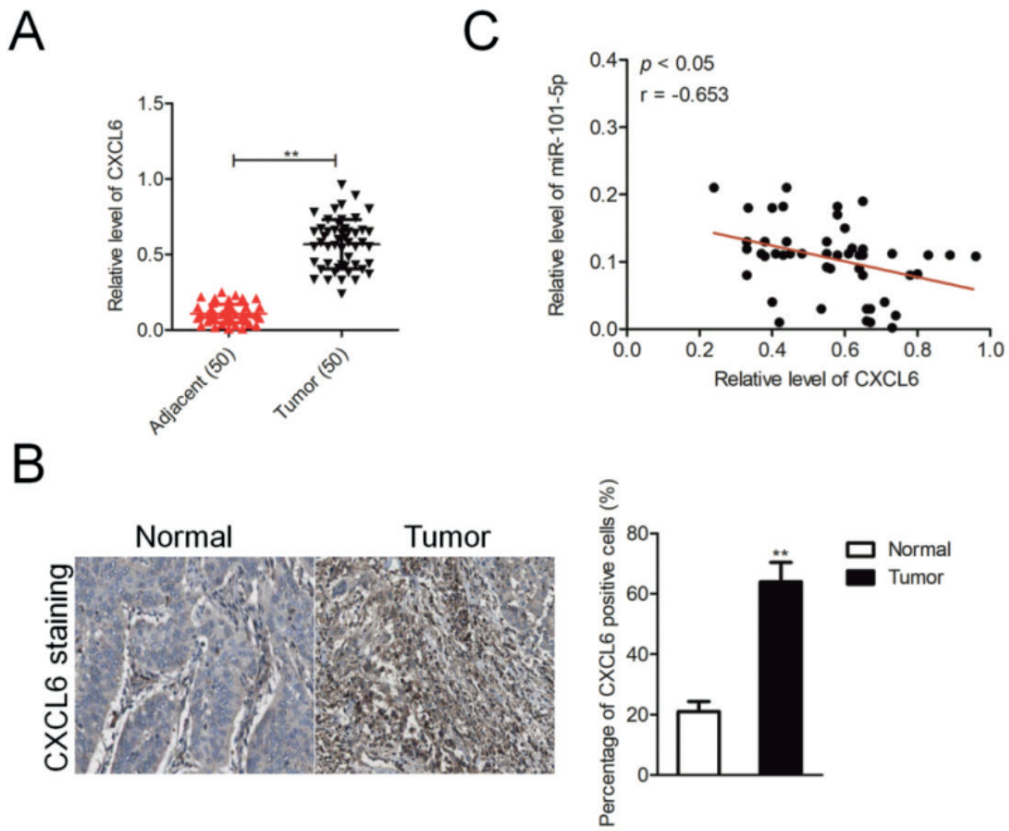

Figure 6. The expression of CXCL6 is inversely correlated with miR-101-5p level in cervical cancer tissue. ***(A)*** The mRNA levels of CXCL6 in 50 pairs of cervical cancer and adjacent normal tissues were detected using qRT-PCR assay. $^{**}p < 0.01$ compared to adjacent; ***(B)*** The protein expression of CXCL6 in cervical cancer tissues was detected using the immunohistochemical staining. $^{**}p < 0.01$ compared to normal; ***(C)*** Spearman's correlation analysis of the association between the level of CXCL6 and miR-101-5p level in human cervical cancer tissues (n=50).

with the level of miR-101-5p in cervical cancer tissues. The main limitation of our study is that we only analyzed fewer cervical cancer tissues and cell lines. Therefore, more elaborate studies are necessary for further explore the potential therapeutic and prognostic role of miR-101-5p in the progression of cervical cancer.

Conclusions

Our results demonstrate that miR-101-5p serves as a tumor suppressor in cervical cancer by targeting CXCL6 and represents a potential therapeutic target for cervical cancer treatment.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Wei H, Zhang JJ, Tang QL. MiR-638 inhibits cervical cancer metastasis through Wnt/beta-catenin signaling pathway and correlates with prognosis of cervical cancer patients. Eur Rev Med Pharmacol Sci 2017; 21: 5587-5593.
2) Yan D, Zhu D, Zhao X, Su J. SHP-2 restricts apoptosis induced by chemotherapeutic agents via Parkin-dependent autophagy in cervical cancer. Cancer Cell Int 2018; 18: 8.
3) Yuan Y, Min SJ, Xu DQ, Shen Y, Yan HY, Wang Y, Wang W, Tan YJ. Expressions of VEGF and miR-21 in tumor tissues of cervical cancer patients with HPV infection and their relationships with prognosis. Eur Rev Med Pharmacol Sci 2018; 22: 6274-6279.
4) Shi X, Ran L, Liu Y, Zhong SH, Zhou PP, Liao MX, Fang W. Knockdown of hnRNP A2/B1 inhibits cell proliferation, invasion and cell cycle triggering apoptosis in cervical cancer via PI3K/AKT signaling pathway. Oncol Rep 2018; 39: 939-950.
5) Poudyal D, Herman A, Adelsberger JW, Yang J, Hu X, Chen Q, Bosche M, Sherman BT, Imamichi T. A novel microRNA, hsa-miR-6852 differentially regulated by Interleukin-27 induces necrosis in cervical cancer cells by downregulating the FoxM1 expression. Sci Rep 2018; 8: 900.
6) Li Y, Liang Y, Sang Y, Song X, Zhang H, Liu Y, Jiang L, Yang Q. MiR-770 suppresses the chemo-resistance and metastasis of triple negative breast cancer via direct targeting of STMN1. Cell Death Dis 2018; 9: 14.
7) Zhao J, Li B, Shu C, Ma Y, Gong Y. Downregulation of miR-30a is associated with proliferation and invasion via targeting MEF2D in cervical cancer. Oncol Lett 2017; 14: 7437-7442.
8) Yu G, Jia B, Cheng Y, Zhou L, Qian B, Liu Z, Wang Y. MicroRNA-429 sensitizes pancreatic cancer cells to gemcitabine through regulation of PDCD4. Am J Transl Res 2017; 9: 5048-5055.
9) Vetter G, Saumet A, Moes M, Vallar L, Le Bechec A, Laurini C, Sabbah M, Arar K, Theillet C, Lecellier CH, Friederich E. miR-661 expression in SNAI1-induced epithelial to mesenchymal transition contributes to breast cancer cell invasion by targeting Nectin-1 and StarD10 messengers. Oncogene 2010; 29: 4436-4448.
10) Zhang LY, Liu M, Li X, Tang H. miR-490-3p modulates cell growth and epithelial to mesenchymal transition of hepatocellular carcinoma cells by targeting endoplasmic reticulum-Golgi intermediate compartment protein 3 (ERGIC3). J Biol Chem 2013; 288: 4035-4047.
11) Han L, Chen W, Xia Y, Song Y, Zhao Z, Cheng H, Jiang T. MiR-101 inhibits the proliferation and metastasis of lung cancer by targeting zinc finger E-box binding homeobox 1. Am J Transl Res 2018; 10: 1172-1183.
12) Zhang X, He X, Liu Y, Zhang H, Chen H, Guo S, Liang Y. MiR-101-3p inhibits the growth and metastasis of non-small cell lung cancer through blocking PI3K/AKT signal pathway by targeting MALAT-1. Biomed Pharmacother 2017; 93: 1065-1073.
13) Nerviani A, Pitzalis C. Role of chemokines in ectopic lymphoid structures formation in autoimmunity and cancer. J Leukoc Biol 2018; 104: 333-341.
14) Karin N. Chemokines and cancer: new immune checkpoints for cancer therapy. Curr Opin Immunol 2018; 51: 140-145.
15) Gao D, Fish EN. Chemokines in breast cancer: regulating metabolism. Cytokine 2018; 109: 57-64.
16) Spaks A. Role of CXC group chemokines in lung cancer development and progression. J Thorac Dis 2017; 9: S164-S171.
17) Emmanouil G, Ayiomamitis G, Zizi-Sermpetzoglou A, Tzardi M, Moursellas A, Voumvouraki A, Kouroumalis E. Angiodrastic chemokines in colorectal cancer: clinicopathological correlations. Anal Cell Pathol (Amst) 2018; 2018: 1616973.
18) Li J, Tang Z, Wang H, Wu W, Zhou F, Ke H, Lu W, Zhang S, Zhang Y, Yang S, Ni S, Huang J. CXCL6 promotes non-small cell lung cancer cell survival and metastasis via down-regulation of miR-515-5p. Biomed Pharmacother 2018; 97: 1182-1188.
19) Ma JC, Sun XW, Su H, Chen Q, Guo TK, Li Y, Chen XC, Guo J, Gong ZQ, Zhao XD, Qi JB. Fibroblast-derived CXCL12/SDF-1alpha promotes CXCL6 secretion and co-operatively enhances metastatic potential through the PI3K/Akt/mTOR pathway in colon cancer. World J Gastroenterol 2017; 23: 5167-5178.
20) Xu L, Duda DG, di Tomaso E, Ancukiewicz M, Chung DC, Lauwers GY, Samuel R, Shellito P, Czito BG, Lin PC, Poleski M, Bentley R, Clark JW, Willett CG, Jain RK. Direct evidence that bevacizumab, an anti-VEGF antibody, up-regulates SDF1alpha, CXCR4, CXCL6, and neuropilin 1 in tumors from patients with rectal cancer. Cancer Res 2009; 69: 7905-7910.
21) Zhu YM, Bagstaff SM, Woll PJ. Production and upregulation of granulocyte chemotactic protein-2/CXCL6 by IL-1beta and hypoxia in small cell lung cancer. Br J Cancer 2006; 94: 1936-1941.
22) Verbeke H, Struyf S, Berghmans N, Van Coillie E, Opdenakker G, Uyttenhove C, Van Snick J, Van Damme J. Isotypic neutralizing antibodies against mouse GCP-2/CXCL6 inhibit melanoma growth and metastasis. Cancer Lett 2011; 302: 54-62.
23) Zhang H, Hou L, Li CM, Zhang WY. The chemokine CXCL6 restricts human trophoblast cell migration and invasion by suppressing MMP-2 activity in the first trimester. Hum Reprod 2013; 28: 2350-2362.
24) Xu H, Yuan Y, Wu W, Zhou M, Jiang Q, Niu L, Ji J, Liu N, Zhang L, Wang X. Hypoxia stimulates invasion and migration of human cervical cancer cell lines

HeLa/SiHa through the Rab11 trafficking of integrin alphavbeta3/FAK/PI3K pathway-mediated Rac1 activation. J Biosci 2017; 42: 491-499.

25) Wang J, Li H. CircRNA circ_0067934 silencing inhibits the proliferation, migration and invasion of NSCLC cells and correlates with unfavorable prognosis in NSCLC. Eur Rev Med Pharmacol Sci 2018; 22: 3053-3060.

26) Hua FF, Liu SS, Zhu LH, Wang YH, Liang X, Ma N, Shi HR. MiRNA-338-3p regulates cervical cancer cells proliferation by targeting MACC1 through MAPK signaling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 5342-5352.

27) Jin W, Chen F, Wang K, Song Y, Fei X, Wu B. miR-15a/miR-16 cluster inhibits invasion of prostate cancer cells by suppressing TGF-beta signaling pathway. Biomed Pharmacother 2018; 104: 637-644.

28) Hujie G, Zhou SH, Zhang H, Qu J, Xiong XW, Hujie O, Liao CG, Yang SE. MicroRNA-10b regulates epithelial-mesenchymal transition by modulating KLF4/KLF11/Smads in hepatocellular carcinoma. Cancer Cell Int 2018; 18: 10.

29) Hou R, Wang D, Lu J. MicroRNA-10b inhibits proliferation, migration and invasion in cervical cancer cells via direct targeting of insulin-like growth factor-1 receptor. Oncol Lett 2017; 13: 5009-5015.

30) Chen Z, Han S, Huang W, Wu J, Liu Y, Cai S, He Y, Wu S, Song W. MicroRNA-215 suppresses cell proliferation, migration and invasion of colon cancer by repressing Yin-Yang 1. Biochem Biophys Res Commun 2016; 479: 482-488.

31) Choi DH, Park SJ, Kim HK. miR-215 overexpression distinguishes ampullary carcinomas from pancreatic carcinomas. Hepatobiliary Pancreat Dis Int 2015; 14: 325-329.

32) Hou Y, Li L, Ju Y, Lu Y, Chang L, Xiang X. MiR-101-3p Regulates the viability of lung squamous carcinoma cells via targeting EZH2. J Cell Biochem 2017; 118: 3142-3149.

33) Zhang JG, Guo JF, Liu DL, Liu Q, Wang JJ. MicroRNA-101 exerts tumor-suppressive functions in non-small cell lung cancer through directly targeting enhancer of zeste homolog 2. J Thorac Oncol 2011; 6: 671-678.

34) Tian H, Huang P, Zhao Z, Tang W, Xia J. HIF-1alpha plays a role in the chemotactic migration of hepatocarcinoma cells through the modulation of CXCL6 expression. Cell Physiol Biochem 2014; 34: 1536-1546.