


## miR-186-3p attenuates the tumorigenesis of cervical cancer via targeting insulin-like growth factor 1 to suppress PI3K-Akt signaling pathway

Xiurong Lu <sup>\*</sup>, Xiao Song, Xiaohui Hao, Xiaoyu Liu, Xianyu Zhang, Na Yuan, Huan Ma, and Zhilin Zhang

Department of Radiotherapy, The First Affiliated Hospital of Hebei North University, Zhangjiakou, Hebei, China

### ABSTRACT

miR-186-3p acts as a tumor suppressor in various cancers. This study aimed to explore the expression levels of miR-186-3p and its role in cervical cancer. We analyzed the effects of miR-186-3p and insulin-like growth factor 1 (IGF1) on the proliferation, invasion, and apoptosis of cervical cancer cells in vitro by regulating the PI3K/Akt signaling pathway. In cervical cancer tissues and cells, miR-186-3p was downregulated, and IGF1 was upregulated. In addition, miR-186-3p inhibited cell proliferation and invasion and enhanced apoptosis of cervical cancer cells. Moreover, our results showed that miR-186-3p inversely regulated the mRNA expression of IGF1 through direct contact. Knockdown of IGF1 reversed the results of miR-186-3p inhibitor in cervical cancer cells. In addition, the PI3K/Akt signaling pathway was activated by the miR-186-3p inhibitor, although partially arrested by IGF1 knockdown. The PI3K/Akt signaling pathway inhibitor suppressed miR-186-3p inhibitor-stimulated cell proliferation in cervical cancer. In conclusion, miR-186-3p inhibits tumorigenesis of cervical cancer by repressing IGF1, which inactivates the PI3K/Akt signaling pathway, implicating miR-186-3p as a potential new target for the treatment of cervical cancer.

### ARTICLE HISTORY

Received 27 May 2021  
Revised 25 August 2021  
Accepted 29 August 2021

### KEYWORDS



Cervical cancer; miR-186-3p; IGF1; PI3K/AKT; tumorigenesis

## Introduction

According to cancer statistics reported in 2020, cervical cancer (CC) ranks as the second leading cause of death among women aged 20–39 years suffering from cancer. Approximately 10 patients diagnosed with cancer die from CC per week [1]. The survival rate of most cancer patients has improved, except for patients with the uterine cervix and uterine corpus over the past few decades. Between 1975–1977 and 2006–2012, the survival for CC decreased from 69.1% to 68.8% [2]. Hence, further advances in treatments are crucial to suppress tumor growth, prolong survival, and improve the quality of life of CC patients. Moreover, it has been reported that genetic and epigenetic changes, particularly microRNAs, act in the screening and progression of CC [3].

miRNAs have a significant impact on translation inhibition, mRNA decay, and mRNA deadenylation through the combination with the 3′-untranslated region (3′ UTR) of most protein-coding genes [4,5].

Several studies have shown that miRNAs are involved in the occurrence and development of tumors by regulating the proliferation, differentiation, apoptosis, angiogenesis, metastasis, and metabolism of tumor cells [6]. For instance, overexpression of miR-25-3p significantly promoted the growth and invasion of gastric cancer cells in vitro and regulated the growth of gastric cancer cells in vivo [7]. MiR-214-3p upregulates migration, invasion, and epithelial-mesenchymal transformation of colon cancer cells [8]. MiR-186 has been extensively studied in recent years. However, most studies have focused on miR-186-5p. It has been suggested that miR-186-5p exists as a tumor suppressor factor in CC. Moreover, miR-186-5p downregulation leads to a decline in cell proliferation, migration, and invasion and an increase in apoptosis [9,10]. Regarding miR-186-3p, only one study has identified that it suppresses tumor growth and regulates glycolysis by directly targeting epiregulin in estrogen receptor-positive breast cancer cells [11]. However, the detailed characterization of miR-186-3p in CC is unclear.

\*CONTACT Xiurong Lu  [xiurong\\_lu2@163.com](mailto:xiurong_lu2@163.com)  Department of Radiotherapy, The First Affiliated Hospital of Hebei North University, No.36 Changqing Road, Qiaoxi District, Zhangjiakou, 075000, Hebei, China

The insulin-like growth factor 1 (IGF1) gene is located on chromosome 12q23.2 and consists of seven exons. It encodes proteins that function and have structure similar to insulin, and growth hormones require IGF1 to function [12]. Interest in IGF1 and its effect on carcinogenesis has increased recently. Several studies showed that circulating levels and tissue expression of IGF1 are upregulated in patients with CC [13,14]. IGF1 has been implicated in promoting mitogenic and metastatic cancer cells, which enhances CC invasiveness and proliferation [15,16]. Only one study, which was conducted to analyze the effect of miR-186-5p on IGF1, showed that miR-186-5p induced significant apoptosis of neurons by directly targeting and suppressing IGF1 expression [17]. However, the effect of the interaction between miR-186-3p and IGF1 on tumor growth in CC has not yet been established.

Therefore, this study aimed to explore how miR-186-3p and IGF1 affect CC progression and their possible mechanisms of action. We hypothesized that miR-186-3p might suppress CC by targeting IGF1. In CC, miR-186-3p expression was decreased and IGF1 expression was increased. Further functional experiments confirmed that the inhibitory effect of miR-186-3p on the viability, proliferation, invasion of CC, and the promotion of apoptosis were achieved by targeting and inhibiting IGF1. Our study reveals for the first

time the effect of miR-186-3p/IGF1 on CC and its mechanism of action, providing a valuable direction for the diagnosis and treatment of CC.

## Materials and methods

### Tissue samples

Fifty patients with CC aged 28–81 years (mean age, 52 years) were included in this study. The samples were stored at  $-80^{\circ}\text{C}$  after liquid nitrogen refrigeration. All study participants provided their samples with informed consent. All experiments followed the ethics committee. The clinicopathological features of all patients are summarized in Table 1.

### Cell culture and treatment

The cell culture conditions were  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . The human cervical surface epithelial cell line (HcerEpic), human CC cell lines (HeLa, CaSki, SiHa, and C33A), and embryonic kidney cell line (HEK293T) acquired from ATCC (USA) were cultured in a plate with RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, USA).

The miR-186-3p inhibitor, miR-186-3p mimic, and paired miRNA negative control (inhibitor-NC and mimic-NC) were designed and synthesized by Shanghai Genechem (Shanghai, China).

**Table 1.** The relationship between miR-186-3p or IGF1 expression and clinicopathological characteristics in 50 patients with cervical cancer.

| Characteristic        | N  | miR-186-3p expression |              | P-value | IGF1 expression |              | P-value |
|-----------------------|----|-----------------------|--------------|---------|-----------------|--------------|---------|
|                       |    | High (n = 25)         | Low (n = 25) |         | High (n = 25)   | Low (n = 25) |         |
| Age (years)           |    |                       |              | 0.382   |                 |              | 0.145   |
| ≥50                   | 31 | 17                    | 14           |         | 18              | 13           |         |
| <50                   | 19 | 8                     | 11           |         | 7               | 12           |         |
| Tumor size (cm)       |    |                       |              | 0.765   |                 |              | 0.371   |
| ≥ 5                   | 17 | 8                     | 9            |         | 10              | 7            |         |
| < 5                   | 33 | 17                    | 16           |         | 15              | 18           |         |
| TNM stage             |    |                       |              | 0.004   |                 |              | 0.023   |
| I–II                  | 28 | 19                    | 9            |         | 10              | 18           |         |
| III–IV                | 22 | 6                     | 16           |         | 15              | 7            |         |
| Distant metastasis    |    |                       |              | 0.012   |                 |              | 0.031   |
| No                    | 35 | 22                    | 13           |         | 14              | 21           |         |
| Yes                   | 15 | 3                     | 12           |         | 11              | 4            |         |
| Lymph node metastasis |    |                       |              | 0.011   |                 |              | 0.002   |
| No                    | 23 | 16                    | 7            |         | 6               | 17           |         |
| Yes                   | 27 | 9                     | 18           |         | 19              | 8            |         |
| Histological grade    |    |                       |              | 0.003   |                 |              | 0.018   |
| Well                  | 18 | 14                    | 4            |         | 5               | 13           |         |
| Moderately/Poorly     | 32 | 11                    | 21           |         | 20              | 12           |         |

The P-value was calculated by Chi-square test or Fisher's exact test.

Before transfection, HeLa and SiHa cells were seeded at a density of  $5 \times 10^4$  cells/well for 12 h. At approximately 40–60% confluence, the cells were transfected with miR-186-3p mimic, miR-186-3p inhibitor, mimic-NC, or inhibitor-NC using Lipofectamine 3000 (Invitrogen, USA). To knock down the expression of IGF1, specific IGF1 interfering oligonucleotides (si-IGF1) were purchased from Shanghai Jikai Gene Chemistry Co. (Shanghai, China). Then, si-IGF1 was used to treat CC cell lines. Nonspecific control siRNA and reagent controls were used in all the experiments. After transfecting the cells for 48 h, the cell suspension was collected for subsequent experiments. For pathway exploration, LY294002 (Medchem Express, USA) was used as a PI3K inhibitor at a concentration of 50  $\mu$ M. The cells were pretreated with LY294002 for 2 h.

#### Quantitative real-time polymerase chain reaction (qRT-PCR)

miR-186-3p expression levels were assessed by qRT-PCR. Total miRNAs were acquired using a miRNeasy Mini kit (Qiagen, Germany) from CC tissues or cell lines. cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions. A miRNA-specific TaqMan MiRNA Assay kit (Applied Biosystems, USA) was used for qRT-PCR. The  $2^{-\Delta\Delta C_t}$  method was applied, which was standardized using U6 small nuclear RNA.

For the mRNAs, the expression level of IGF1 was determined by qRT-PCR in the CC tissues and cells. Briefly, total RNA was extracted from tissue samples and cells using TRIzol reagent (Takara, Japan). The RNA content was quantified, and cDNA was synthesized using the Reverse Transcriptase kit (Takara, Japan) following the manufacturer's instructions. The SYBR Green kit (Takara, China) was used for the determination of cDNA templates. All primers used in this study are listed in Table 2. The  $2^{-\Delta\Delta C_t}$  method [18] was used to analyze the mRNA levels of IGF1 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control.

**Table 2.** PCR primers used in this study.

| Gene  | Primer sequence   |
|-------|---|
| IGF1  | Forward:5'-TCGCATCTCTTCTATCTGGCCCTGT-3<br>Reverse:5'-GCAGTACATCTCCAGCCTCCTCAGA -3 |
| GAPDH | Forward:5'-GGAAGGTGAAGGTCGGAG TCA-3<br>Reverse:5'-GTCATTGATGGCAACAATATCCAC T-3    |
| U6    | Forward:5'-CTCGCTTCGGCAGCACA-3<br>Reverse:5'-AACGCTTCACGAATTTGCGT-3               |

#### Cell proliferation assay

CC cell proliferation was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [8] and enzyme-linked immunosorbent assay (ELISA) – bromodeoxyuridine (BrdU) assays [19]. Briefly, cells seeded in a specific plate at a density of  $4 \times 10^4$  cells/well were analyzed at 24, 48, and 72 h after transfection. Every time point of detection followed a 5-h incubation with 20  $\mu$ L of MTT (KeyGEN BioTECH, China). The absorption degree at 570 nm was examined using an EXL-800 microplate reader (BIOTEK, USA). BrdU incorporation assay was used to analyze cell proliferation using the BrdU Cell Proliferation Assay kit (Cell Signaling Technology, USA). For the assay, cells were incubated with 1X BrdU solution for 6 h at 37°C to induce proliferation and incorporation of BrdU. Thereafter, the labeled cells were cleaned and cultured with detection antibody solution for 1 h. Next, the secondary antibody solution was added, and the plate was incubated for 30 min. The absorbance at 450 nm was read using an EXL-800 microplate reader (BIOTEK, USA).

#### Transwell assay

CC cell invasion was measured as described previously [20]. Briefly, serum-free RPMI-1640 medium (Gibco, USA) containing  $2 \times 10^5$  transfected HeLa and SiHa cells were placed on the upper chamber of the cell culture insert with Matrigel. Thereafter, 600  $\mu$ L of the complete medium was loaded below the cell-permeable membrane. The cells were incubated for 24 h in 5% CO<sub>2</sub> at 37°C. The cells gently migrating from the permeable membrane were treated for 30 min in 70% ethanol and 10 min in 0.1% crystal violet. Invasive cells in five random fields were counted using an inverted light microscope.

### **Caspase-3 activity assay**

Caspase-3 Activity ELISA kit (Cell Signaling Technology, USA) was used to detect caspase-3 activity [21]. Caspase-3 activity was assessed using an ultraviolet spectrophotometer (Thermo, USA) at 405 nm. To conduct this experiment, 6-well plates ( $1 \times 10^5$  cells/well) were used.

### **Dual-luciferase reporter assay**

The luciferase assay was performed as previously described [22]. HeLa and SiHa cells in the exponential growth period were used for the luciferase assay. The amplified wild-type (Wt) or mutant-type (Mut) IGF1 3' UTR segment was inserted into the pGL3 luciferase reporter. These reporter plasmids were co-transfected with miR-186-3p into HeLa and SiHa cells using Lipofectamine 3000 (Invitrogen, USA). The Dual-Luciferase Reporter Assay System (Promega, USA) was used for analysis after 72-h-cell incubation.

### **RNA-pull down assay**

This assay was performed following the method of Lal et al. [23]. Briefly, cells transfected with either biotinylated-miR-186-3p (Bio-miR-186-3p) or negative control oligo (Bio-NC) (25 nM; Roche, USA) were seeded in six-well plates. Thereafter, lysis buffer was utilized for cell lysis, and the cytoplasmic lysate was split into two parts: 95% of the lysate was processed for biotin pull-down assay using streptavidin-coated magnetic beads (Invitrogen, USA), RNA was extracted using the RNeasy Kit (Invitrogen, USA), and the remaining 5% of the lysate was used to extract RNA as an input standard. The levels of mRNA in the miR-186-3p or miR-NC pull-down were analyzed by qRT-PCR. GAPDH mRNA was used to standardize mRNA levels of the target genes. Then, the mRNA gathered by bio-miR-186-3p or bio-NC pulldown was detected by the ratio of the control-normalized pull-down RNA to the control-normalized input levels.

### **Western blotting**

Western blotting was performed as described previously [24]. Total proteins from each sample were

dissolved in radioimmunoprecipitation assay lysis buffer and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, these separated samples were transferred to polyvinylidene difluoride membranes (Millipore, USA). Nonspecific binding was blocked with 5% fat-free milk at room temperature. Primary antibodies, including IGF1 (1:400; Abcam, USA), PI3K (1:1000; Cell Signaling Technology, USA), Akt (1:1000; Cell Signaling Technology, USA), p-Akt (1:1000; Cell Signaling Technology, USA), t-Akt (1:1000; Cell Signaling Technology, USA), and GAPDH (1:1,000; Abcam, USA) were used to detect the corresponding antigen. Following incubation with secondary antibody (1:5000; Cell Signaling Technology, USA) at room temperature for 1 h, protein levels were analyzed.

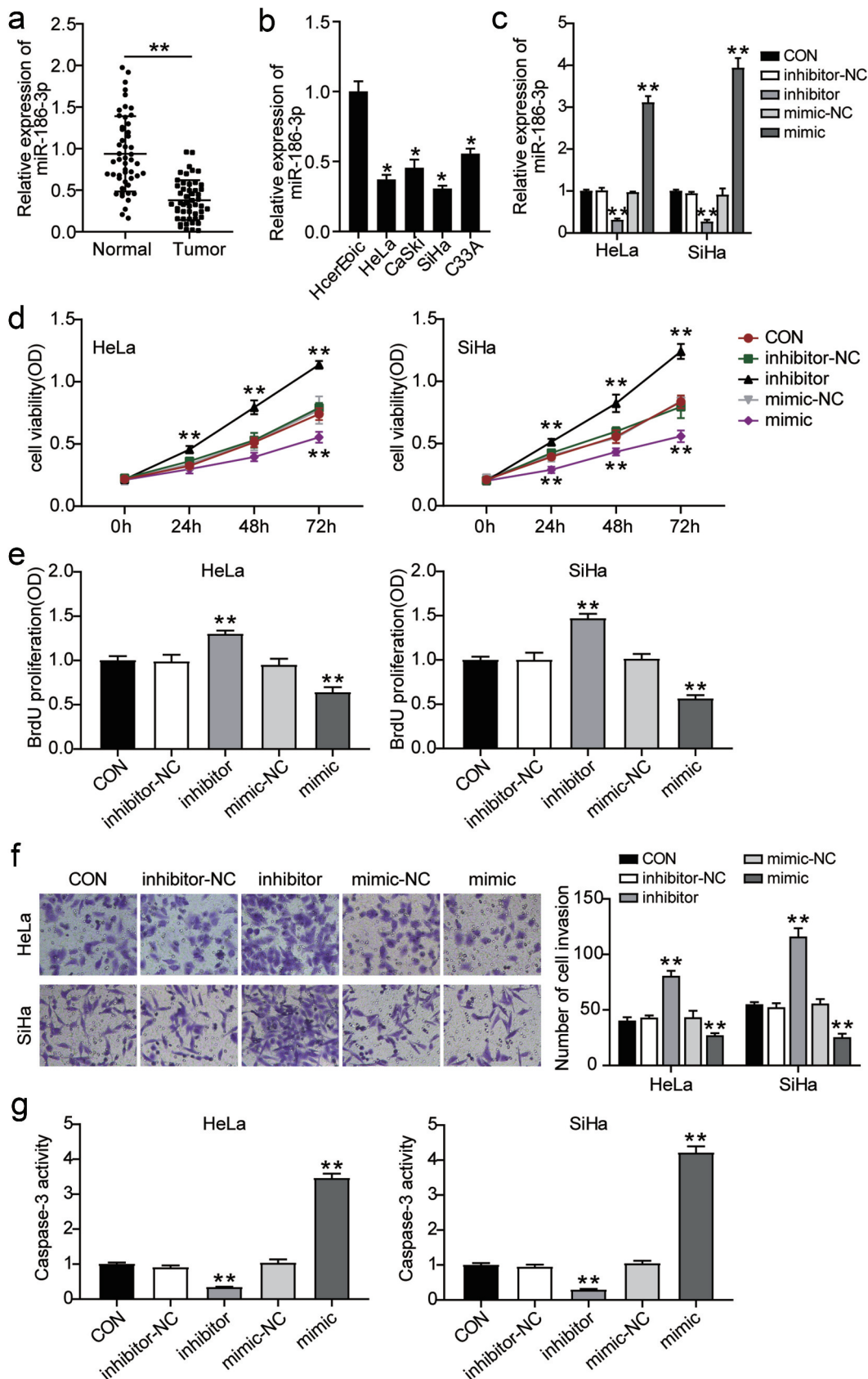
### **Statistical analysis**

GraphPad Prism 6 (GraphPad Software, USA) was used for data analysis. All results were expressed as means  $\pm$  standard deviations (SD). Student's t-test was used to analyze the differences between two groups. Multiple comparisons were assessed by one-way ANOVA with LSD post hoc test.  $P < 0.05$  was considered statistically significant.

## **Results**

### **Downregulation of miR-186-3p affects CC cell proliferation, invasion and apoptosis**

To explore the effect of miR-186-3p on CC, we detected the expression of miR-186-3p in CC tissues and cell lines and analyzed the effect of miR-186-3p on the CC cell viability, proliferation, migration, and apoptosis in vitro through MTT assay, BrdU assay, transwell assay, and caspase-3 activity, respectively. The antitumor effects of miR-186-3p in CC have been clarified. Initially, miR-186-3p levels in CC tissues and CC cells were quantified for the preliminary investigation of the role of miR-186-3p in CC pathological processes. As shown in [Figure 1a](#), miR-186-3p levels reduced (over 50%) in the CC tissues compared to the adjacent normal tissues. The median expression level of miR-186-3p was used as a cutoff point to



**Figure 1. MiR-186-3p is downregulated and correlated with cell proliferation, invasion and apoptosis of cervical cancer.** (a) MiR-186-3p expression by qRT-PCR in cervical cancer tissues and the adjacent normal tissues. \*\* $P < 0.001$  vs. adjacent normal tissues. (b) MiR-186-3p expression by qRT-PCR in the human cervical surface epithelial cell line (HcerEpic) and four cervical cancer cell lines

(HeLa, CaSki, SiHa, and C33A).  $**P < 0.001$  vs. HcerEpic cell. (c) miR-186-3p expression by qRT-PCR in the HeLa and SiHa cells transfected with miR-186-3p inhibitor or mimic.  $**P < 0.001$  vs. vs. CON. (d, e) MTT (d) and BrdU incorporation (e) assays of cell viability after HeLa and SiHa cells transfected with miR-186-3p inhibitor or mimic.  $*P < 0.05$ ;  $**P < 0.001$  vs. CON. (f) Transwell assay in HeLa and SiHa cells transfected with miR-186-3p inhibitor or mimic. Left panel: representative images of the lower chamber (invading cells). Right panel: graph represents number of cell invasion.  $*P < 0.05$ ;  $**P < 0.001$  vs. CON. (g) Caspase-3 activity ELISA assay in HeLa and SiHa cells transfected with miR-186-3p inhibitor or miR-NC.  $**P < 0.001$  vs. CON. miR, microRNA; CON, control; NC, negative control.

divide all 50 patients into two groups: CC patients who expressed miR-186-3p at levels less than the cutoff value were assigned to the low expression group ( $n = 25$ ), and those with expression levels above the cutoff value were assigned to the high expression group ( $n = 25$ ). The relationship between miR-186-3p expression levels and different clinicopathological factors is shown in [Table 1](#). Decreased miR-186-3p expression in CC was significantly associated with the tumor node metastasis (TNM) stage, distant metastasis, lymph node metastasis, and histological grade. However, no significant correlation was observed between miR-186-3p expression and other clinicopathological variables, such as age and tumor size. In addition, we observed that the miR-186-3p level in four CC cell lines was significantly decreased by approximately 50% compared to that in HcerEpic cells ([Figure 1b](#)). As a result, miR-186-3p exhibited the lowest level in HeLa and SiHa cell lines, which were selected for the following experiments.

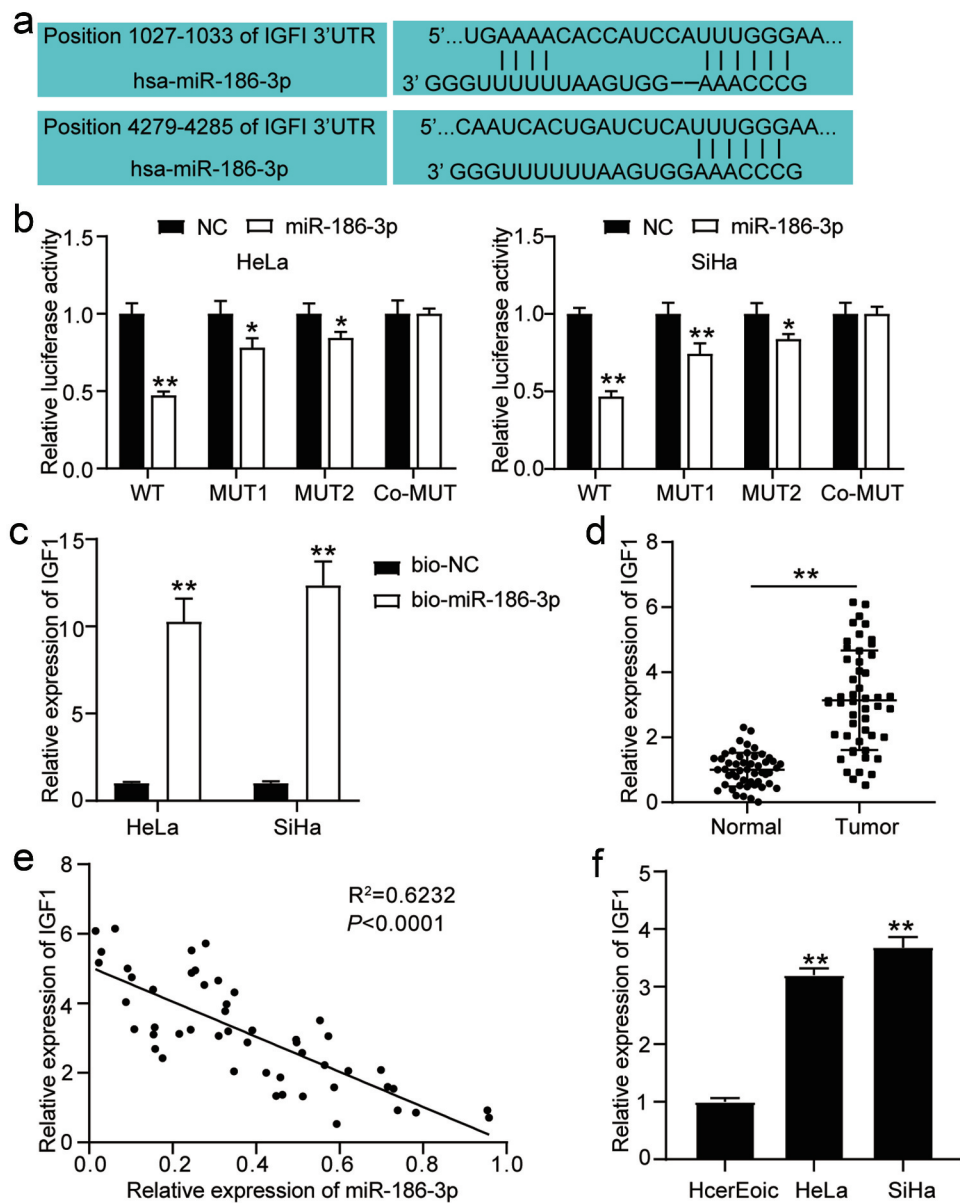
Furthermore, qRT-PCR analysis confirmed the transfection efficiency and showed that miR-186-3p level was repressed by treatment with miR-186-3p inhibitor and enhanced by miR-186-3p mimic ([Figure 1c](#)). The MTT and BrdU incorporation assays demonstrated that the miR-186-3p inhibitor enhanced CC cell proliferation, whereas the miR-186-3p mimic suppressed it ([Figure 1d and 1e](#)). Moreover, miR-186-3p downregulation increased the invasiveness of CC cells in vitro, and miR-186-3p upregulation showed the opposite effect ([figure 1f](#)). Caspase-3 activity was also evaluated. The inhibitor of miR-186-3p decreased caspase-3 activity, and a miR-186-3p mimic increased caspase-3 activity, a critical executioner participated in 'extrinsic' and 'intrinsic' apoptosis ([Figure 1g](#)). These findings demonstrated that upregulation of miR-186-3p inhibits tumorigenesis in CC, and

downregulation of miR-186-3p promotes tumor progression.

### **miR-186-3p targets IGF1 in CC cells**

To elucidate the mechanism of miR-186-3p in CC, luciferase and RNA pull-down assays were used to analyze the binding relationship between miR-186-3p and IGF1. The results showed that miR-186-3p inhibited IGF1. Bioinformatics analysis was then used to identify the possible molecular targets of miR-186-3p. The prediction results showed that the 3' UTR of IGF1 contained two binding sites for miR-186-3p ([Figure 2a](#)). To determine whether IGF1 was the direct target of miR-186-3p, we constructed luciferase reporters containing the IGF1-3' UTR with a conserved miR-186-3p binding sequence or two mutated binding sequences of miR-186-3p. As a result, miR-186-3p significantly attenuated the luciferase activity of the wild-type but not the 3' UTR of IGF1 two mutated binding sequences of miR-186-3p ([Figure 2b](#)). To further confirm the physical binding of IGF1 with miR-186-3p, we conducted an RNA pull-down assay in HeLa and SiHa cells transfected with Bio-miR186-3p. As displayed in [Figure 2c](#), IGF1 mRNA levels were significantly enriched by bio-miR186-3p pulldown compared to bio-NC. These results suggest that miR-186-3p physically interacts with IGF1.

Thereafter, we examined the expression of IGF1 in CC tissues and cell lines. It was demonstrated that the IGF1 level was enriched in the CC tissues compared to the adjacent normal tissues ([Figure 2d](#)). Furthermore, IGF1 upregulation in CC was significantly associated with the TNM stage, distant metastasis, lymph node metastasis, and histological grade, except with age or tumor size ([Table 1](#)). In addition, IGF1 expression was significantly higher in HeLa and SiHa cells ([figure 2e](#)). IGF1 levels were



**Figure 2. IGF1 is a direct target of miR-186-3p in cervical cancer cell.** (a) IGF1 was predicted as miR-186-3p target. Top and bottom, predicted two miR-186-3p binding site in the 3' UTR of IGF1. (b) Dual-luciferase reporter assay. NC RNA was set to 1.0 in each experiment, and the luciferase activity with miR-186-3p was normalized relative to NC RNA. \* $P < 0.05$ ; \*\* $P < 0.001$  vs. NC. (c) RNA-pull down assay. qRT-PCR of IGF1 expression in HeLa and SiHa cells transfected with Bio-NC and Bio-miR186-3p. \*\* $P < 0.001$  vs. bio-NC. (d) Comparison of IGF1 expression in the cervical cancer tissues and the adjacent normal tissues. \*\* $P < 0.01$  vs. adjacent normal tissues. (e) Pearson's correlation analyses showing a negative correlation of miR-186-3p and IGF1 mRNA levels in cervical cancer tissues. (f) qRT-PCR of IGF1 in HcerEpic, HeLa and SiHa cells. \*\* $P < 0.001$  vs. HcerEpic cells. IGF1, insulin-like growth factor 1; 3'UTR, 3'untranslated region; miR, microRNA; NC, negative control; WT, wild-type; MUT, mutant-type; bio-NC, biotinylated- negative control oligo.

negatively correlated with miR-186-3p expression in CC tissues (Figure 2f). In summary, miR-186-3p negatively regulated IGF1 expression through binding to the 3' UTR sequence in CC.

### **IGF1 mediates the functional effects of miR-186-3p in CC cells**

To further study the effect of miR-186-3p on the regulation of IGF1 in CC cells, we analyzed the

changes in viability, proliferation, invasion, and apoptosis of CC cells after co-low expression of miR-186-3p and IGF1. The results showed that the effect of miR-186-3p on CC cells was mediated by GINS2. First, to determine whether IGF1 contributes to the miR-186-3p reaction against CC, si-IGF1 and miR-186-3p inhibitors were co-transfected into HeLa and SiHa cells. The mRNA levels of IGF-1 and miR-186-3p were measured using qRT-PCR. Western blotting was performed to detect the protein levels of IGF-1. After transfection, the relative expression of IGF1 and miR-186-3p in si-IGF1-transfected and miR-186-3p inhibitor-transfected HeLa and SiHa cells significantly decreased, indicating that miR-186-3p and IGF1 expression was effectively suppressed (Figure 3a and 3 b). The proliferation of CC cell lines was significantly inhibited in the si-IGF1 group. However, cell proliferation was significantly upregulated by co-transfection of miR-186-3p inhibitor with si-IGF1 compared to CC cells transfected with si-IGF1 (Figure 3c and 3d). Transwell assays revealed that the invasion capacity of the CC cells was significantly suppressed by transfection with si-IGF1. Inhibition of miR-186-3p promoted CC cell invasion via IGF1 inhibition (Figure 3e). Furthermore, figure 3f shows that miR-186-3p inhibition suppressed caspase-3 activity, whereas additional IGF1 downregulation partially promoted caspase-3 activity in HeLa and SiHa cells. Therefore, these data indicate that miR-186-3p inhibits CC cell growth and invasion and promotes apoptosis by suppressing IGF1 expression.

#### **MiR-186-3p suppress PI3K/Akt pathway by downregulating IGF1 expression**

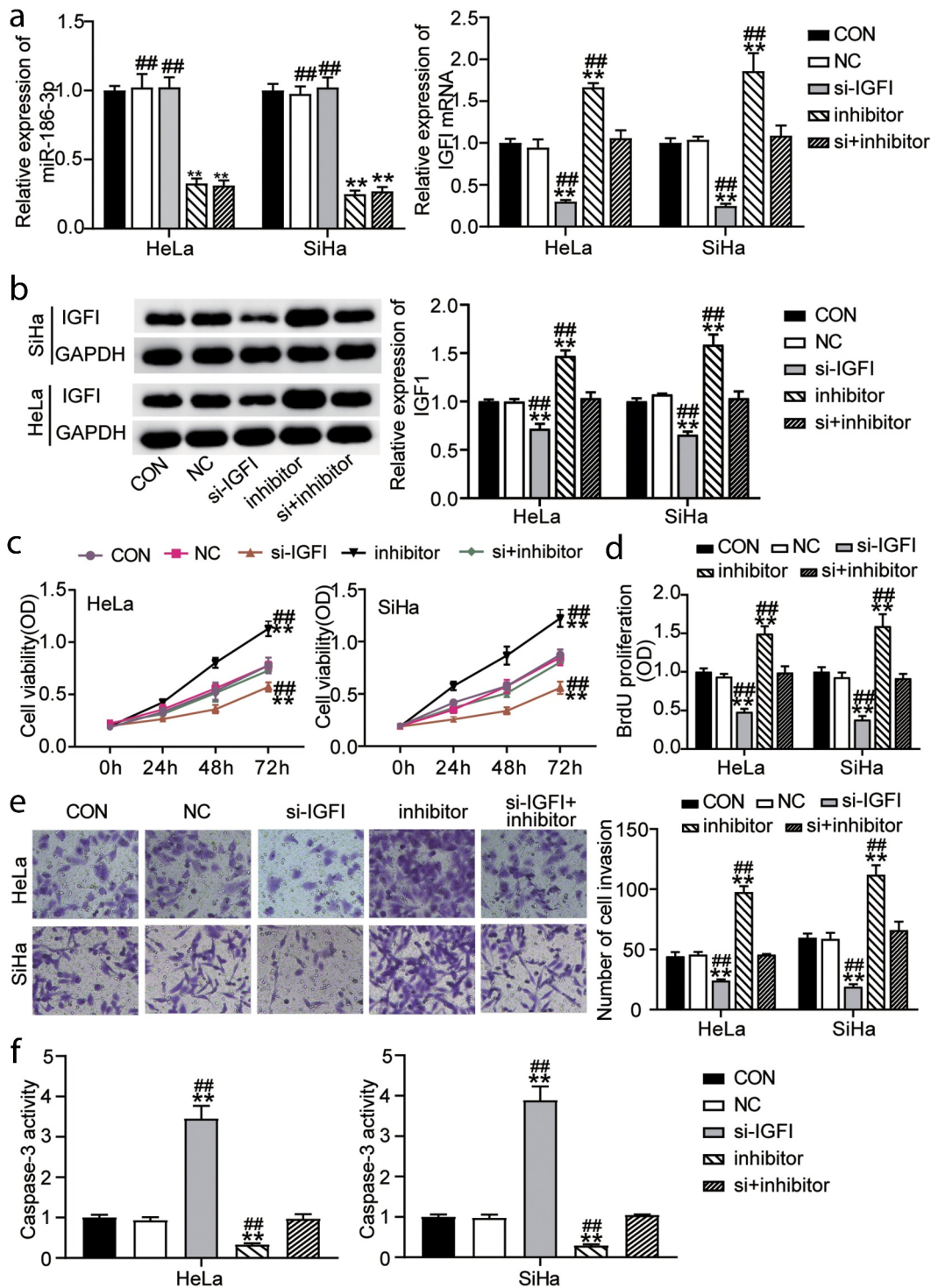
Next, the downstream signaling pathways of miR-186-3p/IGF1 affecting the biological functions of CC cells were identified. Western blot and MTT analysis showed that silencing miR-186-3p or IGF1 inhibited PI3K/Akt pathway activation. Previous studies have shown that the inactivation of the PI3K/Akt pathway inhibits carcinogenesis and the development of cancer [25,26]. In addition, the PI3K/AKT pathway is an indispensable pathway mediated by numerous cellular signals, including IGF-1 [27]. Hence, we further investigated whether miR-186-3p/IGF1 exerts its function via the PI3K/Akt signaling pathway. As

shown in Figure 4a and 4b, the protein expression of PI3K and phosphorylation of AKT were decreased by IGF1 knockdown. In contrast, the miR-186-3p inhibitor upregulated the protein levels of PI3K and phosphorylation of AKT. In addition, si-IGF1 suppressed the activation of the PI3K/AKT pathway induced by the miR-186-3p inhibitor. Therefore, we examined whether the PI3K signaling inhibitor LY294002 could suppress CC cell proliferation. As shown in Figure 4c, the results of the MTT assays indicated that CC cell proliferation was increased after miR-186-3p downregulation. Moreover, LY294002 suppressed the increase in CC cell proliferation following miR-186-3p inhibition. These results revealed that miR-186-3p prevents cell proliferation through the inactivation of the PI3K/AKT signaling pathway via inhibition of IGF1 in CC cell lines (Figure 5).

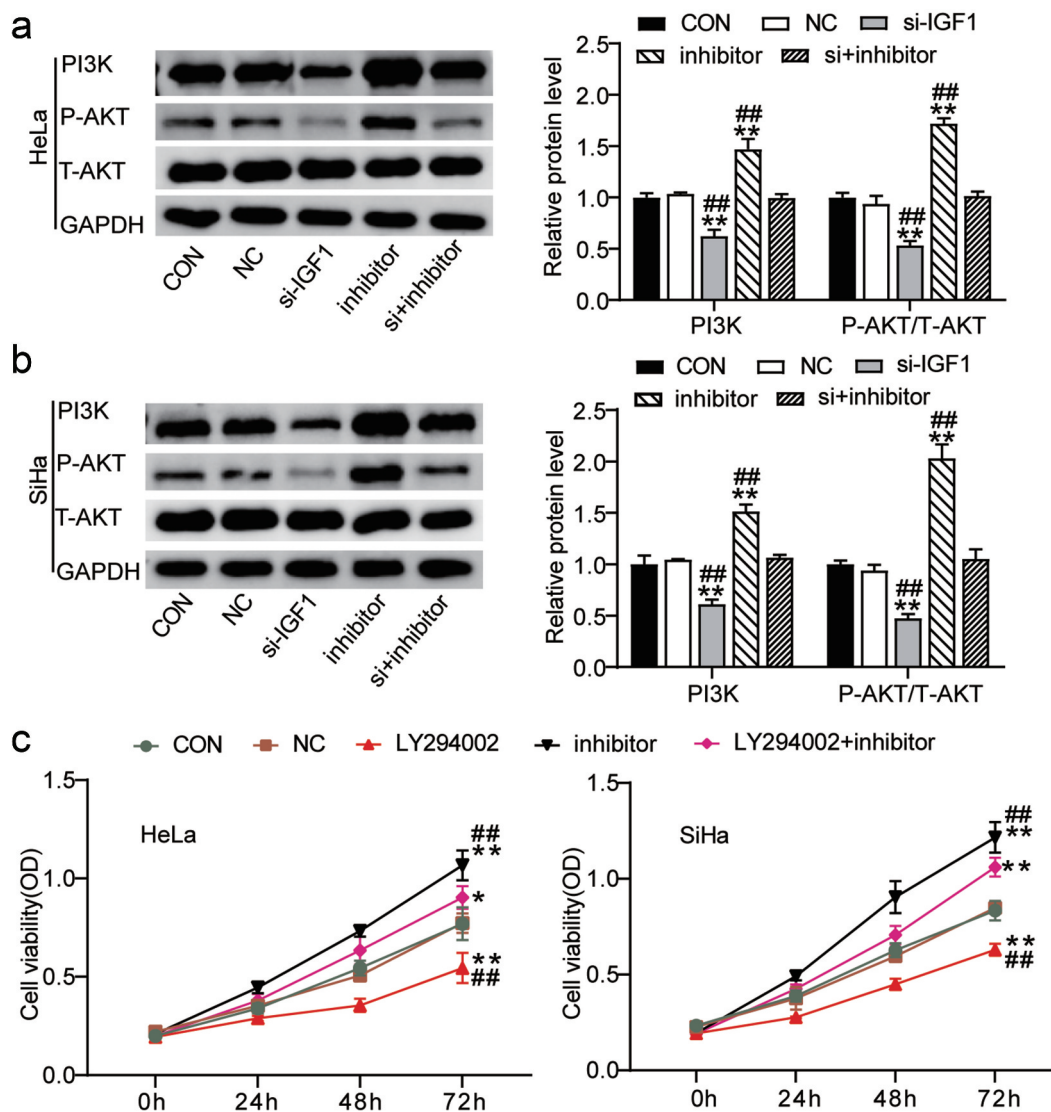
#### **Discussion**

miRNAs are regarded as critical regulators of cancer. Several studies have reported that miR-186 is downregulated in numerous types of human cancers, including non-small cell lung cancer [28], colon carcinoma [29], acute myeloid leukemia [30], esophageal squamous cell carcinoma [31], bladder cancer [32], gastric cancer [33], and hepatocellular carcinoma [34]. In addition, cell life processes in cancers are affected by miR-186 [35]. In non-small cell lung cancer, miR-186 was upregulated and targeted Rho-associated protein kinase 1 to suppress the proliferation and migration of cancer cells [36]. In bladder cancer, upregulated miR-186 decreased the proliferation and invasion of cells through the downregulation of nucleosome-binding protein 1 [32]. In CC, high expression of miR-186 targets Kazrin-F to promote apoptosis and suppress cell proliferation, colony formation, migration, and invasion of CC cells [32]. Zhang et al. [37] found that miR-186 reversed the function of the INK4 locus (ANRIL), leading to the suppression of CC. The above data support miR-186 as a key regulator and candidate therapeutic target in the tumor treatment. As for miR-186-3p, only one study has identified that miR-186-3p suppresses tumor growth and regulates glycolysis by





**Figure 3. IGF1 mediates the functional effects of miR-186-3p on cervical cancer cell.** (a) qRT-PCR of MiR-186-3p (left panel) and IGF1 (right panel) expression in HeLa and SiHa cells transfected with si-IGF1 or miR-186-3p inhibitor. (b) western blot analyses of IGF1 expression in miR-186-3p inhibitor- or si-IGF1-transfected HeLa and SiHa cells. (c, d) MTT (c) and BrdU incorporation (d) assays of cell viability after HeLa and SiHa cells transfected with si-IGF1 or miR-186-3p inhibitor. (e) Transwell assay in HeLa and SiHa cells transfected with si-IGF1 or miR-186-3p inhibitor. Left panel: representative images of the lower chamber (invading cells). Right panel: graph represents number of cell invasion. (f) Caspase-3 activity ELISA assay in HeLa and SiHa cells transfected with si-IGF1 or miR-186-3p inhibitor. \* $P < 0.05$ ; \*\* $P < 0.001$  vs. CON. # $P < 0.05$ ; ## $P < 0.001$  vs. si+inhibitor. CON, control; NC, negative control; si-, small interfering RNA; IGF1, insulin-like growth factor 1; miR-, microRNA.

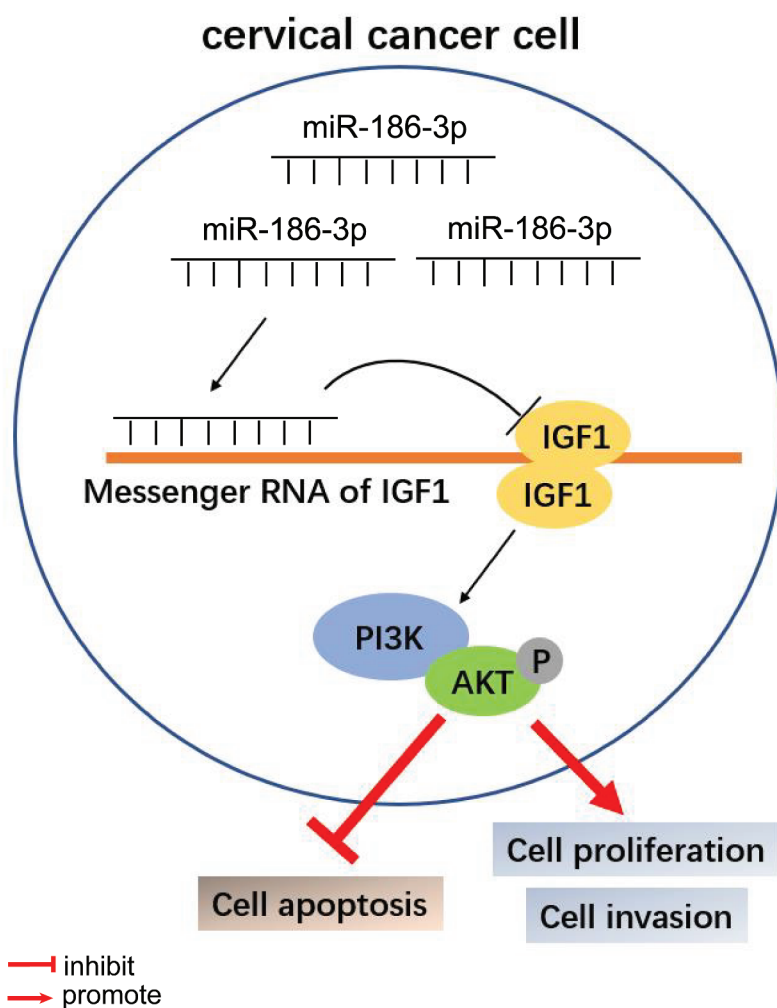


**Figure 4. MiR-186-3p suppress PI3K/Akt pathway through downregulating IGF1 expression.** (a, b) western blot analyses of PI3K, P-AKT and T-AKT expression in miR-186-3p inhibitor- or si-IGF1-transfected HeLa (a) and SiHa (b) cells. \* $P < 0.05$ ; \*\* $P < 0.001$  vs. CON. # $P < 0.05$ ; ## $P < 0.001$  vs. si+inhibitor. (c) MTT assays of cell viability after HeLa and SiHa cells pretreated by PI3K signaling inhibitor (LY294002) following transfection with miR-186-3p inhibitor. \* $P < 0.05$ ; \*\* $P < 0.001$  vs. CON. # $P < 0.05$ ; ## $P < 0.001$  vs. LY294002+ inhibitor. P-AKT, phosphorylated-AKT. T-AKT, total-AKT; CON, control; NC, negative control; si-, small interfering RNA; IGF1, insulin-like growth factor 1.

directly targeting epiregulin in estrogen receptor-positive breast cancer [11]. Based on the findings stated above, we speculated that miR-186-3p plays pivotal roles in modulating CC pathological processes. Our data demonstrated the downregulation of miR-186-3p in CC and CC cell lines. In functional studies, miR-186-3p downregulation remarkably contributed to CC cell proliferation. However, miR-186-3p suppression significantly inhibited apoptosis and reduced the expression of apoptosis-related factors. These results suggest

that miR-186-3p can be regarded as an antitumor agent in CC.

To further determine the mechanisms and targets of miR-186-3p, we performed bioinformatics, dual-luciferase reporter, and biotinylated-miRNA pull-down assays to confirm the predicted binding ability of miR-186-3p on IGF-1. Previous studies have shown that IGF1, a mitogenic and anti-apoptotic peptide, is upregulated in CC [13]. IGF1 stimulates cell growth and invasiveness in CC in a dose-dependent



**Figure 5.** Model explaining the antitumor effect of miR-186-3p in cervical cancer.

manner [15]. Furthermore, IGF1 inhibition induced an increase in the G2M/S fraction, increased apoptosis, and decreased invasive ability of CC stem cells [38], suggesting that IGF1 has a significant effect on the progression of CC. Moreover, additional studies have shown that IGF1 expression is upregulated in other cancers (including hepatocellular carcinoma), predicting a lower 10-year survival rate [39]. Similar to the previous study, our observations showed that IGF1 significantly decreased in the CC tissues and cells. In addition, IGF1 was negatively correlated with miR-186-3p expression in cancer tissues. Inhibition of miR-186-3p significantly increased IGF1 expression in CC. Furthermore, miR-186-3p reduction promoted CC cell proliferation and invasiveness and inhibited apoptosis. IGF1 knockdown displayed the opposite effects. Our observation

revealed for the first time that miR-186-3p acts as a carcinoma inhibitor in CC, at least in part, through the negative regulation of IGF1.

To further elucidate the possible molecular mechanisms, we explored the PI3K/Akt signaling pathway, which is activated by IGF1 and regulates fundamental cellular processes, including cell proliferation, differentiation, apoptosis, metastasis, and epithelial-mesenchymal transition [40]. A recent report found that the PI3K/Akt pathway was activated in endometriosis, and the expression of p-Akt and p-PI3K was upregulated [41]. In addition, other studies have shown the importance of the PI3K/Akt pathway in the progression of tumors, especially CC [42,43]. Recent studies have indicated that CC cells express high levels of PI3K, Akt, and phosphorylated Akt [44–46]. Moreover, treatment with PI3K/Akt pathway inhibitors prevented cell proliferation [47], induced apoptosis [48], and restored

metastasis [49] in CC. These results illustrated that the PI3K/Akt pathway may be a novel biomarker for predicting the prognosis of CC and a potential target for developing new treatment for such tumors [50]. This study found that inhibition of miR-186-3p significantly increased PI3K expression and phosphorylated AKT in CC cell lines. Moreover, this effect was reversed by the addition of IGF1 knockdown. Inhibition of miR-186-3p remarkably enhanced CC cell proliferation. LY294002 suppressed the PI3K/AKT signaling pathway and decreased the tumorigenic impact of the miR-186-3p repressor on CC cell proliferation. These findings strongly suggest that the negative function of miR-186-3p in the tumorigenesis of CC is mediated by the PI3K/Akt pathway. A possible explanation might be related to the fact that miR-186-3p targets and binds to IGF1 and inhibits the activation of specific receptor tyrosine kinases (RTKs), thereby blocking the PI3K/AKT pathway, leading to increased apoptosis and decreased tumor formation, as shown in the present study (Figure 5).

## Conclusions

In conclusion, our study suggests that miR-186-3p inhibits proliferation, promotes apoptosis of CC cells, and inactivates the PI3K/AKT signaling pathway by targeting IGF1. In addition, miR-186-3p serves as a potentially promising target for combating CC in the future. However, further *in vivo* studies are needed to confirm the functions of miR-186-3p in CC.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XS and XH performed the experiments and data analysis. XL and XZ conceived and designed the study. NY and HM made the acquisition of data. ZZ did the analysis and interpretation of data. XL wrote and edited the manuscript. All authors read and approved the manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Hebei North University (Hebei, China). All patients signed written informed consent.

## Disclosure statement

The authors declare that they have no conflict of interests.

## ORCID

Xiurong Lu  <http://orcid.org/0000-0001-8531-1219>

## References

- [1] Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin.* 2020;70(2020):7–30.
- [2] Jemal A, Ward EM, Johnson CJ, et al. Annual report to the nation on the status of cancer, 1975–2014, featuring survival. *J Natl Cancer Inst.* 2017;109(9). DOI:10.1093/jnci/djx030
- [3] Laengsri V, Kerdpin U, Plabplueng C, et al. Cervical cancer markers: epigenetics and microRNAs. *Lab Med.* 2018;49(2):97–111.
- [4] Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol.* 2014;15(8):509–524.
- [5] Croce CM, Calin GA. miRNAs, cancer, and stem cell division. *Cell.* 2005;122(1):6–7.
- [6] Wang Y, Wang L, Chen C, et al. New insights into the regulatory role of microRNA in tumor angiogenesis and clinical implications. *Mol Cancer.* 2018;17(1):22.
- [7] L. Ning M, Zhang Q, Zhu F, et al. miR-25-3p inhibition impairs tumorigenesis and invasion in gastric cancer cells *in vitro* and *in vivo*. *Bioengineered.* 2020;11(1):81–90.
- [8] Han B, Ge Y, Cui J, et al. Down-regulation of lncRNA DNAC3-AS1 inhibits colon cancer via regulating miR-214-3p/LIVIN axis. *Bioengineered.* 2020;11(1):524–535.
- [9] Liu C, Wang J, Hu Y, et al. Upregulation of kazrin F by miR-186 suppresses apoptosis but promotes epithelial-mesenchymal transition to contribute to malignancy in human cervical cancer cells. *Chin J Cancer Res.* 2017;29(1):45–56.
- [10] Liu Y, Guo R, Qiao Y, et al. LncRNA NNT-AS1 contributes to the cisplatin resistance of cervical cancer through NNT-AS1/miR-186/HMGB1 axis. *Cancer Cell Int.* 2020;20(1):190.
- [11] He M, Jin Q, Chen C, et al. The miR-186-3p/EREG axis orchestrates tamoxifen resistance and aerobic glycolysis in breast cancer cells. *Oncogene.* 2019;38(28):5551–5565.
- [12] Murray PG, Clayton PE. Endocrine control of growth, *American journal of medical genetics. Part C, Seminars*

- in medical genetics, 163c (2013) 76–85. doi: [10.1002/ajmg.c.31357](https://doi.org/10.1002/ajmg.c.31357)
- [13] Sharma M, Satyam A, Abhishek A, et al. Molecular and circulatory expression of insulin growth factors in Indian females with advanced cervical cancer. *Asian Pac J Cancer Prev.* 2012;13(12):6475–6479.
- [14] Lee SW, Lee SY, Lee SR, et al. Plasma levels of insulin-like growth factor-1 and insulin-like growth factor binding protein-3 in women with cervical neoplasia. *J Gynecol Oncol.* 2010;21(3):174–180.
- [15] Shen MR, Hsu YM, Hsu KF, et al. Insulin-like growth factor 1 is a potent stimulator of cervical cancer cell invasiveness and proliferation that is modulated by alphavbeta3 integrin signaling. *Carcinogenesis.* 2006;27(5):962–971.
- [16] Renehan AG, Zwahlen M, Minder C, et al. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet.* 2004;363(9418):1346–1353.
- [17] Wang R, Bao H, Zhang S, et al. miR-186-5p promotes apoptosis by targeting IGF-1 in SH-SY5Y OGD/R Model. *Int J Biol Sci.* 2018;14(13):1791–1799.
- [18] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>(-Delta Delta C(T))</sup> Method. *Methods.* 2001;25(4):402–408.
- [19] Zhuang Z, q Xiao H, Hu S, et al. Down-regulation of microRNA-155 attenuates retinal neovascularization via the PI3K/Akt pathway. *Mol Vis.* 2015;21:1173–1184.
- [20] Guo L, Fu J, Sun S, et al. MicroRNA-143-3p inhibits colorectal cancer metastases by targeting ITGA6 and ASAP3. *Cancer Sci.* 2019;110(2):805–816.
- [21] Yang ZC, Ma J. Actein enhances TRAIL effects on suppressing gastric cancer progression by activating p53/Caspase-3 signaling. *Biochem Biophys Res Commun.* 2018;497(4):1177–1183.
- [22] Geng X, Sun Y, Fu J, et al. MicroRNA-17-5p inhibits thyroid cancer progression by suppressing Early growth response 2 (EGR2). *Bioengineered.* 2021;12(1):2713–2722.
- [23] Lal A, Thomas MP, Altschuler G, et al. Capture of microRNA-bound mRNAs identifies the tumor suppressor miR-34a as a regulator of growth factor signaling. *PLoS Genet.* 2011;7(11):e1002363.
- [24] Song W, Zhang T, Yang N, et al. Inhibition of micro RNA miR-122-5p prevents lipopolysaccharide-induced myocardial injury by inhibiting oxidative stress, inflammation and apoptosis via targeting GIT1. *Bioengineered.* 2021;12(1):1902–1915.
- [25] Wang Y, Liu C, Xie Z, et al. Knockdown of TRIM47 inhibits breast cancer tumorigenesis and progression through the inactivation of PI3K/Akt pathway. *Chem Biol Interact.* 2020;317:108960.
- [26] Hu GF, Wang C, Hu GX, et al. AZD3463, an IGF-1R inhibitor, suppresses breast cancer metastasis to bone via modulation of the PI3K-Akt pathway. *Ann Transl Med.* 2020;8(6):336.
- [27] Zheng Y, Wu C, Yang J, et al. Insulin-like growth factor 1-induced enolase 2 deacetylation by HDAC3 promotes metastasis of pancreatic cancer. *Signal Transduct Target Ther.* 2020;5(1):53.
- [28] Cai J, Wu J, Zhang H, et al. miR-186 downregulation correlates with poor survival in lung adenocarcinoma, where it interferes with cell-cycle regulation. *Cancer Res.* 2013;73(2):756–766.
- [29] Chen F, Zhou C, Lu Y, et al. [Expression of hsa-miR-186 and its role in human colon carcinoma cells]. *Nan Fang Yi Ke Da Xue Xue Bao.* 2013;33:654–660.
- [30] Zhang TJ, Wang YX, Yang L, et al. Down-regulation of mir-186 correlates with poor survival in de novo acute myeloid leukemia. *Clin Lab.* 2016;62(1+02/2016):113–120.
- [31] He W, Feng J, Zhang Y, et al. microRNA-186 inhibits cell proliferation and induces apoptosis in human esophageal squamous cell carcinoma by targeting SKP2. *Lab Invest.* 2016;96(3):317–324.
- [32] Yao K, He L, Gan Y, et al. MiR-186 suppresses the growth and metastasis of bladder cancer by targeting NSBP1. *Diagn Pathol.* 2015;10(1):146.
- [33] Liu L, Wang Y, Bai R, et al. MiR-186 inhibited aerobic glycolysis in gastric cancer via HIF-1 $\alpha$  regulation. *Oncogenesis.* 2016;5(5):e224.
- [34] Ruan T, He X, Yu J, et al. MicroRNA-186 targets Yes-associated protein 1 to inhibit Hippo signaling and tumorigenesis in hepatocellular carcinoma. *Oncol Lett.* 2016;11(4):2941–2945.
- [35] Wang Z, Sha HH, Li HJ. Functions and mechanisms of miR-186 in human cancer. *Biomed Pharmacother.* 2019;119:109428.
- [36] Cui G, Cui M, Li Y, et al. MiR-186 targets ROCK1 to suppress the growth and metastasis of NSCLC cells. *Tumour Biol.* 2014;35(9):8933–8937.
- [37] Zhang JJ, Wang DD, Du CX, et al. Long noncoding rna anril promotes cervical cancer development by acting as a sponge of miR-186. *Oncol Res.* 2018;26(3):345–352.
- [38] Javed S, Bhattacharyya S, Bagga R, et al. Insulin growth factor-1 pathway in cervical carcinoma cancer stem cells. *Mol Cell Biochem.* 2020;473(1–2):51–62.
- [39] Han T, Zhou Y, Li D. Relationship between hepatocellular carcinoma and depression via online database analysis. *Bioengineered.* 2021;12(1):1689–1697.
- [40] Zhang L, Zhou F, ten Dijke P. Signaling interplay between transforming growth factor- $\beta$  receptor and PI3K/AKT pathways in cancer. *Trends Biochem Sci.* 2013;38(12):612–620.
- [41] Zhou X, Chen Z, Pei L, et al. MicroRNA miR-106a-5p targets forkhead box transcription factor FOXC1 to suppress the cell proliferation, migration, and invasion of ectopic endometrial stromal cells via the PI3K/Akt/mTOR signaling pathway. *Bioengineered.* 2021;12(1):2203–2213.

- [42] Janku F, Yap TA, Meric-Bernstam F. Targeting the PI3K pathway in cancer: are we making headway? *Nat Rev Clin Oncol.* 2018;15(5):273–291.
- [43] Yang J, Nie J, Ma X, et al. Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Mol Cancer.* 2019;18:26.
- [44] Wang L, Liu Y, Zhou Y, et al. Zoledronic acid inhibits the growth of cancer stem cell derived from cervical cancer cell by attenuating their stemness phenotype and inducing apoptosis and cell cycle arrest through the Erk1/2 and Akt pathways. *J Exp Clin Cancer Res.* 2019;38(1):93.
- [45] Liao S, Xiao S, Zhu G, et al. CD38 is highly expressed and affects the PI3K/Akt signaling pathway in cervical cancer. *Oncol Rep.* 2014;32(6):2703–2709.
- [46] Lee CM, Fuhrman CB, Planelles V, et al. Phosphatidylinositol 3-kinase inhibition by LY294002 radiosensitizes human cervical cancer cell lines. *Clin Cancer Res off J Am Assoc Cancer Res.* 2006;12(1):250–256.
- [47] Zhang XY, Zhang HY, Zhang PN, et al. Elevated phosphatidylinositol 3-kinase activation and its clinicopathological significance in cervical cancer. *Eur J Obstet Gynecol Reprod Biol.* 2008;139(2):237–244.
- [48] Li R, Song Y, Zhou L, et al. Downregulation of RAGE inhibits cell proliferation and induces apoptosis via regulation of PI3K/AKT pathway in cervical squamous cell carcinoma. *Onco Targets Ther.* 2020;13:2385–2397.
- [49] Zhang L, Zhou J, Qin X, et al. Astragaloside IV inhibits the invasion and metastasis of SiHa cervical cancer cells via the TGF- $\beta$ 1-mediated PI3K and MAPK pathways. *Oncol Rep.* 2019;41:2975–2986.
- [50] Bahrami A, Hasanzadeh M, Hassanian SM, et al. The potential value of the pi3k/akt/mtor signaling pathway for assessing prognosis in cervical cancer and as a target for therapy. *J Cell Biochem.* 2017;118(12):4163–4169.