KLF14 targets ITGB1 to inhibit the progression of cervical cancer via the PI3K/AKT signalling pathway

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Abstract

Background

Our study aimed to explore whether Krüppel-like factor 14 (KLF14) inhibits the proliferation and promotes the apoptosis of cervical cancer cells through integrin β1 (ITGB1).

Methods

Immunohistochemistry was used to explore the expression of KLF14 in cervical cancer tissues and adjacent samples.The effect of KLF14 on the proliferation of cervical cancer cells was verified by Cell Counting Kit-8 (CCK-8) assays, colony formation assays and an animal experiment (involving subcutaneous tumour formation in nude mice). The effect of KLF14 on cervical cancer cell apoptosis was detected by flow cytometry. To explore the underlying mechanisms, western blotting was conducted to detect the expression of ITGB1 in KLF14-overexpressing cervical cancer cells. Moreover, downstream related proteins were verified to further confirm that KLF14 targets ITGB1 to affect the apoptosis of cervical cancer cells. We conducted relevant rescue experiments, flow cytometry was used to verify the effect of overexpression of ITGB1 and simultaneous overexpression of ITGB1 and KLF14 on apoptosis of cervical cancer cells, and western blot analysis was used to investigate the expression of downstream related proteins after overexpression of ITGB1 and simultaneous overexpression of ITGB1 and KLF14.

Results

The expression of KLF14 in cervical cancer tissues was lower than that in paracancerous tissues. KLF14 inhibited the proliferation and promoted the apoptosis of cervical cancer cells. Mechanistically, ITGB1 expression was significantly downregulated in KLF14-overexpressing cervical cancer cells. KLF14 targets ITGB1 to regulate its downstream PI3K/AKT signalling pathway. The upregulation of ITGB1 can inhibit the apoptosis of cervical cancer cells by affecting the downstream PI3K/AKT signalling pathway, and the upregulation of KLF14 can reverse the effect of ITGB1 upregulation on cervical cancer cell apoptosis to a certain extent.

Conclusions

KLF14 inhibits the progression of cervical cancer by targeting ITGB1 via the PI3K/AKT signalling pathway.

Background

The Global Cancer Observatory reported that cervical cancer was the fourth most common cancer in women worldwide in 2020[1]. The incidence and mortality of cervical cancer are especially high in transitioning countries[2]. With the improvement of treatment methods and the advent of the human papillomavirus (HPV) vaccine, the treatment and prevention of cervical cancer have improved greatly[3]. However, due to the invasion, metastasis and recurrence of tumours, cervical cancer still has a poor prognosis, posing a serious threat to women's health.

Krüppel-like factor 14 (KLF14), a member of the SP/KLF14 gene family, is a transcription factor involved in the regulation of many physiological and pathological processes[4-5]. Most recent studies have focused on the role of KLF14 in cell metabolism[6]. Some studies have shown that KLF14 is closely related to type 2 diabetes and can improve insulin sensitivity and insulin resistance[7,8]. In addition, KLF14 is associated with a number of cardiovascular diseases and prevents and treats atherosclerosis by increasing cholesterol excretion and reducing inflammation[9]. Recently, KLF14 was also found to be associated with the occurrence and progression of tumours. By reducing LDHB, glycolysis can be regulated to cut off the supply of cancer cells, thus inhibiting the progression of colon cancer[10]. KLF14 is regulated as a target gene of lncRNA DGCR5 to inhibit the progression of hepatocellular carcinoma (HCC)[11]. At present, there are relatively few studies on the role of KLF14 in cervical cancer. Integrins are heterodimeric cell surface receptors and play an important role in cell differentiation, migration, proliferation, adhesion and tumour progression[12,13]. With deepening research on the integrin signalling pathway and related molecules[14], integrins have become a hot target in antitumour therapy. Integrin β1 (ITGB1), a member of the integrin (ITG) family, regulates tumour invasion, migration, and apoptosis[15,16]. On this basis, we considered whether KLF14 might be associated with ITGB1, thereby affecting the development of cervical cancer.

In this study, we investigated whether KLF14 can inhibit the proliferation and promote the apoptosis of cervical cancer cells. Moreover, we further explored whether KLF14 regulates ITGB1 and plays an inhibitory role in the progression of cervical cancer by regulating the PI3K/AKT signalling pathway.

Materials And Methods

1. Cell culture

Human cervical cancer cells, including SiHa and HeLa cells, HeLa cells were purchased from the American Type Culture Collection (ATCC, USA), SiHa cells were purchased from the Chinese Tissue Culture Collections (CTCC,China). These cells were cultured in DMEM (Gibco,USA) containing 10% foetal bovine serum (FBS, Gibco,USA) and 1% penicillin-streptomycin and incubated in a constant-temperature incubator at 37°C with 5% CO_2.

2. Cell transfection

Construction of KLF14 overexpression lentivirus stably transfected cell lines: KLF14 overexpression lentivirus and its negative control lentivirus were constructed by GeneChem (Shanghai, China). KLF14 overexpression lentivirus and its negative control lentivirus were transfected into SiHa or HeLa cells. The cells were screened by treatment with 2.5 μg/mL puromycin, and the expression level of KLF14 was verified by western blotting. In subsequent experiments, cells were precultured with doxorubicin (DOX)-containing medium for 48 h to induce KLF14 expression.

Construction of ITGB1 overexpression lentivirus stably transfected cell lines: ITGB1 overexpression lentivirus and its negative control lentivirus were constructed by GeneChem (Shanghai, China). The ITGB1 overexpression lentivirus and its negative control lentivirus were transfected into SiHa cells. The cells were screened by treatment with 2.5 μg/mL puromycin, and the expression level of ITGB1 was verified by western blotting.

Cotransfection of cells with lentiviruses targeting ITGB1 and KLF14: The KLF14 overexpression lentivirus and its negative control lentivirus were transfected into SiHa cells transfected with Lv-ITGB1. The expression levels of ITGB1 and KLF14 were detected by western blotting after puromycin screening.

3.Immunohistochemistry

A human cervical cancer tissue microarray (OD-CT-RpUtr03-004) was purchased from Shanghai Xinchao Biotechnology Co., Ltd. The tissue chip contained 31 cervical cancer tissues and their paired adjacent tissues, along with clinical data including age, sex, tumour-node-metastasis (TNM) stage and clinical stage for the corresponding patients. The tissue chip was baked, dewaxed and hydrated with ethanol, followed by antigen repair and endogenous peroxidase blocking. The tissue chip was covered with a 1:500 dilution of KLF14 primary antibody(1:500,Sigma-Aldrich,#HPA044729) and incubated overnight at 4°C. The next day, the chip was incubated with secondary antibody for 30 minutes at room temperature. The sections were stained with DAB and haematoxylin, dehydrated, cleared and sealed. Finally, the images were observed under a microscope, photographed and graded.

4. Cell Counting Kit-8 (CCK-8) assay

Cells of the Lv-control group and Lv-KLF14 group were seeded in 96-well plates at a density of 5000 cells per well, and the blank group with only added culture medium was set. Each condition was plated in 6 replicate wells. After approximately 4-6 h, when the cells were completely attached to the well, the absorbance value of each well at 450 nm was detected as the cell state at 0 h. Then, all the medium in the well was removed, the medium was replaced with DOX-containing medium, and the cells were placed into an incubator for culture. After 24 h or 48 h, CCK-8 reagent (#CK04,Dojindo) was added to each well in a dark environment, and the cells cultured immediately in the incubator for 2 h. The absorbance value at 450 nm was assessed to determine the cell state at 24 h or 48 h.

5. Colony formation assay

Cells of the Lv-control group and Lv-KLF14 group were evenly seeded on petri dishes at a density of 2000 cells per dish and cultured in medium containing DOX for approximately 14 days. When obvious colonies appeared, the medium in the dish was discarded, and the cells were washed with PBS, fixed with methanol for 15-20 minutes, and then stained with 1% crystal violet for 20 minutes. After washing with PBS, the cells were photographed and counted.

6. Flow cytometry

Cells of the Lv-control group and Lv-KLF14 group were cultured in six-well plates. The ITGB1-overexpressing cells were cultured in standard medium, while KLF14-overexpressing cells were cultured in medium containing DOX. After overnight incubation in the incubator, the original medium was discarded, and the cells were digested with trypsin after PBS rinsing and collected into EP tubes. Centrifugation was conducted at 1500 RPM for 5 minutes, and the cell precipitate was retained. Then, the instructions of the APC Annexin Ⅴ Apoptosis Detection Kit with PI (#640932,BioLegend,USA)were followed; the steps included cell resuspension, double staining and constant temperature incubation. Finally, the apoptosis rate was detected by flow cytometry. The experiment was repeated three times.

7. Establishment of a subcutaneous tumorigenesis model in nude mice

BALB/C female nude mice aged 4-5 weeks were purchased from Vital River Lab Animal Technology Co., Ltd. SiHa cells (5×10^6 cells/group) of the Lv-KLF14 group were injected subcutaneously into the axilla of the right upper limb of nude mice with 200 μl PBS suspension as the positive group. SiHa cells (5×10^6 cells/group) of the Lv-control group were also injected subcutaneously into the left upper arm axilla of nude mice with 200 μl PBS suspension as the negative group. Six groups were set up. After the injection, the nude mice were fed water with DOX for approximately one week. Approximately two months later, the nude mice were sacrificed, and the tumours that had formed under the skin were removed, photographed, weighed and frozen at -80°C. This experiment was approved by the Ethics Committee of the First Affiliated Hospital of Shandong First Medical University.

8. Western blotting

RIPA lysis buffer was used to extract cell protein samples, separated the cell proteins using 10% SDS-PAGE, and then transferred to PVDF membranes. After transfer, the cells were blocked with 5% skim milk powder for 1 h. The primary antibodies were as follows: FLAG (1:1000, #14793S, CST), ITGB1 (1:1000, #4706, CST), AKT (1:400, #4691, CST), p-AKT (1:400, #4060, CST), PI3K (1:250, #4249, CST), Bax (1:400, #89744, CST), PDK1 (1:400, #5662, CST). The secondary antibody was horseradish peroxidase-labelled goat anti-rabbit antibody or anti-mouse antibody. The reference antibody was beta-actin (1:1000, #AF7018, Affinity). Finally, the protein expression level was observed on a chemiluminescence imager with an enhanced chemiluminescence (ECL) kit (Millipore,USA). The greyscale values of the protein bands were analysed by ImageJ software.

9. Statistical analysis

Data analysis was performed with GraphPad Prism 8.0. The differences between groups were analysed by Student's t-test, one-way ANOVA and multiple t-test. The data were considered statistically significant when the P value was less than 0.05.

Results

1.KLF14 is expressed at low levels in cervical cancer tissues

To assess the expression level of KLF14 in cervical cancer tissues, we performed immunohistochemistry analysis of tissue microarray containing 31 cervical cancer tissues and their adjacent tissues.We scored each tissue point (total score = staining intensity * positive rate).KLF14 expression level in cervical cancer tissues was lower than that in adjacent tissues in 22 cases, higher than that in adjacent tissues in 7 cases. There were 2 cases of delamination.Therefore, the KLF14 expression level in cervical cancer tissues was lower than that in adjacent tissues (Figure 1A, B).

2. KLF14 inhibited the proliferation of cervical cancer cells in vitro

For the following experiments, SiHa and HeLa cells were selected to be transfected with Lv-control and Lv-KLF14. Western blotting was used to verify KLF14 transfection efficiency. In both SiHa and HeLa cells, the expression of KLF14 in the Lv-KLF14 group was significantly higher than that in the Lv-control group (Figure 2A). In the colony formation assay, SiHa and HeLa Lv-KLF14 cells showed lower colony formation rates than Lv-control cells during the same period of time (Figure 2B). In the CCK-8 assay, the absorbance value at 450 nm of SiHa and HeLa cells in the Lv-KLF14 group were lower than those in the Lv-control group after 24 h. Therefore, the proliferation rates of SiHa and HeLa cells in the Lv-KLF14 group were lower than those in the Lv-control group during the same period of time (Figure 2C). In general, we believe that KLF14 has an inhibitory effect on the proliferation of cervical cancer cells.

3. KLF14 inhibited the progression of cervical cancer in vivo

SiHa cells from the Lv-KLF14 group and Lv-control group were suspended in 200 μl PBS and injected subcutaneously to establish a subcutaneous tumorigenesis model in nude mice. The results showed that tumours in the Lv-KLF14 group were smaller than those in the Lv-control group (Figure 3A). The tumour weight in the Lv-KLF14 group was significantly lower than that in the Lv-control group (Figure 3B).

4. KLF14 promotes the apoptosis of cervical cancer cells

To verify the effect of KLF14 on the apoptosis of cervical cancer cells, flow cytometry was performed on cervical cancer cells from the Lv-control group and Lv-KLF14 group, including SiHa cells and HeLa cells. The results showed that the apoptosis rate of both SiHa cells and HeLa cells in the Lv-KLF14 group was higher than that in the Lv-control group (Figure 4A-C), suggesting that KLF14 can promote the apoptosis of cervical cancer cells.

5. KLF14 regulates ITGB1 and affects the apoptosis of cervical cancer cells

ITGB1 is a member of the ITG family that is closely related to apoptosis. Therefore, we considered that KLF14 might associate with ITGB1 to promote the apoptosis of cervical cancer cells, thereby inhibiting the development of cervical cancer. Western blotting was used to verify the expression level of ITGB1 in the Lv-control group and Lv-KLF14 group, and the results showed that the expression level of ITGB1 in the Lv-KLF14 group was lower than that in the Lv-control group (Figure 5A). In addition, four groups were established: Lv-control (ITGB1), Lv-ITGB1, Lv-control (KLF14)+ Lv-ITGB1, and Lv-ITGB1 + Lv-KLF14. Flow cytometry confirmed that when only ITGB1 was overexpressed (Lv-ITGB1), the apoptosis rate of cervical cancer SiHa cells was lower than that of the Lv-control (ITGB1) group, indicating that ITGB1 inhibits the apoptosis of cervical cancer cells. However, when ITGB1 and KLF14 constructs were cotransfected, the above effect was reversed, and the apoptosis rate of cervical cancer cells overexpressing both ITGB1 and KLF14 (Lv-ITGB1 + Lv-KLF14) was higher than that of the Lv-control (KLF14)+ Lv-ITGB1 group (Figure 5B), indicating that KLF14 may regulate ITGB1 and that KLF14 can promote apoptosis and reverse the effect of ITGB1 on apoptosis of cervical cancer cells.

6. KLF14 targets ITGB1 and inhibits the development of cervical cancer through the PI3K/AKT signalling pathway

To understand the underlying mechanism, we carried out further exploration. Western blot results showed that PI3K, PDK1, AKT and p-AKT were downregulated when KLF14 was overexpressed in both SiHa and HeLa cells. Compared with the Lv-control group, the expression of Bax in SIHA cells was upregulated in the Lv-KLF14 group, while the expression of Bax in HeLa cells showed no significant difference between the Lv-control group and the Lv-KLF14 group. (Figure 6A). SiHa cells were used for the rescue experiments, and four groups were established: Lv-control (ITGB1), Lv-ITGB1, Lv-control (KLF14)+ Lv-ITGB1, and Lv-ITGB1 + Lv-KLF14. The western blot results showed that when only ITGB1 was overexpressed, the expression of PDK1, AKT and p-AKT was upregulated compared to that in the Lv-control (ITGB1) group, while Bax was downregulated. However, when Lv-KLF14 and Lv-ITGB1 constructs were cotransfected, the above phenomenon was reversed. Compared to that in the Lv-control (KLF14)+Lv-ITGB1 group, the expression of PDK1, AKT and p-AKT in the cotransfection group was downregulated, while Bax was upregulated (Figure 6B). Therefore, we believe that KLF14 and ITGB1 may be correlated and affect the development of cervical cancer through the PI3K/AKT signalling pathway.

Discussion

Cervical cancer is one of the most common malignancies in women worldwide[17]. Various factors lead to the occurrence of cervical cancer. Although the development of HPV vaccines has prevented cervical cancer to some extent, the incidence and mortality rates of cervical cancer are still high[18]. We aimed to find more effective biomarkers for the diagnosis and treatment of cervical cancer. KLF14 plays an important role in a variety of physiological and pathological processes. In recent years, the number of studies exploring the relationship between KLF14 and cancer has increased. Zhou J et al. found that lncRNA HAND2-AS1 inhibits the progression of colon cancer by regulating the expression of KLF14[19]. Luo et al. demonstrated that KLF14 can regulate the antioxidant

response by regulating the HO-1 pathway in castrate-resistant prostate cancer, thus providing a treatment target for castrate-resistant prostate cancer patients[20]. In addition, KLF14 has been reported to be associated with HCC and breast cancer[11,21]. In this study, we set out to explore whether KLF14 is also associated with cervical cancer. We verified that the proliferation of KLF14-overexpressing cervical cancer cells was inhibited according to CCK-8 and colony formation assays. The establishment of a subcutaneous neoplasia model in nude mice demonstrated that KLF14 inhibited the progression of cervical cancer in vivo. Immunohistochemical assays confirmed that the expression of KLF14 in cervical cancer tissues was lower than that in paracancerous tissues. Flow cytometry also showed that KLF14-overexpressing cervical cancer cells had a higher rate of apoptosis than negative control cells. These results suggested that KLF14 plays an inhibitory role in the progression of cervical cancer and may be helpful in the treatment of cervical cancer in the future.

The relationship between ITGB1, a member of the ITG family, and tumours has been increasingly studied in recent years. Min et al. found that ITGB1 overexpression can regulate the Notch signalling pathway and thus significantly promote the proliferation of glioma cells[22]. Liang et al. confirmed that ITGB1 is a target of miR-493-5p and that patients with high expression of ITGB1 and low expression of miR-493-5p in NSCLC have a shorter survival period and poorer prognosis than those with other expression patterns[23]. Guo et al. showed that THBS4 can interact with ITGB1 and activate the downstream PI3K/AKT pathway, thereby promoting the proliferation and metastasis of HCC[24]. In this study, western blotting was used to verify that when KLF14 was overexpressed, ITGB1 expression was downregulated in SiHa and HeLa cervical cancer cells and affected molecules in the PI3K/AKT pathway, including PI3K, PDK1, AKT, p-AKT and Bax. Subsequently, we used western blotting to verify that PDK1, AKT and p-AKT were upregulated and Bax was downregulated when ITGB1 alone was overexpressed. This effect was reversed when ITGB1 and KLF14 were simultaneously overexpressed. Flow cytometry was used to confirm that the apoptosis rate of SiHa cells decreased when ITGB1 was overexpressed, while the above effects could be reversed when KLF14 and ITGB1 were overexpressed simultaneously, and the apoptosis rate of SiHa cells increased to a certain extent. The above results indicated that KLF14 promotes the apoptosis of cervical cancer cells by downregulating ITGB1 expression via the PI3K/AKT signalling pathway.

Inevitably, there are many shortcomings related to our research. First, the KLF14 expression levels of cervical cancer cells were not compared with those of normal cervical cells due to the difficulty in obtaining normal cervical cells. Second, the number of cases used for immunohistochemistry analysis was limited, and assessment of more cases would provide stronger evidence. Third, the rescue experiments only used SiHa cervical cancer cells for verification, which may not be comprehensive because different cells have different characteristics and may show different experimental phenomena. In addition, we demonstrated that KLF14 regulates ITGB1 through the PI3K/AKT pathway, but the deeper connection between the two is unclear. These limitations need to be addressed in the future.

Conclusion

In conclusion, this study confirmed that KLF14 inhibits the proliferation and promotes the apoptosis of cervical cancer cells. It was also found that ITGB1 may play an important role in this process, as KLF14 inhibits the progression of cervical cancer by targeting ITGB1 through the PI3K/AKT signalling pathway.

Abbreviations

Krüppel-like factor 14 : KLF14

integrin β1 : ITGB1

Immunohistochemistry: IHC

tumour-node-metastasis :TNM

Cell Counting Kit-8 : CCK-8

human papillomavirus : HPV

hepatocellular carcinoma : HCC

integrin : ITG

Doxorubicin : DOX

phospho-AKT: p-AKT

phosphoinositide-3 kinase : PI3K

Declarations

Availability of data and materials

All data generated or analyzed in this study are available in the article.

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Authors' contributions

XL and XD designed the experiment. XL wrote the manuscript and analysed the data. XL, XD, HY, RG, and MW performed the experiments. LC provided funding and revised the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

The animal experiment in this study was approved by the Ethics Committee of Qianfoshan Hospital in Shandong Province（SYDWLS2020016）. Human cervical tissue samples were obtained from Shanghai Xinchao biological sample bank. The collection of cases in the human cervical tissue chip (OD-CT-RpUtr03-004) was approved by the Ethics Committee of Shanghai Xinchao Biotechnology Co., Ltd.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

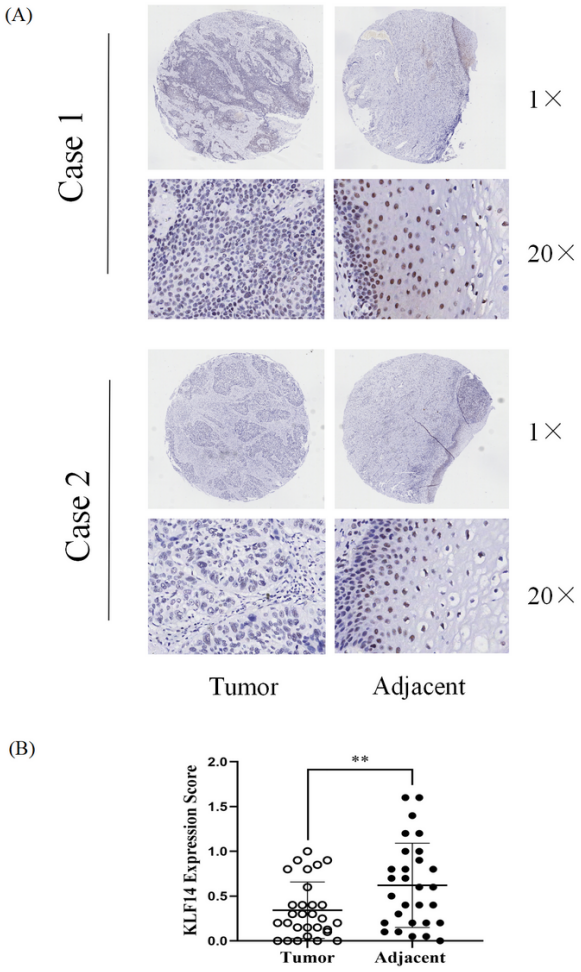

Figure 1

KLF14 is expressed at low levels in cervical cancer tissues. A.The immunohistochemical results showed that the expression level of KLF14 in cervical cancer tissues was lower than that in adjacent tissues. B.We scored each tissue point (total score = staining intensity * positive rate). Quantitative statistics of immunohistochemical results (**P < 0.01).

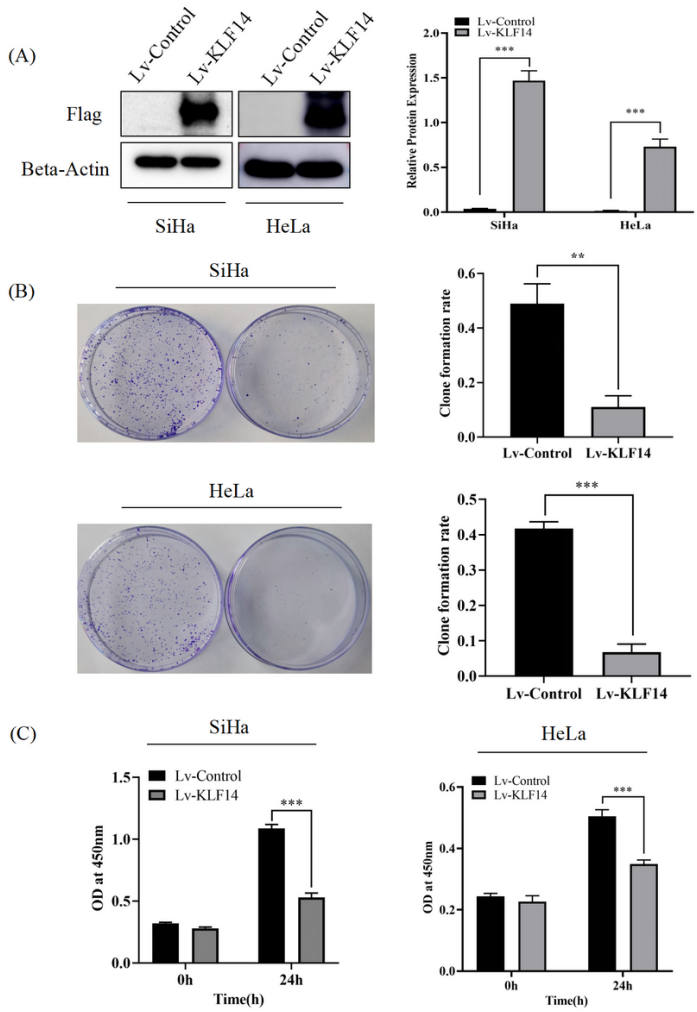

Figure 2

KLF14 inhibited the proliferation of cervical cancer cells in vitro. A. After transfection with Lv-KLF14, the western blot results showed that the expression of KLF14 was significantly increased in SiHa and HeLa cells. B. The colony formation assay verified that the colony formation rate of the Lv-KLF14 group was lower than that of the Lv-control group. C. The CCK-8 assay demonstrated that the proliferation of the Lv-KLF14 group was lower than that of the Lv-control group within 24 h. These results suggest that KLF14 has an inhibitory effect on the proliferation of cervical cancer SiHa cells and HeLa cells (**P < 0.01, ***P < 0.001).

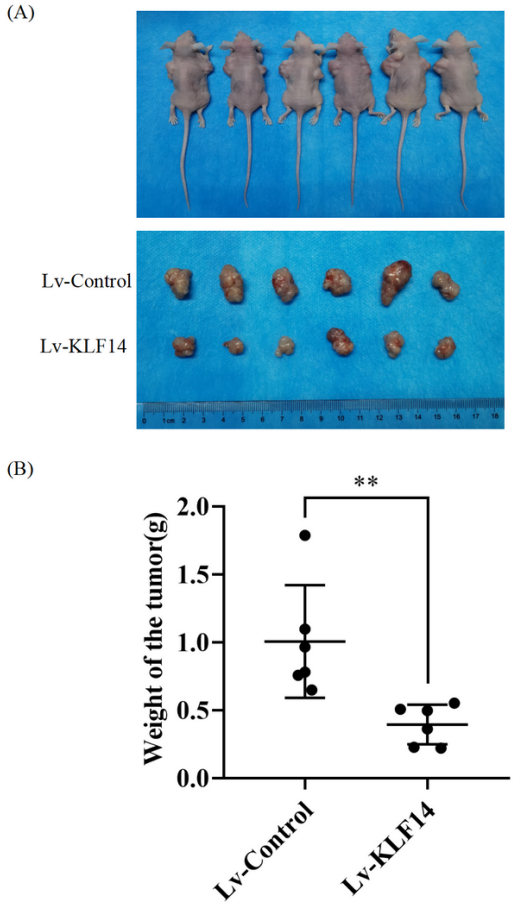

Figure 3

KLF14 inhibited the progression of cervical cancer in vivo. A. SiHa cells (5×106 cells/group) of the Lv-KLF14 group were injected subcutaneously into the axilla of the right upper limb of nude mice with 200 μl PBS suspension. The Lv-control group were injected subcutaneously into the left upper arm axilla of nude mice with 200 μl PBS suspension.The volume of subcutaneous tumours in the Lv-KLF14 group was smaller than that in the Lv-control group. B. The weight of subcutaneous tumours in the Lv-KLF14 group was lower than that in the Lv-control group (**P < 0.01).

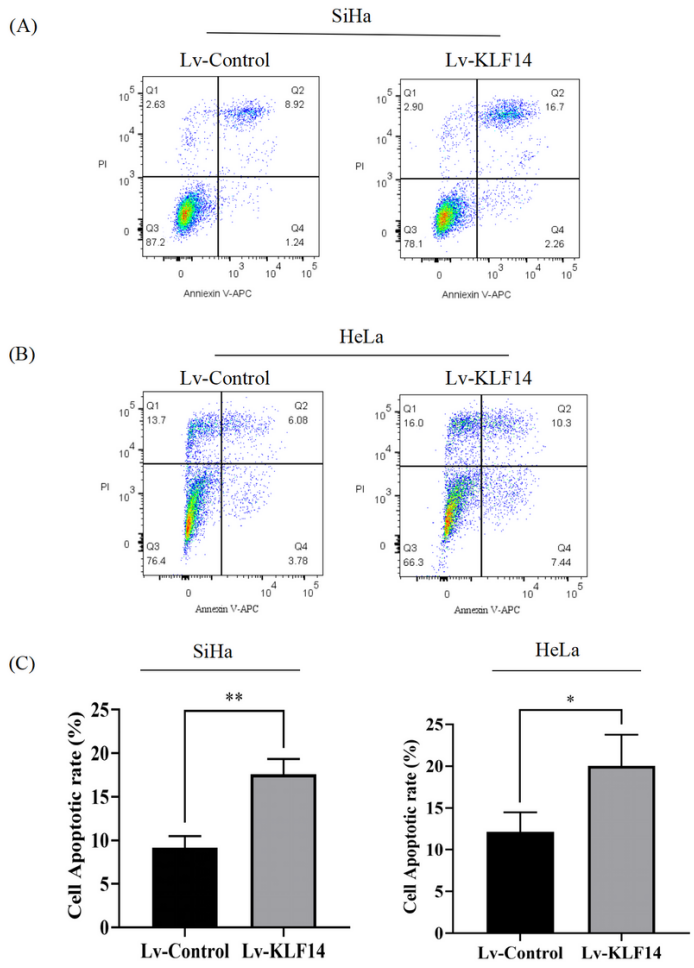

Figure 4

KLF14 promotes the apoptosis of cervical cancer cells. A. Flow cytometry was used to detect apoptosis, and the results showed that the apoptosis rate of SiHa cells in the Lv-KLF14 group was higher than that of those in the Lv-control group. B. The apoptosis rate of HeLa cells in the Lv-KLF14 group was higher than that of those in the Lv-control group. C. Quantitative statistics of the apoptosis rate of SiHa cells and HeLa cells (*P < 0.05, **P < 0.01).

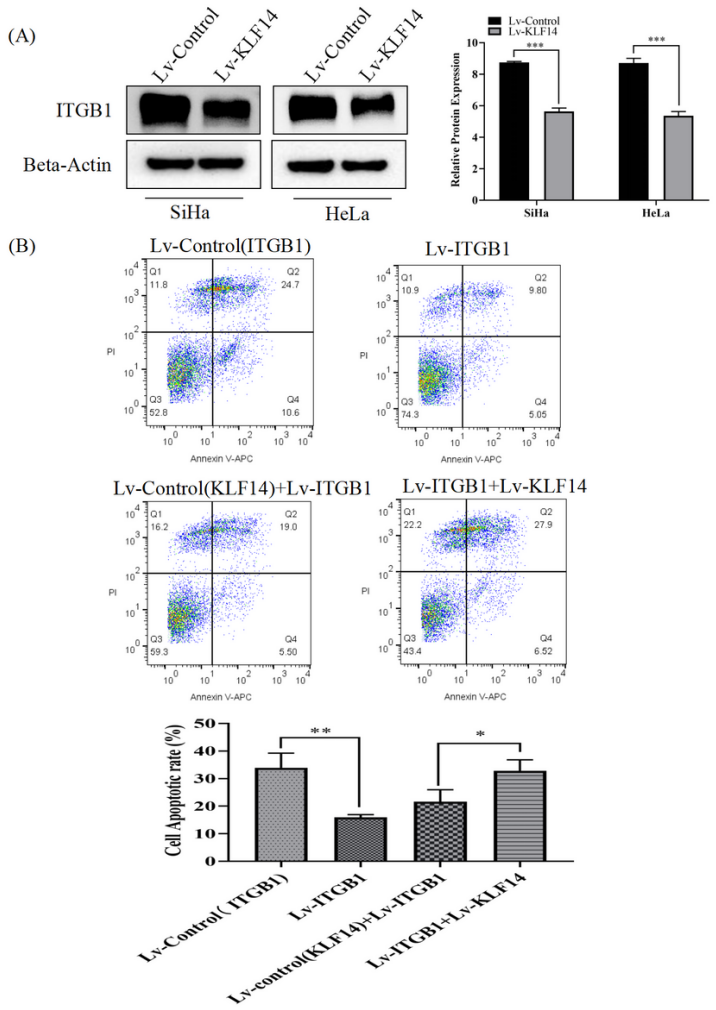

Figure 5

KLF14 regulates ITGB1 and affects the apoptosis of cervical cancer cells. A. The western blot results showed that the ITGB1 expression level in the Lv-KLF14 group was lower than that in the Lv-control group. B. Four groups were set up: Lv-control (ITGB1), Lv-ITGB1, Lv-control (KLF14)+ Lv-ITGB1, and Lv-ITGB1 + Lv-KLF14. Flow cytometry confirmed that the apoptosis rate of SiHa cervical cancer cells in the Lv-ITGB1 group was lower than that of those in the Lv-control (ITGB1) group. The apoptosis rate of SiHa cells in the Lv-ITGB1 + Lv-KLF14 group was higher than that of those in the Lv-control (KLF14) + Lv-ITGB1 group (*P < 0.05, **P < 0.01,***P < 0.001).

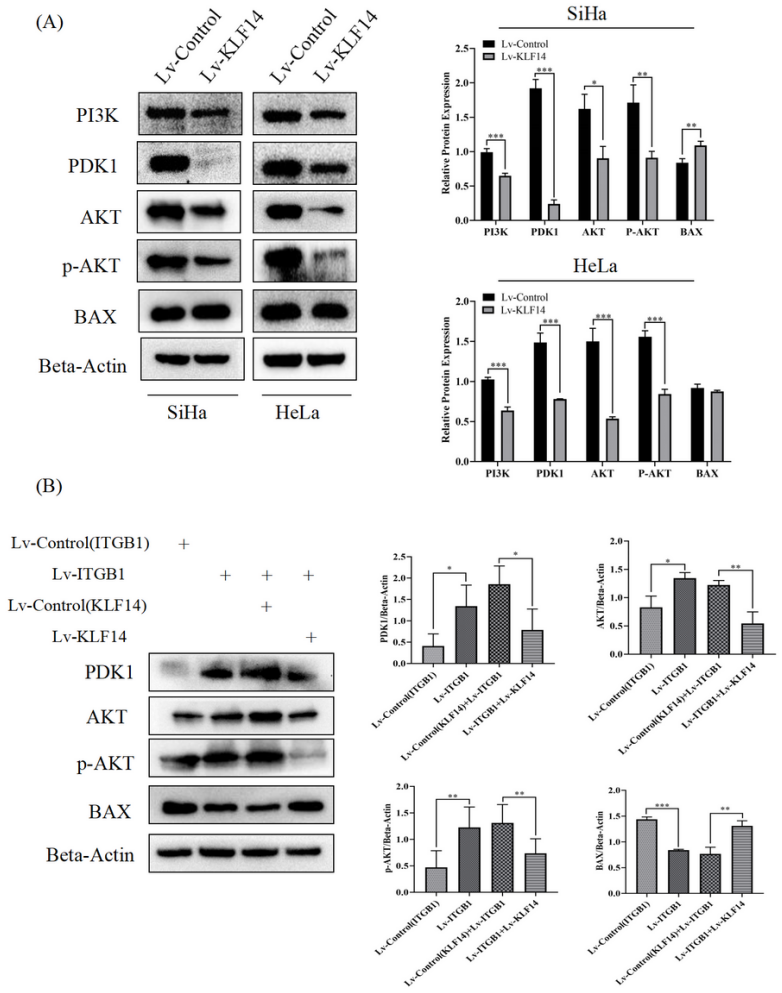

Figure 6

KLF14 targets ITGB1 and inhibits the development of cervical cancer through the PI3K/AKT signalling pathway. A. When KLF14 was overexpressed, the western blot results showed that PI3K, PDK1, AKT and p-AKT were downregulated and Bax was upregulated in SiHa cells compared to the Lv-control group cells. PI3K, PDK1, AKT and p-AKT were downregulated, and Bax showed no significant difference in HeLa cells compared to the Lv-control group cells. B. Four groups were set up: Lv-control(ITGB1), Lv-ITGB1, Lv-control(KLF14)+Lv-ITGB1, and Lv-ITGB1+Lv-KLF14. When ITGB1 was overexpressed, compared to Lv-control (ITGB1), the western blot results showed that the expression of PDK1, AKT and p-AKT was upregulated, while Bax was downregulated. However, when Lv-KLF14 and Lv-ITGB1 were cotransfected, the expression of PDK1, AKT and p-AKT was downregulated compared to that in the Lv-control (KLF14)+Lv-ITGB1 group, while Bax was upregulated (*P < 0.05, **P < 0.01, ***P < 0.001).