# miRNA-221 knockdown suppress cell proliferation and cell cycle in cervical cancer

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**Abstract:** miRNA-221 was a carcinogenic factor in many cancers, however, it is limited that the correlation between miRNA-221 and progression of cervical cancer. So we here aimed to determine the function of miRNA-221 in cervical cancer proliferation and apoptosis. Interestingly, we found that the miRNA-221 was significantly up-regulation in cervical cancer tissues. miRNA-221 knockdown inhibited the cell proliferation and improved the cell apoptosis of cervical cancer cells. Further, miRNA-221 down-regulation suppressed the activation of PI3K and AKT, and stimulated PTEN and P16 proteins expression. Together, our results suggested that miRNA-221 knockdown had suppressed the cell proliferation and stimulated cell apoptosis via PTEN/PI3K/AKT/P16 signaling pathway.

**Key words:** miRNA-221, cervical cancer, PTEN, P16

## Introduction

Cervical cancer is one of the most common gynecologic malignancies, with approximately 500 thousand new cases worldwide and about 300 thousand deaths each year. In recent years, related reports had shown that the incidence of cervical cancer had been younger. Cervical cancer is known to be associated with high-risk HPV infection. However, studies have found that single high-risk HPV infection is not enough to cause cervical cancer, genetic factors and immune factors also play an important role in the development of cervical cancer (1-3). The study of the mechanism of cervical cancer development and diagnosis and treatment of cervical cancer is the focus of medical research.

The discovery of miRNAs offers hope for treatment of cervical cancer patients. The relative studies found that the abnormal expression of miRNAs is closely related to the occurrence and development of most tumors (4-6). MiR-221 is highly expressed in many tumors such as melanoma, breast cancer and prostate cancer, suggesting that miR-221 is a tumor related factor (7-9). Up-regulation of miR-221 interfered with growth factor signaling pathway and promotes cell proliferation (10, 11). However, The reports which the correlation between miR-221 and cervical cancer has been limited. In our present study, we firstly evaluated the miR-221 expression in cervical cancer and adjacent normal tissues, secondly, we studied the effects and mechanism of miR-221 inhibition in cell proliferation and apoptosis of cervical cancer cells in vitro study.

## Material and Methods

### Clinical data

The 20 pairs of cervical cancer and adjacent normal tissues which were from cervical cancer patients who were treated in our hospital from 2014 to 2016 The tissues were divided into 2 parts, one part were store at -80 ℃ until used and another part were fixed by faure Marin solution and paraffin imbedding.

**H&E staining**

The tissues were fixed by 4% formaldehyde; Routine dehydration, embedded section (4 μm), Hematoxylin and eosin were used to stain the cervical tissue specimens. The relative kits were purchased from Sigma. The detailed operation procedures shall be carried out in strict accordance with the reagent instructions.

**In Situ hybridization (ISH)**

The tissues were fixed by 10% formaldehyde, Routine dehydration, embedded section (4 μm), the slices were fixed on a slide packed with poly lysine and baked overnight at 65 ℃; Xylene dewaxing 20 min, 37 ℃ dry 5 min, add pepsin work fluid, digest 10 min at 37 ℃, step by step alcohol dehydration and air drying. Dripping miR-221 probe, adding cover glass, In situ hybridization PCR apparatus, the procedure is 95 ℃, denatured 5 min, and crossing at 37 ℃ for 2 h. The sections were took out, and cleaned by TBS buffer solution and took the cover glass out, and added 5~6 drops PanWash and 2~3 drops enzyme conjugate on every sections. After cultured at 37 ℃ for 30 min, washing by TBS buffer solution for 3 min, coloring by NBT/BCIP for 20~30 min, The dark incubation, discarding the staining solution, ion water rinse each section by adding 2 ~ 3 drops counterstain, neutral balata, microscopy, light microscopy and photographed.

**Cell culture and grouping**

The Hela and Siha cells were purchased from ATCC (USA). The Hela and Siha cells were cultured in DMEM medium (contained with 10% FBS, 1% Penicillin and 1% streptomycin), and stayed in 37℃ and 5% $CO_2$ incubator. The cells were respectively divided into 3 groups: NC groups (treated with normal treatment), BL groups (transfected with empty vector) and miR-221 inhibitor (transfected with miR-221 inhibitor).

**miR-221 inhibitor transfection**

The good growth of Hela and Siha cell count after digestion, the cell density of 2 × $10^5$ / hole inoculated into 6 well plates, cultured box is arranged on the 37 C and 5% $CO_2$ and saturated humidity, when the cells reached 80% confluence, the cells

transfected with Lipofectamine 2000 miR-221 inhibitor according to the instructions. The transfected cells were cultured for 4 h, then the whole medium was changed. The transfected negative control nucleotide fragment was negative control (BL), and cultured in incubator.

**MTT assay**

The transfected cells were digested and counted, the cells were inoculated in 96 well plates as $2.5\times10^4$/ hole, after cultured for 72 h to MTT assay, meanwhile, blank control group was set up. 20 μl MTT (5mg/ml) were added in every holes, continuing culturing for 4 h at 37 ℃, Removing the culture medium and add 150 μL DMSO to each pore, Placed on shaking table, shocking at room temperature for 5 min, The D value at 492 nm was measured with an enzyme marker. Cell proliferation rate was calculated.

**Cell apoptosis by flow cytometry**

The cells of difference groups were collected, washing by PBS, The cells were collected by trypsin digestion without EDTA, and the suspension was made by adding PBS. According to Annexin-V kit instructions, binding buffer before adding 500 μL cell suspension, then add 5 μL FITC labeled Annexin-V and 5 μL PI mix, dark incubation at room temperature for 15 min, cell apoptosis was analyzed by flow cytometry.

**Cell cycle by flow cytometry**

The cells of difference groups were cultured for 24 h, after that, the cells were collected, centrifugal for 5 min as 1000 r/min, rejection the supernatant, washing by PBS at 2 times, centrifugal for 5 min as 1200 r/min, rejection the supernatant, adding the 70 % ethanol (4 ℃) to fix, color by Propidium iodide (PI) for 30 min, The distribution of cell cycle was analyzed by flow cytometry and MUTCYCLE software.

**The relative proteins expressions by WB assay**

The total proteins were extracted from the cells of difference groups, and the concentration was measured by BCA methods. 25 μg protein samples were added into every hole, and then, Electrophoretic separation of proteins. After electrophoresis, the

protein was transferred to poly (vinylidene fluoride) (PVDF) membrane by semi dry transfer method. The skimmed milk powder was closed at room temperature for 1 h, then, The primary antibody was incubated with the membrane at 4 ℃overnight (1: 1000), and second anti-body (1:10000) was incultured at room temperature for 2~3 h, chemiluminescence imaging, and analysis of band gray values. The GAPDH was a reference in this experiment.

**Statistical analysis**

All data are expressed as the means± SD of at least three independent experiments. Data were analyzed using independent-samples t tests between two groups and one-way analysis of variance (ANOVA) for more groups with Dunnett's test (dose 0 as the control group). A value of $P<0.05$ was considered significant.

## Results

**Clinical data and analysis**

Compared with adjacent normal tissues, the cancer tissues were significantly invasion and migration (Figure 1A). Meanwhile, the miR-221 expression of cancer tissues were significantly up-regulation compared with that of adjacent normal tissues by ISH assay (P＜0.05, Figure 1B).

Figure 1

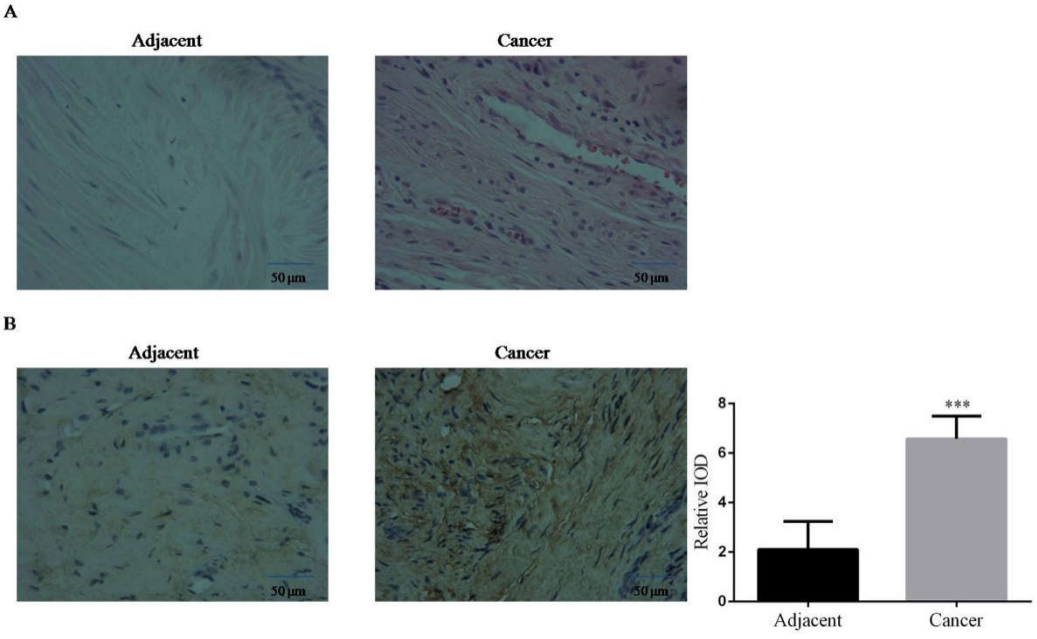

Figure 1. Clinical data

1A. The pathology of cervical cancer and adjacent normal tissues by H&E staining (×200)

1B. The miR-221 expression of difference tissues by ISH assay (×200)

***: P＜0.05, compared with adjacent normal tissues

## The cell proliferation by MTT assay

The cell proliferation rate of miR-221 inhibitor groups were significantly suppressed compared with that of NC groups in Hela and Siha cells (P＜0.05, respectively). The relative data were shown in Figure 2 and Figure 3.

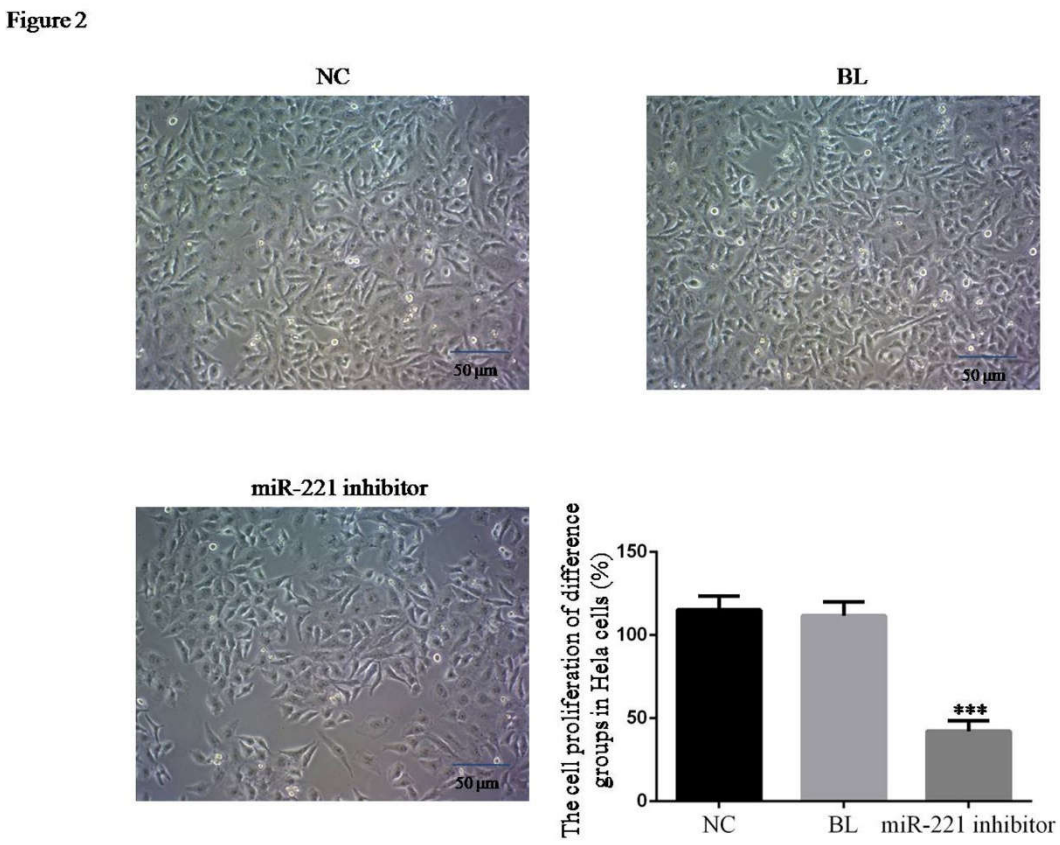


Figure 2. The cell proliferation rate of difference groups in Hela cells

***: P＜0.05, compared with NC group

Figure 3

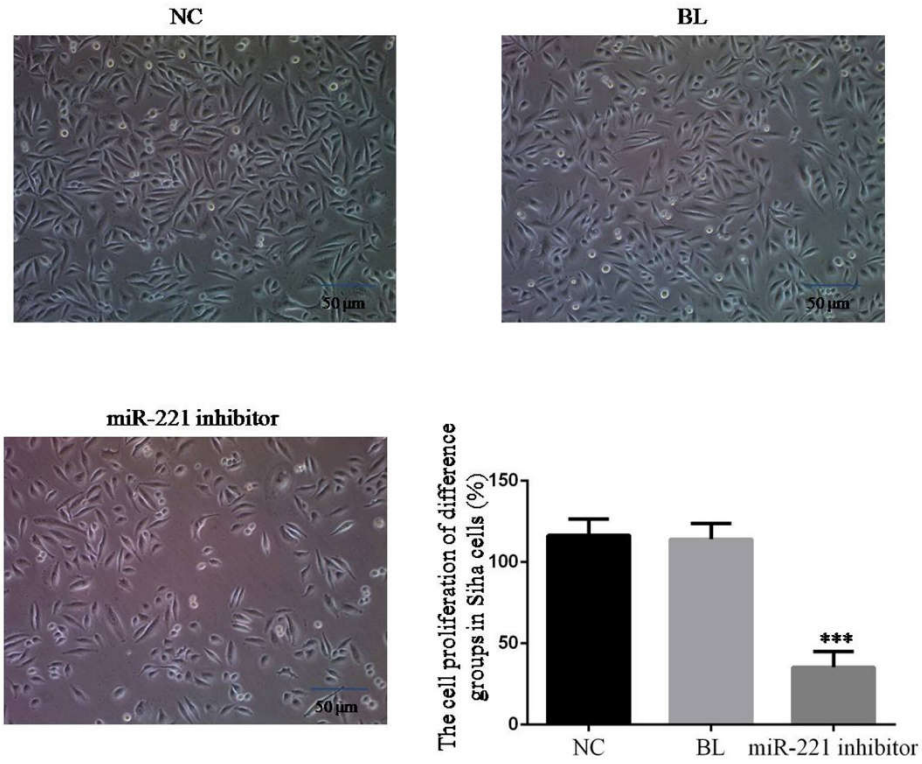


Figure 3. The cell proliferation rate of difference groups in Siha cells

***: P＜0.05, compared with NC group

**The cell apoptosis by flow cytometry**

The cell apoptosis rate of miR-221 inhibitor groups were significantly improved compared with that of NC groups in Hela and Siha cells (P＜0.05, respectively). The relative data were shown in Figure 4 and Figure 5.

Figure 4

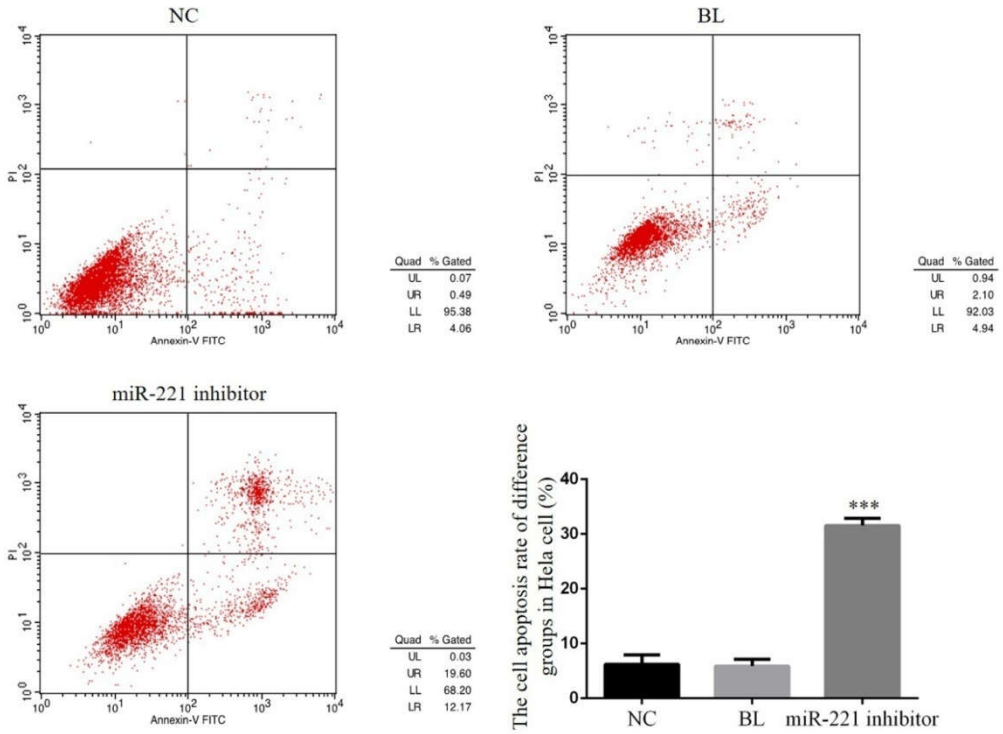


Figure 4. The cell apoptosis rate of difference groups in Hela cell

***: P＜0.05, compared with NC group

Figure 5

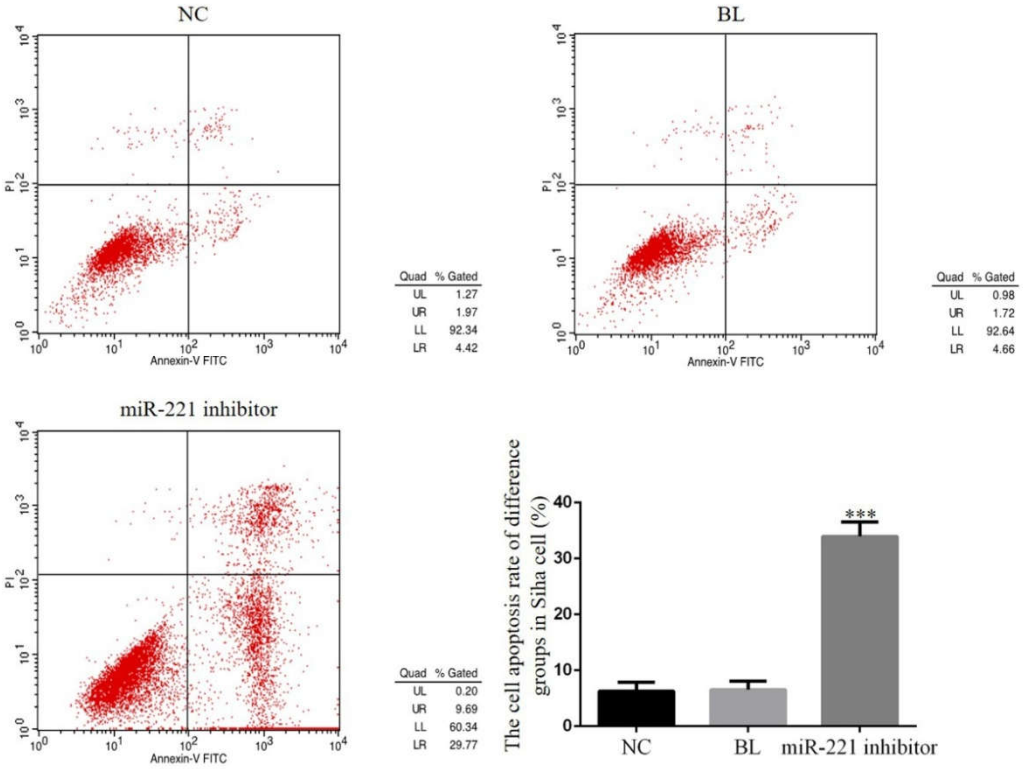

Figure 5. The cell apoptosis rate of difference groups in Siha cell

***: P＜0.05, compared with NC group

**The cell cycle byby flow cytometry**

The G1 phase rate of miR-221 inhibitor groups were significantly improved compared with that of NC groups in Hela and Siha cells (P＜0.05, respectively). The relative data were shown in Figure 6＆ Figure 7.

Figure 6

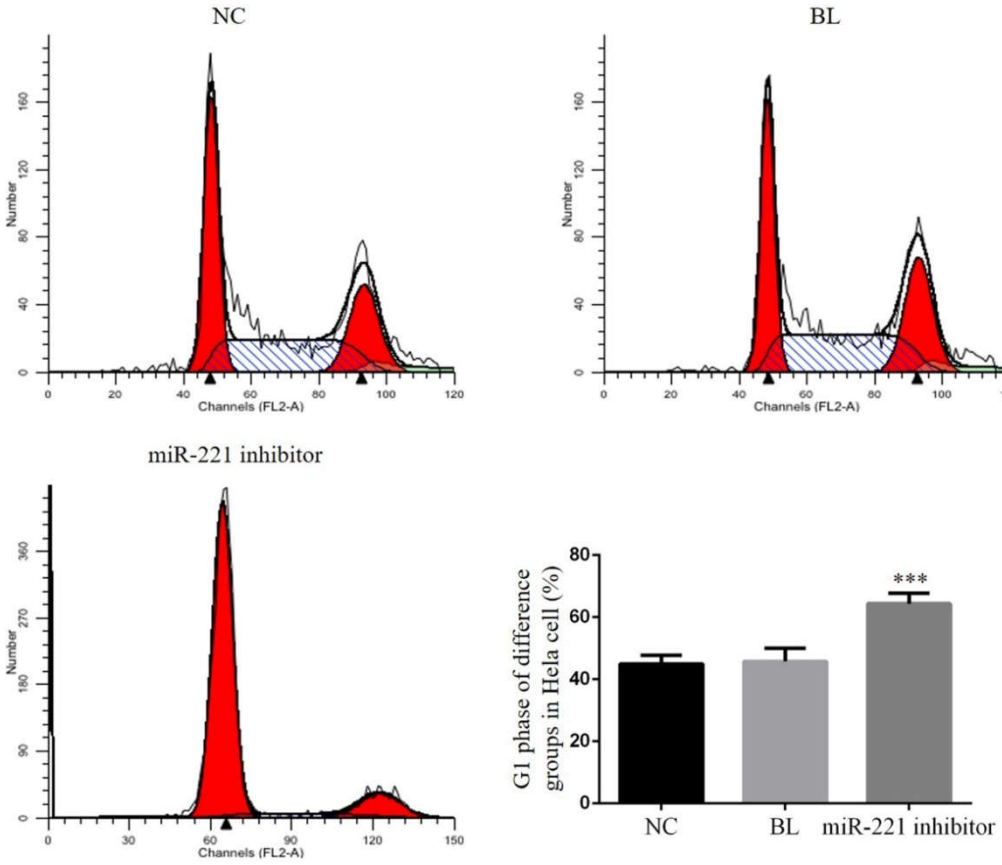


Figure 6. The G1 phase rate of difference groups in Hela cell

***: P＜0.05, compared with NC group

Figure 7

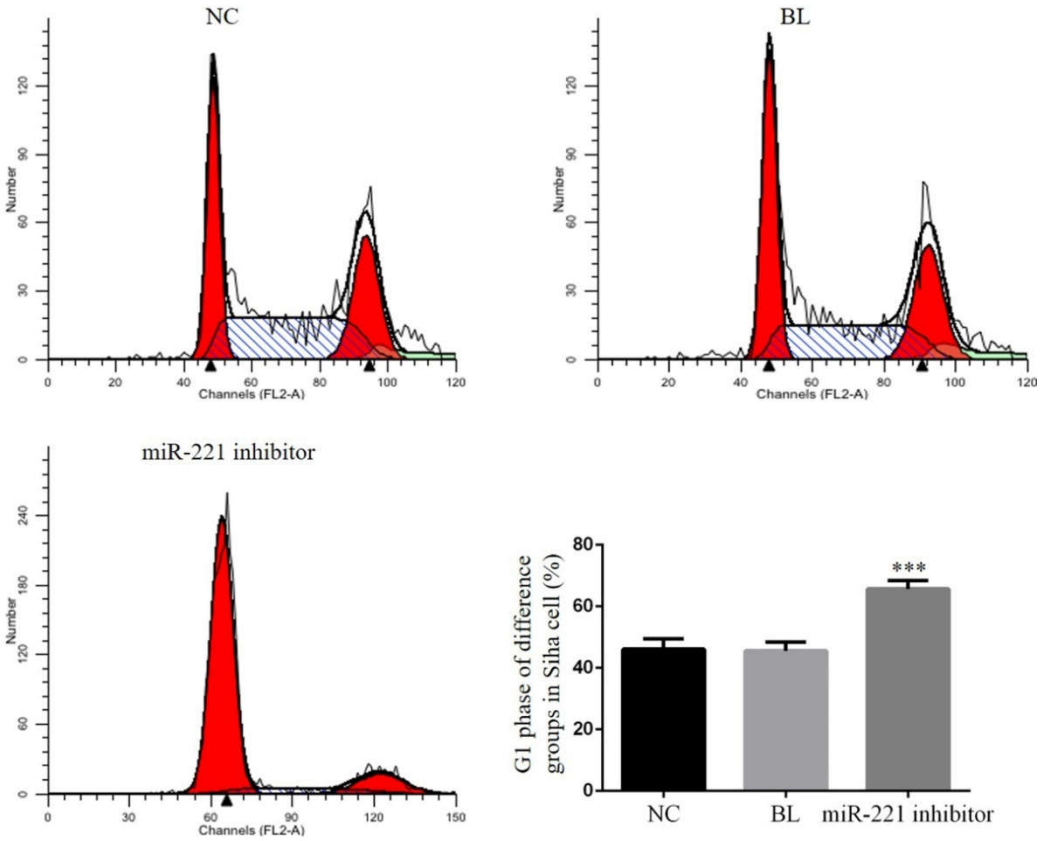


Figure 7. The G1 phase rate of difference groups in Siha cell

***: P＜0.05, compared with NC group

**The relative proteins expressions by WB assay**

Compared with NC groups, the PTEN and P16 proteins expressions of miR-221 inhibitor groups were significantly up-regulation and the PI3K and AKT proteins expressions of miR-221 inhibitor groups were significantly suppressed in Hela and Siha cells (P＜0.05, respectively). The data were shown in Figure 8& Figure 9.

Figure 8

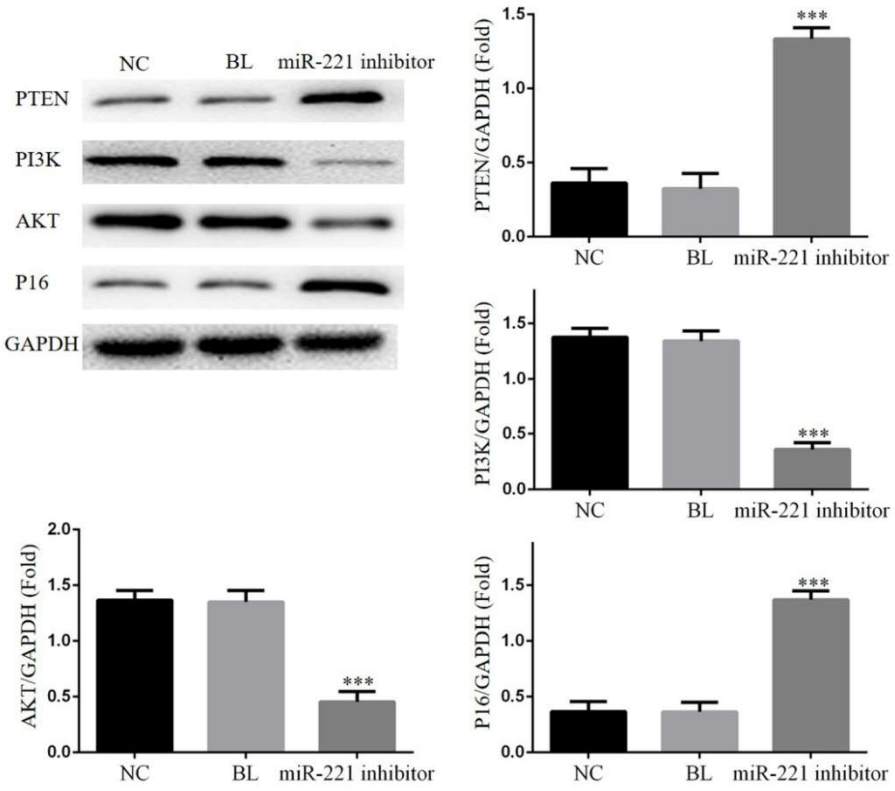


Figure 8. The relative proteins expressions of Hela cell

***: P＜0.05, compared with NC group

Figure 9

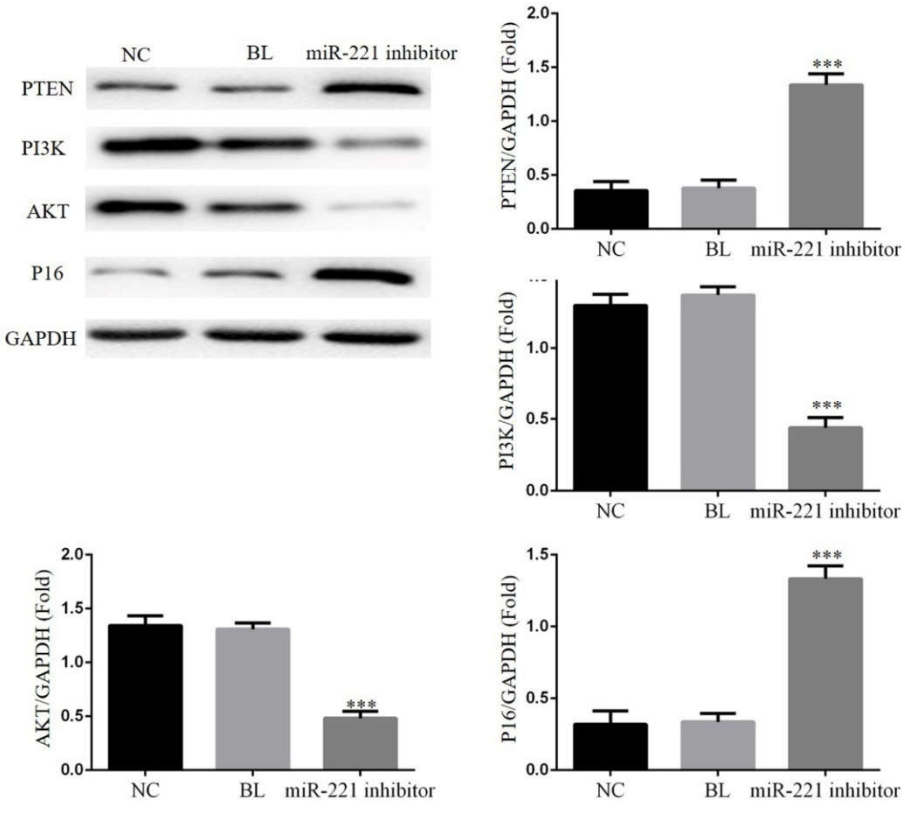

Figure 9. The relative proteins expressions of Siha cell

***: P＜0.05, compared with NC group

## Discussion

Previous studies (12-14) had shown that miRNAs plays an important role in cancer cell proliferation, metastasis, invasion, apoptosis, regulation of cycle, angiogenesis and drug resistance of cancer cells. Relative study found that miR-221 was significantly up-expression in prostate cancer and papillary thyroid carcinoma; miR-221 was a tumor promoting factor (15). Some previous studies (16-18) found that miR-221/222 over-expression promoted leukemia, breast cancer and bladder cancer. Those studies suggested that miR-221, as a tumor promoting factor, was closely related to the occurrence, development, proliferation and drug resistance of various tumors, but limited studies have been done in cervical cancer. In our present study, we evaluated the miR-221 expression in cervical cancer and adjacent normal tissues; the results were shown that miR-221 expression was significantly up-regulation in cervical cancer tissues. In the vitro study, down-regulation of miR-221 expression significantly inhibited proliferation and clonogenic formation of cervical cancer cell lines HeLa and Siha, and the results showed that miR-221 was a tumor promoting factor. Flow cytometry showed that down regulation of miR-221 expression significantly induced apoptosis in cervical cancer cells and increased the G1 phase ratio. The study was found that miR-221 over-expression could target the expression of apoptosis promoting gene PUMA in glioma cells. Studies in non small cell lung cancer have found that down regulation of miR-221 /222 expression activates TNF related apoptotic pathways by promoting the expression of PTEN and TIMP-3 (19, 20). In our present study, miR-221 down-regulation stimulated the PTEN and P16 protein and suppressed the PI3K and AKT protein activities, the results suggested that inhibition of miR-221 expression might activate apoptotic pathways and induce apoptosis.

PTEN is an important regulatory protein of phosphatidylinositol 3-kinase

(PI3K)/AKT signal transfuction pathways (21). Absence or low expression of PTEN could inhibit PI3K kinase activity, the dephosphorylation of PIP3, the expression of the catalytic subunit of the PIP3 amplification leads to AKT phosphorylation, which play an important role in the proliferation of malignant tumor cell, apoptosis, protein synthesis and transfer of a variety of physiological activities, which is an important mechanism of PTEN regulating proliferation and apoptosis tumor cells, previous studies have shown that inhibition of PTEN/PI3K/AKT signaling pathway can inhibit tumor cell proliferation and metastasis (22).At present, the deletion and mutation of P16 had been confirmed in many malignant tumors, especially in lung cancer tissues. P16 is extensively absent or mutated (23, 24). In our present study, the results were shown that P16 protein expression was stimulated with miR-221 inhibitor.

In conclusion, miR-221 was a tumor promoting factor in cervical cancer, miR-221 inhibitor had effects to suppress cell proliferation and improve cell apoptosis by stimulating G1 phase in cervical cancer cell lines (Hela and Siha).

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