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Original Article

The effect of ASPP2 on the proliferation and apoptosis of cervical cancer Hela cells

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Abstract: The P53 apoptosis-stimulating protein 2 (ASPP2) plays a tumor suppressor role in various tumors, but there are few studies on this gene and cervical cancer. This study explored ASPP2's effect on the proliferation and apoptosis of cervical cancer cells. The tumor tissues and adjacent tissues of patients with cervical cancer were collected for an analysis of ASPP2's mRNA and protein expressions using qRT-PCR and western blot. Human cervical epithelial (HCerEpiC) cells and human cervical cancer Hela and Caski cells were cultured in vitro. The Hela cells were divided into pIRES2-NC and pIRES2-ASPP2 transfection groups followed by an analysis of the expressions of ASPP2 and the cleaved caspase-3 protein using western blot, apoptosis using flow cytometry, cell proliferation using EdU staining, and caspase-3 enzyme activity. Compared with the adjacent tissues, the ASPP2 expression in cervical cancer tissues was significantly reduced. There was no significant difference in the prognosis between the patients with a low ASPP2 expression and the patients with a high ASPP2 expression (P=0.225). The ASPP2 expression in the cervical cancer Hela and Caski cells was significantly lower than it was in the HCerEpiC cells. The transfection of pIRES2-ASPP2 significantly up-regulated ASPP2 expression in the Hela cells, increased cleaved caspase-3 protein expression and caspase-3 activity, reduced cell proliferation, and increased cell apoptosis. ASPP2 expression is associated with cervical cancer. The overexpression of ASPP2 significantly reduces the proliferation of cervical cancer cells, promotes apoptosis, reduces the malignant biological characteristics of cervical cancer Hela cells, and is therefore involved in the inhibition of cervical cancer.

Keywords: ASPP2, cervical cancer, proliferation, apoptosis

Introduction

Cervical cancer (CC) is a common malignant tumor in women, and its incidence has increased year by year. Among female reproductive system malignant tumors, the incidence of cervical cancer is the highest [1]. About 500,000 women worldwide are diagnosed with cervical cancer each year, and 40-50 year old women are at a high-risk for cervical cancer. In recent years, the incidence of cervical cancer has gotten higher and higher, and the disease population is younger, posing a serious threat to the lives and health of female patients [2, 3].

The P53 gene is an important tumor suppressor gene, and the decreased expression of P53 or a gene mutation is related to the occurrence and progression of various tumors such as liver cancer, pancreatic cancer, and lung cancer [4-6]. The apoptosis stimulating protein 2 of p53 (ASPP2), also known as full-length 53BP2, is encoded by the p53BP2 gene and consists of 1128 amino acids. It is the most structurally intact protein in the ASPP family [7, 8]. ASPP2 can enhance the anti-cancer effect of the P53 gene by regulating cell cycle arrest and apoptosis, and it acts as a tumor suppressor gene in the occurrence and progression of various tumors such as breast cancer, liver cancer, and lung cancer. However, whether it plays a role in cervical cancer has not yet been studied [9, 10]. In this study, the tumor tissues and adjacent tissues of cervical cancer patients were collected to assess the expression of ASPP2. In addition, ASPP2 overexpression was performed in cervical cancer Hela cells to evaluate its effects on the proliferation and apoptosis of cervical cancer cells.

Materials and methods

Main reagents and materials

Human normal cervical epithelial (HCerEpiC) cells were obtained from Beijing Beina Bio; human cervical cancer Hela and Caski cells were obtained from the Hunan Fenghui Yinjia Science And Technology Co. Ltd; RPMI 1640 medium, fetal bovine serum (FBS) was purchased from Gibco (USA); Lipo 2000 was purchased from Invitrogen; an Ace qPCR RT Kit and SYBR dye were purchased from Toyobo, Japan; pIRES2 plasmid was purchased from Beijing BioVector; rabbit anti-human ASPP2, β-actin polyclonal antibody was purchased from American Abcam; rabbit anti-human cleaved caspase-3 polyclonal antibody was purchased from Cell Signaling Technology; HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody was purchased from Jackson ImmunoResearch; an Annexin V/PI apoptosis detection reagent was purchased from Beijing Suo Labao Bio; an EdU stained cell proliferation assay kit was purchased from Guangzhou Ruibo Biological; a caspase-3 enzyme activity detection kit was purchased from Jiangsu Biyuntian Biological.

Clinical data

40 patients with cervical cancer who were treated in our hospital from January 2016 to November 2018 were enrolled in the study. The specimens of cervical cancer tissue confirmed by histopathological examination were collected, and adjacent tissues at least 2 cm away from the tumor were collected as controls. All tissue specimens were confirmed by pathological examination, and the samples were obtained with informed consent and reviewed by the hospital ethics committee. Tissue specimens were stored in liquid nitrogen immediately after collection.

Cell culture

HCerEpiC, Hela, and Caski cells were cultured in an RPMI 1640 medium containing 10% FBS in an incubator (model: FORMA 3131, manufacturer: Thermo, USA) containing 5% CO_2 at 37°C until the cells were full. After that, the cells were collected by 0.125% trypsin digestion, subcultured at a ratio of 1:4 to 1:5, and the cells in the log phase were subjected to the experiments.

Over-expression plasmid construction and cell transfection

pIRES2 was used as the eukaryotic expression plasmid, Xho I and BamH I were used as restriction enzymes, and the ASPP2 gene was amplified using cDNA as a template. A gel-recovery kit was used to collect the target fragment after gel electrophoresis. The competent cell JM109 was transformed, and the positive strain was selected using an ampicillin-resistant solid culture plate. The recombinant plasmid containing the target fragment was amplified and cultured, and the target fragment of the ASPP2 gene was confirmed by sequencing.

pIRES2-NC and pIRES2-ASPP2 were transfected into HeLa cells using Lipo 2000 reagent. After 72 hours, the cells were collected for apoptosis detection, or they were cultured with EdU for 48 h for a cell proliferation analysis.

qRT-PCR detection of gene expression

The reverse transcription reaction was conducted in a total volume of 10 μL, including 1 μg total RNA, 2 μL RT Buffer (5 ×), 0.5 μL oligo dT+ Random primer Mix, 0.5 μL RT Enzyme Mix, 0.5 μL RNase inhibitor, ddH_2O with the reaction conditions at 37°C for 15 min, 98°C for 5 min, and the obtained cDNA was used for a PCR amplification reaction in a total of 10 μL including 5.0 μL of 2 × SYBR Green Mixture, 0.5 μL of 2.5 μm/L of primers, 1.0 μL of cDNA, and 3.0 μL of ddH_2O. The PCR conditions were: 95°C for 5 min, 95°C for 15 sec, 60°C for 60 sec, 40 cycles and performed on a Bio-Rad CFX96 real-time PCR instrument (model: CFX96, manufacturer: Bio-Rad, USA), and the fluorescence data were collected and analyzed. The primer sequence for ASPP2-F: GTGTTGCAGTTAGGCTATTTTGAGC; R: GTGGTGTACTTACCTAAAATGACATAC; GAPDH-F: GAGCCACATCGCTCAGACAC; R: GGTGCAGGAGGCATTGCTGA.

Western blot

The total protein was isolated using a RIPA lysis buffer and quantified using a BCA assay. 40 μg was separated in SDS-PAGE gel (12% separation gel, 5% concentrated gel), transferred to a PVDF membrane (90 min), blocked with 5% skim milk powder for 60 min at room temperature, and incubated with primary antibodies (ASPP2, cleaved caspase-3, β-actin dilution ratio of 1:2000, 1:1000, 1:8000) at 4°C over-

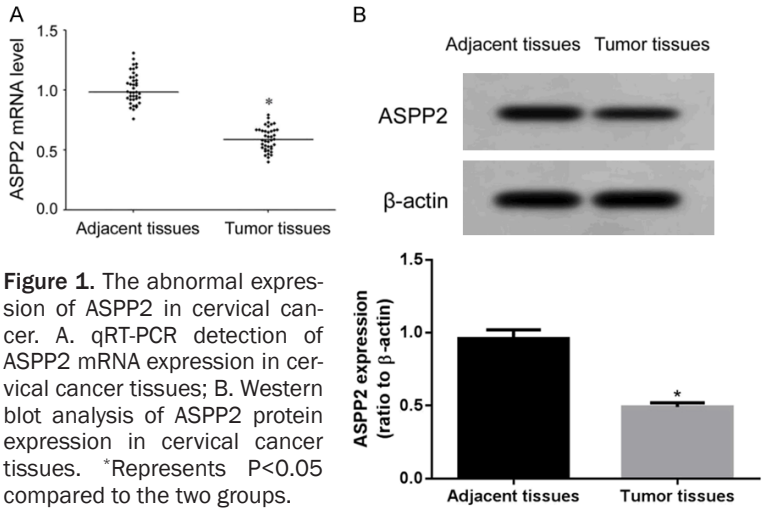

Figure 1. The abnormal expression of ASPP2 in cervical cancer. A. qRT-PCR detection of ASPP2 mRNA expression in cervical cancer tissues; B. Western blot analysis of ASPP2 protein expression in cervical cancer tissues. *Represents P<0.05 compared to the two groups.

night. After rinsing with PBST 3 times, the membrane was incubated with an HRP-conjugated secondary antibody (1:15000) for 60 min at room temperature and then washed 3 times with PBST. After that, an ECL chemiluminescence solution was added for 2 min incubation at room temperature followed by being exposed and developed.

Flow cytometry detection of apoptosis

The cells were collected by trypsinization, washed twice with PBS and then 100 μL of binding buffer, 5 μL Annexin V-FITC and 5 μL PI were added and incubated in the dark for 10 min. After that, 400 μL of binding buffer was added to resuspend the cells for an analysis of the cell apoptosis using an LC 500MCL flow cytometer.

Flow cytometry detection of cell proliferation

The cells of each transfection group were collected by trypsinization, resuspended in a DMEM complete medium containing 10% FBS, incubated with 10 μM of EdU at 37°C for 2 h, cultured for 48 h, washed twice with PBS, and collected by trypsinization. After the digestion was terminated, the cells were transferred to a centrifuge tube, centrifuged to remove the supernatant, and washed once with PBS. After paraformaldehyde fixation, glycine neutralization, and 0.1% Triton X-100 permeabilization, 500 μL of Apollo staining solution was added and incubated at room temperature. After 10 min, the cells were washed with PBS and the cell proliferation was determined using flow cytometry.

Detection of caspase-3 enzyme activity

According to the instructions of the kit, the brief steps were as follows: the pNA standard was diluted by concentration to prepare the standards with concentrations of 200, 100, 50, 25, 12.5, 6.25, and 0 μM, and the absorbance at 405 nm was measured as A405 to make a standard curve. 100 μL of caspase lysis buffer was added to the cell pellet, lysed on ice for 20 min, followed by centrifugation at 12000 g for 10-15 min at 4°C. The isolated protein was quantified using a BCA kit. 65 μL of assay buffer, 25 μL of lysate supernatant, and 10 μL c-DEVD-pNA (2 mM) were added to a 96-well plate, incubated at 37°C for 2 h, followed by measuring the absorbance at 405 nm (A405) when the color changes significantly. The experimental group A405/control group A405 × 100% was defined as the relative enzyme activity unit.

Statistical analysis

The data were analyzed by SPSS 18.0 software. The measurement data are presented as the mean ± standard deviation (SD). Student's *t* test was performed for a comparison of the measurement data between the two groups. The comparison between the measurement data of multiple groups was first analyzed using one-way ANOVA with the Bonferroni correction as a post hoc analysis. A Mann-Whitney U test was used to compare the expressions of ASPP2 mRNA in the cervical cancer tissues. The Kaplan-Meier method was used to establish the survival curve of the patients which was assessed using a log-rank test; P<0.05 indicated a statistically significant difference.

Results

Abnormal expression of ASPP2 in cervical cancer

The expression of ASPP2 mRNA in the cervical cancer patients was significantly lower than it was in the adjacent tissues (**Figure 1A**). Meanwhile, the ASPP2 protein expression was

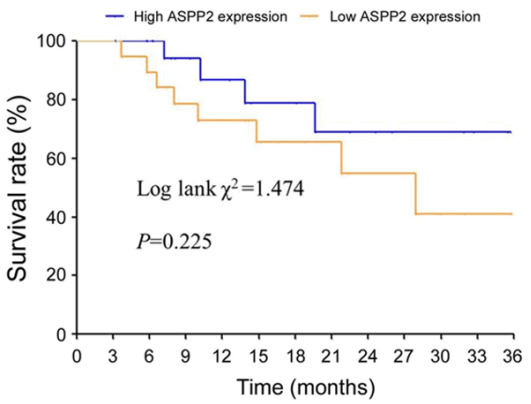

Figure 2. The relationship between the expression of ASPP2 and the survival and prognosis of patients with cervical cancer.

significantly lower in the tumor tissues than it was in the adjacent tissues (**Figure 1B**).

The relationship between ASPP2 expression and survival and prognosis

The cervical cancer patients were divided into an ASPP2 high expression group and an ASPP2 low expression group based on the median mRNA expression of ASPP2, and the relationship between ASPP2 expression and survival and prognosis was analyzed. The survival curve analysis showed no significant difference in survival and prognosis between patients with low ASPP2 expression and those with high ASPP2 expression (Log-rank test χ^2=1.474, P=0.225) (**Figure 2**).

Reduced expression of ASPP2 in cervical cancer cells

The ASPP2 mRNA expression was significantly lower in cervical cancer Hela and Caski cells than it was in human normal cervical epithelial HCerEpiC cells (**Figure 3A**). Meanwhile, the ASPP2 protein expression in cervical cancer Hela and Caski cells was significantly lower than it was in human cervical epithelial HCerEpiC cells (**Figure 3B**).

The overexpression of ASPP2 inhibits the proliferation and induces the apoptosis of cervical cancer Hela cells

The ASPP2 mRNA expression in cervical cancer Hela cells was significantly increased in the pIRES2-ASPP2 transfected group compared with the pIRES2-NC group (**Figure 4A**). In addition, the ASPP2 and cleaved caspase-3 protein expressions in the Hela cells of the pIRES2-ASPP2 transfected group was significantly higher than it was in the pIRES2-NC group (**Figure 4B**). In addition, the transfection of pIRES2-ASPP2 significantly increased the caspase-3 enzyme activity in the cervical cancer Hela cells (**Figure 4C**). A flow cytometry analysis showed that the transfection of pIRES2-ASPP2 significantly attenuated the proliferative capacity (**Figure 4D**) and increased the apoptosis (**Figure 4E**) of Hela cells.

Discussion

The P53 gene is the most common tumor suppressor gene found to be associated with human tumors, and it is divided into a wild type and a mutant type [6, 11, 12]. The wild-type P53 gene detects and repairs DNA damage, scavenges DNA-damaged cells, and induces cell cycle arrest and cell apoptosis. The P53 gene mutation is associated with the development, progression, metastasis, and prognosis of various tumors such as lung cancer, breast cancer, kidney cancer, oral cancer, etc. [5, 13-15].

The ASPP family is a new family of tumor-associated genes discovered in 2003, including the ASPP1, ASPP2, and ASPP family (iASPP) proteins. The members of this family share a common structure, namely an ankyrin, a repeat, SH3 domain and proline-rich region [7, 8]. The ASPP1 and ASPP2 proteins have 48% sequence homology and homology at the N-terminus and C-terminus, both of which can enhance P53-induced cell apoptosis, but have no effect on the cell cycle. ASPP1 and ASPP2 can bind to P53 and specifically enhance the binding of P53 to the promoter of the pro-apoptotic gene, and selectively promote the transcriptional activity of the P53 protein on apoptotic genes [16], thereby promoting apoptosis and functioning as a tumor suppressor gene [10, 17]; iASPP is a proto-oncogene that competes with ASPP1 and ASPP2 for binding to the P53 protein and inhibits P53-induced apoptosis, leading to tumorigenesis [18, 19]. In this study, the tumor tissues and adjacent tissues of cervical cancer patients were collected to compare the differences in ASPP2 expression. In addition, the role of ASPP2 gene over-expression in cervical cancer Hela cells was investigated.

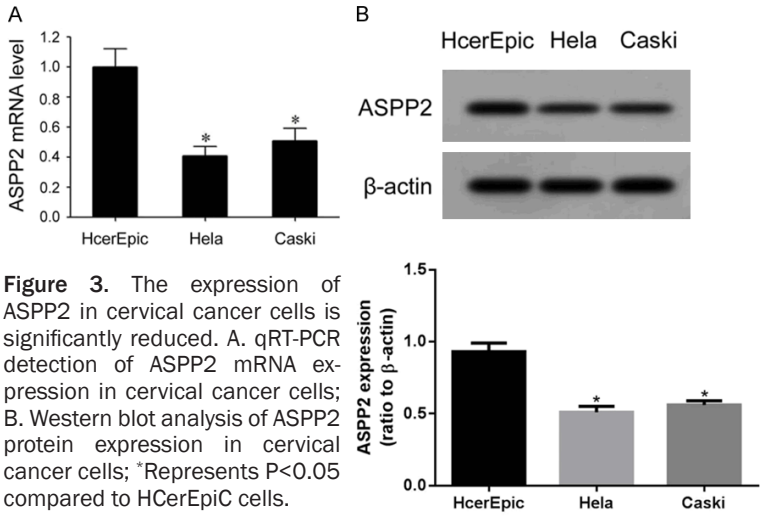

Figure 3. The expression of ASPP2 in cervical cancer cells is significantly reduced. A. qRT-PCR detection of ASPP2 mRNA expression in cervical cancer cells; B. Western blot analysis of ASPP2 protein expression in cervical cancer cells; *Represents P<0.05 compared to HCerEpiC cells.

The results from the clinical samples showed that ASPP2 mRNA and protein expression was significantly lower in the patients' cervical cancer tissues than it was in the adjacent non-cancerous tissues, suggesting that a decreased expression of ASPP2 might be involved in the pathogenesis of cervical cancer. In the survival rate analysis, the ASPP2 expression was divided into a low expression group and a high expression group. The results showed that the survival rate of patients with a lower ASPP2 expression was lower than those with a higher ASPP2 expression. But there was no significant difference between them, which may be related to the small sample size and low statistical test efficiency. The results of the cell culture in vitro also showed that, compared with normal cervical epithelial HCerEpiC cells, the expressions of ASPP2 mRNA and protein in cervical cancer Hela and Caski cells were significantly decreased, indicating that the expression of ASPP2 was decreased in relation to cervical cancer, which was consistent with the patients' data. In a study of the relationship between ASPP2 and tumor incidence, the results of Yin et al. [20] showed that ASPP1 and ASPP2 expressions were significantly decreased in the tumor tissues of patients with colorectal cancer compared with the adjacent tissues, but the iASPP expression was significantly increased. The expression of iASPP was correlated with pathological grade. The decrease of ASPP2 expression and the expression of the carcinoembryonic antigen showed a significantly negative correlation. The results of Song et al. [21] showed that ASPP2 expression was significantly abnormal in the tumor tissues of patients with pancreatic cancer compared with the adjacent tissues. Wang et al. [22] showed that, compared with normal peripheral blood mononuclear cells (PBMC), breast cancer Bcap-37, MDA-MB-231, MCF-7, HBL-100 cells showed significantly reduced expression levels of ASPP2. The results of Meng et al. [23] showed that, compared with non-cancerous lesions in the stomach, ASPP2 expression was significantly decreased in gastric cancer patients, and was significantly lower in gastric cancer patients with *Helicobacter pylori* than it was in patients free of *Helicobacter pylori*. In the positive group, *Helicobacter pylori* may participate in the promotion of gastric cancer by down-regulating ASPP2 expression and up-regulating iASPP expression. The above results indicate that ASPP2 expression is associated with the pathogenesis of intestinal cancer, pancreatic cancer, breast cancer and gastric cancer, but there are few studies in cervical cancer. This study found an abnormal ASPP2 expression in cervical cancer tissues, indicating that ASPP2 might play a role in cervical cancer.

To further assess ASPP2's effect on the biological effects of cervical cancer cells, ASPP2 was overexpressed in cervical cancer Hela cells. The results showed that the transfection of pIRES2-ASPP2 significantly up-regulated the expression of the ASPP2 gene and protein in Hela cells, indicating that the overexpression was successful. The results of the flow cytometry analysis showed that compared with the negative control group, the proliferative ability of the Hela cells in the ASPP2 overexpression group was significantly decreased, but the intracellular caspase-3 enzyme activity and cell apoptosis were significantly increased, indicating that ASPP2 overexpression inhibits Hela cell proliferation and promotes cell apoptosis. In a study of ASPP2 regulating the biological effects of tumor cells, Xu et al. [9] found that SAPP2 can inhibit the tumor stem cell characteristics of liver cancer cells and can inhibit the resistance of liver cancer cells to chemotherapeutic drugs by inhibiting the Src/FAK/Snail signaling pathway axis. Song et al. [21] showed that the down-regulation of ASPP2 expression in pancreatic cancer cells can significantly promote cell proliferation, activate the AMPK signaling pathway, enhance autophagy, and

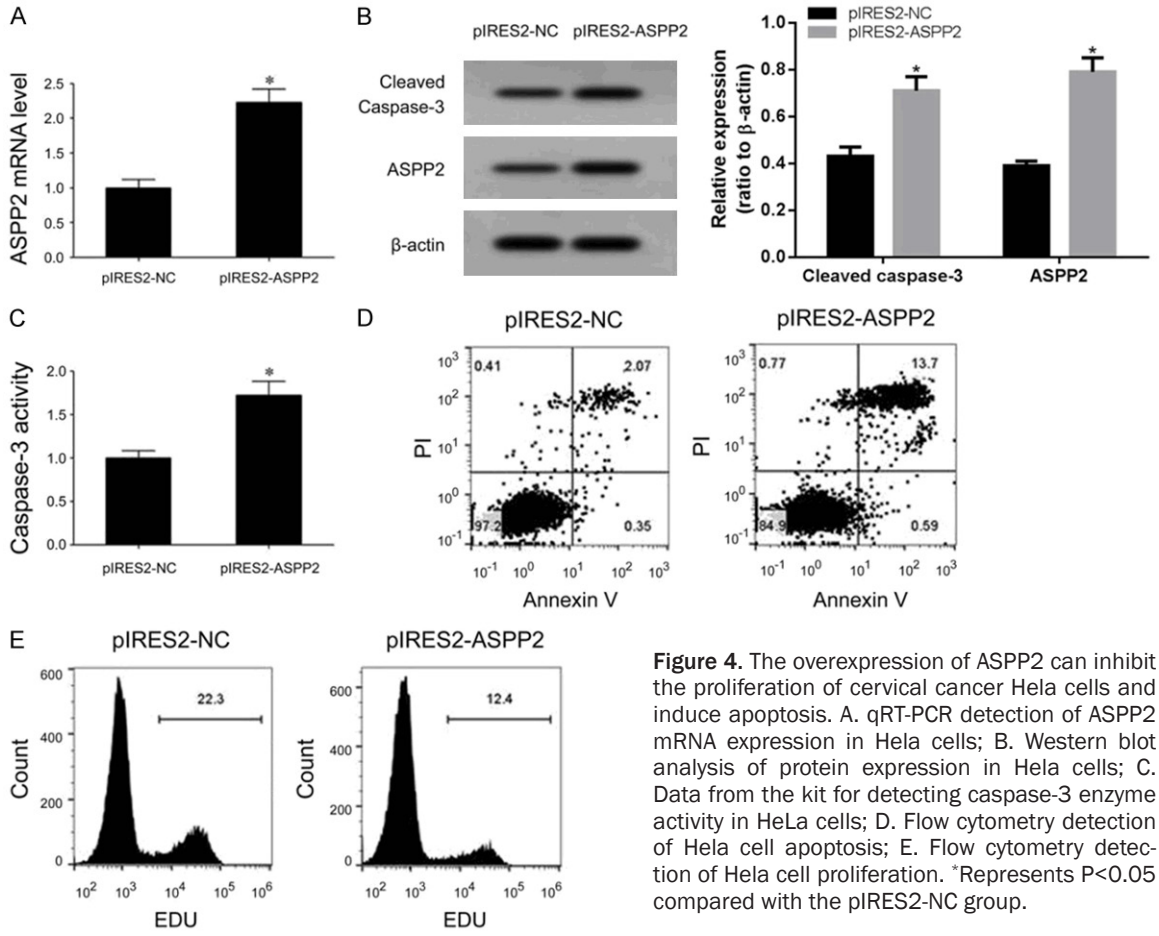

Figure 4. The overexpression of ASPP2 can inhibit the proliferation of cervical cancer Hela cells and induce apoptosis. A. qRT-PCR detection of ASPP2 mRNA expression in Hela cells; B. Western blot analysis of protein expression in Hela cells; C. Data from the kit for detecting caspase-3 enzyme activity in HeLa cells; D. Flow cytometry detection of Hela cell apoptosis; E. Flow cytometry detection of Hela cell proliferation. *Represents P<0.05 compared with the pIRES2-NC group.

increase pancreatic the drug resistance of the cancer cell anti-tumor drug gefitinib (gemcitabine). The results of this study showed that the decreased expression of ASPP2 was associated with the pathogenesis of cervical cancer. Increasing the expression of ASPP2 significantly attenuated the proliferative capacity of cervical cancer Hela cells, promoted apoptosis, and attenuated the malignant biological characteristics of cervical cancer Hela cells. At present, research on the relationship between ASPP2 and cervical cancer has not been reported. This study found that the decrease of ASPP2 expression is related to cervical cancer. Increasing the expression of ASPP2 can reduce the tumor suppressive effect in the malignant biological characteristics of cervical cancer cells.

Conclusion

ASPP2 is associated with the pathogenesis of cervical cancer. The overexpression of ASPP2 can significantly reduce the proliferation of cervical cancer cells, promote apoptosis, reduce the malignant biological characteristics of cervical cancer Hela cells, and is therefore involved in inhibiting cervical cancer.

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Disclosure of conflict of interest

None.

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