Abstract. Cervical cancer is a common tumor of the reproductive system; however, to the best of the authors' knowledge, the regulation and underlying mechanism of p53 apoptosis-stimulating protein 2 (ASPP2) in cervical cancer has yet to be elucidated. Therefore, the present study aimed to explore the role of ASPP2 in cervical cancer. Tumor tissues were collected for the detection of ASPP2 expression. Experiments wherein ASPP2 was overexpressed were designed to upregulate the expression of ASPP2. The levels of autophagy were subsequently assessed by examining LC3B level via immunofluorescence. Cell Counting Kit-8 assay was then performed to estimate the level of cell proliferation. The cell proliferation level was also measured by EdU staining, and TUNEL assay was used to detect the level of apoptosis. The expression levels of ASPP2, Beclin1 and associated proteins were detected using reverse transcription-quantitative PCR and western blotting analyses. ASPP2 was observed to be markedly reduced in patients with cervical cancer and in cervical cancer cell lines. Overexpression of ASPP2 was found to suppress the expression of Beclin1, and autophagy was also inhibited in cervical cancer cells. Overexpression of ASPP2 also inhibited cell proliferation and promoted apoptosis of cervical cancer cells via the inhibition of autophagy. Additionally, overexpression of ASPP2 was shown to enhance the TNF-related apoptosis-inducing ligand-induced apoptosis of cervical cancer cells via inhibiting autophagy. Taken together, the results of the present study have shown that ASPP2 exerted antitumor effect in cervical cancer by inhibiting cell proliferation and promoting apoptosis partly through inhibiting autophagy. These findings may be useful for the provision of potential targets for cervical cancer therapy.

Introduction

Cervical cancer is one of the most common gynecological malignant tumors (1). In recent years, its incidence rate has tended towards an increase in younger women, and the mortality rate associated with cervical cancer is increasing year by year, posing a serious threat to health and life of women (1). Around 570,000 newly diagnosed cases and 311,000 deaths worldwide are reported in 2018 (2). At present, the most commonly used treatment methods in the clinic are surgery and chemoradiotherapy (3,4). However, one adverse effect of using chemoradiotherapy is the damage caused by radiotherapy to the ovarian function of young patients, which is not negligible; furthermore, the commonly used chemotherapy drugs, such as platinum drugs, are far from ideal when applied in the treatment of cervical cancer due to their toxicity and other side effects, and also because of the development of drug resistance (5,6). Therefore, an exploration of the pathogenesis and targeting factors of cervical cancer would be conducive to the development of effective tumor suppressor drugs with low toxicity.

p53 apoptosis-stimulating protein 2 (ASPP2) is a member of the ASPP family, which has characteristic sequences of ankyrin repeats, an SH3 domain and a proline-rich region (7). ASPP2 can directly interact with p53 family members and selectively enhance their transcriptional activities toward pro-apoptosis genes (8). ASPP2 is commonly considered as a tumor suppressor by promoting cancer cell apoptosis and inhibiting cell migration and growth (9-11). Previous studies have shown that the expression of ASPP2 is downregulated in breast cancer, liver cancer and other malignant tumors, and its downregulated level is associated with an advanced tumor stage and poor prognosis, suggesting that this gene may be an important target for tumor therapy (12-14). It has been previously observed that ASPP2 is also downregulated in cervical cancer (15); however, to the best of the authors' knowledge, the direct molecular regulation and underlying mechanism of ASPP2 in cervical cancer have yet to be completely elucidated. In addition, previous studies have revealed the involvement of ASPP2 in the regulation of autophagy in hepatocellular carcinoma, colorectal cancer and which was associated with BECN1-dependent autophagy initiation (16-18), thus, whether
ASPP2 got involved in cervical cancer development by regulating autophagy raised our interest. Therefore, the aim of the present study was to perform a systematic analysis of ASPP2 in order to unravel the underlying mechanism of cervical carcinogenesis, and to provide new leads for novel treatment strategies.

Materials and methods

Patient samples. Tissue samples from patients were collected between March 2018 and September 2019 at Yancheng First People’s Hospital (Yancheng, China). Those who received preoperative chemotherapy or radiotherapy or had concurrent or successive primary malignancies were excluded. The patients were aged 30-69 years old. A total of 30 patients diagnosed with cervical cancer were included in the present study, and tumor tissues and para-tumor tissues were collected for the subsequent investigation. All experiments were approved [approval no. (2018)-(k022)] by the ethics committee of Yancheng First People's Hospital (Yancheng, China). All participants signed informed consent forms and gave their approval for this article to be published.

Cell culture and treatment. Human cervical squamous cell carcinoma cell lines (Ca-Ski, Hela, SiHa and c-33A) were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). A non-cancerous ectocervical epithelial cell line (Ect1/E6E7) was obtained from American Type Culture Collection (ATCC) and maintained in Gibco® Keratinocyte-Serum Free medium with 0.1 ng/ml human recombinant epidermal growth factor, 0.05 mg/ml bovine pituitary extract, and 0.4 mM calcium chloride. Ca-Ski cells were cultured in the Gibco® RPMI-1640 medium and the other cells were cultured in the Gibco® DMEM medium (Thermo Fisher Scientific, Inc.) supplemented with 10% Gibco FBS (Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. All cell lines were incubated at 37˚C in an incubator (Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin supplemented with 10% Gibco FBS (Thermo Fisher Scientific, Inc.) for 1 h at room temperature, cells were incubated with anti-LC3B antibody (1:50; cat. no. ab192890; Abcam) at 4˚C overnight. After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated secondary antibody (1:200; cat. no. ab150077; Abcam) at room temperature for 2 h. The nuclei were stained by DAPI (0.01 mg/ml; Vector Laboratories, Inc.). The images were observed under a fluorescence microscope (Nikon Corporation).

EdU staining. The cell proliferation level was measured using EdU staining. An EdU staining kit (cat. no. CA1170) was purchased from Beijing Solarbio Science & Technology Co., Ltd., and the experiments were performed according to the instructions provided with the kit. Briefly, cells at the exponential growth stage were collected and seeded into 96-well plates at a density of 1x10^4 cells per well. The cells were cultured to the normal growth stage. Subsequently, 100 µl EdU culture medium (50 µM concentration) was added to each well, and the cells were incubated for 2 h at 37˚C. The cells were then washed 1 or 2 times, and 4% paraformaldehyde (50 µl) was added to the cells for an incubation for 30 min at 37˚C. Subsequently, 100 µl 1X Apollo® staining solution was added to each well, and the plates were then incubated in the dark at room temperature for 30 min; the staining solution was then discarded. Subsequently, the cells were washed 2-3 times with 100 µl 0.5% Triton X-100/PBS, and 0.01 mg/ml DAPI solution was added to each well; the plates were then incubated in the dark at room temperature for 30 min before discarding the staining solution. Finally, the cells were observed under a fluorescence microscope (Olympus FV500; Olympus Corporation).

Reverse transcription-quantitative (RT-q) PCR analysis. TRIzol® isolation reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added to lyse the cells and tissues. Total RNA of the c-33A cells and tissues was extracted using the phenol-chloroform method after complete lysis had occurred. The molecular weight and concentration of the extracted RNAs were analyzed using 1% agarose gel electrophoresis, and by...
measuring the ratio of the absorbance at 260-280 nm with an ultraviolet spectrophotometer (Nanodrop2000; Thermo Fisher Scientific, Inc.) respectively. The RNA was reverse-transcribed into complementary (c)DNA according to the poly-A tailing method using a miScript SYBR® Green PCR kit (Qiagen GmbH) according to the manufacturer's instructions. The cDNA samples were then assessed using RT-qPCR with an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.), as recommended by the manufacturer. The PCR thermocycling conditions were as follows: 95˚C for 5 min; 95˚C for 30 sec and 60˚C for 30 sec, for a total of 40 cycles. The relative amount of mRNA was quantified using the $2^{-\Delta\Delta Cq}$ method (21), and β-actin was used as an internal control. The primer sequences were as follows: ASPP2 forward, 5'-GAA GAC TCG GTG AGC ATG CG-3' and reverse, 5'-GCG ATA CGC TCT GAG CCA GT-3'; β-actin forward, 5'-AGC GAG CAT CCC CCA AAG TT-3' and reverse, 5'-GGG CAC GAA GGC TCAT CAT T-3'. The RT-qPCR experiments were independently performed three times.

Western blot analysis. The samples (c-33A cells or tissues) were denatured in a boiling water bath for 10 min. Protein concentrations were measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.). Proteins (30 µg) from each sample were separated using SDS-PAGE (10%). The protein bands were transferred onto a polyvinylidene difluoride membrane at a constant current of 300 mA for 2 h at room temperature. Primary antibodies including anti-ASPP2 (1:10,000; cat. no. ab32124), Bax (1:1,000; cat. no. ab32503; all from Abcam), cleaved caspase-3 (1:1,000; cat. no. 9661), caspase-3 (1:1,000; cat. no. 9662), cleaved poly ADP-ribose polymerase (PARP; 1:1,000; cat. no. 5625), PARP (1:1,000; cat. no. 9542, all from Cell Signaling Technology, Inc.), Death receptor 4 (DR4; 1: 500; cat. no. ab216662), DR5 (1:1,000; cat. no. ab8416) and GAPDH (1:2,500; cat. no. ab9485; all from Abcam) were added, followed by incubation overnight at 4˚C on a shaker. The membrane was washed five times with Tris-buffered saline containing 0.1% Tween 20 (TBST) (5 min each wash). HRP-conjugated secondary antibodies (1:20,000; cat. no. ab205718; Abcam) were added, followed by incubation for 1 h at room temperature. The membrane was then washed five times with TBST for 5 min each wash and developed with enhanced chemiluminescent liquid (cat. no. P0018A; Beyotime Institute of Biotechnology). The images were analyzed using Quantity One 4.62 analysis software (Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were independently performed three times, and the data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 8.0.1 software (GraphPad Software, Inc.). Comparison between two groups were performed with unpaired Student’s t-test, while comparisons among three or more groups were performed with one-way ANOVA followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

**ASPP2 is downregulated in cervical cancer.** To explore the roles of ASPP2 in the process of cervical cancer, the expression level of ASPP2 in cervical cancer tissues were first measured using RT-qPCR and Western blot analysis. ASPP2 is significantly downregulated in cervical cancer tissues compared to normal tissues. The mRNA and protein levels of ASPP2 were lower in cervical cancer tissues and cell lines compared to normal tissues and cell lines. These results suggest that ASPP2 plays a role in the progression of cervical cancer.
examined using RT-qPCR and western blot analyses, respectively. As shown in Fig. 1A and B, ASPP2 expression in the cancer tissues was decreased compared with normal tissues. To further investigate the expression of ASPP2, a range of cervical cancer cell lines, including HeLa, SiHa, c-33A and Ca-ski cells, were examined. The results obtained revealed that ASPP2 expression was decreased in the human cervical cancer cell lines, particularly in c-33A cells, compared with Ect1/E6E7 cells, thus c-33A cells were used for the subsequent experiments (Fig. 1C and D).

Overexpression of ASPP2 suppresses Beclin1 expression and autophagy in cervical cancer cells. To further investigate the effects of ASPP2 in cervical cancer cells, western blot analysis was used to determine the expression level of ASPP2 after cell transfection. Transfection with Ov-ASPP2 led to a significant upregulation of ASPP2 expression (Fig. 2A). Given that a previous study demonstrated that ASPP2 is able to bind to Beclin1 and to inhibit Beclin1 expression (15), the western blotting results in the present study also indicated that overexpressing ASPP2 caused a significant downregulation of Beclin1 expression (Fig. 2B). Subsequently, immunofluorescence assay was subsequently used to investigate the expression of LC3B. Overexpression of ASPP2 led to a decrease of LC3B-positive cells; these effects were partly weakened through additional treatment with Tat-BECN1, the inducer of autophagy (Fig. 2C). These results were further confirmed by detecting the expression levels of LC3II/I, p62 and Beclin1. As revealed in Fig. 2D, Ov-ASPP2 upregulated p62 expression in the c-33A cells, whereas the expression levels of LC3II/LC3I and Beclin1 were suppressed. By contrast, these
trends were reversed following treatment of the cells with Tat-BECN1. Collectively, these findings demonstrated that overexpression of ASPP2 inhibited autophagy.

**Overexpression of ASPP2 ameliorates the proliferation of cervical cancer cells through inhibiting autophagy.** Subsequently, CCK-8 assay was performed to assess the level of cell proliferation. The results obtained showed that Ov-ASPP2 led to an inhibition of the proliferation of the c-33A cells at 24, 48 and 72 h compared with the Ov-NC group (Fig. 3A). Additionally, the results of the EdU staining revealed that overexpression of ASPP2 ameliorated proliferation of the cervical cancer cells via inhibiting autophagy (Fig. 3B). Moreover, western blot assay revealed that the expression levels of Ki67 and PCNA were significantly decreased in the Ov-ASPP2 group compared with the Ov-NC group, whereas these trends were reversed following treatment of the cells with Tat-BECN1 (Fig. 3C).

**Overexpression of ASPP2 promotes apoptosis through inhibiting autophagy.** To verify the effect of ASPP2 on the apoptosis of c-33A cells, TUNEL staining assay was performed. These experiments revealed that the ratio of apoptosis (where green staining represents the apoptotic cells) in the Ov-ASPP2 group was higher compared with that in the Ov-NC and control groups, whereas Tat-BECN1 treatment led to a clear reduction in the extent of cell apoptosis (Fig. 4A). These results were further confirmed by detecting the expression of apoptosis-associated proteins. As demonstrated in Fig. 4B, Ov-ASPP2 led to an upregulation of the expression levels of Bax, cleaved-caspase3/caspase3 and cleaved-PARP/PARP in the c-33A cells, although the expression of the anti-apoptosis protein, Bcl-2, was suppressed; these findings were found to be reversed upon treating the cells with Tat-BECN1. Collectively, these results demonstrated that overexpression of ASPP2 promoted apoptosis through inhibiting autophagy.

**Overexpression of ASPP2 leads to an enhancement in the apoptosis of TRAIL-induced cervical cancer cells via inhibiting autophagy.** The results of the aforementioned studies have strongly suggested that ASPP2 could promote apoptosis through inhibiting autophagy, and the inhibition of autophagy may lead to an increase in TRAIL-induced cell sensitivity and the promotion of apoptosis of cancer cells (22). Therefore, TUNEL staining was performed to verify the effects of ASPP2 on the apoptosis of TRAIL-induced c-33A cells. The ratio of apoptosis in the Ov-ASPP2 or TRAIL group was found to be higher than that in the control group, and the combined treatment of TRAIL and Ov-ASPP2 transfection presented a synergistic effect, whereas additional treatment
with Tat-BECN1 caused a clear reduction in cell apoptosis in the TRAIL-induced c-33A cells (Fig. 5A and B). The western blot assay results showed that the expression levels of DR4 and DR5 were upregulated in the TRAIL and Ov-ASPP2 + TRAIL treatment groups, whereas these trends were reversed in the Ov-ASPP2 + TRAIL + Tat-BECN1 group (Fig. 5C).

Discussion

In the current study, the data obtained have implied that ASPP2 is a key factor involved in the apoptosis of cervical cancer cells. ASPP2 was downregulated in cervical cancer. ASPP2 overexpression could suppress cervical cancer cell autophagy, proliferation and promote apoptosis, which was partly abolished by Tat-BECN1, an inducer of autophagy via activating Beclin1, thus it was suggested that ASPP2 may exert its anti-tumor effect partly via regulating autophagy. In addition, the present study also addressed that ASPP2 could enhance the sensitivity of TRAIL-induced cervical cancer cells to apoptosis, disclosing the specific role of ASPP2 in cervical cancer.

Patients presenting with cervical cancer have shown a trend towards women of a younger age in recent years, and herpes simplex virus type II, human papilloma virus, human cytomegalovirus and fungal infection are all known to cause cervical cancer (23). ASPP2 is a p53-selective...
stimulator (24). Binding of the protein to its relevant binding sites on wild-type p53 induces transactivation of the pro-apoptotic promoter region and promotes cell apoptosis, thereby inhibiting the occurrence of cervical cancer. When ASPP2 expression is low, however, the binding ability of wild-type p53 to DNA is altered, and the ability of wild-type p53 to induce apoptosis is consequently inhibited (11,25). The synergistic effects of ASPP2 and p53 have been shown to have a certain influence on the occurrence of apoptosis in various types of malignant tumor cells (10). From the perspective of the mechanism underlying ASPP2’s influence on cervical cancer, enhancing the binding of ASPP2 to p53 and increasing the stimulatory role of ASPP2, and thereby its ability to induce the p53 gene, are promising strategies that may lead to the development of novel methods for treating cervical cancer.

Liang et al (26) showed that Beclin-1 is highly expressed in normal breast epithelial tissues, although the expression level was decreased, or the protein was not expressed, in breast cancer tissues. Beclin-1 transfected into the human breast cancer MCF7 cell line was shown to stimulate autophagy of the MCF7 cells; furthermore, the proliferation of the MCF7 cells was inhibited in vitro, and the tumorigenicity of the cell line was inhibited. It is also discovered that Beclin 1 is not only an autophagy gene essential for early embryonic development, but also a haploinsufficient tumor suppressor, as the heterozygous disruption of the beclin 1 gene will promote tumorigenesis (27, 28). In the present study, overexpression of ASPP2 led to an inhibition of autophagy, which was reversed by Tat-BECN1, the inducer of autophagy via activating Beclin1, indicating that ASPP2 may inhibit autophagy partly via regulating Beclin1, which subsequently contributing to the suppression of cell apoptosis; however, it needs to be further verified in future studies.

TRAIL is a signaling molecule involved in the regulation of apoptosis, as well as being a type II transmembrane protein, which can interact with DRs, thereby activating the caspase cascade protease family and inducting apoptosis (29,30). Recent studies have shown that the combination of TRAIL and chemotherapeutic drugs leads to an improvement in the sensitivity of tumor cells to TRAIL. TRAIL is a member of the tumor necrosis factor superfamily, which is able to induce tumor cell apoptosis through binding to DRs on cell membranes with low toxicity to normal tissues and cells (31-33).

Figure 5. Overexpression of ASPP2 leads to an enhancement in the apoptosis of TRAIL-induced cervical cancer cells via inhibiting autophagy. (A and B) TUNEL staining was performed to verify the effect of ASPP2 on the apoptosis of TRAIL-induced c-33A cells. (C) Expression levels of DR4 and DR5 were detected by western blot analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. control; +++P<0.001 vs. Ov-ASPP2, +++P<0.001 vs. Ov-ASPP2 + TRAIL. TRAIL, TNF-related apoptosis-inducing ligand; ASPP2, p53 apoptosis-stimulating protein 2; Ov-ASPP2, overexpression of ASPP2; Ov-NC, overexpression-NC; DR, death receptor.
has an intracellular death domain, which is able to effectively transmit apoptotic signals and induce apoptosis. When the concentration of TRAIL is low, TRAIL can competitively bind to the inducible receptor, and under these circumstances, the ability of TRAIL to induce death of the cells is not obvious. When the concentration of TRAIL is high, however, TRAIL can bind to the DRs and induce apoptosis. In our experiments, it was shown that overexpression of ASPP2 was able to enhance the apoptosis of TRAIL-induced cervical cancer cells via inhibiting autophagy.

In conclusion, the present study has shown that ASPP2 expression was decreased in cervical cancer, and that the mechanism of ASPP2 action is dependent on inhibiting autophagy and promoting the apoptosis of cervical cancer. Although the underlying mechanism has not been fully delineated and the clinical information of these patients are limited such as prognosis, the present study has at least suggested that the mechanism involves ASPP2 regulating cervical cancer progression via the targeting of Beclin1. These findings may lead to the identification of novel targets for the diagnosis of cervical cancer, and novel strategies for the treatment of cervical cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL designed the present study. FJ and GB collected, analyzed and interpreted the data. FJ and GB confirm the authenticity of all the raw data. FJ wrote the article. JL critically revised the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved [approval no. (2018)-(k022)] by the ethics committee of Yancheng First People's Hospital (Yancheng, China). Written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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