PTEN downregulation induces apoptosis and cell cycle arrest in uterine cervical cancer cells

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Abstract. The tumor suppressors PTEN and p53 are often downregulated in various human cancer types, which has been associated with a poor prognosis. Recent evidence implies that PTEN downregulation may induce growth arrest of kidney cells and cancer cells. In the present study, the role of PTEN in the proliferation and survival of cervical cancer cells was investigated. It was found that PTEN silencing promoted apoptosis and cell-cycle arrest, accompanied by a significant decrease in the proportion of cells in the S1 phase of the cell cycle. Moreover, PTEN silencing in cervical cancer cells increased levels of p53, p27, p21, phospho-ERK and cleaved caspase-3, and decreased levels of cyclin A2 and cyclin D1. Furthermore, PTEN knockdown significantly impacted the viability of cervical cancer cells. P53 silencing did not affect the ability of PTEN knockdown to induce apoptosis in cervical cancer cells. Taken together, the present study results imply that PTEN silencing induces apoptosis and decreases proliferation in cervical cancer cells; hence, PTEN inhibition may represent a promising strategy for the treatment of cervical cancer.

Introduction

Uterine cervical cancer is the fourth most common cancer and the leading cause of death from cancer among women worldwide (1). Although cervical cancer incidence and mortality rates have decreased in recent years because of screening programs and early detection of preinvasive cervical lesions, cervical cancer remains the second most common female cancer and the third leading cause of cancer-associated death among women. The mortality rate of cervical cancer is exceptionally high in developing countries (1). The standard of care for patients with recurrent cervical cancer is platinum-based chemotherapy; however, chemotherapy is mostly palliative, and new targeted therapeutic agents are urgently needed (2).

Phosphatase and tensin homolog deleted in chromosome 10 (PTEN) is an important tumor suppressor that is frequently mutated or downregulated in various human cancer types (3-9). PTEN mutation or downregulation can significantly enhance carcinogenesis and worsen treatment outcomes (3-9). In a recent study, in contrast to previous reports, high PTEN expression was associated with a worse prognosis in patients with wild-type p53 breast cancer (10).

PTEN and p53 interact closely, with PTEN regulating p53 function and p53 enhancing PTEN transcription (11). In PTEN-/-mice, the loss of PTEN dramatically decreased p53 protein levels (12). Nevertheless, acute loss of PTEN increased the levels and enhanced the function of p53 in prostate cancer cells (13). In a xenograft model of prostate cancer, long-term PTEN inhibition with the water-soluble vanadium-based complex VO-OHpic significantly decreased the tumor burden and prolonged the survival of tumor-bearing mice (14). Furthermore, PTEN downregulation in renal epithelial cells resulted in dedifferentiation and growth arrest (15). These anti-proliferative effects of PTEN silencing were p53-dependent (15).

However, the role of PTEN in cervical cancer remains unclear. In the present study, the effects of PTEN silencing on the proliferation, apoptosis and cell cycle of cervical cancer cells were investigated. The mechanisms underlying the effects of PTEN downregulation on the proliferation of cervical cancer cells were also explored.

Materials and methods

Cell lines and cultures. The human cervical cancer cell lines HeLa and CaSki (Korean Cell Line Bank) were used. Both cell lines were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% (v/v) fetal bovine serum (FBS, Welgene, Inc.), 1% (v/v) penicillin and streptomycin. Cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

Chemicals and reagents. To minimize the possibility of cross-reactivity, cells were transfected with two different small interfering (si)RNAs targeting PTEN: siRNA1, CCA GUC AGA GGC GCU AUG UdTdT; and siRNA2, CAA GAU GUU

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UGA AAC UAU (16,17). A p53-targeting siRNA (CUA CUU CCU GAA AAC GdTdT) was also used (18). A commercial scrambled siRNA was used as a negative control (Bioneer Corporation). Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) was used as the transfection reagent. Opti-MEM (minimal essential medium; Gibco; Thermo Fisher Scientific, Inc.) was used to prepare the siRNA-lipid complexes. For cell cycle analyses, a FITC BrdU Flow kit (BD PharMingen; BD Biosciences) was used. An APO-BrdU TUNEL Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to assess apoptosis. An Annexin V-FITC kit (BD PharMingen; BD Biosciences) was used to visualize apoptotic cells under a fluorescence microscope. An EZ-cyTox kit (DoGenBio) was used to evaluate cell viability. The following primary antibodies were used: PTEN (1:1,000; cat. no. SC-6817-R; Santa Cruz Biotechnology, Inc.), cyclin D1 (1:1,000; cat. no. SC-718; Santa Cruz Biotechnology, Inc.), p53 (1:1,000; cat. no. SC-126; Santa Cruz Biotechnology, Inc.), actin (1:5,000; cat. no. SC-1615; Santa Cruz Biotechnology, Inc.), p27 (1:1,000; cat. no. 3686; Cell Signaling Technologies, Inc.), p21 (1:1,000; cat. no. 2947; Cell Signaling Technologies, Inc.), cyclin dependent kinase (cdk)-6 (1:1,000; cat. no. 3136; Cell Signaling Technologies, Inc.), cyclin E (1:1,000; cat. no. 4129; Cell Signaling Technologies, Inc.), cyclin A (1:1,000; cat. no. 4656; Cell Signaling Technologies, Inc.), cyclin B1 (1:1,000; cat. no. 4138; Cell Signaling Technologies, Inc.), PARP (1:1,000; cat. no. 9542; Cell Signaling Technologies, Inc.), caspase-3 (1:1,000; cat. no. 9665; Cell Signaling Technologies, Inc.), AKT (1:2,000; cat. no. 4691; Cell Signaling Technologies, Inc.), pAKT (Thr308) (1:1,000; cat. no. 13038; Cell Signaling Technologies, Inc.), ERK1/2 (1:1,000; cat. no. 9122; Cell Signaling Technologies, Inc.), pERK1/2 (1:1,000; cat. no. 4370, Cell Signaling Technologies, Inc.), Bax (1:1,000; cat. no. 5023; Cell Signaling Technologies, Inc.), and MDM2 (1:1,000; cat. no. ab16895; Abcam). Goat anti-mouse IgG-HRP (1:5,000; cat. no. sc-2005) and mouse anti-rabbit IgG-HRP (1:5,000; cat. no. sc-2357) secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.

PTEN downregulation by siRNA. To synchronize the cell cycle, HeLa and CaSki cells were cultured in 100-mm culture dishes ($1x10^6$ cells/dish) under serum-starvation and antibiotic-free conditions. After 48 h, the cell culture medium was refreshed and the cells were maintained under serum-starvation and antibiotic-free conditions. Cells were transfected with siRNAs targeting PTEN and a negative control using the Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. Briefly, siRNA-lipid complexes were prepared in 500 μl Opti-MEM media by mixing 20 μl transfection reagent and 20 μM siRNAs (PTEN and negative control) for 5 min at room temperature. The mixtures were added to the cells in 10 ml culture medium. After 6 h of transfection, the medium was replaced with RPMI-1640 containing 10% (v/v) FBS, 1% (v/v) penicillin and streptomycin. Cells were incubated in complete growth medium for 48 h.

Western blot analysis. Transfected cells were harvested and lysed in RIPA buffer supplemented with protease inhibitors. Protein concentration was determined using BCA protein assay (Thermo Fisher Scientific, Inc.). After incubation for 30 min on ice, cell lysates containing 10-30 μg protein per well were resolved by 10-12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dried skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (pH 7.6) overnight at 4˚C with agitation. Subsequently, membranes were incubated with primary antibodies overnight at 4˚C with agitation. After three washes with 1X Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 1 h at room temperature. Signals were developed with a chemiluminescence reagent (Advansta); chemiluminescence was detected using an Amersham Imager 600 (GE Healthcare Biosciences AB).

Flow cytometry. Bromodeoxyuridine (BrdU) incorporation analysis was conducted to evaluate cell-cycle progression. Briefly, serum-starved siRNA-transfected cells ($5x10^5$ cells in 100-mm culture dish) were cultured in complete growth media for 48 h and then labeled with 10 μM BrdU at 37˚C for 60 min. After labeling with BrdU, cells were harvested and immunostained with FITC-conjugated anti-BrdU according to the manufacturer's instructions. BrdU incorporation was evaluated by flow cytometry (BD-FACSCalibur; BD Biosciences).

Apoptosis was assessed by measuring the amount of 5-bromo-2-deoxyuridine 5-triphosphate (BrdUTP) incorporated into the DNA using flow cytometry. Transfected cells were harvested, fixed with paraformaldehyde [1% (w/v) in PBS], and labeled with 8.0 μl BrdUTP (APO-BrdU TUNEL Assay kit) and 31.25 μl H_2O according to the manufacturer's instructions. Stained cells were analyzed on a BD-FACSCalibur, (BD Biosciences).

Visualization of apoptotic cells by fluorescence microscopy. To confirm the role of PTEN silencing in apoptosis, Annexin V-FITC staining was performed, followed by fluorescence microscopy (magnification, x200). Briefly, transfected HeLa cells were washed twice with PBS and once with Annexin V binding buffer. Subsequently, cells were stained with Annexin V-FITC (1:10 in Annexin V binding buffer) for 15 min at room temperature. Cells were washed and observed under a confocal microscope (LSM 700; Carl Zeiss AG). ImageJ software was used for quantification.

MTT assay. The viability of HeLa and CaSki cells was analyzed following transfection with PTEN and control siRNAs. Briefly, siRNA-transfected cells were incubated with water-soluble tetrazolium salts (1/10 of the volume of the culture medium; EZ-cyTox, DoGenBio) at 37˚C for 2 h. Optical absorbance at 450 nm was measured using a microplate reader. The optical absorbance of the culture medium alone was measured as blank.

Statistical analysis. Data were expressed as mean ± standard deviation. All experiments were performed more than three times. Independent-t tests and Mann-Whitney U tests were performed using SPSS 17.0 (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

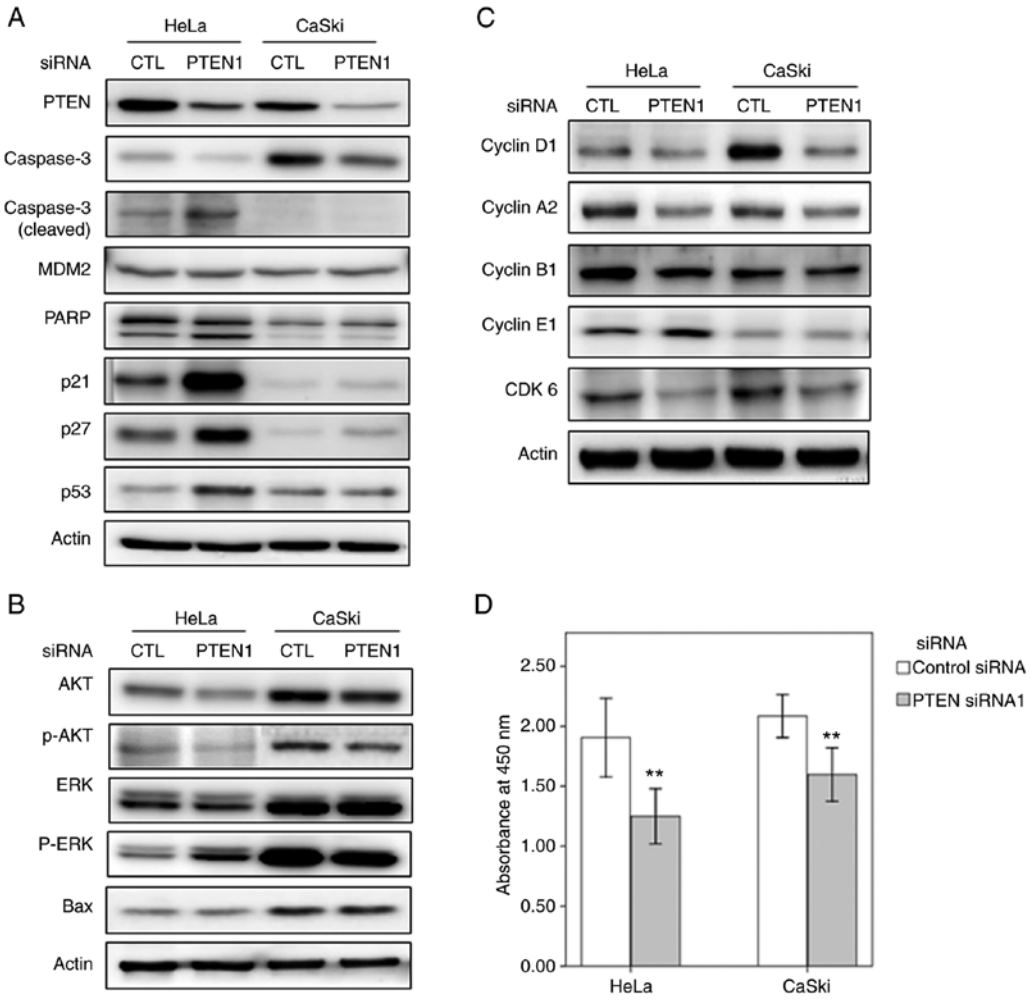

Figure 1. PTEN silencing regulates the expression levels of proteins involved in apoptosis and the cell cycle. (A-C) Western blots showing the levels of proteins involved in apoptosis (caspase-3, MDM2, PARP, p21, p27, p53 and Bax), cell cycle (cyclin D1, cyclin A2, cyclin B1, cyclin E1 and CDK6), and AKT and ERK signaling pathways in cervical cancer cells transfected with PTEN siRNA1. (D) Cell viability of HeLa and CaSki cells transfected with PTEN-targeting and control siRNAs (error bars represent 95% confidence intervals). **P<0.01 vs. control siRNA. siRNA, small interfering RNA; CTL, control siRNA; PTEN1, PTEN siRNA1.

Results

PTEN downregulation enhances the expression of apoptosis-associated proteins. In order to assess the effects of PTEN downregulation on the expression levels of proteins involved in cellular fate, western blotting was performed to measure the levels of proteins associated with cellular apoptosis and senescence. Notably, PTEN silencing in HeLa cells increased the levels of p53 and p53-associated proteins (Fig. 1A). Although PTEN silencing in CaSki cells did not alter the levels of p53, levels of p21 and p27 were increased in PTEN siRNA-transfected cells.

p53 is regulated by the extracellular signal regulated kinase (ERK) and is essential for the activation of downstream ERK pathway components. Activation of caspase-3 and poly (ADP-ribose) polymerase (PARP) by ERK induces apoptosis (19,20). PARP is a substrate for caspase-3, and PARP cleavage promotes apoptosis by inhibiting DNA repair (21). In the study, PTEN silencing in HeLa cells increased the levels of phosphorylated (p)ERK, p53, caspase-3 and cleaved PARP; Bax, levels remained unchanged (Fig. 1B). It was also investigated whether PTEN downregulation affects cell proliferation and viability. Indeed, PTEN silencing inhibited the proliferation of HeLa and CaSki cells, implying that PTEN downregulation induces apoptosis in cervical cancer cells (Fig. 1D).

PTEN downregulation represses the expression of cell cycle-associated proteins. To achieve cellular senescence, an increased amount of Cdk inhibitor p21, a target of p53, can inactivate both cyclin E and cyclin D1-associated kinase (22,23).

In order to examine the role of PTEN in cell-cycle regulation in cervical cancer cells, the levels of cell-cycle-associated proteins were analyzed after PTEN silencing. The levels of Cdk6, cyclin D1 and cyclin A2 were decreased after PTEN silencing (Fig. 1C). However, PTEN downregulation did not alter the levels of cyclin E1 and cyclin B1 in HeLa or CaSki cells (Fig. 1C).

Furthermore, flow cytometry analyses revealed that the proportion of cells in the G_0/G_1 and sub-G_1 phases was increased after PTEN silencing. On the other hand, the proportion of cells in the S-phase was decreased when PTEN was downregulated (Figs. 2 and S1). These findings imply that PTEN downregulation promotes overexpression of p53 and p21, and causes apoptosis and G_1 cell-cycle arrest in HeLa cells (4,13,15,24).

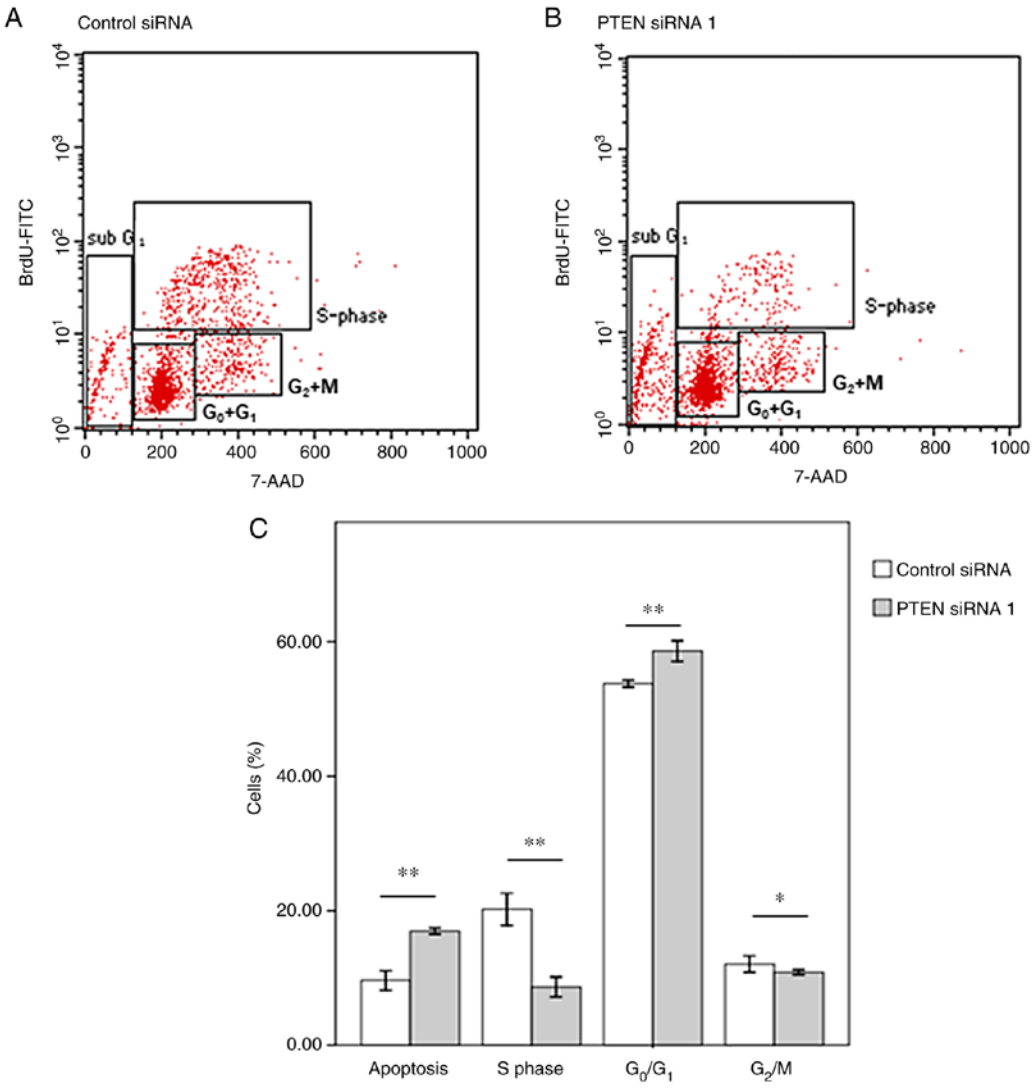

Figure 2. PTEN silencing regulates cell-cycle progression in HeLa cells. The cell-cycle phase in control (A) and PTEN siRNA1-transfected (B) cells. (C) Mean percentage of the cell population in each cell-cycle phase (error bars represent 95% confidence intervals). *P<0.05 and **P<0.01 vs. control siRNA. siRNA, small interfering RNA.

PTEN downregulation induces apoptosis independently of p53. In order to confirm the role of PTEN in the apoptosis of cervical cancer cells, TUNEL assay and flow cytometry were performed using PTEN siRNA-transfected HeLa cells. As shown in Fig. 3A and B, PTEN silencing increased the proportion of HeLa cells undergoing apoptosis. To investigate the role of p53 in the apoptosis of cells with PTEN downregulation, HeLa cells were co-transfected with siRNAs targeting PTEN and p53. Cells co-transfected with PTEN-targeting and scrambled siRNAs were used as a control. PTEN promoted apoptosis in cervical cancer cells regardless of the levels of p53 (Fig. 3B and C). These results imply that p53 is not essential for the induction of apoptosis in HeLa cells in response to PTEN downregulation.

Discussion

Regulation of DNA damage during the cell cycle is essential for cell survival. Cells with unrepaired DNA damage undergo apoptosis or cell-cycle arrest due to the activation of DNA-damage-response pathways (25).

PTEN and p53 are crucial for DNA damage repair and the maintenance of genomic integrity (26). Although PTEN and p53 are often downregulated in cervical cancer, somatic mutations in PTEN are rare, ranging from 0 to 2% (7,27); p53 mutations have been reported in 13.3% of cervical adenocarcinomas and 5.9% of cervical squamous cell carcinomas (28). Although downregulation of PTEN and p53 has been reported in cervical cancer (8,29), no somatic mutations in these genes were detected in HeLa and CaSki cells (30,31), allowing cancer cells to survive despite having unresolved DNA damage. In the present study, PTEN downregulation promoted apoptosis and cell-cycle arrest in cervical cancer cells (Figs. 1 and 2), implying that loss of PTEN impairs the ability of cells to repair DNA damage. Unrepaired DNA damage in cervical cancer cells may promote apoptosis by activating DNA-damage-response pathways.

The PTEN, PI3K and AKT signaling pathways are critical regulators of cell proliferation and apoptosis (3,32). The downregulation of PTEN is associated with the activation of PI3K and AKT signaling, cell proliferation and a worse prognosis

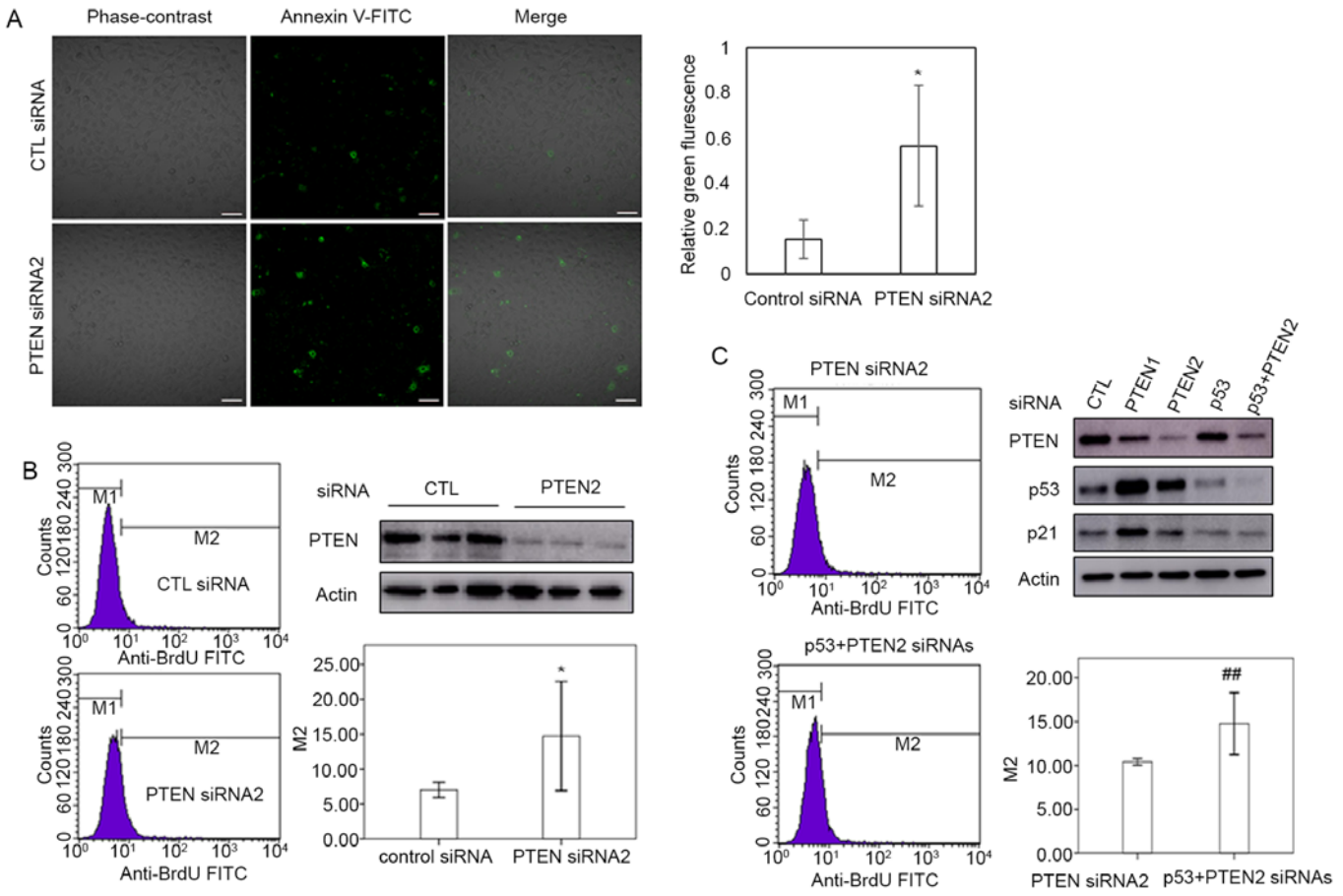

Figure 3. PTEN downregulation induces apoptosis independently of p53 in HeLa cells. (A) Apoptotic cells were visualized by fluorescence microscopy. Cells transfected with control and PTEN siRNA2 were fixed and stained with Annexin V-FITC. The graph shows quantitative analysis of relative Annexin V fluorescence intensity. ImageJ software was used for quantification. (B) Flow cytometry analysis of control and PTEN siRNA2-transfected cells. PTEN expression in control and PTEN siRNA2-transfected cells was evaluated by western blotting. The graph shows the percentage of BrdU staining in control and PTEN siRNA-transfected cells. (C) Flow cytometry analysis of cells transfected with control siRNA + PTEN siRNA2 or PTEN siRNA2 + p53 siRNA (error bars represent 95% confidence intervals). Western blots showing the expression levels of PTEN, p53, p21 and actin. The graph shows the percentage of BrdU-positive cells in the control + PTEN siRNA2 and p53 + PTEN siRNA2 groups. *P<0.05 vs. control siRNA; $^{\#\#}$P<0.01 vs. control siRNA + PTEN siRNA2. Scale bar, 50 μm. siRNA, small interfering RNA; CTL, control siRNA; C, control siRNA; PTEN1, PTEN siRNA1; PTEN2, PTEN siRNA2.

in various cancer types (3-5). However, Tan and Chiu (20) reported that the activation of ERK inhibited the AKT pathway. In the present study, loss of PTEN activated the ERK pathway; nevertheless, the expression levels of MDM2 and phospho-AKT were unchanged regardless of the PTEN status in HeLa cells (Fig. 1A and B).This implies that PTEN regulates p53 levels independently of MDM2 and AKT, consistent with a previous report (12). In contrast to the present observations in HeLa cells, PTEN silencing did not affect AKT activation or MDM expression in CaSki cells. Thus, the regulation of the PTEN-ERK-p53-PARP axis may differ depending on cell type.

Although the loss of both PTEN and p53 enhances tumor aggressiveness, the inactivation of PTEN with intact p53 induces cellular senescence (13). Similar regulatory mechanisms have been reported for BRCA1/2 and p53 in breast cancer (33,34). Furthermore, PTEN inhibition in hepatocarcinoma cells expressing low levels of PTEN has been shown to drive senescence (35). Although PTEN downregulation is common in cervical cancer (8,9), somatic mutations in PTEN are rare (7,27). Therefore, most cervical cancer have intact PTEN, but expressed at low levels, similar to reports regarding hepatocarcinoma (35). In the present study, PTEN inhibition in cervical cancer cells expressing low levels of PTEN promoted cellular senescence and apoptosis, which was independent of p53 status. Therefore, PTEN downregulation in cervical cancer may result in p53 upregulation, thereby inducing apoptosis in cervical cancer cells (Fig. 1).

In conclusion, the present data imply that PTEN downregulation in cervical cancer cells induces cell-cycle arrest and apoptosis. Furthermore, decreased cell viability was observed as a result of PTEN downregulation in cervical cancer cells. Taken together, the present study findings may present a possible strategy for cervical cancer treatment via the regulation of PTEN. More studies will need to follow that will not only detail the effects of PTEN regulation but also reinforce some of the limitations of the present study, which include lack of various forms of analysis and clear mechanistic study. The limitations of the present study will be addressed in a subsequent study, which will mainly focus on elucidating the role of p53 in cervical cancer cell apoptosis by checking gene expression levels of components that are essential for apoptosis induced by PTEN downregulation.

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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

JWS and JYY conducted the experiments and analyzed the results. JWS designed the study and wrote the manuscript. SHK performed analysis and interpretation of data, and the overall editing of the manuscript. JYY and SHK confirmed the authenticity of all the raw data. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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