科技部補助專題研究計畫成果報告
期末報告

原薯蕷皂合併免疫療法與放射治療對子宮頸癌細胞治療協同效應之分子機轉研究

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中 華 民 國 107 年 10 月 01 日

中 文 摘 要 ： Protodioscin (PD)中文名稱為原薯蕷皂苷，主要來自穿龍薯蕷(Dioscorea nipponica)以及蒺藜(Tribulus terrestris)根莖部所萃取的天然物之一。過去研究PD被發現的生物功能中包括抗腫瘤功能，但是目前PD對於人類子宮頸癌的癌細胞之抗癌特性及分子機制仍不清楚。本研究利用protodioscin處理人類子宮頸癌細胞，以MTT、annexin-V雙染方式證實protodioscin會誘導子宮頸癌細胞凋亡，同時protodioscin也會誘導caspase-3、-8、-9和PARP(poly(ADP-ribose) polymerase)活化。Protodioscin也會誘導粒線體膜電位改變、Bax蛋白表現增加、Bcl-2蛋白表現降低，造成cytochrome c釋放。另外也發現在protodioscin的處理下可誘發內質網壓力(ER stress)，當細胞產生ER stress時，GRP78、PERK、p-eIF-2α、ATF-4 和CHOP磷酸化都有明顯增加的現象。同時我們發現protodioscin也會誘導ROS (Reactive oxygen species)產生，處理ROS抑制劑(NAC)會明顯減緩PD 誘導內質網壓力和細胞凋亡。轉殖GRP78/CHOP-siRNA可以有效抑制PD誘導內質網壓力的細胞凋亡。此外PD明顯誘導p38和JNK活化，共同處理JNK抑制劑 (SP600125) 或p38抑制劑 (SB203580)會減緩PD誘導細胞內質網壓力和凋亡作用。另外，PD會增加細胞核內ATF4和CHOP蛋白表現及增進ATF4結合到CHOP啟動子。綜合以上結果證實PD當作抗癌藥物的發展上深具潛力。

中 文 關 鍵 詞 ： 原薯蕷皂苷,活性氧分子, 內質網壓力,細胞凋亡,MAPK,子宮頸癌

英 文 摘 要 ： Protodioscin (PD) is a steroidal saponin with anti-cancer effects on a number of cancer cells, but the anti-tumor effects and mechanism of action of PD on human cervical cancer cells is unclear. We demonstrated that PD inhibits cell viability, causes a loss of mitochondrial function, and induces apoptosis, as evidenced by up-regulation of caspase-8, -3, -9, -PARP, and Bax activation, and down-regulation of Bcl-2 expression. PD was shown to induce ROS and the ER stress pathway, including GRP78, p-eIF-2α, ATF4, and CHOP. Pre-treatment with NAC, a ROS production inhibitor, significantly reduced ER stress and apoptosis-related proteins induced by PD. Transfection of GRP78/CHOP-siRNA effectively inhibited PD-induced ER stress-dependent apoptosis. Moreover, treatment with PD significantly increased p38 and JNK activation. Co-administration of a JNK inhibitor (SP600125) or p38 inhibitor (SB203580) abolished cell death and ER stress effects during PD treatment. In addition, PD induced the expression of nuclear ATF4 and CHOP, as well as the binding ability of ATF4 to the CHOP promoter. Conclusion: Our results suggest that PD is a promising therapeutic agent for the treatment of human cervical cancer.

英 文 關 鍵 詞 ： Protodioscin; reactive oxygen species; endoplasmic reticulum stress; apoptosis; MAPK; cervical cancer

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計畫主持人：應宗和

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計畫參與人員：王佩涵、林佳良

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中　華　民　國　　年　　月　　日

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中文摘要

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Protodioscin (PD) is a steroidal saponin with anti-cancer effects on a number of cancer cells, but the anti-tumor effects and mechanism of action of PD on human cervical cancer cells is unclear. We demonstrated that PD inhibits cell viability, causes a loss of mitochondrial function, and induces apoptosis, as evidenced by up-regulation of caspase-8, -3, -9, -PARP, and Bax activation, and down-regulation of Bcl-2 expression. PD was shown to induce ROS and the ER stress pathway, including GRP78, p-eIF-2α, ATF4, and CHOP. Pre-treatment with NAC, a ROS production inhibitor, significantly reduced ER stress and apoptosis-related proteins induced by PD. Transfection of GRP78/CHOP-siRNA effectively inhibited PD-induced ER stress-dependent apoptosis. Moreover, treatment with PD significantly increased p38 and JNK activation. Co-administration of a JNK inhibitor (SP600125) or p38 inhibitor (SB203580) abolished cell death and ER stress effects during PD treatment. In addition, PD induced the expression of nuclear ATF4 and CHOP, as well as the binding ability of ATF4 to the CHOP promoter. Conclusion: Our results suggest that PD is a promising therapeutic agent for the treatment of human cervical cancer.

Key word: Protodioscin; reactive oxygen species; endoplasmic reticulum stress; apoptosis; MAPK; cervical cancer

前言

Cervical cancer is the third leading cause of mortality in women worldwide, even though strategies for prevention and treatment have rapidly developed in recent decades [1]. Nevertheless, effective treatment options for cervical cancer patients are limited. The current approach to treating cervical cancer includes surgery, radiation treatment, and cytotoxic chemotherapy [2]. Identification of the molecular mechanisms underlying novel targeted therapeutic strategies is therefore an important objective for improved treatment of cervical cancer patients.

Protodioscin (PD) is the main steroidal saponin of the Tribulus and Dioscoreae families, such as the Dioscoreae rhizome (Shan-yao in Chinese) [3]. Dioscoreae rhizome is not only an important food in China, but according to traditional Chinese medicine, the Dioscoreae rhizome is good for the stomach and spleen, and it has been shown that PD significantly improves glucose intolerance, metabolic syndrome, and renal injury [4]. PD also revealed potential neuroprotection against the ischemia-reperfusion injury [5]. In addition, PD exhibits growth inhibition on HL-60 cells by inducing apoptosis [6] and anti-cancer effects in several types of cancer cells [7]. In the current study, the anti-tumor effect and molecular mechanisms initiated by PD in cervical cancer cells have not been investigated.

Imbalances in reduction-oxidation, Ca^{2+} homeostasis, and erroneous protein folding induce the accumulation of misfolded or unfolded proteins in the endoplasmic reticulum (ER). Such an accumulation is referred to as the unfolded protein response (UPR); the UPR results in ER stress [8]. Recent studies have demonstrated that ER stress is involved in cancer, metabolic disorders, inflammatory diseases, and neuro-degenerative diseases [9]. ER stress involves the following three specific stress transducers: protein kinase RNA-like endoplasmic reticulum kinase (PERK); inositol-requiring enzyme 1 (IRE1); and activating transcription factor 6 (ATF6). PERK and IRE1 are activated by disassociating from GRP78 (BiP), then induce phosphorylation of the eukaryotic translation initiation factor 2 subunit α (eIF-2α)/ATF-4/CHOP signaling pathway, which has multiple downstream targets that stimulate apoptosis and cell death [10]. For example, apoptotic cell death ensues following ATF4-CHOP-mediated induction of several pro-apoptotic genes and suppression of the synthesis of anti-apoptotic Bcl-2 proteins [11]. CHOP induces the production of ROS in the ER by inhibition of cellular anti-oxidant enzymes [12]. Therefore, inhibition of oxidative stress is considered a potential anti-tumor therapy for cervical cancer.

Reactive oxygen species (ROS) are molecules derived from oxygen that have accepted extra electrons and can oxidize other molecules [13]. Superoxide, hydrogen peroxide, and hydroxyl radicals are the most-well studied ROS in cancer cells [14]. A previous study has shown that ROS are capable of causing extensive damage to DNA, proteins, and lipids, and thus it is believed that ROS is tumorigenic by increasing genomic instability [15]. Basal levels of ROS are essential for cell physiologic function, whereas overflowing ROS formation induces apoptosis and cell cycle arrest in cancer [16, 17]. Previous studies have demonstrated that ROS induces ER stress-dependent apoptosis through depletion of calcium stores in the ER via inhibition of Ca2+-ATPase [18]. Therefore, the present study showed that ROS generation and the ER stress pathway are required for PD-induced apoptosis via activation of the p38/JNK and GRP78/p-eIF2-α/ATF4/CHOP axis in human cervical cancer cells.◦

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研究方法

[1] Chemicals and reagents：Antibodies against β-actin, Bax, Bcl-2, cytochrome c, p-ERK, ERK, p-JNK, JNK, p-p38, p38, siRNAs-CHOP (siCHOP), and siRNA-GRP78 (siGRP78), and horseradish peroxidase-conjugated anti-mouse, -goat, and -rabbit secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, cleaved-PARP, GRP78, PERK, p-eIF-2α, eIF2-α, ATF4, CHOP, and IRE-1α were purchased from Cell Signaling Technology (Beverly, MA, USA). Protodioscin, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), N-acetylcysteine (NAC), and JC-1 were purchased from Sigma (St. Louis, MO, USA). SP600125 (a JNK inhibitor) and SB203580 (a p38 inhibitor) were bought from Calbiochem (San Diego, CA, USA). The ER-ID® Red assay kit was purchased from Enzo Life Sciences (NY, USA).

[2] Cells and cell culture：The human cervical cancer cell lines, HeLa and C33A, were purchased from the Bioresources Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan). HeLa cells were maintained in Dulbecco’s modified Eagle’s/Ham’s F-12 medium (DMEM/F12; Gibco-Invitrogen Corporation) and C33A cells were maintained in minimum essential medium (MEM; Gibco-Invitrogen Corporation). Cell culture media were supplemented with 10 % fetal bovine serum (FBS; Gibco-Invitrogen Corporation) and 1 % penicillin/streptomycin (Hyclone, Logan, UT, USA) in a humidified 5% CO2 atmosphere at 37 ˚C.

[3] Cell viability assay：Cell viability was measured using the MTT assay. The cells (1 × 105/mL) were seeded in 24-well plates and cultured with 0.1% DMSO or PD (2, 4, and 8 μM) for 24 h. At the end of treatment, the media were replaced with fresh medium and the MTT reagent (0.5 mg/mL), and incubated at 37 ˚C for 4 h. The production of formazan followed solubilization with 1 mL of isopropanol, and the color intensity was measured at 570 nm using a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). The experiments were performed in triplicate.

[4] Annexin V-FITC/PI double-stained assays：Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection kit (BD Bioscience, Becton Dickinson Co., USA)) as previously reported [19]. The cells (2 × 105/mL) were seeded in 6-cm dishes and cultured with 0.1% DMSO or PD (2, 4, and 8 μM) for 24 h. At the end of treatment, the cells were collected, then fixed and stained in 1X binding buffer (10 mM

HEPES/ NaOH, 140 mM NaCl, and 2.5 mM CaCl2 [pH 7.4]) with 5 μl of PI solution and 5 μl of FITC-conjugated Annexin V for 30 min in the dark at room temperature. The apoptotic cells were detected with a FACS Calibur flow cytometry (BD FACSCalibur, Becton Dickinson Co., Franklin Lakes, NJ, USA) and the data were analyzed by Cell Quest software.

[5] Measurement of mitochondrial membrane potential (MMP) by flow cytometry：The mitochondrial membrane potential was measured by flow cytometry using JC-1 reagent [20]. Briefly, PD-treated cells were incubated with 30 μM JC-1 reagent for 20 min at 37 ˚C in the dark. After incubation, the cells were washed with PBS and analyzed within 30 min using a FACS Calibur flow cytometry (BD FACSCalibur, Becton Dickinson Co., Franklin Lakes, NJ, USA).

[6] Flow cytometry and fluorescence microscopy using ER-ID red staining：Cells were seeded at a density of 2×105 cells/mL in 6-cm culture dishes for 24 h, then treated with various concentrations of PD for the indicated times. The cells were incubated with ER-ID® Red assay kit (Enzo Life Sciences, Inc., Farmingdale, NY. USA) at 37 ˚C for 15 min in the dark. The ER stress level was quantified using FACS Calibur flow cytometry. The cells incubated with ER-ID red detection reagent and nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) for 15 min. The cells were visualized using fluorescence microscopy (Olympus, Hamburg, Germany).

[7] Measurement of intracellular ROS assay：Intracellular ROS production was measured, as described previously [21]. The cells (2 × 105/mL) were seeded in 6-cm dishes and cultured with 0.1% DMSO or PD (2, 4, and 8 μM) for 24 h. At the end of treatment, the cells were collected and resuspended in DCF-DA (10 μM), then incubated at 37 ˚C for 15 min. The cells were then rinsed with PBS before flow cytometry. The cells were detected using FACS Calibur flow cytometry and the data were analyzed with Cell Quest software. Data are expressed as the percentage of cells with intact ROS levels.

[8] Western Blotting：The cells were homogenized with 200 μL of lysis buffer. Cell debris was removed by centrifugation at 13,000 g for 20 min at 4 ˚C, and the protein concentration was determined using the Bradford assay. Samples were run on 8%-12% SDS-PAGE and subsequently electro-transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 2 h with 5% non-fat dry milk buffer. After blocking, the membranes were incubated with primary antibodies (1:1000) overnight. After washing, the membranes were incubated with HRP-conjugated anti-mouse (1:10000), -goat (1:10000), or -rabbit antibody (1:10000) at room temperature for 2 h. The reaction was visualized using an enhanced chemiluminescence (ECL) reagent (Millipore, Billerica, MA, USA), and detected using a ImageQuant LAS 4000 Mini (GE Healthcare Life Sciences, Marlborough, MA, USA).

[9] Chromatin immunoprecipitation (ChIP) assay: The cells were twice-washed with PBS, and cross-linked with 1 % formaldehyde for 10 min at room temperature. The formaldehyde was removed, then 0.125 M glycine was added for 5 min at room temperature. The cells were twice-rinsed with ice-cold PBS and a proteinase inhibitor, and the cells were collected in ice-cold PBS, then resuspended in lysis buffer. The cells were sonicated to produce DNA fragments 200-500 bp in length, followed by centrifugation to collect the supernatant. The DNA immunoprecipitation was performed with ATF4 antibody overnight. Then, cross-links were removed from the sample and DNA was isolated. PCR reactions were conducted with PCR Master Mix (Promega), which consisted of 35 cycles at 95˚C × 30 s, 50˚C × 30 s, and 72˚C × 1 min, followed by 10 min at 72˚C. PCR using primers for CHOP promoter regions were as follows: CHOP-1-F, 5’-GCCTCCAGAGTAGCTGGGAT-3’ and -R, 5’-CTTCTTAAAGAGGTCTCCTGGC-3’; and CHOP-2-F, 5’-GCCCCGCCCTCTCTCCTCTCC-3’ and -R, 5’-GTGGCTTTGGGTCACGAG-3’. The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The input DNA and rabbit IgG-pull DNA served as controls for all experiments.

[10] Statistical analysis: The results are presented as the mean ± standard error (SE) from three independent experiments. Data were analyzed using Instat software (GraphPad Prism4, San Diego, CA, USA). Student's t-test or one-way analysis of variance (ANOVA) with a post-hoc analysis using Tukey's multiple-comparison test was used for evaluating parametric data. A $p < 0.05$ or $p < 0.01$ was considered to be statistically significant.

實驗結果

PD inhibits cell viability and induces apoptosis in human cervical cancer cells

Human cervical cancer HeLa and C33A cells were treated with increasing concentrations of PD (2-16 μM) for 24 and 48 h, and cell viability was determined using the MTT assay. PD inhibited the cell viability by both cervical cancer cell lines in a concentration- and time-dependent manner (Fig. 1B and 1C). The apoptotic effect of PD on human cervical cancer cells was determined using Annexin V-FITC/PI double-staining, which showed that PD induced apoptosis in HeLa and C33A cells (Fig. 1D). To clarify the molecular mechanisms underlying PD-induced apoptosis, we showed that PD markedly induced the expression of cleaved-caspase-8, -9, and -3, and -PARP in treated cells (Fig. 1E). In addition, HeLa and C33A cells were pre-treated for 2 h with a pan-caspase inhibitor (Z-VAD), then incubated with PD (4 μM) for 24 h. Z-VAD significantly reversed PD inhibition of cell viability by the MTT assay (Fig. 1F). These findings suggest that PD-induced caspase activation to induce the apoptosis pathway in human cervical cancer cells.

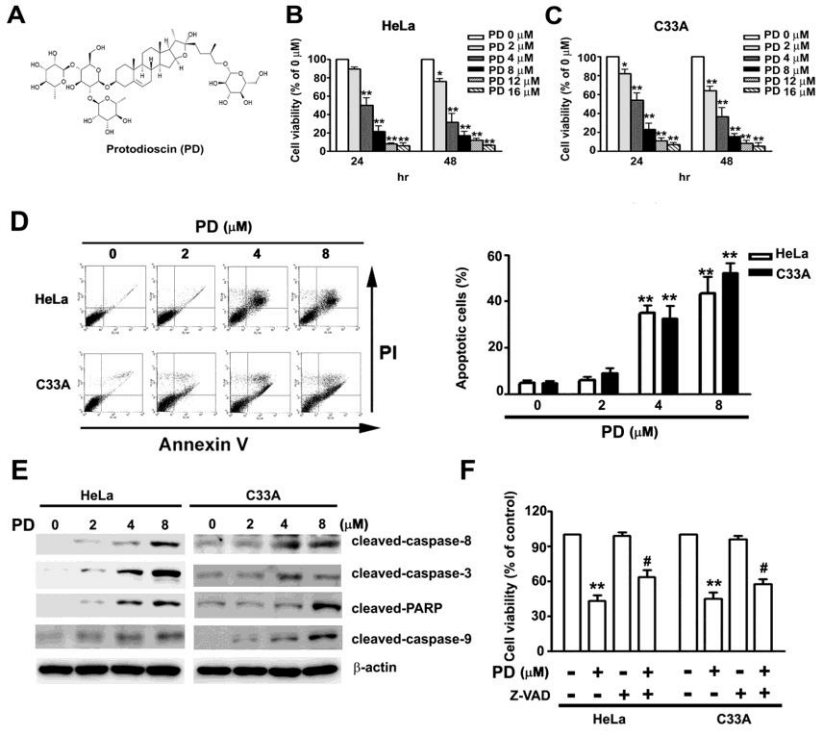

Figure 1. PD inhibits cervical cancer cell viability and induces cell apoptosis. **(A).** The structure of protodioscin (PD). **(B.C).** The effect of PD on cell viability of human cervical cancer cells for 24 and 48 h, respectively. Cell viability was determined by the MTT assay. **(D).** Apoptotic cells were detected with Annexin V/PI staining by flow cytometry analysis. **(E).** Cell lysates were analyzed by Western blotting. **(F).** HeLa and C33A cells were pre-treated with Z-VAD (20 μM) for 2 h, then incubated with PD (4 μM) for 24 h. Cell viability was determined using the MTT assay. **, P<0.01, control versus PD; #, P<0.01, PD versus Z-VAD-FMK plus PD. Data are presented as the mean ± SE of at least three independent experiments.

Effect of mitochondrial dysfunction-mediated apoptosis by PD treatment in cervical cancer cells

Mitochondria has a key role in the intrinsic pathway of apoptosis through sabotage of the mitochondrial membrane potential (MMP) [22]. We analyzed the effect of PD on the ΔΨm for changes in JC-1 fluorescence

intensity using flow cytometry. Loss of the ΔΨm in HeLa and C33A cells increased following PD treatment in a dose-dependent fashion (Fig. 2A), suggesting that mitochondria-mediated apoptosis is involved in activation of a series of molecular events by the Bcl-2 family of proteins, which are important regulators of mitochondrial permeability. The level of Bcl-2 protein expression decreased in response to PD treatment, while Bax and cytochrome c expression were increased (Fig. 2B). These results clearly demonstrated that PD-induced apoptosis involves the intrinsic and extrinsic caspase-cascades apoptosis pathways.

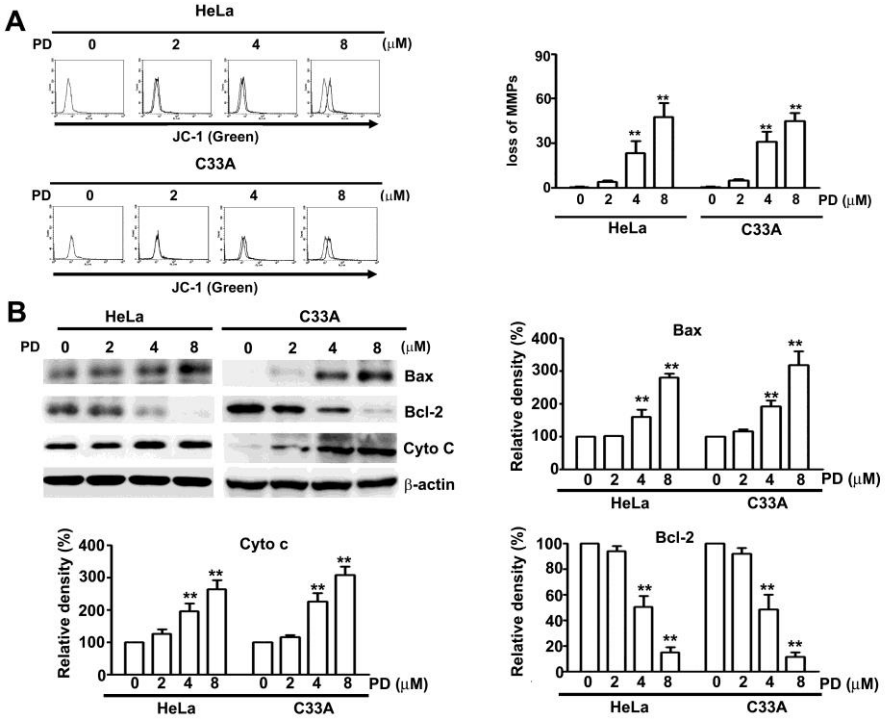

Figure 2. PD induces mitochondria dysfunction in HeLa and C33A cells. **(A).** HeLa and C33A cells were treated with various concentrations (0, 2, 4, and 8 μM) of PD for 24 h, then the mitochondrial membrane potential analyzed with JC-1 staining by using flow cytometry. (**B).** Cell lysates were subjected to Western blotting with anti-Bax, anti-Bcl-2, anti-cytochrome c, and anti-β-actin antibodies. β-actin was used as the loading control. Data are presented as the mean±SE of at least three independent experiments. **, P<0.01, compared with the control (0 μM)

PD induces the endoplasmic reticulum stress pathway in human cervical cancer cells

The ER is essential for most cellular activities and survival [23]. The role of ER stress in PD-induced cervical cancer cell apoptosis was further investigated. We found that PD significantly increased the fluorescent intensity of ER-ID red dye by fluorescence microscopy (Fig. 3A, upper). Quantification was performed with flow cytometry (Fig. 3A, lower) in both cell lines. Next, we investigated the effect of PD on activation of ER stress markers. While the levels of IRE-1α and eIF-2α protein were not altered by PD, the PERK, ATF4, CHOP, and phosphorylated eIF-2α levels were significantly increased following PD treatment in both cell lines (Fig. 3B). The results suggest that PD induced apoptosis through the GRP78-dependent ER stress signaling pathways.

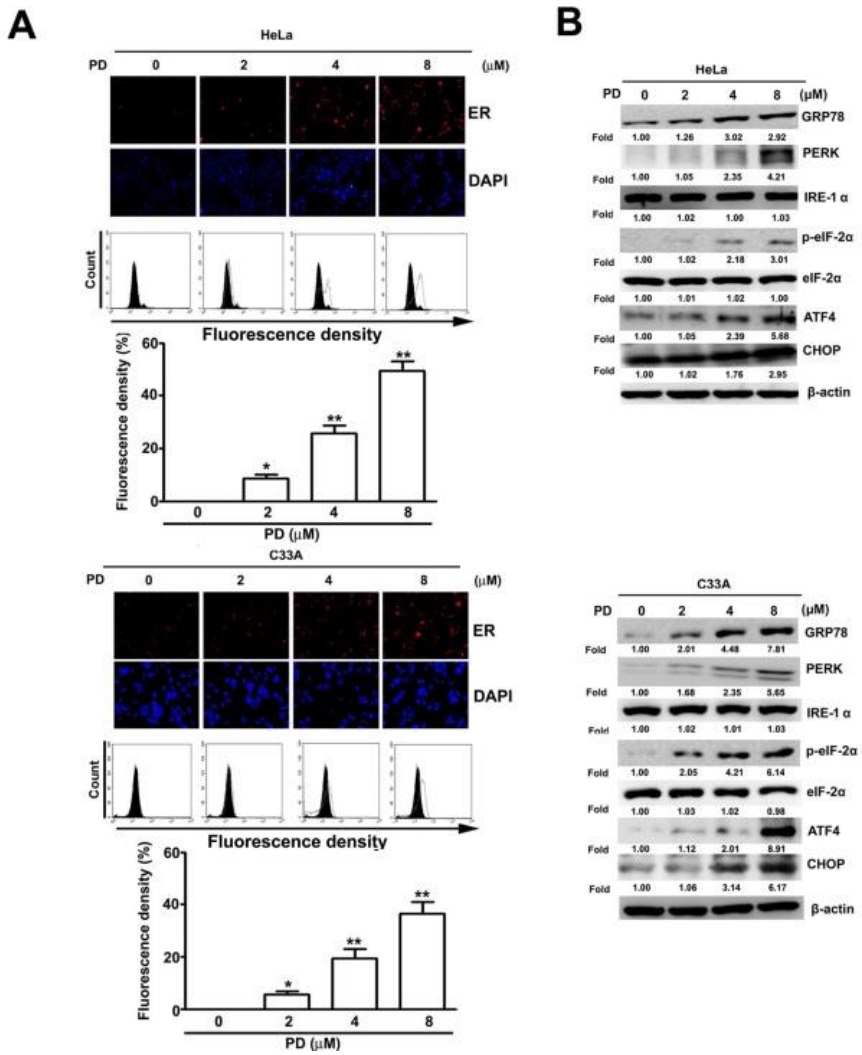

Figure 3. PD induces endoplasmic reticulum stress in HeLa and C33A cells**. (A)** HeLa and C33A cells were treated with various concentrations (0, 2, 4, and 8 μM) of PD for 24 h, then the ER stress analyzed with the ER-ID red assay kit using fluorescence microscopy (upper) and flow cytometry (lower). **(B)** The protein expression of ER stress markers (GRP78, PERK, IRE1-α, p-eIF-2α, eIF-2α, ATF4, and CHOP). β-actin was used as the loading control.

To examine the role of GRP78 or CHOP in PD-induced apoptosis, specific siRNAs for GRP78 and CHOP were used to inhibit GRP78 or CHOP expression in HeLa cells. The combination of siRNA-CHOP or siRNA-GRP78 with PD significantly reversed cell viability (Fig. 4A), the number of apoptotic cells (Fig. 4B), and decreased ER stress levels (Fig. 4C) compared with PD alone. Western blots revealed that the combination of siRNA-CHOP with PD reduced cleaved caspase-8, -3, and -9 in HeLa cells compared with PD alone (Fig. 4D). The results were similar to siRNA-GRP78-treated cells (Fig. 4E). These results suggest that GRP78 and CHOP may play an important role in PD induction of ER stress-mediated apoptosis.

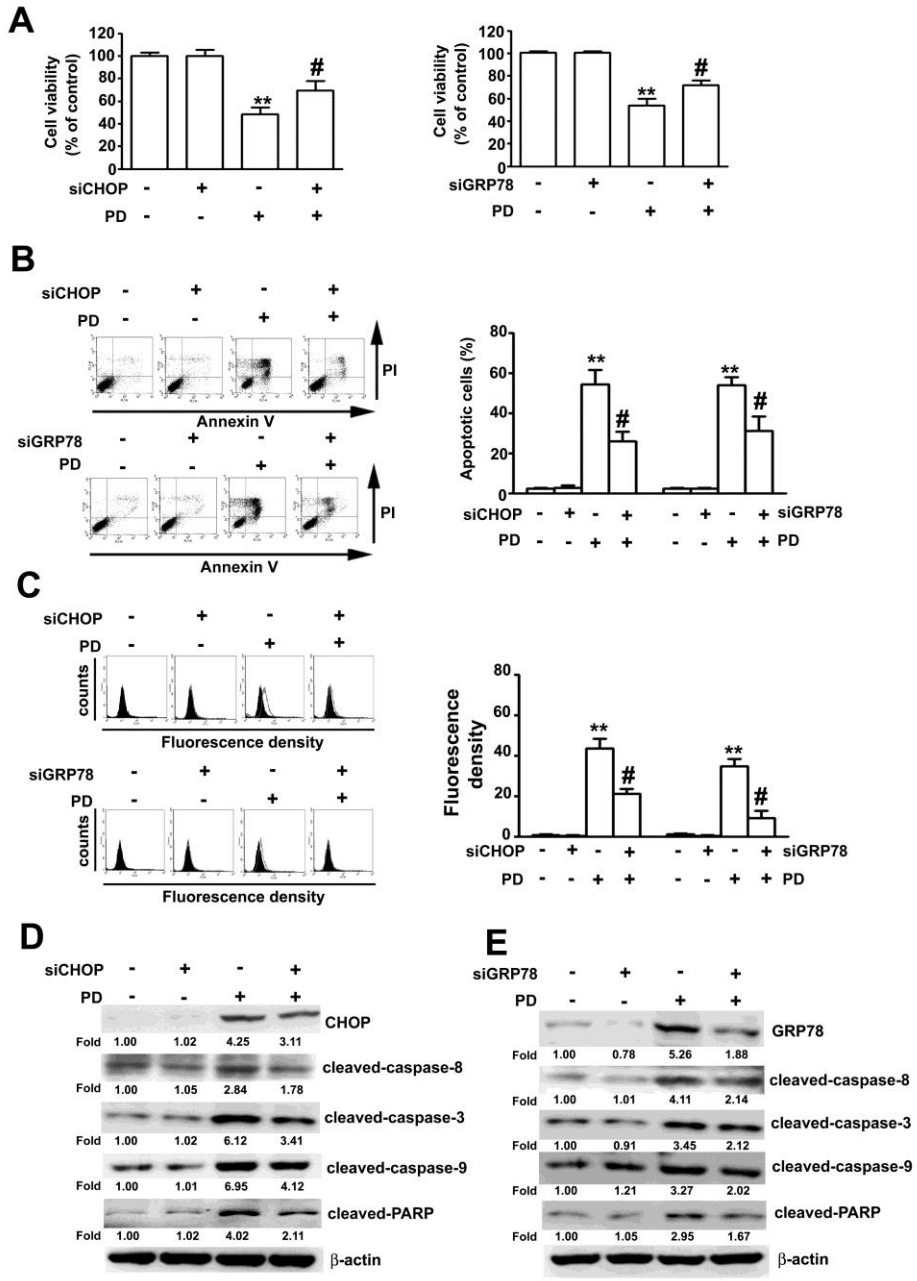

Figure 4. PD induces apoptosis through the GRP78/CHOP pathway in HeLa and C33A cells. HeLa cells were treated with PD (4 μM) in the absence or presence of si-CHOP or si-GRP78 (100 nM). **(A)** Cell viability was determined by using MTT assay. **(B)** The apoptotic cells were detected with Annexin V/PI stain by flow cytometry analysis. **(C).** The ER stress analyzed with ER-ID red assay kit using flow cytometry. **(D, E).** Cell lysates were subjected to Western blotting with anti-CHOP, anti-GRP78, anti-cleaved-caspase-8, anti-cleaved-caspase-3, anti-cleaved-PARP and anti-cleaved-caspase-9 antibodies. β-actin was used as the loading control. Data are presented as the mean±SE of at least three independent experiments. **, $P<0.01$, control versus PD; #, $P<0.01$, PD versus siRNA plus PD

Induction of the ER stress response and apoptosis by PD is dependent on JNK and p38 activation

MAPKs are involved in many aspects of the control of cellular proliferation and apoptosis and have been implicated in the regulation of gene expression in the ER stress signaling cascade in cervical cancer [24, 25]. We performed Western blotting to analyze MAPK signaling pathways induced by PD in cervical cancer cells,

and found that PD induced apoptosis through activation of JNK and p38 expression (Fig. 5), but did not alter the activation of ERK expression in HeLa and C33A cells (Fig. 5).

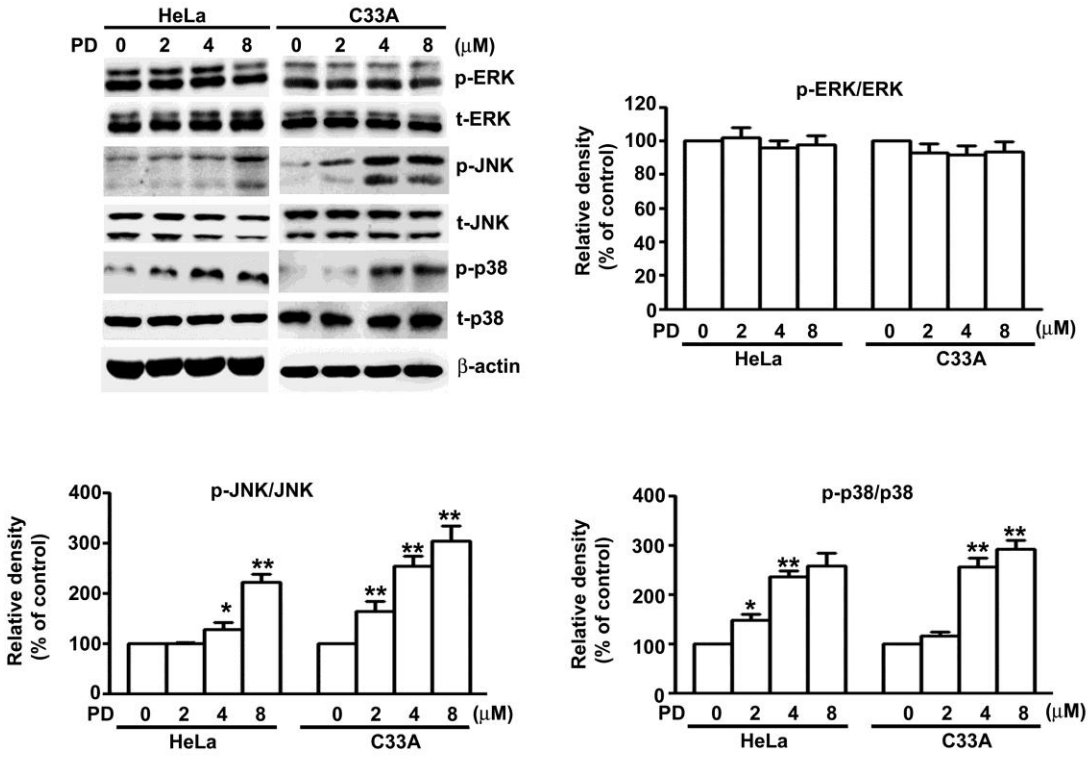

Figure 5. PD induced JNK and p38 activation in HeLa and C33A cells. HeLa and C33A cells were treated with various concentrations (0, 2, 4, and 8 μM) of PD for 24 h. Cell lysates were subjected to Western blotting for detecting the MAPK-related protein expression. The histogram represents the densitometric analysis of p-ERK/ERK, p-JNK/JNK, and p-p38/p38 protein expression. β-actin was used as the loading control. Data are presented as the mean±SE of at least three independent experiments. **, P<0.01, compared with the control (0 μM).

To further investigate the role of JNK and p38 pathways in PD-induced apoptosis, HeLa cells were pre-treated with SB203580 (10 μM) or SP600125 (2 μM), then PD (4 μM) was added for 24 h. We found that SB203580 or SP600125 treatment significantly reversed the PD-induced decrease in cell viability (Fig. 6A), cell apoptosis (Fig. 6B), ER stress (Fig. 6C), and mitochondrial dysfunction (Fig. 6D) compared with PD alone. In addition, SP600125 treatment inhibited activation of JNK expression, attenuated the PD up-regulation of GRP78, CHOP, ATF4, cleaved caspase-9, cleaved-PARP, and Bax, and significantly increased Bcl-2 expression compared with PD alone (Fig. 6E, left). Similar results were obtained with SB203580 (Fig. 6E, right), suggesting that activation of the p38 and JNK signaling pathways may result in induction of ER stress-dependent apoptosis in cervical cancer cells.

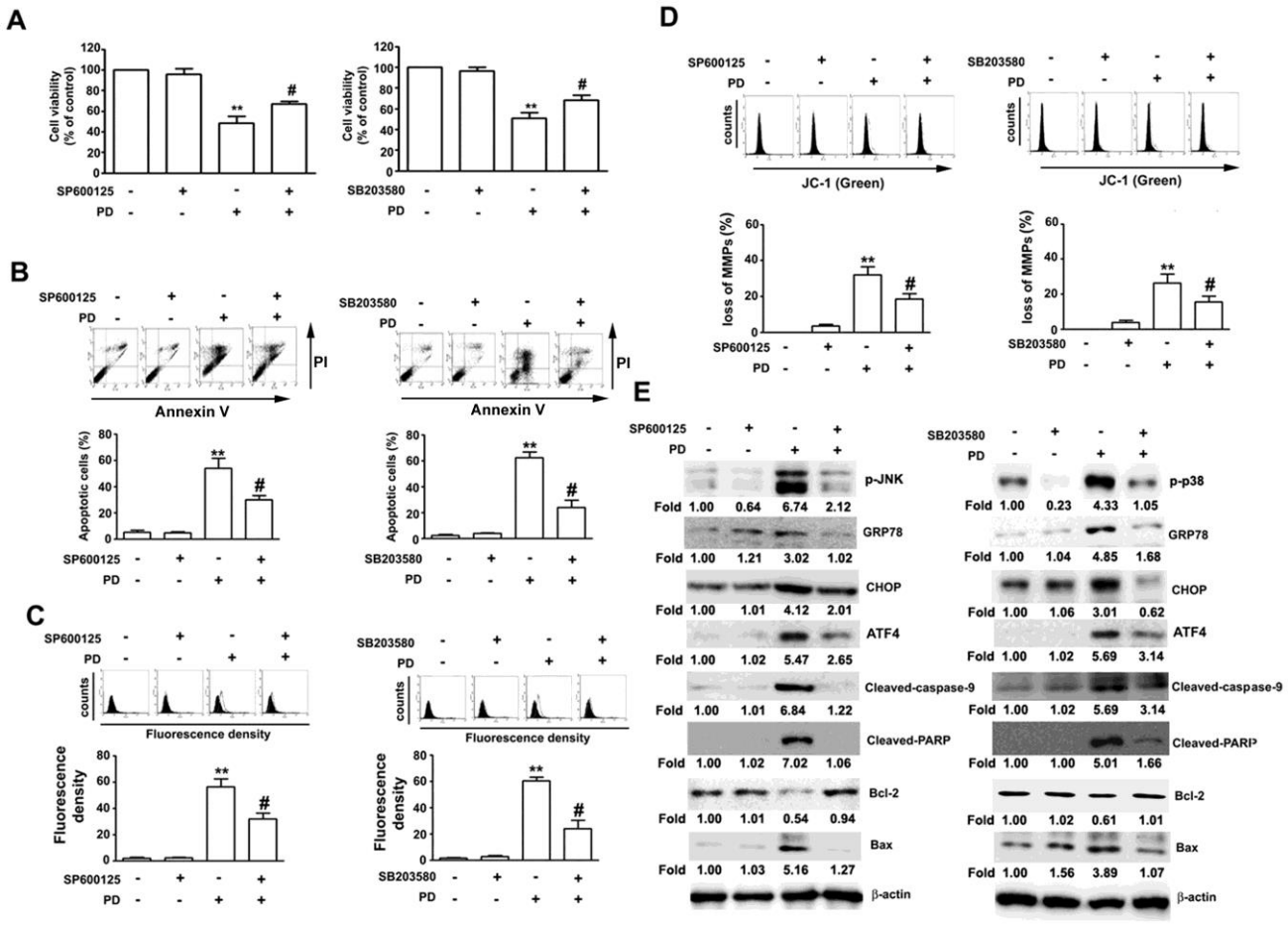

Figure 6. PD-induced ER stress-mediated apoptosis dependent on JNK and p38 activation in HeLa cells. HeLa cells were pre-treated with SP600125 (2 μM) and SB203580 (10 μM) for 2 h prior to PD (4 μM) treatment for 24 h. **(A)** Cell viability was measured by the MTT assay. **(B)** Staining with Annexin V-FITC/ PI and analyzed by flow cytometry. **(C)** The ER stress analyzed with the ER-ID red assay kit using flow cytometry. **(D)** The mitochondrial membrane potential was analyzed with JC-1 staining using flow cytometry. **(E)** The protein expression of ER stress (GRP78, ATF4, and CHOP) and apoptotic or anti-apoptotic markers (Bcl-2, Bax, cleaved-caspase-9, and cleaved-PARP) were determined by Western blotting. β-actin was used as the loading control. Data are presented as the mean ± SE of at least three independent experiments. **, $P<0.01$, control versus PD; #, $P<0.01$, PD versus SP600125 or SB203580 plus PD.

PD increased intracellular ROS levels in human cervical cancer cells

ROS generation is known to be an important factor in tumor cell death [26,27]. To further confirm the role of ROS on PD-mediated apoptosis, HeLa and C33A cells were treated with PD to elevate levels of intracellular ROS production (Fig. 7A). As shown in Fig. 7B, NAC (ROS scavengers) significantly decreased PD-induced ROS production in HeLa and C33A cells; however, NAC significantly reversed the cell growth (Fig. 7C), ER stress (Fig. 7D), and cell apoptosis (Fig. 7E) by PD compared with PD alone. To further investigate the role of ROS on ER stress in PD-induced apoptosis, we measured ER stress and apoptosis markers, and showed that NAC treatment significantly decreased ER stress (GRP78, PERK, p-eIF-2α, CHOP, and ATF4) and apoptosis marker expression (cleaved caspase-3, -8, and -9, -PARP, Bax, and cytochrome c), and increased anti-apoptosis marker expression (Bcl-2) in HeLa cells following PD treatment compared with PD alone (Fig. 7F). In addition, we also found that combined treatment of NAC and PD further reduced

activation of p38 and JNK expression (Fig. 7G). Taken together, the above findings suggest that involvement of ROS in triggering ER stress by PD promotes apoptosis.

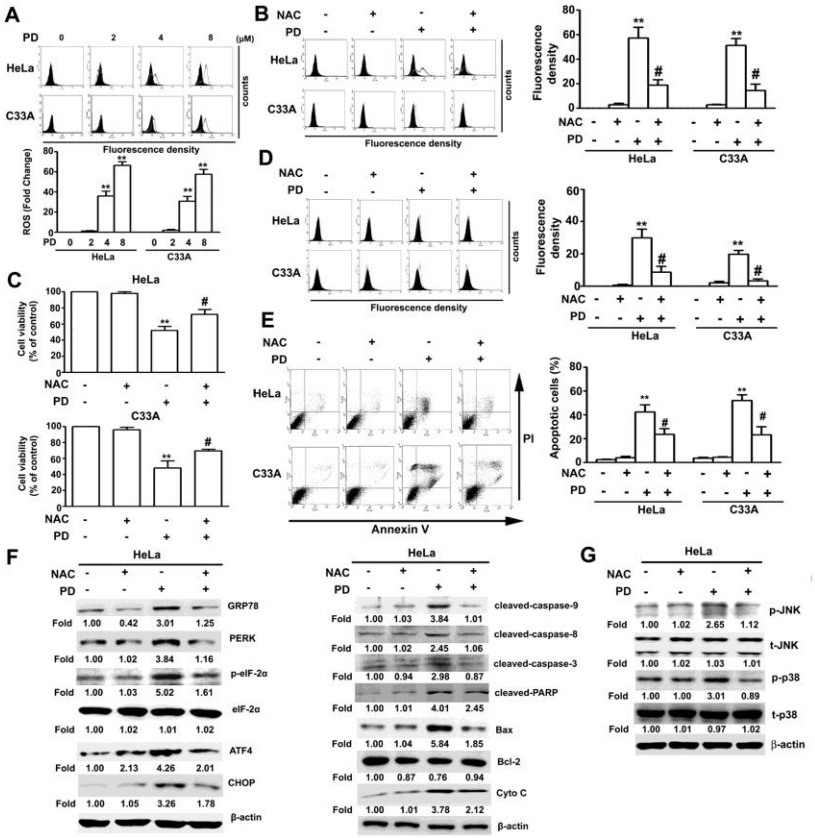

Figure 7. PD triggers ROS generation and ER stress-mediated apoptosis in HeLa and C33A cells**. (A)** HeLa and C33A cells were treated with PD (4 μM) for 24 h, then the ROS generation detected with DCF-DA staining using flow cytometry analysis. **(B)** HeLa and C33A cells were pretreated with NAC (2 mM) for 2 h, then incubated with PD (4 μM) for 24 h were detected with DCF-DA staining using flow cytometry analysis. **(C)** Cell viability was determined by using the MTT assay. **(D)** The ER stress detected with the ER-ID red assay kit using flow cytometry. **(E)** The apoptotic cells were measured by Annexin V/PI stain using flow cytometry analysis. **(F)** Effects of NAC was involved in PD-induced ER stress, apoptosis-related regulatory proteins, **(G)** p-JNK, JNK, p-p38, and p38 protein expression were measured by Western blotting. β-actin was used as the loading control. Data are presented as the mean ± SE of at least three independent experiments. **, P<0.01, control versus PD; #, P<0.01, PD versus NAC plus PD.

ATF4 is involved in PD-induced regulation of CHOP transcription

CHOP is required for ER stress-mediated cell death in response to a variety of pathologic conditions, and the role of ATF4 binding to the CHOP promoter is important for initiating ER stress-induced cell death [28]. To determine whether or not ATF4 binds to the CHOP promoter by PD treatment *in vitro*, we identified two putative ATF4-binding sites (promoter-2, 5'-GTTTCACCA-3' between nucleotides -750/-741; promoter-1, 5'-CATTGCATCATC-3' between nucleotides -330/-318) of the CHOP promoter, as measured by the ChIP assay (Fig. 8A). The results showed that ATF4 directly binds to the CHOP promoter-1 region, not the CHOP promoter-2 region, by PD treatment (Fig. 8B). Following the experiment, we pre-treated with SP600125, SB203580, NAC, and siGRP78, then treated with PD for 24 h. The results showed that SP600125,

SB203580, NAC, and siGRP78 treatment significantly decreased ATF4 binding to the CHOP promoter-1 region (Fig. 8C). The results suggested that ATF4 binding to the CHOP promoter was essential for PD induced ROS-mediated ER stress-dependent apoptosis.

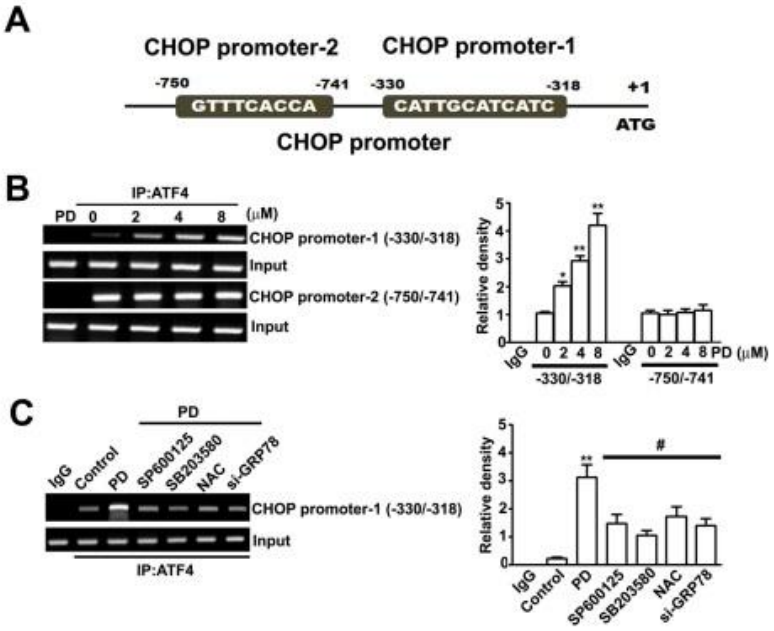

Figure 8. ATF4 binding to the CHOP promoter was involved in PD-induced ER stress-mediated apoptosis in cervical cancer cells. **(A)** Schematic representation of binding sites for ATF4 within the genes encoding the promoter regions of CHOP (-330/-318 and -750/-741). **(B)** The association between ATF4 and CHOP in HeLa cells was examined by the chromatin immunoprecipitation (ChIP) with anti-ATF4, and the PCR analysis of the genes encoding the promoter regions of CHOP (-330/-318 and -750/-741) **(C)** The HeLa cell were pre-treated with SP600125, SB203580, NAC, and siGRP78, then incubated with PD. The PCR analysis of the genes encoding the promoter regions of CHOP (-330/-318) after ChIP in the presence of anti-ATF4 antibody. **, P<0.01, control versus PD; #, P<0.01, PD versus SP600125, SB203580, NAC or siGRP78 plus PD

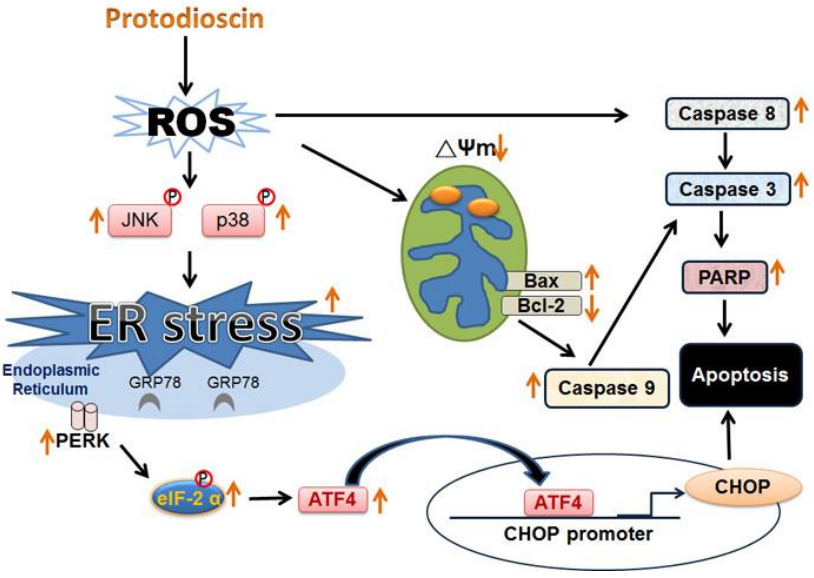

Figure. 9. A schematic representation showing PD induced the ROS-mediated endoplasmic reticulum dependent on apoptosis of human cervical cells via activation of the JNK/p38 activation pathways

討論

Currently, natural compounds have involved pre-clinical or clinical trials with chemopreventive or therapeutic agents for human cervical cancer [29]. Therefore, the search for novel anti-tumor agents has become a subject of great interest. Previous reports have shown that PD, a bioactive natural compound of furostanol saponins, has pharmacologic activities, including antioxidant, anti-inflammatory, and anti-cancer activities [30, 31]. The detailed effects and underlying mechanisms of PD on human cervical cancer cells remain to be investigated. Based on the current study, we suggest that PD inhibits cell growth, leads to mitochondrial dysfunction, induces an ER stress response, and generates ROS leading to cell apoptosis in human cervical cancer cells by activating the JNK and p38 pathways and ROS regulates the GRP78/p-eIF-2α/ATF4/CHOP axis. Here, we defined the involvement of a molecular switch, p38, and JNK in the ER stress response induces apoptosis in PD-treated human cervical cancer cells, thus providing a therapeutic option in the treatment of cervical cancer, as schematically summarized in Figure 9.

ER stress related to cell death of cancer cells involves the UPR-mediated cell signal by different stress stimuli, leading to UPR activation and restoration of ER homeostasis. These UPR signaling pathways are regulated by PERK, IRE1α, and ATF-6. Under normal conditions, GRP78 constitutively binds to ATF6, PERK, and IRE1, which are maintained in an inactive state. Under conditions of ER stress, sequestration of GRP78 by unfolded proteins activates these sensors and initiates the UPR [32]. Following induction of GRP78, ER stress signals lead to activation of ATF4/CHOP, which has been reported to sensitize cells to apoptosis [33]. Similar to the above results, silibinin-induced ER stress-dependent apoptosis in choriocarcinoma cells is closely related to the GRP78 signaling pathways [34]. It has also been reported that CHOP, the ER stress-induced transcription factor, not only down-regulates Bcl-2 expression, but also leads to translocation of Bax from the cytosol to the mitochondria [35]. Additionally, ATF4 has known pro-apoptotic functions that regulate expression of CHOP upon ER stress [36]. In our experiments, we found that PD treatment increased GRP78 dependence on ATF4 regulation of CHOP expression, and knockdown of GRP78 or CHOP by siRNA reversed the increased ER stress-related protein expression by PD and decreased ER stress-induced apoptosis in cervical cancer cells. Therefore, PD induces apoptosis through the ER stress-dependent apoptosis pathway via induction of GRP78/PERK/ATF4/CHOP pathways.

Reactive oxygen species (ROS) are by-products of oxygen metabolism that occur under cellular oxidative stress conditions and play a critical role in the maintenance of homeostasis [37]. Targeting ROS is an important therapeutic strategy for cancer, as exemplified by cancer drugs [38]. It has been shown that ROS activates GRP78/PERK signal transduction, which induces apoptosis via ER stress in cervical cancer cells [39]. This finding is consistent with a recent report that luteolin, a common dietary flavonoid, induces the ER stress response and mitochondrial dysfunction by increasing intracellular ROS levels in glioblastoma cells [40]. Another study reported that JS-K, a glutathione S transferase (GST)-activated nitric oxide (NO) donor prodrug, promotes apoptosis by inducing ROS production in human prostate cancer cells [41]. It was shown that arsenic sulfide induces apoptosis and autophagy through the accumulation of ROS in osteosarcoma cells [42]. Based on the above findings, our results further confirmed that PD induction of ROS is a sound strategy for cervical cancer therapy.

The MAPK pathways have long been implicated in regulating apoptosis, cellular metabolism,

differentiation, and tumor progression [43]. It has recently been shown that the MAPK pathways are targets for development for therapeutic agents against cervical cancer [44]. Recently, it has been shown that methyl protodioscin (MPD) induces apoptosis by increasing the levels of phosphorylated JNK and p38 pathway [45]. Naphtho[1,2-β] furan-4,5-dione (NFD) induces cell apoptosis through activation of JNK and ERK pathways, while ERK protects cells from apoptosis by regulating Bcl-2 family proteins in MDA-MB-231 cells [46]. The polypeptide fraction from Arca subcrenata activates the JNK1/2 and p38 pathways and inactivates the ERK1/2 pathway, and ROS plays a critical role in polypeptide fraction-inhibited growth and induced apoptosis on the MAPK pathways of HeLa cells [47]. In agreement with this finding, we also observed up-regulation of p38 and JNK phosphorylation in PD-treated cervical cancer cells. JNK or a p38 inhibitor significantly reversed PD treatment and triggered signaling to induce ER stress.

Based on all of the above results, it was suggested that PD triggered ER stress-dependent apoptosis, which could be mediated by activation of the JNK and p38 pathways. Our findings contribute significantly toward an understanding of the anti-cancer effect of PD and warrants further evaluation of PD as a new anti-cancer agent and may open interesting perspectives to the strategy in human cervical cancer treatment.

106年度專題研究計畫成果彙整表

計畫主持人：應宗和	**計畫編號：**106-2320-B-040-002-
計畫名稱：原薯蕷皂合併免疫療法與放射治療對子宮頸癌細胞治療協同效應之分子機轉研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
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		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
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		積體電路電路布局權			0		
		著作權			0		
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國外	學術性論文	期刊論文			1	篇	Protodioscin Induces Apoptosis Through ROS-Mediated Endoplasmic Reticulum Stress via the JNK/p38 Activation Pathways in Human Cervical Cancer Cells.Cell Physiol Biochem. 2018;46(1):322-334
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		專書			0	本	
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		技術報告			0	篇	
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				已獲得	0		
			新型/設計專利		0		
		商標權			0		

		營業秘密	0		
		積體電路電路布局權	0		
		著作權	0		
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		博士後研究員	0		
		專任助理	1		聘任一位研究助理
	非本國籍	大專生	0		
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		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：■已發表　□未發表之文稿　□撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   子宮頸癌病患接受放射線治療後常見復發，因此如何治療子宮頸癌病患的化療和放療之效果，是重要的問題。本研究結合以臨床醫師與基礎教師合作開發以原薯蕷皂合併免疫療法與化療和放療為策略之子宮頸癌輔助性治療。未來也許可以成為一個有效的策略來改善現
   在化療和放療所面臨的抗藥性以及副作用所帶來的影響。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：□否　□是
   說明：（以150字為限）