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ORIGINAL RESEARCH PAPER

Parthenolide induces apoptosis and autophagy through the suppression of PI3K/Akt signaling pathway in cervical cancer

Sridharan Jeyamohan · Rajesh Kannan Moorthy · Mahesh Kumar Kannan · Antony Joseph Velanganni Arockiam

Abstract

Objective To investigate the effect of parthenolide on apoptosis and autophagy and to study the role of the PI3K/Akt signaling pathway in cervical cancer.

Results Parthenolide inhibits HeLa cell viability in a dose dependent-manner and was confirmed by MTT assay. Parthenolide (6 μM) induces mitochondrial-mediated apoptosis and autophagy by activation of caspase-3, upregulation of Bax, Beclin-1, ATG5, ATG3 and down-regulation of Bcl-2 and mTOR. Parthenolide also inhibits PI3K and Akt expression through activation of PTEN expression. Moreover, parthenolide induces generation of reactive oxygen species that leads to the loss of mitochondrial membrane potential.

Conclusion Parthenolide induces apoptosis and autophagy-mediated growth inhibition in HeLa cells by suppressing the PI3K/Akt signaling pathway and mitochondrial membrane depolarization and ROS generation. Parthenolide may be a potential therapeutic agent for the treatment of cervical cancer.

Introduction

Cervical cancer is an aggressive and metastatic disease and, during carcinogenesis, many oncogenes and tumor suppressor genes are deregulated (Karlidag et al. 2007). Cervical cancer affects women worldwide and currently ranks as the second leading cause of cancer mortality among women (Ferlay et al. 2010). Human papillomavirus (HPV) is a major risk factor for cervical cancer (Ellenson and Wu 2004). Parthenolide is a sesquiterpene lactone found in the medicinal plant, feverfew, (*Tanacetum parthenium).* It has been used for the treatment of fever, headache and arthritis for many years (Knight 1995). Parthenolide has an anti-cancer activity against colorectal cancer, melanoma, cholangiocarcinoma, pancreatic cancer, breast cancer, prostate cancer and others (Zhang et al. 2004; D'Anneo et al. 2013a, b; Kim et al. 2005; Liu et al. 2010; Sun et al. 2010).

Autophagy plays a dual role in cancer and can act as a tumor suppressor or tumor initiator. Dysfunction of

S. Jeyamohan · R. K. Moorthy · M. K. Kannan · A. J. V. Arockiam (✉)
Molecular Oncology Laboratory, Department of Biochemistry, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamilnadu 620 024, India
e-mail: ajvelanganni@gmail.com; velanganni@bdu.ac.in

autophagy is associated with chromosome instability, DNA damage, cell death, cell proliferation, development and innate immunity (Shintani and Klionsky 2004). Several anti-cancer drugs that promote autophagy mediate cell death in many cancer cells. Moreover, autophagy promotes tumor progression by elimination of damaged organelles, providing ATP and other macromolecules as an energy source for the survival of cancer cells (Janku et al. 2011; Kepp et al. 2011). The PI3K/Akt signaling pathway regulates autophagy in several pathways and is also a chemotherapeutic target to induce autophagy cell death of cancer cells (Sun et al. 2013).

Parthenolide-induced autophagic cell death remains unclear and the exact mechanism behind its involvement with apoptosis and autophagy is not clearly understood. Moreover, there are few reports on parthenolide-induced non-apoptotic mechanisms in cervical cancer. In the present study, we have investigated the effects of parthenolide on autophagy, apoptosis and PI3K/Akt signaling pathway in cervical cancer.

Materials and methods

Chemicals and reagents

Parthenolide ($C_{15}H_{20}O_3$; MW 248.32), DAPI (4,6-diamino-2-phenylindole), DCFH-DA (2′,7′-dichlorodihydrofluoresceine diacetate), propidium iodide (PI), JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanineiodide) and the autofluorescent agent, monodansylcadaverine (MDC), were purchased from Sigma–Aldrich Co. Parthenolide stock solution was prepared in dimethyl sulfoxide (DMSO). MTT, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) were purchased from Invitrogen. Antibodies against Bcl-2, Bax, caspase-3, p-Akt (ser473), phosphatase and tensin homolog (PTEN) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were kind gifts from Prof. Sathees C Ragavan (Indian Institute of Science, India) and Beclin-1, ATG5 and ATG3, were kind gifts from Prof. Sudhandhiran (University of Madras, India).

Cell culture

Human cervical cancer cell line, HeLa, was purchased from the National Centre for Cell Science, India. Cells were grown in DMEM supplemented with 10 % (v/v) FBS and 1 % (w/v) penicillin/streptomycin at 37 °C in a humidified atmosphere 95 % air and 5 % CO_2.

MTT assay

HeLa cells were seeded at 10^4 cells/well in 96 well plates. After incubation overnight, cells were incubated with different concentration of parthenolide (0–10 μM) for 24 h. DMSO (0.02 %) (v/v) was used as control. The medium was removed and 200 μl fresh medium and 20 μl freshly prepared MTT (5 mg/ml PBS) were added to each well and the plate was incubated in a dark at 37 °C for 4 h. The culture medium was removed and 200 μl DMSO was added to each well to dissolve the crystal formation and the absorbance was read at 570 nm using a microplate reader.

Acridine orange/ethidium bromide (AO/EB) dual staining

Cells were grown in 6 well plates and incubated with 6 μM (IC_{50}) parthenolide for 24 h. The medium was removed and cells were washed with PBS and stained with 10 μl AO/EB (1 mg/ml) for 5 min. Morphological changes were visualized with a cell imaging station (Life Technologies, USA).

DAPI staining

Nuclear fragmentation and chromatin condensation were analyzed using DAPI staining. Cells were cultured in 6 well plates and incubated with 6 μM parthenolide for 24 h. The medium was removed and cells were washed with PBS and stained with 10 μl DAPI (100 μg/ml) and incubated for 30 min at 37 °C. The stained cells were visualized with a cell imaging station.

Determination of mitochondrial membrane potential ($\Delta\psi$m)

The alteration of mitochondrial membrane potential in HeLa cells were analyzed using a fluorescent probe, JC-1. [In healthy conditions, JC-1 aggregates within the mitochondria and emits red (aggregated form) fluorescence. During apoptosis, the $\Delta\psi$m collapse and JC-1 accumulates in depolarized mitochondria and emits from red to green (monomeric form). Consequently, the

loss of mitochondrial membrane potential is shown by a decrease in the ratio of red to green fluorescence.] Briefly, HeLa cells were incubated with 6 µM parthenolide for 24 h. Cells were then washed with PBS and stained with JC-1 for 1 h in dark at 37 °C. Stained cells were visualized with a cell imaging station.

Detection of intracellular ROS accumulation

The intracellular reactive oxygen species accumulation was analyzed by DCFH-DA staining. This dye readily diffuses into cells and yields DCFH which is further oxidized by intracellular ROS to transform non-fluorescent DCFH to highly fluorescent DCF. Briefly, cells were plated onto 6 well plates and incubated with 6 µM parthenolide for 24 h. The medium was removed and cells were washed with PBS and stained with 100 µl DCFH-DA (50 µM) for 30 min in dark at 37 °C. The fluorescence was detected by a cell imaging station.

Autophagy detection

The autofluorescent agent, MDC, was used to analyze the formation of autophagolysosome during autophagy process. Cells were treated with 6 µM parthenolide for 24 h. Cells were washed twice with PBS and 50 µM MDC was added and kept for 30 min in 5 % CO_2. After 30 min cells were washed with three times with PBS and the fluorescence was examined by a cell imaging station.

Assay of scratch wound healing

HeLa cells were seeded in 6 well plates, after reaching confluence, cells treated with or without parthenolide. A wound was created by using micropipette p200 tip in the middle area of confluent cells. Cell migration was evaluated with time and images were taken using phase-contrast microscopy with a cell imaging station.

Quantitative real-time PCR

HeLa cells were incubated with 6 µM parthenolide for 24 h. After incubation, total RNA was isolated using Trizol. Equal quantities of RNA (2 µg) from each sample were used to synthesize cDNA with a cDNA synthesis kit. Real-time PCR assay was carried out using SYBER FAST qPCR master mix kit (Kappa Biosystems) in 20 µl by the Step one plus RT-PCR (Applied Biosystem): 40 cycles at 95 °C for 15 s, 60 °C for 45 s and 72 °C for 15 s. Quantitative real-time PCR primers are listed in (Supplementary Table 1). The β-actin gene was used for RNA template normalization.

Western blotting

After parthenolide-treatment, cells were collected and washed with ice-cold PBS in twice then lysed the cells with RIPA lysis buffer: 20 mM Tris HCl, pH 8, 150 mM NaCl, 0.5 % sodium deoxycholate, 5 mM EDTA, 1 % Nonidet P-40, 0.1 % SDS, supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cell lysate was clarified by centrifugation at $12,000 \times g$ for 10 min at 4 °C. Equal amounts of total protein (50 µg per lane) were separated electrophoretically by SDS-PAGE (10 and 15 % gels), and transferred onto 0.2 µm nitrocellulose membrane. The membrane was blocked in blocking buffer (5 % (v/v) non fat milk in TBS) for 1 h at room temperature and then was incubated appropriate primary antibodies overnight at 4 °C. All primary antibodies were used at 1:1000 dilutions. After washing, the membranes were incubated with the corresponding secondary antibodies included alkaline phosphatase-conjugated goat anti-mouse IgG and anti-rabbit IgG for 2 h at 4 °C. Signals were visualized by using a chromogenic substrate BCIP/NBT (Amresco, USA).

Statistical analysis

Statistical significant were analyzed for three independent experiments (n = 3) by One-way ANOVA using GraphPad Prism software version 6.0. Statistical significant was given at a level of $*P < 0.05$.

Results

MTT assay

MTT assay was performed to determine the cytotoxic effect of parthenolide on HeLa cells. Parthenolide effectively inhibited proliferation of HeLa cells in a dose-dependent manner over 24 h (Fig. 1). The IC_{50} value of parthenolide was 6 µM. DMSO was used as

Fig. 1 MTT assay. Cells were treated with parthenolide (0–10 μM) for 24 h; the cytotoxicity of parthenolide was identified by MTT assay. Significant differences between control (DMSO) and treated cells are indicated by *$P < 0.05$
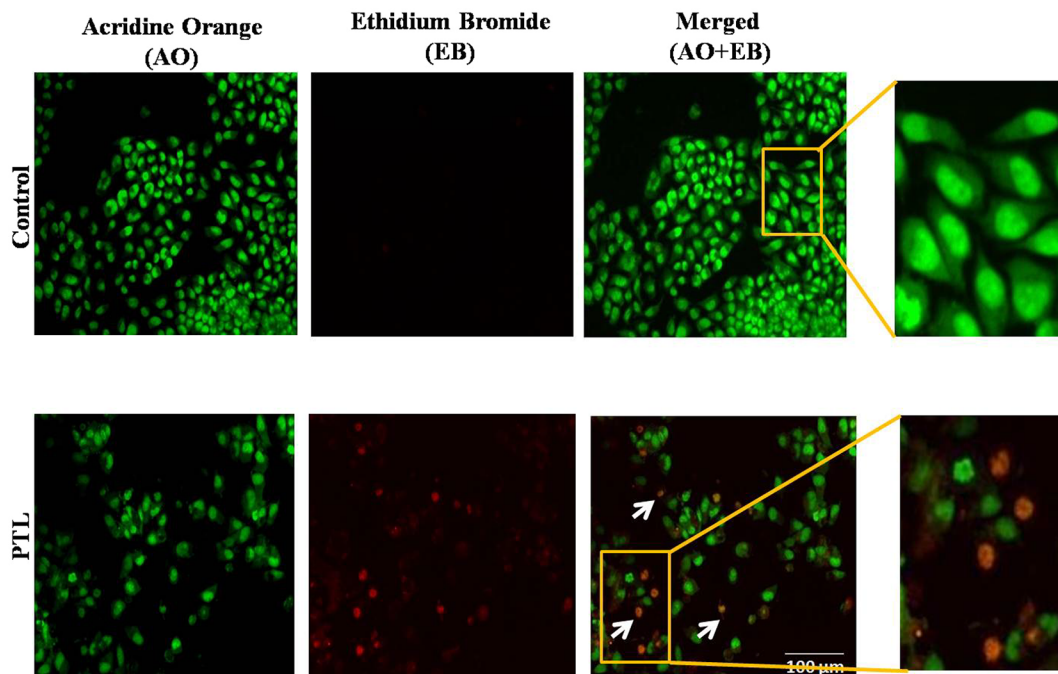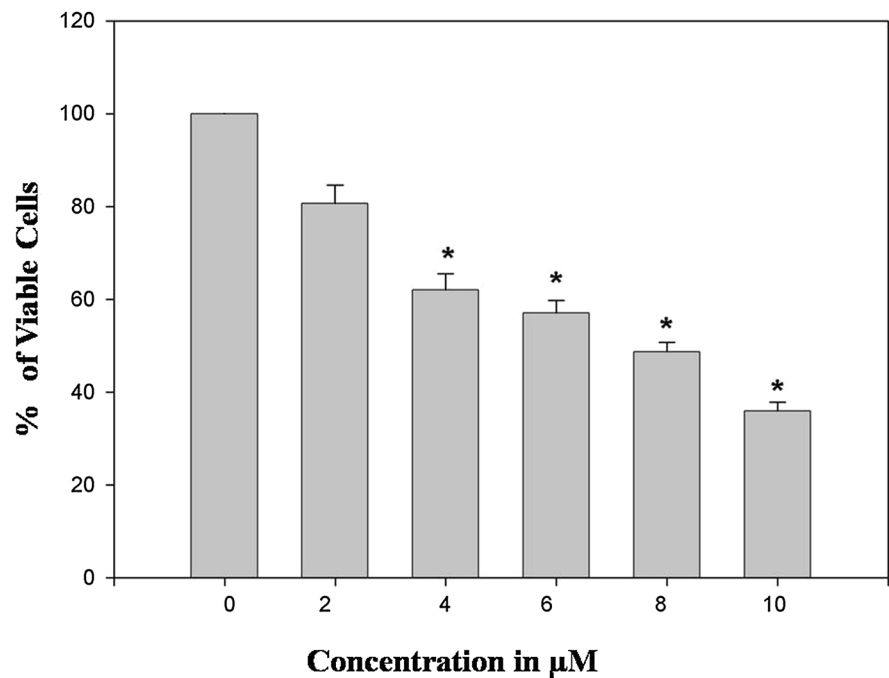
Fig. 2 Morphological changes in the HeLa cells. Cells were treated with 6 μM parthenolide and morphological changes were observed with a cell imaging station (×20) after staining with AO/EB. Control cells appeared *green* and parthenolide treated cells showed apoptotic bodies in *orange* (indicated by *arrows*)

control and did not have any anti-proliferation effect on the HeLa cell line. Parthenolide was used at 6 μM in further experiments.

Parthenolide induces apoptosis and morphological changes in HeLa cells

Cells were treated with 6 μM parthenolide for 24 h and stained with AO/EB. Nuclei of viable cells were intact and appeared green; dead cells appeared bright orange (Fig. 2). There were no morphological changes observed in control cells, which were appeared green.

### Parthenolide promotes DNA damage and nuclear fragmentation in HeLa cells

HeLa cells were treated with 6 μM parthenolide for 24 h. DNA damage was analyzed by DNA-binding stain, DAPI. Control cells appeared blue and had uniform nuclear shape. There were no nuclear fragmentation and DNA damage in control cells. However, most of the parthenolide treated HeLa cells showed damaged DNA and fragmented nuclei (Supplementary Fig. 1). These results confirmed that parthenolide directly damaged the DNA and induced apoptosis in dose-dependent manner.

Parthenolide induces loss of mitochondrial membrane potential ($\Delta\psi$m)

Loss of mitochondrial membrane potential is an early event in apoptosis. HeLa cells were treated with 6 μM parthenolide for 24 h and stained with JC-1. In parthenolide-treated cells, the $\Delta\psi$m was lost and JC-1 aggregated so that the cells appeared green (depolarized). There were no change in $\Delta\psi$m in control cells which had a red fluorescence (energized) (Supplementary Fig. 2). These results indicated that parthenolide induced the loss of $\Delta\psi$m and activated the mitochondrial-mediated apoptotic pathway in HeLa cells.

Parthenolide induces intracellular ROS generation in HeLa cells

DCFH-DA is an indicator of intracellular ROS generation in cells. The ROS level in parthenolide treated HeLa cells were analyzed for 24 h by DCFH-DA. Treated cells produced intracellular ROS and appeared
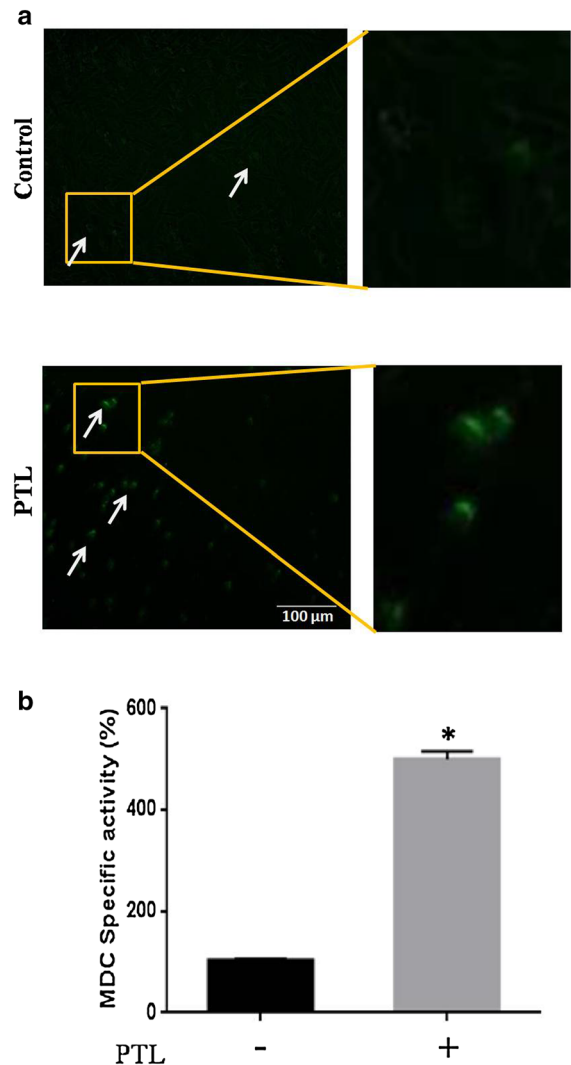
Fig. 3 Parthenolide-induced autophagosome formation. HeLa cells were treated with parthenolide (6 μM) for 24 h and stained with 50 μM MDC and images were taken with a cell imaging station (×20). In parthenolide treated cells, **a** green fluorescence particle indicated autophagosome formations. **b** MDC fluorescence quantification data (*$P < 0.05$)

green and the ROS levels in control cells were also examined. Parthenolide treated cells showed that there were increased level of ROS generation when compared to the control cells (Supplementary Fig. 3a, b).

Parthenolide induced autophagy in HeLa cells

MDC is a specific marker for a detection of autophagy vacuoles formation and it appears as a dot-like

structure in the cytoplasm. In parthenolide-treated cells there were a number of a dot-like structures compared to control cells (Fig. 3a, b). This result indicated that parthenolide strongly induced autophagy in HeLa cells.

Parthenolide inhibits proliferation of HeLa cells

Assay of scratch wound healing was performed with time and images were taken (Fig. 4a, b). In control cells, scratch was almost closed at 24 h. However, in
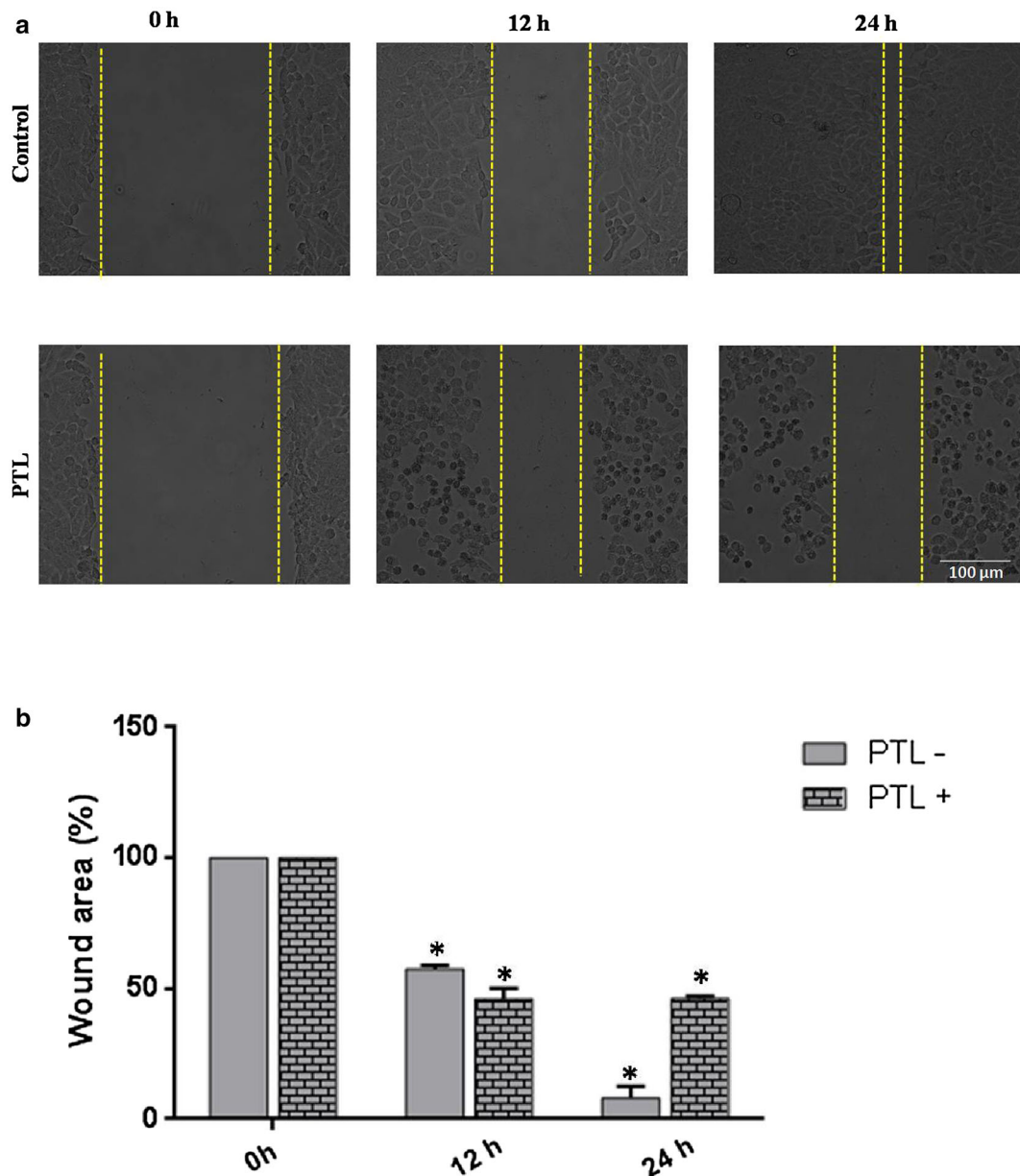
Fig. 4 Assay of scratch wound healing. HeLa cells were treated with or without 6 µM parthenolide. a Wounds were created in the cultured cells and images were taken with a cell imaging station ($\times 20$) after 0, 12 and 24 h of parthenolide treatment respectively. b Wound closure area quantification data ($*P < 0.05$)

parthenolide-treated cells, the scratch did not close at 24 h. These data suggested that parthenolide suppressed the proliferation of HeLa cells.

Parthenolide regulates apoptotic gene expression in HeLa cells

The effect of parthenolide on the expression of anti-apoptotic Bcl-2, pro-apoptotic Bax and caspase-3 were analyzed by real-time PCR and western blotting. Anti-apoptotic Bcl-2 mRNA and protein expression decreased and Bax, caspase-3 mRNA and protein expression increased in HeLa cells (Fig. 5a–c). Our results indicate that parthenolide triggers the intrinsic pathway-mediated apoptosis by decreased Bcl-2 expression and increased Bax, caspase-3 expression in HeLa cells.

Parthenolide induces autophagy and deregulates PI3K/Akt signaling pathway in HeLa cells

The PI3K/Akt signaling pathway is an important pathway for regulating autophagy. PI3K, p-Akt,

PTEN, ATG3, ATG5, Beclin-1 and mTOR gene expressions were analyzed. In parthenolide-treated cells, expression of ATG5, Beclin-1 and PTEN mRNA was up-regulated and mTOR, Akt and PI3K mRNA expression were down-regulated.

In addition, ATG3 protein expression was upregulated and p-Akt protein expression was down-regulated when compared to control cells (Fig. 6a–d). These results suggest that parthenolide inhibits the PI3K/Akt signaling pathway and thus induces autophagy in HeLa cells.

Discussion

Parthenolide plays an important anti-tumor role in many cancer cells exerting its effects through various molecular mechanisms (Mathema et al. 2012). Parthenolide induces apoptosis by the activation of caspase family members, causes loss of mitochondrial membrane potential and the release of pro-apoptotic proteins in colorectal cancer (Zhang et al. 2004). It
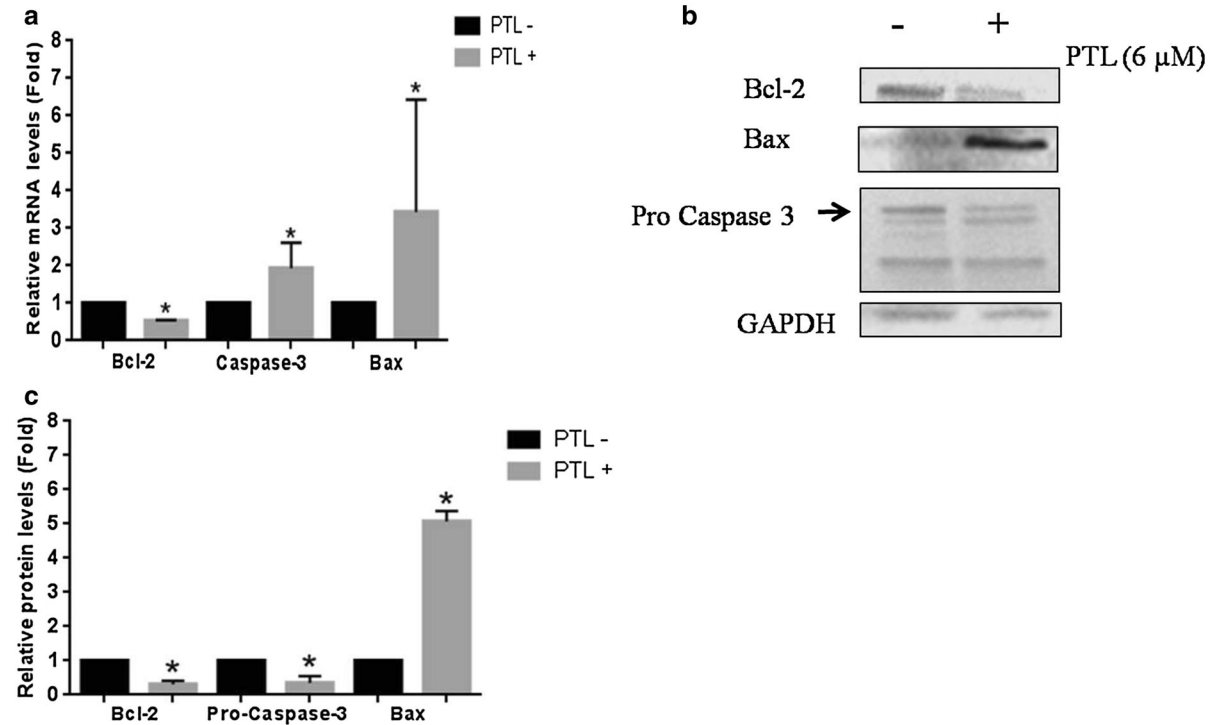
Fig. 5 The apoptotic genes expressions in HeLa cells. Parthenolide regulates Bcl-2, Bax and caspase-3 **a** mRNA and **b** protein expressions. **c** Protein quantification data (*$P < 0.05$) in HeLa cells. Cells were treated with 6 µM parthenolide for 24 h. The expressions of Bcl-2, Bax and caspase-3 mRNA and protein were determined by real-time PCR and western blot. β-actin and GAPDH were used to normalize the gene expressions
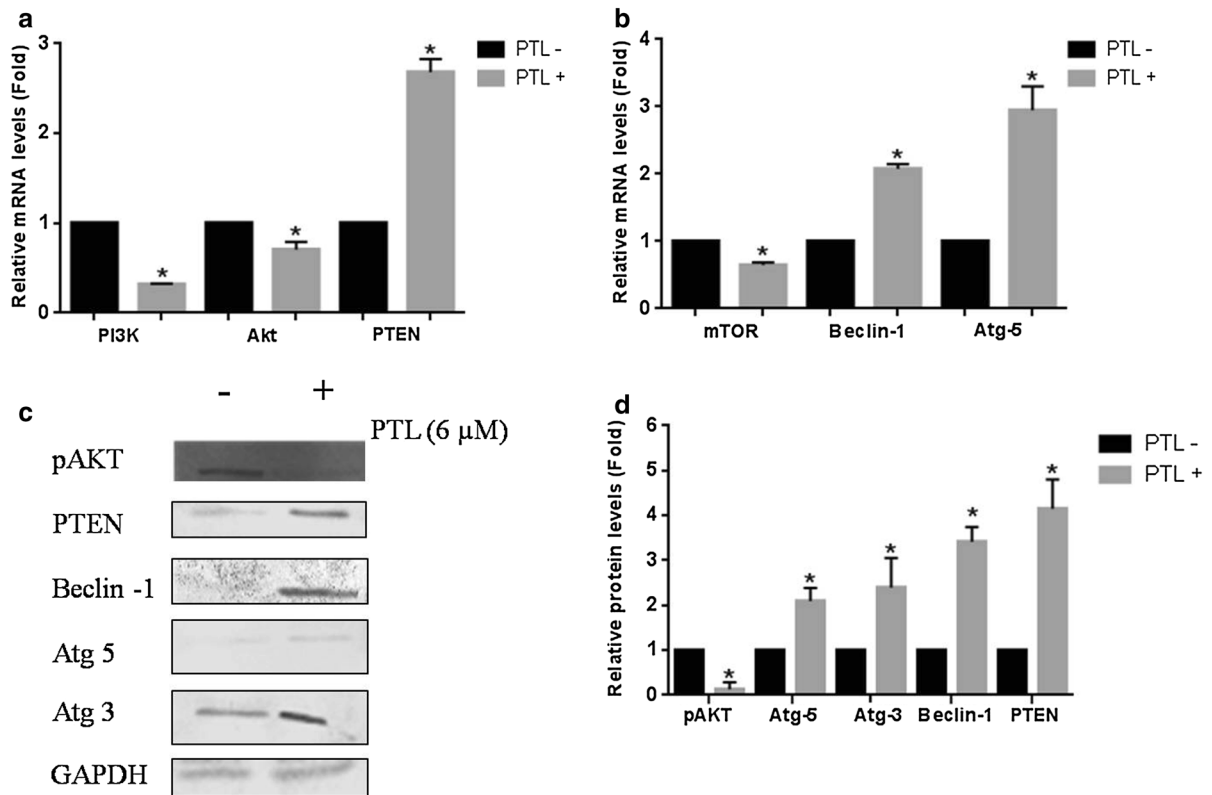
Fig. 6 The autophagy and PI3K/Akt-regulated genes expressions in HeLa cells. Parthenolide modulates **a** PTEN, PI3K, Akt. **b** mTOR, Beclin-1, ATG-5 mRNA and **c** PTEN, p-Akt, Beclin-1, ATG-5 and ATG-3 protein expressions in HeLa cells. **d** Protein quantification data (*$P < 0.05$). β-Actin and GAPDH were used as internal controls

also induces cell cycle arrest and increases the expression of TNFRSF10B/DR5 and PMAIP1/NOXA in human non-small cell lung cancer cells by the activation of the endoplasmic reticulum stress pathway (Zhao et al. 2014). Parthenolide also inhibits the activation of Jun *N*-terminal kinase (JNK), p38 and leads to sensitization of ultraviolet radiation B (UVB)-induced apoptosis in JB6 cells (Won et al. 2004). Parthenolide, as a potent inhibitor of NF-kB, inhibits the IkB kinase complex with sustained cytoplasmic retention of NK-kB; the lack of NF-kB activity leads to apoptosis in cancer cells (Kwok et al. 2001). Therefore, parthenolide induces apoptosis in cancer cells by various mechanisms but how parthenolide induces apoptosis and autophagy in cervical cancer cells remains unclear. In this study, we demonstrate the effect of parthenolide on autophagy and apoptosis in cervical cancer HeLa cells.

ROS generation has a vital role during the induction of apoptosis by anticancer drugs. The increase of ROS

generation can cause loss of $\Delta\psi m$ that leads to apoptosis (Suen et al. 2008). Parthenolide induces apoptotic cell death via the formation of ROS, activation of caspase-3 and modulation of Bcl-2 family proteins in hepatic stellate cells (Kim et al. 2012). The present study strongly suggests that the parthenolide-induced, intrinsic pathway-mediated cell death through ROS generation leads to mitochondrial dysfunction in HeLa cells. Hence, we propose that ROS generation is an important feature for parthenolide-induced apoptosis.

The anti-apoptotic gene, Bcl-2, and pro-apoptotic gene, Bax, have been analyzed in several cancers. They play an important role in cancer development. Inhibition of Bcl-2 or induction of Bax results in mitochondrial dysfunction and causes apoptosis (Dong et al. 2005; Wang et al. 2009). Caspase-3 is responsible for proteolytic cleavage of many proteins and it modulates several upstream genes involved in cell death and triggers apoptosis. Bcl-2 and caspase-3

proteins are the important regulators of the mitochondrial-mediated apoptosis. In our study, Bcl-2 mRNA and protein expression were down-regulated whilst Bax and caspase-3 expression were upregulated in parthenolide-treated HeLa cells.

The ubiquitin-like conjugation system, such as LC3-I/II (Atg8), is an important process in autophagy. During autophagosome formation, LC3-I is converted into LC3-II through the accumulation of ATG5, ATG3, Beclin-1 also leads to this conversion (Mizushima et al. 2011). Parthenolide increases ROS generation and activates JNK, down-regulates NF-kB and activates the autophagic process due to increased expression of Beclin-1 and the conversion of microtubule-associated protein light chain 3-I (LC3-I) to LC3-II in triple-negative breast cancer (D'Anneo et al. 2013b). Our results indicate that parthenolide up-regulated the expression of ATG3, ATG5, and Beclin-1 and autophagosome formation in HeLa cells. Collectively, these results suggest that parthenolide induces autophagy in HeLa cells. The PI3K/Akt signaling pathway is activated and negatively regulates autophagy by inhibiting mTOR expression in cancer cells. PTEN is a potent inhibitor of PI3K/Akt signaling pathway and acts as a tumor suppressor but, in many cancers, PTEN is frequently down-regulated (Di Cristofano et al. 1998; Arico et al. 2001). Our results also show that parthenolide inhibits the PI3K/Akt signaling pathway and mTOR expression by the activation of PTEN gene expression.

In conclusion, parthenolide effectively inhibits the proliferation of cervical cancer cells (HeLa) in a dose-dependent manner. It induces loss of mitochondrial membrane potential, enhances intracellular ROS generation and finally triggers apoptosis and activates autophagy by the inhibition of PI3K/Akt signaling pathway through the activation of PTEN expression. To the best of our knowledge, these are new findings. We propose that parthenolide induces apoptosis and autophagy in HeLa cells and thus may be used as a novel therapeutic agent for the treatment of cervical cancer. However, the crosstalk between apoptosis and autophagy is uncertain in parthenolide treatment. Hence, further study is required to investigate this phenomenon.

Supporting Information Supplementary Table 1—Quantitative real-time PCR primers used in this study.

Supplementary Fig. 1—Chromatin condensation and nuclear fragmentation in HeLa cells.

Supplementary Fig. 2—Determination of mitochondrial membrane potential ($\Delta\psi$m).

Supplementary Fig. 3—Parthenolide induced ROS generation in HeLa cells.

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