

Resveratrol induces cell death through ROS-dependent downregulation of Notch1/PTEN/Akt signaling in ovarian cancer cells

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Received October 12, 2018; Accepted February 12, 2019

DOI: 10.3892/mmr.2019.9962

Abstract. Resveratrol, a natural polyphenol compound, has been reported to exert anticancer activity in various cancer cells. The present study investigated the effect and underlying mechanisms of resveratrol in the human ovarian cancer cell lines, A2780 and SKOV3. Treatment with resveratrol induced apoptotic cell death in dose- and time-dependent manners, as well as a transient increase of reactive oxygen species (ROS) generation. Resveratrol-induced cell death was attenuated by the antioxidant, N-acetylcysteine (NAC), suggesting that ROS were involved in the observed cell death. Treatment with resveratrol resulted in a ROS-dependent decrease of Notch1 signaling. When cells were transfected to overexpress Notch1 using EF.hICN1.CMV.GFP, resveratrol-induced cell death was blocked. Western blot analysis demonstrated that resveratrol also upregulated phospho-phosphatase and tensin homolog (p-PTEN) and downregulated phospho-Akt (p-Akt). Overexpression of p-Akt by transfection with a constitutively active form (caAkt), and the p-PTEN inhibitor SF1670 prevented resveratrol-induced cell death. The caspase-3 inhibitor z-DEVD-FMK significantly attenuated the resveratrol-induced caspase-3 cleavage. Taken together, the results of the present study suggest that resveratrol induces caspase-dependent cell death through suppression of Notch1 and PTEN/Akt signaling and it is mediated by increased ROS generation in human ovarian cancer cells.

Introduction

Ovarian cancer is one of the most common gynecological cancers (1,2). Ovarian cancer patients have a very high death

rate because they do not exhibit initial symptoms, and the cancer is usually found only after metastasis. Between 1999 and 2014, there were 2,413 cases reported with 1,021 deaths from ovarian cancer, showing a 57.7% survival rate in Korea (3). Current cancer treatments involve various methods including surgery, chemotherapy, radiation, and immune therapy. However, the rate of mortality remains high due to drug resistance and undesired side effects (1). Therefore, it is necessary to understand the mechanisms of ovarian cancer cell death and find new and safer agents.

Resveratrol (3,5,4'-trans-trihydroxystilbene) is a polyphenolic phytoalexin. It is abundantly found in a variety of food sources including grapes, berries and peanuts. Resveratrol is known to have cardioprotective, anti-oxidant and anti-inflammatory effects (4). It also provides antitumor activities in various cancers such as breast, prostate, lung, colon, and liver (5-10). Its antitumor activities have been suggested to be closely related with generation of reactive oxygen species (ROS) and Akt signaling (11,12).

Notch signaling is a cell contact-dependent pathway involved in various differentiation processes. Recent studies have suggested that Notch signaling and related transcriptional factors act as upregulators in the death of invasive human cancer cells (13,14). However, the role of Notch signaling in resveratrol-induced death in human ovarian cancer cells is not clear.

In the present study, we demonstrated that resveratrol induced human ovarian cancer cell death through ROS/Notch1/PTEN/Akt signaling. The results suggest that resveratrol can be considered as a potential therapeutic agent for treating human ovarian cancer.

Materials and methods

Reagents. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), SF1670 and resveratrol were purchased from Sigma-Aldrich Chemical (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). EF.hICN1.CMV.GFP was purchased from Addgene (Cambridge, MA, UK). Z-DEVD-FMK was purchased from Calbiochem (San Diego, CA, USA). Antibodies (anti-cleaved-Notch1, anti-total-Notch1, anti-phospho-PTEN, anti-phospho-Akt, anti-total-Akt, anti-cleaved-caspase-3)

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Key words: resveratrol, reactive oxygen species, Notch1, ovarian cancer, apoptosis

were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH was procured from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). All other chemicals were of the highest commercial grade available.

Cell culture. A2780 and SKOV3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 75-cm² culture flasks (Costar, Cambridge, MA, USA). The cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in a humidified incubator filled with 95% air and 5% CO₂. When cells were grown to reach confluence, they were detached using 0.02% EDTA-0.05% trypsin solution and subculture was performed.

Measurement of cell viability. MTT assay was performed to determine cell viability. After washing out the culture media bathing the cells, fresh culture media containing 0.5 mg/ml of MTT was added to each well. After incubating for 2 h, the media was removed by aspiration and the formazan crystals produced by viable cells in each well were solubilized in dimethyl sulfoxide. A 0.1 ml aliquot of each sample was then transferred to 96-well plates and the absorbance of each well was measured with ELISA Reader (FLUOstar OPTIMA; BMG Labtech GmbH, Ortenberg, Germany) at 550 nm.

Measurement of apoptosis. Cell apoptosis was evaluated using FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Changes in FITC Annexin V fluorescence was measured using a FACSsort Becton Dickinson Flow Cytometer and data were analyzed with CELLQuest Software (FACSCalibur™ and Cellquest™; BD Biosciences, Franklin Lakes, NJ, USA). The Annexin V binding assay was carried out according to the manual provided by the manufacturer. After exposure to experimental protocols, cells were washed twice with physiological buffer solution (PBS). Cells were then detached by treatment with 0.025% trypsin and harvested by washing and centrifugation with cold PBS. Cells were then resuspended in Annexin V binding buffer and incubated for 15 min with binding solution containing FITC Annexin V and propidium iodide in the dark. Flow cytometric analysis was performed with the excitation filter at 488 nm. The proportion of apoptotic cells was estimated as the quadrant statistics of the early and late apoptotic region to the entire cell population.

Measurement of reactive oxygen species (ROS). The changes in cellular ROS level were measured using DCFH-DA. DCFH-DA itself is a non-fluorescent ester. As it is highly permeable to the cell membrane, it readily accumulates in the intracellular space. Within the cells, it is hydrolyzed to DCFH by the cellular esterases. In the presence of cellular peroxidase and ROS, DCFH is then rapidly oxidized to a highly fluorescent DCF. Thus, cellular DCF fluorescence is an excellent indicator that reflects the change in the intracellular ROS level. Changes in DCF fluorescence was assayed using FACSsort Becton Dickinson Flow Cytometer (BD Biosciences) and data were analyzed with CellQuest software.

Western blot analysis. After exposure to experimental protocols, cells were collected and disrupted in lysis buffer composed of 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4. After removal of cell debris by centrifugation, the resulting supernatants were resolved on a 10% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with different primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (goat Anti-Rabbit IgG, goat anti-mouse IgG; Santa Cruz Biotechnology). The signal was visualized using an enhanced chemiluminescence (ECL; Bio-Rad, Hercules, CA, USA).

Transfection. To modulate the activity of Akt, cells were transfected transiently with the constitutively active form of Akt. Cells were grown on 6-well plastic plates to reach 70% of confluency. Using Lipofectamine (Invitrogen; Thermo Fisher Scientific), 2 µg cDNA was transiently transfected according to manufacturer's guidelines. After 4-h incubation at 37°C, cells were maintained in normal culture media for 24 h. To overexpress intracellular Notch1, we transferred EF.h1CN1. CMV.GFP (Addgene, Cambridge, MA, USA) according to the manufacturer's instructions.

Statistical analysis. The data are expressed as means ± SEM and the difference between two groups was evaluated using Student's t-test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Resveratrol inhibits cell viability and induces apoptosis. We investigated the effect of resveratrol on the viability of the human ovarian cancer cells, A2780 and SKOV3. After exposure of cells to 0-200 µM of resveratrol for 24 and 48 h, MTT assay was performed to examine the cell viability. Resveratrol significantly decreased the viability of both cell lines in time- and dose-dependent manners. The concentrations of resveratrol to show 50% inhibition of cell viability (IC₅₀) were 46.6±6.2 and 116.6±12.8 µM in A2780 and SKOV3 cells, respectively. Cell viability was 42-58% with 100 µM resveratrol after 48 h of exposure (Fig. 1A). Therefore, 100 µM resveratrol for 48 h was used in subsequent experiments.

To examine whether the resveratrol-induced reduction of cell viability was caused by apoptotic cell death, Annexin V/PI staining was performed. Resveratrol treatment increased apoptotic cell population from 0.25% in the control to 43.0% in A2780 cells, and from 1.1 to 35.1% in SKOV3 cells (Fig. 1B). These results suggest that resveratrol-induced cell death of these ovarian cancer cells occurred mainly through apoptosis.

Resveratrol stimulates ROS production in ovarian cancer cells. To determine whether resveratrol stimulated ROS production in ovarian cancer cell lines, A2780 and SKOV3 cells were exposed to resveratrol and changes in DCF fluorescence were measured using flow cytometry. Resveratrol caused a transient

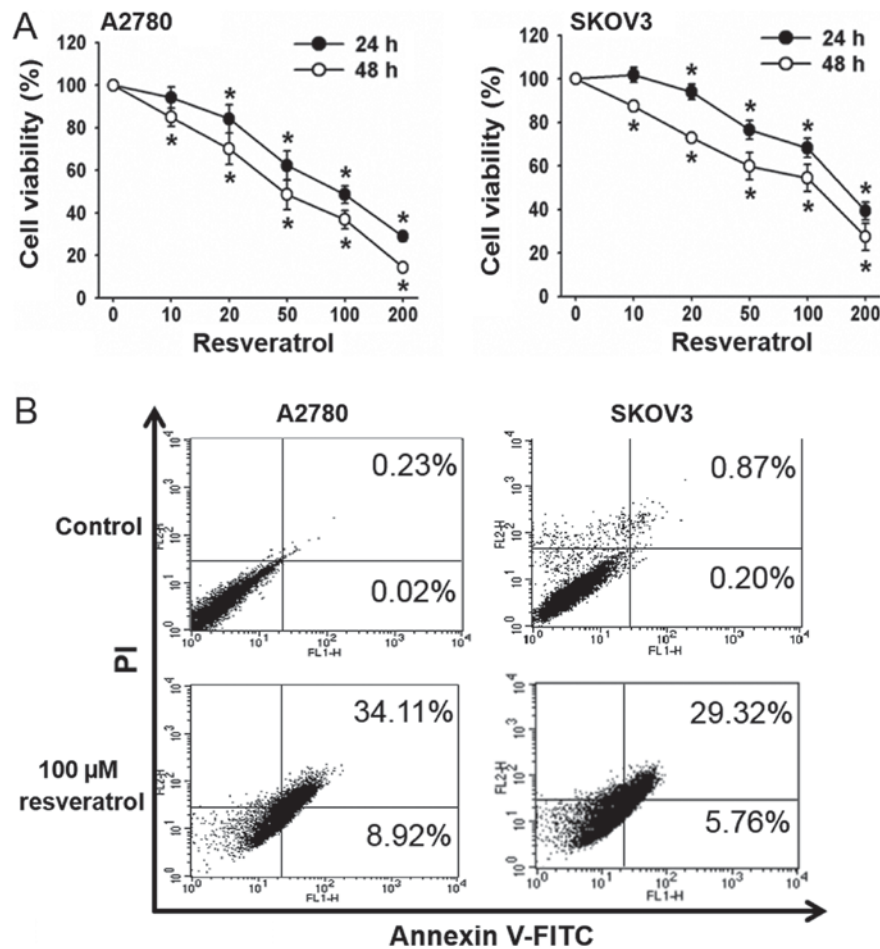


Figure 1. Effect of resveratrol on cell viability and apoptosis. (A) The cells were treated with various concentration of (0-200 μ M) resveratrol for 24 and 48 h. Cell viability was determined by a MTT assay. Data are mean \pm SEM of three independent experiments performed in duplicate. * P <0.05 vs. 0 μ M resveratrol. (B) The cells were treated with 100 μ M resveratrol for 48 h. Cell apoptosis was measured by Annexin V staining and flow cytometry.

increase in ROS generation with a maximum rise after 1 h of treatment (Fig. 2A).

To examine the role of ROS production in resveratrol-induced cell death, the effect of the antioxidant, NAC, on cell viability was determined. Resveratrol-induced cell death was significantly decreased by NAC (Fig. 2B), indicating that this process was associated with ROS generation.

Resveratrol decreases Notch1 expression in ovarian cancer cells. Notch1 signaling has been reported as an important signaling cascade that determines cancer cell fate. Thus, we evaluated the role of Notch1 expression in resveratrol-induced cell death. The expression of Notch1 in resveratrol-treated cells was assessed by western blot analysis using the primary antibody for cleaved notch1 which detects Notch1 intracellular domain (NICD1). As shown in Fig. 3A, resveratrol decreased the expression of Notch1 in a time-dependent manner in both ovarian cancer cell lines.

To determine whether Notch1 signaling was involved in resveratrol-induced cell death, viability was determined in cells transfected with EF.hICN1.CMV.GFP. The overexpression of Notch1 was assessed by western blot analysis (Fig. 3B) and cell viability was analyzed by the MTT assay. Both ovarian cancer cell lines overexpressing Notch1 exhibited resistance to resveratrol-induced cell death (Fig. 3C).

To examine whether resveratrol suppressed Notch1 expression through the stimulation of ROS production, cells were pretreated with NAC before exposure to resveratrol, and changes in Notch1 expression were examined by western blot analysis. As shown in Fig. 3D, the resveratrol-induced decrease in Notch1 expression was prevented by treatment with NAC. These results suggest that the decrease of Notch1 is associated with ROS production and critically implicated in resveratrol-induced cell death.

Resveratrol induces cell death through PTEN/Akt signaling. PTEN/Akt signaling plays important roles in cell proliferation, survival, and differentiation. ROS generation and Notch1 are intimately related with PTEN/Akt signaling. Therefore, we evaluated whether resveratrol-induced cell death was associated with this signaling. After exposure of cells to 100 μ M resveratrol for different time periods, phosphorylation of PTEN and Akt were analyzed by western blot analyses. Resveratrol induced upregulation of p-PTEN and downregulation of p-Akt in a time-dependent manner (Fig. 4A).

To confirm whether PTEN/Akt signaling was involved in resveratrol-induced cell death, cells were pretreated with a PTEN inhibitor, SF1670, or transfected with a constitutively active form of Akt (caAkt), and cell viability was measured. The transfection efficiency was estimated to be >70% by

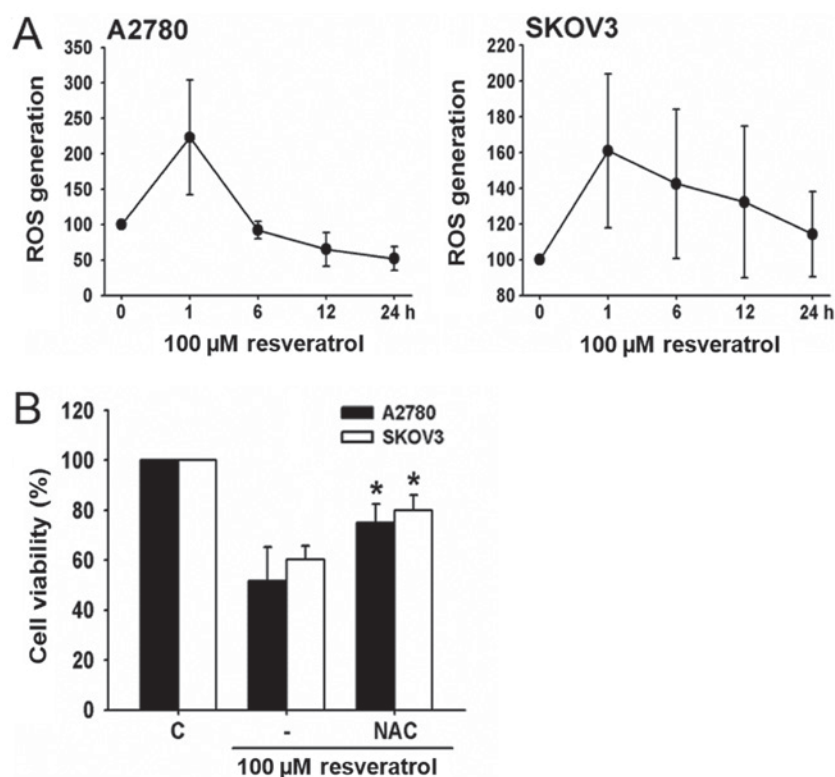


Figure 2. Role of ROS generation in resveratrol-induced cell death. (A) The cells were loaded with DCFH-DA for 1 h, and treated with 100 μ M resveratrol for various times. DCF fluorescence intensity was measured by flow cytometry. Data are mean \pm SEM of three independent experiments performed in duplicate. (B) The cells were treated with 100 μ M resveratrol in the presence or absence of 2 mM (NAC). Cell viability was determined by MTT assay. Data are mean \pm SEM of three independent experiments performed in duplicate. * P <0.05 vs. resveratrol alone. NAC, *N*-acetylcysteine; ROS, reactive oxygen species.

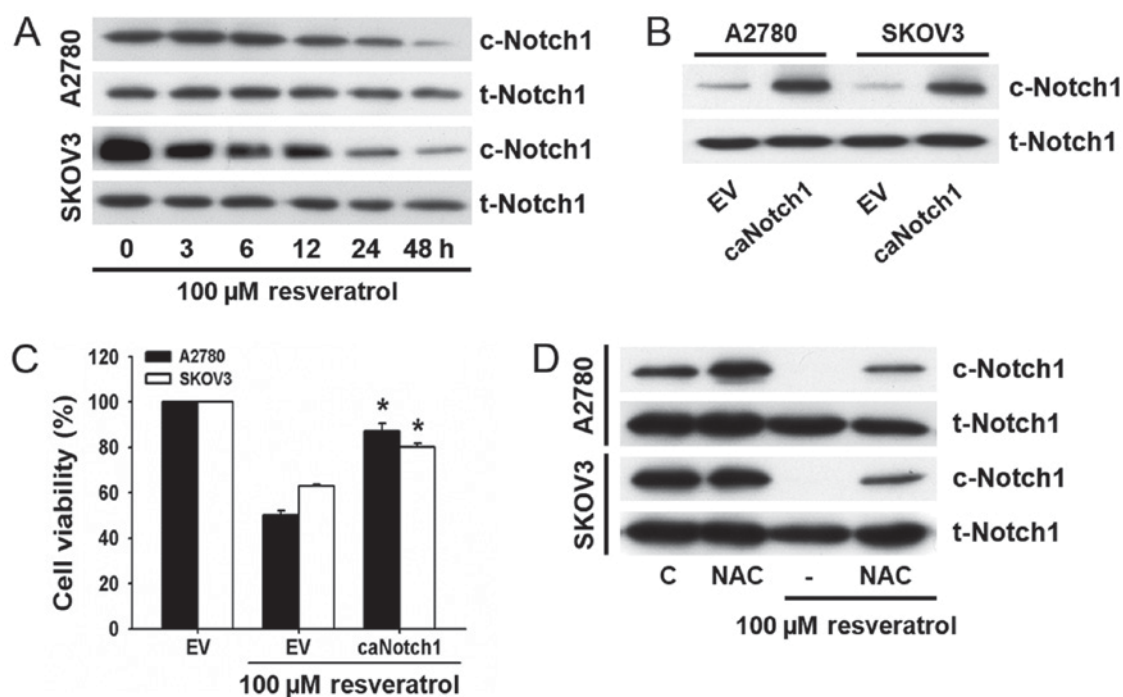


Figure 3. Role of Notch1 expression in resveratrol-induced cell death. The cells were treated with 100 μ M resveratrol for various times. (A) Expression of cleaved-Notch1 (c-Notch1) protein was measured by western blot analysis using the specific antibodies. The total-Notch1 (t-Notch1) was used as a loading control. (B) The cells transfected with empty vector (EV), and EF.hICN1.CMV.GFP (caNotch1) and expression of Notch1 was measured by western blot analysis. (C) The cells transfected with EV, and caNotch1 were treated with 100 μ M resveratrol for 48 h, and cell viability was determined by MTT assay. Data are mean \pm SEM of three independent experiments performed in duplicate. * P <0.05 vs. EV with resveratrol. (D) The cells were treated with 100 μ M resveratrol in the presence or absence of 2 mM NAC, and expression of c-Notch1 protein was estimated by western blot analysis. The t-Notch1 was used as a loading control. Western blot data were presented when at last three out of four to five independent experiments showed reproducible patterns.

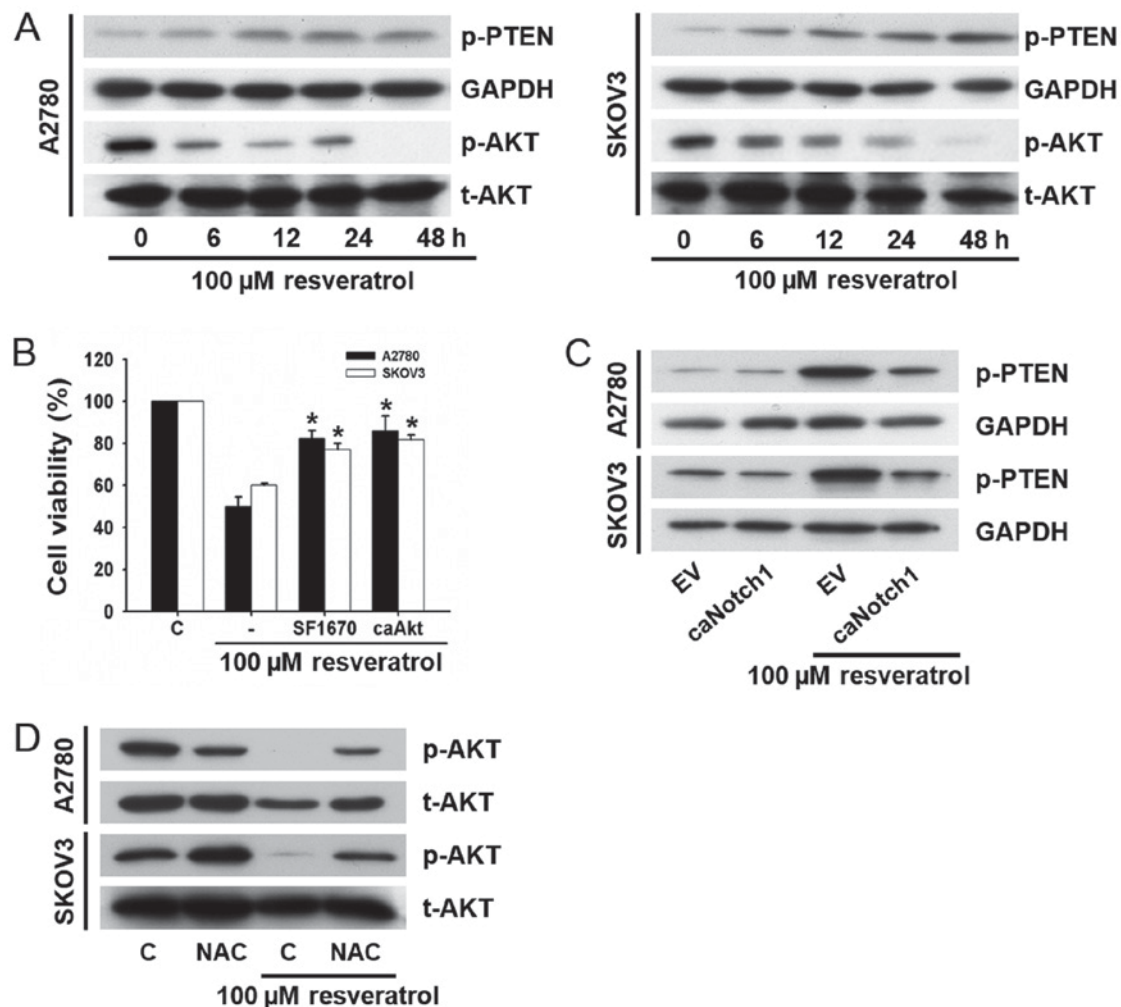


Figure 4. Role of PTEN/Akt signaling in resveratrol-induced cell death. (A) The cells were exposed to 100 μ M resveratrol for various times. Expression of phospho-PTEN (p-PTEN) and phospho Akt (p-Akt) were evaluated by western blot analysis. (B) The cells transfected with constitutively active forms of Akt (caAkt) or pretreated PTEN inhibitor (SF1670) were exposed to 100 μ M resveratrol for 48 h, and cell viability was determined by MTT assay. Data are mean \pm SEM of three independent experiments performed in duplicate. * P <0.05 compared with resveratrol alone. C, control cells. (C) The cells transfected with EV, and caNotch1 were treated with 100 μ M resveratrol for 48 h. Expression of p-PTEN and p-Akt was estimated by western blot analysis. (D) The cells were treated with 100 μ M resveratrol in the presence or absence of 2 mM NAC, and expression of p-Akt protein was estimated by western blot analysis. The t-Akt was used as a loading control. Western blot analysis data were presented when at last three out of four to five independent experiments showed reproducible patterns.

immunofluorescence, and the expression of p-Akt was increased compared with cells transfected with the empty vector (data not shown). Resveratrol-induced cell death was significantly suppressed both by treatment with SF1670 and transfection with caAkt (Fig. 4B).

To investigate whether resveratrol-induced downregulation of p-Akt was attributable to Notch1 signaling, cells were transfected with EF.hICN1.CMV.GFP before exposure to resveratrol and changes in PTEN phosphorylation were determined by western blot analysis. Resveratrol induced upregulation of p-PTEN was prevented by transfection with EF.hICN1.CMV.GFP (Fig. 4C).

To determine whether resveratrol-induced downregulation of p-Akt through the stimulation of ROS generation, cells were pretreated with NAC before exposure to resveratrol, and changes of p-Akt expression were examined by western blot analysis. As shown in Fig. 4D, the resveratrol-induced downregulation of p-Akt was prevented by treatment with NAC. These results suggest that resveratrol-induced cell death

is closely related to the Notch1-dependent upregulation of p-PTEN and downregulation of p-Akt. In addition, it is mediated by increased ROS generation.

Resveratrol induces caspase-dependent cell death. The caspase family of enzymes is essential in the apoptotic pathway. In the process, caspase-3 plays a critical role in the execution phase of apoptosis. We examined the role of caspase-3 in resveratrol-induced cell death.

Resveratrol increased the cleaved caspase-3 level in a time-dependent manner in both ovarian cancer cell lines (Fig. 5A). To examine further the role of activated caspase-3 in resveratrol-induced cell death, the effect of a caspase inhibitor (Z-DEVD-FMK) was examined. When cells were pretreated with Z-DEVD-FMK, the effect of resveratrol on cell viability was significantly attenuated (Fig. 5B).

To investigate whether resveratrol-induced cleavage of caspase-3 was attributable to Akt signaling, cells were transfected with caAkt before exposure to resveratrol, and

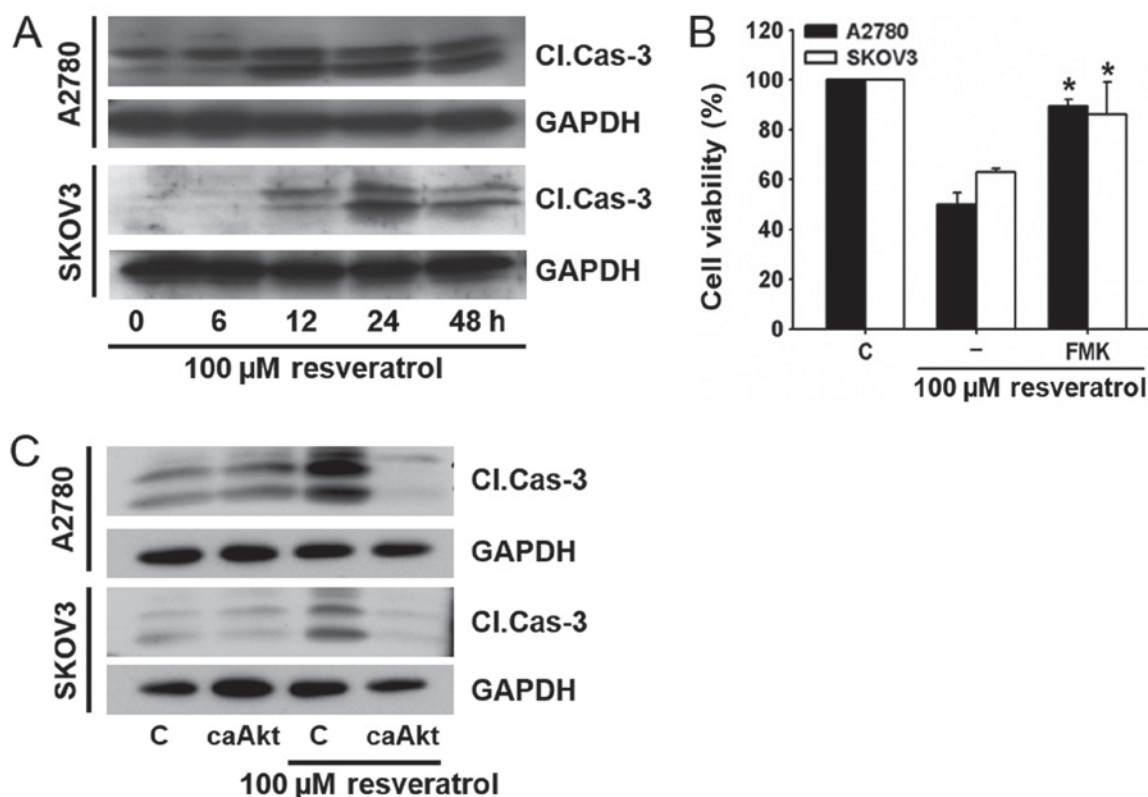


Figure 5. Role of caspase-3 in resveratrol-induced cell death. (A) The cells were exposed to 100 μ M resveratrol for various times, and the cleaved caspase-3 (Cl. Cas-3) was measured by western blot analysis. The GAPDH was used as a loading control. (B) The cells were treated with 100 μ M resveratrol in the presence or absence of 10 μ M Z-DEVD-FMK, and cell viability was determined by MTT assay. Data are mean \pm SEM of three independent experiments performed in duplicate. * $P < 0.05$ compared with resveratrol alone. (C) The cells transfected with constitutively active form of Akt (caAkt) was exposed to 100 μ M resveratrol for 48 h, and cleaved caspase-3 (Cl. Cas-3) was estimated by western blot analysis. The GAPDH was used as a loading control. Western blot analysis data were presented when at last three out of four to five independent experiments showed reproducible patterns.

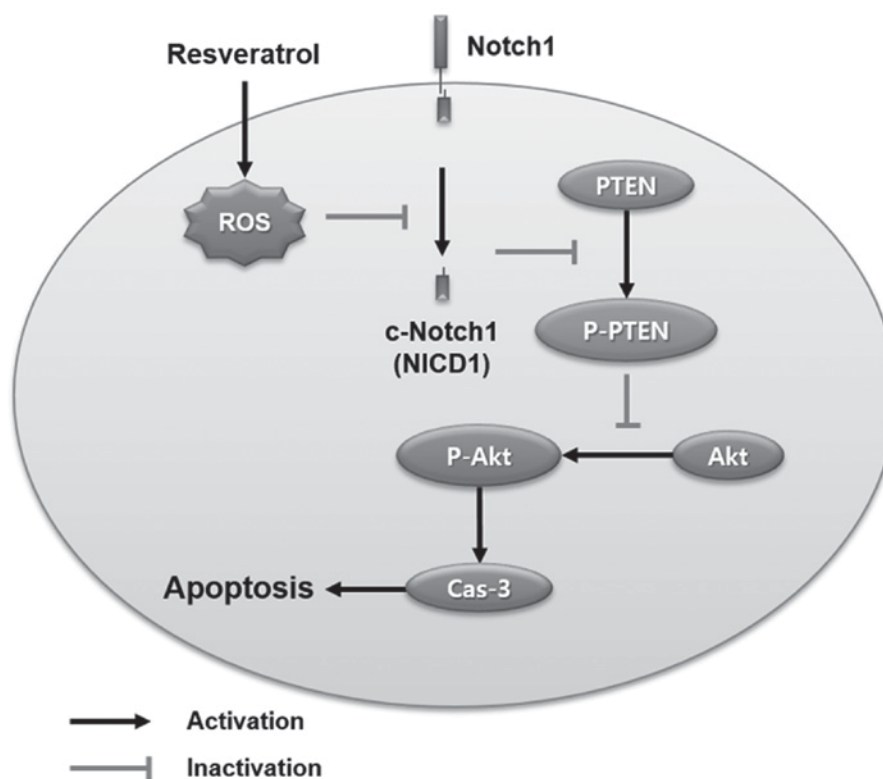


Figure 6. Proposed model of resveratrol-induced cell death in human ovarian cancer cell lines. Resveratrol leads to an increase ROS generation followed by inhibits Notch1 signaling pathway. These signal transductions lead to up-regulation of PTEN and down-regulation of Akt. Decrease of p-Akt induced cell death through a cleavage of caspase-3 dependent mechanism in human ovarian cancer cell.

the change of caspase-3 cleavage was measured by western blotting. As shown in Fig. 5C, resveratrol-induced cleavage of caspase-3 was significantly decreased by transfection with caAkt. These results suggest that resveratrol-induced cell death is associated with Akt signaling-dependent cleavage of caspase-3.

Discussion

Resveratrol is a type of polyphenol found in many plants, including berries, peanuts, raspberries, and grapes (15). Resveratrol has wide-ranging effects such as antioxidant, anti-inflammatory, and antitumor properties in various cancers models. It was reported that ovarian cancer cell is more susceptible to ROS when compared with normal epithelial cells (16-18). However, the precise cellular mechanism of resveratrol-induced cell death has not yet been clarified. The present study provided us with clues to understand the molecular mechanisms of the resveratrol-induced antitumor activity in human ovarian cancer cells.

Intracellular ROS play a pivotal role in cell signaling and homeostasis (19). Although resveratrol is well-known for its antioxidant activity, it may also behave as a pro-oxidant that are responsible for antitumor activity in some cancer cells. We consider that resveratrol has both antioxidant and pro-oxidant properties, depending on the cell type, drug concentration, and other experimental conditions (20-23). In the present study, resveratrol stimulated ROS production, and the antioxidant, NAC, prevented resveratrol-induced cell death (Fig. 2). These results strongly suggest that resveratrol-induced cell death is associated with ROS production. Similar results have been reported in other ovarian cancer cells exposed to resveratrol (24).

Notch signaling promotes cell growth, migration, invasion, and apoptosis in various cancer cells (25,26). Notch receptors are assembled as a large extracellular ligand-binding domain, a single-pass transmembrane domain, and Notch intracellular domain (NICD). When γ -secretase bind to Notch receptors NICD is released. Released NICD translocates to the nucleus and activates transcription of downstream target genes. Notch signaling has emerged as a potential target for cancer therapies. However, the effect of resveratrol on Notch signaling is not clear yet.

In the present study, we used the cleaved-Notch1 primary antibody to detect NICD1. Resveratrol decreased the c-Notch1 protein level (Fig. 3A). In addition, resveratrol-induced cell death was prevented by overexpression of Notch1 (Fig. 3C). The correlation between ROS generation and Notch1 signaling is very intricate and involves many steps. Notch1 suppresses ROS generation (27) and, conversely, ROS generation regulates Notch1 signaling (28). In our study, resveratrol seemed to suppress Notch1 signaling through ROS generation (Fig. 3D).

PTEN/Akt signaling works to regulate cellular responses to various extracellular stimuli (29). This signaling is associated with cancer cell proliferation, invasion, tumorigenesis, and drug resistance in many different cell types (30-32). Activated Akt can inhibit the release of cytochrome *c* and thus the cleavage of caspase-3, thereby inhibiting apoptosis and promoting cancer cell survival. Resveratrol inhibits Akt signaling thereby inducing apoptosis in several types of cancer cells. The cleavage of Notch by γ -secretase releases the

Notch intracellular domain into the cytoplasm. The released domain can suppress PTEN, an inhibitor of Akt. Hence, downregulation of Notch signaling inhibits Akt signaling by PTEN (33,34). In the present study, resveratrol upregulated p-PTEN and downregulated p-Akt in time-dependent manners (Fig. 4A). Resveratrol-induced cell death was prevented by a PTEN inhibitor, SF1670, or transfection with caAkt (Fig. 4B). In addition, resveratrol-induced upregulation of p-PTEN was prevented by overexpression of Notch1 (Fig. 4C). Resveratrol-induced downregulation of p-Akt was prevented by treatment with NAC (Fig. 4D). Although data were not presented in our results pretreatment with SF1670 and/or transfection of caAkt did not affect ROS generation. These results suggest that ROS generation resides on upstream of resveratrol-induced changes in Notch1/PTEN/Akt signaling pathway.

Caspase-3 plays a critical role during the execution phase in various forms of apoptosis. Caspase-3 is present as an inactive pro-enzyme and is activated by proteolytic cleavage. This cleavage is initiated by ligands of many cell surface receptors in a complex associated with the cytoplasmic death domain that triggers the release of cytochrome *c* from mitochondria. Cytochrome *c* binds with apoptotic protease activation factor 1, which then activates caspase-9 that, in turn, cleaves caspase-3 (35,36). There is also an induction of caspase-3 (37). In the present study, resveratrol increased the cleavage of caspase-3 as demonstrated by western blotting (Fig. 5A). Resveratrol-induced cell death was prevented by a selective caspase-3 inhibitor, Z-DEVD-FMK (Fig. 5B). In addition, resveratrol-induced cleavage of caspase-3 was blocked by transfection with caAkt (Fig. 5C).

In conclusion, the present study demonstrated that resveratrol induced human ovarian cancer cell death through ROS-dependent Notch1/PTEN/Akt signaling. Related signaling mechanisms are summarized in Fig. 6. Our results suggest that resveratrol could be considered as a potential candidate for treating human ovarian cancer, and Notch1 signaling could be a potential target for further investigation.

Acknowledgements

Not applicable.

Funding

The current study was supported by a 2-Year Research Grant of Pusan National University.

Availability of data and materials

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

Authors' contributions

JHP carried out western blotting and collected data. THK carried out assays for cell viability, apoptosis and ROS generation. THK and JSW participated in experiment design and the draft preparation. All authors have read and approved the final 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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