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Mitochondrial dysfunction and cytochrome c translocation induced by resveratrol are dependent on NF-κB activity and facilitate TRAIL sensitivity in human lung cancers

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent that has the potential to sensitize a wide variety of cancer or transformed cells by inducing apoptosis. However, resistance to TRAIL is a growing concern, but it can be mitigated by employing different combination therapies. Resveratrol is a polyphenolic compound annex the outpouring of p53 and its pro-apoptotic modulator PUMA. Here, we showed the p53-independent activation of apoptosis by decrease the expression of phosphorylated Akt-mediated suppression of NF-κB activation that is also substantiated with the downregulation of anti-apoptotic factors Bcl-2 and Bcl-xl in Non-Small Cell lung adenocarcinoma cells (NSCLC), resulting in an attenuation of TRAIL resistance in combined treatment. Furthermore, apoptosis was induced in TRAIL-resistant lung cancer cells via a co-treatment of resveratrol and TRAIL, which was assessed by the loss of mitochondrial membrane potential, resulting in the translocation of cytochrome c from the mitochondria into the cytosol due to mitochondrial dysfunction. Moreover, autophagy flux was not affected by resveratrol-induced TRAIL-mediated apoptosis of lung cancer cells. Overall, targeting the NF-κB (p65) pathway via resveratrol attenuates TRAIL resistance and induces TRAIL-mediated apoptosis which could be the eminent therapeutic strategy to treat lung cancer.

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Introduction

Cancer is a concerning disease and a leading cause of death. There are ongoing basic research studies and clinical trials for the developing of treatment options to ameliorate cancer [1]. Currently, lung adenocarcinoma is one of the leading causes of cancer deaths. Several available anticancer therapy regimens include radiotherapy, surgery, chemotherapeutic drugs like Doxorubicin Hydrochloride, Cisplatin, 5- fluorouracil, paclitaxel etc. and immune checkpoint inhibitor like PD-1/PD-L1 and CTLA-4/B7-1/B7-2 or a combination of treatments in patients with advanced progressive tumors that are resistant to monotherapy. Small molecules like tyrosin kinase inhibitors- gefitinib, erlotinib, sorafenib, VEGF Receptor 2 inhibitor-apatinib, PI3K inhibitors, Bcl-2 inhibitors, serine/threonine kinase inhibitor-temsirolimus etc. are popular molecular targeted therapy regimen for cancer patient. Additionally, combination therapies with potent chemotherapeutic agents can exert potential benefits against cancers such as non-small cell lung cancer [2-4].

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in cancer or transformed cells but not in normal cells. The death-inducing signaling complex is activated by binding to its death receptors, DR4 and DR5, which then associates with the adaptor molecules FADD and caspase-8, resulting in the TRAIL-mediated activation of caspase-9 and subsequent activation of caspase-3 to induce apoptosis [5-7]. The release of cytochrome c and Smac/DIABLO from the mitochondria modulates caspase-3 activation and TRAIL via an intrinsic pathway when apoptosis is being induced [8]. Although TRAIL selectively affects cancer or uncontrolled proliferating cells while leaving normal cells unharmed, many cancer cells can become resistant to TRAIL through mechanisms that are not fully understood. Several studies have shown that genetic or epigenetic modification of TRAIL receptors, an increased

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expression of decoy receptors, or a decreased expression of DR4/DR5 correlates with TRAIL resistance [9, 10]. Furthermore, proteins, such as Bcl-2, Bcl-xl, anti-apoptotic c-FLIP, and pro-apoptotic protein Bax, and Bak, which is considered an apoptotic regulatory protein, may affect the functions of TRAIL [8, 11, 12]. TRAIL receptor-mediated activation of NF-κB (p65) also contributes to the apoptotic resistance of TRAIL in different cancer cells [13, 14]. Overall, studies on TRAIL resistance as well as effective and favorable combination drug therapies have been performed to improve the outcomes of certain cancer treatments [15-17].

Apoptosis is characterized by specific cell morphologies that include cell shrinkage, blebs, nucleus fragmentation, chromatin condensation, and distinct apoptotic body formation [18, 19]. Apoptosis can occur through two major pathways including the extrinsic cell death receptor-mediated pathway or the mitochondria-mediated pathway via the activation of caspase [20]. The activation of apoptotic pathways plays a major protective role in preventing the spread of cancer. Studies have also shown that numerous anticancer drugs may damage the mitochondria, therefore affecting the mitochondrial membrane potential (MMP), resulting in the release of cytochrome c and subsequent activation of apoptosis via the caspase cascade [21, 22]. Additionally, the p53 transcription factor can induce cell cycle arrest, DNA damage, cellular senescence, and apoptosis in different cancer cell types [23, 24]. p53 independent apoptosis cascade formation also now considerable issue [25-27]. NF-κB (p65), a pro inflammatory transcription factor that is activated by numerous inflammatory agents, tumor promoters, carcinogens, and the tumor microenvironment could also promote tumorigenesis. Proteins regulated by NF-κB may also contribute to cellular proliferation, apoptosis suppression, angiogenesis, and metastasis, and the suppression of NF-κB (p65) has been shown to provide effective therapeutic benefits in cancer treatments [28, 29]. NF-κB (p65) regulates anti-apoptotic

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proteins Bcl-2 and Bcl-xl, both of which localize to the mitochondrial outer membrane where they play a significant role in promoting cellular survival and inhibiting pro-apoptotic proteins such as Bax and Bak, which are known to induce apoptosis via permeabilization and the release of cytochrome c [15, 30]. Finally, the Akt cell survival signaling pathway also plays a key role in the regulation of apoptosis via NF-κB (p65) [31, 32].

Autophagy is the homeostatic and adaptive system that results in the lysosomal digestion of cytoplasmic components and organelles [33, 34]. Autophagy begins with the maturation of autophagosomes, double-membrane vesicles that engulf cytoplasmic components, which then fuses with the lysosome, resulting in the subsequent degradation and recycling of its cargo [35, 36]. The formation of autophagosomes is regulated by the Atg12-Atg5-Atg16 complex and microtubule-associated protein light chain 3 (LC3-I)-phospholipid conjugates (LC3-II), both of which could be used as markers for autophagy [37-39]. Furthermore, the autophagy marker p62 is incorporated into autophagosomes by interaction with LC3, which is then degraded during autophagy. Overall, autophagy and apoptotic signaling pathways govern cell survival and cell deaths [40, 41].

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a naturally occurring phytochemical that is produced by plants and is mostly found in grape skins, mulberries, raspberries, peanuts, and red wine [42, 43]. Resveratrol has been shown to have diverse biological properties, including anti-inflammatory, anti-oxidant, antiviral, neuroprotective, antifungal, and anticancer properties [44, 45]. Furthermore, resveratrol strongly inhibits the initiation, promotion, and progression of tumors such as leukemia, skin, pancreas, breast, lung, prostate, bladder, and colon cancers [46-48]. However, the molecular mechanisms that govern its anticancer activity are still unclear, but it is hypothesized to be associated with cell-cycle arrest and the induction of apoptosis by

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regulating the expression of NF-κB, Bcl-2, and Bcl-xl, or through the loss of mitochondrial function and the release of cytochrome c [49, 50]. Furthermore, p53-independent activation of apoptosis has been shown to be induced by resveratrol; however, p53 is likely to be required for resveratrol-induced apoptosis in many cancer cell lines [27, 51, 52]. In cases of cancer cells that are resistant to TRAIL, a combination therapy of resveratrol with TRAIL can exert potential benefits by attenuating its resistance to TRAIL and enhancing the activation of apoptosis [53-55].

Although resveratrol annex the outpouring of p53 & its pro apoptotic modulator PUMA, Here, we demonstrate that p53-independent activation of apoptosis by decrease the expression of phosphorylated Akt -mediated suppression of NF-κB activation results in the downregulation of Bcl-2, Bcl-xl and release of cytochrome c due to mitochondrial dysfunction in A549 human lung adenocarcinoma cells, therefore attenuating TRAIL resistance.

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Results

Resveratrol enhances TRAIL-mediated apoptosis in lung adenocarcinoma A549 and HCC-15 cells

The effects of resveratrol on TRAIL-induced apoptosis were demonstrated using two lung adenocarcinoma cell lines. A549 and HCC-15 cells were pretreated with serial concentrations of resveratrol for 12 h followed by treatment with and or TRAIL protein for an additional 2.5 h. Changes in cell morphologies were confirmed under a light microscope to determine viable treatment conditions. As shown in Fig. 1 and 2, treatment of resveratrol or TRAIL alone only slightly affected cell viability, since minor morphological changes were observed when compared to the control group, therefore indicating that A549 and HCC-15 cells were resistant to TRAIL. However, co-treatment of TRAIL with different concentrations of resveratrol significantly reduced cell viability as shown by the observation of apoptotic cell morphologies (Fig. 1A, B and 2A, B). The combined treatment of TRAIL with resveratrol decreased cell viability and increased the percentage of A549 and HCC-15 cells undergoing apoptosis (Fig. 1C and 2C). The lactate dehydrogenase (LDH) levels in A549 cells demonstrated that resveratrol induced apoptosis in a dose-dependent manner when combined with TRAIL, whereas resveratrol by itself did not induce cell death (Fig. 1D). Furthermore, the combination treatment of TRAIL and resveratrol activates caspase-8 and cleaved caspase-3 compare with the single treatment of either resveratrol or TRAIL (Fig. 1E and 2D).

Collectively, these results indicated that resveratrol with exposure of TRAIL treatment induces TRAIL-mediated apoptosis in TRAIL-resistant human lung adenocarcinoma cells.

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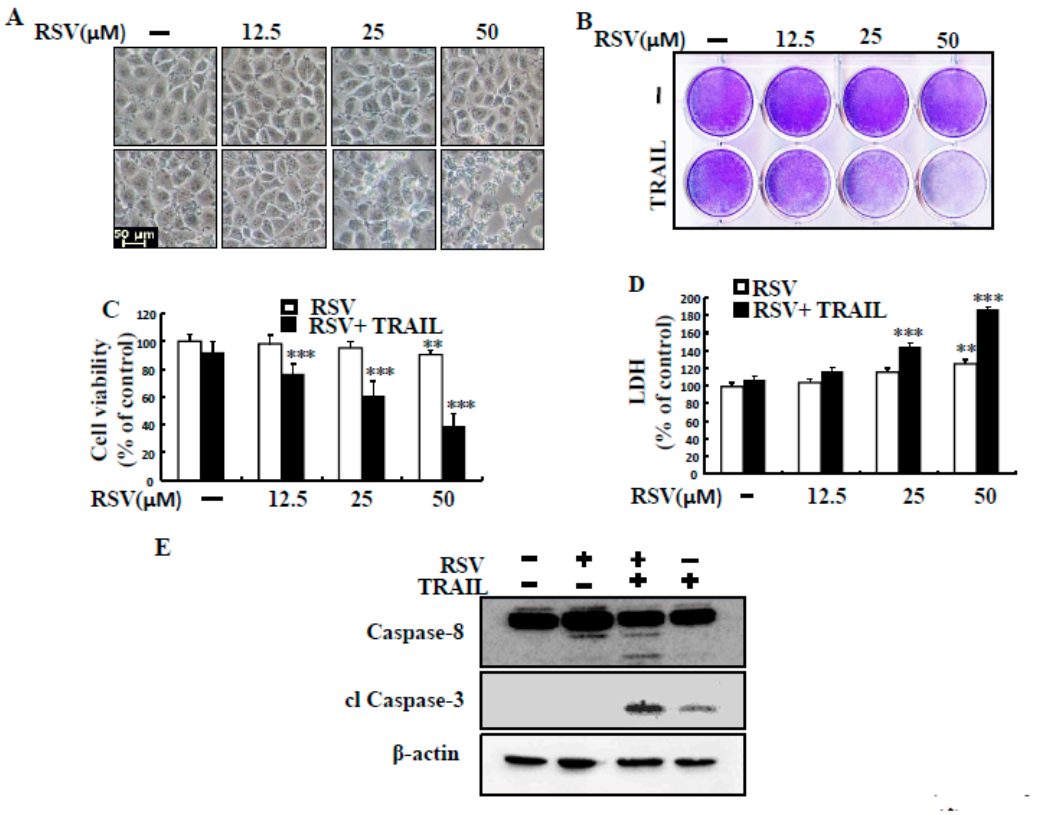

Figure1. Resveratrol enhances TRAIL-mediated apoptosis in A549 cells.

Cells were pretreated with resveratrol at serial concentrations (0, 12.5, 25, and 50 μM) for 12 h followed by treatment with 100 ng/ml of TRAIL protein for an additional 2.5 h. (A) Cell morphology were photographed under light microscope (×100) in A549 Cells, scale bar 50 μm; (B) Cell viability determined with crystal violet staining in A549 Cells; (C) Bar graph showing the cell viability by MTT assay in A549 Cells. * p<0.05 ** p<0.01, *** p < 0.001: significant differences between control and each treatment group, The results represent the means of at least 3 independent experiments.; (D) Release of LDH into cell culture during co-treatment measured from supernatant; (E) A549 cells were pre-treated with resveratrol for 12 h and then exposed to 100 ng/ml TRAIL for an additional 1 h. cl caspase 3 and caspase 8 activation was observed by western blot analysis. β-actin was used as loading control; RSV-Resveratrol.

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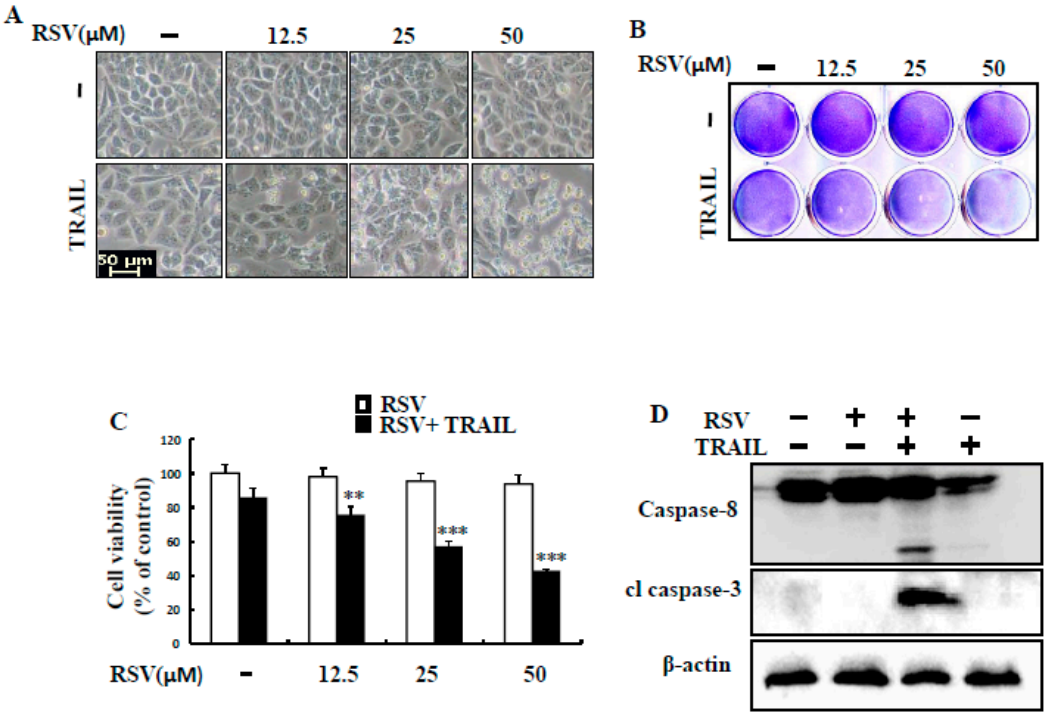

Figure2. Resveratrol enhances TRAIL-induced apoptosis in HCC15 cells.

Cells were pretreated with resveratrol with varying concentrations (0, 12.5, 25, and 50 μM) for 12 h followed by treatment with 100 ng/ml of TRAIL protein for an additional 2.5 h. (A) Cell morphology were captured under light microscope (×100) in HCC15 Cells, scale bar 50 μm; (B) Cell viability evaluated with crystal violet staining in HCC15 Cells; (C) Bar diagram shows the average density of cells by MTT assay in HCC15 Cells. * p<0.05 ** p<0.01, *** p < 0.001: significant differences between control and each treatment group. The results represent the means of at least 3 independent experiments. (D) cl caspase 3 and caspase 8 activation observed by western blot analysis. HCC15 cells were pre-treated with resveratrol 50 μM for 12 h and then exposed to 100 ng/ml TRAIL for an additional 1 h. β-actin was used as loading control

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Autophagy flux is induced by resveratrol treatment in A549 cells

To evaluate the effects of resveratrol on the induction of autophagy flux, A549 cells were pretreated with different concentrations of resveratrol for 12 h. Cell lysates were then used for western blot analysis. The formation of the autophagosome is mediated by the Atg12-Atg5-Atg16 complex and microtubule associated protein light chain 3 (LC3-I)-phospholipid conjugates (LC3-II). P62 is the well-known autophagy substrate; p62 is incorporated with autophagosomes by interacting with LC3 and is efficiently degraded by autophagy. As shown in Fig. 3A, the conversion rate of LC3-I to LC3-II increased as evidenced by the formation of autophagosomes. Furthermore, p62 expression decreased as a result of resveratrol treatment in a dose-dependent manner, therefore indicating a complete activation of autophagy flux via lysosomal degradation. Additionally, increases in p62/SQSTM1 mRNA expression in A549 cells treated with resveratrol in a dose-dependent manner were also observed, therefore demonstrating the activation of autophagy flux (Fig. 3B). Densitometry analysis indicated down expression of p62 (Fig. 3C). As shown in Fig. 3D, A549 cells treated with resveratrol showed increased amounts of punctate, therefore suggesting the activation of autophagy. Finally, transmission electron microscopy was performed to confirm the formation of double-membrane autophagosome, which contained numerous autophagic vacuoles and empty vacuoles in cells treated with 50 μM of resveratrol, whereas very few vacuoles were observed in untreated cells (Fig. 3E). These findings suggest that resveratrol could induce autophagy in TRAIL-resistant A549 cells.

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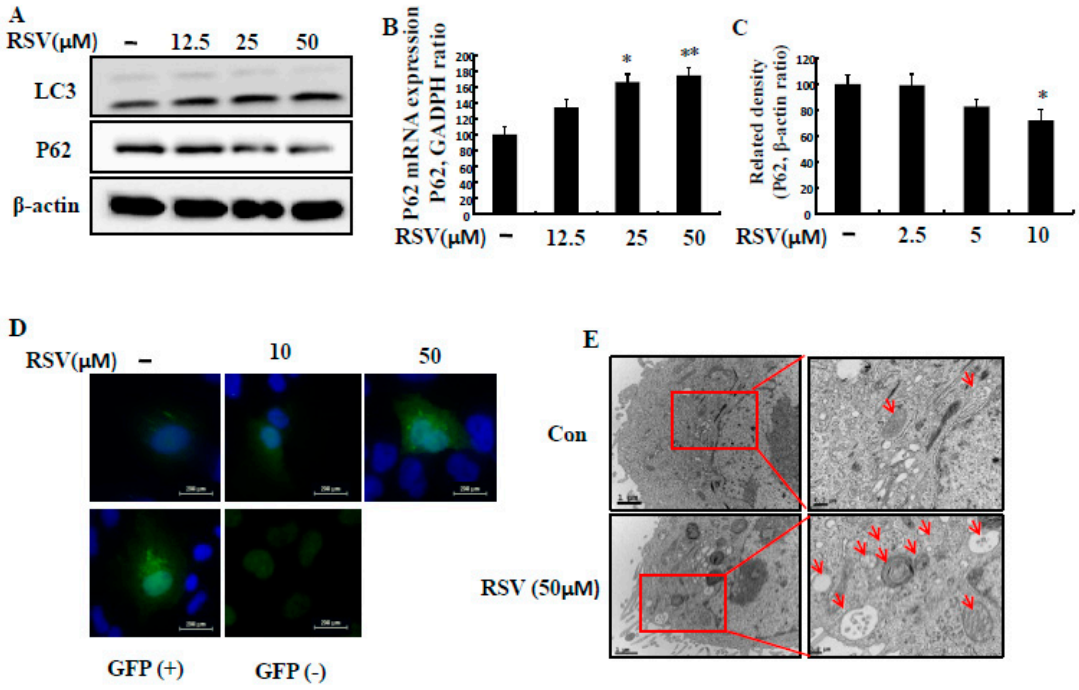

Figure 3. Autophagy flux is induced by resveratrol treatment in A549 cells

A549 cells were pretreated with resveratrol at indicated concentrations (0, 12.5, 25, and 50 μM) for 12 h. (A) Cells were harvested and examined by Western blotting to determine the expression levels of autophagy marker LC3-II, p62; (B) Real-time PCR for p62/SQSTM1 gene mRNA expression in A549 cells; (C) Densitometry analysis of p62 with β-actin; (D) A549 cells were mixed with a titration (30MOI) of BacMam GFP-LC3B virus over 18 h and were then treated with resveratrol 10 μM and 50 μM for 12 h. Negative control reagent GFP(-) and positive control reagent GFP(+) (CQ) treated at the same time. Green punctate indicates the formation of autophagosome, scale bar 200 μm. (E). TEM pictures shows the ultrastructure of cells treated with 50 μM resveratrol for 12 h. Arrows indicate autophagosomes, including residual digested material and empty vacuoles; * $p<0.05$ ** $p<0.01$, significant differences between control and each treatment group. The results represent the means of at least 3 independent experiments; CQ:

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chloroquine, RSV-resveratrol.

TRAIL-mediated apoptosis by resveratrol is independent of autophagy flux

To confirm the role of autophagy in resveratrol-mediated TRAIL induction of apoptosis, we investigated the effect of chloroquine on A549 cells (Fig. 4). A549 cells were treated with chloroquine for 1 h, followed by a resveratrol treatment for 12 h, and or TRAIL protein for an additional 1 h. Cell lysates were used for western blotting to determine changes in LC3-II and p62 expression levels. Autophagy induction was demonstrated by the confirmation of autophagy flux in cells treated with chloroquine. Furthermore, chloroquine treatment resulted in the accumulation of membrane-bound LC3-II and p62, which were not observed in the control group (Fig. 4A and 4B). To confirm the role of autophagy on A549 cell, chloroquine was used to demonstrate the effects of resveratrol on TRAIL-induced apoptosis. A549 cells were treated with chloroquine for 1 h, and then treated with resveratrol for 12 h, followed by a TRAIL treatment for an additional 2.5 h. As an autophagy inhibitor chloroquine was unable to abolish cell death, which was induced by combined treatment of resveratrol and TRAIL during autophagy activation. Cell death was observed as demonstrated by cell morphological changes and crystal violet staining in cells treated with both resveratrol and TRAIL (Fig. 4C, 4D). Furthermore, chloroquine did not have an effect on apoptosis that was initiated by resveratrol and TRAIL treatments, as indicated by the percentage of viable cells remaining (Fig. 4E). Overall, TRAIL-mediated apoptosis via resveratrol is independent of autophagy flux.

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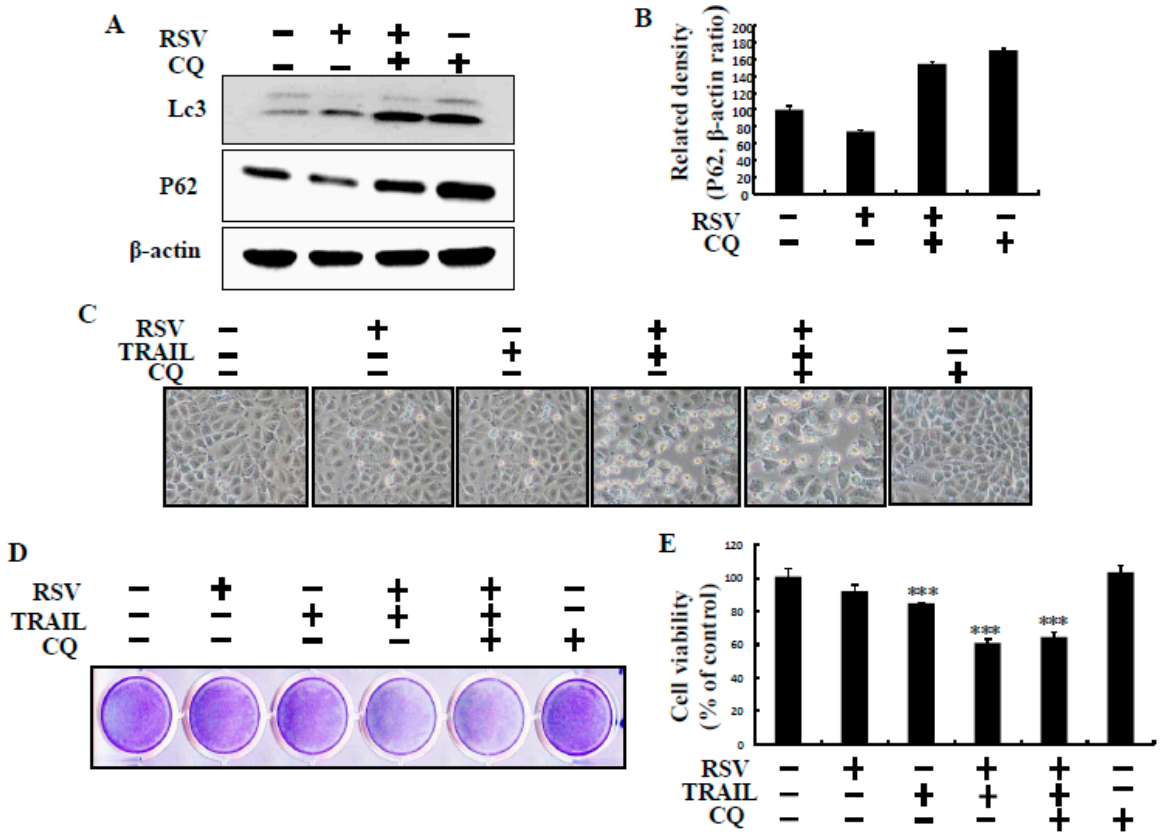

Figure 4. **TRAIL-mediated apoptosis by resveratrol is independent of autophagy flux**

A549 adenocarcinoma cells were also pretreated with and or chloroquine (30 μM) for 1 h followed by treatment with resveratrol (50 μM) for 12 h. (A) Cell lysates were prepared for western blotting and analyzed LC3-II, p62 expression level. (B). Related density of p62 were measured by representative three independent test. (C). Then, cells were pretreated with and or chloroquine (20 μM) for 1 h followed by resveratrol (50 μM) for 12 h finally with and or 100 ng/ml of TRAIL protein for an additional 2.5 h. Cell morphology photographed under light microscope (×100); (D) Cell viability measured by crystal violet assay; (E) Bar graph shows the average density of crystal violet staining dye; ** p<0.01, *** p < 0.001: significant differences between control and each treatment group. The results represent the means of at least 3 independent experiments.

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Resveratrol-enhanced TRAIL-mediated apoptosis is independent of p53

To investigate the molecular mechanism resulting in A549 cell death via co-treatment with resveratrol and TRAIL, we analyzed the contribution of p53 to apoptosis (Fig. 5). A549 cells were pretreated with different concentrations of resveratrol for 12 h and then additionally treated with and or TRAIL protein for 1 h. Western blotting revealed that Resveratrol treatment increased the expression levels of tumor suppressor proteins acetyl p53, p53, and PUMA (Fig. 5A). The combined treatment of resveratrol and TRAIL further increased the expression levels of acetyl p53, p53, and PUMA when compared to cells treated with only resveratrol (Fig. 5B). Pifithrin α, a pharmacological inhibitor of p53, was used to confirm the expression of p53 acetylation, p53, and PUMA. Through immunoblotting, we observed that pretreatment of pifithrin α suppresses the p53 acetylation, p53, and PUMA expression (Fig. 5C). To investigate the role of p53 in the apoptotic cascade, cells were treated with and or pifithrin α for 1 h, followed by resveratrol in a indicated concentration for 12 h, and then were finally treated with TRAIL protein for an additional 2.5 h. Blocking of cell death was not observed as demonstrated by a lack of morphological changes and crystal violet staining in cells treated with pifithrin α, resveratrol and TRAIL compared to the pifithrin α untreated group, therefore demonstrating that pifithrin α had no influence on cell death induced by resveratrol and TRAIL co-treatment (Fig. 5D & E). Furthermore, no differences were observed in the percentage of viable cells in the pifithrin α-treated group when compared to the pifithrin α untreated group (Fig. 5F). Pifithrin α also did not affect the activation and function of caspase-8 and cleaved caspase-3 that was initiated by the resveratrol and TRAIL co-treatment (Fig. 5G). Overall, these findings suggest that resveratrol-induced TRAIL-mediated apoptosis is independent of p53.

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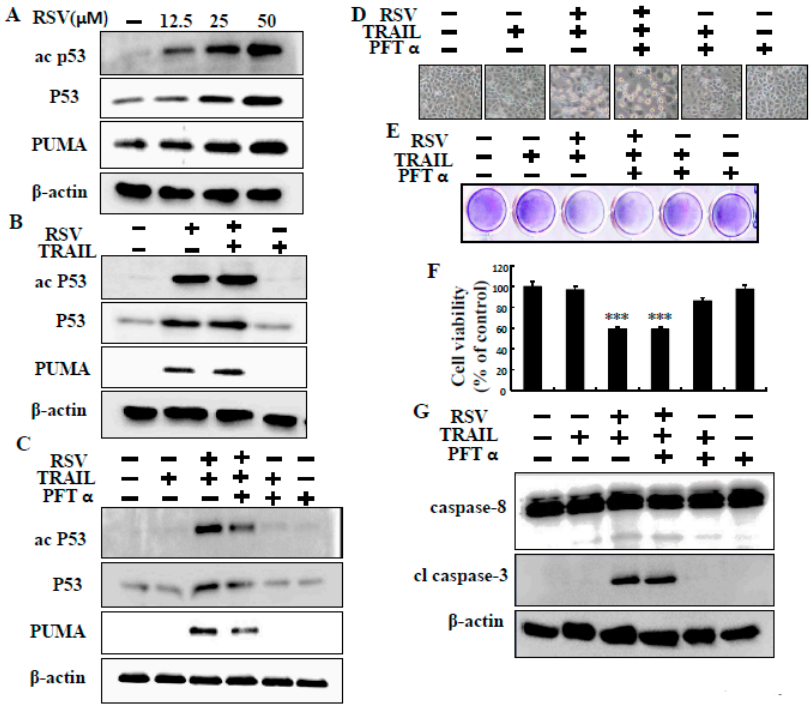

Figure 5. Resveratrol-enhanced TRAIL-mediated apoptosis is independent of p53

A549 cell were treated with resveratrol concentrations of (0,12.5,25,and 50 μM) for 12 h then incubated additional 1h and or with TRAIL protein (100 ng/ml) (A). Enhanced expression of ac p53, p53, PUMA; (B). Overexpression of p53 & PUMA observed in combined treatment. Again cells were pretreated with PFT α (40 μM) for 1 h followed by treated with resveratrol (50 μM) for 12 h, then treated with TRAIL protein for an additional 1 h; (C, G) Finally cell lysates used for western blotting and analyzed the expressions of ac p53, p53, PUMA, caspase 8 and cl caspase-3; (D). Cell morphology were photographed under light microscope in (×100); (E). Cell viability examined with crystal violet assay in A549 Cells; (F). Bar diagram showing the average density of crystal violet dye in A549 Cells; ** $p<0.01$, *** $p < 0.001$: significant differences between control and each treatment group. The results represent the means of at least 3 independent experiments. CQ- Chloroquine. PFT α- Pifithrin α; ac p53- Acetyl p53; PUMA- p53 upregulated modulator of apoptosis.

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Knockdown of p53 is unable to block apoptosis

To confirm the role of resveratrol in TRAIL-mediated apoptosis, we used a p53 knockout cell line as well as cells treated with p53 siRNA, which surely confirmed that p53 or PUMA did not maintain apoptotic stimulation (Fig. 6). A549 cells were transfected with p53 siRNA or a negative control siRNA (NC) for 24 h and then treated with 50 μM resveratrol for 12 h with and or TRAIL protein (100ng/ml) for an additional 2.5 h. We observed that p53 siRNA transfected co-treated cells did not block cellular apoptosis which was induced by resveratrol and TRAIL co-treatment. Furthermore, treatment with only the p53 siRNA or negative control siRNA had no effect on cell viability (Fig. 6A). Next, cells that were transfected with p53 siRNA were treated with resveratrol for 12 h then additionally with TRAIL for another 1 h. We observed that acetyl p53 and PUMA were downregulated in cells transfected with the p53 siRNA following co-treatment with resveratrol and TRAIL protein when compared to the non-transfected group. However, caspase-8 and cl-caspase-3 were not blocked in cells transfected with the p53 siRNA (Fig. 6B). Next, we used a p53 knockout cell line to evaluate the role of p53 in resveratrol-induced TRAIL-mediated apoptosis to confirm the results observed with the p53 siRNA. Cells were pretreated with resveratrol for 12 h, followed by additional treatment with TRAIL for 2 h. When we analyzed for cell viability, no significant differences were observed in the p53 knockout cells when compared to wild type cells after treatment with resveratrol and TRAIL (Fig. 6C). Moreover, in western blot analysis $p53^{+/+}$ and $p53^{-/-}$ HCT116 human colon carcinoma cells that were co-treated with resveratrol and TRAIL showed block the expression of p53 and PUMA when compared to wild type cells (Fig. 6D). Finally, caspase-8 and cleaved caspase-3 were also activated in both p53 wild type and knockout cell lines (Fig. 6E). Overall, these results demonstrate that knockdown of p53 is unable to block resveratrol-induced TRAIL-mediated

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apoptosis.

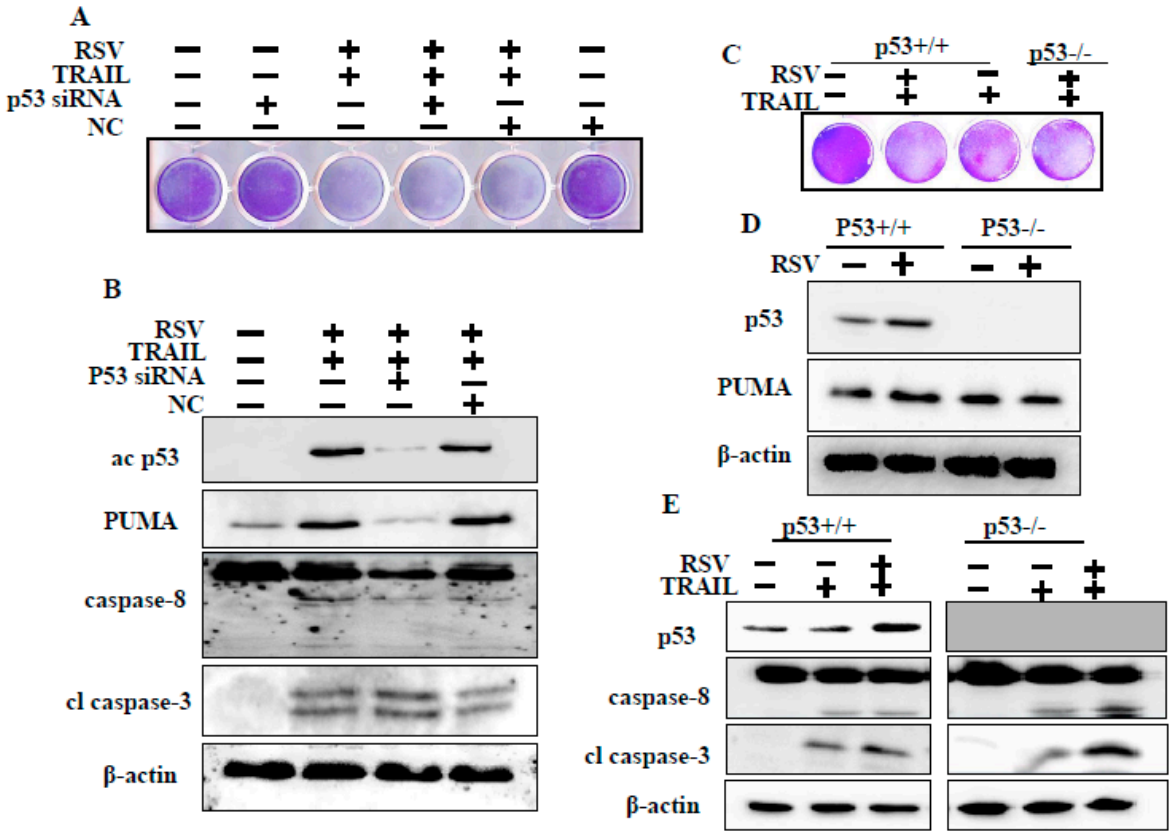

Figure 6: Knockdown of p53 is unable to block apoptosis

A549 cells were transfected by p53 siRNA and NC siRNA (85nM) for 24 h then treated with resveratrol (50μM) for 12 h then additional 1 h and or 2.5 h for TRAIL treatment (100ng/ml); (A) Cell viability determined by crystal violet staining; (B) Western blot analysis determined the expression of ac p53, PUMA, caspase 8, cl caspase 3. β-actin was used as loading control; (C). HCT 116 colon carcinoma p53+/+ and p53-/- cell line were pretreated with resveratrol (50μM) for 12 h followed by TRAIL treatment for an additional 1h. Cell viability was determined by crystal violet staining assay; (D, E) Cell lysates were applied for immunoblotting and analyzed the expression of p53, PUMA, caspase 3, cl caspase 3. β-actin was used as loading control; NC-negative control.

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Downregulation of NF-κB (p65) by resveratrol attenuates TRAIL resistance

To demonstrate exact the molecular mechanisms that are related to resveratrol-induced TRAIL-mediated apoptosis in A549 cells, we performed further immunoblotting (Fig. 7). Cells that were pretreated with resveratrol as described above showed that resveratrol decrease the expression of phosphorylated Akt-mediated suppression of NF-κB (p65) activation in a dose-dependent manner (Fig. 7A). Furthermore, we confirmed the relation between NF-κB (p65) and p53 by using p53 siRNA and a p53 knockout cell line. Lysate from resveratrol treated $p53^{+/+}$ and $p53^{-/-}$ cells were applied for immunoblotting, and we observed that NF-kB (p65) was inactivated when compared to control cells in both cell line (Fig. 7B). Furthermore, p53 siRNA and NC siRNA treatments also showed inactivation of NF-κB(p65) when compared to untreated cells proved NF-κB (p65) signaling did not regulated by p53 (Fig. 7C). Furthermore, cells co-treated with resveratrol and TRAIL also resulted in the suppression of NF-kB (p65) activity when compared to those treated only with resveratrol or TRAIL (Fig. 7D). Next, LY294002 was used and result showed that LY294002 pretreated resveratrol and TRAIL co-treated cells undergo more apoptosis than resveratrol and or TRAIL treated alone. Western blot result also observed higher blocking of NF-κB (p65) activation in LY294002 and resveratrol co-treatment than mono-treatment. These data confirmed the potential relationship to decrease the expression of phosphorylated Akt-mediated suppression of NF-κB (p65) activation by resveratrol (Fig. 7E and F). We then focused on Bcl-2 and Bcl-xl, both of which localize to the mitochondrial outer membrane. Here, they play a significant role in promoting cellular survival and inhibiting Bax and Bak, both of which promote permeabilization and the release of cytochrome c for the initiation of apoptosis. Cells were pretreated with resveratrol as described above, and cell lysates were applied for immunoblotting. Both Bcl-2 and Bcl-xl were significantly downregulated in a

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dose-dependent manner, whereas Bax remain unchanged (Fig. 7G). Resveratrol also decrease the Bcl2/Bax ratio in a dose-dependent manner (data not shown). Overall, we confirmed that the suppression of NF-kB (p65) activation by resveratrol attenuates TRAIL resistance and enhances TRAIL-mediated apoptosis via inhibition of anti-apoptotic signals.

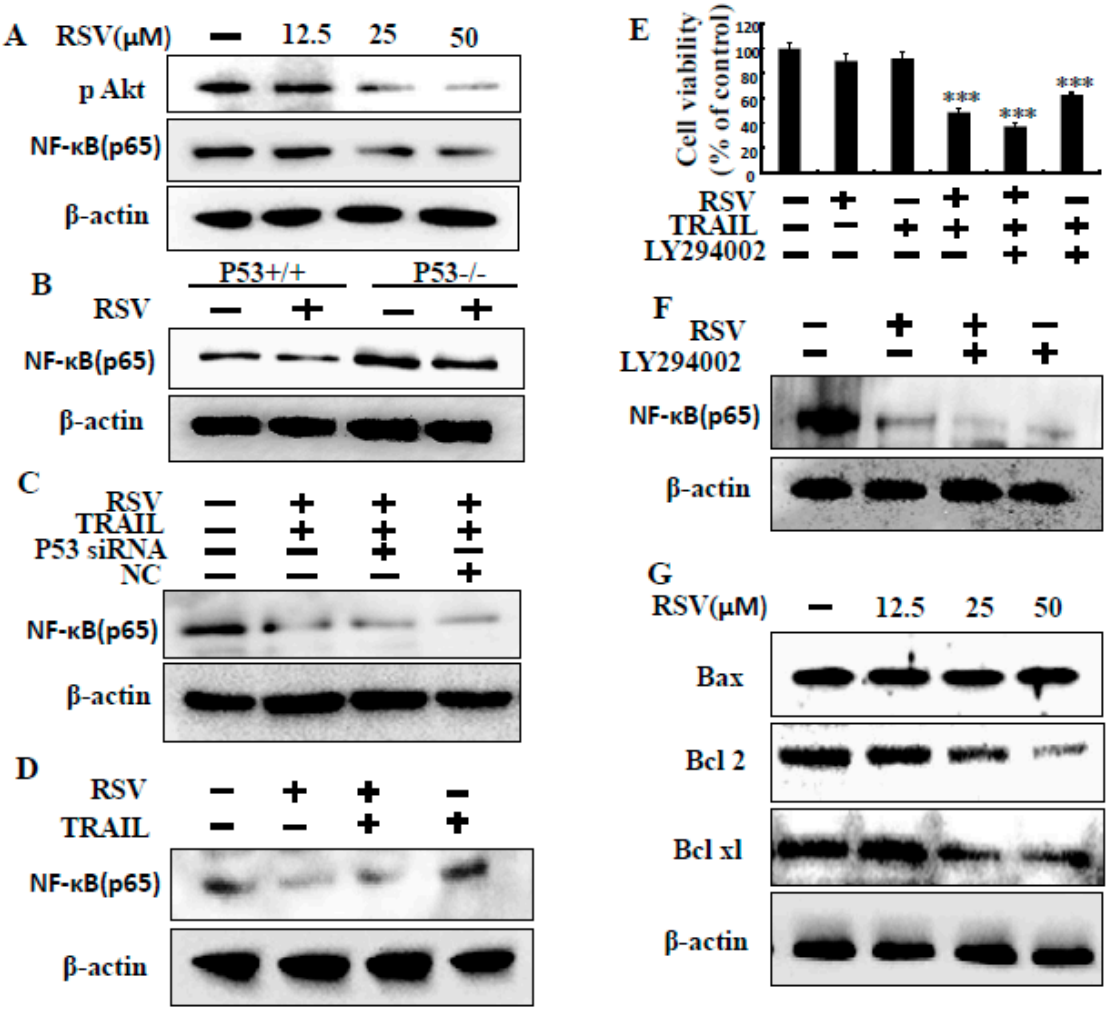

Figure 7: Downregulation of NF-κB (p65) by resveratrol attenuates TRAIL resistance

A549 cells were pretreated with resveratrol by serial concentration of (12.5, 25, 50 μM) for 12 h. (A and G). Cell lysates were used for western blotting and observed the expression level of pAkt, NF-κB (p65), Bax, Bcl-2, Bcl-xl dose dependently; (B) p53 knockout cell line was treated with 50 μM resveratrol for 12 h and observed the expression level of NF-κB (p65) by western

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blotting; (C). Cells were transfected with p53 siRNA and/or NC siRNA (85nM) for 24 h then treated with resveratrol 12 h followed by 1h additional TRAIL (100ng/ml) treatment and expression level of NF-κB (p65) was determined by immunoblotting; (D) NF-κB (p65) activity was measured by resveratrol (50 μM) and TRAIL 100 ng/ml combined treatment; (E). Cells were pretreated with LY294002 for 1h then treated with and or resveratrol (50 μM) for 12 h finally additional 2.5 h for TRAIL (100ng/ml) treatment. Bar diagram showing the average density of MTT assay in A549 Cells; ** p<0.01, *** p < 0.001: significant differences between control and each treatment group. The results represent the means of at least 3 independent experiments. (F). Cells were pretreated with LY294002 for 1h then treated with and or resveratrol (50 μM) for 12 h. NF-κB(p65) activity was measured by western blot analysis. NF-κB (p65)-nuclear factor kappaB, Bcl-2-B-cell lymphoma 2 protein, Bax-Bcl-2 associated X protein.

Cytochrome c translocation through mitochondrial dysfunction by resveratrol sensitizes TRAIL

Next, we confirmed the mitochondrial integrity by investigating the mitochondrial membrane potential (MMP) during the initiation of apoptosis (Fig. 8). Cells were pretreated with resveratrol for 12 h, followed by an additional 1 h treatment with and or TRAIL. JC-1, a lipophilic cationic dye, was used to determine the loss of MMP. In untreated cells, JC-1 normally aggregates within intact mitochondria and emits a red fluorescence in untreated cells. These aggregates were decreased in cells treated with resveratrol, and were mostly absent in the co-treated group. On the other hand, JC-1 produces green fluorescence was observed prominently in resveratrol and TRAIL co-treated group indicated the collapse of MMP due to mitochondrial dysfunction (Fig. 8A). The red-to-green fluorescent ratio confirmed the loss of MMP in cells that were co-treated

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(Fig. 8B). Owing to mitochondrial damage in resveratrol-treated A549 cells, cytochrome c was released dose dependent manner and also translocation of cytochrome c was observed from the mitochondria into the cytosol during resveratrol treatment. However, no changes in Bax localization were observed. In translocation test it showed that RSV treatment Bax translocation into mitochondria was absent. So the Bax does not require in resveratrol induced TRAIL mediated cell death (Fig. 8C and D).

Overall, resveratrol facilitate the TRAIL based cancer treatment more active in human lung cancer cells by attenuating expression of phosphorylated Akt-mediated suppression of NF-κB activation leads to mitochondrial dysfunction and cytochrome c translocation.

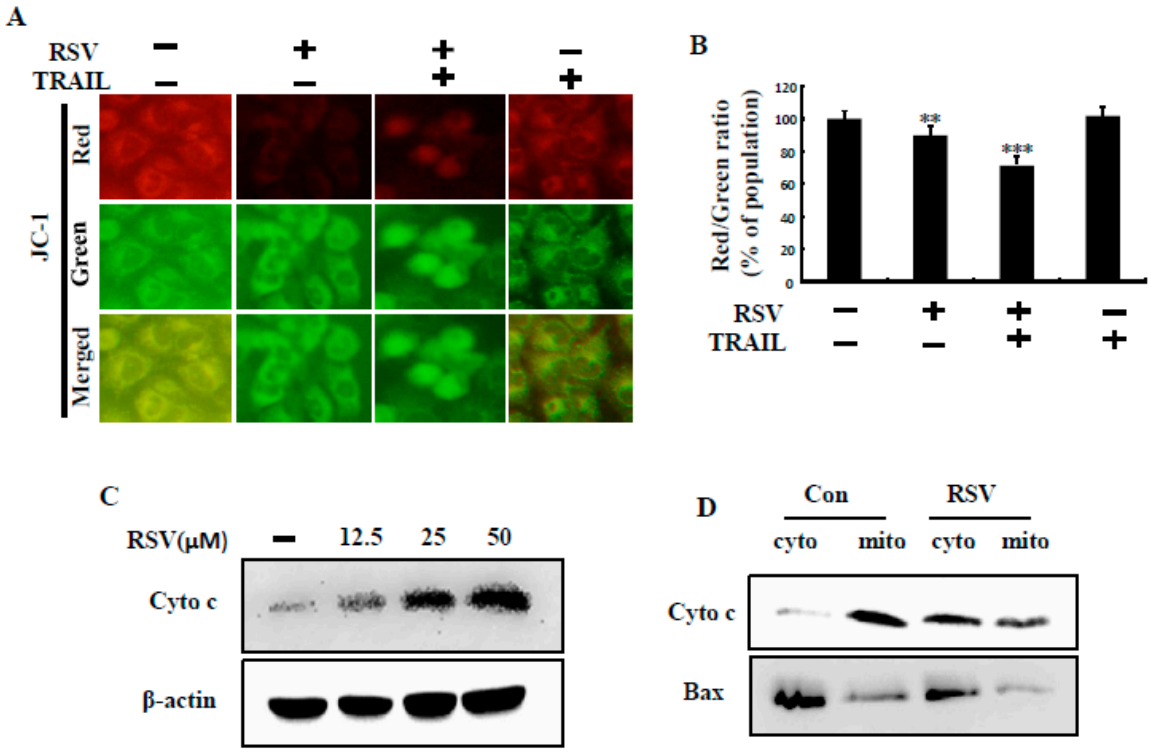

Figure 8: Cytochrome c translocation through mitochondrial dysfunction by resveratrol sensitizes TRAIL

A549 cells were cultured in glass slide and treated with resveratrol 50 μM for 12 h then

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additional treatment of and/or TRAIL 100 ng/ml for 1 h. (A) JC-1 aggregated cell morphological images were taken under fluorescence microscope; (B). Bar diagram showing the loss of MMP by resveratrol (50 μM). ** $p<0.01$, *** $p < 0.001$: significant differences between control and each treatment group. The results represent the means of at least 3 independent experiments. (C). Cell lysates transferred for western blotting showed the gradual expression of cytochrome c by dose dependent manner; (D). Mitochondrial or cytosolic proteins expression of cytochrome c and Bax were detected by western blot analysis.

Discussion

Although numerous invasive and noninvasive therapeutic interventions have been developed for cancers, tumors still display highly complex behaviors and resistance to therapeutic actions. To meet the current demand for anticancer therapies, our goal was to demonstrate the molecular mechanisms underlying resveratrol-induced TRAIL-mediated apoptosis in NSCLC. Herein, we conclude that a combination treatment using resveratrol and TRAIL is effective against TRAIL-resistant lung cancer cells via targeting suppression of NF-κB (p65) activity which potentiate mitochondrial dysfunction and apoptosis.

TRAIL sensitizes a wide variety of cancer cells or transformed cells by apoptosis; however, TRAIL resistance in different cancer cells is a growing concern for cancer chemotherapies [9, 56, 57]. In our study, we also observed that a single treatment with resveratrol or TRAIL did not or only slightly increase cell death, whereas a combined treatment enhanced apoptosis in a dose-dependent manner. This was shown by the activation of caspase-8 and cleaved caspase-3 in A549 and HCC-15 cells that were treated with both resveratrol and TRAIL (Fig. 1 and 2); therefore demonstrating that TRAIL resistance in lung cancer cells can be attenuated by a combined

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treatment. We then investigated the molecular mechanism underlying this apoptotic pathway. Previous reports have suggested that resveratrol has the capability to induce autophagy and might also play a role in enhancing apoptosis [58-60]. However, our western blot results revealed that treatment with different concentrations of resveratrol increased LC3-II accumulation and decreased p62 expression levels in A549 cells, thus confirming the activation of complete autophagy flux by lysosomal degradation (Fig. 3). Furthermore, to demonstrate the role of autophagy in resveratrol-induced TRAIL-mediated apoptosis, we used chloroquine, a pharmacological autophagy inhibitor. Our result demonstrated that pretreatment with chloroquine did not rescue the cells undergoing apoptosis, suggesting that the apoptotic pathway was independent of autophagy flux (Fig. 4). To investigate the specific mechanism involved in resveratrol-induced TRAIL-mediated apoptosis, we analyzed p53 and PUMA, both of which have been previously shown to have a potential role in apoptosis [27, 50, 61, 62]. Our findings demonstrate that resveratrol enhanced p53 acetylation, p53, and PUMA expression in a dose-dependent manner and was triggered by the combined treatment of resveratrol and TRAIL. To confirm the exact contribution of p53 during apoptosis, we used pifithrin α, p53 siRNA and p53 knockout cell line. Although knock down of the upregulated expression of acetylated p53, p53, and PUMA, but in case of cell viability rescue of cells undergo apoptosis does not appeared. Furthermore, caspase-8 and cleaved caspase-3 remained unchanged in the co-treated groups (Fig. 5, 6). Overall, these results demonstrated that resveratrol-induced TRAIL-mediated apoptosis is independent of p53.

Additionally, NF-κB (p65) signaling may contribute to cellular proliferation, suppression of apoptosis, angiogenesis, and metastasis. The suppression of NF-κB (p65) has been shown to be an effective therapeutic in cancer treatments [63]. However, NF-κB (p65) has also been shown to

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contribute to TRAIL resistance [14]. NF-κB (p65) regulates Bcl-2 and Bcl-xl, both of which localize to the mitochondrial outer membrane where they play a significant role in promoting cellular survival and inhibiting the actions of Bax and Bak, which are known to promote permeabilization and the release of cytochrome c for the initiation of apoptosis [15, 30, 64, 65]. Our data suggest that resveratrol treatment suppress expression of phosphorylated Akt, which is involved in cell survival, thus resulting to the supression of NF-κB (p65) activation. PI3K/pAkt inhibitor LY294002 combined with resveratrol and TRAIL significantly enhance the cells undergo apoptosis and suppression of NF-κB (p65) activation was also prominent. To confirm the relationship between p53 and NF-κB(p65), we used $p53^{+/+}$ and $p53^{-/-}$ HCT 116 cells and transfected them with a p53 siRNA. In both cases, NF-κB(p65) had no effect on p53 expression. A combined treatment of resveratrol and TRAIL also suppressed NF-κB (p65). The downregulation of Bcl-2 and Bcl-xl via resveratrol treatment also supported its role in an anti-cancer mechanism, especially since Bax remained unchanged (Fig. 7). Furthermore, the aggregation of JC monomers was an effective indicator for the MMP. The loss of MMP was due to the collapse of cell integrity during the initiation of apoptosis. Our data showed that the loss of MMP was highest in the resveratrol and TRAIL co-treated group, which lead to the release of cytochrome c. The translocation of cytochrome c was observed from the fragile mitochondria into the cytoplasm in the treated group, whereas no changes in Bax localization were observed (Fig. 8). Overall, these studies conclude that resveratrol could attenuate TRAIL resistance in TRAIL-resistant cancer cells as well as simultaneously enhancing TRAIL-mediated apoptosis via targeting the NF-κB (p65) signaling pathway. Finally, resveratrol potentiate TRAIL based cancer treatment more effective and it could be eminent therapeutic strategy in lung cancer treatment regimen.

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MATERIALS AND METHODS

Cell culture

Cancer cells originating from lung (A549, HCC15) tumors were obtained from the American Type Culture Collection (Global Bioresource Center, Manassas, VA, USA). P53-containing ($p53^{+/+}$) and p53 knockout ($p53^{-/-}$) HCT116 human colon carcinoma cell lines were provided by Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). Cells were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 µg/ml penicillin-streptomycin). Cells were incubated at 37 °C in a 5% CO_2 environment.

Reagents

Resveratrol, chloroquine (20 µM), and Pifithrin α were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRAIL (100 ng/ml) was purchased from Abfrontier (Geumcheon-gu, Seoul, South Korea).

Cell viability test

A549 cells were seeded at a density of 1.0×10^4 cells onto 12-well plates and incubated at 37 °C for 24 h. The A549 cells were pretreated with Resveratrol at serial concentrations of (0, 12.5, 25, 50µM) following 12 h then recombinant TRAIL protein (100 ng/ml) was added and co-incubated for additional 2.5 h. Additionally cells were pretreated with pifithrin α (40 µM) and chloroquine (20 µM) for 1 h, followed by Resveratrol treatment. Cell morphology was assessed by taking photographs under inverted microscopy (Nikon, Japan). Cell viability was measured by adding 50 µl of 5 mg/ml methyl-thiazolyl-tetrazolium (MTT) to each well, and the plates were incubated at 37˚C for 2 h. Then, 500 µl dimethyl sulfoxide was added to each well after removal of the old medium, and absorbance was measured by spectrophotometer (Bio-Rad, Hercules, CA, USA) at

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570 nm. Cell viability was also determined by using the crystal violet staining method. In brief, cells were stained with a staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde) for 10 min at room temperature, washed four times with PBS, and dried. Cells were then lysed with 1% SDS solution. The absorbance value was then measured at a wavelength of 550 nm using a plate reader. Cell viability was expressed as relative dye intensity compared with that of the control.

Lactate dehydrogenase assay

Cytotoxicity from supernatants assessed by using a lactate dehydrogenase (LDH) cytotoxicity detection kit (Takara Bio, Inc., Tokyo, Japan) according to the manufacturer's protocol. LDH activity was determined by measuring absorbance at 490 nm, using a microplate reader (Spectra Max M2, Molecular Devices, Sunnyvale, CA, USA).

Western blot assay

Treated Cells were washed in cold PBS, harvested by re-suspending in lysis buffer [25 mM HEPES (pH 7.4), 100 mM EDTA, 5 mM $MgCl_2$, 0.1 mM DTT, and a protease inhibitor cocktail], or using a Nuclear/Cytosol Fractionation kit 261 (BioVision, Milpitas, CA, USA) and sonicated to prepare A549 cell lysates. Proteins (20-35 μg) were separated in 10%–15% SDS gels and transferred into nitrocellulose membranes. After incubation with indicated concentration of primary antibody in dilution buffer (1% milk with PBS-Tween) and secondary antibody (1:5000) membranes were developed with enhanced chemiluminescence reagents. Primary antibodies (1:1000) were used for immunoblotting: LC3, p62 Sigma-Aldrich (St. Louis, MO, USA), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), acetyl p53 ((epitomics), PUMA (CALBIOCHEM); p53, Bcl-2, Bcl xl, NF-κB (p65) and Bax (1:500) ; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), cytochrome c, caspase-8 (BD pharmingen, USA),

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and ß-actin Sigma-Aldrich (1:1000) (St. Louis, MO, USA). Images were obtained using a Fusion-FX7 imaging system (Vilber Lourmat, Marne-la-Vallée, France). For each blots we confirmed the data at least 3/4 independent experiments.

TEM (Transmission Electron Microscopy) analysis

TEM samples were analyzed by Transmission Electron Microscope (JEM-2010, JEOL) installed in the Center for University-Wide Research Facilities (CURF) at Chonbuk National University. After fixation of samples in 2 % glutaraldehyde (EMS, USA) and 2 % paraformaldehyde (EMS, USA) in 0.05 sodium carcodylate buffer (pH7.2) (EMS, USA), specimens were post fixed in 1% osmium tetroxide (EMS, USA), dehydrated in graded ethanol and propylene oxide (EMS, USA). The cells were embedded in Epoxy resin (Embed 812, NMA; Nadic methyl anhydride, DDSA; Dodenyl Succinic Anhydride, DMP-30) (EMS, USA). Ultrathin sections were cut on an LKB-III ultratome (LEICA, Austria) and were stained with 0.5% uranyl acetate (EMS, USA) and lead citrate (EMS, USA). The images were taken on a Hitachi H7650 electron microscope (Hitachi, Japan) at an accelerating voltage of 100 kV.

BacMam transduction

Wild-type or mutant GFP-tagged LC3B was expressed in cells by adding the appropriate concentrations of the appropriate virus from the Premo Autophagy Sensor LC3B-GFP kit (BacMam 2.0) (Life Technologies P36235) to the growth medium as indicated in the figure legends.

Mitochondrial Membrane Potential assay

Resveratrol treated A549 cell were incubated in media containing 10 µM JC-1 at 37 °C for 30 min at RT, washed with PBS, and transferred to a clear 96-well plate. J-aggregates in intact mitochondria were evidence as red fluorescence with emission at 583 nm, excitation 490nm indicating high or normal MMP. Green fluorescence with emission at 525 nm, excitation 490 nm

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indicates low MMP when JC-1 remains in the monomeric form in the cytoplasm. A549 cells were cultured onto cover slips in a 24-well plate, incubated in media containing 10 μM JC-1 at RT for 30 min in dark condition, and washed with PBS. Finally, cells were mounted with DakoCytomation fluorescent medium (Dako, Carpinteria, CA, USA) and visualized under a fluorescence microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total ribonucleic acid (RNA) was extracted from A549 cells using the easy-spin™ Total RNA Extraction Kit (Intron Biotechnology, Seoul, Korea). The cDNA synthesis was carried out following the instructions in the TaKaRa PrimeScript TM 1st strand cDNA synthesis kit (TaKaRa Bio, Tokyo, Japan). For qRT-PCR, 1 μl of gene primers with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA) in 20 μl of reaction volume was applied. The primers were: p62/SQSTM1 (forward: 5′CTCCCCAGACTACGACTTGTGT3′, reverse: 5′TCAACTTCAATGCCCAGAGG3′), and β-actin (as an internal control) (forward: 5′GCAAGCAGGAGTATGACGAG3′, reverse: 5′CAAATAAAGCCATGCCAATC3′). All reactions with iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) were performed on the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA).

RNA interference

A549 cells were transfected with p53 small interfering RNA (siRNA ID 106140; ambion, by life Technologies Corporation) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. At 24-h post transfection, the knockdown efficiency at protein level was determined by immunoblotting and cell viability test. Scrambled siRNA (Invitrogen) was used as a negative control.

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Statistical analysis

All data are expressed as means ± standard deviation (SD). For multiple comparisons, one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test was used. All statistical analysis was performed using GraphPad Prism software. Statistical significance was indicated by a *P* value less than 0.05 (*), 0.01 (**), or 0.001 (***).

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Conflict of interest

The authors declare no conflict of interest.

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