Original Article

DOI: 10.5582/bst.2016.01170

Induction of apoptosis by ethanol extract of *Evodia rutaecarpa* in HeLa human cervical cancer cells *via* activation of AMP-activated protein kinase

Seon Young Park[1,*], Cheol Park[2,*], Shin-Hyung Park[3], Su-Hyun Hong[4], Gi-Young Kim[5], Sang Hoon Hong[1,], Yung-Hyun Choi[4,6,**]**

[1] *Department of Internal Medicine, Dongeui University College of Korean Medicine, Busan, Korea*;
[2] *Department of Molecular Biology, College of Natural Sciences and Human Ecology, Dongeui University, Busan, Korea;*
[3] *Department of Pathology, Dongeui University College of Korean Medicine, Busan, Korea;*
[4] *Department of Biochemistry, College of Korean Medicine, Dongeui University, Busan, Korea;*
[5] *Department of Marine Life Sciences, School of Marine Biomedical Science, Jeju National University, Jeju, Korea;*
[6] *Anti-Aging Research Center, Dongeui University, Busan, Korea.*

Summary **The fruit of *Evodia rutaecarpa* (Juss.) Benth has been used widely in traditional medicine therapy. Although it has been shown to possess many pharmacological activities, the molecular mechanisms of its anti-cancer activity have not been clearly elucidated. In the present study, we investigated the pro-apoptotic effects of an ethanol extract isolated from immature fruits of *E. rutaecarpa* (EEER) in HeLa human cervical cancer cells. EEER treatment decreased the cell viability of HeLa cells in a concentration-dependent manner, which was related to apoptotic cell death resulting from apoptotic body formation, DNA fragmentation, and an increased population of annexin V^+-positive cells. EEER treatment significantly suppressed anti-apoptotic Bcl-2 expression, leading to subsequent loss of mitochondrial membrane potential (MMP), while it did not change expression levels of death receptor (DR)-related proteins. EEER treatment increased activity of caspase-3 and -9 but not caspase-8, and pretreatment of a caspase-3 inhibitor markedly attenuated EEER-induced apoptosis. Furthermore, EEER activated the AMP-activated protein kinase (AMPK) signaling pathway; however, inhibition of AMPK markedly abrogated EEER-induced apoptosis. Overall, the results suggest that the apoptotic activity of EEER may be associated with a caspase-dependent cascade through activation of the intrinsic signaling pathway connected with AMPK activation. *E. rutaecarpa* could be a prospective clinical application to treat human cervical cancer.**

Keywords: *Evodia rutaecarpa*, cervical cancer, apoptosis, caspase, AMPK

1. Introduction

Apoptosis is a well-known type of programmed cell death mediated by extrinsic and intrinsic pathways. The extrinsic pathway is activated by an interaction between DRs and their ligands at the plasma membrane, which causes subsequent activation of caspase-8 (*1*). The intrinsic pathway, also called the mitochondrial pathway, is initiated by the loss of MMP ($\Delta\psi m$) and the release of pro-apoptotic proteins, such as cytochrome *c*, leading to the activation of caspase-9 and -3 (*2*). In particular, mitochondria are emerging as idealized targets for anti-cancer drugs. Whereas oncogenes are frequently mutated in cancers, leading to drug resistance, mitochondria have proved to be invariant targets working across all cancer types (*3,4*). Even though not absolutely essential for the induction of apoptosis, mitochondrial disruption results in excessive

Released online in J-STAGE as advance publication November 27, 2016.

*These authors contributed equally to this works.
***Address correspondence to:*
Dr. Sang Hoon Hong, Department of Internal Medicine, Dongeui University College of Korean Medicine, Busan 47227, Republic of Korea.
E-mail: shhong@deu.ac.kr

Dr. Yung-Hyun Choi, Department of Biochemistry, College of Korean Medicine, Dongeui University, Busan, 47227, Republic of Korea.
E-mail: choiyh@deu.ac.kr

reactive oxygen species (ROS) production and therapy-activating apoptosis in cancer cells (*4,5*). In addition, cancer cells produce adenosine triphosphate (ATP) from mitochondria through oxidative phosphorylation (OXPHOS) in glucose-deprived conditions, implying that targeting the mitochondrial bioenergetic pathway could be an effective strategy to treat cancers (*6*).

AMPK is a metabolic-sensing protein kinase that plays a critical role as an energy sensor in ATP-deprived conditions (*7,8*). An increased ADP/ATP ratio leads to AMPK phosphorylation at Thr172, contributing to activation of ATP-generating catabolic processes, such as fatty acid oxidation and glycolysis, as well as suppression of ATP-utilizing anabolic pathways, including glycogen, protein, and lipid synthesis. Beyond these metabolic effects, interest in the potential involvement of AMPK in the regulation of cancer cell growth has gradually increased since the tumor suppressor liver kinase B1 (LKB1) was identified as the major upstream kinase of AMPK (*9,10*). A variety of studies have reported that AMPK regulates cell proliferation and apoptosis *via* multiple signaling pathways, such as phosphorylation and subsequent stabilization of tumor suppressor p53, upregulation of cyclin-dependent kinase inhibitors, and downregulation of the mammalian target of rapamycin complex-1 activity (*7,11*). In particular, it has been demonstrated that activation of AMPK is responsible for inducing apoptosis *via* the mitochondrial pathway (*12,13*). However, AMPK is also known to play a protective role in cancer cells under metabolic stressed conditions by maintenance of energy homeostasis and modulation of autophagy, making the influence of AMPK on cancer progression controversial (*14,15*).

The immature fruit of *Evodia rutaecarpa* (Juss.) Benth has traditionally been used in Oriental medicine to treat headaches, gastrointestinal disorders, and amenorrhea for thousands of years (*16,17*). It has been reported that the extracts of *E. rutaecarpa* and its alkaloids exhibit anti-obesity, anti-dementia, anti-inflammatory, and anti-oxidant effects (*18,19*). More recently, anti-cancer activities of *E. rutaecarpa* have been suggested. For instance, G2/M phase cell cycle arrest was involved in apoptosis of lung cancer cells induced by evodiamine, which is an *E. rutaecarpa* derived alkaloid based substance. The caspase activity accompanied by c-Jun N-terminal kinase (JNK) activity inhibition was involved in apoptosis induced in both ovarian and colonic cancer cells (*20,21*). In addition, there have been other reports indicating that inhibition of the activity of the mechanistic target of rapamycin (mTOR) signal systems, or insulin-like growth factor 1, is involved in apoptosis of human cancer cells exposed to evodiamine (*22,23*). Furthermore, it has been reported that the inhibition of human cancer cell proliferation by evodiamine is associated with overcoming of multidrug resistance related to nuclear factor-kappa B signaling pathway inhibition and inhibition of invasive activity by calcium/JNK signaling-mediated autophagy induction and to increases in the activity of matrix metalloproteinase (*24-26*) However, to date no systematic study has been conducted on *E. rutaecarpa* extracts *per se*. In the current study, we explored the anti-cancer effects of the ethanol extract of *E. rutaecarpa* immature fruits (EEER) in HeLa human cervical cancer cells and investigated the underlying mechanism. We found that EEER triggered apoptosis in HeLa cells *via* the mitochondrial pathway, indicating that AMPK is a critical regulator of EEER-induced apoptosis. To our knowledge, this is the first study suggesting the involvement of AMPK in the anti-cancer activity of *E. rutaecarpa*.

2. Materials and Methods

2.1. *Preparation of EEER*

For the preparation of EEER, the dried immature fruits of *E. rutaecarpa* (100 g) were provided from Dongeui Korean Medical Center (Busan, Republic of Korea) and pulverized into a fine powder. The powder was then extracted in 1 L of 70% ethanol by sonication for 3 h. After filtering and concentrating the extracts, the remaining powder was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) as a stock solution at a concentration of 100 mg/mL and stored at 4˚C. The stock solution was diluted with medium to the desired concentration prior to use.

2.2. *Cell culture*

HeLa human cervical carcinoma cells were obtained from the American Type Culture Collection (Manassas, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin streptomycin (Gibco BRL) at 37˚C in a humidified atmosphere containing 5% CO_2.

2.3. *Cell viability assay*

To investigate cell viability, the HeLa cells were seeded in a 96-well plate at a density of 2×10^3 cells/well and stabilized for 24 h. The cells were treated with various concentrations of EEER for 24 h with or without 1 h pre-treatment with N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk), a caspase-3 inhibitor (CalBiochem, San Diego, CA, USA), and compound C, an inhibitor of AMPK (Sigma-Aldrich Chemical Co.). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT, Sigma-Aldrich Chemical Co.) working solution (0.5 mg/mL) was then added to the media and incubated for a further 4 h at 37˚C. After incubation, the culture supernatant

was aspirated, and 100 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co.) was added to completely dissolve the formazan crystals. Absorbance of each well was measured at a wave length of 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA) (*27*).

2.4. *Nuclear staining with DAPI*

The HeLa cells were treated with EEER at various concentrations for 24 h with or without 1 h pre-treatment with z-DEVD-fmk or compound C. The cells were then harvested, washed with phosphate-buffered saline (PBS), and fixed with 3.7% paraformaldehyde (Sigma-Aldrich Chemical Co.) for 30 min at room temperature. After washing twice with PBS, the cells were attached on glass slides using cytospin (Shandon, Pittsburgh, PA, USA) and stained with 2.5 μg/mL 4,6-dianmidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) solution for 20 min at room temperature. The stained cells were washed three times with PBS and analyzed using a fluorescence microscope (Carl Zeiss, Deisenhofen, Germany).

2.5. *DNA fragmentation assay*

Following EEER treatment for 24 h at various concentrations with or without 1 h pre-treatment using z-DEVD-fmk or compound C, the cells were lysed in a buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylendiaminetetra acetic acid (EDTA), and 0.5% Triton X-100] for 1 h at room temperature. After centrifugation at 14,000 rpm for 30 min, the supernatant was collected and incubated with proteinase K (Invitrogen, Carlsbad, CA, USA) for 3 h at 50˚C. The fragmented DNA in the supernatant was purified using the same amount of neutral phenol:chloroform:isoamyl alcohol solution (Sigma-Aldrich Chemical Co.) by rotation for 30 min at room temperature. After centrifugation, the supernatant was added to 0.5 M NaCl (final concentration) and 1 volume of isopropanol to precipitate the fragmented DNA and was incubated overnight at 4˚C. The DNA pellet obtained by centrifugation was then dissolved in TE buffer (10 mM Tris-HCl containing 1 mM EDTA) containing RNase A (Sigma-Aldrich Chemical Co.) and was separated on 1% agarose gels. The DNA fragmentation pattern was visualized by an ultraviolet light source after ethidium bromide (EtBr, Sigma-Aldrich Chemical Co.) staining.

2.6. *Flow cytometry analysis*

Changes in the MMP were measured by flow cytometry (Becton Dickinson, San Jose, CA, USA) using 5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma-Aldrich Chemical Co.) and a dual-emission potential-sensitive probe. The cells treated with various concentrations of EEER for 24 h were collected, washed twice with PBS, and incubated with 10 μM JC-1 for 20 min at 37˚C in the dark. After centrifugation, the stained cells were washed once with PBS and were resuspended in PBS. Cells with normal MMP formed aggregates with a high FL-2 fluorescence after JC-1 treatment. However, the loss of MMP changes the FL-2 fluorescence into FL-1 fluorescence because the dye shifts from an aggregate to a monomeric state. Therefore, the increase of FL-1 fluorescence was determined to be the loss of MMP. For Annexin V-propidium iodide (PI) double staining, the cells were challenged with EEER for 24 h with or without 1 h pretreatment with z-DEVD-fmk or compound C. Apoptotic cells were quantitatively identified using an Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson) containing FITC conjugated annexin V and PI, according to the protocols provided by the manufacturer (*28)*. The data were converted to density plots for presentation using CellQuest software.

2.7. *Determination of caspase activity*

The activities of caspases were determined using colorimetric assay kits (R&D Systems, Minneapolis, MN, USA) containing the synthetic tetrapeptides [Asp-Glu-Val-Asp (DEAD) for caspase-3, Ile-Glu-Thr-Asp (IETD) for caspase-8, and Leu-Glu- His-Asp (LEHD) for caspase-9] labeled with p-nitroaniline (pNA), according to the manufacturer's instructions. Briefly, EEER-treated cells were harvested and lysed in the supplied lysis buffer. Supernatants were then collected and incubated at 37˚C with the supplied reaction buffer, dithiothreitol (DTT), and respective substrates. Reaction activities were evaluated by measuring absorbance at 405 nm using an ELISA reader.

2.8. *Western blotting analysis*

To prepare whole cell lysate, cells treated under the indicated condition were lysed with ice-cold lysis buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenymethylsulfonyl fluoride (PMSF), 5 mM DTT], including a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (1 mM Na3VO4, 100 mM NaF, 10 mM NaPP). After centrifugation at 13,000 rpm at 4˚C for 30 min, the supernatants were collected and protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Equivalent amounts of protein were resolved using sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were probed with the specific primary antibodies and corresponding secondary antibodies. The protein-antibody complexes were

detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce, Rockford, IL, USA), according to the manufacturer's protocol. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for the antibody against phospho-acetyl-coenzyme A carboxylase (ACC), which was obtained from Cell Signaling Technology (Beverly, MA, USA). The horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Amersham Life Science Corp. (Arlington Heights, IL, USA).

2.9. *Statistical analysis*

Each result is expressed as the mean ± standard deviation (S.D.) of data obtained from independent triplicate experiments. A statistical analysis was performed using a paired Student's *t*-test. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. *EEER inhibits cell viability and induces apoptosis in HeLa cells*

To investigate the effects of EEER on cell viability, HeLa cells were treated with various concentrations of EEER for 24 h. As shown in Figure 1A, EEER treatment reduced cell viability in a concentration-dependent manner, with an IC_{50} of about 45 μg/mL (Figure 1A). Morphological changes, including membrane blebbing, diminished cell density, and an increased number of floating cells were also observed (Figure 1B). Because several types of cell death, including necrosis, apoptosis, and autophagic cell death, have been reported, we next investigated which type of cell death is induced by EEER. The results showed that EEER treatment significantly increased the number of condensed or blebbing nuclei that generally appear in apoptosis before nuclear fragmentation (Figure 1C). DNA fragmentation was consistently observed with EEER treatment at 20 μg/mL and gradually increased in a concentration-dependent manner (Figure 1D). EEER also enhanced the population of annexin V^+/PI^- cells, which represent early apoptotic cells (Figure 1E). These data collectively suggest that EEER suppressed cell proliferation by inducing apoptosis in HeLa cells.

3.2. *EEER modulates the intrinsic pathway leading to the loss of MMP in HeLa cells*

Given that there are two classical pathways in apoptosis – the extrinsic and intrinsic pathways – we examined which pathway is involved in EEER-induced apoptosis. The extrinsic pathway is initiated by the interactions between the DRs and their corresponding ligands.

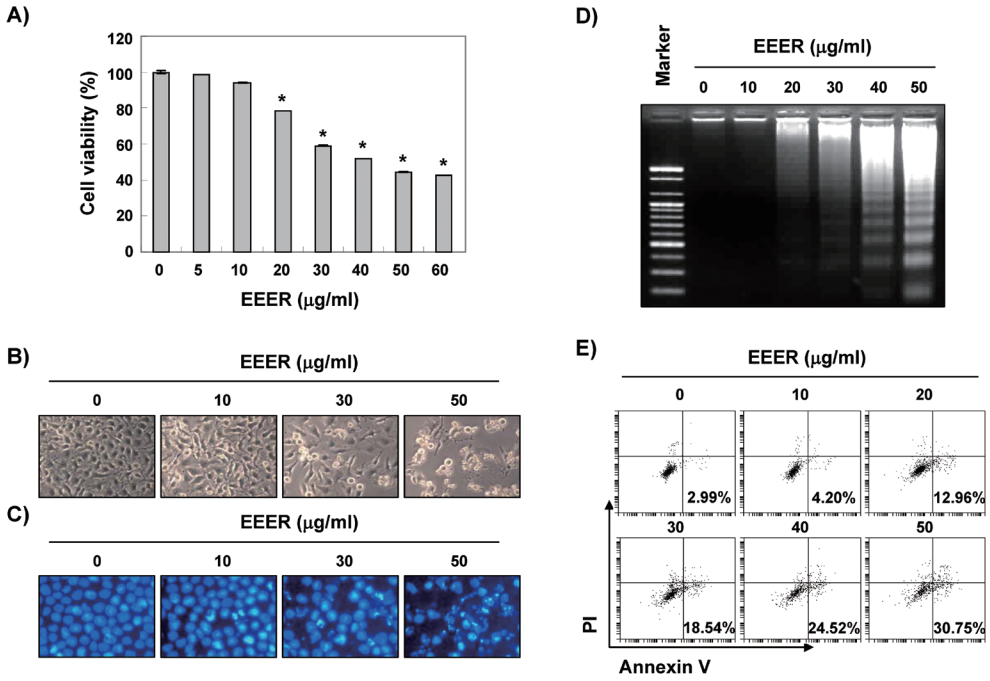

Figure 1. Inhibition of cell viability and induction of apoptosis by EEER in HeLa cells. Cells were treated with various concentrations of EEER for 24 h. **(A)** Cell viability was measured by an MTT assay. Data are expressed as the mean ± S.D. of three independent experiments. Significance was determined by the Student's *t*-test (*$p < 0.05$ *vs*. untreated control). **(B)** Morphological changes were visualized by an inverted microscope (Magnification, ×200). **(C)** Nuclei were stained with DAPI solution and photographed with a fluorescent microscope (Magnification, ×400). **(D)** Cells were collected and DNA was extracted. Fragmented DNA was separated on 1% agarose gel electrophoresis and visualized under UV light after staining with EtBr. **(E)** Cells were stained with FITC-conjugated annexin V and PI for DNA flow cytometry analysis. Percentages of apoptotic cells were determined by counting annexin V^+/PI^- cells. Data are presented as mean ± two independent experiments.

The most well recognized DRs are Fas, DR4, and DR5. They bind to Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) to transfer death signals. Upon ligand binding, an adapter protein Fas-associated death domain (FADD) or TRAIL-associated death domain (TRADD) is recruited to the death receptor and forms a death-inducing signaling complex (DISC) to activate caspase-8 (*1*). Our results showed that the expressions of TRAIL, DR4, DR5, Fas, and FasL were hardly changed by EEER treatment, suggesting that EEER did not regulate the extrinsic pathway (Figure 2A).

Meanwhile, the intrinsic pathway is initiated through mitochondrial membrane permeabilization and release of pro-apoptotic proteins from mitochondria (*2*). Bcl-2 family proteins, including anti-apoptotic Bcl-2 and Bcl-xL, as well as pro-apoptotic Bax, BAD, and Bak, control this process tightly, and the ratio between pro-apoptotic and anti-apoptotic proteins determines the sensitivity to apoptosis (*2*). Our results clearly showed that anti-apoptotic Bcl-2 was markedly downregulated by EEER treatment, even though the expression of pro-apoptotic Bax was not changed (Figure 2A). Accordingly, EEER treatment reduced MMP in a concentration-dependent manner (Figure 2B). Taken together, our results suggest that EEER induced apoptosis *via* the mitochondrial pathway but not *via* the DR-related pathway by an increase of the Bax/Bcl-2 ratio in HeLa cells.

3.3. *EEER-induced apoptosis is regulated by the activation of caspases in HeLa cells*

The extrinsic and intrinsic signaling pathways activate caspase cascades, which is a key hallmark of apoptosis. In particular, the disruption of mitochondria can trigger apoptosis through the activation of caspase-9 and -3, even if it can also induce caspase-independent apoptosis by the translocation of pro-apoptotic proteins, such as apoptosis-inducing factor (AIF) and endonuclease G (endoG), from the mitochondria into the nucleus (*29,30*). To examine whether EEER activates caspases, we investigated the expression and activity of two initiator capsases of the extrinsic and intrinsic apoptosis pathways, caspase-8 and -9, respectively, and caspase-3, a typical effector caspase. As shown in Figure 3A, EEER treatment apparently suppressed the expression of pro-caspase-9 and -3, while it hardly affected that

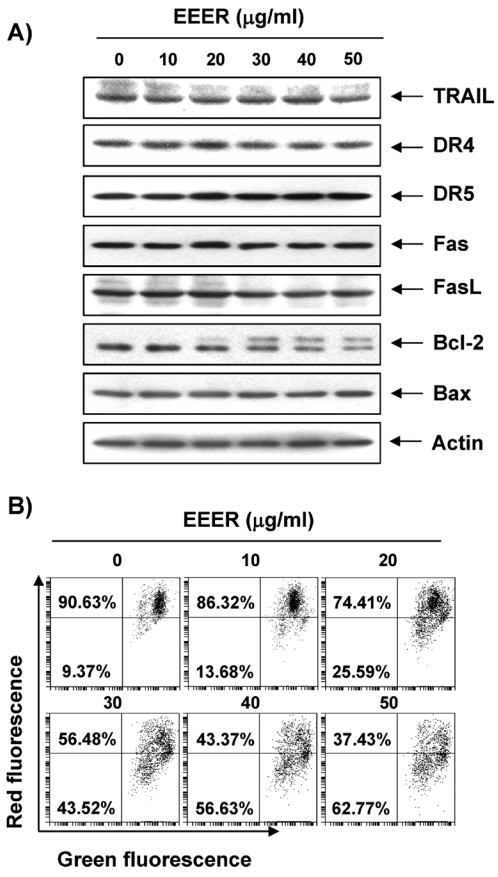

Figure 2. Effects of EEER on expression levels of apoptosis regulators and MMP values in HeLa cells. (A) After treatment with various concentrations of EEER for 24 h, cells were lysed and then equal amounts of cell lysates were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. Actin was used as an internal control. **(B)** To evaluate the changes of MMP, the cells grown under the same conditions as (A) were stained with JC-1 dye and were then analyzed on a DNA flow cytometer. Data are presented as mean ± two independent experiments.

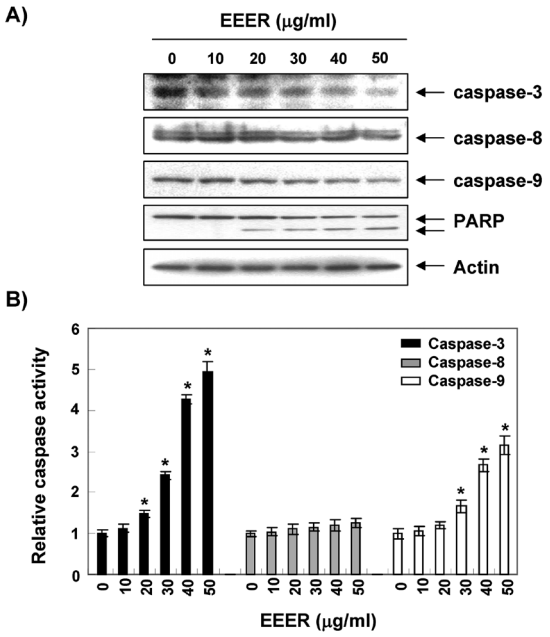

Figure 3. Activation of caspases by EEER treatment in HeLa cells. Cells were treated with various concentrations of EEER for 24 h. **(A)** Cells were lysed and then equal amounts of cell lysates were separated on SDS-polyacrylamide gels and transferred to membranes. Membranes were probed with the indicated antibodies against caspases, and the proteins were visualized using an ECL detection system. Actin was used as an internal control. **(B)** Activities of caspases were evaluated using caspase colorimetric assay kits. Data are expressed as the mean ± S.D. of three independent experiments. Significance was determined by a Student's *t*-test (*$p < 0.05$ *vs*. untreated control).

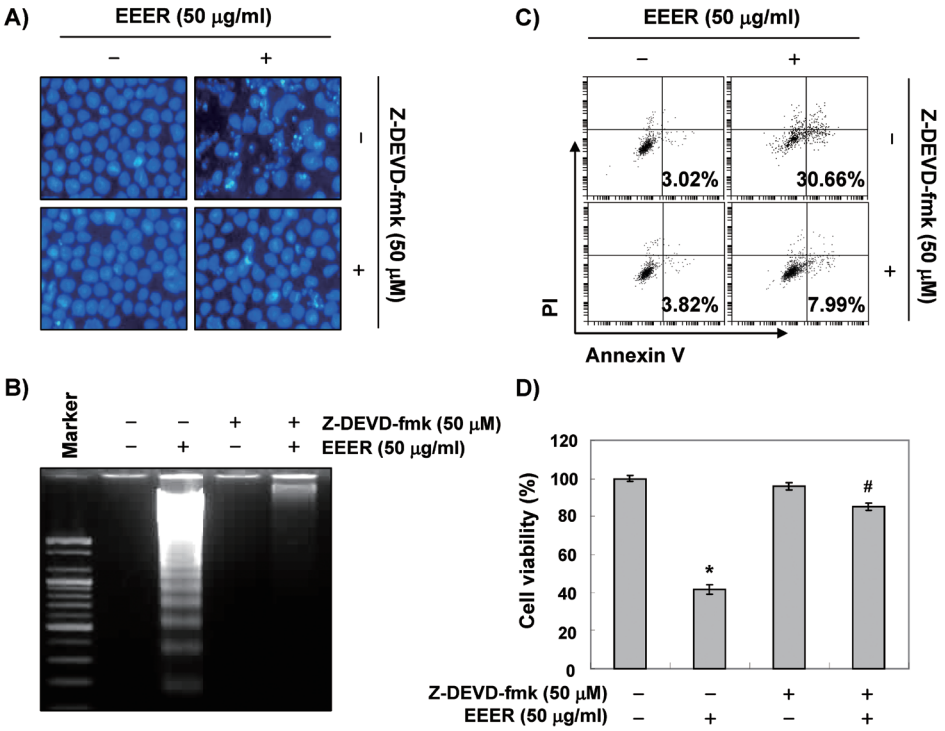

Figure 4. Suppression of EEER-induced apoptosis by inhibition of caspase-3 in HeLa cells. Cells were pre-treated with 50 μM z-DEVD-fmk, a caspase-3 specific inhibitor, 1 h before treatment with 50 μg/mL EEER for 24 h. **(A)** Nuclei were stained with DAPI solution and were photographed with a fluorescent microscope (Magnification, ×400). **(B)** Fragmented DNA was separated on 1% agarose gel electrophoresis and visualized under UV light after staining with EtBr. **(C)** Percentages of apoptotic cells (annexin V^+/PI^- cells) were measured using DNA flow cytometric analysis. Data are presented as mean ± two independent experiments. **(D)** Cell viability was measured using an MTT assay. Data are expressed as the mean ± S.D. of three independent experiments. Significance was determined by a Student's *t*-test (*$p < 0.05$ *vs*. untreated control; #$p < 0.05$ *vs*. EEER-treated cells).

of pro-caspase-8. The subsequent increase of cleaved poly(ADP-ribose) polymerase (PARP), a well-known substrate of caspase-3 (*31*), was also observed (Figure 3A). The *in vitro* activity of caspase-3 and -9 but not of caspase-8 was consistently significantly enhanced by EEER treatment (Figure 3B). These results are in agreement with those in Figure 2, which suggest that EEER regulated the intrinsic pathway without control of the extrinsic pathway.

To verify whether EEER-induced apoptosis is caspase-dependent, we next inhibited the activity of caspase-3 using z-DEVD-fmk. Our results clearly showed that EEER-induced apoptotic body formation and DNA fragmentation were absolutely abrogated by z-DEVD-fmk pre-treatment (Figures. 4A and B). The increased population of annexin V^+/PI^- cells and reduced cell viability were also reversed by the inhibition of caspase-3 activity (Figures. 4C and D). Taken together, our results demonstrate that the activation of caspase cascades following mitochondrial dysfunction is essential for EEER-induced apoptosis in HeLa cells.

3.4. *EEER induces apoptosis through the activation of AMPK in HeLa cells*

Given that mitochondria are the energy source, we

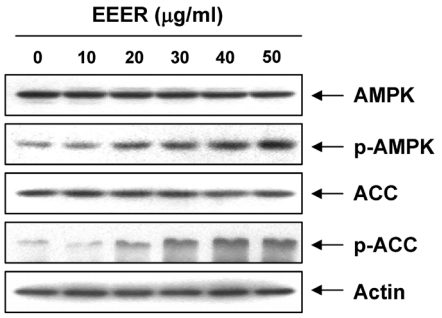

Figure 5. Activation of AMPK by EEER treatment in HeLa cells. Cells were treated with the indicated concentration of EEER for 24 h. Expression levels of p-AMPK, AMPK, p-ACC, and ACC were detected using Western blot analysis. Actin was used as a loading control.

hypothesized that the collapse of MMP would disturb energy homeostasis in HeLa cells. Because AMPK is a representative enzyme activated under low ATP states and is recognized as an emerging target of anti-cancer therapy (*7,8*), we checked activation of the AMPK signaling pathway. Interestingly, AMPK and its downstream target ACC were obviously phosphorylated by EEER treatment in a concentration-dependent manner, indicating that they were converted to the activated state (Figure 5). Previous research has demonstrated that activation of AMPK is responsible

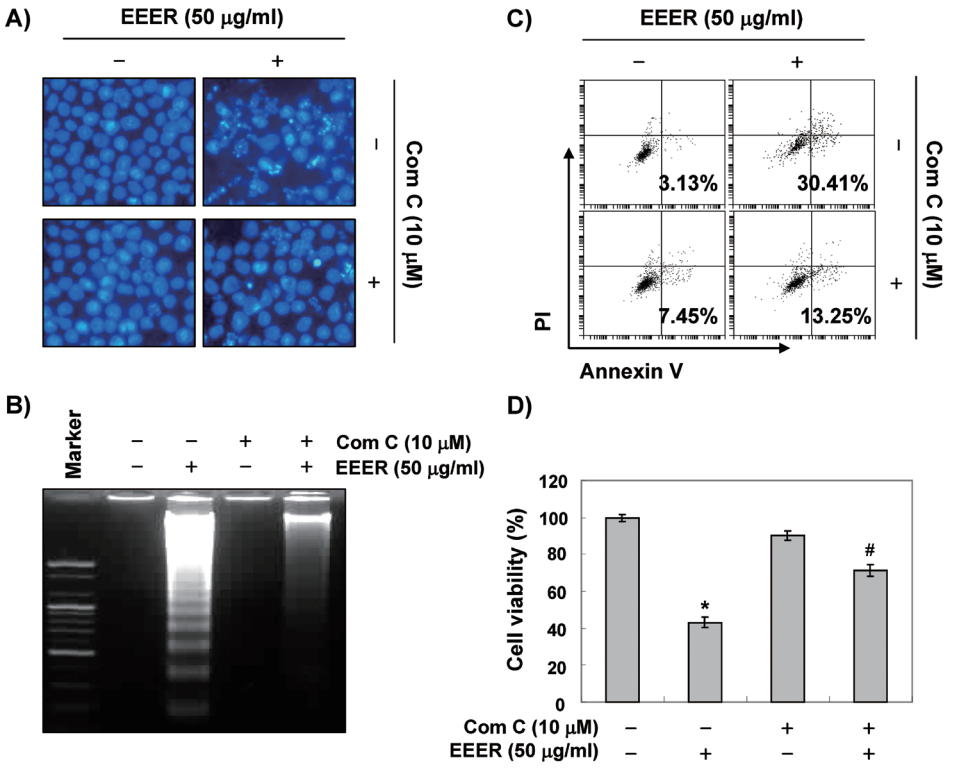

Figure 6. Suppression of EEER-induced apoptosis by inhibition of AMPK in HeLa cells. Cells were pre-treated with 10 μM compound C, an inhibitor of AMPK, 1 h before treatment with 50 μg/mL EEER for 24 h. **(A)** Nuclei were stained with DAPI solution and were photographed with a fluorescent microscope (Magnification, ×400). **(B)** Fragmented DNA was separated on 1% agarose gel electrophoresis and visualized under UV light after staining with EtBr. **(C)** Percentages of apoptotic cells (annexin V^+/PI^- cells) were measured using DNA flow cytometric analysis. Data are presented as mean ± two independent experiments. **(D)** Cell viability was measured using an MTT assay. Data are expressed as the mean ± S.D. of three independent experiments. Significance was determined by a Student's *t*-test ($^*p < 0.05$ *vs*. untreated control; $^{\#}p < 0.05$ *vs*. EEER-treated cells).

for mitochondria-mediated apoptosis (*12,13*). Therefore, we next investigated whether AMPK is involved in EEER-induced apoptosis. Our results showed that the markers of apoptosis increased by EEER treatment, including condensed and fragmented nuclei, DNA ladder, and the increased population of annexin V^+/PI^- cells, which were reversed by pretreatment with compound C, an inhibitor of AMPK (Figure 6A-C). Accordingly, an EEER-induced decrease of cell viability was reversed by the addition of compound C (Figure 6D). These observations collectively suggest that AMPK activation played a crucial role in EEER-induced apoptosis in HeLa cells.

4. Discussion

In the current study, we investigated the anti-cancer activity of EEER and explored the underlying mechanism in HeLa human cervical carcinoma cells. Even though a variety of research has already reported the anti-proliferative effects of *E. rutaecarpa* and its alkaloids in various cancer cells, this is the first study, to our knowledge, to propose AMPK as a critical molecule mediating EEER-induced apoptosis.

Our results clearly show that EEER induced apoptotic cell death in HeLa cells. EEER treatment markedly suppressed the expression of Bcl-2, a critical component of the mitochondrial pathway, and subsequently triggered the loss of MMP but did not modulate the DR-related pathway. The mediators of mitochondria-mediated apoptosis have been determined in various preceding reports. The first candidate released from the mitochondria upon apoptotic stimuli is cytochrome *c*, an essential component of the respiratory chain (*2*). It forms an apoptosome with apoptotic peptidase activating factor-1 (Apaf-1) and pro-caspase-9 to activate caspase-9 and the classical caspase cascade (*30*). Our results consistently showed that EEER activated caspase-9 and -3, while it did not influence the activity of caspase-8, thus supporting our idea that EEER induced mitochondria-mediated apoptosis. The other candidates released from the mitochondria to elicit apoptosis are AIF and endoG, which are especially involved in the caspase-independent pathway (*29,30*). However, based on our present data showing that z-DEVD-fmk completely blocked the apoptotic cell death induced by EEER, we suggest that the role of AIF and endoG in EEER-induced apoptosis might be slight. Notably, several preceding studies have also reported that evodiamine, an alkaloid isolated from *E. rutaecarpa*, induced mitochondria-mediated apoptosis in several cancer cell lines (*20-26*), which supports our current results.

Although the contribution of mitochondria in cancer

cell survival has been underestimated since Warburg suggested the significance of anaerobic glycolysis in cancers in overriding mitochondria as the source of energy (*5,32*), mitochondria are still idealized targets for anti-cancer therapy. When the cancer cells are in a glucose-depleted condition, they maintain ATP synthesis through OXPHOS and activate the catabolic pathways, including the oxidation of fatty acids and amino acids (*6,33*). In addition, cancer cells are more vulnerable to ROS generally generated from mitochondria compared to normal cells (*5,34*). Therefore, a broad range of physical and chemical stimuli causing mitochondrial dysfunction have been reported. First, the signals from the death receptor pathway are known to mediate the mitochondrial pathway. Caspase-8 connects the extrinsic pathway with the intrinsic pathway *via* cleavage of Bid, called truncated Bid (tBid). Binding of tBid to Bcl-2 proteins triggers release of Bax and Bak from Bcl-2, resulting in their indirect activation. In addition, tBid directly interacts with Bax located in the mitochondrial membrane to promote Bax oligomerization and subsequent loss of MMP (*2,35*). However, according to our present data, EEER treatment did not modulate the extrinsic pathway, which excludes tBid from the candidates causing mitochondrial disruption. Second, endoplasmic reticulum (ER) stress can induce permeabilization of the mitochondrial membrane *via* release of calcium into the cytoplasm (*36*). Liu *el al*. (*25*) demonstrated that evodiamine induced calcium/mitochondria-mediated apoptosis in human glioblastoma cells. In addition, cytosolic calcium is reported to activate AMPK *via* the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) pathway (*37*). As we observed that EEER treatment significantly activated the AMPK pathway, these studies strengthen the possibility that EEER-induced mitochondrial dysfunction might be the result of cytoplasmic calcium shifts. Third, accumulating research now indicates that oxidative stress also triggers apoptosis *via* the mitochondrial pathway. Oxidative stress causes severe damage to the electron transfer chain found in the inner mitochondrial membrane, leading to the explosive increase of ROS generation, referred to as ROS-induced ROS release, and resulting in the loss of MMP (*38*). Interestingly, ROS is also known to be an upstream signal of AMPK (*39,40*). Several studies have reported that oxygen deprivation induces autophagy *via* AMPK activation in human cancer cells (*41,42*). Therefore, the EEER-induced collapse of MMP might be the result of excessive generation of ROS.

Communication between mitochondria and AMPK has been suggested by various studies. When the cellular ATP level drops, AMPK is activated to inhibit mTOR signaling, leading to attenuation of protein synthesis (*12,42*). Because the mitochondrial electron transfer chain is the major source of ATP production, mitochondrial dysfunction can drive reduction of ATP synthase, which results in activation of AMPK. Although AMPK might give cancer cells a survival advantage by conservation of energy in ATP-depleted conditions (*14*), it has been generally considered as a tumor suppressor based on the following points: *i*) AMPK deactivates mTOR, which is commonly activated in many cancers; *ii*) most of the tumor suppressor genes, including LKB1 and phosphatase and tensin homolog (PTEN), have been identified as upstream activators of AMPK (*43,44*); and *iii*) AMPK activators, such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and metformin, are reported to inhibit tumorigenesis and tumor growth (*45,46*). Notably, metformin and antroquinonol have been reported to activate AMPK to induce apoptosis by inducing mitochondrial stress (*47,48*). Avicin D also disrupts mitochondrial metabolism, leading to decreased ATP levels and activation of AMPK, which is followed by autophagic cell death (*41*). These results are in agreement with our present data proposing AMPK as a key molecule induceing apoptosis in HeLa cells and supporting the possibility that EEER-induced loss of MMP leads to ATP depletion and the subsequent activation of AMPK. However, there are still other possible activators of AMPK, including ROS generated from the collapsed mitochondrial respiratory complex and CaMKK activated by increased cytosolic calcium, as mentioned above. Therefore, further studies are warranted to determine the precise upstream activator of AMPK.

In conclusion, our present results verified the anti-cancer effects of EEER in HeLa human cervical carcinoma cells. We suggest that AMPK plays a pivotal role in mitochondria-mediated apoptosis in response to EEER treatment. In support of our suggestion, recent clinical trials have demonstrated that metformin, an AMPK activator, decreased the incidence of cancer and cancer-related mortality and improved the disease control rate by itself or in combination with another drug (*49*). Although the active compound of EEER and the exact mechanism through which AMPK is activated should be further elucidated, our results suggest *E. rutaecarpa* is a prospective clinical option to treat human cervical cancer.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2014R1A1A1008460 and 2015R1A2A2A01004633).

References

1. Okada H, Mak TW. Pathways of apoptotic and non-

apoptotic death in tumour cells. Nat Rev Cancer. 2004; 4:592-603.
2. Hensley P, Mishra M, Kyprianou N. Targeting caspases in cancer therapeutics. Biol Chem. 2013; 394:831-843.
3. Ralph SJ, Neuzil J. Mitochondria as targets for cancer therapy. Mol Nutr Food Res. 2009; 53:9-28.
4. Modica-Napolitano JS, Weissig V. Treatment strategies that enhance the efficacy and selectivity of mitochondria-targeted anticancer agents. Int J Mol Sci. 2015; 16:17394-17421.
5. Ralph SJ, Rodríguez-Enríquez S, Neuzil J, Moreno-Sánchez R. Bioenergetic pathways in tumor mitochondria as targets for cancer therapy and the importance of the ROS-induced apoptotic trigger. Mol Aspects Med. 2010; 31:29-59.
6. Solaini G, Sgarbi G, Baracca A. Biochim Biophys Acta. Oxidative phosphorylation in cancer cells. Solaini. 2011; 1807:534-542.
7. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008; 30:214-226
8. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: Metabolism and growth control in tumour suppression. Nat Rev Cancer. 2009; 9:563-575.
9. Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, Cantley LC. The LKB1 tumor suppressor negatively regulates mTOR signaling. LC. Cancer Cell. 2004; 6:91-99.
10. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol. 2003; 13:2004-2008.
11. Monteverde T, Muthalagu N, Port J, Murphy DJ. Evidence of cancer-promoting roles for AMPK and related kinases. FEBS J. 2015; 282:4658-4671.
12. Figarola JL, Singhal J, Tompkins JD, Rogers GW, Warden C, Horne D, Riggs AD, Awasthi S, Singhal SS. SR4 uncouples mitochondrial oxidative phosphorylation, modulates AMP-dependent kinase (AMPK)-mammalian target of rapamycin (mTOR) signaling, and inhibits proliferation of HepG2 hepatocarcinoma cells. J Biol Chem. 2015; 290:30321-30341.
13. Chen H, Wang JP, Santen RJ, Yue W. Adenosine monophosphate activated protein kinase (AMPK), a mediator of estradiol-induced apoptosis in long-term estrogen deprived breast cancer cells. Apoptosis. 2015; 20:821-830.
14. Laderoute KR, Amin K, Calaoagan JM, Knapp M, Le T, Orduna J, Foretz M, Viollet B. 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. Mol Cell Biol .2006; 26:5336-5347.
15. Jeon SM. Regulation and function of AMPK in physiology and diseases. Exp Mol Med. 2016; 48:e245.
16. Jiang J, Hu C. Evodiamine: A novel anti-cancer alkaloid from *Evodia rutaecarpa*. Molecules. 2009; 14:1852-1859.
17. Kim D, Lee YH, Park SH, Lee MJ, Kim MJ, Jang HS, Lee JM, Lee HY, Han BS, Son WC, Seok JH, Lee JK, Jeong J, Kang JS, Kang JK. Subchronic oral toxicity of evodia fruit powder in rats. J Ethnopharmacol. 2014; 151:1072-1078.
18. Ko HC, Wang YH, Liou KT, Chen CM, Chen CH, Wang WY, Chang S, Hou YC, Chen KT, Chen CF, Shen YC. Anti-inflammatory effects and mechanisms of the ethanol extract of *Evodia rutaecarpa* and its bioactive components on neutrophils and microglial cells. Eur J Pharmacol. 2007; 555:211-217.
19. Tan MX, Liu YC, Luo XJ, Li DQ. Studies on the antioxidant activities of total alkaloids from the fruits of *Evodia rutaecarpa* (Juss.) Benth. Adv Mater Res. 2012; 396-398:52-55.
20. Lin L, Ren L, Wen L, Wang Y, Qi J. Effect of evodiamine on the proliferation and apoptosis of A549 human lung cancer cells. Mol Med Rep. 2016; 14:2832-2838.
21. Chen TC, Chien CC, Wu MS, Chen YC. Evodiamine from *Evodia rutaecarpa* induces apoptosis *via* activation of JNK and PERK in human ovarian cancer cells. Phytomedicine. 2016; 23:68-78.
22. Li YL, Pan YN, Wu WJ, Mao SY, Sun J, Zhao YM, Dong JY, Zhang DY, Pan JP, Zhang C, Lin NM. Evodiamine induces apoptosis and enhances apoptotic effects of erlotinib in wild-type EGFR NSCLC cells *via* S6K1-mediated Mcl-1 inhibition. Med Oncol. 2016; 33:16.
23. Huang J, Chen ZH, Ren CM, Wang DX, Yuan SX, Wu QX, Chen QZ, Zeng YH, Shao Y, Li Y, Wu K, Yu Y, Sun WJ, He BC. Antiproliferation effect of evodiamine in human colon cancer cells is associated with IGF-1/HIF-1α downregulation. Oncol Rep. 2015; 34:3203-3211.
24. Sui H, Zhou LH, Zhang YL, Huang JP, Liu X, Ji Q, Fu XL, Wen HT, Chen ZS, Deng WL, Zhu HR, Li Q. Evodiamine suppresses ABCG2 mediated drug resistance by inhibiting p50/p65 NF-κB pathway in colorectal cancer. J Cell Biochem. 2016; 117:1471-1481.
25. Liu AJ, Wang SH, Chen KC, Kuei HP, Shih YL, Hou SY, Chiu WT, Hsiao SH, Shih CM. Evodiamine, a plant alkaloid, induces calcium/JNK-mediated autophagy and calcium/mitochondria-mediated apoptosis in human glioblastoma cells. Chem Biol Interact. 2013; 205:20-28.
26. Zhao LC, Li J, Liao K, Luo N, Shi QQ, Feng ZQ, Chen DL. Evodiamine induces apoptosis and inhibits migration of HCT-116 human colorectal cancer cells. Int J Mol Sci. 2015; 16:27411-27421.
27. You MK, Kim MS, Jeong KS, Kim E, Kim YJ, Kim HA. Loquat (*Eriobotrya japonica*) leaf extract inhibits the growth of MDA-MB-231 tumors in nude mouse xenografts and invasion of MDA-MB-231 cells. Nutr Res Pract. 2016; 10:139-147.
28. Eom DW, Lee JH, Kim YJ, Hwang GS, Kim SN, Kwak JH, Cheon GJ, Kim KH, Jang HJ, Ham J, Kang KS, Yamabe N. Synergistic effect of curcumin on epigallocatechin gallate-induced anticancer action in PC3 prostate cancer cells. BMB Rep. 2015; 48:461-466.
29. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 2001; 412:95-99.
30. Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene. 2004; 23:2785-2796.
31. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. Cancer Res. 1993; 53:3976-3985.
32. Warburg O. On the origin of cancer cells. Science. 1956; 123:309-14.
33. Oronsky BT, Oronsky N, Fanger GR, Parker CW, Caroen SZ, Lybeck M, Scicinski JJ. Follow the ATP: Tumor energy production: A perspective. Anticancer Agents Med Chem. 2014; 14:1187-98.
34. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-

induced ROS release: An update and review. Biochim Biophys Acta. 2006; 1757:509-517.
35. Brenner D, Mak TW. Mitochondrial cell death effectors. Curr Opin Cell Biol. 2009; 21:871-877.
36. Jimbo A, Fujita E, Kouroku Y, Ohnishi J, Inohara N, Kuida K, Sakamaki K, Yonehara S, Momoi T. ER stress induces caspase-8 activation, stimulating cytochrome c release and caspase-9 activation. Exp Cell Res. 2003; 283:156-166.
37. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. 2005; 2:9-19.
38. Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resistance Updates. 2004; 7:97-110.
39. Hwang JT, Lee M, Jung SN, Lee HJ, Kang I, Kim SS, Ha J. AMP-activated protein kinase activity is required for vanadate-induced hypoxia-inducible factor 1alpha expression in DU145 cells. Carcinogenes. 2004; 25:2497-2507.
40. Sid B, Verrax J, Calderon PB. Role of AMPK activation in oxidative cell damage: Implications for alcohol-induced liver disease. Biochem Pharmacol. 2013; 86:200-209.
41. Papandreou I, Lim AL, Laderoute K, Denko NC. Hypoxia signals autophagy in tumor cells *via* AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. Cell Death Differ. 2008; 15:1572-1581.
42. Xu ZX, Liang J, Haridas V, Gaikwad A, Connolly FP, Mills GB, Gutterman JU. A plant triterpenoid, avicin D, induces autophagy by activation of AMP-activated protein kinase. Cell Death Differ. 2007; 14:1948-1957.
43. Feng Z, Hu W, de Stanchina E, Teresky AK, Jin S, Lowe S, Levine AJ. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: Stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res. 2007; 67:3043-3053.
44. Hardie DG. AMP-activated protein kinase as a drug target. Annu Rev Pharmacol Toxicol. 2007; 47:185-210.
45. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, Viollet B, Thompson CB. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. Cancer Res. 2007; 67:6745-6752.
46. Sui X, Xu Y, Yang J, Fang Y, Lou H, Han W, Zhang M, Chen W, Wang K, Li D, Jin W, Lou F, Zheng Y, Hu H, Gong L, Zhou X, Pan Q, Pan H, Wang X, He C. Use of metformin alone is not associated with survival outcomes of colorectal cancer cell but AMPK activator AICAR sensitizes anticancer effect of 5-fluorouracil through AMPK activation. PLoS ONE. 2014; 9:e97781.
47. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res. 2006; 66:10269-10273.
48. Chiang PC, Lin SC, Pan SL, Kuo CH, Tsai IL, Kuo MT, Wen WC, Chen P, Guh JH. Antroquinonol displays anticancer potential against human hepatocellular carcinoma cells: A crucial role of AMPK and mTOR pathways. Biochem Pharmacol. 2010; 79:162-171.
49. Groenendijk FH, Mellema WW, van der Burg E, Schut E, Hauptmann M, Horlings HM, Willems SM, van der Heuvel MM, Jonkers J, Smit EF, Bernards R. Sorafenib synergizes with metformin in NSCLC through AMPK pathway activation. Int J Cancer. 2015; 136:1434-1444.

(Received September 7, 2016; Revised November 2, 2016; Accepted November 5, 2016)