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

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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 (Δψm) 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...
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.) Bentham 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% CO₂.

2.3. Cell viability assay

To investigate cell viability, the HeLa cells were seeded in a 96-well plate at a density of 2 × 10⁴ 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-benzylxycarbonyl-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-diamidino-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 caspsases 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 phenylmethylsulfonyl 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 IC50 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.

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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 caspases 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...
of pro-caspase-8. The subsequent increase of cleaved poly(ADP-ribose) polymerase (PARP), a well-known substrate of caspase-3, 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 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, 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
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 et 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 inducing 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.

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