**Rhus verniciflua** Extract Modulates Survival of MCF-7 Breast Cancer Cells through the Modulation of AMPK-Pathway

Jung Ok Lee, Ji Wook Moon, Soo Kyung Lee, So Mi Kim, Nami Kim, Seong-Gyu Ko, Hyeon Soo Kim, and Sun Hwa Park

*Institute of Human Genetics, Department of Anatomy, Korea University College of Medicine; 126–1, 5-ga, Anam-dong, Seongbuk-gu, Seoul 136–701, South Korea; and Department of Preventive Medicine College of Traditional Korean Medicine, Kyunghee University; Hongi-dong, Dongdaemun-gu, Seoul 130–701, South Korea.

Received November 14, 2013; accepted February 4, 2014; advance publication released online February 20, 2014

**Abstract**

*Rhus verniciflua* Stokes (RVS) (Anacardiaceae) is a native plant to East Asian countries, including Korea, China, and Japan, and is used as a traditional herbal medicine. Traditionally, RVS was considered to have the function of alleviating blood stasis and purging hardness. Therefore, it could be applied for cancer treatment. RVS was used for treating various stomach diseases, including tumors, in East Asia, including Korea, during the 15th century. Recently, several experimental studies have demonstrated that flavonoids from RVS have effective anti-proliferative and apoptotic activities on various tumor cell lines, including human lymphoma, breast cancer, osteosarcoma, and transformed hepatoma cells. However, the molecular basis for the induction of apoptosis by RVS remains unclear. In this study, we explored the potential role of AMPK in RVS-induced MCF-7 human breast cancer cell apoptosis.

AMPK is a critical enzyme that plays an essential role in cellular energy homeostasis and controls processes related to tumor development, including cell cycle progression, protein synthesis, and survival. Therefore, as an anti-cancer target, AMPK has received intensive attention in recent years. Several metabolic stresses, including hypoxia, exercise, and starvation, lead to the activation of AMPK. AMPK also induces apoptosis in several cell types. These results suggest that AMPK signaling could be a potential therapeutic target for cancer.

In this study, we found for the first time that RVS-induced AMPK activation triggers apoptosis of MCF-7 cells. Reactive oxygen species (ROS) production is involved in AMPK activation by RVS. We provide evidence that supports AMPK-mediated apoptosis in RVS-treated breast cancer cells.

**Key words**  
*Rhus verniciflua*; AMP-activated protein kinase (AMPK); apoptosis; MCF-7

**Rhus verniciflua** Stokes (RVS) is used as an anti-cancer agent in traditional herbal medicine. However, the underlying molecular mechanism of its action is poorly understood. Here, we elucidated the mechanism of the anti-cancer mechanism of RVS in MCF-7 human breast cancer cells. We found that RVS increased the phosphorylation of AMP-activated protein kinase (AMPK) and downstream acetyl-CoA carboxylase (ACC) and suppressed cell viability in an AMPK-dependent fashion. RVS also induced an increase in reactive oxygen species (ROS) levels. RVS-induced AMPK phosphorylation was not observed in the presence of *N*-acetyl-cysteine (NAC), which indicated that ROS is associated with RVS-induced AMPK phosphorylation. In addition, fluorescent staining (Annexin V/propidium iodide) revealed that RVS increased the expression of Annexin V, which indicates that RVS leads to cancer-induced apoptosis. Moreover, RVS increased the phosphorylation of p53 and the expression of Bax. The inhibition of AMPK blocked RVS-induced p53 phosphorylation and Bax expression, which suggests that AMPK is involved in RVS-induced cancer apoptosis. Taken together, these results demonstrate that RVS has anti-tumor effects on MCF-7 cells through an AMPK-signaling pathway.

**Materials and Methods**

**Reagents**

Anti-phospho-AMPKα2 (Thr172), anti-AMPKα2, anti-phospho-p53, anti-Bax, antibodies were purchased from Cell Signaling Technology (New England Biolabs, Beverly, MA, U.S.A.). Anti-phospho-acetyl-CoA carboxylase (ACC) (Ser79) and anti-ACC antibodies were purchased from Millipore (Billerica, MA, U.S.A.). Anti-β-actin antibody and the antioxidant compound *N*-acetyl-cysteine (NAC) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). 5-Aminooimidazole-4-carboxamide ribonucleotide (AICAR) and Compound C, an AMPK inhibitor were obtained from Calbiochem (San Diego, CA, U.S.A.). RVS extract was obtained from the Department of Preventive Medicine, College of Oriental Medicine, Kyunghee University, and Republic of Korea.

**Sample Preparation**

The bark of RVS (Anacardiaceae) was purchased from Omniherb (Gyeongsangbukdo, Korea) and extracted in 2010. The dried *Rhus verniciflua* was ground in a mill and passed through a 50 mesh sieve. One hundred grams of RVS was extracted twice by repeat sonication (30 min with 80% ethanol) at room temperature and then filtered. The filtrates were combined and concentrated using a vacuum evaporator at 40°C and freeze-dried. The extract yield of RVS was 13.7% w/w. The RVS was extracted in 2010 and RVS stock solution was stored at −83°C until used.

**Cell Culture**

MCF-7 cells were grown in RPMI-1640 (GIBCO™, Auckland, NZ, U.S.A.) containing 0.584 g/L of l-glutamate and 4.5 g/L of glucose, 100 g/mL of gentamicin, 2.5 g/L of sodium carbonate, and 10% heat-inactivated fetal bovine serum (FBS).

**Immunoblot Analysis**

Cells were grown on 6, 12-well plates and serum-starved for a minimum of 6 h prior to treatment with the indicated agents. Following treatment of the
cells, the media was aspirated and the cells were washed twice in ice-cold phosphate buffered saline (PBS) and lysed in 60µL of lysis buffer. Then, the samples were briefly sonicated, heated for 5 min at 95°C and centrifuged for 5 min. The supernatants were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) gels and transferred to polyvinylidene difluoride membranes. The blots were incubated overnight at 4°C on a shaker with primary antibodies and then washed 6 times in Tris-buffered saline/0.1% Tween 20 for 1 h prior to being incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature. The blots were then visualized via ECL (Amersham Biosciences, Buckinghamshire, U.K.). In some cases, the blots were stripped and reprobed using other antibodies.

**Assay of Intracellular ROS by Confocal Microscopy**

H$_2$DCFH-DA (Molecular Probe/Invitrogen, Carlsbad, CA, U.S.A.) is a ROS-sensitive probe that can be used for the detection of ROS production in living cells. It diffuses passively into cells where its acetate groups are cleaved by intracellular esterase, which release the corresponding dichlorodihydrofluorescein derivative H$_2$DCF. H$_2$DCF oxidation yields a fluorescent adduct dichlorofluorescein (DCF), which is trapped inside the cell. Cells were treated with RVS for 24 h. Cells were incubated at 37°C with 20µM H$_2$DCFH-DA for 30 min and then the fluorescence was measured using a confocal microscope (Zeiss LSM 510, Carl Zeiss). Using an argon laser, DCF fluorescence was excited at 488 nm and emission was detected using a 530-nm long-pass filter.

**3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide (MTT) Assay**

The MTT assay was used as a crude measure of cell viability. MTT is converted by metabolically active cells into a colored water-insoluble formazan salt. MCF-7 wells were seeded at a density of 1×10$^4$/well in 96-well plates and grown for 24 h. The growth medium was replaced with serum-free medium 24 h prior to treatment. Subsequently, MTT reagents (10µL/well (7.5 mg/mL in PBS)) were added, and the culture was incubated for 30 min at 37°C. The reaction was stopped by the addition of acidified triton buffer (0.1 M HCl and 10% (v/v) Triton X-100 (50µL/well)), and the tetrazolium crystals were dissolved by mixing on a plate shaker at room temperature for 20 min. The samples were then analyzed using a plate reader (Bio-Rad 450, Richmond, CA, U.S.A.) at a 595-nm test wavelength and a 650-nm reference wavelength. At a minimum, the results were obtained from experiments repeated in triplicate.

**4',6-Diamidino-2-phenylindole (DAPI) Staining**

Circular TC-treated cover slips were placed at the bottom of each well of a 24-well plate. In total, 1×10$^4$ of MDA-MB-231-TXSA cells were seeded in each well and incubated for 24 h. The growth medium was removed and replaced with fresh Dulbecco's modified Eagle's medium (DMEM) or DMEM supplemented with 300 µg/mL of RVS. After 24 h, the medium was removed and the cells were incubated for 10 min with 1 µg/mL DAPI stain (Roche Diagnostics, Indianapolis, IN, U.S.A.). The morphology of the cell nuclei was observed using a fluorescence microscope (Olympus BH Series) at an excitation wavelength of 350 nm.

**Apoptosis Analysis**

MCF-7 cells were seeded in 60-mm plates and treated with RVS. After 48 h of treatment, the cells were harvested and stained using Annexin-V Alexa Fluor® 488/PI staining, as described by the Tali™ Apoptosis Kit (Invitrogen, Carlsbad, CA, U.S.A.). Apoptosis was evaluated using the Tali™ Image-based Cytometer.

**Silencing of AMPKα2**

MCF-7 cells were seeded in 96-well plates and allowed to grow to a confluence of 70% for 24 h. Transient transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer’s protocol. Briefly, 100 nm of AMPKα small interfering RNA (siRNA) (NM_001013367; Dharmacon, Lafayette, CO, U.S.A.) and 5 µL of Lipofectamine 2000 were diluted with 95 µL of serum medium (Opti-MEM; Invitrogen) and then mixed. The mixture was incubated for 10 min at room temperature and added drop-wise to each culture well, which contained 800 µL of Opti-MEM (final siRNA concentration, 40 nm). Four hours after the transfection, the medium was changed with fresh complete medium. The transfected cells were cultivated for 48 h prior to conducting the experiments.

**RESULTS**

**RVS Activates AMPKα2 and the Downstream Target of AMPK, ACC, in MCF-7 Cells**

To determine whether RVS is involved in the regulation of AMPKα2, a key molecular target of anti-tumor control, the level of AMPKα2 phosphorylation in RVS-treated MCF-7 cells was examined. Using phosphorylation-specific antibodies, RVS stimulated AMPKα2 activation in a dose-dependent manner (Fig. 1A). AICAR, a known AMPK activator, was employed as a positive control. In addition, the administration of RVS induced a time-dependent increase in AMPKα2 phosphorylation (Fig. 1B). The phosphorylation level of Thr$^{272}$, which is in the active site of the AMPKα2 subunit and is essential for enzyme activity, reached a maximum level 30 min after treatment. Consistent with the increase in AMPKα2 activity, the phosphorylation of ACC-Ser$^{79}$, the best-characterized phosphorylation site by AMPKα2, increased after RVS administration. These results demonstrate that AMPKα2 may mediate an RVS-associated signaling pathway in MCF-7 cell.

**RVS Suppresses Cell Viability through AMPK**

To assess the effect of RVS on cell viability, we examined the cytotoxic effects of serial concentrations (0–300 µg/mL) of RVS in 2-d treatments using an MTT assay. Compared with the untreated cells, the viability of cells significantly decreased after 2 d of treatment with 200 µg/mL RVS (Fig. 2A). The microscopic examination of the drug-treated MCF-7 cell cultures revealed dose-dependent cell death that is characteristic of apoptosis. The treated cells ceased proliferation, rounded-up, and detached from the plate (Fig. 2B). The nuclear examination of the drug-treated cells stained with DAPI, a fluorescent DNA-binding dye, indicated condensed highly stained nuclei characteristic of apoptotic cells (Fig. 2C). To better understand the mechanism by which AMPKα2 alters cell viability, the effect of AMPKα2 knock-down on RVS-induced viability suppression was also determined. The suppression of cell viability was recovered by AMPKα2 knock-down (Fig. 2D). To examine the role of AMPKα2 on RVS-induced viability suppression, RVS mediated AMPKα2 phosphorylation was examined in AMPKα2 knock-down condition. RVS-induced AMPKα2 phosphorylation was decreased in AMPKα2 knock-down condition (Fig. 2E). Taken together, these results indi-
cate that AMPKα2 is involved in RVS-induced cellular apoptosis in MCF-7 cells.

ROS Production Is Involved in RVS-Induced AMPKα2 Activation

ROS are produced by the mitochondria during the execution phase of apoptosis. To clarify whether the ROS produced by RVS accumulated intracellularly, MCF-7 cells pretreated with 5 µmol/L DCFH-DA for 1 h at 37°C were stimulated with 200 µg/mL of RVS for 30 min, and the DCF produced was scanned by confocal microscopy. The intracellular fluorescent intensities were rapidly increased (Fig. 3A). However, the increase in fluorescence observed in the MCF-7 cells exposed to RVS was significantly reduced by pretreatment with NAC (5 mM), a ROS scavenger (Fig. 3A). Next, we attempted to determine whether the phosphorylation of AMPKα2 is related to ROS induction. RVS-induced AMPKα2 phosphorylation was not observed in the presence of NAC (5 mM), which suggests that ROS are associated with RVS-induced AMPKα2 phosphorylation (Fig. 3B). To determine whether H₂O₂ is associated with AMPKα2 phosphorylation, MCF-7 cells were exposed to 100 µM H₂O₂. The Western blot analysis revealed that H₂O₂ induced an increase in the phosphorylation of AMPKα2 (Fig. 3C). To better understand the mechanism by which ROS alters cell viability, the effect of ROS on RVS-induced cell viability suppression was also determined. The suppression of cell viability was recovered by pretreatment with NAC (5 mM) (Fig. 3D). These results indicate that RVS induces an increase in phosphorylation of AMPKα2 through ROS.

RVS-Induced Apoptosis

To determine whether the cytotoxicity of RVS is due to apoptosis, apoptosis analysis was performed in MCF-7 cells after RVS treatment. Apoptotic induction was also analyzed using the Tali™ Apoptosis Kit, where Annexin V and propidium iodide (PI) are used for determination of apoptotic cell death. The treatment with RVS (0 and 200 µg/mL) for 2 d resulted in approximately 24% of the cells staining positive for Annexin V (early apoptosis) (Figs. 4A, B). To further understand the molecular mechanism of RVS-induced apoptosis in MCF-7 cells, we examined the expression of proteins associated with apoptosis after RVS
Fig. 2. RVS Suppresses Cell Viability through AMPKα2

(A) Dose-dependent suppression of viability of MCF-7 cells after RVS treatment. Cells were treated with RVS at the indicated doses for 48h. Values are expressed as the percentage of absorbance (MTT) relative to the control. (B) Dose-dependent morphological changes in RVS-treated MCF-7 cells. MFC-7 cells were exposed to the indicated dose for 2 d and examined by microscopy (20×). (C) The cells were treated with the indicated doses for 48h. Fluorescent micrographs of cells stained with DAPI are shown on the left. Phase contrast photographs are shown on the right (magnification 20×). (D) Effect of AMPKα2 knock-down on the RVS-induced decrease in viability. Cells were transfected with 50 nM AMPKα siRNA and maintained for 48h. Either non-transfected cells or AMPKα2 siRNA-transfected cells were then treated with 200 µg/mL of RVS for 48h. Values are expressed as the percentage of absorbance (MTT) relative to control. (E) Effect of AMPKα2 knock-down on the RVS-induced AMPKα2 phosphorylation. Cells were transfected with 50 nM AMPKα2 siRNA and maintained for 48h. Either non-transfected cells or AMPKα2 siRNA-transfected cells were then treated with 200 µg/mL of RVS for 48h. Cell lysates were analyzed by Western blot using anti-phospho-AMPKα2, anti-AMPKα2, and anti-β-actin antibodies. *p<0.05 vs. basal values.
Fig. 3. ROS Production Is Involved in RVS-Induced AMPK Activation

(A) Microscopic analysis of RVS-induced ROS generation. MCF-7 cells were treated with 200 µg/mL of RVS for 48 h. ROS generation was detected by confocal microscopy. (B) Effect of NAC on RVS-induced AMPKα2 phosphorylation. Cells were pre-treated with NAC (20 mM) for 30 min and then stimulated with 200 µg/mL of RVS for 3 h. Cell lysates were analyzed by Western blot using anti-phospho-AMPKα2, anti-phospho-ACC, anti-AMPKα2, and anti-ACC antibodies. (C) Effect of H2O2 on AMPKα2 phosphorylation. Cells were stimulated with 100 µM H2O2 for the indicated times. Cell lysates were analyzed by Western blot using anti-phospho-AMPKα2, anti-phospho-ACC, anti-AMPKα2, and anti-ACC antibodies. (D) Effect of NAC on RVS-induced cell viability. Cells were pretreated with NAC for 30 min and then treated with 200 µg/mL of RVS for 48 h. Values are expressed as the percentage of absorbance (MTT) relative to control. Data in the bar graphs represent the mean ± S.E.M. values of the ratios of densities (p-AMPKα2/AMPKα2) for at least three dependent Western blot experiments. *p<0.05 vs. basal values.
treatment. RVS up regulated pro-apoptotic Bax expression in a dose-dependent manner (Fig. 4C). The tumor suppressor protein, p53, regulates apoptosis through the transcriptional activation of its target genes and acts as a key facilitator of many cross-talking pathways. After treatment with RVS, the levels of p53 phosphorylation were significantly increased (Fig. 4C). Taken together, these findings suggest that RVS induces apoptosis in MCF-7 cells.

**AICAR Enhances RVS-Induced MCF-7 Breast Cancer Cell Death/Apoptosis** To gain insight into the role of AMPKα2 in RVS-mediated apoptosis, we measured the phosphorylation of p53. The levels of p53 phosphorylation were attenuated by treatment with Compound C, an AMPK inhibitor (Fig. 5A). To ascertain the role of RVS on AMPK phosphorylation, we examined the phosphorylation of AMPKα2 after the co-treatment of RVS and AICAR, an AMPK activator. The phosphorylation level of AMPKα2 was higher in co-treatment condition than individual treatment condition (Fig. 5B). Taken together, these findings support our hypothesis that AMPKα2 is a critical regulator in RVS-mediated cell viability inhibition.

**DISCUSSION**

In this study, we demonstrated that RVS activates AMPKα2 and contributes to the suppression of MCF-7 cell viability. The association of AMPKα2 with the suppression of MCF-7 cell viability has raised several questions regarding the mechanism by which RVS can suppress tumor growth. The results of our study suggest that RVS-induced AMPKα2 activation plays a critical role in the suppression of tumor cell growth.

Our objective was to determine whether the viability of MCF-7 breast tumor cells was directly regulated by RVS and,
The principal finding of this study is that AMPKα2 is involved in RVS-mediated suppression of MCF-7 cell viability. Our results also demonstrated that p53 and Bax are associated with RVS-mediated suppression of MCF-7 cells. p53, a transcription factor that mediates cell responses to many types of harmful stressors, is one of the most widely studied proteins. The activation of p53 induces cell cycle regulation, DNA repair, senescence, and cell death in cells that have been exposed to a variety of insults, including DNA damage, hypoxia, and oxidative stress. Metabolic alterations are a determinate of the response of cancer cells to variable nutrient conditions as well as their regulation of cell proliferation, which indicates that p53 plays an important role in metabolic shifting in cancer cells. Bax forms heterodimers with multiple anti-apoptotic proteins and induces apoptosis through the release of cytochrome c. Moreover, a previous study reported that AMPK activation stimulated Bax translocation, which suggests that AMPKα2 is involved in Bax redistribution to mitochondria.

In the previous study, RVS suppressed inflammation by down-regulation of c-Jun N-terminal kinase (JNK) pathway in lipopolysaccharide (LPS) induced RAW264.7 macrophages. However, ethanol-extracted RVS activated JNK. This discrepancy may be caused by the experimental different conditions, such as cell type, extract method and the concentration of RVS. JNK is well known to be involved in oxidative stress-associated pro-apoptotic processes. It was also reported that activation of stress-activated protein kinases, such as JNK, induced apoptosis in certain cells. In other reports also showed that the AMPKα2 leaded to apoptosis via JNK activation. Taken together, these facts indicated that JNK may be involved in AMPK-mediated apoptosis.

These findings implicate the AMPKα2-mediated p53/Bax pathway in RVS-mediated suppression of tumor viability. In conclusion, we have demonstrated that RVS treatment activated AMPKα2 in MCF-7 cells.

These results showed that the AMPK pathway may exert a profound influence on RVS-mediated inhibition of tumor viability. Future studies will focus on elucidating the mechanism between AMPKα2 and p53/Bax in RVS-mediated signaling.

Acknowledgments This work was supported by Grant 2012R1A1A2041548 from the National Research Foundation of Korea, funded by the Korea Government.
REFERENCES


