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Original Article

Caffeic Acid Induces Autophagy in and Inhibits Migration of Melanoma Cells via Upregulation of 5' Adenosine Monophosphate-Activated Protein Kinase

Tzi-Peng Yang[1], Nu-Man Tsai[1], Chau-Jong Wang[2,3*]

[1] *Department of Medical Laboratory and Biotechnology, Chung-Shan Medical University, Taichung City 402, Taiwan (R.O.C)*

[2] *Institute of Biochemistry, Microbiology and Immunology, Medical College, Chung Shan Medical University, Taichung City 402, Taiwan (R.O.C)*

[3] *Department of Medical Research, Chung Shan Medical University Hospital, Taichung City 402, Taiwan (R.O.C)*

Caffeic acid, an organic compound found in plants, possesses antioxidant, immunomodulatory, anti-inflammatory, and antitumor activities. Melanoma is a tumor of melanocytes which is associated with poor prognosis. Moreover, treatment of malignant melanoma is difficult. Studies have shown that some antioxidants reduce melanoma cell proliferation by inducing autophagy. The aim of this study is to investigate whether caffeic acid inhibits the tumor properties of melanoma cell line and the death-regulation pathway. We treated melanoma B16-F1 cells with caffeic acid and detected cell motility on migration assay and cell death on MTT assay. Caffeic acid treatment induced autophagy in and decreased mobility of B16-F1 cells. Moreover, levels of autophagy regulators phosphorylated-AKT and phosphorylated-AMPK decreased and increased, respectively, in caffeic acid-treated B16-F1 cells. Furthermore, expression of tumor-related protein FASN, which is activated by AKT and inhibited by AMPK, decreased, while expressions of autophagy-related proteins BECN-1 and LC3 increased, in caffeic acid-treated B16-F1 cells. To investigate the role of AMPK in this regulating pathway, we blocked AMPK phosphorylation using compound C. We observed that inhibition of AMPK phosphorylation partially restores caffeic acid-induced suppression of melanoma cell growth and migration, as well as expressions of autophagy-related proteins BECN-1 and LC3. The results of this study indicate that AMPK is a key regulator of caffeic acid-induced autophagy and provide valuable information for the inhibition and chemoprevention of melanoma.

Key words: caffeic acid; melanoma; autophagy; AMPKα; AKT

Introduction

Caffeic acid is an organic compound classified as hydroxycinnamic acid. This yellow solid contains both phenolic and acrylic functional groups (Olthof, Hollman, and Katan 2001). Caffeic acid is found in all plants because it is a key intermediate in lignin biosynthesis (Boerjan, Ralph, and Baucher 2003). Coffee is the primary source of caffeic acid in the human diet (Olthof, Hollman, and Katan 2001). Moreover, caffeic acid is one of the main natural

* Correspondence: Chau-Jong Wang
Institute of Biochemistry, Microbiology and Immunology, Medical College, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, South Dis., Taichung, Taiwan. (R.O.C)
Tel: +886-4-24730022 ext. 11670
Fax: +886-4-23248167
E-mail: wcj@csmu.edu.tw

phenols in argan oil (Charrouf and Guillaume 2007) and is present in many foods such as thyme, sage, spearmint, Ceylon cinnamon, star anise, apples, artichokes, berries, and pears (Santos et al. 2011). Caffeic acid possesses antioxidant, immunomodulatory, and anti-inflammatory activities (Orban et al. 2000; Mirzoeva and Calder 1996; Staniforth, Chiu, and Yang 2006; Neradil, Veselska, and Slanina 2003). It protects skin cells by inhibiting UVB-induced IL-10, MAPK/JNK/P38, and AP1/NF-kB pathways (Staniforth, Chiu, and Yang 2006), indicating that it downregulates immune suppressive cytokine cascades and carcinogenesis-related pathways. Studies have shown that caffeic acid and its derivative caffeic acid phenethyl ester (CAPE) inhibit carcinogenesis (Hirose et al. 1998; Chung et al. 2004; Demestre et al. 2009).

Melanoma refers to a tumor of melanocytes, which produce melanin in the skin and other organs. Melanoma is associated with poor prognosis. Moreover, treatment of malignant melanoma is difficult (Jerant et al. 2000). Many antioxidants inhibit melanoma through RAS/ERK/MAPK, serine/threonine kinase, and AKT/protein kinase B/NF-κB pathways (Demierre 2006). Inhibition of BRAF and MEK induces the binding of LKB1 to AMPK and promotes the phosphorylation of AMPK, thus inducing the apoptosis of melanoma cells (Esteve-Puig et al. 2009). Studies have shown that some chemicals or drugs reduce melanoma cell proliferation by inducing autophagy. Zyflamend, a dietary supplement containing concentrated extracts of 10 herbs, including caffeic acid-rich herbs, and metformin, an oral diabetes medicine, promote the death of melanoma cells by inducing autophagy and apoptosis (Ekmekcioglu et al. 2011; Tomic et al. 2011). Sivridis et al. showed that tumor hypoxia and anaerobic glycolysis exert extensive autophagic effect and that angiogenesis exerts low autophagic effects on nodular cutaneous melanomas (Sivridis et al. 2011). Ma *et al*. measured autophagy in melanoma cells to predict their invasiveness, chemotherapy resistance, and survival (Ma et al. 2011). Thus, autophagy is a potential prognostic factor and therapeutic target for melanoma.

As caffeic acid exerts tumor-suppressive effect on skin cells and some antioxidants inhibit melanoma, we examined whether caffeic acid inhibits tumor properties of a melanoma cell line. We treated B16-F1 melanoma cells with caffeic acid and found that these cells undergo autophagy after 24 h. Moreover, our results showed that caffeic acid-induced autophagy in B16-F1 cells is regulated by Akt/AMPK pathway, which is related to reverted tumor formation and retarded metastasis. Thus, they provide useful information for developing strategies for the chemoprevention of melanoma.

Materials and Methods

Materials

Caffeic acid (Cat No.C-0625; Sigma, St. Louis, MO, USA) was dissolved in DMSO to obtain a 500mM stock solution. Dorsomorphin (compound C; ab120843), a potent, selective, and reversible AMPK inhibitor, was purchased from Abcam (Cambridge, UK). Other reagents were purchased from Sigma or Bio-Rad (Hercules, CA, USA), unless otherwise indicated. Antibodies against AKT, BCL-xL, BCL-2, BAX, becline-1 (BECN-1), LC3, and p53 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and those against phosphorylated-AMPK (p-AMPKα, Thr172), phosphorylated-p53 (Ser15) and FASN were obtained from Cell Signaling Technology (Denver, MA, USA). Anti-β-actin and horseradish peroxidase (HRP)-conjugated secondary antibodies were supplied by Sigma Co.

Methods

Cell Culture and Treatment

Mouse melanoma B16-F1 cells (epithelial cells) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco/ BRL, Gaithersburg, MD) supplemented with 10% FBS and antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin) and cultured at 37°C in 5% CO_2. N ext, these cells were seeded (density, 60×10^4 cells) onto 60-mm Petri dishes for 18 h before treatment with caffeic acid.

Wound Healing and Invasion Assays

Wound healing and invasion assays were performed as described previously (Lin et al. 2011). B16-F1 cells were grown in confluent monolayers on six-well Petri dishes containing serum-free medium for 18 h, after which the medium was replaced with serum-containing medium. Next, the cells were divided into the following groups: untreated control cells, DMSO-treated control cells, 0.2 mM caffeic acid-treated cells, 0.4 mM caffeic acid-treated cells, 0.6 mM caffeic acid-treated cells, and 0.8 mM caffeic acid-treated cells. Cells in the monolayer were disrupted (wounded) by scraping with P200 micropipette tip and washed twice with phosphate-buffered saline (PBS) at indicated time points (0, 8, 16 and 24 h). The number of cells in the denuded (scraped) zone of each Petri dish was counted at 100× magnification (in a blinded manner). Cells in each dish were counted three times to ensure accuracy.

For the invasion assay, caffeic acid-pretreated cells were trypsinized and suspended in DMEM to obtain a single-cell suspension. Next, the cells were added to the upper wells (cell density, 2×10^4 cells/well) of a 48-well Boyden Chamber (Neuro Probe AP48, Neuro Probe, Inc. MD). Migratory cells traversed a polycarbonate filter (pore size, 8 μm) from the upper chamber to the lower chamber. After incubation at 37°C and 5% CO_2 for 5 h, nonmigratory cells on the upper membrane surface were removed with a cotton swab. Cells that had spread to the lower membrane surface were fixed with methanol and stained with Giemsa stain (modified solution; Sigma Co.). The number of migratory cells per field (magnification, 400×) was quantified using a microscope with a 40× objective lens. Cells in 10 random fields of each filter were examined. Each experiment was performed in triplicate. Cell migration is expressed as mean ± standard deviation (SD) of total cells counted per field.

Cell Viability (MTT Assay)

B16-F1 cells were seeded (density, 5×10^4/well) onto 24-well plates and treated with the indicated concentrations of caffeic acid for 24 h. After incubation, cell viability was determined on MTT assay. Briefly, 5 mg/mL MTT solution was added to each well and cells were incubated at 37 °C for 4 h. After washing with PBS, purple blue formazan crystals were dissolved in 1 mL isopropanol and absorbance was measured at 563 nm. Cell viability was proportional to the amount of formazan present in the cells.

AO Staining

Autophagy was assessed by detecting and quantifying AVOs (lysosomes and autophagolysosomes) on immunofluorescence analysis (AO staining). Cells were stained with 1 μM AO and incubated for 17 min, as described previously (Feng et al. 2014). Photographs were obtained using a fluorescence microscope equipped with a 340-/380-nm excitation filter (Nikon Diaphot-300; Nikon, Japan). Percentage of AVO-positive cells was calculated by dividing autophagic cells by the total number of cells, then multiplying by 100. Each experiment was conducted three times and at least 300 cells were counted in each experiment.

Flow Cytometric Analysis of Cell Cycle

Flow cytometric analysis of B16-F1 cells treated with the indicated concentrations of caffeic acid was performed using FAScan (Becton Dickinson, NJ). The cells were washed twice with Dulbecco's PBS and cell suspension was centrifuged at 1,500 g at room temperature for 5 min. Supernatant was discarded and 1 mL 70% methanol was added to the cell pellet. The cells were incubated at -20°Cfor at least 24 h before performing flow cytometric analysis. Next, the cells were incubated with 1 mL cold PI solution (20 μg/mL PI, 20 μg/mL RNase A, and 0.1% Triton X-100) in the dark at room temperature for 15 min. PI was excited at 488 nm and fluorescence signal was subjected to logarithmic amplification with PI fluorescence (red) detected above 600 nm. Cell cycle distribution was expressed as the number of cells versus the amount of DNA, as indicated by fluorescence intensity. The extent of apoptosis was determined by quantifying DNA content in cells in the sub-G1, G0/G1, S and G2/M phases using CellQuest Version 3.3 software (Becton Dickinson, NJ). Percentage of hypodiploid cells (sub-G1 phase cells) among the total number

of cells was calculated and expressed as the percentage of apoptotic cells.

DAPI Staining

Changes in cell morphological characteristics due to apoptosis were examined on fluorescence microscope with DAPI staining. Cells in monolayer were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. The fixed cells were permeabilized three times with 0.2% Triton X-100 in PBS, incubated with 1 μg/mL DAPI for 30 min, and washed three times with PBS. Apoptotic nuclei (intensely stained, fragmented nuclei with condensed chromatin) were examined at 400× magnification using a fluorescence microscope equipped with a 340-/380-nm excitation filter (Diaphot-300). Percentage of apoptotic cells was calculated by dividing the number of apoptotic cells by the total number of cells, then multiplying by 100. Each experiment was conducted three times and at least 300 cells were counted in each experiment.

Immunoblotting Assay

Total proteins were extracted by lysing cells in lysis buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl [pH 7.5]) containing 17 μg/mL leupeptin and 10 μg/mL sodium orthovanadate. Protein concentration was measured using Bradford protein assay kit. Equal amounts of protein (50 μg) were resolved on SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk powder and 0.1% Tween-20 in Tris-buffered saline (TBS), then incubated with the specific primary antibodies at 4 oC overnight. Next, the membranes were washed three times with TBS containing Tween-20 (TBST) and incubated with HRP-conjugated secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Subsequently, the membranes were washed extensively with TBST and reactive signals were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, UK). β-Actin served as an internal control. Band intensities were detected by exposing the membranes to LAS-3000 (Fujifilm, Tokyo, Japan). Protein level was determined by performing densitometric analysis with Multi Gauge V2.2 software (Fujifilm).

Animal Study

All animal care and experimental procedures adhered to the guidelines of the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMC) for the care and use of laboratory animals. As B16-F1 cells were derived from C57/BL6 mice, we used C57/BL6 mice (National Laboratory Animal Center, NLAC, Taiwan) as the animal model. A total of 22 mice were divided into the following groups: (1) control mice, (2) mice injected with B16-F1 cells only, and (3) mice injected with B16-F1 cells and orally treated with 10 mg/day caffeic acid. Washed B16-F1 cells were resuspended in PBS. Cell suspension containing 10^6 cells in 0.1 mL PBS was subcutaneously injected into the right flank of 6-week-old C57/BL6 mice in groups 2 and 3. Mice in group 3 were treated daily with 10 mg/day caffeic acid (equivalent to 40 mg/100 g body weight) (Lin et al. 2011) for 2 weeks. As our preliminary study with this animal model showed the development of numerous cancerous nodules 2 weeks after injection of B16/F1 cells, after 2 weeks mice that had been subcutaneously injected with B16-F1 cells were sacrificed and the sizes and numbers of tumor nodules were measured immediately.

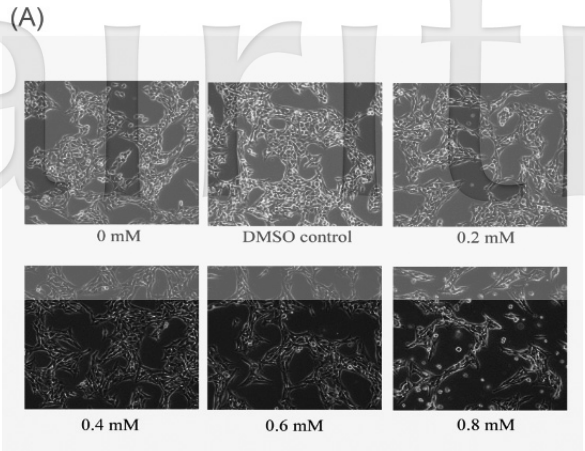
Statistical Analysis

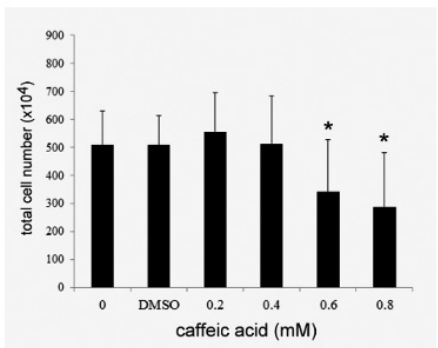
Data were analyzed using unpaired t-test and expressed as mean ± SD. $p < 0.05$ was considered statistically significant.

Results

Caffeic Acid Inhibits the Viability of B16-F1 Melanoma Cells

Caffeic acid inhibits carcinogenesis (Hirose et al. 1998). However, its effect on melanoma cells is unclear. In this study, we treated B16-F1 melanoma cells with caffeic acid and measured the number of live cells and cell survival rate. B16-F1 cells were





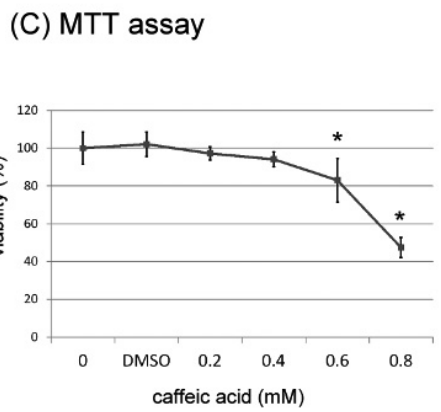


Figure.1. Effects of caffeic acid on the growth of B16-F1 cells. Cells were treated with 0, 0.2, 0.4, 0.6, or 0.8 mM caffeic acid or control solvent (DMSO) for 24 h. (A) Morphology of B16-F1 cells was examined using a reverse-phase microscope; magnification: 100×. (B) Cell growth curve. (C) Cell viability was analyzed on MTT assay. Data are expressed as total cell number in each condition and are presented as mean ± SD of three independent experiments. Results were statistically analyzed with Student's t-test. *p < 0.01 compared with untreated control.

seeded (density, 6×10^5 cells) onto 60-mm Petri dishes and incubated for 18 h until they reached approximately 35%-40% confluence. Next, the cells were treated with 0, 0.2, 0.4, 0.6, or 0.8 mM caffeic acid or DMSO (control) to determine the dose-dependent effects of caffeic acid on melanoma cell viability. After 24 h, cell numbers were as shown in Figures 1A and 1B. Treatment with 0.6mM caffeic acid decreased the number of B16-F1 cells. Moreover, treatment with 0.8 mM caffeic acid decreased the number of B16-F1 cells and resulted in their detachment from the monolayer culture. Further investigation of cell viability was carried out on MTT (Figure 1C), which showed only 50% viability at 0.8 mM compared to control. These results indicated that treatment with caffeic acid inhibits the viability of melanoma cells.

Caffeic Acid Inhibits the Motility of B16-F1 Melanoma Cells

Caffeic acid inhibited the growth of B16-F1 melanoma cells. We next examined whether it affects the motility of these cells. B16-F1 cells were treated with caffeic acid, as described previously, and invasion and wound healing assays were performed on Boyden chamber (Figures 2A and 2B). Results of both assays showed that caffeic acid treatment inhibits the motility of B16-F1 melanoma cells. Thus, caffeic acid treatment decreases both melanoma cell number and cell motility. We also performed zymography to determine the levels of metalloproteases. The results revealed no significant differences between control and caffeic acid-treated cells.

Caffeic Acid Induces Autophagy in B16-F1 Melanoma Cells

Our results showed that caffeic acid affects the viability of melanoma cells. However, the underlying mechanism is unclear. To address this, we examined the effects of caffeic acid on apoptosis of B16-F1 cells by DAPI staining and autophagy induction in B16-F1 cells by AO staining. The results indicated that caffeic acid only slightly induces apoptosis of B16-F1 melanoma cells (Figure 3A) but remarkably induces autophagy in B16-F1 melanoma cells. Moreover, treatment with > 0.6

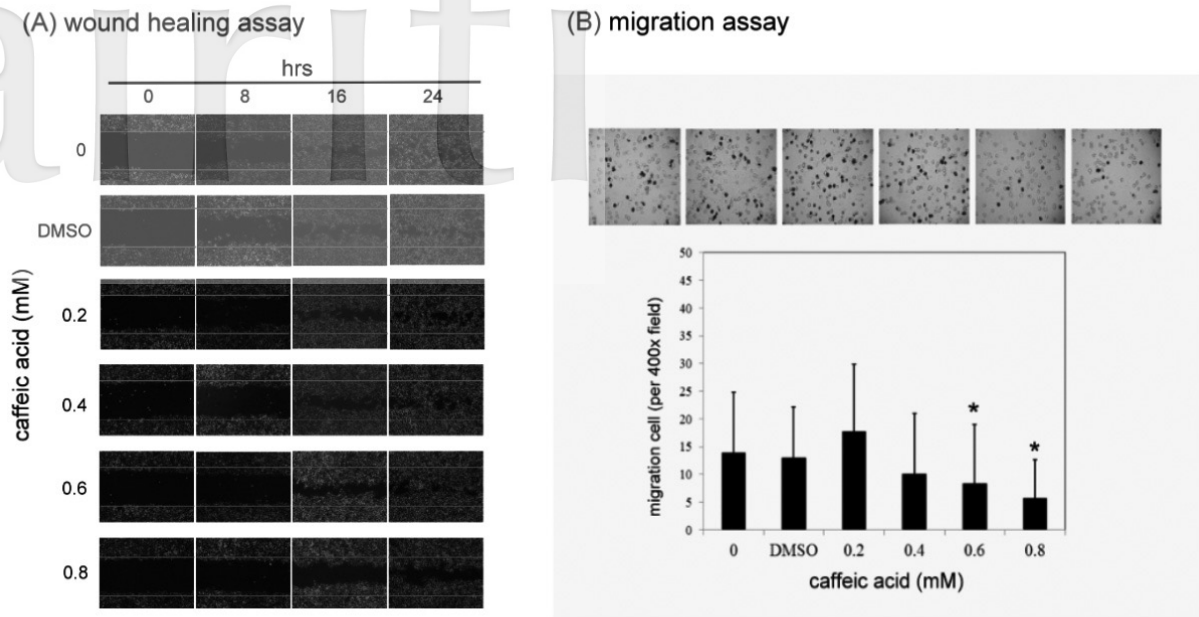


Figure. 2 Caffeic acid inhibited the mobility of B16-F1 cells. B16-F1 cells were treated with 0, 0.2, 0.4, 0.6, or 0.8 mM caffeic acid or control solvent (DMSO) for 24 h. (A) Wound healing assay was performed as described in Materials and Methods. The number of migrated cells was examined under a microscope. (B) B16-F1 cells were plated at a density of 6×105 cells onto a 60 mm dish. After overnight incubation, the cells were treated with various concentrations of caffeic acid for 24 h. The cells were allowed to migrate through 0.8-μM pore polycarbonate filters for 6 h. Results are expressed as mean ± SD of three independent experiments. *p < 0.01 compared with untreated control.

mM caffeic acid significantly increased the number of AO-positive B16-F1 cells (Figure 3B).

AMPK/FASN Pathway Participates in Caffeic Acid-Induced Autophagy

Phosphorylated AKT (p-AKT) is a well-known molecule that regulates melanoma cell proliferation (Pejkova et al. 2016). It reduces autophagy through mTOR and induces tumorigenesis by increasing FASN levels (Menendez and Lupu 2007, 2006). AMPK activation reduces the expression of FASN that promotes the production of fatty acids, which are aliphatic acids fundamental for energy production and storage (Kusunoki, Kanatani, and Moller 2006). To confirm the mechanism underlying caffeic acid- induced autophagy, we examined the expressions of p-AKT, AMPK, and FASN-related proteins. As shown in Figure 4, caffeic acid decreased p-AKT levels in a concentration-dependent manner. In the AMPK/FASN pathway, caffeic acid upregulates p-AMPK expression and downregulates FASN expression. Caffeic acid downregulates the expressions of proliferation- and cell motility-related proteins c-SRC, PLC, and FAK. This indicates that caffeic acid decreases angiogenesis by downregulation of c-SRC and PLC in melanoma cells. Thus, caffeic acid effectively suppresses tumor formation and metastasis by inhibiting the abovementioned molecules. In the present study, caffeic acid treatment upregulated the expressions of autophagy-related proteins BECN-1 and LC3 (Figure 4A) but only slightly affected the expressions of apoptosis-related proteins BCL-2 and BCL-xL.

Time course experiments showed the same pattern. Moreover, treatment of B16-F1 cells with 0.6 mM caffeic acid downregulated the expressions of p-AKT and FASN and upregulated the expressions of p-AMPK, BECN-1, and LC3 (Figure 4B).

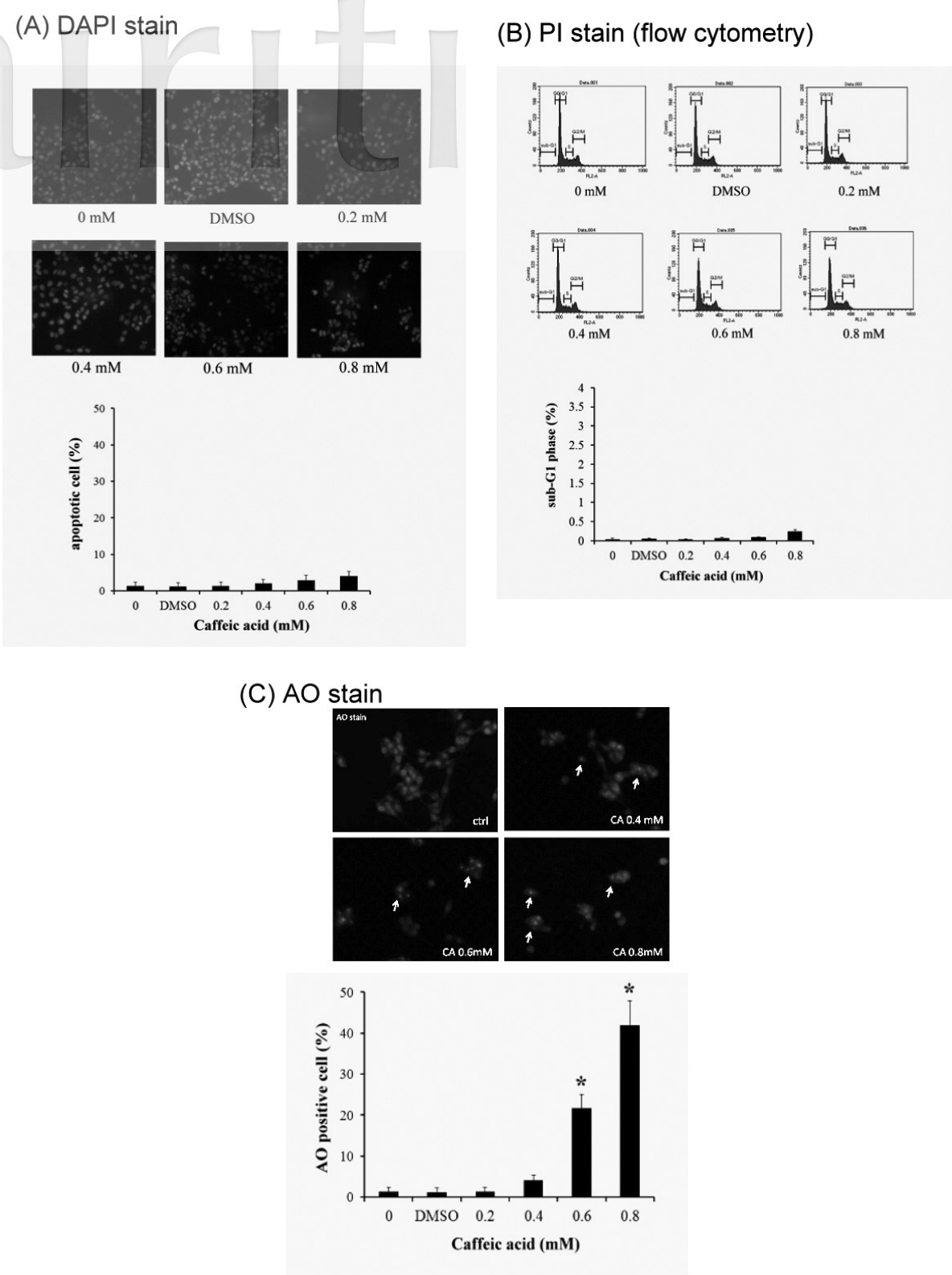


Figure. 3 Effects of caffeic acid on the apoptosis of B16-F1 cells. B16-F1 cells were treated with 0, 0.2, 0.4, 0.6, or 0.8 mM caffeic acid or control solvent (DMSO) for 24 h and used for different assays. (A) Results of DAPI staining were obtained by examining the cells under a microscope.

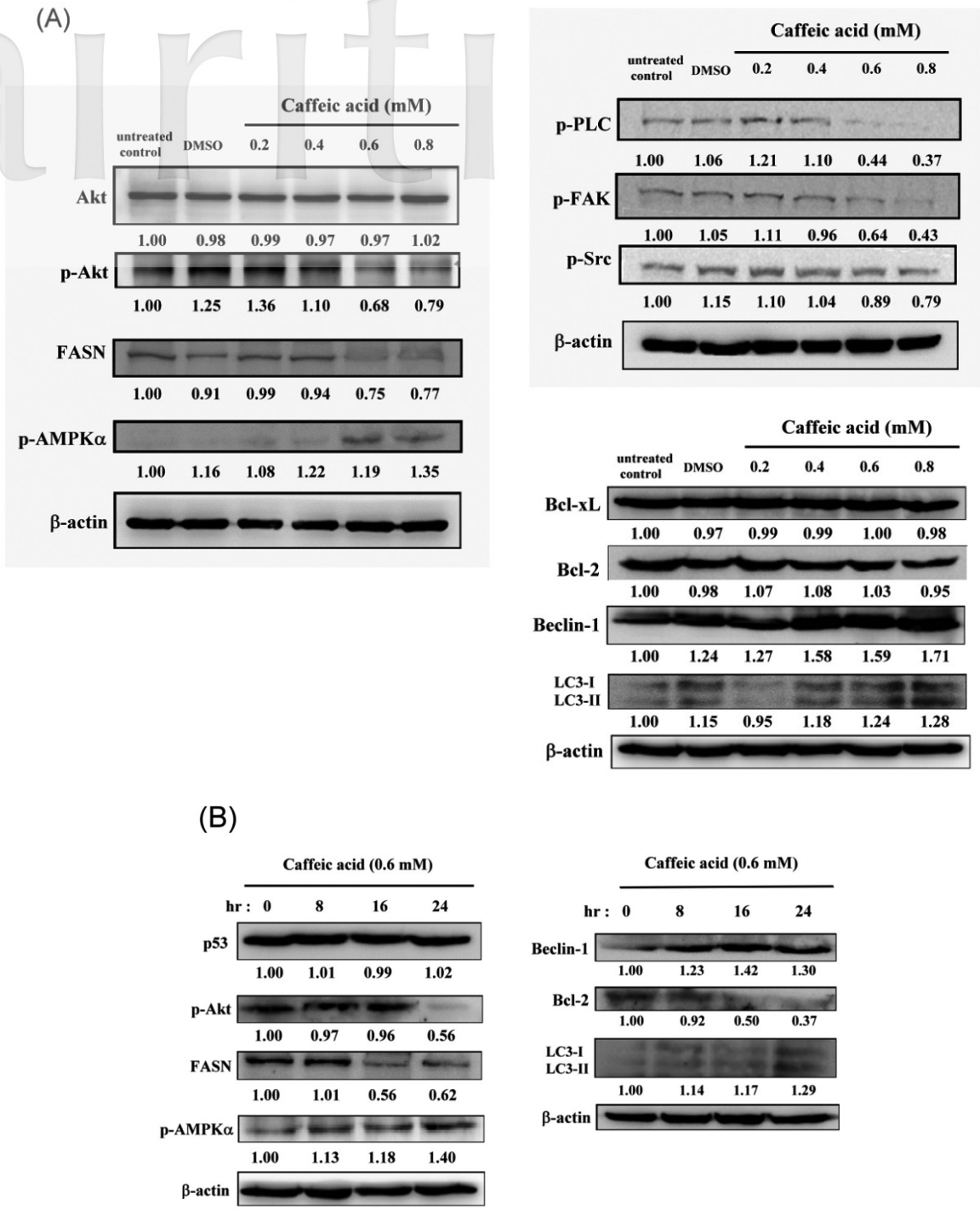


Figure. 4 CImmunoblotting analysis of the expressions of proliferation-, metastasis-, and autophagy-related-proteins in caffeic acid-treated B16-F1 cells. (A) Cultured cells were treated with 0, 0.2, 0.4, 0.6, or 0.8 mM caffeic acid or control solvent (DMSO) for 24 h, (B) cultured cells were treated with 0.6 mM caffeic acid for 0, 8, 16, or 24 h, and whole-cell extracts were prepared, as described in Materials and Methods. Equal amounts of total proteins (100μg) were loaded into each lane of an SDS-polyacrylamide gel. Western blotting was performed using antibodies as figure markers. β-Actin was used as an internal control. All data are presented as fold change relative to that in control cells. Results are representative of three independent experiments, all of which showed similar patterns of change.

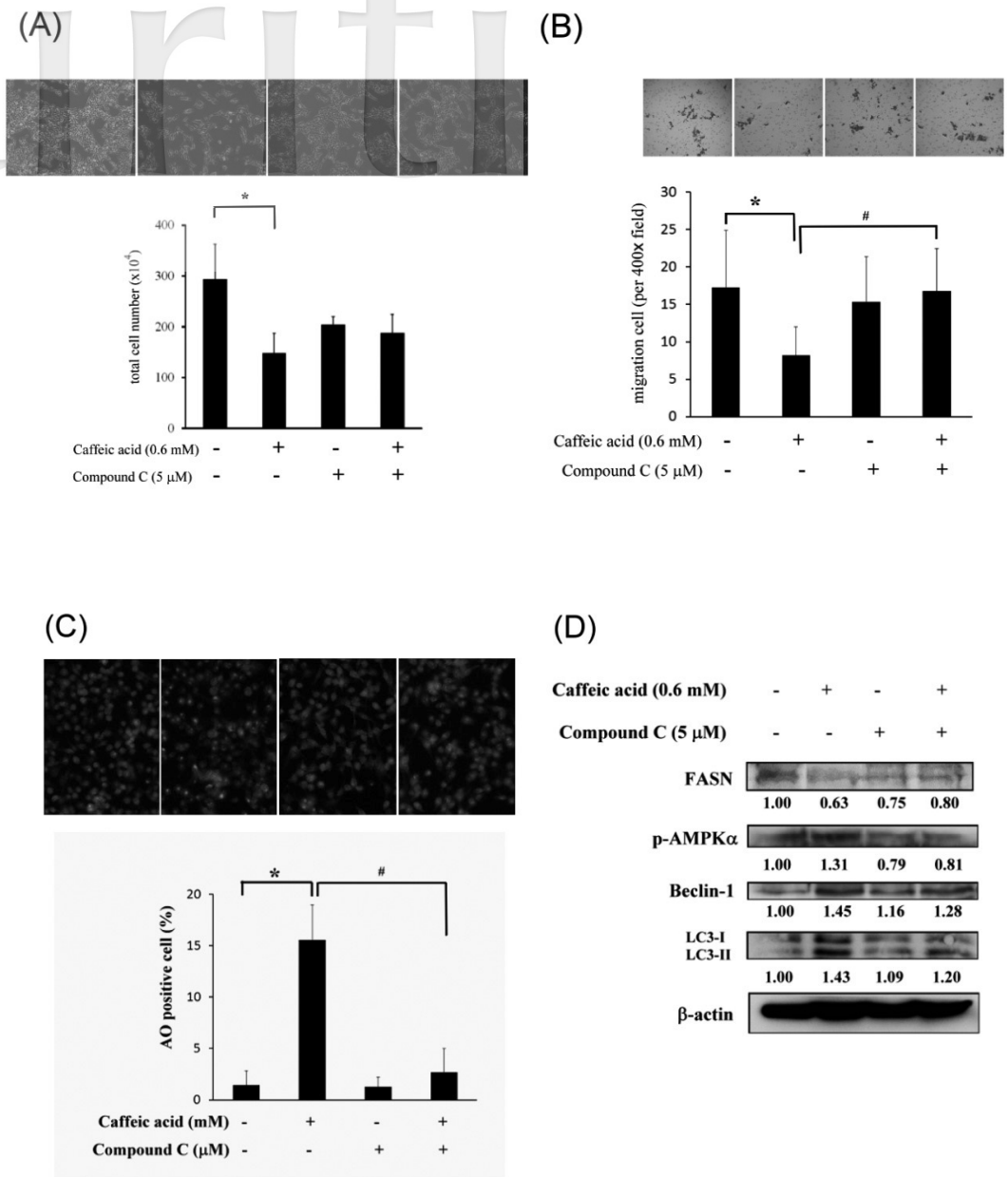


Figure. 5 Effects of compound C (inhibitor of AMPK phosphorylation) on caffeic acid- treated B16-F1 cells. B16-F1 cells were plated (density of 6 × 10^5 cells) onto a 60 mm dish. After overnight incubation, the cells were pre-treated with 5 μM of compound C for 30 min, followed by treatment with 0.6 mM of caffeic acid for 24 h as described in the figure. Results of (A) cell number, (B) migration assay (Boyden chamber), (C) AO staining, and (D) immunoblotting assay.Whole-cell extracts were prepared, as described in Materials and Methods. Equal amounts of total proteins (100 μg) were loaded into each lane of an SDS-polyacrylamide gel. Western and blotting was performed using antibodies against p-AKT, FASN, p-AMPKα, BECN-1, LC3, β-actin. β-Actin was used as an internal control. All data are presented as fold change relative to that of control cells. Data are representative of three independent experiments, all of which showed similar patterns of change. *p < 0.05 for control compared with caffeic acid-treated cells. #p < 0.05 for caffeic acid-treated cells only compared with compound C-pretreated and caffeic acid-treated cells.

AMPK Inhibitor Compound C Partially Restores the Changes Induced by Caffeic Acid

As AMPK regulates caffeic acid-induced autophagy in B16-F1 cells, we examined the effects of compound C (AMPK inhibitor) on caffeic acid-induced autophagy in B16-F1 cells. B16-F1 cells (6×10^5) were seeded onto a 60-mm dish and cultured overnight. Next, these cells were pretreated with 5 μM compound C for 30 min, followed by treatment with caffeic acid, as illustrated in Figure 5A. Compound C pretreatment induced toxicity in some B16-F1 cells but partially restored caffeic acid-induced decrease in cell number compared with caffeic acid only-treated cells (Figure 5A). Based on the results of AO staining and migration assay, compound C pretreatment decreases autophagy and restores motility of B16-F1 cells (Figures 5B and 5C). Results of Western blotting analysis showed that compound C pretreatment downregulates the expressions of p-AMPK and autophagy-related proteins Beclin-1, LC3-I, LC3-II.

Caffeic Acid Decreases Tumor Colonization in B16-F1-Injected Mice

Our results clearly showed that caffeic acid represses the growth and migration of cancer cells *in vitro*. Therefore, we injected C57/BL6 mice with B16-F1 cells, followed by oral treatment with or without caffeic acid at a dose of 10 mg/mouse daily. The mice were examined for tumor dissemination after 2 weeks. C57/BL6 mice injected with B16-F1 cells and not treated with caffeic acid showed

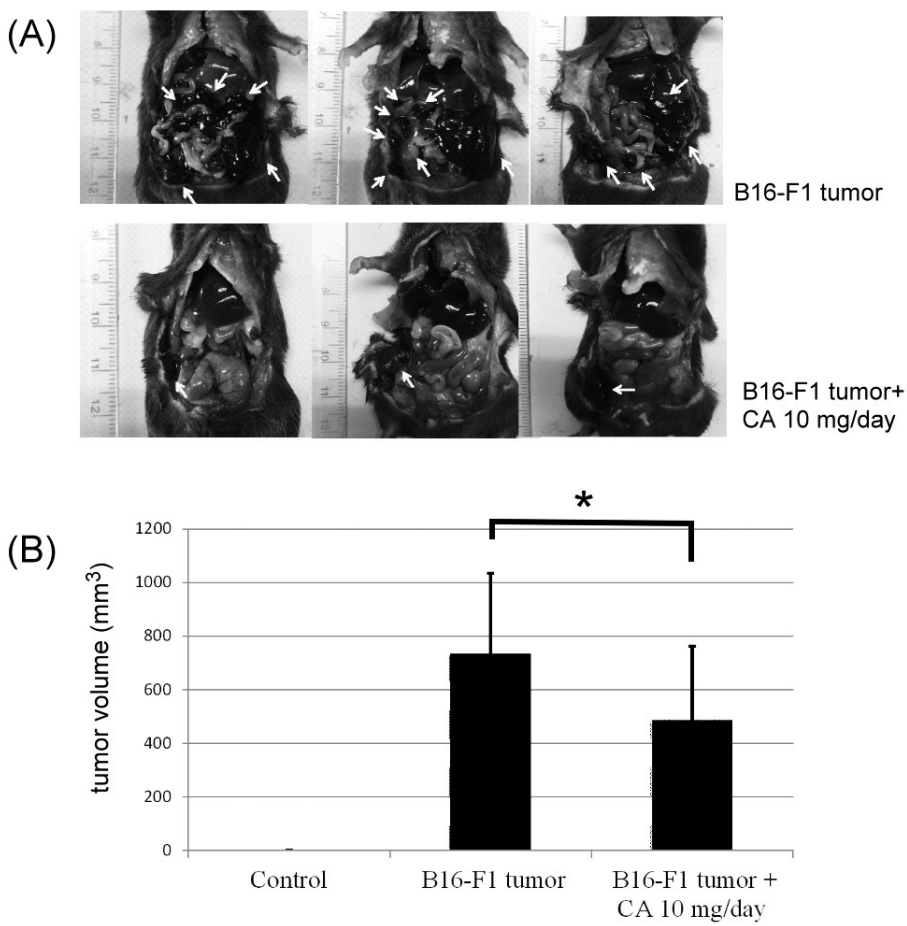


Figure. 6 Effects of caffeic acid on the colonization of B16-F1 melanoma cells. Mice were sacrificed 2 weeks after injection of B16-F1 cells and cell number and tumor volume were measured. *$p < 0.05$ for mice injected with B16-F1 cells only compared with mice injected with B16-F1 cells and orally treated with caffeic acid.

rapid tumor colonization, whereas mice injected with B16-F1 cells and treated orally with caffeic acid showed 80% survival rate (data not shown). Obvious tumor colonies were observed in mice injected with B16-F1 cells and not treated orally with caffeic acid (Figure 6). However, tumor formation was restricted and tumor volume decreased in mice injected with B16-F1 cells and treated orally with caffeic acid. This indicates that caffeic acid exerts a tumor suppressive effect *in vivo*. Moreover, these results demonstrated that caffeic acid-containing foods can be used as a chemopreventive strategy.

Discussion

Caffeic acid is a phytochemical that is widely found in plants (Boerjan, Ralph, and Baucher 2003; Budisan et al. 2017). Both ferulic and caffeic acids effectively inhibit melanin production in B16 melanoma cells. Ferulic acid shows no toxicity against melanoma cells, whereas caffeic acid exerts cellular toxicity at concentrations higher than 0.35mM (Maruyama et al. 2018). In the present study, caffeic acid remarkably inhibited the proliferation and migration of B16-F1 melanoma cells. We treated cells at lower densities than in the previous studies but at the same concentrations of caffeic acid. The resulting inhibitory effect was more severe (data not shown). The findings of the present study indicated that caffeic acid can regulate melanoma cell death, thus highlighting its significance as part of a healthy diet and in tumor chemoprevention. Moreover, we found that caffeic acid-induced autophagy in B16-F1 cells is due to decreasing p-AKT level and increasing p-AMPK level. Activation of the p-AKT pathway induces the tumorigenesis of melanoma (Alers et al. 2012; Demierre 2006; Eggermont and Robert 2012; Halachmi and Gilchrest 2001). The AMPK/LKB1 pathway functions as a tumor repressor and regulates autophagy in epithelial cells (Alers et al. 2012; Brenman 2007; Hezel and Bardeesy 2008; Jung et al. 2010; Miranda et al. 2010; Zheng et al. 2009). Moreover, p-AMPKα1/2 acts as a tumor suppressor and is involved in the reorganization of actin cytoskeleton and motility of polar cells (Miranda et al. 2010). In melanoma cells, AMPK-related kinase NUAK2 affects cell motility and growth (Namiki et al. 2011).

FASN promotes cancer cell growth by protecting cancer cells against hypoxia, low pH, and nutritional deprivation. FASN inhibition reduces intestinal cancer metastasis, breast cancer proliferation and angiogenesis (Lee et al. 2009; Mashima, Seimiya, and Tsuruo 2009). Results of the present study showed that p-AMPK does not absolutely regulate FASN, with no remarkable reaction in compound C experiment. A study by Menendez et al. (2006) demonstrated that both RAS-RAF-MEK-ERK1/2 and PI3K-AKT pathways control FASN. Thus, blockade of AMPK may help to regulate FASN expression through the above-mentioned pathways. In the present study, treatment of B16-F1 cells with compound C and caffeic acid decreased p-AKT levels, suggesting that p-AKT regulates FASN and decreases the effect of AMPK. FASN is involved in p53-induced cell cycle arrest and failed p53 function induces apoptosis (Menendez and Lupu 2007). In the present study, p53 did not affect treatment outcomes. However, further studies should be performed to elucidate the efficacies of p53 and FASN. Determination of the diversity of these regulatory molecules will help in understanding their effects and cell death induction patterns.

In addition to cell survival, cell motility is essential for cancer maintenance. Our data showed that caffeic acid decreases the expressions of basic migration- and adhesion-related molecules such as FAK, SRC, and PLC. Adhesion of cells to the extracellular matrix is mediated by integrin receptors that bind to FAK/SRC complex, thus activating downstream effectors such as RAS/ERK1/2 and PI3K/AKT (Niit et al.). The AKT pathway may downregulate SRC and FAK expressions. Moreover, LKB1 may regulate AMPK phosphorylation to induce SRC and FAK activation, promoting cytoskeleton reorganization and altering cell adhesion. A study involving pharmacological inhibitors suggested the involvement of AMPK, SYK, SRC, and ERK in PKC-dependent adhesion

of THP-1 monocytes onto culture plates. PKC may activate LKB1 and AMPK, phosphorylating and activating SYK, with subsequent activation of SRC and FAK (Chang et al.). Our *in vivo* animal study also showed that oral caffeic acid treatment restricts tumor colonization. These results indicated multiple effects of caffeic acid on melanoma cell death and motility.

Melanoma is associated with complex genetic events. Moreover, treatment of malignant tumor is difficult (Halachmi and Gilchrest 2001). Although some studies have shown that autophagy correlates with melanoma prognosis, other studies have shown that autophagy-induced melanoma cells are more resistant to chemotherapy (Amelio, Melino, and Knight 2011). Upregulation of autophagy marker BECN-1 decreases the survival rate of patients with melanoma and downregulation of this marker decreases chemotherapy resistance and increases patient survival rate (Ekmekcioglu et al. 2011; Tomic et al. 2011). In the autophagy pathway, autophagosomes fuse with lysosomes to form autolysosomes that digest or recycle proteins using enzymes present in lysosomes (Burman and Ktistakis 2010; Yoshimori 2004; Baehrecke 2005). Therefore, the result of autophagy is cell death or overcoming of adverse situation by lysis of death-related proteins. Autophagy is considered a kind of biphasic pattern. Several factors such as cell density, pH, and oxygen metabolism may be associated with cell death induction. Autophagy is a decisive point for cells to overcome stress. In the melanoma tumor, triggering autophagy may increase inhibitory effect on growth. Our findings indicate stationary condition in the system and provide valuable information for tumor inhibition and chemoprevention of melanoma. However, additional studies should be performed to investigate in detail the regulation of factors involved in the pathogenesis of melanoma.

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