Abstract. Torilis japonica extract (TJE) has been reported to possess diverse medicinal properties including anti-inflammatory and antibacterial activities. However, the precise mechanism of its anticancer effect is not understood. Thus, we evaluated the apoptotic effects of TJE and examined its underlying molecular mechanisms in HCT116 colorectal cancer cells. Our results show that TJE induces apoptosis through the generation of intracellular reactive oxygen species (ROS), and that it regulates the mitochondrial outer membrane potential via the AMPK/p38 MAPK signaling pathway. Importantly, ~50% of cancer cells have p53 mutations. Thus, the ability to induce apoptosis in a p53-independent manner would be of great value in cancer treatment. Our results show that not only does TJE regulate the AMPK/p38 signaling pathway, but it induces apoptosis in cells in which p53 has been knocked down using siRNA. Moreover, as in in vitro studies, TJE induced apoptosis and regulated apoptosis related-proteins in an HCT116 xenograft model. Taken together, our results demonstrate that TJE, a natural compound that may provide a substitute for chemotherapeutic drugs, has potential as an anticancer agent.

Introduction

The incidence of cancer continues to increase, largely because of an aging population and increasing rates of cancer-causing behaviors and conditions, particularly smoking and obesity. Colorectal cancer is the third most common cancer in males and the second most common in females, with over 1.2 million new cases and 608,700 deaths in 2008 (1,2). The incidence of colorectal cancer is higher in Westernized areas such as New Zealand, Europe and North America than in Asian countries. However, incidence rates are rapidly increasing in several East Asian countries such as Japan, China and Singapore (3-5). The trend is thought to reflect a change in dietary patterns (6-8). For this reason, interest in alternative medicine for the prevention and treatment of colorectal cancer has increased and research on the effects of various food extracts on colorectal cancer is in progress (8,9).

Induction of apoptosis in cancer treatment. Apoptosis is the process of programmed cell death. In contrast to necrosis, which is a caused by acute cellular injury, apoptosis has many biological advantages. For example, the separation of fingers and toes in a developing human embryo occurs because cells between the digits undergo apoptosis (10,11). Understanding apoptosis in disease conditions is very important as it not only offers insights into the pathogenesis of a disease but may also give clues as to how the disease can be treated. Numerous research studies have demonstrated the death of cancer cells through apoptosis and compounds have been developed to take advantage of this knowledge (7,9,12,13).

Induction of apoptosis in cancer cells is driven by a complex interplay between several proteins. Members of the Bcl-2 family of proteins are key regulators of apoptosis. These proteins are known to regulate mitochondrial function and control the release of apoptosis-inducing factors such as cytochrome c from the mitochondrial inter-membrane space (14-16). The anti-apoptotic proteins Bcl-2 and Mcl-1 are predominantly found in the mitochondria; they inhibit apoptosis by suppressing the release of cytochrome c (17,18). In contrast, the pro-apoptotic proteins Bax, Bak and PUMA mainly induce the release of stimulators of apoptosis and bring about mitochondrial dysfunction after translocating to the mitochondrial outer membrane (19-21). In particular, translocation of Bax and Bak to the mitochondrial outer membrane is required for the release of cytochrome c during apoptosis. Bax and Bak undergo homo- and hetero-oligomerization and bind to the mitochondrial outer membrane. These protein
complexes trigger cytochrome c release into the cytosol by reducing mitochondrial outer membrane permeabilization (22-25). Cytochrome c induces apoptosis by increasing the activity of caspases in the cytoplasm (26). Control of this process using targeted compounds is very important in cancer treatment.

The fruit of *Torilis japonica* can be used as a substitute for *She chuang zi*, which is a traditional Chinese medicine prescribed as an anti-allergic, anti-fungal, anti-bacterial and sedative agent. Previously, we found that a 95% ethanol extract from *Torilis japonica* had beneficial effects on metastasis through regulation of the EGFR signaling pathway in MCF-7 breast cancer cells (27). However, its anti-proliferative and apoptosis-inducing effects have not yet been elucidated.

In this study, we investigated the effects of *Torilis japonica* extract (TJE) extracted from the fruit of *Torilis japonica* on apoptosis in HCT116 and HT-29 colon cancer cells. TJE induced apoptosis through the generation of intracellular reactive oxygen species (ROS) and a reduction in the mitochondrial membrane potential via regulation of the AMPK/p38 MAPK signaling pathway. Moreover, the apoptotic effects of TJE persisted in cells lacking p53. Taken together, our results indicate that TJE may be a novel natural ingredient for cancer therapy that decreases the mitochondrial membrane potential of colorectal cancer cells, thereby inducing apoptosis.

**Materials and methods**

**Plant material and preparation of TJE.** Dried whole fruit of *Torilis japonica* was purchased from Na-nam Pharmacy (Kyung-buk, Korea). Plant material (200 g) was extracted two times with 95% ethanol at room temperature for 3 days and was subsequently filtered. The combined filtrate was concentrated under vacuum at 60°C, and completely dried by freeze drying. The yield was 10% and TJE powder was dissolved in DMSO and filtered by 0.2 µm pore size filter for in vitro studies.

**Reagent.** N-acetyl cystein (NAC), 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DCFH-DA Reagent. and filtrated by 0.2 µm pore size filter for measurement.

**Plant material and preparation of TJE.** Dried whole fruit of *Torilis japonica* was purchased from Na-nam Pharmacy (Kyung-buk, Korea). Plant material (200 g) was extracted two times with 95% ethanol at room temperature for 3 days and was subsequently filtered. The combined filtrate was concentrated under vacuum at 60°C, and completely dried by freeze drying. The yield was 10% and TJE powder was dissolved in DMSO and filtered by 0.2 µm pore size filter for in vitro studies.

**Detection of intracellular ROS by fluorescence microscope.** Cells were seeded 1x10⁵/ml in 12-well plate with cover glasses. After treatment the indicated time and dose at 37°C in a 5% CO₂ atmosphere, the cells were incubated with 10 µM of DCFH-DA for 30 min and fixed with 3.7% formaldehyde for 20 min. Cells were washed with phosphate-buffered saline (PBS) twice and fluorescence was detected by fluorescence microscope (Carl Zeiss, Thornwood, NY, USA).

**Measurement of intracellular ROS levels.** Cells were seeded 1x10⁴/ml in 100 mm plate and incubated for 24 h. After incubation, cells treated with test compound for 6 h at 37°C in a 5% CO₂ atmosphere. Cells were incubated with 40 µM of DCFH-DA for 30 min and harvested by trypsinization, collected by centrifugation, washed with PBS twice, and resuspended in PBS. Fluorescence intensity were analyzed by using flow cytometer (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA).

**Cell proliferation assay (MTT).** Cells were seeded at 4,000/ml each well in 96-well plate, and incubated 24 h. After the incubation, treated with test compound and incubate at 37°C in a 5% CO₂ atmosphere. After 24 h, cells were incubated with 20 µl MTT (5 mg/ml with PBS) solution for 1 h. Optical densities of solution, in each well, were determined by Microplate reader (Bio-Rad Laboratories, Inc., Tokyo, Japan) at 595 nm.

**Determination of apoptosis by Annexin V/PI staining analysis.** Cells were seeded at 1x10⁵/ml in 100 mm plate and incubated for 24 h. After incubation, cells treated with test compound for 24 h at 37°C in a 5% CO₂ atmosphere. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS, and resuspended in binding buffer. Cells were stained with Annexin V and PI for 15 min. Fluorescence intensity were analyzed by using flow cytometer (BD Biosciences).

**Measurement of mitochondria membrane potential.** Cells were seeded at 1x10⁴/ml in 100 mm plate and incubated for 24 h. After incubation, cells treated with test compound for 24 h at 37°C in a 5% CO₂ atmosphere. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS, and resuspended in binding buffer. Cells were stained with JC-1 for 30 min 37°C in a 5% CO₂ atmosphere before the flow cytometer analysis (BD Biosciences).

**Caspase-3 activity assay.** We used caspase-3 activity assay kit (Abcam PLC, Cambridge, UK). Cells were seeded at 1x10⁴/ml in 100 mm plate and incubated for 24 h. After incubation, cells treated with test compound for 24 h at 37°C in a 5% CO₂ atmosphere. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS. Cells were resuspended in lysis buffer and mixed with 2X reaction buffer. Samples were reactivated with DEVD-p-NA for 2 h at 37°C. Optical densities of solution, in each well, were determined by Microplate reader (Bio-Rad Laboratories, Inc.) at 405 nm.

**Fraction of mitochondria and cytosol proteins.** We used Mitochondria/Cytosol Fraction kit (Abcam PLC). Cells were seeded at 1x10⁴/ml in 100 mm plate and incubated for...
24 h. After incubation, cells were treated with test compound for 24 h at 37˚C in a 5% CO₂ atmosphere. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS, and homogenized in ice-cold cytosol extraction buffer mix containing DTT and protease inhibitor using a sonicator. The homogenates were centrifuged at 3,000 rpm for 10 min at 4˚C and supernatants were collection. Supernatant were centrifuged at 13,000 rpm for 30 min at 4˚C and collected supernatant for cytosol proteins and pellets were resuspended with ice cold mitochondria extraction buffer containing DTT and protease inhibitor for mitochondria proteins.

**Protein oligomerization.** Bak and Bax oligomerization were assessed by chemical crosslinking. Briefly, mitochondria protein fractions were resuspended in conjugation buffer. For disulphide-bond formation, mitochondria protein fractions were incubated with the bis(maleimido)hexane (Thermo Fisher Scientific, Rockford, IL, USA) for 1 h at room temperature and the samples analyzed by non-reducing SDS-PAGE.

**Immunoprecipitation (IP) assay.** We used to sure bead protein G magnetic beads kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cells were seeded at 1x10⁵/ml in 100 mm plate and incubated for 24 h. After incubation, cells were treated with test compound for 24 h at 37˚C in a 5% CO₂ atmosphere. Mitochondria/cytosol proteins were fraction and mitochondria proteins were incubated with specific antibody bound magnetic bead. The beads were washed using a magnet and PBS. Target proteins were elusion in 1X sample buffer and analyzed by western blotting.

**Bcl-2 activation assay.** We used Muse Bcl-2 activation dual detection kit (Merck Milipore, Darmstadt, Germany). Cells were seeded at 1x10⁵/ml in 6-well plate and incubated for 24 h. After incubation, cells were treated with test compound for 24 h at 37˚C in a 5% CO₂ atmosphere. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS. Cell were fixed and permeabilized by reagent in the kit. The antibody, which was combined with fluorescence tag, was reacted for 1 h at room temperature with slow agitation, and it was washed once with the assay buffer. Cells were resuspended in assay buffer and analyzed by Muse cell analyzer.

**Transient transfection with small interfering RNA.** Small interfering RNA (siRNA) was purchased by Dharmacon (Chicago, IL, USA). For transient transfection, cells were seeded at 1x10⁵/ml on a 6-well plate with antibiotics free medium. After incubation overnight, targeting siRNA was transfected using the modified formula V = 1/2 (length x width²). After the 3-week treatment, tumor was removed and frozen in liquid nitrogen for western blot analysis or fixed with formalin for immunohistochemistry and H&E staining. All animal experiments were approved by the Ethics Committee for Animal Experimentation, Hannam University.

**Immunohistochemistry.** Tumor specimens from mice were fixed in 10% formaldehyde, embedded in paraffin and sectioned into 5 μm thick slices. Consecutive thin cryosections (5 μm) of OCT compound (Sakura Finetek, Torrance, CA, USA) embedded tumor tissues were fixed in acetone at 4˚C for 10 min. After washing in PBS, sections were treated with 3% H₂O₂ for 10 min to block endogenous peroxidase activity, and the sections were blocked with normal rabbit serum. Then, the sections were blocked and washed in PBS and incubated with specific antibody overnight at 4˚C. Negative controls were incubated with the primary normal serum IgG for the species from which the primary antibody was obtained.

**TUNEL assay.** Levels of apoptosis in distal colon tissue were determined using the TdT-mediated dUTP nick-end labeling (TUNEL) method. Tumor specimens from mice were fixed in 10% formaldehyde, embedded in paraffin and sectioned into 5 μm thick slices. Tissue sections were processed according to manufacturer's instructions for the ApopTag peroxidase in situ apoptosis detection kit (Vector Laboratories, Burlingame, CA, USA).

**Statistical analysis.** Cell viability and caspase-3 activity was statistically analyzed using unpaired t-test (SPSS, Chicago, IL, USA). P<0.05 was considered statistically significant.

**Results**

**TJE suppresses cancer cell proliferation and induces apoptosis by reducing the mitochondrial membrane potential.** We
investigated the anti-proliferative and apoptotic effects of TJE. We treated cells with TJE (10-100 µg/ml) for 12 and 24 h, and then assayed for cellular viability and apoptosis. Cells treated with TJE showed a decrease in viability and an increase in the number of Annexin V-positive cells in a dose-dependent manner. In normal human fibroblasts, however, TJE had no effect on cellular viability (Fig. 1A-C).

To understand the mechanism by which TJE induces apoptosis, we measured the mitochondrial membrane potential after treatment with different concentrations of TJE via JC-1 staining (Fig. 1D). Our results showed that TJE reduced the membrane potential dose-dependently.

**TJE regulates p53 and AMPK expression, p38 activation and levels of pro-apoptotic proteins.** We analyzed the changes in p-AMPKα1, p-p38, p53 and p-p53 levels and the apoptosis-related proteins Bax, Bak, PUMA, cleaved caspase-3, Mcl-1 and Bcl-2 after treatment with different concentrations of TJE by western blotting. Our results showed that TJE strongly activated AMPK, p38 and p53 dose-dependently (Fig. 2A).

Moreover, TJE reduced the expression of Mcl-1 and Bcl-2 and induced the expression and mitochondrial translocation of Bax, Bak and PUMA. The latter three proteins became localized to the outer membrane of the mitochondria, which led to secretion of cytochrome c from the mitochondria to the cytosol by a reduction in the mitochondrial membrane potential (Fig. 2B and C).

**TJE modulates signaling pathways and mitochondrial membrane potential through generation of intracellular ROS.** We examined whether TJE promotes the generation of ROS in HCT116 colon cancer cells. We measured intracellular ROS levels following treatment of cells with TJE (20-80 µg/ml) for 6 h. As shown in Fig. 3A (left panel), TJE increased ROS levels at the indicated concentrations. These effects were completely blocked by co-treatment with NAC, a ROS scavenger (right panel).

To make sure that the increase in intracellular ROS levels was related to TJE-regulated signaling proteins and the induction of apoptosis, we co-treated cells with NAC and then analyzed protein levels and the concentration of...
Annexin V-positive cells. The cells co-treated with NAC and TJE did not undergo increases in AMPK, p38 or p53 phosphorylation and showed decreased expression, oligomerization, and binding of apoptosis-related proteins and increases in the expression of anti-apoptotic proteins in comparison to cells treated with TJE alone (Figs. 3E and 4). In addition, cells co-treated with TJE and NAC did not undergo cell death and did not exhibit a reduction in the mitochondrial membrane potential (Fig. 3F). By contrast, TJE-treated cells displayed increased levels of apoptotic cell death through changes in the mitochondrial membrane potential which led to secretion of cytochrome c from the mitochondria to the cytosol (Fig. 3G).

Generally, cytochrome c in the cytosol initiates caspase cleavage and activates effector caspases such as caspase-3, which in turn induce apoptosis. We demonstrated that TJE induced caspase-3 activation dose-dependently. Co-treatment with NAC abolished this effect (Fig. 3H).

**TJE regulates cytochrome c translocation to the cytoplasm.** To show that TJE treatment induced cytochrome c release from the mitochondria to the cytosol, we co-treated cells with TJE and NAC for 24 h and stained cells in order to visualize the mitochondria and cytochrome c. In TJE-treated cells, cytochrome c translocated from the mitochondria to the cytosol; in contrast, in control cells and those co-treated with both TJE and NAC, cytochrome c remained in the mitochondria (Fig. 5).
proteins. Our results showed that TJE did not induce pro-apoptotic protein expression or downregulation of anti-apoptotic proteins in AMPK siRNA- or p38 siRNA-transfected cells, but the effects of TJE were still seen in p53 siRNA-transfected cells, except those treated with PUMA; PUMA cannot act independently of p53 (Fig. 6C and D). Moreover, TJE induced apoptotic cell death in HT-29 colon cancer cells, which have a mutation in the p53 gene (Fig. 6F).

TJE induces cell death through regulation of intracellular signaling pathways in an HCT116 xenograft model. To analyze the consequences of TJE treatment in an HCT116 xenograft model of tumor growth, we performed histological analysis from control- and TJE (80 mg/kg/day)-treated tumor tissue stained with H&E using the TUNEL assay. The amount of tumor tissue in TJE-treated samples was considerably less than that of control samples (Fig. 7A). The number of TUNEL-positive cells was significantly increased and cancer tissue was degraded in TJE-treated samples (Fig. 7E). In addition, in a similar in vitro experiment, the AMPK/p38 MAPK and p53 signaling pathways were activated, and apoptosis-related proteins showed an increase in expression and translocation to the cytosol (Fig. 7B-D and F).

Discussion
The incidence of colorectal cancer has increased because of changes in dietary patterns and lifestyles (1-5). For this reason,
there has been an increased interest in the effect of compounds extracted from natural sources on the prevention and treatment of colorectal cancer. Such compounds induce apoptosis and arrest metastasis through the regulation of intracellular signaling in cancer cells (27-33). Many researchers have identified food extracts that have been used in cancer chemotherapy experiments.

The fruit of *Torilis japonica* is used as a substitute for *She chuang zi*, which is a traditional Chinese medicine. Previously, we found that ethanol extracts from the fruit of *Torilis japonica* arrested abnormal metastasis through regulation of the EGFR signaling pathway in MCF-7 breast cancer cells (27). In this study, we focused on the effects of TJE on the induction of apoptosis through alterations in the mitochondrial membrane potential in colorectal cancer cells.

In this study, we confirmed that TJE negatively regulated cancer cell viability. Then, we showed that TJE reduced cell viability dose-dependently. Also, it induced apoptotic cell death related proteins through generation of intracellular ROS. Cells were treated with TJE after pretreatment 5 mM NAC for 30 min (A) Bcl-2 activation levels were measured by Muse cell analyzer. **P<0.01 and ***P<0.001 compared to control; *P<0.05, **P<0.01 and ***P<0.001 compared to TJE treated only groups. NS, not significant (each experiment n=3). (B) Oligomerization of pro-apoptotic proteins. Oligomer form was analyzed by non-ionized gel western blotting. (C) Co-binding of Bax and Bak were detected by immunoprecipitation. Co-binding form was analyzed by non-ionized gel western blotting.
Figure 5. TJE regulates cytochrome c translocation to the cytoplasm. (A) Cells were treated with TJE after 5 mM NAC for 30 min. Pre-stained with mito-tracker before fixation and permeabilization of cells and it was reacted with specific antibody. Fluorescence detected by confocal microscope. Cell count was confirmed by DIC image. Magnification, x400. (B) Magnified image of merged image. Magnification, x600.

Figure 6. TJE induces apoptosis via the AMPK/p38 MAPK signaling pathway in a p53-independent manner. Cells were transfected with AMPKα1, p38 and p53 siRNA using DharmaFECT and treated with 60 µg/ml TJE for 6 h after being pretreated with NAC 5 mM for 30 min. (A) Apoptotic cell death was measured by Annexin V/PI staining and flow cytometry. *P<0.01 and **P<0.001 compared to control; †P<0.05 and ‡P<0.01 compared to TJE treated only groups. NS, not significant (each experiment’s n=3). (B) Caspase-3 activities were measured by caspase-3 activity assay. *P<0.05 and †P<0.01 compared to control; ‡P<0.05, §P<0.01 and ¶P<0.001 compared to TJE treated only groups. NS, not significant (each experiment n=3). (C) The protein levels of p-AMPK, p-p38, p-p53 and apoptosis related-proteins were examined by western blotting. (D) Cytochrome c translocation to cytoplasm were detected by a confocal microscope, green fluorescence is cytochrome c and red fluorescence is Mitotracker. (E) Bcl-2 activation was measured by Muse cell analyzer. (F) Measurement of apoptotic cell death in HT-29 colon cancer cells, which are p53 mutant cell line.
death by altering the mitochondrial membrane potential and regulating apoptosis-related proteins including Bax, Bak and PUMA. Induction of intracellular ROS in cancer cells by treatment with natural compounds is an appealing option in the development of anticancer agents. Increasing ROS by treatment with natural products not only controls the cell cycle but also induces apoptotic cell death by regulating intracellular signaling molecules such as the Bcl-2 family and caspases (12,13,28,32-35). Thus, we hypothesized that TJE treatment effects cancer cells through an increase in intracellular ROS. Indeed, our results showed that treatment of cancer cells with TJE induces intracellular ROS and affects apoptosis via a reduction in the mitochondrial outer membrane potential through the regulation of protein signaling.

Previous study demonstrated that AMPK activation by ROS induces apoptosis via the ASK1/p38 MAPK pathway in MCF-7 breast cancer cells (35). In the present study, we found that activation of p38 MAPK by phosphorylation of AMPK regulates the mitochondrial membrane potential and induces apoptosis in a p53-independent manner (28). p53 is known to be involved in cancer cell death and arrest of abnormal cellular proliferation (36-39). However, ~50% of cancer cells have p53 mutations (40). Thus, a p53-independent method of inducing apoptosis would be extremely valuable in cancer treatment. Our results showed that TJE regulates the AMPK/p38 MAPK signaling pathway and induces apoptosis via regulation of the mitochondrial outer membrane potential and translocation of pro-apoptotic proteins to the mitochondria. When we silenced AMPK and p38 using specific siRNAs, TJE did not affect apoptotic cell death or pro-apoptotic protein expression. Interestingly, in cells transfected with p53 siRNA, apoptosis was induced and apoptosis-related proteins were affected, as in cells treated with only TJE. Moreover, in cells in which AMPK and p38 MAPK were
silenced, p53 was not activated. Previous studies found that p38 MAPK activation may be regulated by p53 expression and activation via downstream signaling pathways (41,42). Also, the AMPK/p38 MAPK pathway controls apoptosis-related protein expression and induces apoptotic cell death without p53 activation (28). From our results and those of previous studies, we conclude that TJE induces apoptosis and downregulates the mitochondrial outer membrane potential via the AMPK/p38 MAPK signaling pathway in a p53-independent manner.

As in our in vitro studies, TJE induced apoptosis in a mouse xenograft model. The rate of tumor growth was reduced in the TJE-injected group as compared with the control group. Moreover, the TJE-treated group exhibited an increase in pro-apoptosis protein expression and translocation to the mitochondria. Thus, our in vivo studies confirm that TJE regulates the AMPK/p38 MAPK signaling pathway.

In conclusion, we demonstrated that TJE, a natural compound, has potential as an anticancer agent and may provide a substitute for chemotherapeutic drugs.

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References


