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# Chlorogenic acid regulates apoptosis and stem cell marker-related gene expression in A549 human lung cancer cells

Kazuo Yamagata[1] · Yuri Izawa[1] · Daiki Onodera[1] · Motoki Tagami[2]

**Abstract** Previous studies indicated that chlorogenic acid, a compound present in many fruits and vegetables, has anti-cancer activities. We report that chlorogenic acid regulates the expression of apoptosis-related genes and self-renewal-related stem cell markers in cancer cells. The lung cancer cell line A549 was cultured with or without chlorogenic acid. The presence of chlorogenic acid decreased cell proliferation as measured by MTT activity. Polymerase chain reaction (PCR) showed that treatment of cells with chlorogenic acid reduced the expression of BCL2 but increased that of both BAX and CASP3. Chlorogenic acid enhanced annexin V expression as measured using fluorescently labeled annexin V. Chlorogenic acid also induced *p38 MAPK* and *JNK* gene expression. Meanwhile, several agents, including SB203580 (p38 MAP kinase inhibitor), N-acetylcysteine (antioxidant inhibitor), dipyridamole (phosphodiesterase inhibitor), and apocynin (NADPH-oxidase inhibitor) blocked chlorogenic acid-induced *BAX* gene expression. Chlorogenic acid reduced gene expression levels of stem cell-associated markers *NANOG, POU5F1,* and *SOX2.* Together these results indicate that chlorogenic acid affects the expression of apoptosis-related genes that are part of oxidative stress and p38 MAP-dependent pathways, as well as genes encoding stem cell markers. In conclusion, chlorogenic acid may contribute to the polyphenolic anti-cancer effect associated with consumption of vegetables and fruits.

**Keywords** Apoptosis · Chlorogenic acid · Stem cell markers

**Abbreviations**
| | |
|---|---|
| CSCs | Cancer stem cells |
| NAC | N-acetylcysteine |
| NSCLC | Non-small cell lung cancer |
| ROS | Reactive oxygen species (ROS) |

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## Introduction

Lung cancer is the leading cause of cancer-related mortality throughout the world. Although considerable progress has been made in the treatment of lung cancer, the five-year survival rate is only 15–17% [1]. On the other hand, epidemiological and experimental studies indicated that consumption of fruits and vegetables containing phytochemicals such as polyphenol compounds might reduce the incidence of cancer [2–4]. For example, epidemiological studies suggest that intake of coffee [5, 6] and apples [7] reduces the risk of certain cancers. Chlorogenic acid is a phenolic acid that exists at high levels in coffee beans and fruits such as plum, pear, and apple [12], and is one of many dietary polyphenols that has been shown to reduce the incidence of chemical carcinogenesis in several animal models of cancer [10, 11]. An in vitro study demonstrated that treatment of human colon cancer cells with chlorogenic acid increased reactive oxygen species generation and reduced cell viability [8]. Consumption of chlorogenic acid may decrease colorectal cancer risk, increase colon motility, and enhance antioxidant status [9]. However, the

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molecular mechanisms associated with the anti-carcinogenic properties of chlorogenic acid are not well understood.

Apoptosis is a physiological process that removes abnormal or damaged cells and is critical for the maintenance of tissue homeostasis [13]. Apoptosis is regulated at multiple molecular levels, including anti- and pro-apoptotic members of the BCL2 family such as BCL2- and BCL2-associated X (BAX), respectively, as well as caspase-3 (CASP3) and caspase-7 [14]. The polyphenol curcumin has anti-cancer activity and has been shown to induce apoptosis via mitochondrial pathways in various cancer cell types by modulating the expression of BCL2 protein family members [15, 16]. Furthermore, chlorogenic acid stimulated apoptosis and inhibited cell proliferation in a human acute promyelocytic leukemia cell line [17]. Chlorogenic acid induced the expression of several caspases and mitochondria-dependent pathways to promote apoptotic cell death in U937 human leukemia cells [18]. The effects of these polyphenols suggest that they could have potential as cancer therapeutic agents [19]. On the other hand, cancer stem cells (CSCs) are resistant to general therapies and may be responsible for tumor relapse [20]. Therefore, standard chemotherapy is often ineffective against cancer stem cells (CSCs) and for epithelial–mesenchymal transition (EMT) patients. For patients with progressive cancer, decreases in survival rates are largely due to tumor relapse and metastasis [21]. Previously, anti-apoptotic proteins such as BCL-XL were shown to have anti-apoptotic effects and promote chemoresistance in hyaluronan-mediated-CD44-activated tumor cells [22] In non-small cell lung cancer (NSCLC), apoptosis plays a role in drug resistance [23], whereas expressions of stem cell markers, CD44, NANOG, Oct4 are also related to drug resistance. These findings indicate that expression of stem cell markers may be associated with apoptosis regulation. CD44 is expressed by various cancer cells and is an important stem cell marker [24, 25]. Similarly, the stem cell marker NANOG is a key transcription factor for self-renewal and is required for maintenance of embryonic stem cells [26]. NANOG is also expressed in several tumor cell lines [27]. Meanwhile, the SOX2 gene regulates stem cell function and organogenesis [28] and regulates multiple malignant processes in cancer development in breast cancer [29]. Oct4 is another stem cell marker that contributes to self-reproductive capacity and multipotency [30]. Oct4 interacts with NANOG and SOX2 to regulate differentiation and maintain pluripotency [31]. On the other hand, hypoxia induces stem cell-like properties of cancer cell lines by increasing the expression of stem cell genes (OCT4 gene symbol; POU5F1, SOX2, and NANOG) and the CD133+ stem cell fraction [32].

Anti-cancer activity of polyphenols can regulate the interplay between autophagy [33, 34] and apoptosis [35, 36]. Furthermore, polyphenols have also been implicated in several mechanisms associated with CSCs [37]. Although chlorogenic acid is thought to have anti-cancer properties, the mechanisms of these effects are unclear. We hypothesized that chlorogenic acid affects gene expression of apoptotic-related genes and cancer stem cell markers. In this study we investigated the anti-cancer role of chlorogenic acid in A549 human lung cancer cells by examining the effect of this compound on apoptosis and the expressions of several stem cell markers such as OCT4, SOX2, CD44, and NANOG under ischemic conditions.

## Results

### Effect of chlorogenic acid on A549 cell proliferation

To examine the antitumor effects of chlorogenic acid, A549 cells were treated with 30 or 50 μM chlorogenic acid for 24 h before cell proliferation was assessed using an MTT assay. Chlorogenic acid at 30 and 50 μM inhibited the cell proliferation of A549 cells by 27 and 39%, respectively, relative to untreated cells ($n = 4$, Fig. 1).

### Effect of chlorogenic acid on gene expression of BCL2, BAX, and CASP3 in A549 cells

In addition to A549 cells, we examined the effect of chlorogenic acid on the human lung cancer cell lines Lu65 and Lu 135. Both cell lines showed similar increases in *BAX* gene expression following chlorogenic acid treatment wherein treatment of Lu65 and Lu 135 cells with 30 μM chlorogenic acid increased *BAX* expression by 105 and
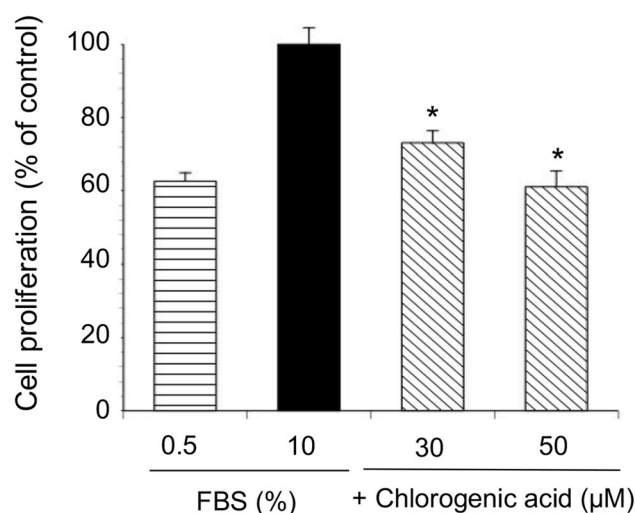


**Fig. 1** Effect of chlorogenic acid on A549 cell proliferation

113%, respectively, relative to untreated control cells, and at 50 µM the increase was 117 and 115% for Lu65 and Lu135 cells, respectively. Meanwhile, similar changes in *BAX* expression following treatment of A549 cells with chlorogenic acid were seen in studies focusing on the anti-cancer activity of polyphenols. Therefore, in this study we used only A549 cells.

In this study, the expression of several genes associated with apoptosis was examined in A549 cells treated for 8 h with chlorogenic acid at increasing concentrations (10, 30, and 50 µM). At 30 and 50 µM, chlorogenic acid significantly ($p < 0.05$) reduced gene expression of *BCL2* to 75 and 69%, respectively ($n = 4$), of that for untreated A549 cells (Fig. 2a). At 10 µM chlorogenic acid, the expression levels did not significantly differ from untreated cells (Fig. 2a). Meanwhile, chlorogenic acid significantly ($p < 0.05$) induced gene expression of *BAX* (30 µM, 110%; 50 µM, 127%) ($n = 4$) relative to untreated cells (Fig. 2b). Additionally, the gene expression of *CASP3* significantly ($p < 0.05$) increased in cells treated with 10

(110%) and 30 µM (115%) chlorogenic acid as compared with untreated A549 cells ($n = 4$, Fig. 2c). However, *CASP3* gene expression was not increased in cells treated with 50 µM chlorogenic acid relative to that in untreated A549 cells. In particular, the ratio of *BAX* to *BCL2* showed a chlorogenic acid concentration-dependent increase in expression (Fig. 2d). These results indicate that chlorogenic acid can enhance expression levels of several genes that play roles in apoptosis of A549 cells.

## Expression of BCL2, BAX, and CASP3 protein in A549 cells treated with chlorogenic acid

To examine the protein expression of BCL2 and BAX, we examined cells that were treated with 2, 10, and 30 µM chlorogenic acid for 24 h. The protein expression levels of BCL2 were significantly ($p < 0.05$) reduced by chlorogenic acid treatment compared with untreated A549 cells (2 µM, 32%; 10 µM, 29%, 30 µM, 77%) ($n = 4$; Fig. 3). In contrast, the protein expression of BAX following



Fig. 2 Effect of chlorogenic acid on *BCL2, BAX,* and *CASP3* gene expression in A549 cells
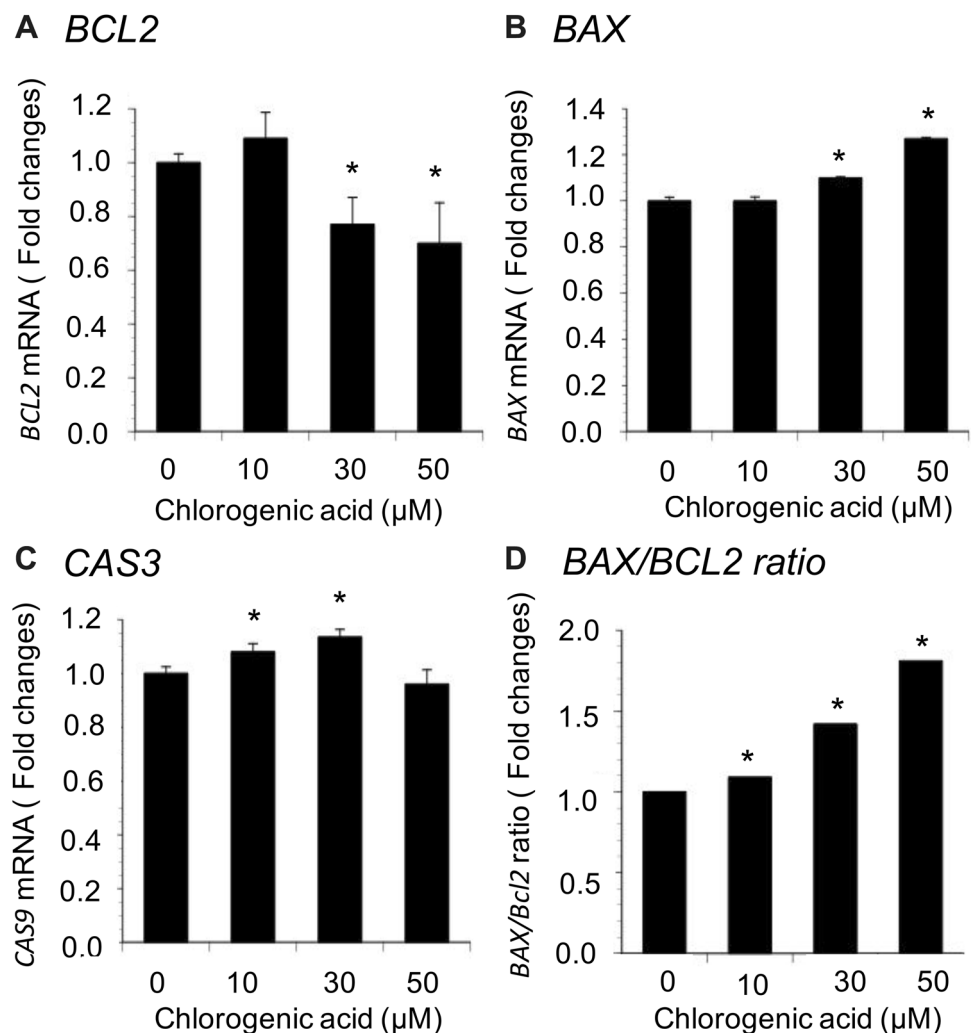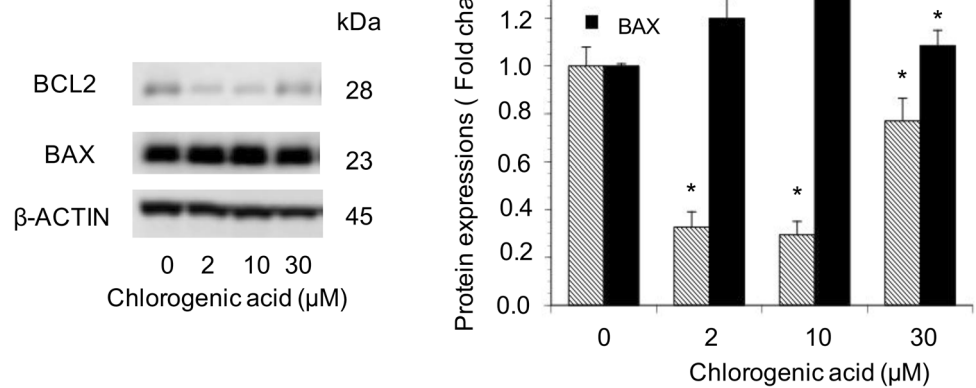
**Fig. 3** Effect of chlorogenic acid on regulation of BAX and BCL2 protein levels in cultured A549 cells



treatment with 2 and 10 μM chlorogenic acid increased by 119 and 134% (*n* = 4), respectively, relative to untreated cells (Fig. 3). The increase in the expression ratio of BAX/BCL2 was also confirmed by western blotting (Fig. 3).

### Effect of chlorogenic acid on fluorescence intensity of annexin V in A549 cells

Apoptosis in chlorogenic acid-treated cells was also assessed by binding of fluoresceinated annexin V. Chlorogenic acid exposure induced the expression of fluoresceinated annexin V in A549 cells (Fig. 4). Specifically, treatment with chlorogenic acid at 100 and 150 μM significantly ($p < 0.05$) increased annexin V upregulation (results not seen at 10, 30, or 50 μM). Chlorogenic acid

inhibited cell proliferation such that the cell number in the chlorogenic acid group may have been reduced relative to that for control conditions. Chlorogenic acid did enhance the fluorescence intensity of annexin V, indicating increased levels of apoptosis (Fig. 4).

### Effect of chlorogenic acid on gene expression of p38 MAP and JUN in A549 cells

To examine the gene expression of *p38 MAP* and *JUN*, we treated A549 cells with 10, 30, or 50 μM chlorogenic acid for 8 h. A549 cells treated with 10 μM chlorogenic acid had expression levels that were similar to that of untreated cells, whereas A549 cells exposed to 30 and 50 μM chlorogenic acid showed significantly ($p < 0.05$) increased

**Fig. 4** Effect of chlorogenic acid on fluorescence intensity of annexin V in A549 cells
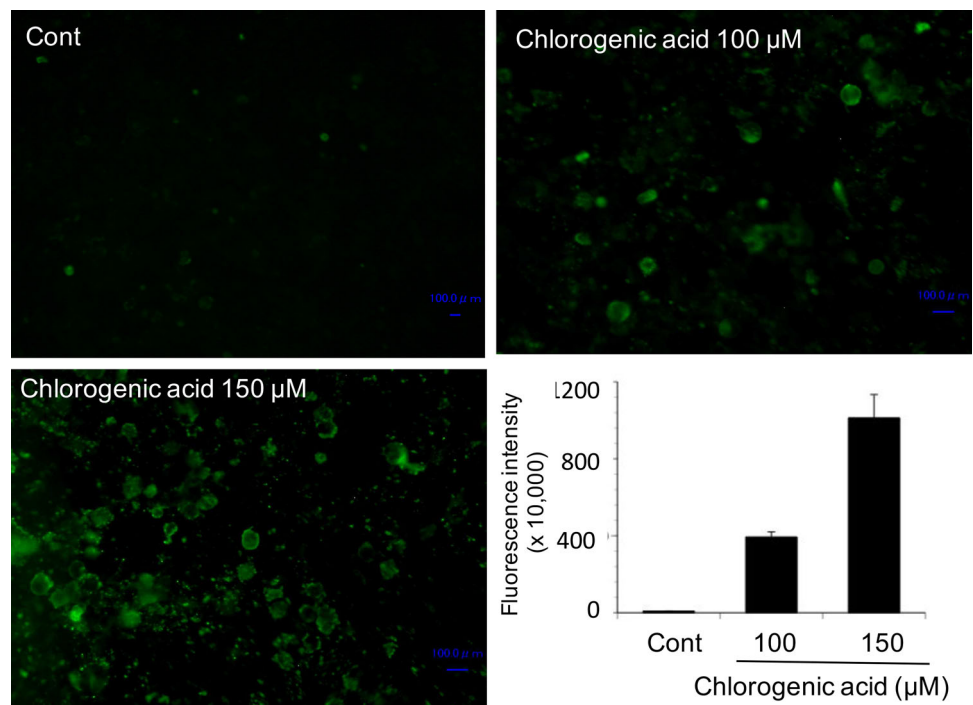
gene expression of *p38 MAP* (30, 118%; 50 μM, 127%) (*n* = 4) and *JUN* (30, 116%; 50 μM, 123%) compared to untreated cells (*n* = 4; Fig. 5).

### Effect of several inhibitors on chlorogenic acid-mediated BAX gene expression in A549 cells

As noted above, treatment of A549 cells with chlorogenic acid increased the expression levels of *BAX* and in turn the number of apoptotic cells. To identify pathways that may be involved in chlorogenic acid-dependent regulation of gene expression, we next assessed the effects of the p38 MAP kinase inhibitor SB203580, the antioxidant inhibitor *N*-acetylcysteine (NAC), the phosphodiesterase inhibitor dipyridamole (DIP), and the NADPH-oxidase inhibitor apocynin (Fig. 6). SB203580, NAC, DIP, and apocynin all significantly (*p* < 0.05) inhibited gene expression of *BAX* relative to untreated cells. In particular, *BAX* gene expression was nearly completely attenuated in the presence of PD98059, NAC, and apocynin. The action of these inhibitors suggests that several kinases and reactive oxygen species (ROS) pathways might be involved in the effects of chlorogenic acid.

### Expression of stem cell marker-related genes following treatment of A549 cells with chlorogenic acid

Given the chlorogenic acid-mediated increase in *BAX* expression, we next examined whether chlorogenic acid affected expression of stem cell markers that may also be associated with the regulation of apoptosis. Treatment of A549 cells for 8 h with increasing amounts of chlorogenic acid (10, 30, and 50 μM) significantly (*p* < 0.05) reduced gene expression of *NANOG* (30 μM, 85%; 50 μM, 79%) (*n* = 4) relative to untreated A549 cells (Fig. 7b). Similarly, gene expression of *POU5F1* and *SOX2* was

significantly (*p* < 0.05) decreased in the presence of chlorogenic acid compared to untreated A549 cells (Fig. 7c, d). These results indicate that chlorogenic acid treatment reduces expression levels of stem cell marker genes in A549 cells.

## Discussion

In A549 human lung cancer cells, our studies indicated that chlorogenic acid decreased cell proliferation and regulated the expression of the apoptosis-related genes *BCL2*, *BAX,* and *CASP*3 as well as genes encoding stem cell markers associated with cancer cell self-renewal: *NANOG*, *POU5F1,* and *SOX2*. In addition, chlorogenic acid induced the expression of annexin V in A549 cells. Enhanced BAX expression in the presence of chlorogenic acid treatment was associated with pathways that regulate oxidative stress and p38 MAP-dependent signaling. Together these results indicate that the chlorogenic acid can affect the expression of apoptosis-related genes and stem cell markers.

The control and reduction of apoptotic cell death can in turn modulate immune responses in cancer development [38, 39]. The BCL2 family, which includes anti-apoptotic BCL2 and pro-apoptotic BAX, along with CASP3, is the main regulatory protein involved in apoptosis [40]. The BAX protein forms a heterodimer with BCL2, and induces apoptosis. In particular, increases in the BCL2/BAX ratio are an essential factor in the suppression of apoptosis [41]. In chronic lymphocytic leukemia, an increase in the BCL2/BAX ratio rendered cells resistant to apoptosis, and the clinical significance of this change was demonstrated by an increase in chemoresistance [42, 43]. Here we found that *BAX* and *BCL2* gene expression in A549 cells was enhanced and reduced, respectively, by chlorogenic acid. In addition, chlorogenic acid increased *CASP3* gene expression levels.

**Fig. 5** Effect of chlorogenic acid on *p38 MAP* and *JUN* gene expression in A549 cells
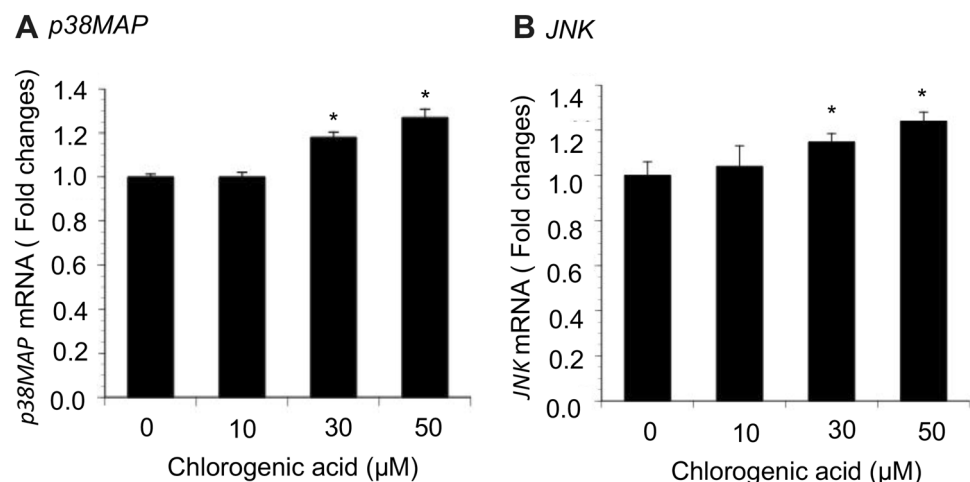
**Fig. 6** Effect of inhibitors on chlorogenic acid-mediated BAX gene expression in A549 cells
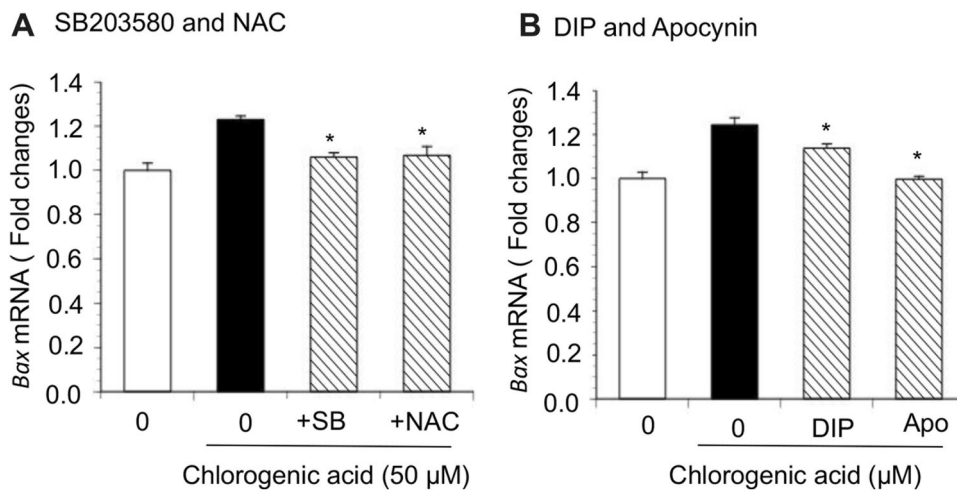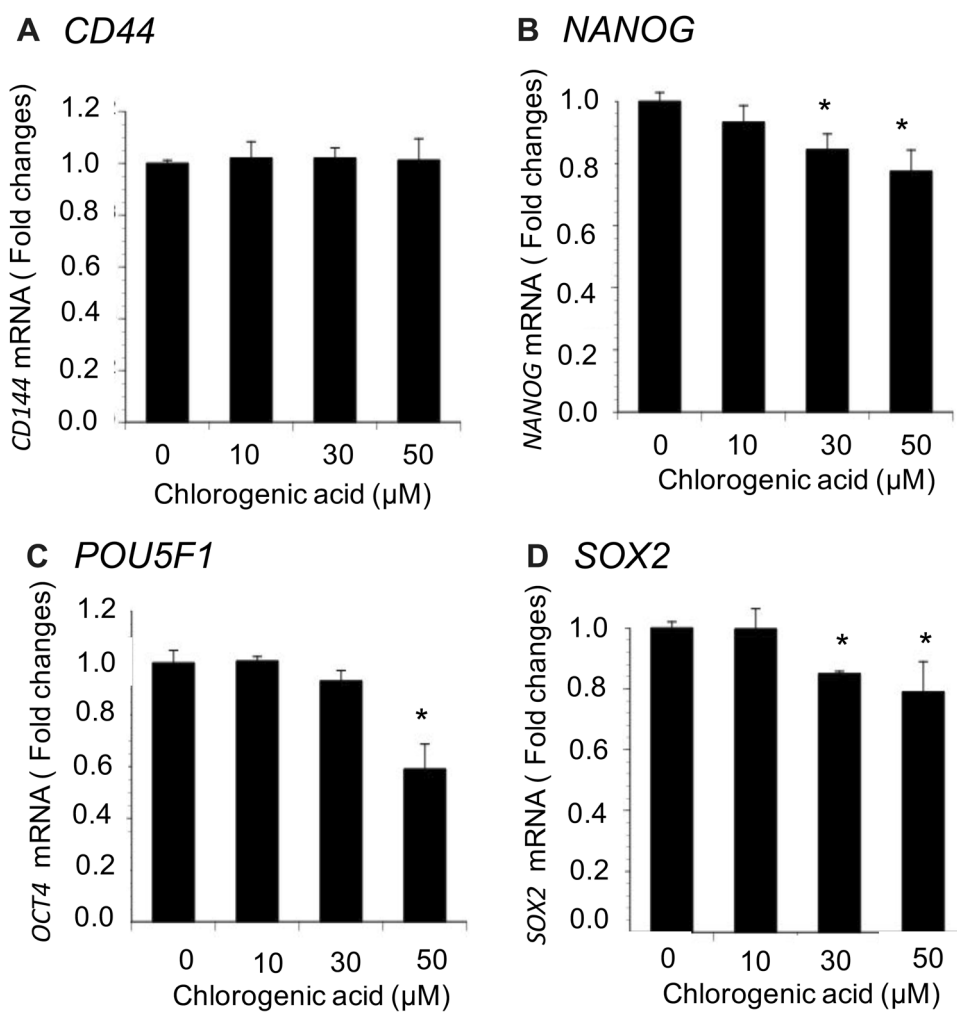


**Fig. 7** Effect of chlorogenic acid on *CD44, NANOG, POU5F1,* and *SOX2* gene expressions in A549 cells



The increase in *BAX* gene expression in A549 cells in response to chlorogenic acid treatment was likely associated with ROS- and p38MAP-mediated pathways as indicated by results from experiments using specific inhibitors and measurements of *p38 MAP* and *JNK* gene expression.

This result is similar to that seen for treatment of A549 cells with another polyphenol, curcumin, which enhances apoptosis via oxidative stress and MAPK signaling pathways [44]. Specifically, curcumin-enhanced apoptosis is associated with reduced amounts of intracellular reactive

oxygen species, increased superoxide dismutase activity, and decreased amounts of malondialdehyde. Furthermore, induction of apoptosis by curcumin is accompanied by activation of the mitogen-activated protein kinase signaling pathway factors c-Jun N-terminal kinase, p38, and extra-cellular signal-regulated kinase. On the other hand, oleanolic acid is abundant in several vegetables [45] and enhances ROS-dependent ASK1 activation that in turn promotes p38 MAPK-dependent apoptosis in cancer cells [46]. These reports, together with our data, suggest that chlorogenic acid mediates its pro-apoptotic effects by regulating oxidative stress and activation of p38 MAPK.

Apoptosis is known to play a role in drug resistance in non-small cell lung cancer (NSCLC) [23]. In NSCLC tumor cells that show upregulated levels of HA-CD44, the anti-apoptotic Bcl2 family member BCL-xL was associated with cellular resistance to both apoptosis and chemotherapeutic agents [27]. In addition, cancer stem cells are associated with cancer drug resistance, metastasis, and recurrence. CD44, NANOG, Oct4, and SOX2 are important stem cell markers [25, 26]. Previous reports indicated that the flavonoid apigenin prevented proliferation and migration of both cancer cells and cancer stem cells [47]. Treatment with apigenin is associated with decreased expression levels of the pluripotency marker Oct3/4 and these decreases might be associated with down-regulation of the PI3K/Akt/NF-κB pathway. Here we investigated A549 cells to determine whether chlorogenic acid influenced the expression of genes related to cancer stem cells. NANOG, POU5F1, and SOX2 gene expressions were decreased following treatment of A549 cells with chlorogenic acid, suggesting that it can suppress the expression of several stem cell markers in A549 cells.

Taken together, the present study investigated the anti-cancer effects of chlorogenic acid in A549 human lung cancer cells. Cancer cells are often resistant to anti-cancer drugs due to their capacity to resist drug-induced apoptosis. Although cytotoxic chemotherapy kills cancer cells, cancer stem cells can survive and retain the ability to convert heterogenic tumor mass [48]. Here, chlorogenic acid stimulated the expression of several apoptosis-associated genes and enhanced annexin V expression. In contrast, inhibitors of the p38 MAP kinase pathway, antioxidants, phosphodiesterase, and NADPH-oxidase blocked chlorogenic acid-induced increases in BAX gene expression. This result is consistent with earlier findings showing that other phytochemicals can block p38 MAP signaling pathway activity. In addition, chlorogenic acid decreased the gene expressions of stem cell markers NANOG, POU5F1, and SOX2. These results indicate that the chlorogenic acid polyphenol that is naturally found in fruits and vegetables may be a candidate compound for preventing lung cancer through the elimination of cancer stem cells.

## Materials and methods

### Materials

The human adenocarcinoma cell line A549, as well as Lu 65 and Lu 135 cells, which were derived from lung cancer tissue, was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Wako Pure Chemical Industries, Ltd. (Japan, Tokyo). Chlorogenic acid and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Super-script III, TRIzol reagent, and DNase were purchased from Life Technologies Japan, Inc. (Tokyo, Japan). GoTaq-Green Master Mix was purchased from Promega KK (Tokyo, Japan). Bradford reagent was obtained from Bio-Rad (Richmond, CA, USA). BAX (SC-493) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). BCL2 (#2876) and β-actin (#4970) antibodies were purchased from Cell Signaling Technology Japan K.K. (Tokyo, Japan). Alexa Fluor 488 conjugate annexin V (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit V13241, Life Technologies Japan) was purchased from Life Technologies. N-acetyl-L-cysteine (NAC), diphenyleneiodonium (DIP), apocynin (NADPH inhibitor), and SB203580 (p38 MAPK inhibitor) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Stock solutions of chlorogenic acid (100 μM) were made using dimethyl sulfoxide (DMSO) and stored at −20 °C. We confirmed that the DMSO vehicle alone had no effect. All other reagents, unless otherwise indicated, were purchased from Life Technologies Japan and Sigma-Aldrich.

### Cells and culture conditions

A549 cells were inoculated on culture plates (Falcon; Becton–Dickinson) and cultured in DMEM containing 10% FBS, penicillin (50 U/mL), and streptomycin (100 μg/mL) and plated on 25-cm$^2$ flasks at a density of $6 \times 10^5$ cells/cm$^2$ until they reached confluence at 37 °C in a CO$_2$ incubator. A549 cells were plated on 100-mm culture dishes (Sumitomo Bakelite Co., Ltd.) or 96-well, 24-well plates (Falcon; Becton–Dickinson) at an initial density of $2 \times 10^5$ cells/cm$^2$ and were grown in DMEM containing 10% FBS until confluence was reached. One day later, A549 cells were exposed to chlorogenic acid (0–100 μM) or FBS-supplemented DMEM lacking chlorogenic acid as a control.

## Cell proliferation determination by MTT assay

For MTT assay, A549 cells were seeded in serum-free and serum-reduced growth medium (1% FBS) and grown overnight. A549 cells were seeded in 96-well plates at 1000 cells/well in DMEM medium supplemented with 1% FBS. After 24 h, chlorogenic acid (10, 30, and 50 μM) or DMSO only (0.1% vol/vol) was added, and the cells were incubated for 24–72 h. Cell growth was determined by MTT assay according to the manufacturer's instructions (Dojindo Molecular Technology, Inc. Kumamoto, Japan).

## Annexin V staining assay for apoptosis analysis

Apoptosis status can be detected by the binding of fluorescein-labeled annexin V. A549 cells were seeded in 10% FBS and grown overnight in serum-reduced growth medium (1% FBS). The cells were then seeded in 24-well plates at 3000 cells/well in DMEM medium supplemented with 1% FBS. After 24 h, A549 cells were stimulated with polyphenol (10, 30, 50 μM) incubated at 37 °C for 24–48 h, and stained for apoptosis using Alexa Fluor 488 conjugate annexin V (Life Technologies Japan). The plates were incubated for 20 min at 4 °C in the dark. For quantification of results, fluorescence intensity was measured under the same exposure conditions and the same area as that used with the BZ-8000 fluorescent microscope (Keyence, Tokyo, Japan). At least 10,000 cells per group were counted.

## RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted from cultured A549 cells following treatment with TRIzol and DNase I for 15 min at room temperature to remove genomic DNA. Synthesis of cDNA was performed by annealing total RNA to 50 μM oligo (dt) (5 min at 65 °C and 5 min at 95 °C) in a reaction mixture containing first strand buffer, DTT (7.5 mM), random primer (250 ng), dNTP (0.5 mM), RNase OUT (1 unit/μL), and Superscript III reverse transcriptase (5 units/μL). Reverse transcription-PCR (RT-PCR) was performed for BCL2, BAX, CASP3, NANOG, POU5F1, and SOX2 as described previously [49]. Reactions were incubated initially at 94 °C for 10 min, 55 °C for 2 min, and 72 °C for 3 min for 35 cycles using the GoTaq-Green Master Mix (Promega K.K., Tokyo, Japan) with a PCR System 9700 (Applied Biosystems) as described previously [50]. Gene products amplified by PCR were evaluated by electrophoresis in 2% NuSieve 3:1 agarose gels (FMC Products, Rockland, ME) and visualized with UV illumination after staining with ethidium bromide. Gene expression levels were calculated relative to 18S ribosomal RNA. In addition, we used quantitative PCR to confirm the RT-PCR results. Quantitative PCR was performed using real-time 'TaqMan TM' technology [51] [50] and the results were evaluated using a Sequence Detector System (Applied Biosystems. CA, USA) as described previously [52]. Primers and the TaqMan probes were selected from GenBank (Accession number BCL2; NM_000633, Bax; NM_138763, CAS3; BC016926, NANOG; NM_024865, POU5F1; NM_002701, SOX2; NM_003106 and designed using the primer design software Primer Express (Applied Biosystems). The forward and reverse primers for BCL2 were 5′-AAGTGAAC ATTTCGGTGACTTCC-3′ and 5′-ACTTCGGTCTCCT AAAAGCAGG-3′, respectively. The TaqMan probe sequence for BCL2 was 5′-CCA-GAGCATCAGG CCGCCAC-3′. For BAX, the forward and reverse primers were 5′-GACGGCAACTTCAACTGGG-3′ and 5′-CAGGGCCTTGAGCACCA-3′, respectively, and the TaqMan probe sequence was 5′-GTCGCCCTTTTCTACTTTGCCAGC-3′. The forward and reverse primers for CASP3 were 5′-GCCTGTTCCATGAAGGCAG-3′ and 5′-CGTATGGA-GAAATGGGCTGTAG-3′, respectively, and the TaqMan probe sequence was 5′-CCATGGACCACGCAG-GAAGGG-3′. The forward and reverse primers for NANOG were 5′-TCAGGACAGCCCTGATTCTTC-3′ and 5′-TGCGACACTCTTCTCTGCAG-3′, respectively, and the TaqMan probe sequence was 5′-CAGTCCCAAAGG-CAAACAACCCACT-3′. For POU5F1, the forward and reverse primers were 5′-ATGTGGTCCGAGTGTGGTTC-3′ and 5′-GCATAGTCGCTGCTTGATCG-3′, respectively, and the TaqMan probe sequence was 5′-TAACCGGCGCCAGAAGGGCA-3′. The forward and reverse primers for SOX2 were 5′-GAATGCCTT-CATGGTGTGGT-3′ and 5′-CGCTTGCTGATCTCC-GAG-3′, respectively, and the TaqMan probe sequence was 5′-AGATGGCCCAGGAGAACCCCAAG-3′. The amplification reaction mixture (50 μL) contained 25 ng of the cDNA sample; 1.25 U AmpliTaqAmpliTaq DNA polymerase; 1X PCR reaction buffer; 200 nM of each primer; 200 μM dATP, dCTP, and dGTP; and 400 μM dUTP; 5.5 mM MgCl$_2$; and 100 nM TaqMan probe (Applied Biosystems). The thermal cycling conditions included 2 min at 50 °C and 10 min at 95 °C. Thermal cycling proceeded with 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

## Protein determination

Protein levels were measured using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA) with BSA as a standard.

## Western blot analysis

A549 cells were washed in ice-cold PBS and immediately resuspended in RIPA buffer containing 25 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 1 mM EDTA (pH 8.0). Cell lysates (15–30 µg protein/line) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes as described previously [53]. Membranes were then incubated with primary antibodies (anti-BCL2, anti-BAX, anti-β-actin) for 1.5–5 h. After two washes, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Life Technologies Japan, Tokyo, Japan), and protein levels were detected using an enhanced chemiluminescence system (Life Technologies Japan) with a C-Digit blot scanner (MS Techno Systems Inc., Tokyo, Japan).

## Statistical analysis

Data are presented as mean ± SE. Significance of differences was determined using Fisher's protected least significant difference (PLSD) method following an analysis of variance (ANOVA).

Confluent A549 cells were exposed to the indicated concentration of chlorogenic acid (0, 10, 30, and 50 µM) for 24 h. Cell growth was determined by MTT assay. FBS; fetal bovine serum. 0.5; 0.5% FBS, 10; 10% FBS, 30; 10% FBS plus 30 µM Chlorogenic acid, 50; 10% FBS plus 50 µM Chlorogenic acid. Columns show mean ± SE ($n = 4$), *$P < 0.05$.

Confluent A549 cells were exposed to the indicated concentration of chlorogenic acid (0, 10, 30, and 50 µM) for 8 h, after which total cellular RNA was used for RT-PCR analysis. Comparison of (A) *BCL2,* (B) *BAX,* (C) *CASP3, and (D) BAX/BCL2* ratio gene expression in A549 cells. Gene expression levels were normalized to 18S ribosomal RNA. Columns show mean ± SE ($n = 4$), *$P < 0.05$.

Confluent A549 cells were exposed to the indicated concentration of chlorogenic acid (0, 10, 30, and 50 µM) for 24 h, after which BCL2 and BAX protein levels were assessed by western blotting. Total protein (30 µg) was analyzed by western blot with an anti-BCL2 antibody or an anti-BAX antibody. Columns show mean ± SE ($n = 4$), *$P < 0.05$, $n = 4$.

Confluent A549 cells were exposed to the indicated concentration of chlorogenic acid (100 or 150 µM) for 24 h. Columns show mean ± SE (n = 4), *$P < 0.05$.

Confluent A549 cells were exposed to the indicated amount of chlorogenic acid (0, 10, 30, and 50 µM) for 8 h, after which total cellular RNA was used for RT-PCR analysis. Comparison of (A) *p38 MAP* gene expression in A549 cells. Gene expression levels were normalized to 18S ribosomal RNA. Columns show mean ± SE ($n = 4$), *$P < 0.05$.

Confluent A549 cells were exposed to 30 µM chlorogenic acid in the presence or absence of the indicated inhibitor for 8 h, after which total cellular RNA was used for RT-PCR analysis. Gene expression levels were normalized to 18S ribosomal RNA. SB; SB203580, NAC; *N*-acetylcysteine Apo; apocynin, DIP; dipyridamole. Columns show mean ± SE ($n = 4$), *$P < 0.05$.

Confluent A549 cells were exposed to the indicated amount of chlorogenic acid (0, 10, 30, and 50 µM) for 8 h, after which total cellular RNA was used for RT-PCR analysis. Comparison of (A) *CD44,* (B) *NANOG,* (C) *POU5F1,* and (D) *SOX2* gene expression in A549 cells. Gene expression levels were normalized to 18S ribosomal RNA. Columns show mean ± SE ($n = 4$), *$P < 0.05$.

## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no competing interests.

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