Quercetin-induced apoptosis of HT-29 colon cancer cells via inhibition of the Akt-CSN6-Myc signaling axis

LIN YANG[1-3], YANQING LIU[1-3], MEI WANG[1,4], YAYUN QIAN[1-3], XIAOYUN DONG[1-3], HAO GU[1-3], HAIBO WANG[1-3], SHIYU GUO[5] and TADASHI HISAMITSU[5]

[1]Institute of Traditional Chinese Medicine & Western Medicine, School of Medicine, Yangzhou University, Yangzhou, Jiangsu 225009; [2]Jiangsu Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Treatment of Senile Diseases, Yangzhou, Jiangsu 225001; [3]Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu 225009; [4]Department of Gastroenterology, The First People's Hospital of Yangzhou, The Second Clinical School of Yangzhou University, Yangzhou, Jiangsu 225000, P.R. China; [5]Department of Physiology, School of Medicine, Showa University, Tokyo 142-8555, Japan

Received August 19, 2015; Accepted September 19, 2016

DOI: 10.3892/mmr.2016.5818

Abstract. Constitutive photomorphogenesis 9 signalosome (CSN) consists of a total of eight subunits (CSN1-CSN8) in mammalian cells. CSN6 may promote carcinogenesis by positively regulating v-myc avian myelocytomatosis viral oncogene homolog (Myc) and MDM2 proto-oncogene stability, and is regarded as a potential target for cancer therapy. Quercetin has a substantial anticancer effect on various human cancer cells. The present study investigated the effects of quercetin on HT-29 human colorectal cancer cell viability, apoptosis and cell cycle arrest using an MTT assay, flow cytometry, transmission electron microscopy and western blotting. It was determined that quercetin inhibited HT-29 cell viability in a dose-dependent manner. Cell shrinkage, chromatin condensation and nuclear collapse were observed in the 50, 100 and 200 μM quercetin groups. The exposure of HT-29 cells to quercetin led to significant cell cycle arrest in the S-phase. Western blot analysis revealed that quercetin reduced the protein expression levels of phosphorylated-Akt and increased CSN6 protein degradation; therefore, affecting the expression levels of Myc, p53, B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein. The overexpression of CSN6 reduced the effect of quercetin treatment on HT-29 cells, suggesting that quercetin-induced apoptosis may involve the Akt-CSN6-Myc signaling axis in HT-29 cells.

Correspondence to: Professor Yanqing Liu, Institute of Traditional Chinese Medicine & Western Medicine, School of Medicine, Yangzhou University, 11 Huaihai Road, Yangzhou, Jiangsu 225009, P.R. China
E-mail: liuyanqing2014@163.com

Key words: quercetin, apoptosis, CSN6, Myc, HT-29 colon cancer cells

Introduction

The incidence of colorectal carcinoma in the United States is among the highest in the world, affecting ~52/100,000 individuals, and the incidence of colorectal cancer in India is among the lowest, affecting ~7/100,000 individuals, suggesting that lifestyle factors may contribute to the development of the disease (1). Previous epidemiological and dietary intervention studies have suggested that diet-derived flavonoids may have a beneficial contribution to cancer therapy, primarily due to their pro-apoptotic or anti-angiogenic activities (2-4). Quercetin (also termed 3,3', 4', 5,7-pentahydroxyflavone) is a ubiquitous flavonoid found in various fruits, vegetables, nuts and red wine. Its antitumor effects have been confirmed in various cancer cells, including leukemia, breast, ovarian, colon, cervical, prostate and lymphoma (5-8). Different molecular mechanisms underlying the antitumor activity of quercetin have been identified, including upregulation of cell cycle inhibitors, downregulation of oncogene expression and the inhibition of glycolysis (5,9-12). However, the precise target for quercetin and its mechanisms of action remain to be elucidated.

The constitutive photomorphogenesis 9 (COP9) signalosome (CSN), is an evolutionarily conserved multiprotein complex that is present in all eukaryotes. It consists of eight subunits termed CSN1-CSN8 (13). Previous studies have identified that CSN6 of the COP9 complex is crucial for proteasome-mediated protein degradation, as it regulates E3 ligases, including MDM2 proto-oncogene and COP1 (14,15). Notably, CSN6 overexpression has been identified in various types of cancer, including glioblastoma, breast cancer, myeloma and leukemia (16). It has been determined that the CSN6-MDM2-p53 signaling axis is important for cell proliferation and has antiapoptotic effects (14). Previous studies have demonstrated that CSN6 prevents MDM2 autoubiquitination at lysine 364, which results in the stabilization of MDM2 and the degradation of p53 (14,17,18). A previous study revealed that the HER2-Akt signaling axis is associated

with CSN6 regulation and that Akt acts as a positive regulator of CSN6 (19). A recent study determined that CSN6 promotes carcinogenesis by positively regulating v-myc avian myelocytomatosis viral oncogene homolog (Myc) stability. Additionally, CSN6 overexpression was positively correlated with Myc protein expression. Additionally, the gene expression signature of Myc target genes have been identified in human breast and pancreatic cancer (20). A previous study also determined that CSN6 overexpression may be is as high as 40% in colon adenocarcinoma (20). The overexpression of Myc may be 70-80% in colorectal cancer (21); however, the function of the Akt-CSN6-Myc signaling axis remains to be elucidated in colorectal cancer.

CSN6 has been identified as a potential novel therapeutic agent in cancer treatment and has thus been widely previously investigated (16). The present study determined that quercetin may reduce the protein expression levels of CSN6 in HT-29 colon cancer cells and that it may be one of the important targets for quercetin-induced apoptosis in HT-29 cells. The present study determined that quercetin may reduce cell viability and induce apoptosis of HT-29 cells by mediating the phosphorylation of Akt and increasing CSN6 protein degradation, which also affected the expression levels of Myc, p53, B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax), indicating that quercetin-induced apoptosis of HT-29 cells may involve the Akt-CSN6-Myc signaling axis.

Materials and methods

Chemicals, reagents and growth media. RPMI-1640 and 10% fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Antibodies against p53 (cat. no. 9282), Bax (cat. no. 2772), Bcl-2 (cat. no. 2872), caspase-3 (cat. no. 9665) and β-actin (cat. no. 4970s) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The antibody against Myc (cat. no. NB600-336) was purchased from Novus Biologicals (Littleton, CO, USA) and antibody against CSN6 (cat. no. LS-C174568) was acquired from LifeSpan BioSciences Inc. (Seattle, WA, USA). The enhanced chemiluminescence kits used for the visualization of the proteins were purchased from GE Healthcare Life Sciences (Chalfont, UK). Quercetin, DMSO and MTT were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). All other reagents were purchased from Beyotime Institute of Biotechnology, Inc. (Jiangsu, China). Quercetin was dissolved in DMSO to a concentration of 100 μM. Further dilutions were performed in cell culture media.

Cell line and culture. The HT-29 human colorectal cancer cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HT-29 cells were cultured in RPMI-1640 containing 10% FBS and were maintained at 37˚C in a humidified incubator in an atmosphere of 5% CO_2.

Cell viability assay. Cell viability was determined using an MTT assay. HT-29 cells were cultured until the log-phase and were subsequently seeded into a 96-well plate at a density of $1.0x10^4$ cells/well overnight prior to treatment with different concentrations of quercetin (12.5, 25, 50, 100 and 200 μM) or DMSO. Following an incubation of 24, 48 or 72 h, the cells were then incubated with medium containing MTT for 4 h and the formazan crystals were dissolved with 150 μl DMSO. The plates were incubated on a shaker for 15 min at room temperature. The absorbance was measured at 490 nm using a microplate reader. The drug dose at which the cell viability was reduced by 50% (IC_{50}) at 48 h of treatment was quantified. The experiments were repeated in triplicate.

Ultrastructures observed by transmission electron microscopy (TEM). Following treatment with quercetin, the cells were washed with PBS, collected by centrifugation (1,500 x *g*, 4˚C, 5 min) and fixed in 2.5% electron microscopy-grade glutaraldehyde. Next, they were rinsed with 0.1 M PBS, fixed in 1% osmium tetroxide, dehydrated through a graded series of ethanol and processed for Epon epoxy embedding. Ultra-thin sections (60 nm) stained with uranyl acetate and lead citrate and were observed using a JEM-1230 electron microscope.

Apoptosis and cell cycle analysis by flow cytometry. Control and quercetin-treated cells were collected, washed twice with ice-cold PBS and resuspended in 500 μl binding buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Next, 5 μl annexin V-fluorescein isothiocyanate (FITC) and 5 μl propidium iodide (PI) were added and the cells were incubated for 15 min at room temperature in the dark. FITC and PI staining was analyzed to determine the apoptotic rate. The percentage of total apoptotic cells was calculated by adding the percentages of early apoptotic gated cells (annexin-V^+) and late apoptotic gated cells (annexin-V^+/PI^+)

For cell cycle analysis, the cells were fixed in 70% ethanol at 4˚C for a minimum of 4 h and washed twice with ice-cold PBS. Subsequently, 100 μl RNase A was added and the cells were incubated in a 37˚C water bath for 30 min. Following incubation, the cells were stained with 400 μl PI for 30 min in dark conditions. The assays were performed in triplicate using a FACSort flow cytometer and quantified using BD CellQuest™ Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. The protein expression levels of Akt, phosphorylated (p-)Akt, CSN6, Myc, Bax, Bcl-2, caspase-3 cleaved caspase-3 and p53 in HT-29 cells were determined using western blotting. Briefly, a cell lysis solution was prepared using an extraction reagents kit (Fermentas; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A 50 μg sample of protein was separated by 10% SDS-PAGE and was transferred onto nitrocellulose membranes (Merck Millipore). The membranes were blocked with 5% not-fat dry milk for 2 h at room temperature and were then incubated with the appropriate primary antibodies in a shaker overnight at 4˚C. Subsequently, the membranes were washed 3 times at room temperature with washing buffer (1X TBS T: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 10 min and then incubated with secondary antibodies (1:1,000; cat. no. G130321; Hangzhou HuaAn Biotechnology Co., Ltd., Hangzhou, China) for 2 h at room temperature. β-actin was used as a loading control. Enhanced chemiluminescence was used to visualize the proteins with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Inc.)

on a Molecular Imager ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Densitometry was performed using 170-9600 Quantity One® 1-D software (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following treatment with quercetin, the total RNA was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) under RNase-free conditions and reverse transcription was performed in a 20 μl reaction with 200 ng total RNA using a two-step reverse-transcription reaction kit (Takara Biotechnology Co., Ltd., Dalian, China). The RT-qPCR was performed on an Applied Biosystems 7500 Real-time PCR system using a SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) in Axygen 96-well reaction plates.

The primers used in the present study were obtained from Sangon Biotech Co., Ltd. (Shanghai, China) and their sequences were as follows: CSN6 (NM_486571), forward (F) 5'-AGAGGCCACAATGCTGTTTG-3' and reverse (R) 5'-CGTGGTCTACACCAATGCGTT-3'; GAPDH (NM_002046), F: 5'-TGGCACCCAGCACAATGAA-3' and R: 5'-CTAAGTCATAGTCCGCCTAGA-3'. GAPDH was used as a housekeeping gene and internal control. The data was analyzed using the $2^{-\Delta\Delta Cq}$ method (22).

Retroviral constructs and transfection. The complete codon sequence of CSN6 (NM_474971) was amplified using Platinum Taq DNA Polymerase high fidelity (Invitrogen; Thermo Fisher Scientific, Inc.) and the following primers: F 5'-GACTCGAGA TGGCGGCGGCGGCGGCGGCGGCTGCAGCTA-3' and R 5'-GAGAATTCTCAGAAAAAGAGCCCGCGCATTCTC CTGCCGA-3'. The PCR product was cloned into *Xho*I and *Eco*RI sites on the retroviral vector MSCV MIGR1 (provided by Professor Duonan Yu, University of Pennsylvania, Philadelphia, PA, USA). Sequence fidelity was confirmed using DNA sequencing by Sangon Biotech Co., Ltd. HT-29 cells were seeded into 6-well plates ($1.0x10^5$ cells/well) overnight and transfected with the recombinant retroviral expression plasmid using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cells were visualized under a fluorescence microscope (Nikon Corporation, Tokyo, Japan) to detect transfection efficiency and were then treated with quercetin for an additional 48 h.

Statistical analysis. The data are expressed as the mean ± standard deviation. Each experiment was repeated at least three times. Statistical comparisons of >2 groups were performed using a one-way analysis of variance, followed by a Bonferroni post-hoc test. All statistical analyses were performed using SPSS version 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Quercetin reduces cell viability of HT-29 cells. The MTT assay revealed that HT-29 cell viability was decreased in a dose-dependent manner with increasing concentration of quercetin. Dose-dependent inhibition of cell viability was also observed in the HT-29 cells. The IC50 value for treatment for 48 h was determined as 81.65±0.49 μM quercetin (Fig. 1). Therefore, it was determined that quercetin exerted an negative activity against viability of colorectal cancer cells.

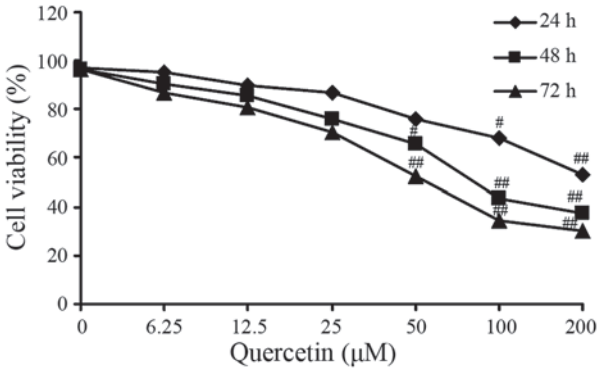

Figure 1. Cell viability of HT-29 cells following treatment with different concentrations of quercetin for varying durations. The data are presented as the mean ± standard deviation (n=3; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. 0 μM quercetin treated group for 24, 48 and 72 h).

Quercetin induces apoptosis in HT-29 cells. As presented in Fig. 2A, ultrastructures in HT-29 cells were observed by TEM 48 h after quercetin treatment. The cells in the control group had intact organelles, with normal nuclei and nucleolus chromatin. However, upon treatment with 50, 100 or 200 μM quercetin, cell shrinkage, chromatin condensation and nuclear collapse were observed (Fig. 2A).

Flow cytometry analysis revealed an increase in the apoptotic rate in treatment groups with higher quercetin concentration compared with the control group (Fig. 2B and C).

Cleaved-caspase-3 is a key factor required for apoptosis and is the active form of pro-caspase-3. Immunoblotting analysis determined that cleaved-caspase-3 was significantly higher in the 50, 100 and 200 μM quercetin treatment groups compared with the control group (0 μM quercetin; $P<0.01$; Fig. 2D and E).

Members of the Bcl-2 family are crucial for the regulation of apoptosis. Therefore, the present study used western blot analysis to determine the protein expression levels of Bax and Bcl-2. Bcl-2 expression decreased and Bax expression increased with increasing concentrations of quercetin compared with the control group (Fig. 2D and E). The protein expression of p53 was significantly increased compared with the control group ($P<0.01$; Fig. 2D)

Effect of quercetin on the cycle progression of HT-29 cells. In order to determine whether the proliferation-inhibiting effect of quercetin on HT-29 cells was a result of cell-cycle arrest, cell-cycle analysis was performed using flow cytometry (Fig. 3A). The proportion of cells in the G0/G1 phase of the cell cycle was significantly increased in the treatment groups exposed to quercetin compared with the control group ($P<0.01$; Fig 3B). The number of cells in the S and G2/M phases in the quercetin treatment groups were significantly decreased ($P<0.05$; Fig. 2B). Therefore, quercetin inhibited the proliferation of HT-29 cells via G0/G1 phase arrest (Fig. 3A and B).

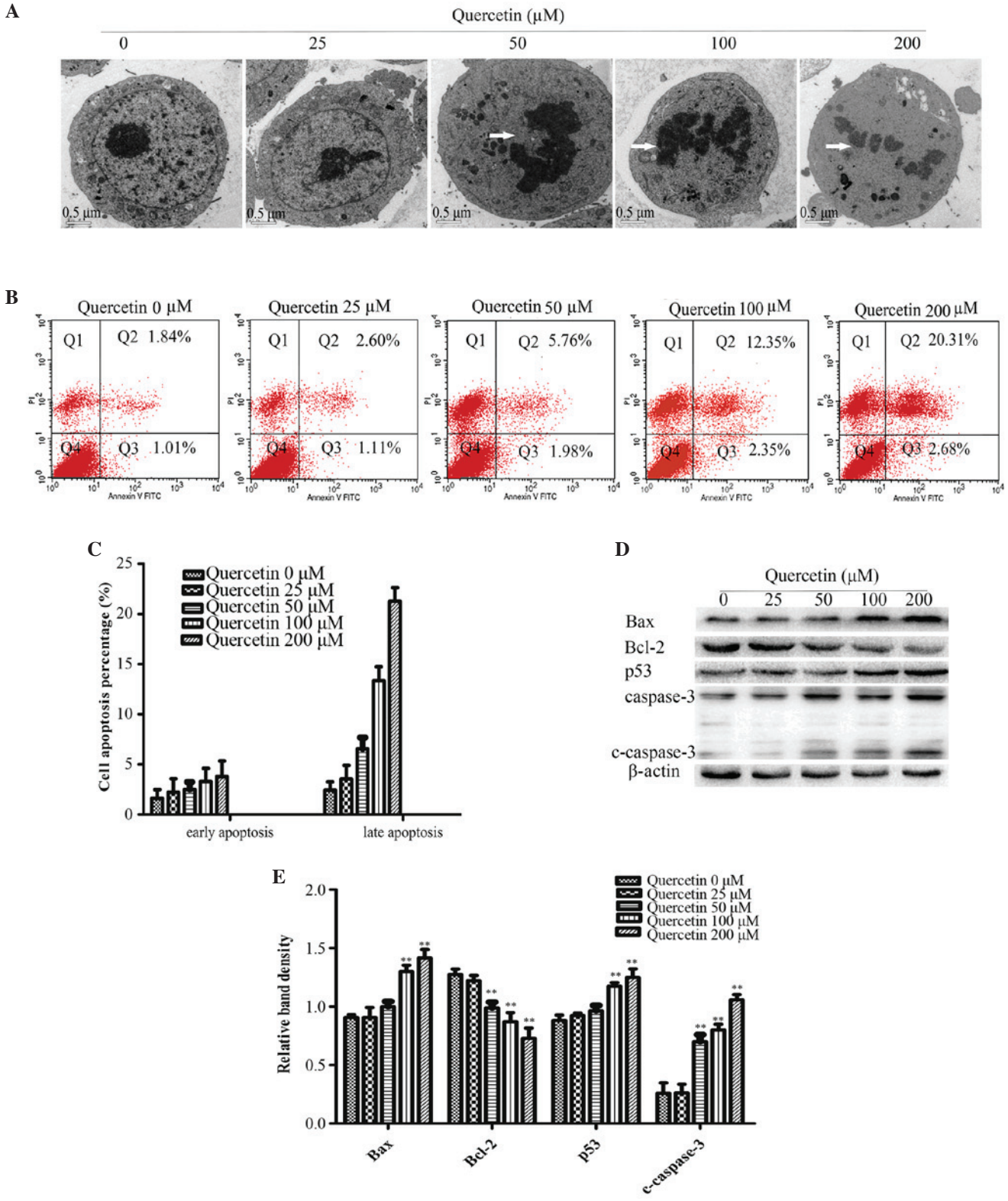

Figure 2. Quercetin-triggered apoptosis in HT-29 cells following 48 h treatment. (A) Transmission electron microscopy images of cellular structures; black arrows show chromatin condensation and nuclear collapse in the different treatment groups. (B) Flow cytometry analysis revealed that quercetin induced apoptosis in a dose-dependent manner. (C) The number apoptotic cells were quantified following flow cytometry. (D) The protein expression levels of Bax, Bcl-2, p53 and c-caspase 3 in HT-29 cells exposed to different concentrations of quercetin were determined by western blot analysis. (E) Quantification of western blotting was normalized against the expression of β-actin and is expressed as the mean ± standard deviation (n=3; **P<0.01 vs. 0 μM quercetin group). Bcl-2, B-cell leukemia/lymphoma 2; Bax, Bcl-2 associated-X protein; c-, cleaved-; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Akt-CSN6-Myc signaling axis mediates quercetin cytotoxicity. To determine the effect of CSN6 on quercetin-induced apoptosis of HT-29 cells, the recombinant retrovirus plasmid MIGR1-CSN6, containing the full-length human CSN6 gene, was constructed. HT-29 cells were transfected with MIGR1-CSN6 and an empty plasmid (MIGR1, used as

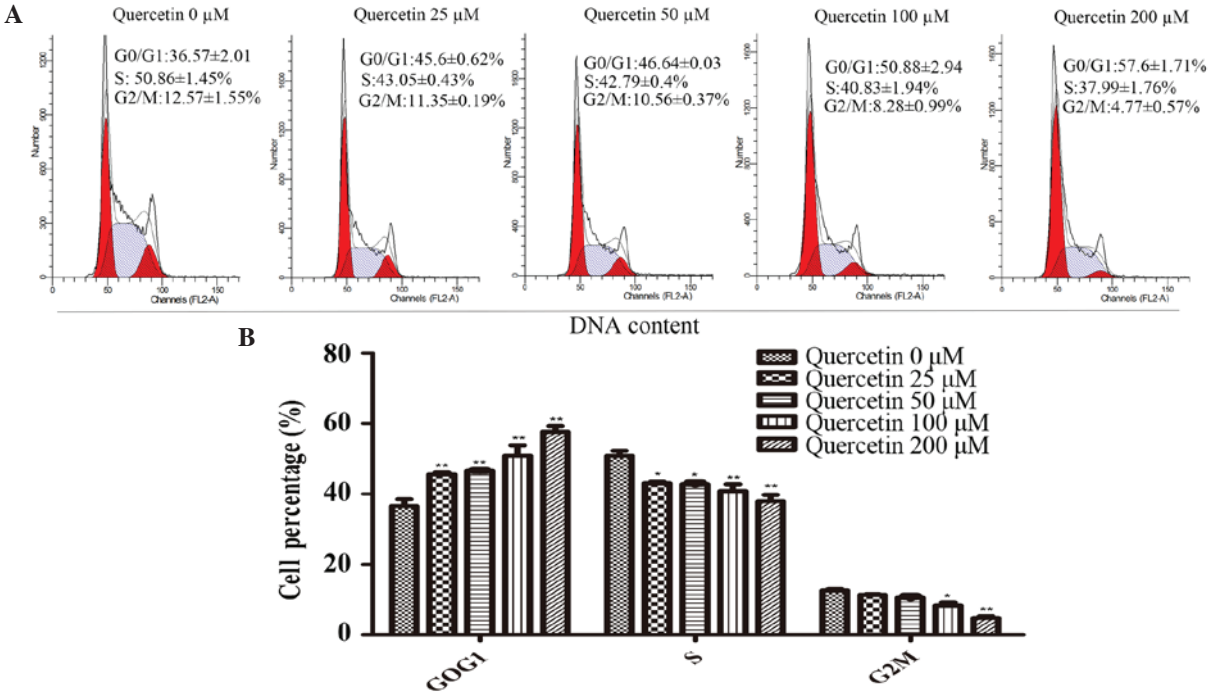

Figure 3. Quercetin altered the cell cycle of HT-29 cells. (A) HT-29 cells were treated with different concentrations of quercetin for 48 h and the cell cycle distribution was examined using propidium iodide staining and flow cytometry. (B) Quantification of the cells in G0/G1, S and G2/M phases. The data are expressed as the mean ± standard deviation (n=3; *P<0.05, **P<0.01 vs. 0 μM quercetin group).

a control), and were separately selected by flow cytometry for GFP^{+} cells. A transduction of ~100% was achieved (Fig. 4A and B). To determine whether the Akt-CSN6-Myc signaling axis was involved in quercetin-induced apoptosis of HT-29 cells, the levels of Akt, p-Akt, CSN6, Myc and p53 were examined using western blotting. The protein expression levels of p-Akt-Thr308, CSN6 and Myc were significantly reduced in the quercetin treatment groups compared with the control group (P<0.01; Fig. 4C and D). Additionally, the mRNA expression of CSN6 was determined using RT-qPCR. No significant difference was identified in terms of CSN6 mRNA expression when quercetin treatment groups were compared with the control group (Fig. 4E). The western blot analysis demonstrated that the protein expression levels of cleaved-caspase 3, p53 and Bax were also downregulated, while Myc and Bcl-2 were upregulated compared with cells treated with 50 μM quercetin alone and empty plasmid controls (Fig. 4F and G). The MTT assay revealed that the overexpression of CSN6 reduced the effect of quercetin on cell viability compared with the empty plasmid MIGR1 (Fig. 4H).

Discussion

Epidemiological evidence has revealed that cancer incidence may be significantly modulated by an increased dietary intake of flavonoids through increased consumption of fruits and vegetables (23). Flavonoids are one of the largest groups of naturally occurring phenols, including flavones, flavanols, isoflavones, flavonols, flavanones and flavanonols (24). These dietary antioxidants have been identified to exert significant antitumor effects and have been extensively investigated (4,25,26).

The present study investigated the effect of quercetin, which is one of the frequently researched flavonoids (27), due to its positive effect on the growth inhibition and the induction of apoptosis in HT-29 cells. The anti-proliferative effects of quercetin were initially assessed following incubation with different concentrations of quercetin for 24, 48 and 72 h. The cell viability of HT-29 cells was significantly inhibited in a time-and dose-dependent manner. Following a 24 h incubation, the inhibitory effect of quercetin on HT-29 cell viability was not evident at low concentrations of quercetin. However, following a 48 h incubation, significant inhibition of cell growth was observed at 50, 100 and 200 μM quercetin (Fig. 1).

Quercetin induces pro-apoptotic signaling pathways, which lead to cell death (5,8,9). In the present study, TEM observation of the quercetin-treated HT-29 cells revealed chromatin condensation, nuclear collapse and apoptotic body formation in cells treated with 50, 100 and 200 μM quercetin (Fig. 2A). Apoptotic cell death was also quantified by determining the percentage of early apoptotic gated cells (Annexin-V^{+}) and late apoptotic gated cells (Annexin-V^{+}/PI^{+}). The results revealed an increase in the percentage of apoptotic cells in quercetin treatment groups in a dose-dependent manner (Fig. 2B and C). Caspase-3 is a key factor in apoptosis execution and cleaved caspase-3 is an activated form of caspase-3. Both the mitochondria-initiated intrinsic apoptotic pathway and the death receptor-triggered extrinsic apoptotic pathway may lead to caspase-3 activation. Therefore, cleaved caspase-3 protein expression levels were evaluated using western blotting, the result revealed that the expression levels of cleaved caspase-3 were significantly increased following quercetin treatment (Fig. 2D and E). Bcl-2 and Bax have also been identified as key proteins for controlling the release of cytochrome *c* and other pro-apoptotic factors from the

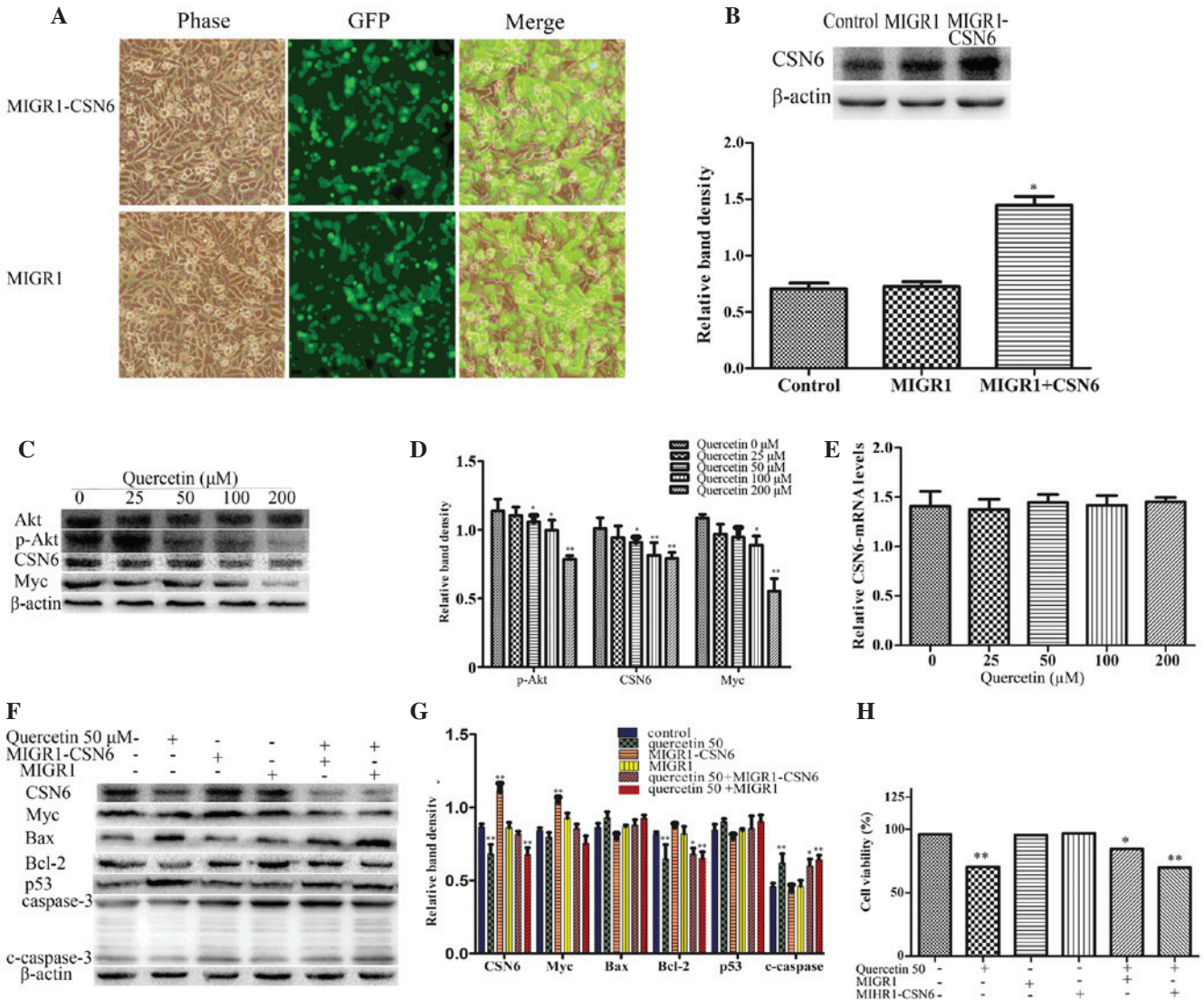

Figure 4. Quercetin-induced apoptosis involves the Akt-CSN6-Myc signaling pathway in HT-29 cells. (A) Overexpression of CSN6 was performed by transfection with the recombinant retroviral expression plasmid and the cells were selected by flow cytometry for GFP+ cells; ~100% transduction efficiency was achieved. (B) Expression levels of CSN6 were analyzed by western blotting in HT-29 cells transfected with control (empty) or CSN6 vectors. The data are expressed as the mean ± standard deviation (n=3; *P<0.05 vs. control). (C and D) Western blot analysis of the protein expression levels of p-Akt, CSN6 and Myc in HT-29 cells treated with different concentrations of quercetin for 48 h. The data are expressed as the mean ± standard deviation (n=3; *P<0.05, **P<0.01 vs. control). (E) The mRNA expression levels of CSN6 were examined by reverse transcription-quantitative polymerase chain reaction using total mRNA of HT-29 cells and compared with the control. (F and G) Western blotting was used to determine protein expression levels of CSN6, Myc, Bax, Bcl-2, p53 and caspase 3 in the CSN6 overexpression cell line and control (empty) cell line, which remained untreated or exposed to 50 μM quercetin for 48 h. The data are expressed as the mean ± standard deviation (n=3; *P<0.05, **P<0.01 vs. untreated control). (H) Cell viability was examined in a cell line overexpressing CSN6 and control (empty) cell line treated with or without 50 μM quercetin for 48 h, *P<0.05 and **P<0.01 vs. untreated control. MIGR1, plasmid; CSN6, COP9 signalosome subunit 6; Akt, Akt serine/threonine kinase 1; p-Akt, phosphorylated-Akt; Myc, v-myc avian myelocytomatosis viral oncogene homolog; Bcl2, B cell leukemia/lymphoma 2; Bax, Bcl2 associated X; c-caspase 3, cleaved-caspase 3.

mitochondria, which leads to subsequent caspase activation and apoptotic cell death (28). The present study determined that quercetin treatment significantly decreased the expression of Bcl-2, while increasing the expression of Bax (Fig. 2D and E). This indicated that quercetin induced apoptosis via the regulation of the expression levels of Bcl-2 family proteins.

In order to determine whether this quercetin-induced inhibition of cell viability was due to cell cycle arrest, PI staining was performed and revealed that quercetin treatment significantly increased cell cycle arrest in the G0/G1 phase of the cell cycle, and that the number of cells in the S and the G2/M phase was reduced (Fig. 3A and B). This result was consistent with the findings of Kim *et al* (29). The immunoblot analysis revealed that the expression levels of p53 increased and those of Myc decreased following treatment with quercetin for 48 h (Figs. 2D and 4C). The upregulation of p53 proteins led to an inhibition of growth and proliferation, involved with the G1 and G2/M phase arrest in cancer cells (30-32). A previous study reported that the downregulation of Myc-associated genes was involved in cell cycle arrest in acute myeloid leukemia (33). The cell cycle arrest of nasopharyngeal carcinoma cells also involved the inhibition of the c-Myc signaling pathway (34).

Additionally, quercetin has been considered a powerful modulator of several cellular signaling pathways, including the phosphatidylinositol-3-kinase (PI3K)-mediated signaling

pathway, which important for quercetin-repressed tumors (35). Akt is a downstream target of PI3K and regulates cell survival through the phosphorylation of downstream substrates that control apoptosis either directly or indirectly. Previous studies have revealed that oncogenic activation through Akt may act as an antiapoptotic signal via the rapid destabilization of p53 (36,37). A previous study revealed that quercetin inhibited lymphoma by downregulating the PI3K-Akt-p53 signaling pathway (38); however, the mechanism by which Akt regulates p53 remains to be elucidated. Previous studies determined that the MDM2-p53 signaling axis may be regulated by CSN6 (14,39), and a subsequent study revealed that the HER2-Akt axis was associated with CSN6 regulation and that Akt is a positive regulator of CSN6 (19). A recent study also demonstrated that CSN6 contributed to carcinogenesis by positive regulation of Myc stability (20). The present study aimed to determine the importance of the Akt-CSN6-Myc signaling axis in quercetin-induced apoptosis of HT-29 cells. The immunoblot analysis revealed that the expression of p-Akt-Thr308 and CSN6 decreased in quercetin treatment groups. The expression of direct or indirect CSN6 target genes, including Myc and Bcl-2 decreased, whereas p53 and Bax increased in HT-29 cells treated with quercetin (Figs. 2D and 4C). In order to determine the effect of CSN6 on quercetin-induced apoptosis, HT-29 cells were transfected with plasmid MIGR1-CSN6 or an empty MIGR1 plasmid and then treated with 50 μM quercetin for 48 h. The MTT assay revealed that the overexpression of CSN6 reduced the effect of quercetin on cell viability compared with the empty MIGR1 plasmid (Fig. 4H). Additionally, the western blot analysis determined that the protein expression levels of cleaved-caspase 3, p53 and Bax were downregulated, whereas the levels of Myc and Bcl-2 were upregulated in the CSN6 overexpression group compared with the control group where cells were treated with quercetin and transfected with an empty plasmid (Fig. 4F and G), indicating that quercetin-induced apoptosis involves the Akt-CSN6-Myc signaling axis in HT-29 cells.

In conclusion, the present study demonstrated that quercetin inhibited cell viability, induced apoptosis and led to cell-cycle arrest in HT-29 cells. The protein expression levels of p-Akt-Thr308 and CSN6 were significantly downregulated following quercetin treatment. Additionally, the expression levels of genes downstream of CSN6, including Myc and Bcl-2 were reduced and the levels of p53 and Bax were increased following treatment with quercetin. The overexpression of CSN6; however, reduced the effect of quercetin treatment on HT-29 cells. Therefore, it is possible that the Akt-CSN6-Myc signaling axis may be a potential target for novel treatment strategies of colorectal cancer.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant nos. 81403232, 81274141 and 81450051), the Natural Science Foundation of Jiangsu Province of China (grant nos. BK2012686 and SBK2014021480) and the Bureau of Traditional Chinese Medicine, Science and Technology Project of Jiangsu Province (grant no. YB2015183).

References

1. Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. CA Cancer J Clin 63: 11-30, 2013.
2. Hirpara KV, Aggarwal P, Mukherjee AJ, Joshi N and Burman AC: Quercetin and its derivatives: Synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability. Anticancer Agents Med Chem 9: 138-161, 2009.
3. Tan WF, Lin LP, Li MH, Zhang YX, Tong YG, Xiao D and Ding J: Quercetin, a dietary-derived flavonoid, possesses antiangiogenic potential. Eur J Pharmacol 459: 255-262, 2003.
4. Prasad S, Phromnoi K, Yadav VR, Chaturvedi MM and Aggarwal BB: Targeting inflammatory pathways by flavonoids for prevention and treatment of cancer. Planta Med 76: 1044-1063, 2010.
5. Murakami A, Ashida H and Terao J: Multitargeted cancer prevention by quercetin. Cancer Lett 269: 315-325, 2008.
6. Kawahara T, Kawaguchi-Ihara N, Okuhashi Y, Itoh M, Nara N and Tohda S: Cyclopamine and quercetin suppress the growth of leukemia and lymphoma cells. Anticancer Res 29: 4629-4632, 2009.
7. Luo H, Jiang BH, King SM and Chen YC: Inhibition of cell growth and VEGF expression in ovarian cancer cells by flavonoids. Nutr Cancer 60: 800-809, 2008.
8. Vidya PR, Senthil MR, Maitreyi S, Ramalingam K, Karunagaran D and Nagini S: The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-kappaB inhibition. Eur J Pharmacol 649: 84-91, 2010.
9. Suolinna EM, Buchsbaum RN and Racker E: The effect of flavonoids on aerobic glycolysis and growth of tumor cells. Cancer Res 35: 1865-1872, 1975.
10. Gibellini L, Pinti M, Nasi M, Montagna JP, De Biasi S, Roat E, Bertoncelli L, Cooper EL and Cossarizza A: Quercetin and cancer chemoprevention. Evid Based Complement Alternat Med 2011: 591356, 2011.
11. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP and Williams RL: Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin and staurosporine. Mol Cell 6: 909-919, 2000.
12. Triantafyllou A, Mylonis I, Simos G, Bonanou S and Tsakalof A: Flavonoids induce HIF-1alpha but impair its nuclear accumulation and activity. Free Radic Biol Med 44: 657-670, 2008.
13. Deng XW, Dubiel W, Wei N, Hofmann K and Mundt K: Unified nomenclature for the COP9 signalosome and its subunits: An essential regulator of development. Trends Genet 16: 289, 2000.
14. Zhao RY, Yeung SC, Chen J, Iwakuma T, Su CH, Chen B, Qu C, Zhang F, Chen YT, Lin YL, *et al*: Subunit 6 of the COP9 signalosome promotes tumorigenesis in mice through stabilization of MDM2 and is upregulated in human cancers. J Clin Invest 121: 851-865, 2011.
15. Choi HH, Gully C, Su CH, Velazquez-Torres G, Chou PC, Tseng C, Zhao R, Phan L, Shaiken T, Chen J, *et al*: COP9 signalosome subunit 6 stabilizes COP1, which functions as an E3 ubiquitin ligase for 14-3-3sigma. Oncogene 30: 4791-4801, 2011.
16. Lee MH, Zhao R, Phan L and Yeung SC: Roles of COP9 signalosome in cancer. Cell cycle 10: 3057-3066, 2011.
17. Fang S, Jensen JP, Ludwig RL, Vousden KH and Weissman AM: Mdm2 is a ring finger-dependent ubiquitin protein ligase for itself and p53. J Biol Chem 275: 8945-8951, 2000.
18. Inuzuka H, Fukushima H, Shaik S and Wei W: Novel insights into the molecular mechanisms governing Mdm2 ubiquitination and destruction. Oncotarget 1: 685-690, 2010.
19. Xue Y, Chen J, Choi HH, Phan L, Chou PC, Zhao R, Yang H, Santiago J, Liu M, Yeung GE, *et al*: HER2-Akt signaling in regulating COP9 signalsome subunit 6 and p53. Cell cycle 11: 4181-4190, 2012.
20. Chen J, Shin JH, Zhao R, Phan L, Wang H, Xue Y, Post SM, Ho Choi H, Chen JS, Wang E, *et al*: CSN6 drives carcinogenesis by positively regulating Myc stability. Nat Commun 5: 5384, 2014.
21. Sikora K, Chan S, Evan G, Gabra H, Markham N, Stewart J and Watson J: c-myc oncogene expression in colorectal cancer. Cancer 59: 1289-1295, 1987.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

23. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M and Boyle P: Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 18: 581-592, 2007.
24. Harborne JB and Williams CA: Advances in flavonoid research since 1992. Phytochemistry 55: 481-504, 2000.
25. Hirpara KV, Aggarwal P, Mukherjee AJ, Joshi N and Burman AC: Quercetin and its derivatives: Synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability. Anti-Cancer Agents Med Chem 9: 138-161, 2009.
26. Zhao D, Beiring B and Glorius F: Ruthenium-NHC-catalyzed asymmetric hydrogenation of flavones and chromones: General access to enantiomerically enriched flavanones, flavanols, chromanones and chromanols. Angew Chem Int Ed Engl 52: 8454-8458, 2013.
27. Thomas S, Quinn BA, Das SK, Dash R, Emdad L, Dasgupta S, Wang XY, Dent P, Reed JC, Pellecchia M, *et al*: Targeting the Bcl-2 family for cancer therapy. Expert Opin Ther Targets 17: 61-75, 2013.
28. Veeriah S, Kautenburger T, Habermann N, Sauer J, Dietrich H, Will F and Pool-Zobel BL: Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. Mol Carcinog 45: 164-174, 2006.
29. Kim HJ, Kim SK, Kim BS, Lee SH, Park YS, Park BK, Kim SJ, Kim J, Choi C, Kim JS, *et al*: Apoptotic effect of quercetin on HT-29 colon cancer cells via the AMPK signaling pathway. J Agric. Food Chem 58: 8643-8650, 2010.
30. Jin Q, Feng L, Behrens C, Bekele BN, Wistuba II, Hong WK and Lee HY: Implication of AMP-activated protein kinase and Akt-regulated survivin in lung cancer chemopreventive activities of deguelin. Cancer Res 67: 11630-11639, 2007.
31. Su RY, Chao Y, Chen TY, Huang DY and Lin WW: 5-Amino imidazole-4-carboxamide riboside sensitizes TRAIL-and TNF(alpha)-induced cytotoxicity in colon cancer cells through AMP-activated protein kinase signaling. Mol Cancer Ther 6: 1562-1571, 2007.
32. Hwang JT, Ha J, Park IJ, Lee SK, Baik HW, Kim YM and Park OJ: Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. Cancer Lett 247: 115-121, 2007.
33. Eriksson A, Kalushkova A, Jarvius M, Hilhorst R, Rickardson L, Kultima HG, de Wijn R, Hovestad L, Fryknäs M, Öberg F, *et al*: AKN-028 induces cell cycle arrest, downregulation of Myc associated genes and dose dependent reduction of tyrosine kinase activity in acute myeloid leukemia. Biochem Pharmacol 87: 284-291, 2014.
34. Hu ZY, Sun J, Zhu XF, Yang D and Zeng YX: ApoG2 induces cell cycle arrest of nasopharyngeal carcinoma cells by suppressing the c-Myc signaling pathway. J Transl Med 7: 74, 2009.
35. Wang K, Liu R, Li J, Mao J, Lei Y, Wu J, Zeng J, Zhang T, Wu H, Chen L, *et al*: Quercetin induces protective autophagy in gastric cancer cells: Involvement of Akt-mTOR-and hypoxia-induced factor 1α-mediated signaling. Autophagy 7: 966-978, 2011.
36. Moll UM and Petrenko O: The MDM2-p53 interaction. Mol Cancer Research 1: 1001-1008, 2003.
37. Mayo LD and Dormer DB: The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. Trends Biochem Sci 27:462-467, 2002.
38. Maurya AK and Vinayak M: Quercetin regresses dalton's lymphoma growth via suppression of PI3K/AKT signaling leading to upregulation of p53 and decrease in energy metabolism. Nutr Cancer 67: 354-363, 2015.
39. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B and Hung MC: HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol 3: 973-982, 2001.