# Role of transcription factor Sp1 in the quercetin-mediated inhibitory effect on human malignant pleural mesothelioma

JUNG-IL CHAE[1*], JIN HYOUNG CHO[1*], KYUNG-AE LEE[2], NAG-JIN CHOI[3], KANG SEOK SEO[4], SANG-BUM KIM[5], SANG-HAN LEE[2] and JUNG-HYUN SHIM[2]

[1]Department of Dental Pharmacology, School of Dentistry, Brain Korea 21 Project, Chonbuk National University, Jeonju 561-756; [2]Department of Biochemistry, College of Medicine, Soonchunhyang University, Cheonan 330-090; [3]Department of Animal Science, College of Agricultural and Life Science, Chonbuk National University, Jeonju 561-756; [4]Department of Animal Science and Technology, Sunchon National University, Suncheon 540-742; [5]Animal Genomics and Bioinformatics Division, National Institute of Animal Science, Rural Development Administration (RDA), Suwon 441-706, Republic of Korea

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**Abstract.** Quercetin (Qu) is found in plants, including red onions and in the skins of red apples, and induces the apoptosis of certain malignant cells. However, no report has been issued on the apoptotic effect of Qu on human malignant pleural mesothelioma. In the present study, it was found that MSTO-211H mesothelioma cell viability was reduced and apoptotic cell death was increased by Qu (20-80 $\mu$M), which was found to have an $IC_{50}$ of 58 $\mu$M. In addition, Qu increased the sub-$G_1$ cell population, and was found to interact with specificity protein 1 (Sp1) and significantly suppressed its expression at the protein and mRNA levels. Furthermore, Qu modulated the levels of Sp1 regulatory genes, such as cyclin D1, myeloid cell leukemia (Mcl)-1 and survivin in MSTO-211H cells. Apoptotic signaling cascades were activated by the cleavage of Bid, caspase-3 and PARP, and by the downregulation of Bcl-xL and the upregulation of Bax in MSTO-211H cells. Our results strongly suggest that Sp1 be considered as a novel molecular target of Qu in human malignant pleural mesothelioma.

## Introduction

Quercetin (Qu) [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one] belongs to an extensive class of polyphenolic flavonoid compounds ubiquitous in plants and plant food sources. Qu is a beneficial chemical compound widely dispersed in the human diet. Accordingly, high concentrations of Qu are found in onions (391.0 mg/kg), broccoli (74.5 mg/kg), apples (50 mg/kg) and beans (11.0 mg/kg) (1) and intakes between 6 and 31 mg/day have been reported (2). Qu has been used as a dietary supplement for many years, and thus, its use does not raise concerns as regards its toxicity. Of note, when Qu was discovered it was believed to be a mutagen, but subsequent studies showed it to be a powerful natural anticancer agent (3). The exact mechanism behind the cancer preventive effect of Qu remians unknown, yet the majority of studies have indicated that its anti-inflammatory and antioxidant properties may be responsible for the beneficial effects (4). Although the link between Qu and cancer has been established in controlled scientific settings, its efficacy in the treatment of cancer in humans is currently unknown.

Human malignant pleural mesothelioma is a rare tumor; however, its incidence is increasing and it has a poor prognosis (5,6). Thus, knowledge of the mechanism of mesothelial carcinogenesis is required to support efforts to develop targeted treatments.

Specificity protein (Sp) is an ubiquitously expressed transcription factor belonging to a family of 8 transcription factors (7), which are ubiquitously expressed in a variety of mammalian cells (8). Furthermore, Sp1 is highly expressed in a number of cancer tissues, such as pancreatic, thyroid, colorectal, breast, hepatocellular, prostate, gastric cancer and lung cancer (7,9). Sp1 was one of the first eukaryotic transactivators to be identified (10). Sp1 proteins have been defined as Sp/Kruppel-like transcription factors (11), and a previous study showed that Sp1 plays an important role in the carcinogenesis and metastasis of human tumors by regulating growth-related signal transductions, apoptosis, tumor suppressor genes, cell cycle control molecules, oncogenes and angiogenesis-related factors (12,13).

Qu is an important dietary flavonoid, found in various vegetables, fruits, seeds, nuts, tea and red wine (14). Qu has been shown to have diverse biological activities, including anti-inflammatory and antitumor properties (15-17). Furthermore,

*Correspondence to:* Professor Jung-Hyun Shim, Department of Biochemistry, College of Medicine, Soonchunhyang University, Ssangyong-dong, Seobuk-gu, Cheonan, Choongnam 331-090, Republic of Korea
E-mail: s1004jh@gmail.com

*Contributed equally

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Qu has been shown to have various beneficial effects, such as antioxidant, cardioprotective, anti-inflammatory and antitumor effects (18). However, the antitumor mechanisms involved and the molecular targets of Qu have not been identified, especially in human malignant pleural mesothelioma. Accordingly, in the present study, we investigated the apoptotic effect of Qu on a human malignant pleural mesothelioma cell line. Our results suggest that Qu be considered as a drug or natural supplement candidate for the prevention of malignant pleural mesothelioma.

## Materials and methods

*Materials*. HyClone RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Thermo Scientific (Logan, UT). The following antibodies were used: 4'-6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), anti-Sp1 (1C6), anti-poly (ADP-ribose) polymerase (PARP) (BD Biosciences, San Diego, CA), anti-cyclin D1 (M-20), anti-myeloid cell leukemia (Mcl)-1, anti-survivin, anti-Bid, anti-Bax, anti-Bcl-xL (Cell Signaling Technology, Inc., Danvers, MA), anti-caspase-3 (H-277) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-β-actin (AC-74) (Sigma-Aldrich, Inc. St. Louis, MO). RNase A was supplied by Sigma-Aldrich.

*Cell culture*. MSTO-211H cells were obtained from the American Tissue Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 medium supplemented with 5% FBS and 100 U/ml each of penicillin and streptomycin at 37˚C in a 5% $CO_2$ incubator.

*MTS assay*. The effects of Qu on cell viability were determined using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit (Promega, Madison, WI). MSTO-211H cells were seeded in a 96-well plate for 24 h and treated with Qu (20-80 $\mu$M) for 24 or 48 h. MTS solution was then added for 2 h at 37˚C in 5% $CO_2$. The absorbance at 490 nm was recorded using a GloMax-Multi Microplate Multimode Reader (Promega).

*DAPI staining*. The level of nuclear condensation and fragmentation was observed by nucleic acid staining with DAPI. MSTO-211H cells were treated with Qu, harvested by trypsinization, and fixed in 100% methanol at room temperature for 20 min. The cells were spread on slides, stained with DAPI solution (2 $\mu$g/ml), and analyzed under a FluoView confocal laser microscope (Fluoview FV10i; Olympus Corporation, Tokyo, Japan).

*PI staining*. Following treatment with Qu (20-80 $\mu$M) for 72 h, the detached MSTO-211H cells (floaters) were collected by centrifugation and combined with adherent cells. The cells were fixed with 70% ice-cold ethanol overnight at -20˚C, and subsequently treated with 150 mg/ml RNase A and 20 mg/ml PI. DNA contents were analyzed by flow cytometry using a MACSQuant Analyzer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

*Western blot analysis*. MSTO-211H cells treated with Qu (20-80 $\mu$M) for 48 h were washed with phosphate-buffered saline (PBS), and were then homogenized with PRO-PREP™ Protein Extraction Solution (Intron Biotechnology, Korea). Extracted proteins were measured using DC protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA). The equal amounts of protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred onto membranes, which were blocked for 2 h at room temperature with 5% non-fat dried milk in PBS containing 0.05% Tween-20, and then incubated overnight at 4˚C with specific antibodies. The protein bands were observed after treating them with horseradish peroxidase-conjugated secondary antibody using a Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL).

*In vitro EGCG-sepharose 4B pull-down assays*. This method has been described previously (19,20). Briefly, MSTO-211H cell lysates were reacted with sepharose 4B beads or Qu-sepharose 4B beads in reaction buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 2 $\mu$g/ml bovine serum albumin, 0.02 mM phenylmethylsulfonyl fluoride and 1X proteinase inhibitor), and washed 5 times with washing buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.02 mM phenylmethylsulfonyl fluoride). Proteins bound to the beads were analyzed by western blot analysis using anti-Sp1 antibody.

*Reverse transcription-polymerase chain reaction (RT-PCR)*. Total RNA was extracted from the cells using TRIzol® Reagent (Life Technologies, Carlsbad, CA), and 2 $\mu$g of RNA were used to synthesize cDNA using the HelixCript™ first-strand cDNA synthesis kit (NanoHelix, Korea). cDNA was obtained by PCR amplification using β-actin-specific and Sp1-specific primers (as described below) using the following PCR conditions: 25 cycles of 1 min at 95˚C, 1 min at 60˚C and 1 min at 72˚C. The β-actin primers used were: forward, 5'-GTG-GGG-CGC-CCC-AGG-CAC-CA-3' and reverse, 5'-CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC-3'; and the Sp1 primers were: forward, ATG CCT AAT ATT CAG TAT CAA GTA and reverse, CCC TGA GGT GAC AGG CTG TGA. PCR products were analyzed by 2% agarose gel electrophoresis.

*Luciferase assay for cyclin D1, Mcl-1 and survivin transactivation*. MSTO-211H cells ($6x10^4$) were added to each well of a 24-well plate and incubated at 37˚C in a humidified atmosphere of 5% $CO_2$ for 24 h. Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The survivin-269 promoter construct was kindly provided by Dr Sung-Dae Cho (Chonbuk National University, Jeonju, Korea). The Mcl-1 promoter was obtained from Addgene (Cambridge, MA). Cells were transfected with 250 ng of cyclin D1 (-1745-luc), Mcl-1 (-325-luc), or survivin (-269-luc) and 20 ng of β-gal using Lipofectamine 2000 reagent (Invitrogen) for 24 h (21). After cafestol and kahweol treatment for 48 h, the cells were disrupted with 100 ml of lysis buffer [0.1 M potassium phosphate (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol (DTT), 2 mM EDTA], and firefly luciferase activity and galactosidase were determined with a Promega luciferase assay kit according to the manufacturer's instructions. Relative luciferase units were calculated by normalizing to the galactosidase activity.

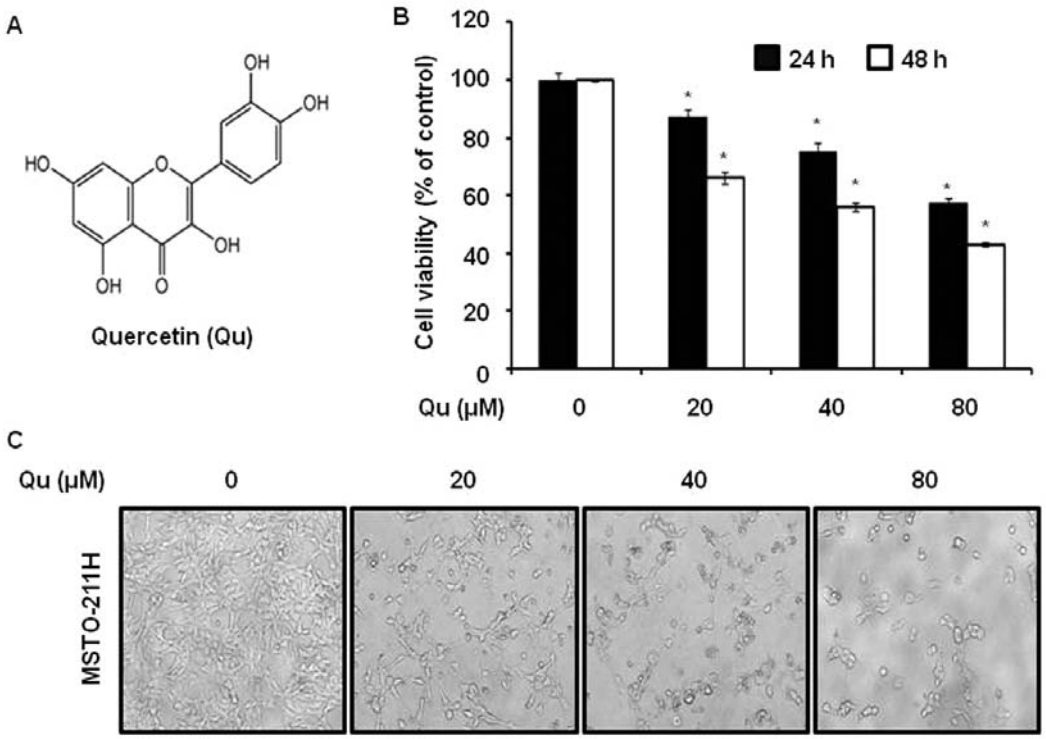


Figure 1. The effect of quercetin (Qu) on MSTO-211H cell viability. (A) Chemical structure of Qu. (B) The effect of Qu on the viability of MSTO-211H cells. MSTO-211H cells ($3x10^3$ cells/200 $\mu$l) were treated with Qu (20-80 $\mu$M) in 5% FBS-RPMI-1640 for various amounts of time. Cell viabilities were estimated using MTS assay kits, as described in Materials and methods. Results are cell viabilities relative to untreated cells, and represent 3 independent experiments. Data are presented as the means ± SD. The asterisk indicates a significant difference compared with the control ($^*P<0.05$). (C) Morphological changes observed in MSTO-211H cells treated or not treated with Qu (20-80 $\mu$M) for 48 h.

*Statistical analysis*. The results are presented as the means ± SD of at least 3 independent experiments performed in triplicate. Data were analyzed for statistical significance using one-way analysis of variance. A P-value <0.05 was considered to indicate a statistically significant difference.

## Results

*Qu reduces the viability of MSTO-211H cells*. To evaluate the effects of Qu (Fig. 1A) on the viability of malignant mesothelioma cells we used a MTS assay. It was found that Qu suppressed cell viability with an $IC_{50}$ of 58 $\mu$M in MSTO-211H cells for 48 h (Fig. 1B). Qu treatment also resulted in a significant concentration-dependent inhibition of cell growth with $IC_{50}$ values of approximately 73 $\mu$M in HT28 cells (another malignant mesothelioma cell line) for 48 h (data not shown). To investigate the morphological changes, MSTO-211H cells were treated with various concentrations (20-80 $\mu$M) of Qu for 48 h. The results obtained showed that the cells had decreased in size and that they had became rounded (Fig. 1C).

*Qu induces the apoptosis of MSTO-211H cells*. The effect of Qu treatment on the initiation of apoptosis in MSTO-211H cells was determined by nuclear morphology using DAPI staining. The Qu treatment of mesothelioma cells increased nuclear condensation and fragmentation when compared to the control group (Fig. 2A). In order to evaluate whether the increase in the sub-$G_1$ cell population induced by Qu was related to apoptosis, the Qu-treated cells were used for PI staining. The number of MSTO-211H cells in the sub-$G_1$ phase increased from 25 to 50% in the presence of 20-80 $\mu$M Qu (Fig. 2B and C).

*Qu regulates Sp1 protein levels in MSTO-211H cells*. Sp1 contributes to cell progression and apoptotic cell death via the regulation of the expression of a number of genes, such as cyclin D1, Mcl-1, survivin, Bcl-xL, Bax and caspase-3 in various cancers (7,13,22). The interaction between Sp1 and Qu was examined by conducting a Qu-sepharose 4B affinity experiment by immunoblotting with anti-Sp1. The results obtained indicated that Qu bound with Sp1 in cell lysates from human MSTO-211H cells (Fig. 3A). Furthermore, we found that Qu at 20, 40 and 80 $\mu$M downregulated Sp1 protein (Fig. 3D) and mRNA levels (Fig. 3B), and Sp1 protein expression level monitoring showed that Qu time-dependently reduced protein levels over 0 to 48 h (Fig. 3C). Qu clearly attenuated the cyclin D1, Mcl-1 and survivin promoter activities (Fig. 4A-C). In addition, significant decreases in the protein levels of cyclin D1, Mcl-1 and survivin were observed following Qu treatment (Fig. 4D).

*Qu regulates the expression of anti-apoptotic and apoptotic molecules in MSTO-211H cells*. The treatment of cells with Qu regulated the expression levels of several apoptosis-related proteins. MSTO-211H cells were treated with various concentrations (20-80 $\mu$M) of Qu for 48 h and harvested. The protein expression levels of Bid, Bcl-xL, caspase-3, PARP and Bax were analyzed by western blot analysis. The results showed

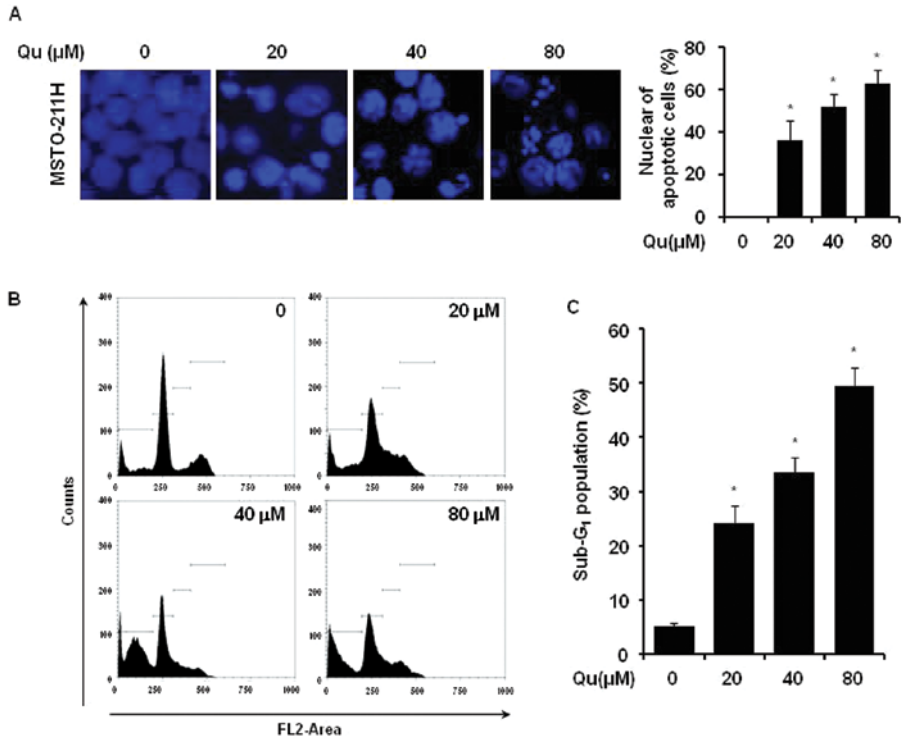


Figure 2. The apoptotic effect of quercetin (Qu) on MSTO-211H cells. (A) Cells were treated or not (DMSO) with Qu (20, 40 or 80 $\mu$M) for 48 h. The cells were harvested and prepared for DAPI staining and PI staining as described in Materials and methods. (B) Analysis of DNA fragmentation and nuclear condensation by fluorescence microscopy (magnification, x600) after Qu treatment. (C) DNA fragmentation and nuclear condensation were quantified. The results are from triplicate experiments and are expressed as the means ± SD. Representative histograms of the sub-$G_1$ population. Qu-treated cells were compared with the untreated cells. The results shown are the averages of 3 independent experiments performed in triplicate. The asterisk indicates a significant difference compared with the control ($^*$P<0.05).

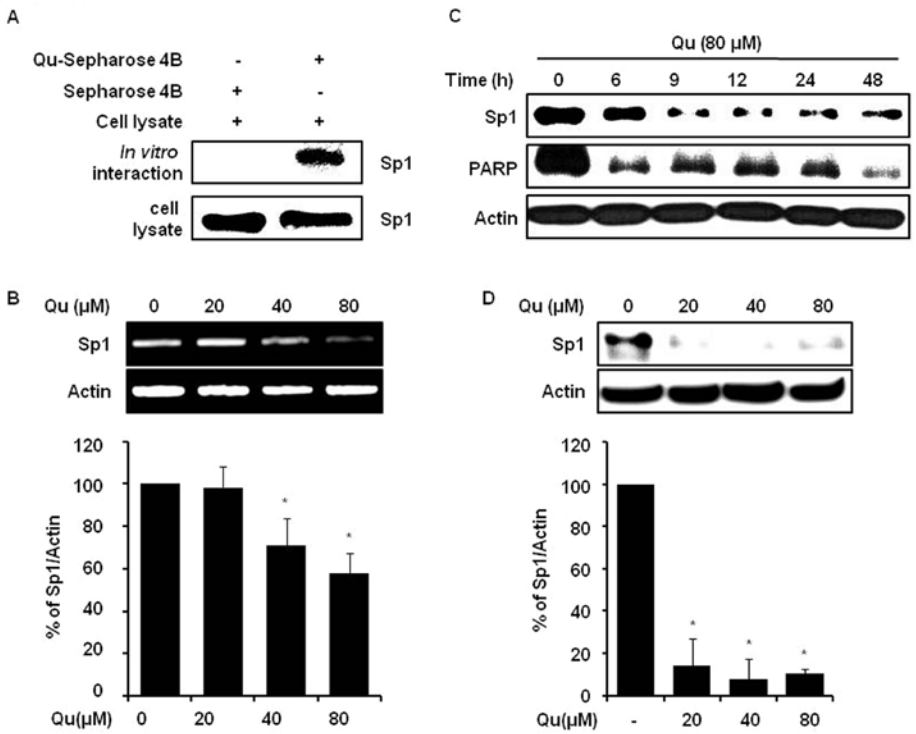


Figure 3. Quercetin (Qu) bound with specificity protein 1 (Sp1) and suppressed Sp1 protein levels. (A) Qu bound with Sp1 *ex vivo*. The *ex vivo* binding of Qu with Sp1 from MSTO-211H cell lysates was confirmed by pull-down assay using Qu-sepharose 4B beads and subsequent western blot analysis. (B) The effect of Qu treatment (20-80 $\mu$M) for 48 h on Sp1 mRNA expression was determined by RT-PCR. The graphs indicate the ratio of Sp1 to actin expression. (C) Time-dependent effects of Qu on Sp1 and PARP protein expression levels were investigated in MSTO-211H cells treated with Qu (20-80 $\mu$M) for 0, 6, 9, 12, 24 or 48 h. (D) The effect of Qu treatment (20-80 $\mu$M) for 48 h on Sp1 protein expression was determined by western blot analysis. Actin was used to ensure equal protein loadings. The data shown are the means ± SD of 3 independent experiments. The asterisks indicate a significant decrease in Sp1 protein expression in Qu-treated cells compared with the untreated cells ($^*$P<0.05).

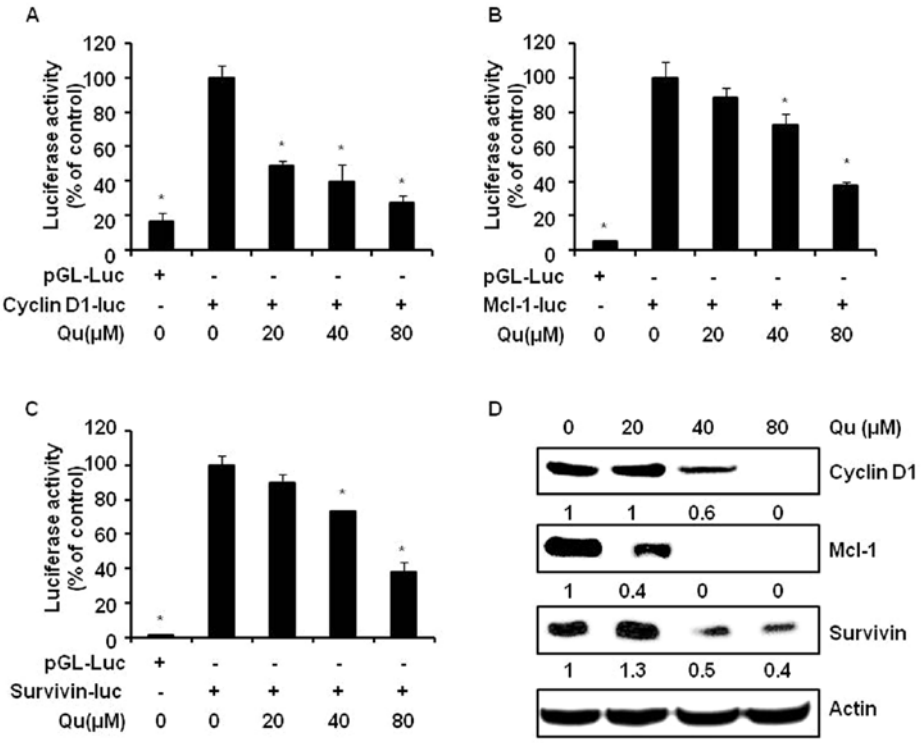


Figure 4. The effect of quercetin (Qu) on specificity protein 1 (Sp1) downstream target proteins. The firefly luciferase (A) cyclin D1, (B) Mcl-1 or (C) survivin reporter gene activity was assessed. For the reporter gene assay, MSTO-211H cells were transfected with a plasmid mixture containing the cyclin D1, Mcl-1, or survivin luciferase reporter gene (250 ng) and the β-gal gene (20 ng) for normalization. At 24 h after transfection, cells were treated with various concentrations of Qu for 48 h. Results are shown as the means ± SD of 3 independent experiments. The asterisks indicate a significant change relative to the Qu-treated cells compared with the untreated cells (*P<0.05). (D) MSTO-211H cells were treated with Qu (20-80 $\mu$M) for 48 h, and the protein expressions of cyclin D1, Mcl-1, and survivin were determined by western blot analysis. A total of 40 $\mu$g of cellular extract per lane was separated on a 12% SDS-PAGE gel as described in Materials and methods. Equal protein loading and protein transfer were confirmed with anti-actin antibody.

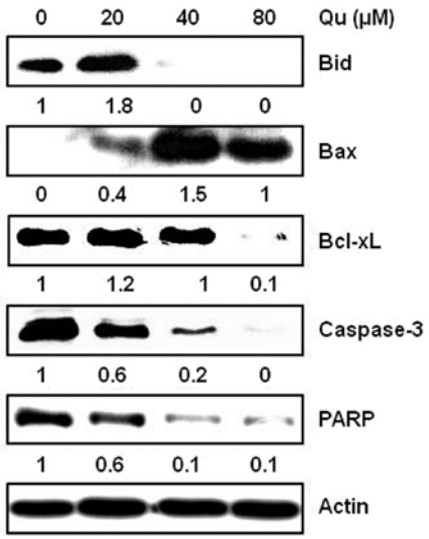


Figure 5. The apoptotic effect of quercetin (Qu) on MSTO-211H cells. The MSTO-211H cells were treated with Qu (20-80 $\mu$M) for 48 h, and the protein expressions of Bid, Bcl-xL, caspase-3, PARP and Bax were determined by western blot analysis. A total of 40 $\mu$g of cellular extract per lane was separated on a 12% SDS-PAGE gel as described in Materials and methods. Equal protein loading and protein transferwere confirmed with anti-actin antibody.

that Qu induced the activation of Bid, caspase-3 and PARP, decreased Bcl-xL, and increased Bax levels in the MSTO-211H cells (Fig. 5).

## Discussion

Malignant pleural mesothelioma and the majority of lung carcinomas are diagnosed at an advanced stage, conferring a poor prognosis (23,24). The incidence of malignant pleural mesothelioma is rising rapidly in many countries and it continues to be a challenging clinical problem. Our results showed that Qu has potential as a chemopreventive and chemotherapeutic agent for malignant pleural mesothelioma and that Sp1 is a potential therapeutic target of Qu.

It has previously been reported that the Sp1 protein is overexpressed in many human tumors (25) and cancer cell lines (26-30) and a number of studies have reported that Sp1 is highly expressed in a variety of human tumors, such as those of the pancreas, colon, lung, breast and prostate (9), demonstrating that the use of natural compounds may be used to inhibit Sp1 expression in cancer. Advances in treatment regimens have only had modest effects, although gene therapy offers a novel therapeutic approach, and has been evaluated in a number of clinical trials. Strategies include the induction of apoptosis, cytokine-based therapy, suicide gene expression, tumor suppressor gene replacement, various vaccination approaches, and the adoptive transfer of modified immune cells. A number of studies have considered the clinical results, limitations and future directions of gene therapy trials for thoracic malignancies (23,24).

Various plants and fruits have been reported to have cancer chemopreventive properties, and thus, investigators have sought to identify new active phytochemicals (31). Qu is an ubiquitous

dietary flavonoid that has recently been described as a potential anticancer agent (32) due to its ability to modulate cell proliferation, survival and differentiation, targeting key molecules responsible for tumor cell growth (33,34).

However, the mechanisms responsible for the antitumor activity of Qu are not yet fully understood. The induction of apoptosis may be one of the mechanisms as Qu has antiproliferative effects (35,36) and can induce death via apoptosis in leukemia (37), breast (38), hepatoma (39), oral (40) and colon (35) cancer cells. However, no previous study has been conducted on the effect of Qu on human mesothelioma cells. In this study, to the best of our knowledge, we demonstrate for the first time that Qu induces apoptotic cell death by inhibiting Sp1 protein expression in a time- and dose-dependent manner in MSTO-211H cells.

Previous studies have identified other flavonoids as having similar effects as Qu. For example, mithramycin A is one of the older chemotherapy drugs, and is known to suppress the expression of Sp1, to inhibit Sp1 binding and to inhibit the transcription of c-myc, p27, p21, cyclin D1, Mcl-1 and survivin selectively (13,41-43). Remarkably, our results indicate that Qu directly binds to Sp1, and possibly prevents the binding of Sp1 by G-C rich promoters. Our data provide evidence that Qu inhibits Sp1 expression at the protein and mRNA levels. Transcriptional response targeting genes containing the Sp1 binding site in their promoters are involved in a number of cellular functions ranging from differentiation to cell cycle progression, proliferation and apoptotic cell death (7). Our results showed that Qu suppressed Sp1 downstream target genes, including cyclin D1, Mcl-1 and survivin in MSTO-211H cells by promoter assay and western blot analyses. The apoptotic effect of Qu on MSTO-211H cells was found to be induced via the inhibition of Sp1 protein expression *in vitro*. Our results from *in vitro* experiments show that the Sp1 protein is a major factor of the antitumor effects of Qu in mesothelioma.

In conclusion, the results from the present study suggest that Qu has therapeutic and chemopreventative benefits and that Sp1 be considered a therapeutic target in malignant pleural mesothelioma and other advanced-stage cancers. Furthermore, our data suggest that Qu be considered a drug or natural supplement candidate for the prevention of malignant pleural mesothelioma.

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