Abstract. Flavonoids are assumed to exert beneficial effects in different types of cancers at high concentrations. Yet, their molecular mechanisms of action remain unknown. The present study aimed to examine the effect of quercetin on proliferation and apoptosis in HER2-expressing breast cancer cells. The anti-proliferative effects of quercetin were examined by proliferation, MTT and clonogenic survival assays. The effect of quercetin on expression of apoptotic molecules was determined by western blotting. Luciferase reporter assay was performed to measure signal transducer and activator of transcription 3 (STAT3) transcriptional activity. ELISA assay was performed to measure intracellular MMP-9 levels. Immunocytochemistry was performed to evaluate the nuclear STAT3 level. The results revealed that quercetin inhibited the proliferation of BT-474 cells in a dose- and time-dependent manner. Quercetin also inhibited clonogenic survival (anchorage-dependent and -independent) of BT-474 cells in a dose-dependent manner. These growth inhibitions were accompanied with an increase in sub-G$_0$/G$_1$ apoptotic populations. Quercetin induced caspase-dependent extrinsic apoptosis upregulating the levels of cleaved caspase-8 and cleaved caspase-3, and inducing the cleavage of poly(ADP-ribose) polymerase (PARP). In contrast, quercetin did not induce apoptosis via intrinsic mitochondrial apoptosis pathway since this compound did not decrease the mitochondrial membrane potential and did not affect the levels of B-cell lymphoma 2 (Bel-2) and Bel-2-associated X protein (BAX). Quercetin reduced the expression of phospho-JAK1 and phospho-STAT3 and decreased STAT3-dependent luciferase reporter gene activity in the BT-474 cells. Quercetin inhibited MMP-9 secretion and decreased the nuclear translocation of STAT3. Our study indicates that quercetin induces apoptosis at concentrations >20 µM through inhibition of STAT3 signaling and could serve as a useful compound to prevent or treat HER2-overexpressing breast cancer.

Introduction

Consumption of particular fruits and vegetables is considered to prevent or even treat various diseases including cancer. For example, the consumption of broccoli (250 g/day) and Brussels sprouts (250 g/day) may decrease colorectal cancer risk (1). Significant efforts have been made to develop plant-derived dietary agents which have beneficial effect on cancer. Quercetin (3,3',4',5,6-pentahydroxyflavone) is a flavonoid that is found in many plants and foods, such as onions, green tea, apples, berries, broccoli, red wine and others (2,3). Quercetin exerts anti-oxidant (4), anti-inflammatory (5), anti-mutagenic (6), and anti-angiogenic activities (2). Moreover, in vitro and in vivo studies have shown that quercetin exhibits various anticancer activities. It was reported that quercetin-3-O-glucoside induced human DNA topoisomerase II inhibition, cell cycle arrest and apoptosis in hepatocellular carcinoma cells (7). It was also reported that quercetin derivatives demonstrated anti-oxidant activity [monochloropivaloyl quercetin (IC$_{50}$=27 µM)] and cytotoxicity in HeLa [chloronaphthoquinone quercetin (IC$_{50}$=13.2 µM)] and NIH-3T3 [tri(diacetylcaffeoyl) quercetin (IC$_{50}$=10.6 µM)] cells (8). Quercetin (40 mg/ml) is reported to

Quercetin induces caspase-dependent extrinsic apoptosis through inhibition of signal transducer and activator of transcription 3 signaling in HER2-overexpressing BT-474 breast cancer cells

HYE-SOOK SEO$^1$, JIN MO KU$^1$, HAN-SEOK CHOI$^{1,2}$, YOUN KYUNG CHOI$^1$, JONG-KYU WOO$^3$, MINSOO KIM$^4$, ILHWAN KIM$^4$, CHANG HYEOK NA$^5$, HANSOL HUR$^4$, BO-HYOUNG JANG$^1$, YONG CHEOL SHIN$^1$ and SEONG-GYU KO$^1$

$^1$Laboratory of Clinical Biology and Pharmacogenomics and Center for Clinical Research and Genomics, College of Korean Medicine, Kyung Hee University, Dongdaemun-gu, Seoul 02447; $^2$Next-Generation Pharmaceutical Research Center, Korea Institute of Toxicology, Yuseong-gu, Daejeon 34114; $^3$College of Veterinary Medicine, Seoul National University, Gwanak-gu, Seoul 08826; Departments of $^4$Science in Korean Medicine, and $^5$Applied Korean Medicine, Graduate School, Kyung Hee University, Dongdaemun-gu, Seoul 02447, Republic of Korea

Received October 19, 2015; Accepted January 11, 2016

DOI: 10.3892/or.2016.4786

Correspondence to: Dr Yong Cheol Shin, Laboratory of Clinical Biology and Pharmacogenomics and Center for Clinical Research and Genomics, College of Korean Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 130-701, Republic of Korea

E-mail: syc99@khu.ac.kr

Key words: breast cancer, quercetin, apoptosis, HER2, p53, STAT3
inhibit the growth of MCF-7 breast cancer cells and to promote apoptosis by inducing G$_0$/G$_1$ phase arrest (9). Quercetin (100 µM) inhibited the growth of colorectal cancer cells, by up-regulation of the expression of tumor-suppressor genes and modulation of cell cycle-related and apoptosis genes (10,11). Moreover, quercetin (2%) inhibited carcinogen-induced rat mammary tumor growth (12).

Apoptosis is a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death (13). The process of apoptosis is associated with various caspases, which are aspartate-specific cysteine proteases and members of the interleukin-1β-converting enzyme family (14,15). Caspases, once activated, play a key role in the intracellular signal cascade for undergoing apoptosis. In most tumor cells, apoptosis occurs via two different signaling pathways: the extrinsic and intrinsic apoptosis pathways. The extrinsic pathway is related to the activation of death receptors, such as Fas and tumor necrosis factor receptors (TNFRs) and the cleavage of caspase-8 and caspase-3 (16-18). The intrinsic pathway is related to changes in the mitochondrial membrane potential (ΔΨm), the mitochondrial permeability transition, and the cleavage of caspase-9 and caspase-3 (19). In both the extrinsic and intrinsic pathways, caspase-3 is responsible for the cleavage of poly(ADP-ribose) polymerase (PARP) during cell death (20).

Overexpression of HER2 is encountered in approximately 25% of invasive breast cancers (21). HER2-positive breast cancers tend to grow more quickly than HER2-negative breast cancers. HER2-positive cancers are associated with frequent recurrence and reduced overall survival, compared to HER2-negative tumor subtypes. The most widely used chemotherapeutic agent is Herceptin (trastuzumab), which acts by attaching itself to HER2 receptors on breast cancer cells and blocking them from receiving growth signals (22,23). Herceptin also causes arrest at the G1 phase of the cell cycle and inhibits the phosphorylation of p27$^{kip}$, resulting in the suppression of cdk2 activity and reduced proliferation (24). Herceptin suppresses angiogenesis by both the induction of anti-angiogenic factors and the repression of pro-angiogenic factors. However, many women do not respond to Herceptin or develop resistance to this drug (25). This has resulted in significant efforts to identify other compounds that can effectively treat HER2-overexpressing breast cancer.

Previously, we reported that phytoestrogen suppresses cell growth and induces apoptosis by inhibiting signal transducer and activator of transcription 3 (STAT3) and/or NF-kB signaling in HER2-overexpressing breast cancer cells (26,27). In the present study, we investigated whether quercetin displays growth-suppressive activity in HER2-overexpressing BT-474 breast cancer cells. For this purpose, we tested the effects of quercetin on the proliferation and apoptosis of BT-474 cells. We also investigated the mechanism by which quercetin regulates the growth of BT-474 cells by analyzing the cell cycle and measuring the levels of apoptotic molecules and intracellular signaling molecules. We also aimed to ascertain whether quercetin inhibits the STAT3 signaling pathway, leading to the growth suppression of HER2-overexpressing breast cancer cells. Our study may advance human health by clarifying the efficacy of quercetin for the prevention and treatment of HER2-positive breast cancer.

Materials and methods

Compounds. Quercetin (3,3’,4’,5,6-pentahydroxyflavone) and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). These compounds were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the controls and each sample did not exceed 0.1%. We found that 0.1% DMSO did not affect the cell growth rate compared to 0% DMSO (no treatment) in the breast cancer cells (data not shown). JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide) was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA). The caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). The STAT3 inhibitor S3I-201 was obtained from Calbiochem (San Diego, CA, USA). An EZ-western chemiluminescent detection kit was purchased from Daeil Lab Service Co. (Seoul, Korea).

Cell cultures. BT474 human breast cancer cells (ATCC, American Type Culture Collection; Manassas, VA, USA) were cultured in RPMI-1640 medium containing 50 U/ml penicillin, 50 mg/ml streptomycin and 10% fetal bovine serum (FBS; Welgene, Daegu, Korea) at 37˚C in an atmosphere of 5% CO$_2$.

Antibodies. Monoclonal or polyclonal antibodies (mouse or rabbit) directed against FAS, cleaved caspase-8, caspase-3, cleaved caspase-3 and PARP [poly(ADP-ribose) polymerase] were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Monoclonal or polyclonal antibodies (mouse or rabbit) directed against Bcl-2, BAX, p53, phospho-p53 (Ser15), p21 and VEGF were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal or polyclonal antibodies (mouse or rabbit) against Bcl-XL and HIF-1α were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Monoclonal or polyclonal antibodies (mouse or rabbit) directed against STAT3, phospho-STAT3 (Tyr705), and phospho-JAK1 (Tyr1022/Tyr1023) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-tubulin antibody was from Sigma Chemical Co. Horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse and rabbit) were purchased from Calbiochem and anti-goat secondary antibody was from Jackson ImmunoResearch (West Grove, PA, USA).

Cell proliferation assay. Cells were seeded in 12-well culture plates at a density of 5x10$^4$ cells/well. After the cells were exposed to different concentrations of quercetin (20-60 µM) and incubated for 3 days, they were harvested by trypsinization, resuspended in 1-2 ml of medium, and counted using a hemocytometer.

MTT assay. Cells were seeded in 96-multi-well culture plates at a density of 3x10$^3$ cells/well and incubated for 24 h at 37°C. Then, they were treated with different concentrations of quercetin (20-60 µM) for 24, 48, or 72 h. After incubation,
MTT reagents (0.5 mg/ml) were added to the each well and the plates were incubated in the dark at 37°C for 2 h. At the end of the incubation, the medium was removed, the resulting formazan was dissolved in DMSO, and the optical density was measured at 570 nm using an ELISA plate reader (fluorescence readers; Molecular Devices, Sunnyvale, CA, USA).

**Clonogenic survival assays (anchorage-dependent and -independent).** For anchorage-dependent colony formation assay, cells were seeded into 6-well culture plates at a density of 5x10^4 cells/well. After overnight incubation, they were treated with different concentrations of quercetin (20-60 µM) or vehicle and maintained for 10 days at 37°C. Cells were fed every 3 days by removing old medium and adding fresh medium containing quercetin. Finally, the plates were stained with hematoxylin and the colony number was determined. For anchorage-independent colony formation assay, soft agar was used. Cells (1x10^5) were suspended in 1 ml of 0.6% soft agar that was layered on top of 1 ml of 1% solidified agar in each well of 12-well plates. The plates were incubated for 15 to 21 days in complete RPMI medium containing quercetin (20-60 µM). The medium was changed every 3 days during this period. At the end of the experiment, tumor cell colonies measuring at least 30 µm were counted using a dissection microscope.

**Cell cycle analyses by flow cytometry.** Cells were harvested with 0.25% trypsin and washed once with phosphate-buffered saline (PBS). After centrifugation, the cells were fixed in cold 95% ethanol with 0.5% Tween-20, and stored at -20°C for at least 30 min. The cells were incubated in 50 µg/ml of propidium iodide (PI) (including 1% of sodium citrate and 50 µg/ml of RNase A) at room temperature in the dark for 30 min. The analysis of apoptotic cells was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the data were analyzed using CellQuest software.

**Analysis of mitochondrial transmembrane potential (ΔΨm).** Cells were seeded at a density of 1x10^5 cells/dish in 100-mm dishes and incubated for 24 h at 37°C. After stabilization, the cells were treated with quercetin (20-60 µM) and vehicle for 72 h. After harvest by removing old medium and adding fresh medium containing quercetin, the next day, the cells were treated with different concentrations of quercetin (20-60 µM) for 24 h and then submitted to the analysis. Mitochondrial transmembrane potential (ΔΨm) was measured at 450 nm on an automated ELISA reader.

**STAT3 luciferase reporter assay.** BT-474 cells were plated and allowed to attach by overnight incubation at 37°C. Cells were transiently transfected with p4xM67-TK-luc plasmid (Addgene plasmid 8688; Addgene, Cambridge, MA, USA) containing four copies of the STAT-binding site (TTCCCGTAA). The next day, the cells were treated with different concentrations of quercetin (20-60 µM) for 24 h and then submitted to the luciferase assays. Luciferase assays were performed using a dual-luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI, USA). Finally, luciferase activities were determined using a luminometer (BMG Labtech, Ortenberg, Germany).

**Statistical analysis.** All experiments were performed in triplicate. The data for the cell proliferation, MTT, ELISA, and STAT3 luciferase reporter assays are expressed as the mean ± standard deviation (SD). The standard deviations for all of the measured biological parameters are displayed in the appropriate figures. A Student's t-test was used for single variable comparisons, and a p-value of <0.05 was considered to be indicative of a statistically significant result.
Figure 1. Effect of quercetin on BT-474 cell growth. (A) BT-474 cells were treated with different doses of quercetin and genistein (0-100 µM). After 72 h, cell viability was assessed using a cell proliferation assay. (B) BT-474 cells were treated with different doses of quercetin (0-100 µM). The relative cell growth rate was measured by MTT assay after 24, 48, and 72 h. The growth rate of the vehicle-treated cells was set to 100%, and the relative decrease in cell viability resulting from the phytoestrogen treatment is expressed as a percentage of the control. (C) BT-474 cells were treated with different doses of quercetin (0-60 µM) for 72 h and photographed by phase-contrast microscopy (original magnification, x40). (D) BT-474 cells were treated with different doses of quercetin (0-20 µM). The relative cell growth rate was measured by MTT assay after 24, 48, and 72 h. Control cells were treated with DMSO alone. Data are shown as the means of three independent experiments (error bars denote SD). *P<0.05, **P<0.01, ***P<0.001.
Results

Quercetin suppresses the growth of BT-474 cells. The effects of quercetin on cell growth were measured by cell proliferation and MTT assays in the BT-474 cells. As shown in Fig. 1A, quercetin and genistein significantly inhibited BT-474 cell proliferation in a dose-dependent manner (20-100 µM) after 72 h of treatment (proliferation assay). Between two phytoestrogens, quercetin had the stronger growth suppressive activity compared to genistein in the BT-474 cells. Therefore, we chose quercetin for our experimental study. In addition, the time-dependent growth suppressive activity of quercetin was measured by the MTT assay (Fig. 1B). As shown in Fig. 1A and B, the proliferation assay appeared to be more sensitive than the MTT assay with respect to measuring the intensity of the cell growth inhibition. Moreover, the growth inhibition induced by quercetin was verified by microscopic observation. As shown in Fig. 1C, quercetin effectively inhibited the growth rate of BT-474 monolayer cells after 72 h of treatment. Of note, quercetin also induced morphological changes in these cells (Fig. 1C). Since lower concentrations of phytoestrogen could stimulate the growth of breast cancer cells through nuclear and membrane estrogen receptors, we performed an MTT assay to measure the growth rate of BT-474 cells under lower concentrations of quercetin (0-20 µM). We found that lower concentrations of quercetin did not affect the cell growth rate (Fig. 1D).

Quercetin inhibits clonogenic survival of BT-474 cells. Next, we investigated the effect of quercetin on clonogenic survival of BT-474 cells using clonogenic survival assays (anchorage-dependent and -independent). As shown in Fig. 2A, quercetin significantly inhibited anchorage-dependent colony formation dose-dependently in the BT-474 cells. Consistently
that quercetin induced an increase in the sub-G$_0$/G$_1$ apoptotic population in the BT-474 cells (Fig. 3).

**Quercetin induces extrinsic apoptosis in BT-474 cells.** Next, we investigated whether apoptosis induced by quercetin occurs via the extrinsic apoptosis pathway in the BT-474 cells. For this purpose, we measured the loss of mitochondrial transmembrane potential (ΔΨm) within the cells using JC-1. JC-1 is able to selectively enter mitochondria and reversibly transforms the color from red to green when the membrane potential decreases. In non-apoptotic cells with high mitochondrial ΔΨm, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. In contrast, in apoptotic cells (particularly mitochondrial-mediated apoptotic cells) with low ΔΨm, JC-1 remains in the monomeric form, which shows only green fluorescence. In our study, quercetin did not induce a low mitochondrial transmembrane potential (ΔΨm), showing relatively weak green fluorescence (DMSO, 3.0%; quer 20 µM, 12.6%; quer 40 µM, 12.2%; quer 60 µM, 14.8%) compared to FCCP (positive control, 87.6%) (Fig. 4A). We also measured the levels of Bcl-2 family members (BAX and Bcl-2) which are important in the intrinsic mitochondrial apoptosis pathway. We found that quercetin failed to decrease the level of Bcl-2 or increase the level of BAX as shown in Fig. 4B and C. These results demonstrate that quercetin does not induce apoptosis via the intrinsic mitochondrial pathway but induces apoptosis via the extrinsic pathway in BT-474 breast cancer cells.

**Quercetin induces apoptosis via the caspase-dependent apoptosis pathway in BT-474 cells.** In this step, we investigated whether quercetin activates the caspase-dependent apoptosis pathway by measuring the expression of caspase-8, caspase-3, and PARP. We observed that quercetin upregulated the levels of cleaved caspase-8 and caspase-3, and induced the cleavage of PARP in the BT-474 cells (Fig. 5A). We also found that the cleavage of caspase-8, caspase-3 and PARP was inhibited by the caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk (Fig. 5B), but quercetin prevented this inhibition and was able to induce the cleavage of caspase-8, caspase-3 and PARP in the presence of Z-IETD-fmk and Z-LEHD-fmk (Fig. 5B). Moreover, the caspase-8 and caspase-9 inhibitors did not suppress cell growth, while quercetin was able to induce apoptosis even in their presence (Fig. 5C). These results confirm that quercetin strongly promoted apoptosis via a caspase-dependent mechanism in the BT-474 cells.

**Effect of quercetin on STAT3 activation in BT-474 cells.** Quercetin upregulated phospho-p53 (p-p53) and p21 (p53 target gene) (Fig. 6A). Quercetin did not affect the p53 level. As shown in Fig. 6B, we aimed to ascertain whether quercetin affects STAT3 signaling measuring levels of p-STAT3 and VEGF (STAT-3 target gene). We found that quercetin reduced the expression of p-STAT3 as well as p-JAK1 (an upstream kinase of STAT3) (Fig. 6B). Quercetin also reduced the level of VEGF (Fig. B). Since STAT3 is a potential modulator of HIF-1α (28), we observed the relationship between STAT3 and HIF-1α. We found that quercetin suppressed the expression of p-STAT3 and HIF-1α that was upregulated by CoCl$_2$ (hypoxia mimic) (Fig. 6C). Immunocytochemical staining indicated...
that quercetin decreased the nuclear localization of STAT3 in the presence and absence of CoCl₂ (Fig. 6D). Fig. 6E showed that quercetin strongly decreased STAT3 transcriptional activity as revealed by transient transfection and luciferase assays. As shown in Fig. 6F, quercetin suppressed the production of STAT3 target gene, MMP-9, as revealed by ELISA assay. These results suggest that quercetin decreases HER2-positive breast cancer cell growth rate at higher concentrations (>20 µM) by inhibiting the STAT3 signaling pathway.

**Discussion**

In the present study, we investigated the mechanism by which quercetin inhibits cell growth and induces apoptosis in HER2-overexpressing BT-474 breast cancer cells. Quercetin significantly inhibited BT-474 cell growth in a dose- and time-dependent manner. Clonogenic survival assays demonstrated that quercetin inhibited anchorage-dependent and -independent colony formation in a dose-dependent manner. These growth inhibitions were related with an increase in the sub-G₀/G₁ apoptotic population in BT-474 cells. Quercetin increased the number of apoptotic cells in a dose-dependent manner, as assessed by FACS analysis. Interestingly, quercetin did not induce apoptosis via the intrinsic mitochondrial apoptosis pathway in the BT-474 cells as revealed by JC-1 dyeing of the cells and western blot analysis; quercetin did not reduce mitochondrial transmembrane potential (ΔΨm) maintaining red fluorescence, and failed to decrease the level of Bcl-2.

Figure 4. Quercetin induces apoptosis via the extrinsic pathway in BT-474 cells. (A) BT-474 cells were incubated with quercetin (0-60 µM) for 72 h and were dyed with JC-1 (4 µg/ml). The data were analyzed by FACS Calibur flow cytometry measuring the green fluorescence and red fluorescence at 514/529 nm (FL-1) and 585/590 nm (FL-2), respectively. The data shown are representative of three independent experiments that gave similar results. (B and C) Analysis of intrinsic apoptosis-related molecules. BT-474 cells were treated with quercetin (0-60 µM) for 24 h. Total proteins were analyzed by western blotting with anti-Bcl-2, anti-BAX, anti-Bcl-xL and anti-tubulin antibodies.

Effect of S3I-201 on STAT3 activation in BT-474 cells. Finally, we investigated whether the STAT3 inhibitor S3I-201 inhibits cell proliferation and STAT3 activation in BT-474 cells. As shown in Fig. 7A and B, S3I-201 decreased cell growth in a dose- and time-dependent manner. Furthermore, S3I-201 reduced the expression of p-STAT3, STAT-3 and VEGF (Fig. 7C). These results demonstrate that STAT3 inhibition induced cell growth inhibition and repressed the expression of oncogenic molecules.
Figure 5. Quercetin induces caspase-dependent apoptosis in BT-474 cells. (A) Quercetin induces apoptosis via a caspase-dependent apoptosis pathway in the BT-474 cells. BT-474 cells were treated with quercetin (0-60 µM) for 24 h. Whole cell lysates were analyzed by western blotting with anti-FAS, anti-cleaved caspase-8, anti-cleaved caspase-3, anti-cleaved PARP and anti-tubulin antibodies. The data shown are representative of three independent experiments that gave similar results. (B) Effect of caspase-8 and caspase-9 inhibitors on quercetin-induced apoptosis in BT-474 cells. BT-474 cells were exposed to 60 µM quercetin with or without the caspase-8 inhibitor (40 µM) or the caspase-9 inhibitor (40 µM) for 24 h, the cell lysates were separated by SDS-PAGE, and western blotting with specific antibodies was performed (anti-cleaved caspase-8, anti-cleaved caspase-3, anti-cleaved PARP, and anti-tubulin). The data shown are representative of three independent experiments that gave similar results. (C) Effect of caspase-8 and caspase-9 inhibitors on BT-474 cell proliferation. BT-474 cells were exposed to 60 µM quercetin with or without the caspase-8 inhibitor (40 µM) or the caspase-9 inhibitor (40 µM) for 72 h and photographed by phase contrast microscopy (original magnification, x40).
Figure 6. Effect of quercetin on STAT3 activation in BT-474 cells. (A) BT-474 cells were treated with quercetin (0-60 µM) for 24 h. Whole cell lysates were analyzed by western blotting with anti-p-p53, anti-p53, anti-p21 and anti-tubulin antibodies. (B) BT-474 cells were treated with quercetin (0-60 µM) for 24 h. Whole cell lysates were analyzed by western blotting with anti-p-JAK1, anti-p-STAT3, anti-STAT3, anti-VEGF, and anti-tubulin antibodies. (C) BT-474 cells were treated with quercetin (60 µM) for 24 h in the presence or absence of CoCl$_2$ (4 h). Whole cell lysates were analyzed by western blotting with anti-phospho-STAT3, anti-HIF-1α, anti-STAT3, and anti-tubulin antibodies. (D) BT-474 cells were treated with quercetin (60 µM) for 24 h in the presence or absence of CoCl$_2$ and then submitted to immunocytochemistry for detection of nuclear STAT3. The data shown are representative of three independent experiments that gave similar results. (E) BT-474 cells were transiently transfected with p4xM67-TK-luc plasmid containing four copies of the STAT-binding site, treated with quercetin (0-60 µM) and submitted to dual-luciferase assay. (F) BT-474 cells were treated with quercetin (0-60 µM) for 24 h and the intracellular MMP-9 concentration was measured by ELISA. Data are shown as the means of three independent experiments (error bars denote SD). *P<0.05, **P<0.01, ***P<0.001.
or increase the level of BAX. Whereas, quercetin induced apoptosis via the caspase-dependent extrinsic apoptosis pathway since quercetin increased the cleavage of caspase-8, caspase-3 and PARP. Moreover, quercetin reversed inhibition of the cleavage of caspase-8, caspase-3 and PARP induced by caspase-8 inhibitor Z-IETD-fmk and the caspase-9 inhibitor Z-LEHD-fmk. These results suggest that quercetin contains a strong apoptotic capacity. The caspases, a family of cysteine-dependent aspartate-directed proteases, are common death proteases (29). Caspases are synthesized as relatively inactive zymogens that become activated by scaffold-mediated transactivation or by cleavage via upstream proteases in an intracellular cascade (29). Once activated, they cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases (29).

Quercetin increased the expression of active p53 (p-p53) and p21 (p53 target gene), suggesting that this compound suppresses HER2-overexpressing breast cancer cell growth via a p53-dependent manner. In agreement with our data, quercetin has been shown to increase the levels of p-p53 and p21 in human lung carcinoma cells (30). The p53 tumor suppressor inhibits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses including DNA damage, growth factor deprivation, hypoxia and oncogene activation (31,32). p53-dependent apoptosis is produced by the caspase proteinases and related to pro-apoptotic proteins such as BAX, NOXA and PUMA (33).

Interestingly, quercetin decreased the expression of p-JAK1 (upstream kinase of STAT3), p-STAT3 and VEGF (STAT3 target gene) suggesting its negative regulation of STAT3 pathway in BT-474 cells. Elevated p-STAT3 expression by CoCl2 was also reduced by quercetin. Quercetin inhibited nuclear localization of STAT3 in the presence or absence of CoCl2 as revealed by immunocytochemistry. Quercetin inhibited the production of MMP-9 as revealed by ELISA assay. The STAT3 inhibitor S3I-201 decreased the cell growth and expression of p-STAT3, STAT-3 and VEGF in the BT-474 cells. These results clearly indicate that quercetin induces growth-suppressive activity by inhibiting the STAT3 signaling pathway. STAT3 is a transcription factor that regulates the gene expression in response to various cellular stimuli and plays an important role in cell growth and apoptosis. STAT3 usually acts as a tumor-promoter, although its role as a tumor-suppressor has been recently reported (33,34). STAT3 accelerates cell proliferation and angiogenesis, inhibits apoptosis, and drives invasion and metastasis (33-35). STAT3 in melanoma tumors is associated with poor prognosis (35-37). Constitutive STAT3 phosphorylation is mediated by several upstream kinases (Jak and Src) and is thought to be a key component of the oncogenic process (38,39). Phytoestrogen (resveratrol) is known to inhibit STAT3 signaling and induces the apoptosis of malignant cells containing activated STAT3 (40). The VEGF promoter contains various transcription factor binding sites, including sites for STAT3 (41) and HIF-1 (42). The physical interaction of STAT3 with HIF-1 controls VEGF transcriptional activation by their binding to the VEGF promoter (28).

Breast cancers with HER2 gene amplification or HER2 protein overexpression are HER2-positive. Approximately, 20-25% of invasive breast carcinomas reveal HER2 overexpression (43). A normal breast cell has 20,000 HER2 receptors, while a breast cancer cell may have up to 1.5 million. HER2 enhances the aggressiveness of breast cancer and is associ-
ated with recurrence when compared to HER2-negative breast cancer. HER2 is a member of the HER/ErbB2/Neu protein family, which also includes HER1/EGFR, HER3 and HER4. HER2 crosstalks with the estrogen receptor (ER) signal transduction pathway (44), and its expression level can be regulated by ER. In the present study, we found that quercetin significantly inhibited the growth and induced apoptosis in HER2-overexpressing breast cancer cells. This indicates that quercetin could be a useful natural therapy that inhibits HER2-overexpressing breast cancer. Quercetin could be a promising target for the treatment and prevention of HER2-overexpressing breast cancer.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2015R1C1A201051539). This research was also supported by a grant funded by the Traditional Korean Medicine & R&D Project of the Ministry of Health and Welfare (HI12C1889 and HI11C2110).

References

1. Walters DG, Young PJ, Agus C, Knize MG, Boobis AR, Gooderham NJ and Lake BG: Cruciferous dietary consumption alters the metabolism of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in humans. Carcinogenesis 25: 1659-1669, 2004.
2. Igura K, Otta T, Kuroda Y and Kaji K: Resveratrol and quercetin inhibit angiogenesis in vitro. Cancer Lett 171: 11-20, 2001.
3. Hertog MG and Hollman PC: Potential health effects of the dietary flavonol quercetin. Eur J Clin Nutr 50: 63-71, 1996.
4. Coskun O, Kanter M, Korkmaz A and Oter S: Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. Pharmacol Res 51: 117-123, 2005.
5. Boots AW, Wilms LC, Swennen EL, Kleinjans JC, Bast A and Haenens GR: In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. Nutrition 24: 703-710, 2008.
6. Geetha T, Malhotra V, Chopra K and Kaur IP: Antimutagenic and antioxidant/prooxidant activity of quercetin. Indian J Exp Biol 43: 61-67, 2005.
7. Sudan S and Rupasinghe HP: Quercetin-3-O-glucoside induces human DNA topoisoform II inhibition, cell cycle arrest and apoptosis in hepatocellular carcinoma cells. Anticancer Res 34: 1691-1699, 2014.
8. Danilehová M, Veverka M, Sturdík E and Jantová S: Antioxidant action and cytotoxicity on HeLa and NIH-3T3 cells of new quercetin derivatives. Interdiscip Toxicol 6: 209-216, 2013.
9. Deng XH, Song HY, Zhou YF, Yuan GY and Zheng FJ: Effects of quercetin on the proliferation of breast cancer cells and expression of survivin in vitro. Exp Ther Med 6: 1155-1158, 2013.
10. van Erk MJ, Roepman P, van der Lende TR, Stierum RH, van Ommen B: Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer-related mechanisms in colon cancer cells in vitro. Eur J Nutr 44: 143-156, 2005.
11. Murtaza I, Marra G, Schlabach R, Patrignani A, Künzli M, Wagner U, Sabates J and Dutt A: A preliminary investigation demonstrating the effect of quercetin on the expression of genes related to cell-cycle arrest, apoptosis and xenobiotic metabolism in human CO15 colon-adenocarcinoma cells using DNA microarray. Biotechnol Appl Biochem 45: 29-36, 2006.
12. Verma AK, Johnson JA, Gould MN and Tanner MA: Inhibition of 7,12-dimethylbenz(a)anthracene- and N-nitrosomethylurea-induced rat mammary cancer by dietary flavonol quercetin. Cancer Res 48: 5754-5758, 1988.
13. Elmore S: Apoptosis: A review of programmed cell death. Toxicol Pathol 35: 495-516, 2007.
14. Fan T, Han LH, Cong RS and Liang J: Caspase family proteases and apoptosis. Acta Biochim Biophys Sin (Shanghai) 37: 719-727, 2005.
15. Bosch M, Poultet NS, Vatocyc S and Franklin-Tong VE: Initiation of programmed cell death in self-incompatibility: Role for cytokinetic modifications and several caspase-like activities. Mol Plant 1: 879-887, 2008.
16. Zhang A, Wu Y, Lai H, Wang J, Li Y and Smulson M: Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 274: 7939-7948, 1999.
17. Bouloures AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S and Smulson M: Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP 3 mutant increases rates of apoptosis in transfected cells. J Biol Chem 274: 7939-7948, 1999.
18. Igura K, Otta T, Kuroda Y and Kaji K: Resveratrol and quercetin inhibit angiogenesis in vitro. Cancer Lett 171: 11-20, 2001.
19. Wilson CA, Cajuiles EE, Green JL, Olsen TM, Chung YC, Damore MA, Dering J, Calzone FJ and Slamon DJ: HER-2 overexpression differentially alters transforming growth factor-beta responses in luminal versus mesenchymal human breast cancer cells. Breast Cancer Res 7: R108-R1097, 2005.
20. Joshi P, Brown NE, Kliner SR and Nutt A: Growth differentiation factor 15 (GDF15)-mediated HER2 phosphorylation reduces trastuzumab sensitivity of HER2-overexpressing breast cancer cells. Biochem Pharmacol 82: 1090-1099, 2011.
21. Famoni RE, Daga A, Malatasta P and Florio T: Preclinical studies identify novel targeted pharmacological strategies for treatment of human malignant pleural mesothelioma. Br J Pharmaco 166: 532-553, 2012.
22. Tokunaga E, Oki E, Nishiida K, Koga T, Egashira A, Morita M, Kakeji Y and Maehara Y: Trastuzumab and breast cancer: Developments and current status. Int J Clin Oncol 11: 199-206, 2006.
23. Dean-Colomb W and Esteva FJ: HER2-positive breast cancer: Hereceptin and beyond. Eur J Cancer 44: 2806-2812, 2008.
24. Seo HS, Choi HS, Choi HS, Choi YK, Um YJ, Choi I, Shin YC and Ko SG: Phytoestrogens induce apoptosis via extrinsic pathway, inhibiting nuclear factor-kappaB signaling in HER2-overexpressing breast cancer cells. Anticancer Res 31: 3301-3313, 2011.
25. Seo HS, Choi HS, Kim SR, Choi YK, Woo SM, Shin I, Woo JK, Park SY, Shin YC and Ko SG: Apigenin induces apoptosis via extrinsic pathway, inducing p53 and inhibiting STAT3 and NF-kB signaling in HER2-overexpressing breast cancer cells. Mol Cell Biochem 369: 319-334, 2012.
26. Jung JE, Lee HG, Cho IH, Chung DH, Yoon SH, Yang YM, Lee JW, Choi S, Park JW, Ye SK, et al: STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. FASEB J 19: 1296-1298, 2005.
27. Earnshaw WC, Martins LM and Kaufmann SH: Mammalian caspases: Structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68: 383-424, 1999.
28. Kuo PC, Liu HF and Chao JJ: Survivin and p53 modulate quercetin-induced cell growth inhibition and apoptosis in human lung carcinoma cells. J Biol Chem 279: 55873-55885, 2004.
29. Schulier M and Green DR: Mechanisms of p53-dependent apoptosis. Biochem Soc Trans 29: 684-688, 2001.
30. Southall D and White E: p53-dependent apoptosis pathways. Adv Cancer Res 82: 55-84, 2000.
31. de la Iglesia N, Konopka G, Puram SV, Chan JA, Bachoo RM, You MJ, Levy DE, Depinho RA and Bonni A: Identification of a Pten-regulated STAT3 brain tumor suppressor pathway. Genes Dev 22: 449-462, 2008.
32. Lewis HD, Winter A, Murphy TF, Tripathi S, Pandey VN and Bignon YP: STAT3 inhibition in prostate and pancreatic cancer lines by STAT3 binding sequence oligonucleotides: Differential activity between 5' and 3' ends. Mol Cancer Ther 7: 1543-1550, 2008.
33. Kortylewski M, Jove R and Yu H: Targeting STAT3 affects melanoma on multiple fronts. Cancer Metastasis Rev 27: 1543-1550, 2008.
37. Xie TX, Huang FJ, Aldape KD, Kang SH, Liu M, Gershenwald JE, Xie K, Sawaya R and Huang S: Activation of stat3 in human melanoma promotes brain metastasis. Cancer Res 66: 3188-3196, 2006.

38. Sellers LA, Feniuk W, Humphrey PP and Lauder H: Activated G protein-coupled receptor induces tyrosine phosphorylation of STAT3 and agonist-selective serine phosphorylation via sustained stimulation of mitogen-activated protein kinase. Resultant effects on cell proliferation. J Biol Chem 274: 16423-16430, 1999.

39. Zhang Y, Turkson J, Carter-Su C, Smithgall T, Levitzki A, Kraker A, Krolewski JJ, Medveczky P and Jove R: Activation of Stat3 in v-Src-transformed fibroblasts requires cooperation of Jak1 kinase activity. J Biol Chem 275: 24935-24944, 2000.

40. Kotha A, Sekharam M, Cilenti L, Siddiquee K, Khaled A, Zervos AS, Carter B, Turkson J and Jove R: Resveratrol inhibits Src and Stat3 signaling and induces the apoptosis of malignant cells containing activated Stat3 protein. Mol Cancer Ther 5: 621-629, 2006.

41. Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, et al: Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. Oncogene 21: 2000-2008, 2002.

42. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL: Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16: 4604-4613, 1996.

43. Tolaney SM and Krop IE: Mechanisms of trastuzumab resistance in breast cancer. Anticancer Agents Med Chem 9: 348-355, 2009.

44. Buzdar AU: Role of biologic therapy and chemotherapy in hormone receptor- and HER2-positive breast cancer. Ann Oncol 20: 993-999, 2009.