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1,2,3,4,6-Penta-O-galloyl-β-D-glucose, Quercetin, Curcumin and Lycopene Induce Cell-Cycle Arrest in MDA-MB-231 and BT474 Cells through Downregulation of Skp2 Protein

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ABSTRACT: The F-box protein S-phase kinase-associated protein 2 (Skp2), which acts as an oncogene through targeting p27 for degradation, is overexpressed in many different human cancers. Skp2 can play an important role in breast cancer progression and may also be a novel molecular target for the treatment of breast cancer, especially estrogen receptor (ER)/human epidermal growth factor 2 (HER2) negative breast cancers. Unfortunately, specific drugs that target Skp2 are unavailable at present. Therefore, it is important to explore whether commonly used chemopreventive agents may downregulate Skp2 expression. In this study, we examined the effects of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (pentagalloylglucose, 5gg), quercetin, curcumin and lycopene on the expression of Skp2 in MDA-MB-231 (ER/HER2-negative) and BT474 (ER-negative/HER2-positive) cells. We found that all four phytochemicals studied induced cell growth inhibition in MDA-MB-231 cells. The mechanism of the initial growth inhibitory events involves blocking the cell cycle progression. Further, we found that quercetin and curcumin induced growth arrest by inhibition of Skp2, and induced p27 expression in MDA-MB-231 cells. However, the decrease in Skp2 levels in cells treated with 5gg or lycopene did not translate to p27 upregulation. Consequently, the downregulation of Skp2 did not always correlate with the upregulation of p27, suggesting that phytochemical-dependent downregulation of Skp2 can influence cell growth in several ways. Several studies have demonstrated that Skp2 directs the ubiquitylation and subsequent degradation of forkhead box protein O1 (FoxO1). Furthermore, our results reveal that FoxO1 protein was increased after 5gg, quercetin, curcumin and lycopene treatment. The therapeutic strategies designed to reduce Skp2 may therefore play an important clinical role in treatment of breast cancer cells, especially ER/HER2-negative breast cancers.

KEYWORDS: Skp2, pentagalloylglucose, quercetin, curcumin, lycopene, anticancer

1. INTRODUCTION

Breast cancer is by far the most common cancer in women in industrialized countries and the second leading cause of cancer-related deaths in women. The prognosis and clinical management of patients with breast cancer are commonly determined by traditional clinicopathological factors such as tumor size and grade, lymph node status, and the expression of receptors to estrogen (ER), progesterone, and HER2.[1] Nevertheless, patients can display different response to therapy and present with significantly different outcomes despite similar clinicopathological features. Therefore, an understanding of the molecular mechanisms involved in breast cancer formation and progression should be helpful in developing more effective treatments for breast cancer.

The F-box protein S phase kinase-associated protein 2 (Skp2) was originally identified as a protein associated with the S-phase promoting kinase cyclin A-cyclin-dependent kinase 2 (Cdk2). It appears to play a particularly important role in the regulation of mammalian cell cycle progression. Levels of Skp2 decrease as cells exit the cell cycle and increase as cells re-enter the cycle. Interestingly, levels of the SCF^{Skp2} components Skp1, Roc1/Rbx1 and Cul1 do not change significantly during the cell cycle, whereas the expression of Skp2 is cell cycle regulated. It can first be detected at the transition between the G_1 and S phases; amounts of Skp2 then accumulate during S/G_2 and drop as cells proceed through the M phase. At present, little is known about the mechanisms underlying Skp2 periodicity.

Overexpression of SCF components has been found in many human cancers.[2] The F-box protein Skp2, which acts as an oncogene through targeting p27 for degradation, is overexpressed in a number of human cancers including oral squamous carcinoma,[3] small cell lung carcinoma,[4] breast cancer[5,6] and prostate cancer.[7] An inverse relationship between levels of Skp2 and p27 is observed in a large variety of human cancers, and high expression of Skp2 correlates with poor prognosis.[8] In addition, Skp2 mediates the degradation of other cell cycle regulatory proteins that may contribute to cancer progression, including cyclin E, p57, p21, p130, FoxO1, c-Myc and E2F1.[9]

Targeted therapy with tamoxifen and trastuzumab (Herceptin) is available for estrogen receptor-positive (ER-positive) and HER2-overexpressing breast tumors, respectively.[10,11] However, no specific therapy has been identified in those tumors that are both ER- and HER2-negative. Skp2 plays an important role in breast cancer, and is also considered to have strong independent prognostic potential. Signoretti et al. have previously demonstrated

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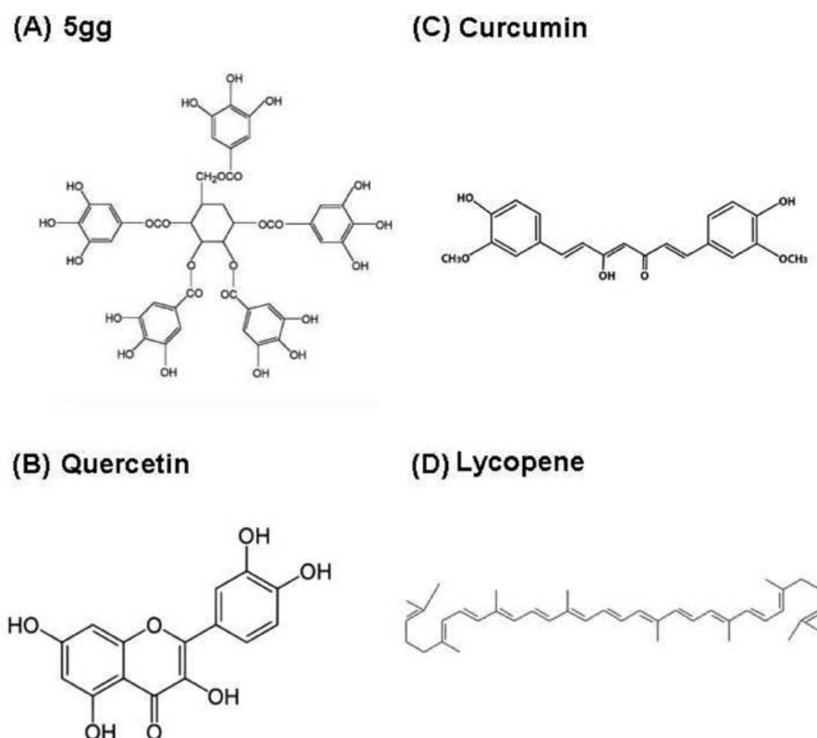

Figure 1. Chemical structure of (A) 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (pentagalloylglucose, 5gg), (B) quercetin, (C) curcumin, and (D) lycopene.

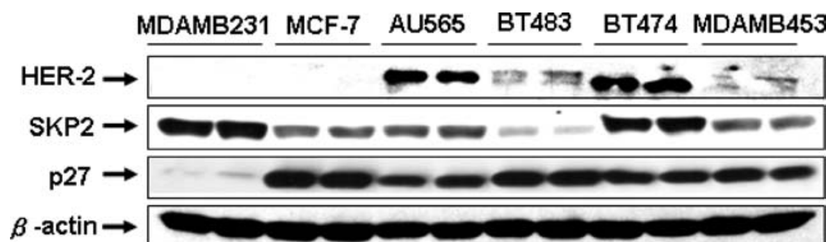

Figure 2. Levels of HER-2, Skp2 and p27 in breast cancer cell lines. Western blot analysis was employed to determine the expression levels of Skp2 and p27 in ER/HER2-negative (MDA-MB231), ER-positive (MCF-7), and HER2- positive (AU565,BT483, BT474, and MDA-MB-453) cells. At least two independent cell lysate preparations from each cell line were used in the analysis. β-Actin was utilized to ensure even loading.

that Skp2 is highly expressed in 61−67% of ER-negative tumors, and it is also expressed at higher levels in 15−23% of ER-positive tumors.[5] Since Skp2 expression is associated with lack of both ER and HER2, and Skp2 inhibition affects the *in vitro* growth of ER/HER2-negative breast cancer cells.[5,12] Thus, Skp2 can contribute to identify prognostic biomarkers that can aid in the development of targeted therapeutic strategies for ER/HER2-negative breast cancer. Unfortunately, specific drugs that target Skp2 are unavailable at present. Therefore, it is important to explore whether commonly used chemotherapies may down-regulate Skp2 expression. However, the regulatory effects of many of these drugs on the expression of Skp2 are currently unknown.

There has been considerable interest in the use of phytochemicals for the treatment of breast cancer. Epidemiological studies have shown that the consumption of vegetables, fruits and tea is associated with a decreased risk of cancer and cardiovascular diseases, and phytochemicals are believed to play an important role in preventing these diseases.[13−15] Phytochemicals in human diet may reduce the risk of various cancers, especially breast and prostate cancers, and prevent menopausal symptoms.[16−18] These data indicate that certain phytochemicals may be used as chemopreventive agents.

Our previous study has demonstrated that EGCG attenuated Skp2 protein levels, thereby inhibiting p27 ubiquitination and promoting p27 accumulation in estrogen-stimulated MCF-7 cells.[19] In an attempt to search for compounds more effective than EGCG, we examined the inhibitory effect of phytochemicals on the expression of Skp2 protein. In the present study, we examined the effect of other natural phytochemicals, such as 5gg, quercetin, curcumin, and lycopene (Figure 1), on Skp2 gene expression in MDA-MB-231 and BT474 cells. 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (pentagalloylglucose, 5gg) is structurally similar to (−)-epigallocatechin gallate (EGCG) in that it contains galloyl groups and an important component in traditional Chinese crude drugs.[20] Quercetin is a flavonoid mostly found in onions, extra-virgin olive oil and broccoli.[21] Curcumin is a polyphenol derived from *Curcuma longa*, commonly called turmeric.[22] Lycopene is the most abundant carotenoid in tomatoes.[23] All these compounds have been shown to be breast

cancer-preventive agents, albeit by several different mechanisms. Although the anticancer activities of the four phytochemicals have over the years attracted the attention of many researchers, the detailed mechanisms of the four phytochemicals on cancer prevention are not fully deciphered.

2. MATERIALS AND METHODS

2.1. Chemicals. 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose was isolated from the leaves of *Macaranga tanarins* (L.) MUELL. *et* ARG. as described previously.[24] Lycopene, curcumin, quercetin, cycloheximide, LLnL, and MTT were purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies for Skp2, cullin1, p21, p27 (C-19), cyclin A, cyclin D, cyclin E, cdk2, and cdk4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for phospho-RB were purchased from Cell Signaling Technology (Beverly, MA). β-Actin antibody was from Abcam Inc. (Cambridge, MA). Anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents and chemicals were purchased from Sigma and were of analytical grade.

2.2. Cell Culture. The human breast cancer cell lines used in this study were MDA-MB-231, MDA-MB-453, AU565, BT483, BT474, and MCF-7. MCF-7 was cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and 1% penicillin−streptomycin, and other cell lines were cultured in DMEM/F-12. These cells were grown at 37 °C in a humidified atmosphere of 5% CO2.

2.3. Immunoblotting (IB, Western Blotting). Cells were treated with various agents as indicated in figure legends. After treatment, cells were placed on ice, washed with cold PBS, and lysed in lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 25 mM NaF, 0.5 mM sodium orthovanadate, 1 mM DTT, 1 μg/mL pepstanin, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.1 mg/mL phenylmethylsulfonyl fluoride]. Cell lysates were centrifuged twice at 12,000 rpm for 30 min at 4 °C. Protein content was determined against a standardized control, using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Each lane was loaded with 50 μg of protein separated on sodium dodecyl sulfate−polyacrylamide gel (SDS−PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membrane (Immobilon^P, Millipore, Bedford, MA, USA). The membrane was preincubated in phosphate buffered-saline (PBS) containing 0.01% Tween-20, 1% bovine serum albumin (BSA), and 0.2% NaN_3 overnight at 4 °C. It was then incubated with a different primary antibody, followed by secondary anti-rabbit/goat/mouse IgG conjugated with horseradish peroxidase. The immunoreactive bands were visualized with enhanced chemiluminescent reagents (ECL, Amersham).

2.4. MTT Assay. Cells were seeded at 2×10^4 cells/well in a 24-well plate for 24 h, treated with varying concentrations of 5gg, quercetin, curcumin, or lycopene, and incubated for an additional 48 h. The effect of 5gg, quercetin, curcumin, or lycopene on cell growth was examined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 20 μL of MTT solution (5 mg/mL, Sigma Chemical Co.) was added to each well and incubated for 4 h at 37 °C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 500 μL of dimethyl sulfoxide (DMSO). Finally, the absorbance was monitored by a microplate reader at a wavelength of 550 nm.

2.5. Flow Cytometry. Cells (1×10^6) were cultured in 10 cm Petri dishes and incubated for various times. They were then harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of iced 100% ethanol at −20 °C. After being left to stand overnight, cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37 °C for 30 min. Then 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry (BD Biosciences, San Jose, CA).

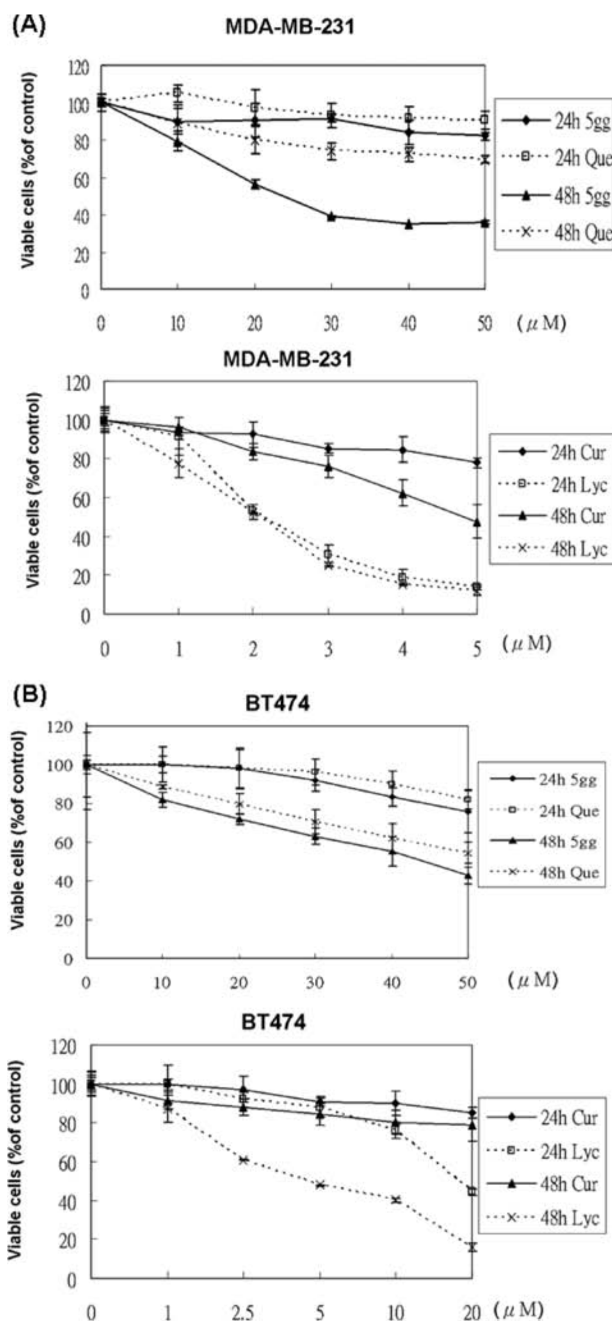

Figure 3. Effects of 5gg, quercetin, curcumin, or lycopene on cell proliferation in MDA-MB231 (A) and BT474 (B) cells. Cells were cultured in DMEM/F-12 supplemented with 10% fetal calf serum for 24 h. After 24 h culture, cells were synchronized by serum-free medium for 24 h, followed by addition of 5% FCS and 5gg, quercetin, curcumin or lycopene of different concentrations, as indicated. Cell growth inhibition was determined by MTT assays. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. Data are means of three independent experiments. Bars represent the ±SE.

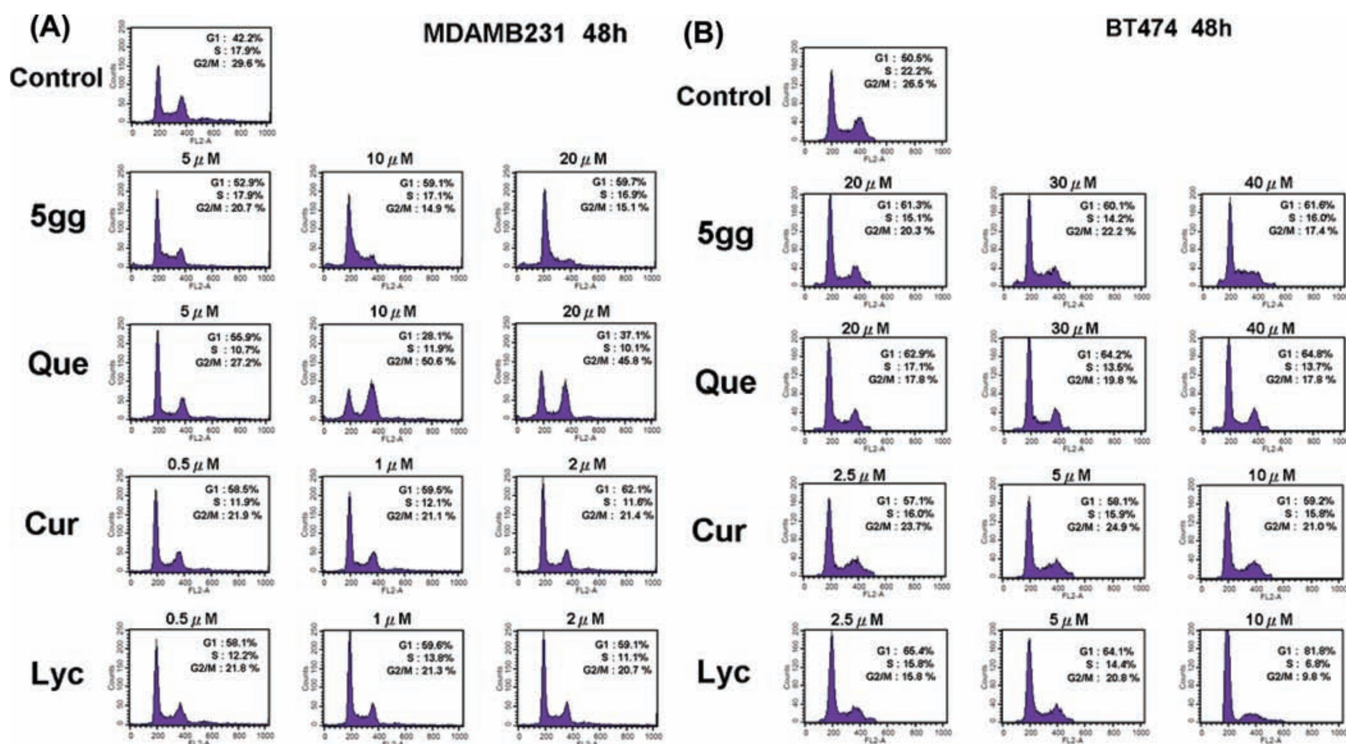

Figure 4. Changes in cell cycle phase distribution following 5gg, quercetin, curcumin, or lycopene treatment. MDA-MB231 and BT474 cells were cultured in DMEM/F-12 supplemented with 10% fetal calf serum for 24 h. After 24 h culture, cells were synchronized by serum-free medium for 24 h, followed by addition of 5% FCS and 5gg, quercetin, curcumin or lycopene of different concentrations for 24 or 48 h. Cells were harvested and stained with propidium iodide, and the cell cycle distribution was analyzed by flow cytometry.

2.6. RNAi Suppression of Skp2. Logarithmically growing MDA-MB-231 cells were seeded at a density of 1.0 × 10^5 cells/well in six-well plates in serum-containing medium. The Skp2 p45 siRNA gene silencer (human) dsRNA was obtained from Santa Cruz Biotechnology (sc-36499). MDA-MB-231 cells were transfected with dsRNAs using siRNA Transfection Reagent (Santa Cruz) and incubated for 6 h. Afterward, the cells were analyzed by immunoblot for Skp2 and p27 expression. Cell viability was assessed using Trypan blue. This experiment was repeated three times.

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen) as recommended by the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) 18 primer by incubating the reaction mixture (20 μL) at 37 °C for 60 min. Amplification of cDNA was performed by polymerase chain reaction (PCR) in a final volume of 50 μL containing 2 μL of RT product, dNTPs (each at 200 μM), 1× reaction buffer, a 1 μM concentration of each primer (Skp2, forward 5′-ACAGTGAGAACATCCCCCAG-3′, reverse 5′- GGTCCATAAATGATCGTGGG-3′; p27, forward 5′-AAACGTGCGAGTGTCTAACGGGA, reverse 5′- CGCTTCCTTATTCCTGCGCATTG-3′; GAPDH, forward 5′- TGAAGGTCGGTGTGAACGGATTTGGC-3′, reverse 5′-CATGTAGGCCATGAGGTCCACCAC-3′), and 50 units/mL Pro Taq DNA polymerase. After an initial denaturation for 5 min at 95 °C, 35 cycles of amplification (95 °C for 30 s, 58 °C for 1 min, and 72 °C for 2 min) were performed, followed by 72 °C for 10 min. A 5 μL sample of each PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

2.8. Statistical Analysis. All values were expressed as mean ± SE. Each value is the mean of at least three separate experiments in each group. The differences in the effects of compound treatment when compared with vehicle-treated control values were analyzed by *t* test as appropriate.

3. RESULTS

3. 1. Relationship between Skp2 and p27 Expression in Different Breast Cancer Cells. The role of Skp2 as an oncogene responsible for downregulation of p27 protein levels is well established in a wide variety of cancers, including early breast cancer. To address a possible correlation between Skp2 and p27 expression in breast cancer cell, we first analyzed Skp2 and p27 protein levels in ER/HER2-negative (MDA-MB231), ER-positive (MCF-7), and HER2-positive (AU565, BT483, BT474, and MDA-MB-453) breast cancer cells. Using Western blot analysis, we detected Skp2 expression in all cell lines (Figure 2). The highest expression was found in MDA-MB231 and BT474 cells. While high levels of Skp2 accompanied low levels of p27 in MDA-MB-231 cells, the opposite condition was found in MCF-7, AU565, BT483, and MDA-MB-453 cells (i.e., low Skp2 levels with high p27 levels). These results suggest a significant inverse correlation between p27 and Skp2 protein expression in MDA-MB231, MCF-7, AU565, BT483, and MDA-MB-453 cells. In contrast, high levels of Skp2 and p27 were observed in BT474 cells. Next, we selected MDA-MB231 and BT474 cells, which have the highest Skp2 expression among the six cell lines, to carry out a series of experiments to determine whether downregulation of Skp2 by phytochemicals could influence the growth of breast cancer cells.

3.2. Lycopene Preferentially Inhibited Skp2-Overexpressing Breast Cancer Cells. To assess the biological activity of 5gg, quercetin, curcumin, and lycopene in terms of cell proliferation, MDA-MB-231 and BT474 cells were treated with these plant phytochemicals of different concentrations for 24 and 48 h. Figures 3A and 3B show the growth response of MDA-MB-231 and BT474 cells, respectively, to the four phytochemicals of

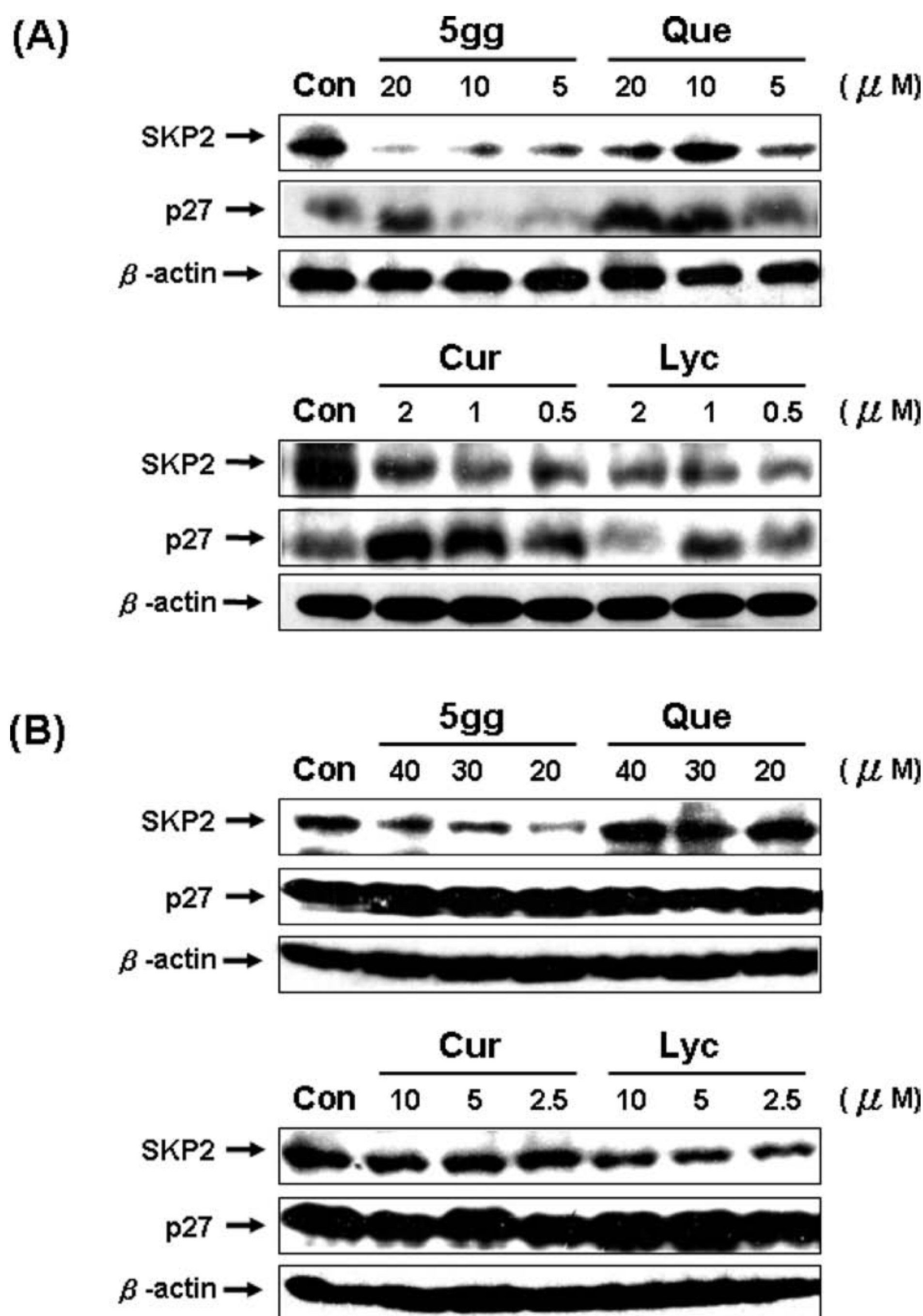

Figure 5. Effect of 5gg, quercetin, curcumin, and lycopene on the expression of Skp2 and p27 protein. (A) MDA-MB-231 and (B) BT474 cells were cultured in DMEM/F-12 supplemented with 10% fetal calf serum for 24 h. After 24 h culture, cells were synchronized by serum-free medium for 24 h, followed by addition of 5% FCS and 5gg, quercetin, curcumin or lycopene of different concentrations for 48 h. Immunoblotting was employed to measure protein levels of Skp2, p27 and β-actin.

varying concentrations. The growth of the tested cell lines was inhibited by 5gg, quercetin, curcumin, and lycopene in a dose- and time-dependent manner but to varying extents. MDA-MB-231 cells showed higher susceptibility to 5gg, curcumin, and lycopene than did BT474 cells, resulting in lower cell viability. In contrast, quercetin showed higher cytotoxicity against BT474 cells. At a 5 μM concentration, lycopene blocked 86% of growth in MDA-MB-231 cells (48 h). However, under the same conditions, curcumin inhibited only 43% of growth in MDA-MB-231 cells. Moreover, high-dose (20−50 μM) 5gg treatment significantly enhanced growth inhibition of MDA-MB-231 cells, but low-dose 5gg treatment did not. Overall, these results suggest that lycopene preferentially suppresses the growth of ER/HER2-negative breast cancer cells.

3.3. 5gg, Quercetin, Curcumi, and Lycopene Induced Cell Cycle Arrest in Skp2-Overexpressing Cells. To elucidate the mechanism underlying the antiproliferative effect of 5gg, quercetin, curcumin and lycopene, cell cycle progression in MDA-MB-231 and BT474 cells was investigated. The percentages of cell cycle were determined by flow cytometry. After a 24 h

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treatment exposure to 10−20 μM 5gg, around 60% of cells were observed at G1 phase compared with 40% of control cells (data not shown). In contrast, the cell cycle distribution of MDA-MB-231 and BT474 cells was not significantly altered by quercetin, curcumin or lycopene treatment at 24 h (data not shown). After 5gg or lycopene treatment for 48 h, the two breast cancer cells were arrested in G1 phase. However, quercetin arrested MDA-MB-231 cells at G2/M phase, whereas BT474 cells were arrested at G1 phase. Curcumin induced G1-phase cell cycle arrest in MDA-MB-231 cells, but not in BT474 cells (Figures 4A and 4B). These results show that phytochemicals induce cell growth inhibition accompanied by blocking of cell cycle progression. Next, we investigated the effect of phytochemicals on intracellular expression of Skp2 and p27 by Western blot analysis. In MDA-MB-231 cells, we found that upregulation of p27 was accompanied by a reduction in Skp2 after quercetin and curcumin exposure. A significant decrease in Skp2 levels was also observed in 5gg- and lycopene-treated cells, but this was associated with a slight decrease in p27 levels (Figure 5A). In BT474 cells, downregulation of Skp2 was also observed in 5gg- and lycopene-treated cells while no significant change in p27 protein level was detected (Figure 5B). However, the protein levels of p27 and Skp2 did not significantly change in quercetin- and curcumin-treated cells. The downregulation of Skp2 did not always correlate with the upregulation of p27, suggesting that phytochemicals dependent on Skp2 downregulation can influence cell growth in several ways.

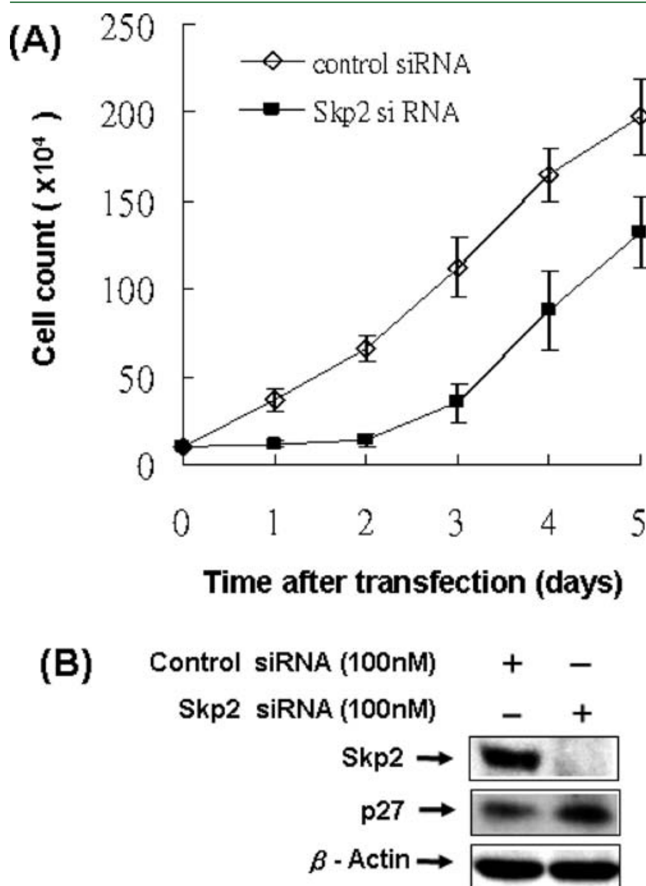

Figure 6. Growth curve of human breast cancer MDA-MB-231 cells transfected with siRNA2. (A) Growth curve of MDA-MB-231 cells. Cells were transfected with S-phase kinase-associated protein 2 (Skp2), small interfering RNA (siRNA) or control siRNA and counted in triplicate wells at indicated times after transfection. The representative data of three independent experiments are shown. (B) Western blot analysis of Skp2 and p27 protein expression was performed 48 h after transfection.

3.4. Growth Inhibition of MDA-MB-231 by Skp2 RNA Interference. We examined the effects of RNA silencing of Skp2 on the growth of breast cancer in MDA-MB-231 cells. As shown in Figure 6A, the cell growth in Skp2 siRNA transfectants was affected compared with the control siRNA transfectants. RNA interference was carried out to investigate whether p27 protein levels would increase when MDA-MB-231cells, which had high protein levels of Skp2 and low levels of p27, were transfected with Skp2 siRNA. Skp2 siRNA knocked down the protein levels of Skp2 in MDA-MB-231 cells compared with control siRNA transfectants. As expected, it also increased the protein levels of p27 (Figure 6B).

3.5. Effects of 5gg, Quercetin, Curcumin, and Lycopene on mRNA Level of Skp2 in MDA-MB-231 Cells. To address the mechanism by which phytochemicals decreased the protein level of Skp2, we first examined the mRNA level of Skp2. A striking decrease in Skp2 mRNA was found in 5gg-, curcumin- and lycopene-treated cells (Figure 7), suggesting that the downregulation of Skp2 mRNA was, at least in part, attributable to the decrease in Skp2 protein expression. However, no significant change in Skp2 mRNA levels was observed in quercetin-treated cells. These results indicated that quercetin-induced decrease in Skp2 protein levels involves a post-transcriptional mechanism. Furthermore, experiments would be required to validate this possibility.

3.6. Effects of 5gg, Quercetin, Curcumin and Lycopene on Expression Levels of Cell Cycle Regulatory Proteins in Skp2-Overexpressing Cells. To determine the specific cell cycle regulatory proteins responsible for the induced cell cycle arrest following 5gg, quercetin, curcumin, or lycopene treatment, Western blot analysis using antibodies specific to cyclin B, cyclin D, cyclin E, Cdk2, Cdk4 and FoxO1 was performed. As shown in

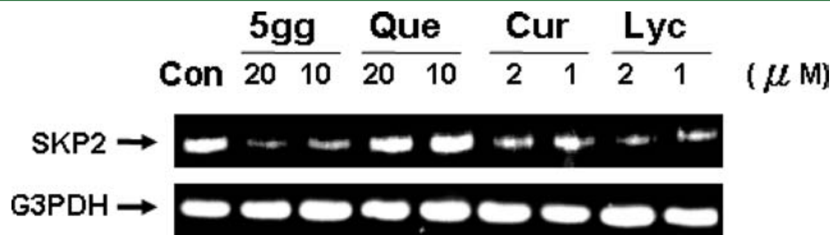

Figure 7. Effects of 5gg, quercetin, curcumin, and lycopene on Skp2 mRNA level in MDA-MB-231 cells. Cells were treated with Me_2SO alone (CON), or phytochemicals of varying concentrations for 48 h. Total RNA was isolated, and the mRNA expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as described under Materials and Methods.

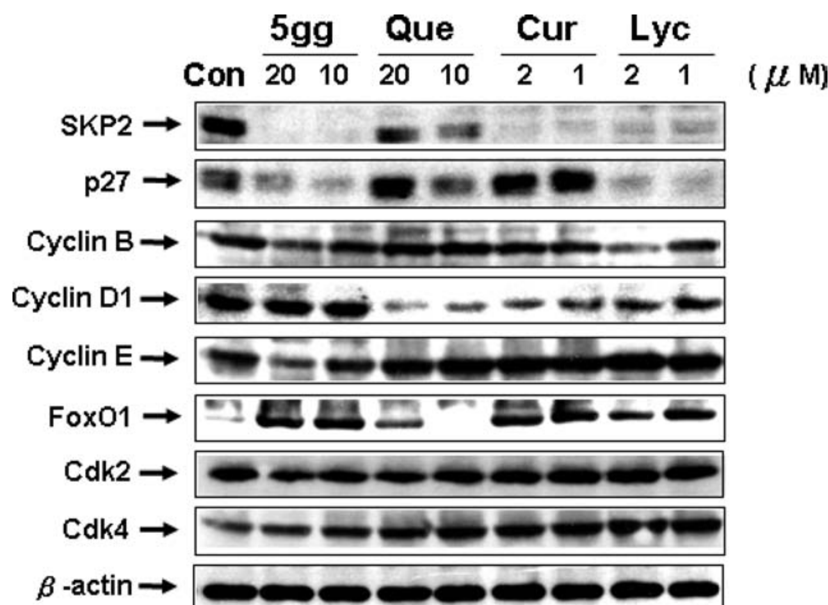

Figure 8. Effects of 5gg, quercetin, curcumin, and lycopene on expression levels of cell cycle regulatory proteins in MDA-MB-231 cells. Cells were treated with Me_2SO alone (CON), or phytochemicals of varying concentrations for 48 h. Total cell lysates were prepared, and Western blot analysis was performed. The experiment was performed thrice with each antibody, with each yielding similar results. β-Actin was used as an internal control for equivalent protein loading.

Figure 8, expression of cyclin B, cyclin E, Cdk2 and Cdk4 was not affected by 5gg, quercetin, curcumin, or lycopene. Conversely, the protein level of FoxO1 was increased. We also examined the expression of Skp2 and found that this protein was decreased after 5gg, quercetin, curcumin, and lycopene treatment. These data indicated that 5gg, quercetin, curcumin, and lycopene modulated the expression of cell cycle regulatory proteins, thus inducing cell cycle arrest.

3.7. Enhancing Effects of Tamoxifen in Phytochemicals. Tamoxifen is a very effective agent used in the treatment of breast cancer.[25] To extend the study of synergistic effects, we examined whether tamoxifen could enhance anticancer activities by 5gg, quercetin, curcumin or lycopene. Tamoxifen significantly and synergistically enhanced growth inhibition of MDA-MB-231 cells by 5gg. After 48 h of incubation with 15 μM 5gg alone, the number of viable cells was 77.3% of that in the control while the number of viable cells after treatment with 5 μM tamoxifen alone was 94.8%. Combined treatment using 15 μM 5gg and 5 μM tamoxifen reduced the number of cells to 36.4% (Figure 9). These findings indicate that 5gg and tamoxifen have a synergistic effect on the inhibition of MDA-MB-231 cell growth. In contrast, combined treatment using 5 μM tamoxifen and 15−20 μM quercetin, 5 μM tamoxifen and 1.5−2 μM curcumin, or 5 μM tamoxifen and 1.5−2 μM lycopene did not show synergistic growth inhibition.

3.8. Effect of Various Phytochemicals on Skp2-Overexpressing Human Breast Cancer Cells. Phytochemicals are naturally occurring plant polyphenols found abundantly in diets rich in fruit, vegetables and plant-derived beverages such as tea. Depending on their structure, some phytochemicals inhibit tyrosine kinase and serine/threonine kinase activities.[26] In order to determine the biological activities involved, we examined the inhibitory activities of various phytochemicals on the protein level of Skp2 in Skp2-overexpressing breast cancer cells.

We treated separately the Skp2-overexpressing human breast cancer MDA-MB-231 and BT474 cells with 21 compounds at 37 °C for 48 h in the presence of 5% serum. After treatment, the Skp2 protein was analyzed by Western blot assay. As shown in Figure 10, we found that the Skp2 protein in MDA-MB-231 cells could be degraded in the presence of lycopene, curcumin, quercetin, kaempferol, 5gg, resveratrol, apigenin, and ellagic acid. In BT474 cells, however, Skp2 expression protein was only reduced after 5gg and lycopene treatment.

4. DISCUSSION

Breast cancer is the most common cancer in women and the second leading cause of cancer-related deaths in women. Estrogen receptor (ER) expression and HER2 amplification define specific subsets of breast tumors for which specific therapies exist. Targeted therapy with tamoxifen and trastuzumab (Herceptin) is available for estrogen receptor-positive (ER-positive) and HER2-overexpressing breast tumors, respectively. However, no specific therapy has been identified in tumors that are both ER- and HER2-negative. The S-phase kinase-associated protein Skp2 is required for the ubiquitin-mediated degradation of p27 and is a proto-oncoprotein. Clinical studies have demonstrated that Skp2 is highly expressed in ER/HER2-negative (MDA-MB-231) and ER-positive tumors (MCF-7). Thus, Skp2 can contribute to identify prognostic biomarkers that can aid in the development of targeted therapeutic strategies for ER/HER2-negative and ER-positive breast cancers. At present, there are no specific drugs that can target Skp2 *in vivo*. Routine clinical use of small interfering RNA (siRNA), which can effectively downregulate Skp2 levels *in vitro*,[27,28] is not yet feasible. Thus, studies are now being undertaken to examine the effects of different chemotherapies on Skp2 expression.

Epidemiological studies have shown that the intake of certain vegetables, fruits, and tea in the daily diet provides effective cancer prevention.[29,30] These effects have been attributed to the flavonoids and related flavanols in these plants. In a recent study

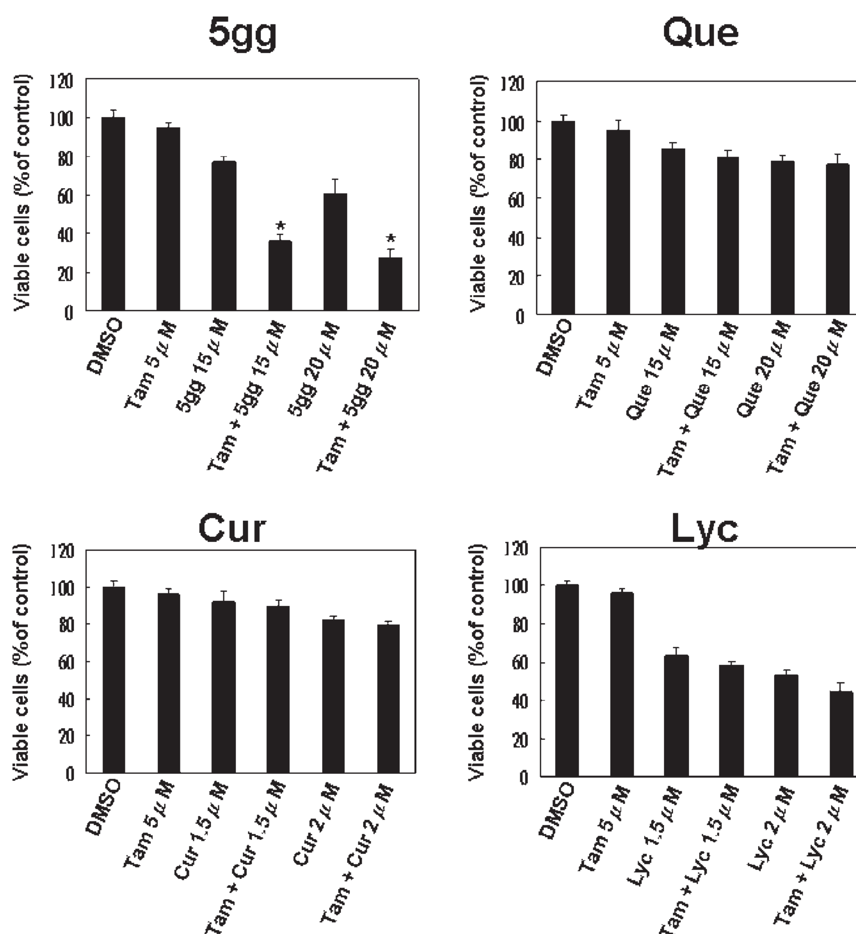

Figure 9. Effects of 5gg, quercetin, curcumin, lycopene, and tamoxifen on cell proliferation. MDA-MB-231 cells were cultured in DMEM/F-12 supplemented with 10% fetal calf serum for 24 h. After 24 h culture, cells were synchronized by serum-free for 24 h, followed by addition of 5% FCS and 5gg, quercetin, curcumin, lycopene, or tamoxifen of different concentrations, as indicated. Cell growth inhibition was determined by MTT assays. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. Data are means of three independent experiments. Bars represent the SE. The combination of phytochemicals and tamoxifen was more effective than either agent alone. *$p < 0.05$.

from our laboratory, we found that EGCG downregulated Skp2 in estrogen-stimulated MCF-7 cells in a dose-dependent manner, leading to increased expression of p27 levels. EGCG provided deep insights into this concept, opening the door to the development of potent agents that can pharmacologically target Skp2.[19]

In this study, we examined the effects of four phytochemicals on the expression of Skp2 in MDA-MB-231(ER/HER2-negative) and BT474 (HER2-positive) cells. Each of the phytochemicals used in our study has been shown to inhibit the growth and proliferation of several cancer cell lines and tumors in animal models;[31−37] hence, they are good chemopreventive candidates. However, the molecular mechanisms involved remain largely unknown. We found that all four phytochemicals studied induced cell growth inhibition in MDA-MB-231 cells. The mechanism of the initial growth inhibitory events involves blocking of cell cycle progression. 5gg, curcumin and lycopene induced G1 phase arrest while quercetin caused G2/M phase arrest. In BT474 cells, 5gg, quercetin and lycopene induced both G1 phase arrest and cell growth inhibition. In contrast, curcumin at <10 μM did not induce G1 phase arrest and cell growth inhibition. In both cell lines, lycopene was the most potent with regard to inhibition of cell growth. Further, we found that quercetin and curcumin induced growth arrest by inhibition of Skp2, and induced p27 expression in MDA-MB-231 cells. Our findings are consistent with previous research showing that Skp2-deficient mice exhibit cellular accumulation of p27.[38] However, the decrease in Skp2 levels in cells treated with 5gg or lycopene did not translate to p27 upregulation. These findings are in contrast to our previous observations in estrogen-stimulated MCF-7 cells, whereby estrogen treatment increased Skp2 levels, resulting in p27 downregulation; whereas blocking by EGCG repressed Skp2 levels, leading to p27 upregulation. Similar results have been found in other clinical studies. Davidovich et al.[12] reported that despite the decrease in Skp2 levels after doxorubicin-based chemotherapy, p27 levels were not increased in tumors. Bar-On et al.[39] have also demonstrated that the changes in p27 levels in doxorubicin-treated cells were not associated with the expected changes in Skp2 levels. Therefore, it is noteworthy that, despite the lack of changes in p27, Skp2 levels were independently associated with a significantly better disease-free survival. This finding evidences that Skp2 has other important oncogenic effects and the reduction in Skp2 levels is a rational therapeutic objective. Skp2 has been reported as the specific ubiquitin ligase not only for p27 but also cyclin E, p21, p57, p130, E2F1, Forkhead box O (FoxO), Forkhead box O3 (FoxO3), human Orc1 (hOrc1p), Cdk9, Cdt1,

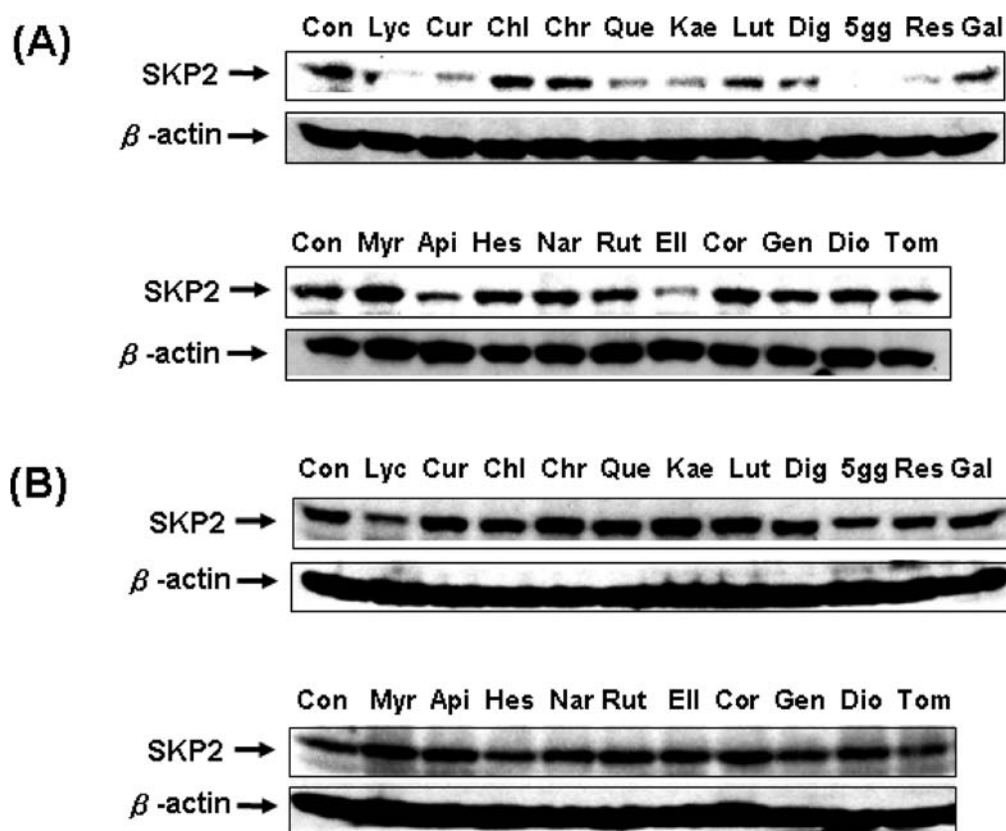

Figure 10. Effect of various phytochemicals on the expression of Skp2 protein. (A) MDA-MB-231 and (B) BT474 cells were incubated with Me_2SO (Con) or lycopene (2 μM), curcumin (2 μM), chlorogenic acid (2 μM), chrysin (20 μM), quercetin (20 μM), kaempferol (20 μM), luteolin (20 μM), diosgenin (20 μM), 5gg (20 μM), resveratrol (20 μM), galangin (20 μM), myricetin (20 μM), apigenin (20 μM), hesperetin (20 μM), narigenin (20 μM), rutin (20 μM), ellagic acid (20 μM), corilagin (20 μM), genisten (20 μM), diosmetin (20 μM), and tomatidine (20 μM) at 37 C for 48 h. Immunoblotting was used to measure protein levels of Skp2 and β-actin.

c-Myc, and B-Myb.[38] Therefore, it is still important to understand that accumulation of other known Skp2 substrates may also contribute to the apoptosis and growth inhibition induction by downregulation of Skp2. A recent report reveals that Skp2 directs the ubiquitylation and subsequent degradation of FoxO1.[38] Additionally, our results reveal that FoxO1 protein was increased after 5gg, quercetin, curcumin and lycopene treatment. The issue of whether or not FoxO1 is a direct target for the four phytochemicals needs further study.

In the clinic, endocrine therapy is an important intervention for women with breast cancers that express estrogen receptor, and treatment with tamoxifen has enhanced patient survival.[38] However, 5-year tamoxifen use has become associated with a number of serious side effects. Therefore, alternative interventions such as herbal substances are needed to replace or to supplement current regimens. There are grounds for the belief that multiple drugs in breast cancer may be more effective than single agents. In accordance with this concept, the ideal regimen would contain at least two potent drugs. For instance, the combination of tamoxifen and docetaxel synergistically inhibited the growth of three ER-negative cancer cell lines (MDA-MB-231, CEM-VBLr and MCF-7ADr).[40] Similarly, Chisholm et al.[38] demonstrated synergistic cytotoxicity when MDA-MB-231 cells were treated with tamoxifen and EGCG. Synergism has also been reported *in vivo*, as complete inhibition of DMBA-induced mammary tumors in rats was achieved following treatment with both tamoxifen and 6-MCDF.[41] Another important finding of this study was that tamoxifen significantly and synergistically enhanced growth inhibition of MDA-MB-231 cells by 5gg. Although combined treatment using 5gg and tamoxifen has produced interesting results, the mechanisms involved have not been well identified.

In conclusion, these findings provide additional insights into the induction of cell cycle arrest in breast cancer cells by phytochemicals through direct downregulation of Skp2 expression. The results presented here indicate that these phytochemicals are of potential value for chemoprevention of breast cancer.

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