Curcumin inhibits proliferation and migration by increasing the Bax to Bcl-2 ratio and decreasing NF-κBp65 expression in breast cancer MDA-MB-231 cells

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Abstract. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), is extracted from the plant *Curcuma longa*. It has cytotoxic effects and induces apoptosis in many human cancer cells but the molecular mechanisms are not fully understood. In the present study, we evaluated the effects of curcumin on human breast cancer MDA-MB-231 cells. The cytotoxic effects of curcumin on MDA-MB-231 cells were measured by MTT assay. The percentages of cell cycle were determined by flow cytometry. The protein expressions of p21, 53, Bax and Bcl-2 were examined by Western blotting. The results show that curcumin inhibits the proliferation of MDA-MB-231 cells and induces G2/M arrest in a dose-dependent manner. Curcumin increased the protein expressions of p21 and Bax, but decreased the protein expression of p53 and Bcl-2 in MDA-MB-231 cells. Our results show that one molecular mechanism of curcumin inhibits the proliferation of MDA-MB-231 cells either through up-regulating p21 expression and then inducing apoptosis, or through up-regulating the Bax to Bcl-2 ratio and then inducing apoptosis. Our results also show that curcumin inhibits the migratory activity of MDA-MB-231 cells through down-regulating the protein expression of NF-κBp65. Accordingly, the therapeutic potential of curcumin for breast cancer deserves further study.

Introduction

Curcumin (diferuloylmethane; 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a natural polyphenol derived from plants especially in several *curcuma* species, commonly known as turmeric. It has been widely used in the human diet for centuries (1). It is documented that curcumin induces cytotoxicity in many human cancer cell lines *in vitro* (2-4). That curcumin inhibits the proliferation of breast cancer cells *in vitro* is documented as well (5). Curcumin also exerts multiple suppressive effects on human breast carcinoma cells (6). That dietary curcumin inhibits chemotherapy-induced apoptosis *in vivo* was documented by Somasundaram *et al* (7). However, the efficacy and molecular mechanisms of curcumin in breast cancer are not fully elucidated. In the present study, we evaluated the effects and molecular mechanisms of curcumin on human breast cancer MDA-MB-231 cells.

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Key words: curcumin, MDA-MB-231, Bax, Bcl-2, NF-κBp65

Materials and methods

Chemicals and reagents. Curcumin was purchased from Herbasin Co. (Shenyang, China), aprotinin, antipain, sodium deoxycholate, leupeptin, propidium iodide (PI), sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A and MTT [3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazolium-romide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). L-15 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Human breast cancer cell line (MDA-MB-231 cells). The human breast cancer cell line (MDA-MB-231 cells) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C in humidified air atmosphere (CO_2 not required), in L-15 medium (Sigma Chemicals Co., St. Louis, MO), contained with 10% heat-inactivated FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin; 10 mg/ml streptomycin). All data presented in this report are from at least three independent experiments.

The effects of curcumin on the viability of MDA-MB-231 cells. The MDA-MB-231 cells were plated in a density of $1x10^5$ cells/well and grown for 24 h. The various concentrations of curcumin (0, 10, 20 and 30 μg/ml) were added and cells were grown for 24, 48, 72 h, while only adding 0.2% DMSO (solvent) for the control regimen. For determining cell viability,

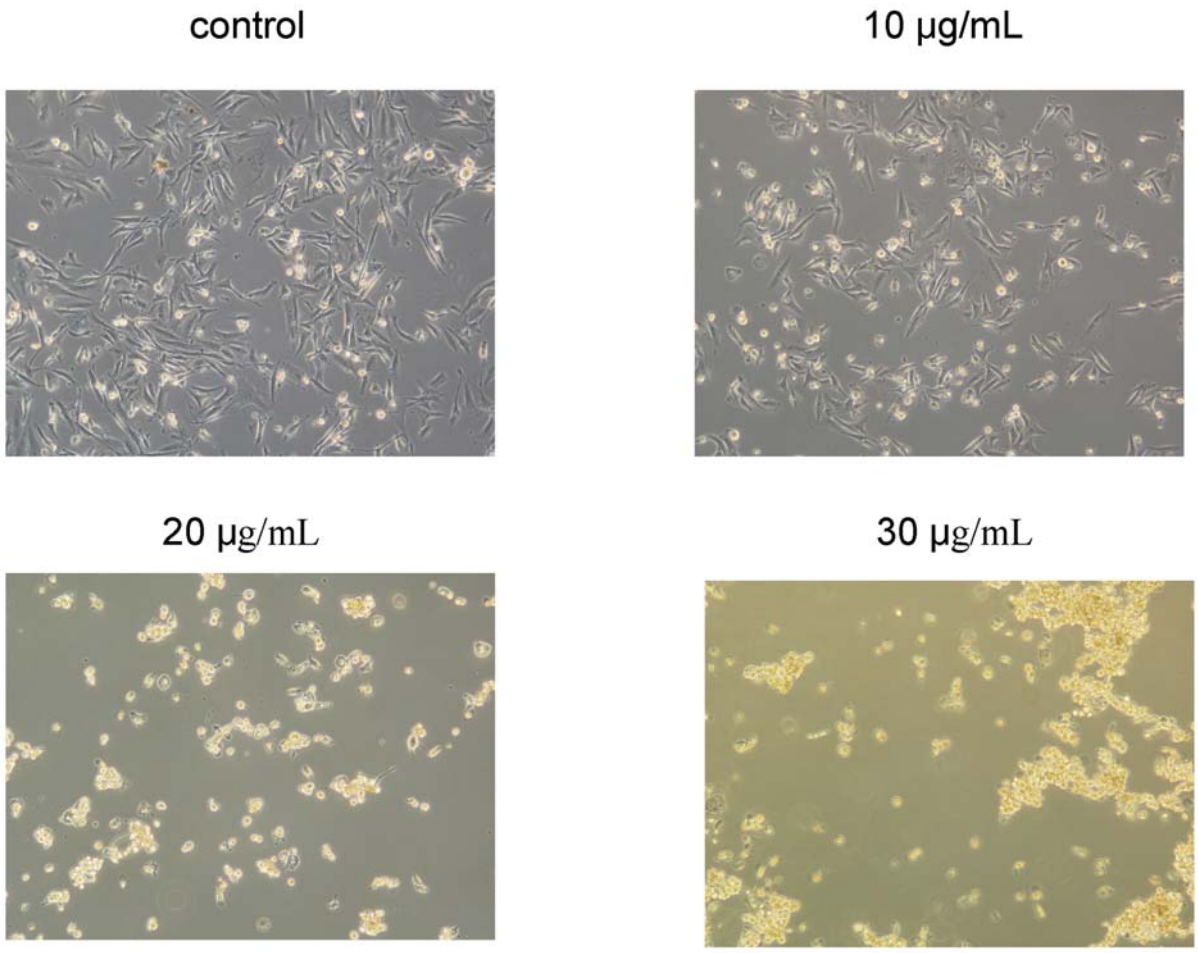

Figure 1. MAD-MB-231 cells were treated with various concentrations of curcumin for 48 h and examined under contrast-phase microscope (x100) and photographed.

after 24, 48, 72 h of culture, the viability was evaluated by MTT assay in triplicate. Briefly, MDA-MB-231 cells were seeded in a 6-well plate at a density of $1x10^5$ cells/well and allowed to adhere overnight. After removing the medium, 2,000 μl of fresh medium per well, containing 10 mM HEPES, was then added. Then 200 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the wells and the plate was incubated for 30 min at 37˚C in the dark. The medium was removed and 1,000 μl DMSO was added to the wells. Absorbance was measured using an ELISA plate reader at 590 nm (8).

The cell cycle analysis for MDA-MB-231 cells treated with curcumin by flow cytometry assay. The percentage of cells in sub-G1, G0/G1, S and G2/M phases were determined by flow cytometry as previously described (9). Briefly, $1x10^6$ MDA-MB-231 cells/10-cm dish were incubated with curcumin at 0, 5, 10, 15 and 20 μg/ml concentrations for 48 h before the cells were harvested by centrifugation. After being harvested the cells were washed with PBS and then fixed gently (drop by drop) in 70% ethanol (in PBS) in ice overnight and then resuspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase (Sigma) and 0.1% Triton X-100. After 30 min at 37˚C in the dark, the cells were analyzed with flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. Then cell cycle and apoptosis were determined and analyzed using ModFit software. The average of the percentage for each phase in the cell cycle was representative of three independent experiments.

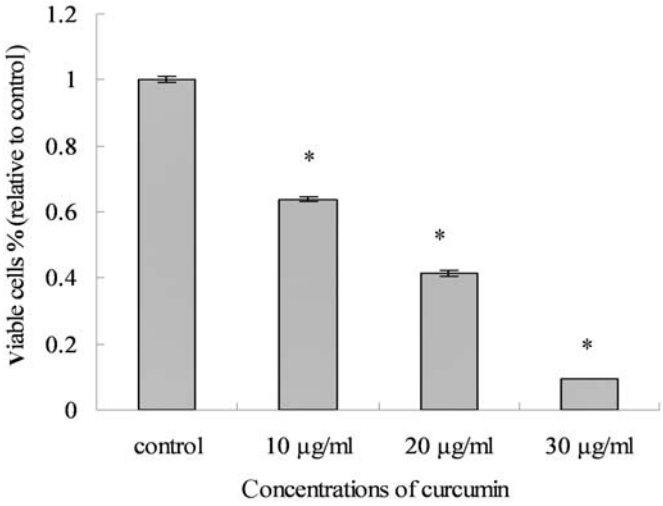

Figure 2. The MDA-MB-231 cells were plated in a density of $1x10^5$ cells/well and grown for 24 h. The various concentrations tanshinone IIA were added and cells grown for 48 h, while only adding DMSO 0.2% (solvent) for the control regimen. After 48 h of culture, the viability was evaluated by MTT assay in triplicate. Each point is mean ±SD of three experiments. *P<0.05.

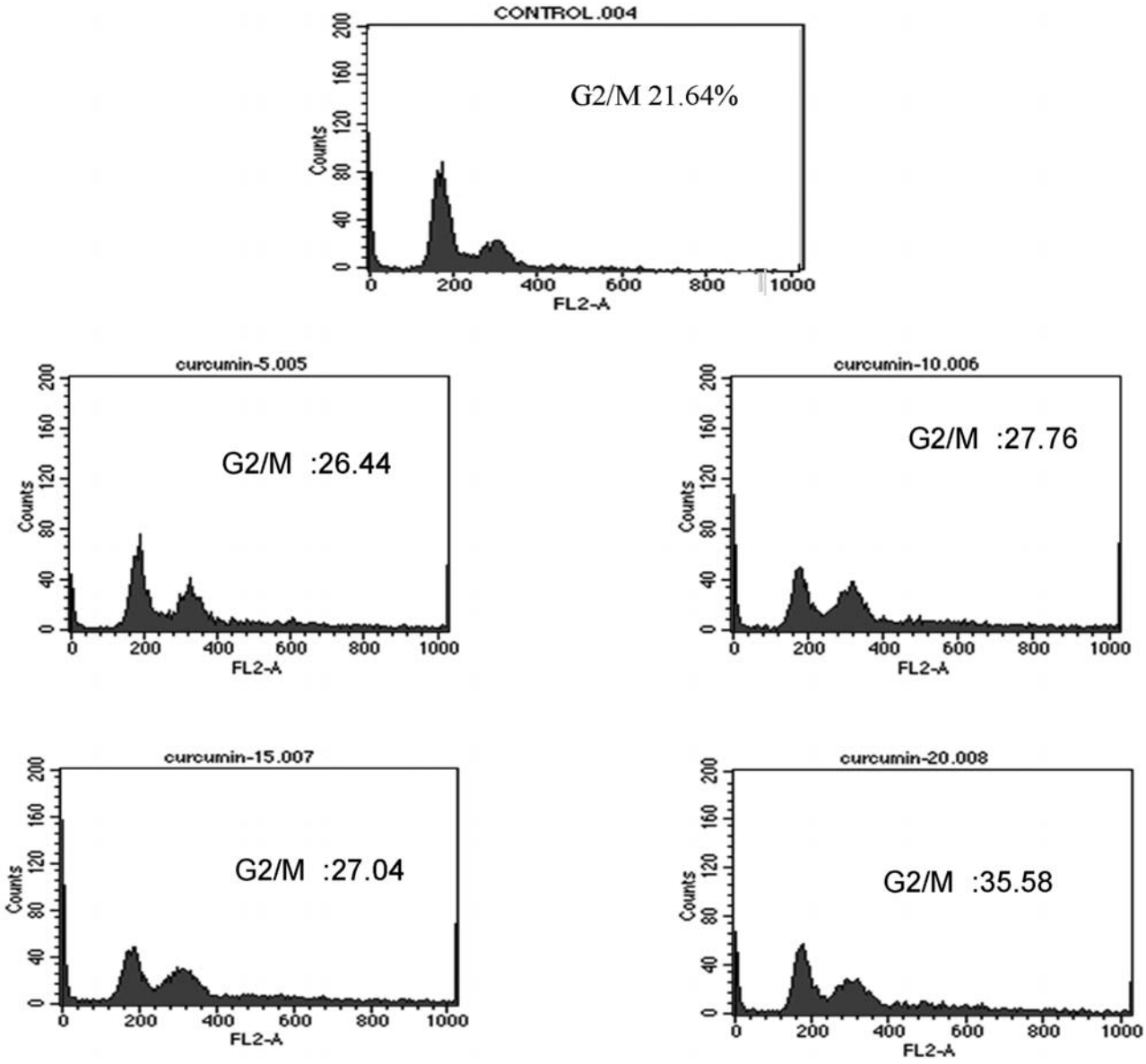

Figure 3. MDA-MB-231 cells were treated with curcumin for 24 h. Then cell cycle percentage was checked by FACS.

Protein preparation. About 1x10^6 cells/10-cm dish were incubated with curcumin at 0, 5, 10 and 20 μg/ml concentrations for 24 h before cells were harvested by centrifugation. About 1x10^6 cells/10-cm dish were incubated with curcumin at a 20 μg/ml concentration for different durations (0, 6, 12 and 24 h) before the cells were harvested by centrifugation. Protein was extracted as previously described (10). Briefly, cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 0.25% sodium deoxycholate, EGTA, DTT, PMSF, sodium orthovanadate, sodium fluoride 1 mM each, aprotinin, leupeptin and antipain 5 μg/ml each) for 30 min at 4˚C. Lysates were immediately centrifuged at 13,000 x g for 20 min at 4˚C and supernatant was collected, aliquoated (50 μg/tube) and stored at -80˚C until assay. The protein concentrations were measured with Bradford methods (11).

Western blotting for examining the effect of curcumin on the expressions of bax, bcl-2, p21, 53 and ß-actin in MDA-MB-231 cells. All samples were separated by sodium dodecylsulfate polyacrylamide (SDS-PAGE) gel electrophoresis (10 and 13%) (Bio-Rad Life Science Products, Hercules, CA, USA) as previously described (10). The SDS-separated proteins were followed by equilibration in transfer buffer [50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS, 20% methanol and electro transferred to Immobilon-P Transfer Membrane (Millipore Co., Bedford, MA, USA)]. Then the blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl, Sigma Chemical Co.) containing 0.05% Tween-20 for 1 h, washed and incubated with antibodies to bax, bcl-2, p21, 53 and ß-actin (Upstate, Lake Placid, NY, USA) at 4˚C overnight. After incubating with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the signal was visualized by enhanced chemiluminescence (ECL, Amerham Pharmacia Biotech). The detection of ß-actin was used as an internal control in all data for Western blotting.

The effects of curcumin on migration of MDA-MB-231 cells were assayed by modify Boyden chamber. Chemotactic migration of MDA-MB-231 cells were assayed by modify

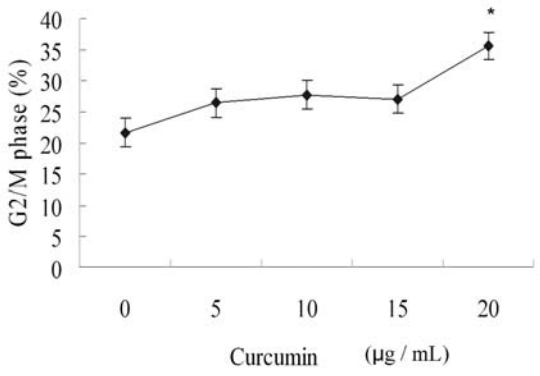

Figure 4. MDA-MB-231 cells were treated with curcumin for 24 h and then cell cycle change was checked by FACS. When the concentration of curcumin increased, the G2/M phase arrest was noted.

Boyden chamber. Briefly, MDA-MB-231 cells were suspended in serum-free RPMI-1640. MDA-MB-231 cells were added to the upper wells (24-well Boyden micro chambers) at $5x10^4$ cells/well in the presence or absence of curcumin while fibronectin (10 mg/ml) was present in the lower chamber. If the cells migrate, they traverse a polycarbonate filter from the upper chamber to the lower chamber. After 24 h at 37˚C in 5% CO_2, the cells that traversed to the lower chamber and spread on the lower membrane surface were collected and the number of migratory cells per membrane was enumerated by trypan blue (BioWest) exclusion.

Statistical analysis. Values are presented as mean ±SD. The Student's t-test was used to analyze the statistical significance. P-values <0.05 were considered significant for all tests.

Results

Morphological changes of human breast cancer MDA-MB-231 cells in response to various concentrations of curcumin. The MDA-MB-231 cells were treated with various concentrations (0, 10, 20 and 30 μg/ml) of curcumin for 48 h. Cells were examined under contrast-phase microscope (x100) and photographed (Fig. 1). The results indicate that the number of viable cells decrease as the concentration increases, suggesting that curcumin induces cell death on MDA-MB-231 cells.

The effects of curcumin on the viability of MDA-MB-231 cells. The MDA-MB-231 cells ($1x10^5$ cells/well) were plated in L-15 medium + 10% FBS with various concentrations (0, 10, 20 and 30 μg/ml) of curcumin for 48 h. Then cells were collected by centrifugation and viable cells were determined by MTT assay as described in Materials and methods. The viable cell percentages relative to control were 100±2.04, 63.74±2.38, 41.61±4.78, 9.42% respectively. When cultured with various concentrations of curcumin (0, 10, 20 and 30 μg/ml) for 48 h (Fig. 2). The IC_{50} was 16.25 μg/ml, when MDA-MB-231 cells were treated with curcumin for 48 h. The proliferation of colo 205 was inhibited by curcumin in a dose- and time-dependent manner.

Curcumin induces G2/M phase arrest in MDA-MB-231 cells. The results of FCM show that percentages of G2/M were

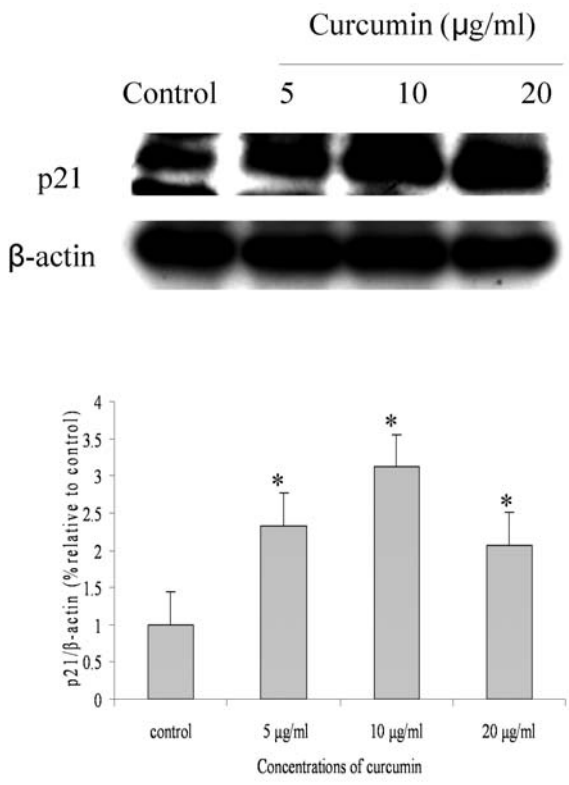

Figure 5. The protein expression of p21, Bax, p53, Bcl-2 and ß-actin for MDA-MB-231 cells were treated with various concentrations (control, 5, 10 and 20 μg/ml) of curcumin for 24 h. Curcumin up-regulated the protein expression of p21.

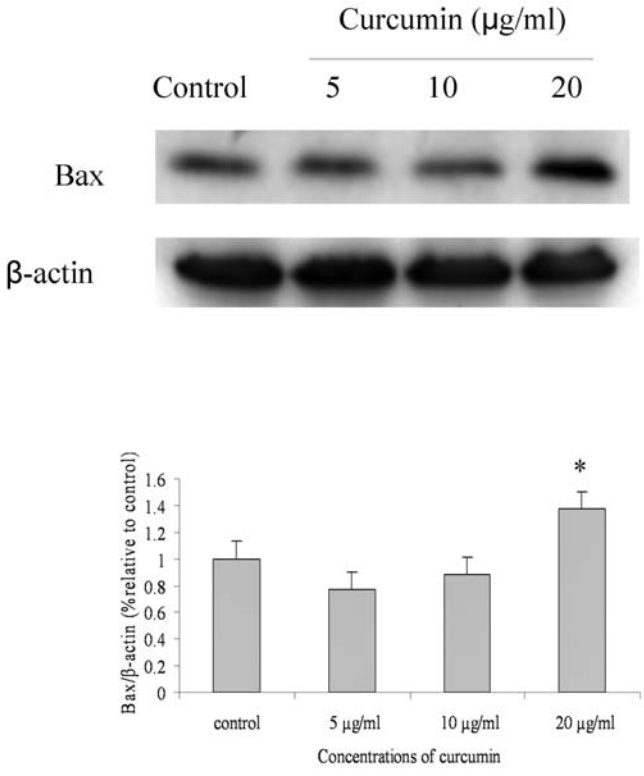

Figure 6. The protein expression of p21, Bax, p53, Bcl-2 and ß-actin for MDA-MB-231 cells were treated with various concentrations (control, 5, 10 and 20 μg/ml) of curcumin for 24 h. Curcumin up-regulated the protein expression of Bax.

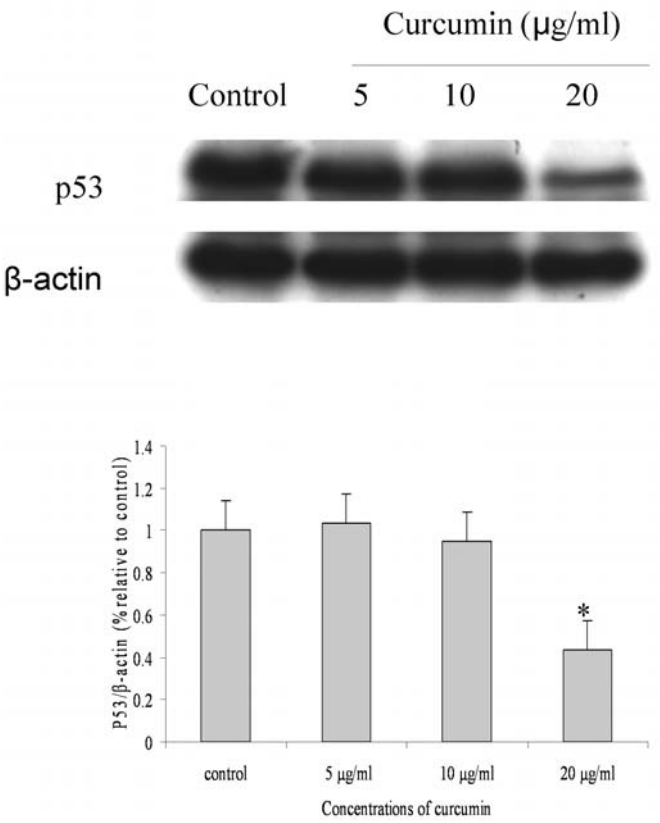

Figure 7. The protein expression of p21, Bax, p53, Bcl-2 and ß-actin for MDA-MB-231 cells were treated with various concentrations (control, 5, 10 and 20 μg/ml) of curcumin for 24 h. Curcumin down-regulated the protein expression of p53.

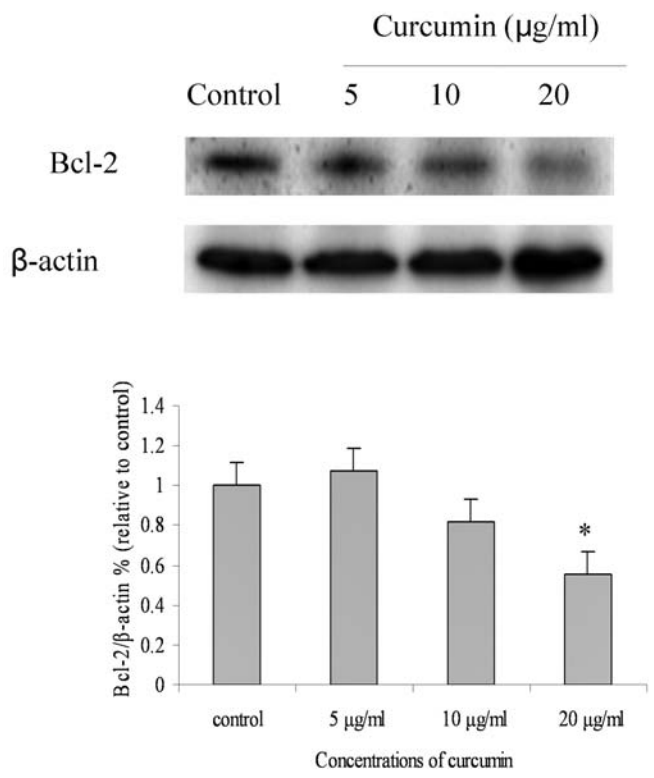

Figure 8. The protein expression of p21, Bax, p53, Bcl-2 and ß-actin for MDA-MB-231 cells were treated with various concentrations (control, 5, 10 and 20 μg/ml) of curcumin for 24 h. Curcumin down-regulated the protein expression of Bcl-2.

21.64, 26.44, 27.76, 27.04 and 35.58% respectively, when MDA-MB-231 cells were cultured with various concentrations (control, 5, 10, 15 and 20 μg/ml) of curcumin for 48 h (Fig. 3). Curcumin induced G2/M arrest in a dose-dependent manner (Fig. 4).

Western blotting for examining the effects of curcumin on bax, bcl-2, p21, 53, NF-κBp65 and ß-actin of MDA-MB-231 cells. MDA-MB-231 cells were left in concentrations of curcumin (0, 5, 10 and 20 μg/ml) for 24 h and then harvested for Western blotting examinations. The results indicate that curcumin increases the expression of p21 (Fig. 5) and Bax (Fig. 6). Curcumin decreased protein expression of p53 (Fig. 7) and Bcl-2 (Fig. 8). MDA-MB-231 cells were exposed to curcumin (20 μg/ml) for different durations (0, 6, 12 and 24 h) and then cells were harvested for Western blot examinations. The results indicate that curcumin increases the expression of p21 (Fig. 9) but decreases it for p53 (Fig. 10) and NF-κBp65 (Fig. 11).

The effect of curcumin on the migration ability of MDA-MB-231 cells. The effects of curcumin on the migration ability for MDA-MB-231 cells through matrigel were examined. The results show that curcumin inhibits the migration of MDA-MB-231 cells in a dose-dependent manner. When the dose of curcumin increased to 20 μg/ml it inhibited migration significantly (Fig. 12).

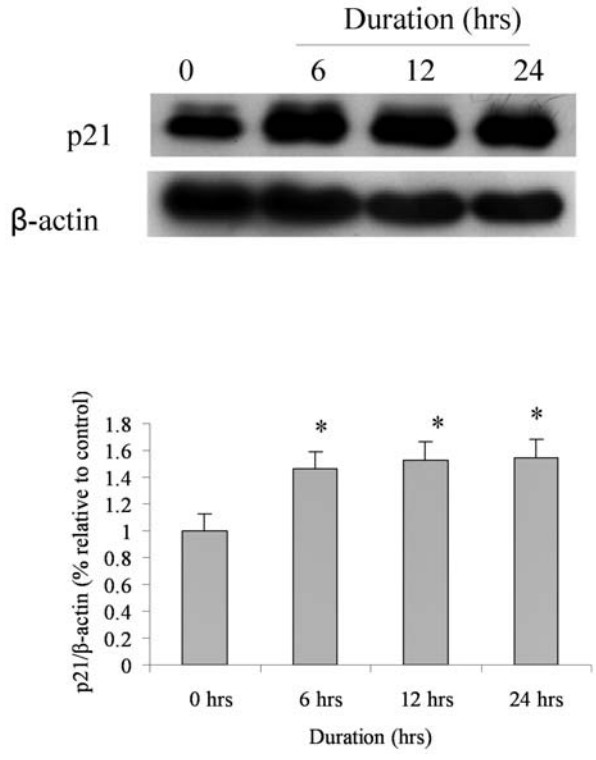

Figure 9. The protein expression of p21, Bax, p53, Bcl-2 and ß-actin for MDA-MB-231 cells were treated with curcumin (20 μg/ml) for different durations (0, 6, 12 and 24 h). Curcumin up-regulated the protein expression of p21.

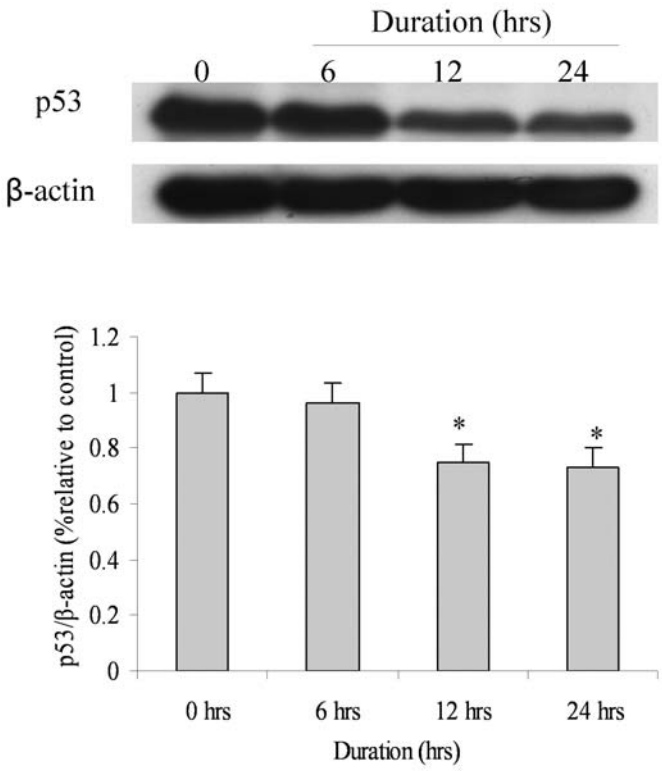

Figure 10. The protein expression of p21, Bax, p53, Bcl-2 and ß-actin for MDA-MB-231 cells were treated with curcumin (20 μg/ml) for different durations (0, 6, 12 and 24 h). Curcumin down-regulated the protein expression of p53.

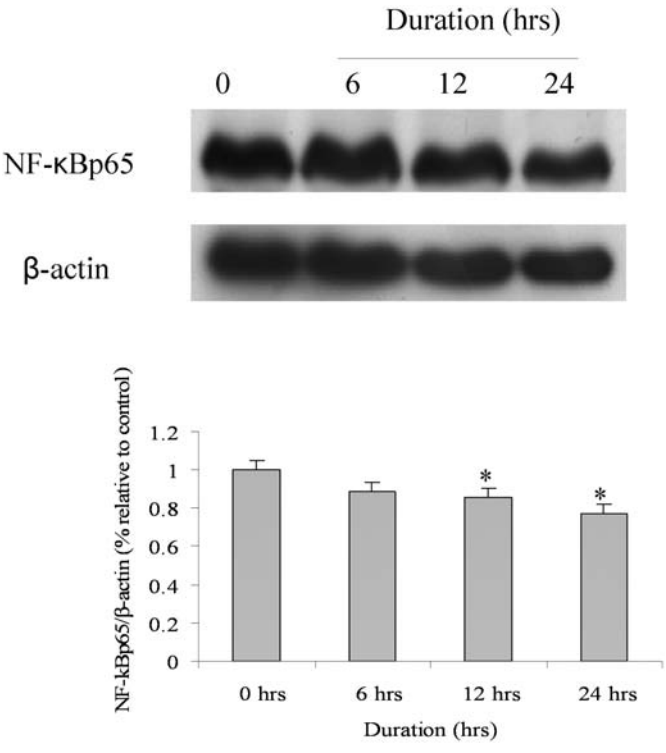

Figure 11. The protein expression of p21, p53, NF-κBp65 and ß-actin for MDA-MB-231 cells were treated with curcumin (20 μg/ml) for different durations (0, 6, 12 and 24 h). Curcumin down-regulated the protein expression of NF-κBp65.

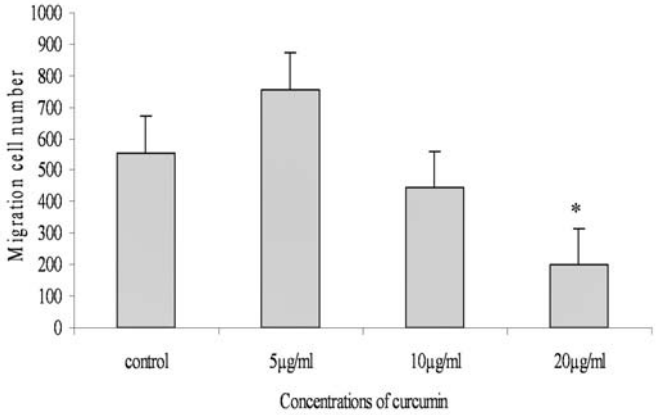

Figure 12. MDA-MB-231 cells ($5x10^4$/well/24 well) were treated with various concentrations of curcumin. Migration ability was checked by migration and invasion assays. The results show that curcumin (20 μg/ml) inhibits the migration of MDA-MB-231 cells significantly.

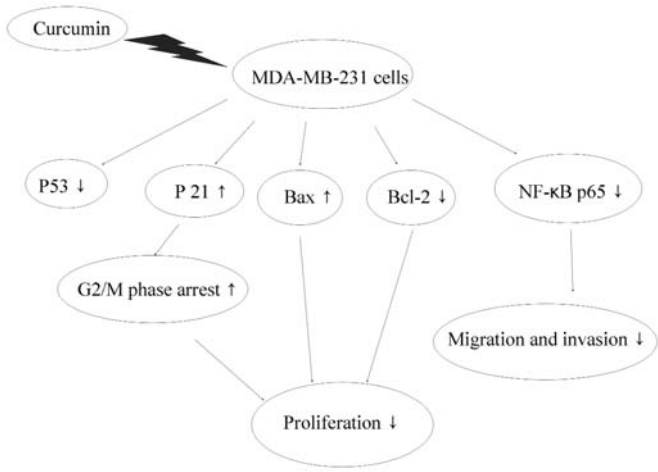

Figure 13. The proposed anti-proliferation and migration activity signal pathways of curcumin in human breast cancer MDA-MB-231 cells.

Discussion

In the present study, the proliferation of MDA-MB-231 cells was inhibited by curcumin in a dose-dependent manner. This agrees with other studies that curcumin induces cytotoxicity and apoptosis in human breast cancer MDA-MB-231 cells (12). It is documented that the increase of Bax and decrease of Bcl-2 protein expression induces apoptosis in cancer cells (13-15). Our results show that curcumin increases the protein level of Bax but decreases it for Bcl-2 in MDA-MB-231 cells. One molecular mechanism for curcumin is inhibiting the proliferation of MDA-MB-231 cells may be through increasing the Bax to Bcl-2 ratio. It is documented that *p21* (*WAF1*, *Cip-1*) has the potential to induce apoptosis (16,17). Our results also show that curcumin increases the protein level of p21 but decreases it for p53 in MDA-MB-231 cells. One possible reason for curcumin-induced apoptosis in MDA-MB-231 cells is the up-regulation of the expression of p21 which is p53 independent, and then induces apoptosis. This does not agree with another study stating that curcumin induces apoptosis in human breast cancer MCF-7 cells through p53-dependent Bax

induction (18). The results also show that curcumin inhibits the migration of MDA-MB-231 cells. That curcumin inhibits NF-κB activation is documented (19). It is documented that the decrease of protein expression of NF-κBp65 inhibits the metastasis of cancer cells (20,21). Our results also show that curcumin decreases the protein level of NF-κBp65 in MDA-MB-231 cells. One molecular mechanism of curcumin inhibits migration of MDA-MB-231 cells through decreasing protein expression of NF-κBp65. Taken together, these data suggest that curcumin has significant therapeutic potential for MDA-MB-231 cells. The molecular mechanisms may be through increasing the Bax to Bcl-2 ratio and the protein expression of p21. Curcumin inhibits the migratory ability of MDA-MB-231 (Fig. 13) through decreasing the protein expression of NF-κBp65.

Acknowledgements

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