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Title: Curcumin inhibits cell proliferation of MDA-MB-231 and BT-483 breast cancer cells mediated by down-regulation of NFκB, cyclinD and MMP-1 transcription

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Keywords: Curcumin, NFκB ,MMPs, cyclinD1/CDK

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curcumin treatment group compared with control group. Our finding extrapolates the antitumor activity of curcumin in mediating the breast cancer cell proliferative rate and invasion by down-regulating the NFκB inducing genes.

Figure1

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Fig 1 A

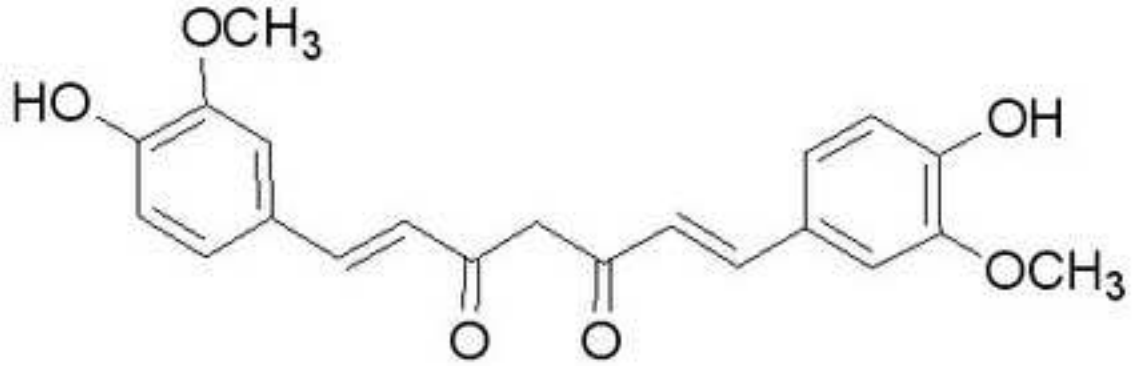

Fig 1 B

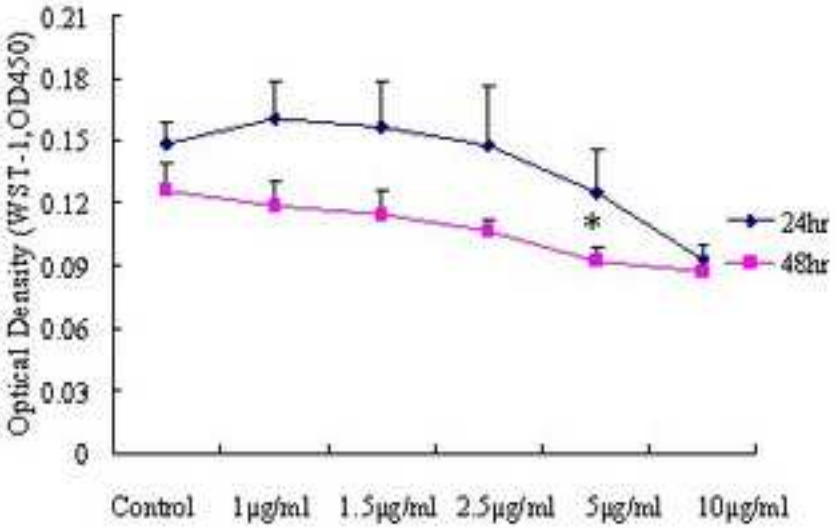

Fig 1 C

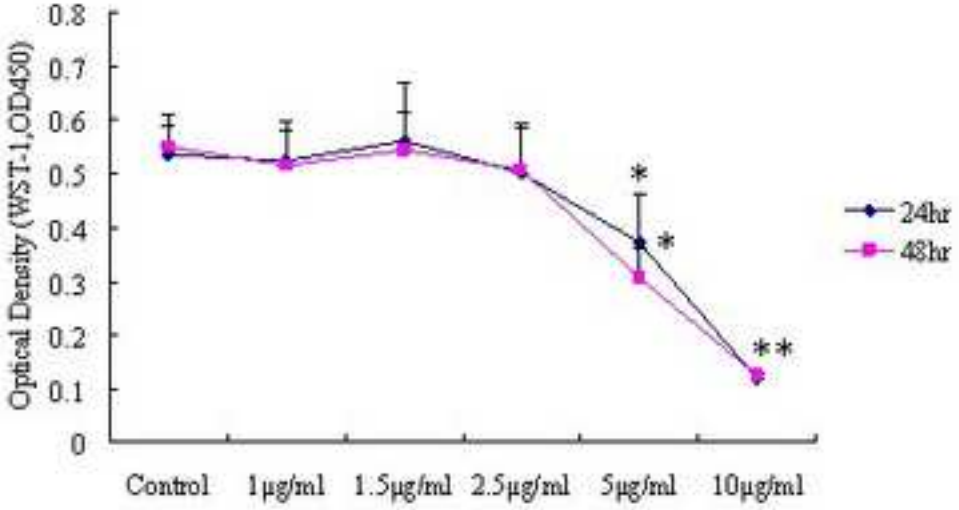

Figure2
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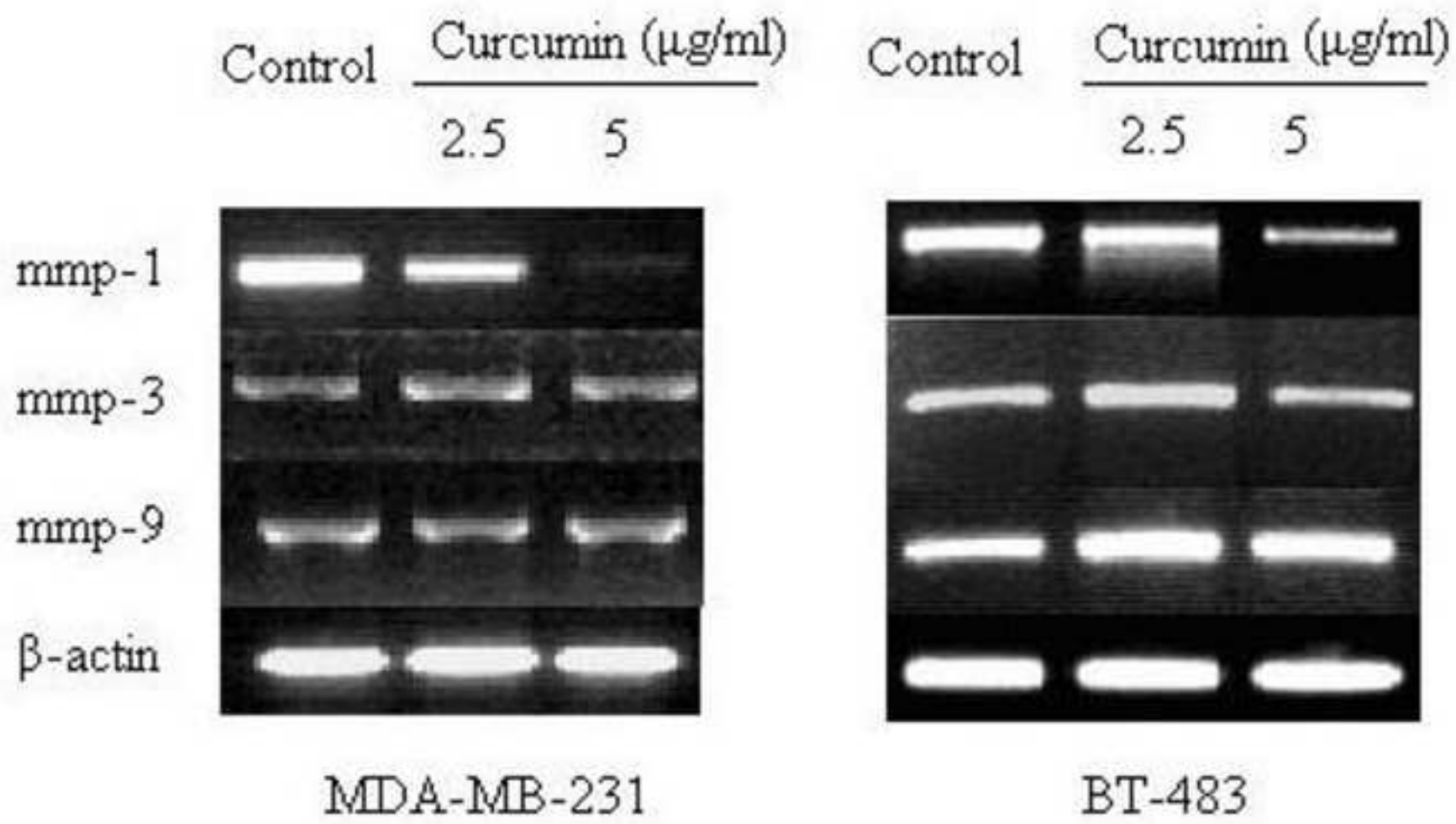
Fig 2



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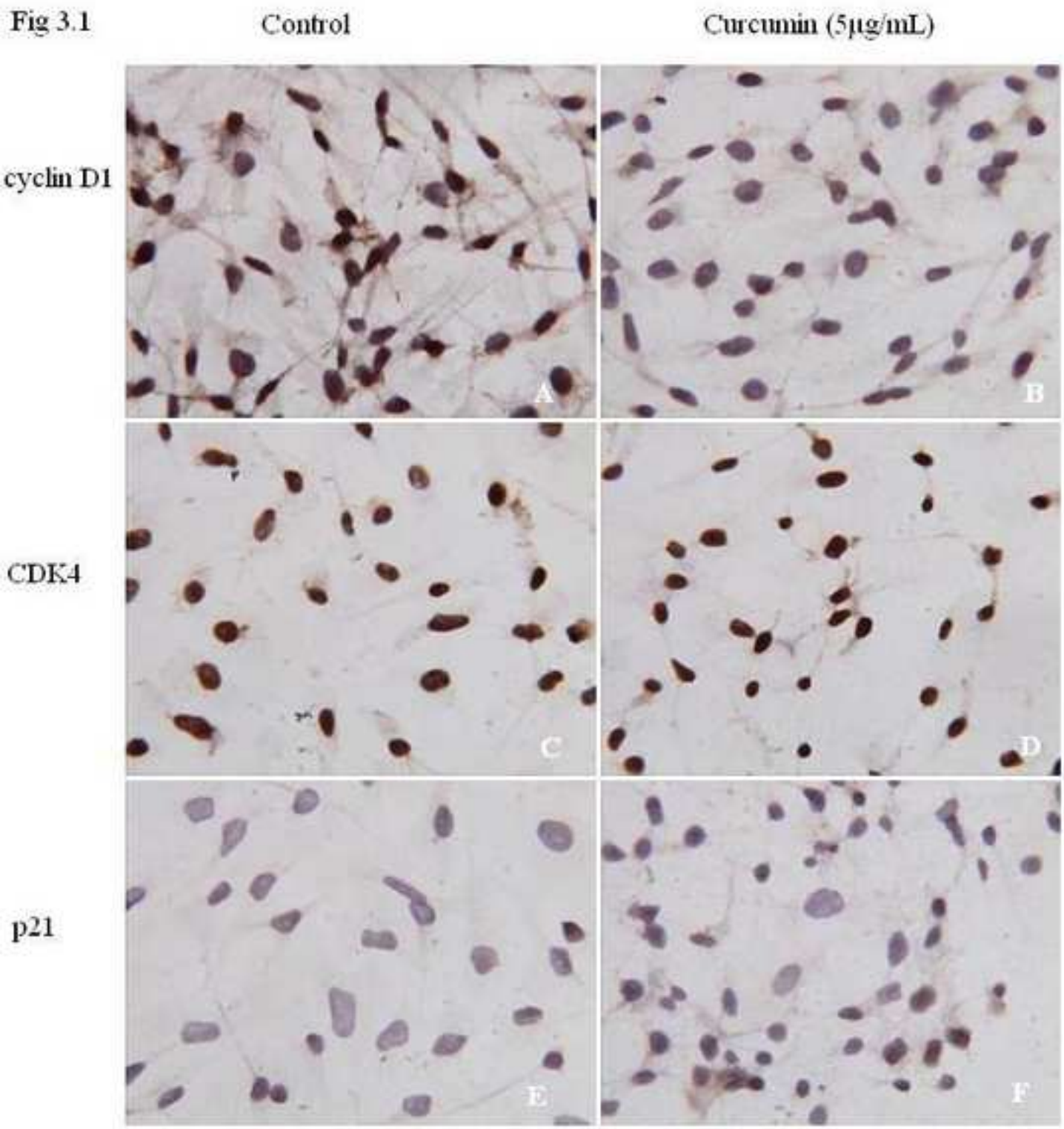

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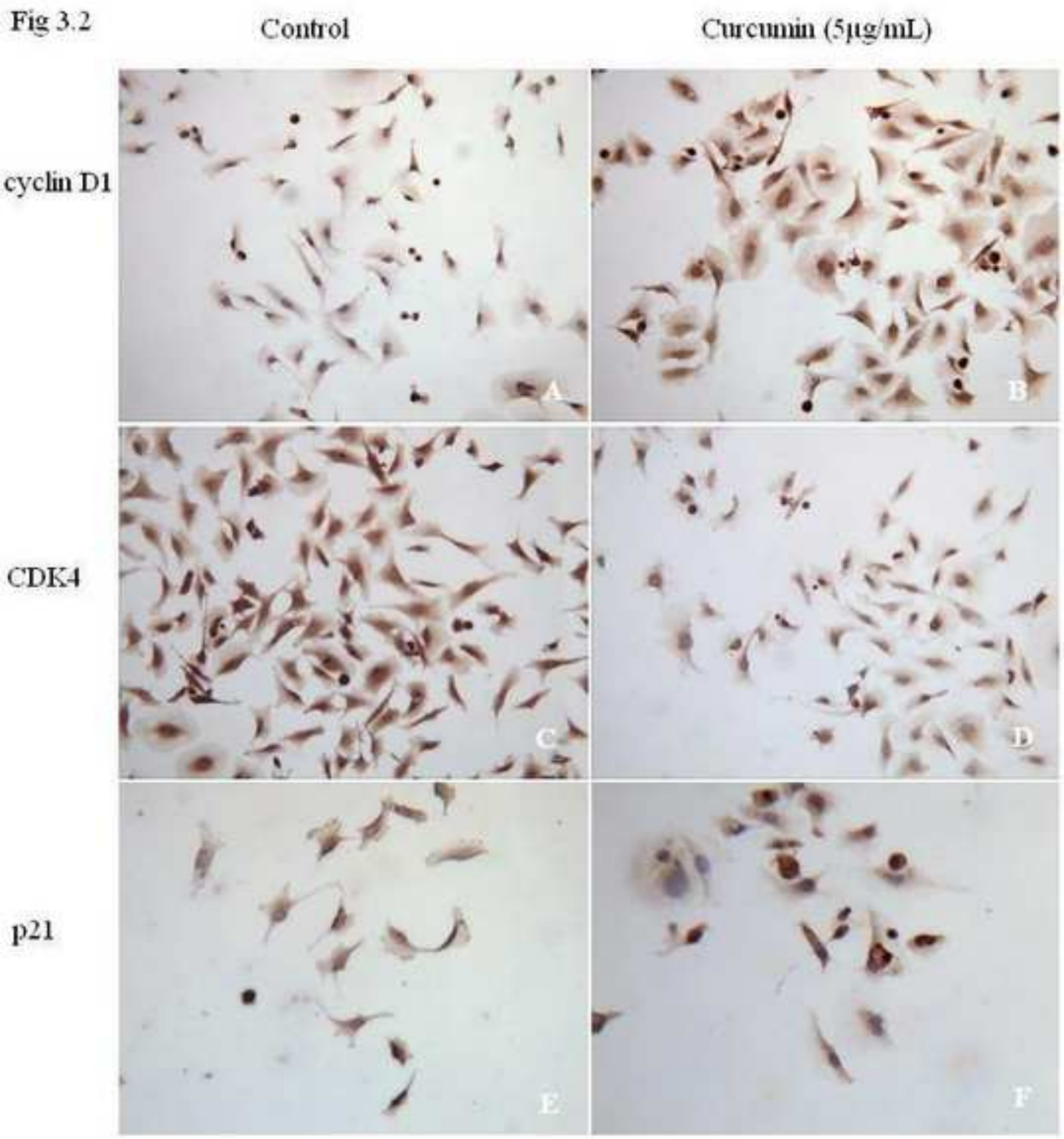

Figure4

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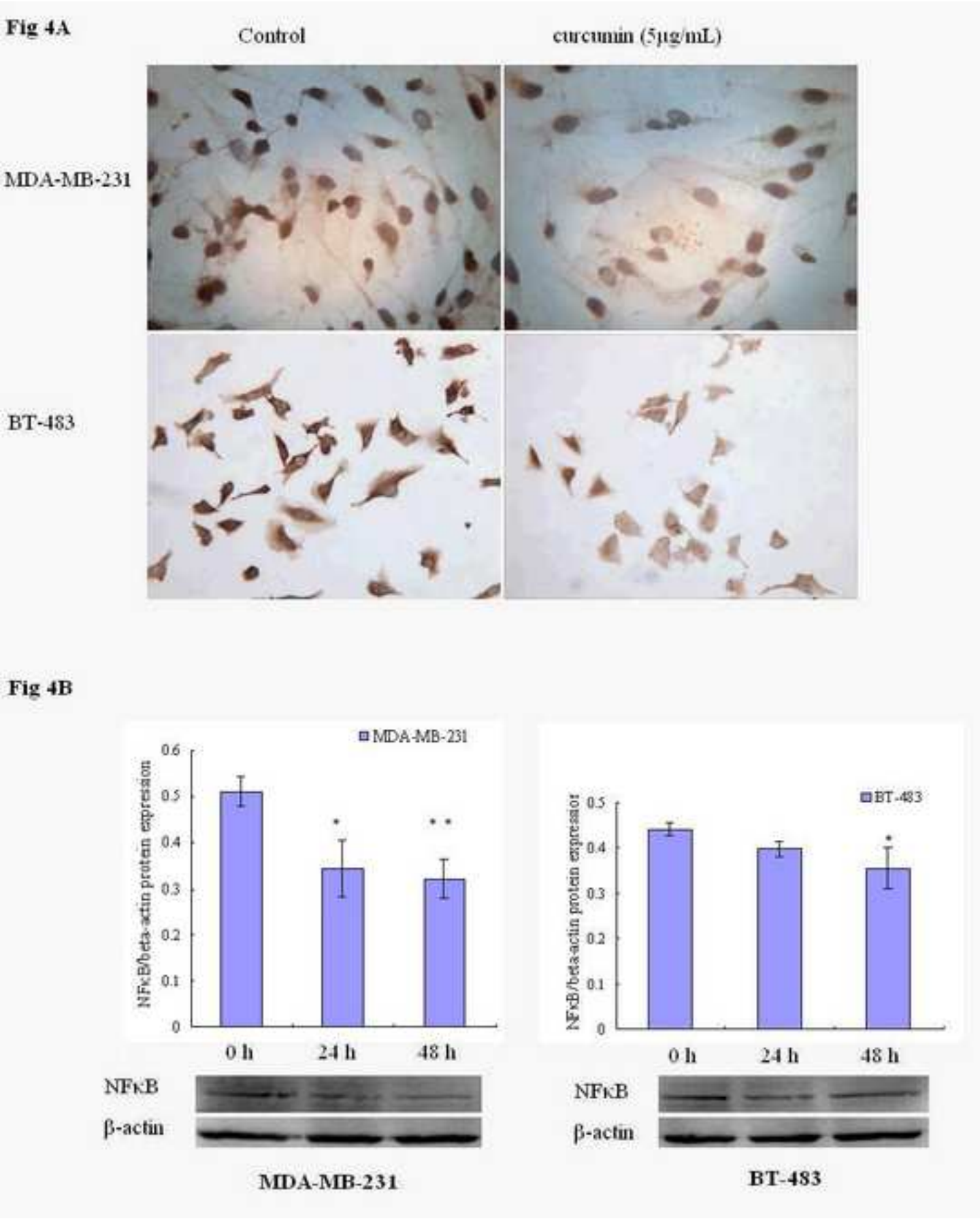

Curcumin inhibits cell proliferation of MDA-MB-231 and BT-483 breast cancer cells through down-regulation of NFκB, cyclinD/CDK4 and MMP-1 transcription

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Curcumin inhibits cell proliferation of MDA-MB-231 and BT-483 breast cancer cells mediated by down-regulation of NFκB, cyclinD and MMP-1 transcription

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Abstract:

Curcumin, an active constituent of turmeric, has been shown to possess inhibitory effect of cell proliferation and induction of apoptosis towards a board range of tumors. Cell inhibition activities of curcumin are behaved differently in various cell types. To investigate the mechanism basis for the cell inhibition of curcumin on breast cancer cell lines, we examine curcumin effect on NFκB, cell cycle regulatory proteins and matrix metalloproteinases (MMPs) in two breast cancer cell lines (MDA-MB-231 and BT-483). Cell proliferation was performed by water soluble tetrazolium WST-1 assay. The effect of curcumin's on the activity of matrix metalloproteinase-1, 3, 9 were analyzed by RT-PCR. Cell cycle regulatory protein including cyclin D1, CDK4 and p21 were examined by immunochemistry. The expressions of NFκB in breast cancer cells treated with curcumin were studied by immunochemistry and western blot. The results from WST-1 cell proliferation assay showed that curcumin exhibited the anti-proliferation effect on MDA-MB-231 and BT-483 cells in a time- and dose-dependent manner. In response to the treatment, while, the expression of cyclin D1 had declined in MDA-MB-231 and the expression of CDK4 in BT-483 had declined. MMP1 mRNA expression in BT-483 and MDA-MB-231 had significantly decreased in curcumin treatment group compared with control group. Our finding extrapolates the antitumor activity of curcumin in mediating the breast cancer cell proliferative rate and invasion by down-regulating the NFκB inducing genes.

Keyword: *Curcumin, NFκB ,MMPs, CyclinD1/CDK4,*

Introduction

The first-line treatment of breast cancer is usually relied on surgery and radiotherapy, and is frequently supplemented by adjuvant chemo- or hormonotherapies. Unfortunately, breast cancer is highly resistant to chemotherapy, It has an urgent need in developing novel anticancer agents presenting low cytotoxic side-effects, but effective inhibit tumor cell proliferation pathway[1, 2]. Curcumin, a phenolic compound extract from rhizome of the plant Curcuma longa is found to have inhibitory effects towards a board range of tumors. In breast cancer, curcumin's anticancer effect has been anticipated in relating to induce apoptosis at G2 phase of cell cycle via a p53-dependent pathway [3], but it's role in G1/S transition of breast cancer has not been established well. Based on the evolving understanding of molecular basis of breast cancer, it has been explained that pathways activated by NFκB up-regulation are implicated not only in cell proliferation including cyclin D1 [4] but also regulate promoters that encode several MMPs. Therefore, in this study, we attempt to investigate the cell inhibition activity of curcumin on the NFκB and its regulatory genes including G1 cyclins and MMPs in BT-438 breast cancer cells and MDA-MB-231 breast cancer cells. Estrogen Receptor (ER) positive breast cancers are often beneficial from anti-hormonal drugs on either inhibiting interaction of oestradiol with ER or interrupting oestradiol synthesis. However, ER negative breast cancer, being lack of functional estrogen receptor, cannot be targeted by these therapies and are more aggressive with poorer prognoses[5]. In our study, we used the MDA-MB-231 and BT-483 human breast cancer cell line as the model of ER-negative and ER-positive breast cancers respectively in comparing.

Materials and Methods

Curcumin(Molecular Weight 368.38) purchased from Sigma Chemicals (St. Louis, MO) was accurately weighed and dissolved in dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO) to a concentration of 500μg/mL as a stock solution. It was diluted (50μg/mL) in DMEM and Leibovitz's L-15 supplemented with fetal bovine serum (10%) (Invitrogen, Carlsbad, California, US) respectively. The dilution (50μg/mL) was further diluted with supplemented medium to a

series of concentrations.

Both of ER-positive breast cancer cell line BT-483 and ER-negative breast cancer cell line MDA-MB-231 were bought from the American Type Culture Collection (Rockville, MD, USA). BT-483 were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%), l-glutamine (2 mM) and antibiotics (penicillin100 units/ml; streptomycin 100 mg/ml) at 37°C in an atmosphere of 5% CO_2, while MDA-MB-231 cells were grown in Leibovitz's L-15 medium with 2 mM L-glutamine, supplemented with fetal bovine serum (10%), l-glutamine (2mM) and antibiotics (penicillin100 units/ml; streptomycin 100 mg/ml) at 37°C without CO_2. Cells were cultured at a density of 5×10^3 cells/well into 96-well plates. Cell growth was followed for 24 and 48 hr after stimulation in the presence or absence of curcumin (1, 1.25, 2.5, 5, 10, 20 μg/mL, namely 2.7, 4.1, 6.8, 13.6, 27, 54μM,). Medium alone was a negative control. Cell proliferation was assessed in vitro using the water soluble tetrazolium WST-1 (Roche, Indianapolis, IN).

Human breast cancer lines MDA-MB-231 and BT-483 were cultured with curcumin at the concentration of 2.5 μg/mL and 5μg/mL respectively for 48 hours in standard humidified incubator. Total RNA was extracted by Geneaid® RNA Mini Kit (cultured cells) (Geneaid, Taiwan) according to the manufacture's instructions. The mRNA levels of the MMPs were determined by RT-PCR. Primer sequences for MMP-1, 3, 9 were previous described[6]. The relative target mRNA expression level was normalized by β-actin (forward 5'-CCTCTATGCCAACACAGTGC-3' reverse 5'-ATACTCCTGCTTGCTGATCC -3') in the same sample. PCR products were electrophoretic displayed on a 2% agrose gel with ethidium bromide staining and captured in gel documentation system (Bio-Rad, Hercules, CA, USA). Nuclear proteins were extracted from cultured cells as well. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Sample proteins were resolved by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and then

electrophoretically transferred to nitrocellulose. The primary antibody of NFκB were added and incubated at 4˚C overnight. Then membranes were incubated with corresponding secondary antibodies for 1 hour. Protein bands were revealed by the ECL PLUS kit (GE. Healthcare Bio-Sciences, NJ, USA). The amount of NFκB protein was corrected by the signal of β-actin in the same sample. Quantification was achieved by quantifying the densities of the bands with the program of Quantity One.

The immunocytochemical studies for both cell lines were performed culturing in 24-well plate with glass-cover slides at a density of 10^5 cells/well for 24 hours. The application of curcumin (5μg/mL) was followed by 48 hours incubation. The cells were fixed and immunostained with the following antibodies: Cyclin D1, p21, CDK4 and NFκB. Staining was performed using 2% DAB substrate-chromogen solution. The intensity of DAB staining was examined under a standard light microscope using × 40 magnification.

Statistical analysis was performed using SPSS 16.0 (SPAA Inc, Chicago, IL, USA) including the ANOVA test for pairwise comparisons. The continuous variables were analyzed by independent-sample t test. All P values reported were two-sided with $P < 0.05$ considered significant. Experimental results were presented as the mean ± SD.

Results and Disscussion:

Effects of curcumin on breast cancer cell proliferation

Treatment of breast cancer cell BT-483 and MDA-MB-231 with curcumin exhibited inhibitory effect on cell growth (Figure 1). Curcumin with the dose as low as 1.25μg/ml was found to be effective on BT-483 and treatment with 5μg/ml for 48 h could result in a 60–70% inhibition. Curcumin with the dose as low as 2.5μg/ml was quite effective on MDA-MB-231 and treatment with 5μg/ml for 48 h resulted in a 50–60% inhibition. Thus we selected 5μg/mL concentration of curcumin as the optimal concentration for the subsequent experiments.

Fig. 1. (A) Chemical structure of curcumin. (B) Effects of curcumin on MDA-MB-231 and (C) BT-483 cell growth. Cultured cells were treated with various concentrations (0, 1, 1.5, 2.5, 5 and 10 μg/ml) of curcumin for 24, and 48 hours as described in the text. The cell viability of cells was counted by WST-1 assay. The results were represented the mean ± S.D. of three independent experiments and the significant difference was established at *$p < 0.05$, **$p < 0.001$ compared with the control group for the indicated time. DMSO served as the solvent control.

Curcumin decreased Matrix metalloproteinases-1 expression in MDA-MB231 and BT-483 breast cancer cells

To determine whether curcumin can down regulate MMPs expression in breast cancer cells, BT-483 and MDA-MB-231 were treated with 2.5μg/mL, 5μg/mL curcumin for 48 hours, and then the mRNA was isolated and probed with MMP-1, MMP-3 and MMP-9 cDNA respectively. Curcumin at the concentration of 2.5μg/mL and 5μg/mL can down-regulated MMP-1 mRNA in BT-483 breast cancer cell and MDA-MB-231 breast cancer cell. In this study, 2.5μg/mL and 5μg/mL curcumin have not showed inhibitory effect on MMP-3 or MMP-9 mRNA expression

Fig 2. Curcumin at 2.5 μg/mL and 5μg/mL can down-regulate MMP-1 mRNA in both cell lines, but no inhibitory effect on MMP-3 mRNA or MMP-9 mRNA expression.

Immunochemical detection of cyclin D1, CDK4 and p21 in MDA-MB231 and BT-483 breast cancer cells

MDA-MB-231 were incubated with curcumin at the 5μg/mL (Fig 3.1 B, D, F) and without curcumin (Fig 3.1 A, C, E) for 48 hours. The intensity of DAB staining was examined under a light microscope on × 40 magnification. The intensity of the stain was assessed by their thickness and darkness of the DAB precipitate.

BT-483 were incubated with curcumin at the concentration of 5μg/mL (Fig 3.2 B, D, F) or without curcumin (Fig 3.2 A, C, E) for 48 hours. The intensity of DAB staining was examined under a light microscope with × 40 magnification. The intensity of the stain was assessed by their thickness and darkness of the DAB precipitate.

Effect of curcumin on NFκB protein of MDA-MB231 and BT-483 breast cancer cells.

To investigate whether curcumin inhibit NF-κB in BT-483 and MDA-MB-231 human breast cancer, we perform immunostaining and western blot of NF-κB p65 protein. As shown in Fig. 5. Treatment of BT-483 and MDA-MB-231 cell lines with curcumin at the concentration of 5μg/mL for 12, 24, 48 hours respectively shown a reduced expression of nuclear NF-κB.

Fig. 4. Time course of curcumin treatment on NF-κB expression in MDA-MB-231 and BT-483 cells. (A)Cultured cells were treated with curcumin (5 μg/mL) for 48 h. The control group cells express strong nuclear and cytoplasmic p65 immunoreactivity (cells with strong nuclear staining, with or without minor cytoplasmic staining).Cells treated with curcumin shows decreased nuclear and cytoplasmic p65 immunopositivity compared to the controls.

(B) Cells were incubated with curcumin for three periods of time (0, 24 and 48 h). Nuclear extracts were prepared and assayed for NF-κB P65 by Western-blot as described in method section. All the samples were probed with beta-actin to normalize protein loading. The intensity of each western blotting band was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA). The results were represented the mean ± S.D. of three independent experiments and the significant difference was established at *$p < 0.05$, **$p < 0.005$ compared with the control group.

Treatment of breast cancer cell BT-483 and MDA-MB-231 with curcumin exhibited inhibitory effect on cell growth (Figure 1). In mammalian cells, the D cyclins with their catalytic partners, CDK4 and CDK6, function as the cells leave G_0 and progress through G_1 into S phase[7-9].

Three D-type cyclins, designated D1, D2, and D3, have been identified to date. D-cyclin isoforms have implicated a tissue and cell specificity and may play a different role during normal and tumor development. In majority of human breast cancers, deregulated overexpression of cyclin D1 is found[9]. The presence of specific CDK inhibitory proteins regulates the activities of cyclins and CDKs. Whether curcumin modulate its anti-proliferative effects through the regulation of cyclin D1 was examined. Immunochemistry was carried out after treatment with 5μg/mL. The result showed that curcumin decreased cyclin D1 expression (Fig3.1B) in MDA-MB-231 while the decrease of cyclin D1 has not been observed in BT-483. Recently, the role of cyclin D3 in breast cancer progression has been suggested[10]. Cyclin D3 share a similar function as well as cyclin D1 in regulating the G1 check-point of the cell cycle. Though cyclin D3 share a similar function of cycline D1 inregulating the G1 check-pint of the cell cycle, the role of cyclin D3 in breast cancer progression had just been recently suggested. Garcia-Morales[11] have identified that ER-positive MCF-7 breast cancer cells growth inhibition by rapamycin is due to a G1 arrest that specifically caused by cyclin D3 down-regulation but not cyclin D1. In our study, down-regulated cyclin D1 expression has not been found in ER-positive breast cancer BT-483, wherease a lower level of CDK4 expression has been found in BT-483 treated with curcumin. It straightly indicates the decrease of CDK4 occurred in BT-483 could be interpreted as a consequence of the cyclin D3 loss but not the cyclin D1.

Proteins termed CDK inhibitory proteins including INK4 proteins and the Kip/Cip proteins can also bind to cyclin-Cdk complexes. Kip/Cip proteins, comprised of p27, p21, and p57, can inhibit each of the cyclin-CDK complexes being essential for G1 progression and S phase entry [12]. $p21^{cip1/waf1}$ is known to inhibit the kinase activity of the CDK4–Cyclin D1. Inhibition by p27 is another generally proposed mechanism to explain the inactivity of D-type cyclin-CDK4 complexes [12]. p27 concentration allows the nuclear import and activation of cyclin D3-CDK4 complexes [13]. We investigated curcumin's regulatory effect on $p21^{cip1/waf1}$ by immunochemistry. As results shown in Figure3F, we observed curcumin induced high level of

$p21^{cip1/waf1}$ protein expression in MDA-MB-231 but failed to see such difference in case of between ER-positive BT-483 cells no matter it treated with curcumin or not. The possibility that curcumin inhibits cyclin D3-CDK4 complex to bind p27 in breast caner related to ER status should be tested in the future study.

MMPs are a family of proteolytic enzymes that can degrade extracellular matrix (ECM) components including collagen, fibronectin, and laminin. Due to the significant role that MMPs play in cancer as well as additional human pathologies, giving interest has focused on identifying natural and synthetic compounds that can inhibit MMP activities. Curcumin has been reported that it can inhibit the activity of MMP2 in MDA-MB-231 cell line by western blot [14]. Our RT-PCR result showed that curcumin can inhibit the mRNA expression of MMP-1 in MDA-MB-231 and BT-483 while having no effect on the MMP-3, 9.

Constantly activated NF-κB directly induce genes that promote cell proliferation including cyclin D1 and the cyclin-dependent kinase inhibitors p21[4]. The transcription factor NFκB has also been shown to be critically involved in the regulation of MMP-1, MMP-3[15]. Curcumin has been reported to have a synergistic effect against the growth of both ER-positive and ER-negative cells[14]. We proposed that curcumin may suppress NF-κB which in turn suppressing MMPs, cyclin D1. Under the normal conditions, NF-κB as an inactive heterotrimer present in the cytoplasm. Cytokines such as TNF-α and environmental hazards like ionizing radiation or toxins trigger the translocation of NF-κB from the cytoplasm to the nucleus. Activation of this transcription factor protects a variety of cell types from the damage that induced by pro-apoptotic stimuli[16]. It is therefore, inhibition of NF-κB activity is not only desirable in the treatment of inflammation but also in cancer therapy. Curcumin decreased nuclear and cytoplasmic p65 immunopositivity of both BT-483 and MDA-MB-231 cells. Curcumin inhibited intranuclear expression of NF-κB p65 in MDA-MB-231 and BT-483 cells

were also confirmed by western blot.

Breast cancer often progresses from the estrogen-dependent, nonmetastatic phenotype to the estrogen-independent, metastatic phenotypes. This progression is usually accompanied by altered function of the estrogen receptor or outgrowth of ER-negative cancer cells with generally high constitutive activity of NF-κB[17]. Although many estrogen receptor-positive breast cancers initially respond to antihormones drugs, responses are commonly incomplete when resistance ultimately emerging. Preclinical studies suggest that treatment strategies designed to prevent or interrupt activation of NF-κB in cell-line models of ER-positive breast cancers can restore their sensitivity to such standard endocrine agents as tamoxifen. On the other hand, activated ER can inhibit both NF-κB activation and the ability of NF-κB to regulate gene expression[18]. Further studies are necessary to investigagte how curcumin suppress the activation of NF-κB via inhibition of osteoclastogenesis in ER-positive breast cancer cell. Conceivably, our results show that curcumin can treat both ER-negative breast cancer (with generally high constitutive activity of NF-κB) and perhaps hormone-resistant ER-positive breast cancer via inhibiting the activation of NF-κB.

In summary, our finding extrapolates the antitumor activity of curcumin in mediating the breast cancer cell proliferative rate and invasion by down-regulating the NF-κB B inducing genes. Our findings suggest curcumin could be a potentially therapeutic agent for both ER-positive and ER-negative breast cancer.

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