

## RESEARCH ARTICLE

**Garcinol, an Acetyltransferase Inhibitor, Suppresses Proliferation of Breast Cancer Cell Line MCF-7 Promoted by 17 $\beta$ -Estradiol**Xia Ye<sup>&</sup>, Lei Yuan<sup>&</sup>, Li Zhang, Jing Zhao, Chun-Mei Zhang, Hua-Yu Deng\***Abstract**

The acetyltransferase inhibitor garcinol, a polyisoprenylated benzophenone, is extracted from the rind of the fruit of *Garcinia indica*, a plant found extensively in tropical regions. Anti-cancer activity has been suggested but there is no report on its action via inhibiting acetylation against cell proliferation, cell cycle progression, and apoptosis-inhibition induced by estradiol (E<sub>2</sub>) in human breast cancer MCF-7 cells. The main purposes of this study were to investigate the effects of the acetyltransferase inhibitor garcinol on cell proliferation, cell cycle progression and apoptosis inhibition in human breast cancer MCF-7 cells treated with estrogen, and to explore the significance of changes in acetylation levels in this process. We used a variety of techniques such as CCK-8 analysis of cell proliferation, FCM analysis of cell cycling and apoptosis, immunofluorescence analysis of NF- $\kappa$ B/p65 localization, and RT-PCR and Western blotting analysis of ac-H3, ac-H4, ac-p65, cyclin D1, Bcl-2 and Bcl-xl. We found that on treatment with garcinol in MCF-7 cells, E<sub>2</sub>-induced proliferation was inhibited, cell cycle progression was arrested at G0/G1 phase, and the cell apoptosis rate was increased. Expression of ac-H3, ac-H4 and NF- $\kappa$ B/ac-p65 proteins in E<sub>2</sub>-treated MCF-7 cells was increased, this being inhibited by garcinol but not ac-H4. The nuclear translocation of NF- $\kappa$ B/p65 in E<sub>2</sub>-treated MCF-7 cells was also inhibited, along with cyclin D1, Bcl-2 and Bcl-xl in mRNA and protein expression levels. These results suggest that the effect of E<sub>2</sub> on promoting proliferation and inhibiting apoptosis is linked to hyperacetylation levels of histones and nonhistone NF- $\kappa$ B/p65 in MCF-7 cells. The acetyltransferase inhibitor garcinol plays an inhibitive role in MCF-7 cell proliferation promoted by E<sub>2</sub>. Mechanisms are probably associated with decreasing ac-p65 protein expression level in the NF- $\kappa$ B pathway, thus down-regulating the expression of cyclin D1, Bcl-2 and Bcl-xl.

**Keywords:** Garcinol - acetylation - histone - NF- $\kappa$ B/ac-P65 - breast cancer

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**Introduction**

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) play an important role in the regulation of eukaryotic gene transcription. Histone acetylation and deacetylation is a dynamic modification process in the nucleus, which has a close relation with the gene activation or gene silencing, with hyperacetylated histones being associated with transcriptionally active genes, while hypoacetylated histones associated with transcriptionally repressed ones. This constitutes one of the epigenetic control of gene expression. Disorders and imbalances of oncogenes and tumor suppressor genes are closely related to abnormal histone acetylation modifications in a growing number of malignancies including breast cancer. An increasing number of studies also demonstrated that some abnormal nonhistone acetylation modification plays a crucial role in cancer development and progression (Singh et al., 2010; Mooney et al., 2010).

Breast cancer remains a major cause of cancer-related deaths in women (Jemal et al., 2009; Minatoya et al., 2013). Estrogen is an important factor promoting cell proliferation in estrogen receptor (ER) positive breast cancer, which is associated with abnormal changes in acetylation levels in current study (Kininis et al., 2007). Estrogen may affect the acetylation levels of histones or nonhistones of oncogenes, thus boost oncogene expression, tumor cell transformation, accelerate proliferation and inhibit apoptosis. CyclinD1 may be an important cell cycle-regulatory gene, an important basis of promoting tumor cell proliferation, and Bcl-2, Bcl-xl belong to antiapoptotic members of apoptosis-regulatory gene, which all may be affected by estrogen through abnormal acetylation modifications.

Ligand-activated ERs mediate transcription by directly interacting with specific estrogen response elements (EREs) or indirectly interacting with other transcription factors, such as AP-1, Sp-1, nuclear factor- $\kappa$ B (NF- $\kappa$ B) et al. It has been reported that activation of NF- $\kappa$ B plays a

significant role in tumor cells proliferation in response to estrogen exposure (Rubio et al., 2006). Estrogen receptor coactivator, p300/CBP (cAMP-responsive element binding protein)-binding protein) may be important for estrogen mediating the acetylation of NF- $\kappa$ B. Acetylation of NF- $\kappa$ B/p65 at discrete sites regulates distinct nuclear functions of NF- $\kappa$ B, and acetylation of lysine 310 is required for enhanced transactivation potential of NF- $\kappa$ B/p65 (Chen et al., 2002), promoting relevant target genes expression (Sung et al., 2008). There may be a critical relationship between the protein expression of CyclinD1, Bcl-2, Bcl-x1 and the enhanced acetylation of lysine 310 of NF- $\kappa$ B/p65.

In this study, we assessed the effects of acetyltransferase inhibitor Garcinol on E<sub>2</sub>-induced cell proliferation and E<sub>2</sub>-inhibited cell apoptosis in human breast cancer MCF-7 cells, detected the changes in the acetylated levels of histone H3, H4 and nonhistone NF- $\kappa$ B/p65 as well as the expression of CyclinD1, Bcl-2 and Bcl-x1, and tried to explore the significance of changes in acetylation levels in estrogen promoting proliferation of breast cancer cells. There are little literatures on Garcinol's action through inhibiting acetylation against cell proliferation in human breast cancer cells.

## Materials and Methods

### Reagents

40-mmol/L solution of Garcinol Enzo Life Science and 7.34 $\times$ 10<sup>7</sup>-nmol/L solution of 17 $\beta$ -Estradiol Cayman were prepared in 100% dimethyl sulfoxide, stored at -20°C, and then diluted as needed in cell culture medium.

### Cell culture

Human breast cancer cell line MCF-7 was provided by the Department of Pathophysiology, Chongqing Medical University and was maintained in phenolred-free medium (DMEM) (Gibco), supplemented with 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin and 10% CDNBCS (Gibco, New Zealand) at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

### Removal of sex hormones by Charcoal-Dextran (Han et al., 2002)

Charcoal was washed twice with cold sterile water immediately before using. 5 g charcoal suspension in 500 mg dextran T40 of the same volume as serum was centrifuged at 1000 $\times$ g for 10 min. Supernatants were aspirated, and the serum was mixed with the charcoal precipitation. This charcoal-serum mixture was maintained in suspension by mixing at 56°C for 30 min. This suspension was centrifuged at 1000 $\times$ g for 20 min. This procedure was repeated twice, and the supernatants were filtered through a 0.22  $\mu$ m cellulose acetate-filter. The charcoal dextrantreated NBCS was stored at -20°C until needed.

### Concentration of 17 $\beta$ -Estradiol (E<sub>2</sub>) assay

We have accomplished the appropriate concentration of E<sub>2</sub> assay in our previous study (Zhang et al., 2013). The effect of E<sub>2</sub> on promoting proliferation of MCF-7 cells

was investigated with CCK-8 assay and the concentration (100 nmol/L) of E<sub>2</sub> in the following experiments was determined according to the rate of proliferation.

### Cell proliferation assay

Cells were seeded at a density of 2 $\times$ 10<sup>4</sup>/ml in three sterilized 96-well culture plates, per well containing 200  $\mu$ l suspensions. These groups were set up in this study: vehicle control group containing only 200  $\mu$ l DMEM medium, the blank control group consisting of 200  $\mu$ l cell suspension, the E<sub>2</sub> treatment group containing 100 nmol/L concentration of E<sub>2</sub>, the E<sub>2</sub> plus different concentrations of Garcinol (10  $\mu$ mol/L, 20  $\mu$ mol/L, 30  $\mu$ mol/L, 40  $\mu$ mol/L, 50  $\mu$ mol/L) treatment group. After incubation for 24 h, 48 h, 72 h, 10  $\mu$ l of CCK8 solution (Beyotime, China) was added to the culture medium, and incubated further for 2 h at 37°C. Absorbance was determined at 450 nm wavelength and inhibition ratio was calculated according to the absorbance. Each treatment had five replicate wells and the amount of DMSO in reaction mixture never exceeded 0.1%. Moreover, each experiment was repeated at least three times.

### Flow cytometric analysis of cell cycle

Cell cycle analysis was performed using the standard propidium iodide method. In brief, cells were trypsinized, washed with cold PBS, fixed in 70 % ethanol at 4°C overnight, and cellular DNA were stained with PI for 30 min. Finally, cell samples were analyzed by Becton-Dickinson Flow Cytometer (BD Biosciences, USA). Each experiment was performed at least in triplicates.

### Flow cytometric analysis of cell apoptosis

Apoptotic cells were differentiated from viable or necrotic ones by combined application of annexin V-FITC and propidium iodide (PI). The samples were washed twice and adjusted to a concentration of 1 $\times$ 10<sup>6</sup> cells/ml with cold PBS. 10  $\mu$ l of annexin V-FITC and 10  $\mu$ l PI were added into 100  $\mu$ l of cell suspension followed by incubation for 15 min at room temperature in the dark. Then, 400  $\mu$ l of binding buffer was added to each sample without washing and analyzed by flow cytometry. Experiments were performed at least in triplicates.

### Immunofluorescence analysis of NF- $\kappa$ B/p65 localization

The effect of Garcinol on the E<sub>2</sub>-induced nuclear translocation of p65 was examined by using standard immunofluorescence techniques. MCF-7 cells were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized with 0.5% Triton X-100 for additional 15 min at 25°C, then blocked with 10% goat serum for 1h. Finally, cells were incubated with NF- $\kappa$ B/p65 (1:50 in blocking buffer) antibody at 4°C overnight. After appropriately washing with PBS, cells were incubated with goat anti-rabbit IgG-FITC (1:45 in blocking buffer) (Bioss, China) in a humid chamber at 37°C for 1h. Nuclei were stained with PI for 30 sec at room temperature in the dark, washed extensively, and examined under a confocal laser scanning microscope (Sp2, Leica, Germany). FITC shew green fluorescence and PI shew red fluorescence.

**Reverse transcription-polymerase chain reaction analysis**

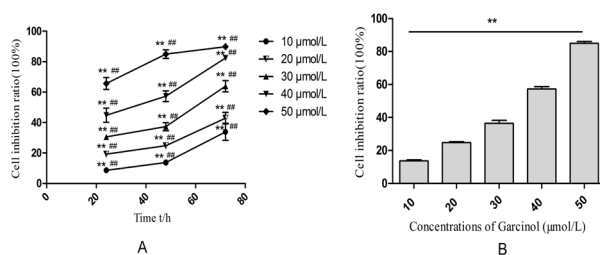
Total RNA was isolated using RNAPure rapid extraction kit (Bioteke Corporation, Beijing, China) according to the manufacturer's protocol, from MCF-7 cells. The first-strand cDNA was synthesized from 1  $\mu$ g of total RNA using a Prime Script kit (Bioteke Corporation, Beijing, China). CyclinD1, Bcl-2, Bcl-x1 gene expressions were quantified by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).  $\beta$ -actin was used as an endogenous control. RT-PCRs were performed with primers listed in Table.1, The PCR conditions were: 94°C for 3 min, followed by 35 cycles of 95°C for 50 sec, 59.5°C for 45 sec and 72°C for 40 sec. Five microliters of the PCR product were separated by electrophoresis in 1.5% agarose gel and visualized by GoodView staining. Gene expression analysis was performed with the Quantity One Software (Bio-Rad, Hercules, CA, USA).

**Western blot analysis**

Total protein extracts of each treatment group cells were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF (Millipore). After blocking, the PVDF membranes were probed with rabbit anti-human ac-H3 K9 (diluted 1:1000), ac-H4 K5 (diluted 1:1000), NF- $\kappa$ B/ac-p65 K310 (diluted 1:500) and CyclinD1 (diluted 1:1000) monoclonal

**Table 1. Sequences of the Primer Pairs for CyclinD1, Bcl-2, Bcl-x1 and Human Housekeeping Genes used for RT-PCR**

Gene	Primers
CyclinD1	F: 5'-GCGAGGAACAGAAGTGCG-3'
	R: 5' - TGG AGTTGTCGGTGTAGATGC-3'
Bcl-2	F: 5'-ATGTGTGTGGAGAGCGTCAAC-3'
	R: 5'-AGACAGCCAGGAGAAATCAAAC-3'
Bcl-x1	F: 5'-CAGGCGACGAGTTTGAAGTGC GGTA -3'
	R: 5' - ATGCCCGTCAGGAACCAG -3'
$\beta$ -actin	F: 5'-CTG GGACGACATGGAGAAAA-3'
	R: 5'-AAGGAAGGCTGGAAGAGT GC-3'



**Figure 1. The Garcinol Effects on MCF-7 Cell Proliferation in the Presence of E<sub>2</sub>.** Cells were treated with 100 nmol/L E<sub>2</sub> plus various concentrations (10-50  $\mu$ mol/L) of Garcinol for 24 h, 48 h, 72 h, respectively. The CCK-8 assay was carried out as described in materials and methods. a Comparative analysis of the antiproliferative effect of Garcinol on MCF-7 cells treated with E<sub>2</sub>. (\*\* $p$ <0.01, analysis of variance, among different concentration group. ## $p$ <0.01, analysis of variance, among different time group). b The inhibition ratio of various concentrations (10-50  $\mu$ mol/L) of Garcinol on the proliferation of MCF-7 cells treated with E<sub>2</sub> for 48 h. (\*\* $p$ <0.01, analysis of variance, among different concentration group)

antibodies (Abcam), rabbit anti-human Bcl-2 (diluted 1:1000), Bcl-x1 (diluted 1:1000) polyclonal antibodies (Santa Cruz) followed by incubation with secondary antibody. The immunoreactive bands were visualized using an ECL kit, according to the manufacturer's instructions. Proteins expression analysis was performed with the Quantity One Software (Bio-Rad).

**Statistical analysis**

Experiments were performed in triplicates, and data were expressed as the mean $\pm$ SD where applicable, and all statistical analyses were performed by SPSS 17.0 using one-way ANOVA for 3-group comparisons and t tests for 2-group comparisons. The probability of  $p$ <0.05 was considered to be statistically significant.

**Results****Garcinol inhibited proliferation of MCF-7 cells treated with E<sub>2</sub>**

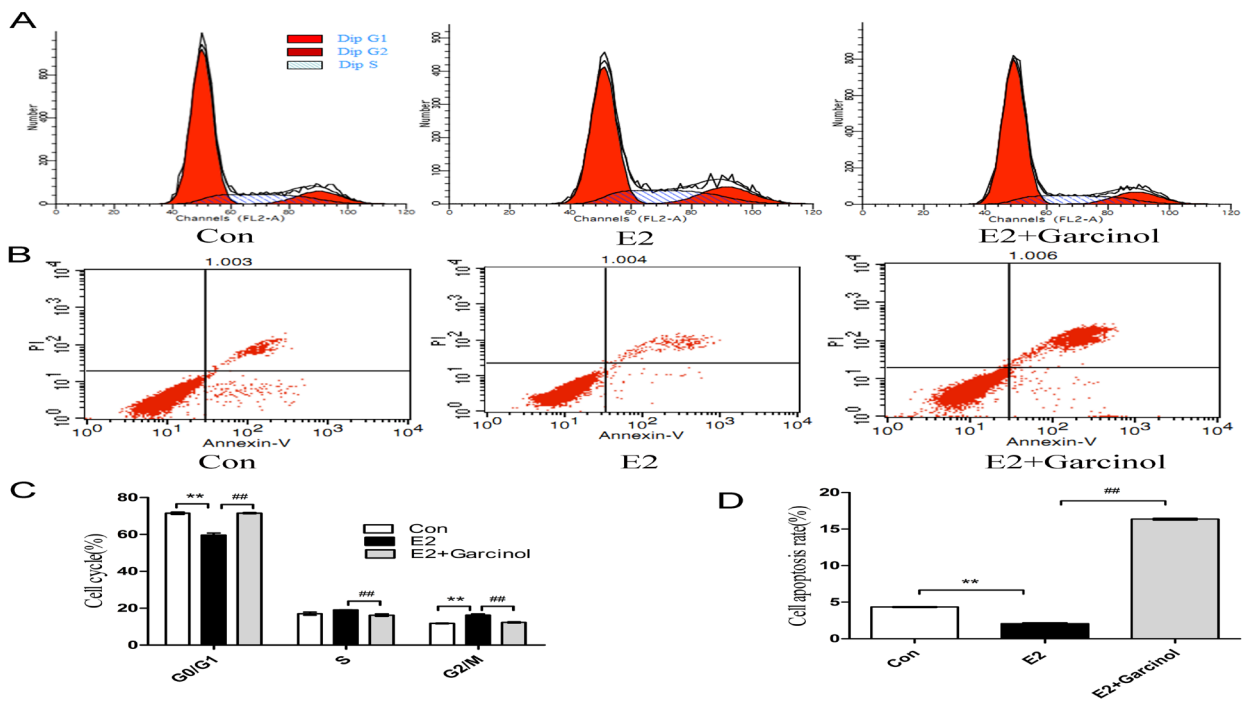
Garcinol inhibited MCF-7 cells proliferation induced by E<sub>2</sub> in a time- and dose-dependent manner. As shown in Figure1a, we first investigated the anti-proliferative properties of Garcinol by increasing doses of Garcinol for 24 h, 48 h and 72 h respectively for MCF-7 cells, which were exposed 100 nmol/L E<sub>2</sub> all the time. Then the effects of treatments on cell proliferation were assessed by CCK-8 assay. The optional concentration of Garcinol (35  $\mu$ mol/L for 48 h) in the following experiment was determined according to the inhibition ratio.

**Garcinol regulated cell cycle progression and cell apoptosis in MCF-7 cells treated with E<sub>2</sub>**

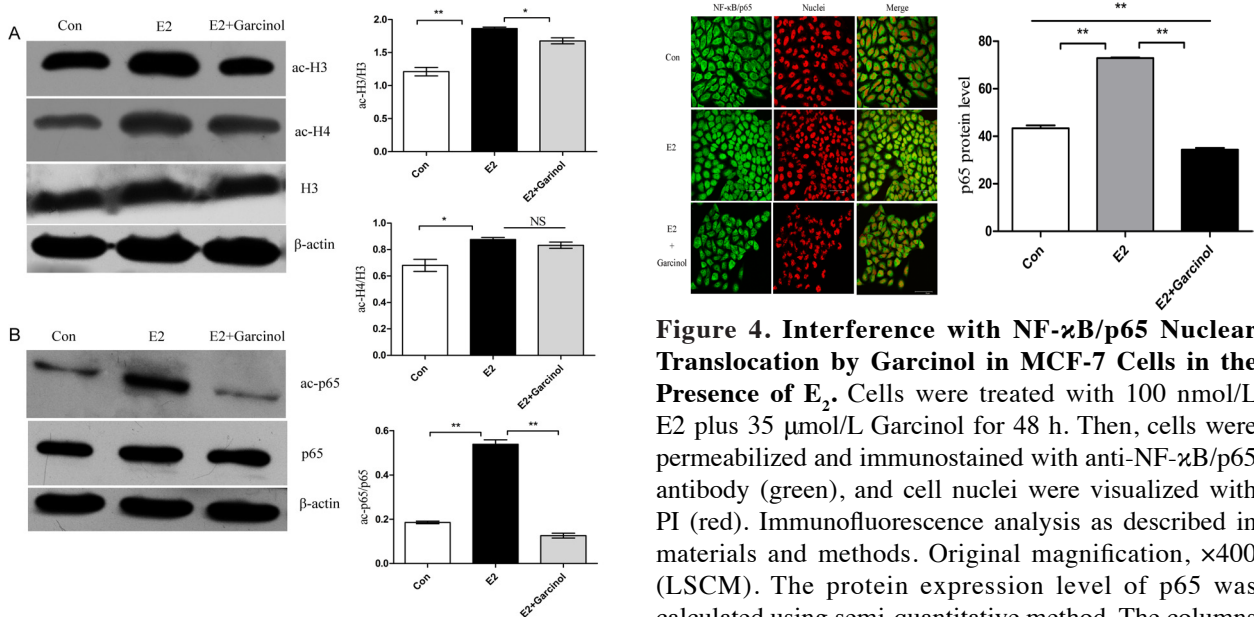
To investigate the effects of Garcinol on cell cycle and apoptosis in MCF-7 cells treated with E<sub>2</sub>, cells were given differential treatment followed by flow cytometry analysis. As shown in Figure 2, treatment with 100 nmol/L E<sub>2</sub> for 48 h, the percentage of G0/G1 phase significantly decreased from 71.5 $\pm$ 0.12 to 59.6 $\pm$ 0.19 ( $p$ <0.01), but G2/M phase increased from 11.7 $\pm$ 0.03 to 16.3 $\pm$ 0.01 ( $p$ <0.01), compared with Con. Correspondingly, the apoptosis rate decreased from 4.3 $\pm$ 0.112 to 2.1 $\pm$ 0.20 ( $p$ <0.01). However, treatment with 35  $\mu$ mol/L acetyltransferase inhibitor Garcinol for 48 h, the cell cycle progression of MCF-7 cells treated with E<sub>2</sub> was arrested at G0/G1 phase with the percentage of 70.6 $\pm$ 0.06 along with the increasing apoptosis rate of 16.35 $\pm$ 0.224 ( $p$ <0.01).

**Garcinol inhibited acetylation of H3, H4, p65 in MCF-7 cells treated with E<sub>2</sub>**

Based on the previous demonstration, p300/CBP and PCAF mediate hyperacetylation of histone H3, H4 induced by estrogen in MCF-7 cells. As shown in Figure3, Western blot assay showed that lower basal expression of ac-H4 compared with ac-H3 in Con, treatment with 100 nmol/L E<sub>2</sub> for 48 h led to up-regulation of ac-H3, ac-H4, NF- $\kappa$ B/ac-p65 ( $p$ <0.05) in MCF-7 cells compared with Con. Treatment with E<sub>2</sub> plus 35  $\mu$ mol/L Garcinol, the protein expressions of ac-H3, NF- $\kappa$ B/ac-p65 in MCF-7 cells were decreased ( $p$ <0.01) but there was no significant changes in ac-H4 ( $p$ >0.05).



**Figure 2. The Garcinol Effects on MCF-7 Cell Cycle Progression and Apoptosis in the Presence of E<sub>2</sub>.** MCF-7 cells were treated with 100 nmol/L E<sub>2</sub> plus 35 μmol/L Garcinol for 48 h. Cell cycle and apoptosis analysis by flow cytometry was carried out as described in materials and methods. Data are presented as the relative fluorescence intensity of cell sub-populations in the two-dimensional profile (a and b) or bar diagram (c and d). c Comparative analysis of various cell cycle among Con, E<sub>2</sub> and E<sub>2</sub> plus Garcinol groups. d Comparative analysis of cell apoptosis ratio among Con, E<sub>2</sub> and E<sub>2</sub> plus Garcinol groups. (\*\**p*<0.01 vs Con, ##*p*<0.01 vs E<sub>2</sub>). Experiments were performed in triplicates

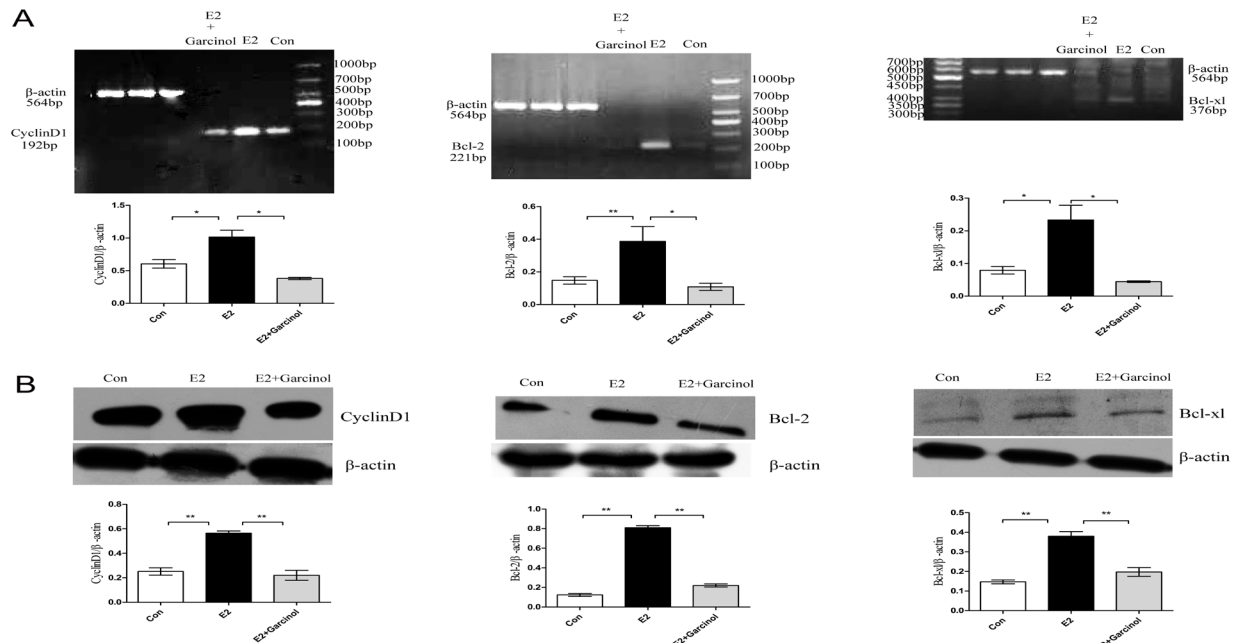


**Figure 3. Garcinol Decreases the Protein Expression Levels of ac-H3, ac-H4, and NF-κB/ac-p65 in MCF-7 cells in the presence of E<sub>2</sub>.** Cells were treated with 100 nmol/L E<sub>2</sub> plus 35 μmol/L Garcinol for 48 h. Then, cells were lysed and immunoblotted. The blot was probed with β-actin antibody for normalization and with anti-ac-H3 (K9), anti-ac-H4 (K5) and anti-H3 antibodies **A**), anti-NF-κB/ac-p65 (K310) and anti-p65 antibodies **B**). a Garcinol decreased the protein expression levels of ac-H3 and ac-H4 which were overexpressed by E<sub>2</sub> in MCF-7 cells. b Garcinol decreased the protein expression level of ac-p65 which was overexpressed by E<sub>2</sub>. Each blot represents three independent experiments. \*\**p*<0.01 vs Con or E<sub>2</sub>+Garcinol, \**p*<0.05 vs Con or E<sub>2</sub>+Garcinol. NS represents *p*>0.05

**Figure 4. Interference with NF-κB/p65 Nuclear Translocation by Garcinol in MCF-7 Cells in the Presence of E<sub>2</sub>.** Cells were treated with 100 nmol/L E<sub>2</sub> plus 35 μmol/L Garcinol for 48 h. Then, cells were permeabilized and immunostained with anti-NF-κB/p65 antibody (green), and cell nuclei were visualized with PI (red). Immunofluorescence analysis as described in materials and methods. Original magnification, ×400 (LSCM). The protein expression level of p65 was calculated using semi-quantitative method. The columns indicate the mean fluorescence intensity of the protein expression levels. \*\**p*<0.01 vs Con or E<sub>2</sub>+Garcinol. Experiments were performed in triplicates

*Garcinol inhibited E<sub>2</sub>-induced nuclear translocation of p65*

p65 is a subunit of NF-κB that has nuclear localization signals and retained in the cytoplasm by I-κBα. We examined whether Garcinol inhibited the nuclear translocation of p65 induced by E<sub>2</sub>. As shown in Figure4, treatment with 100 nmol/L E<sub>2</sub> for 48 h significantly enhanced p65 translocation (*p*<0.01) from the cytoplasm to the nucleus along with the increased numbers of cells



**Figure 5. The Garcinol Effects on the Expression of CyclinD1, Bcl-2 and Bcl-xl in MCF-7 Cells in the Presence of E<sub>2</sub>.** Cells were treated with 100 nmol/L E<sub>2</sub> plus 35  $\mu$ mol/L Garcinol for 48 h. **A)** Garcinol decreased the mRNA expression levels of CyclinD1, Bcl-2 and Bcl-xl which were overexpressed by E<sub>2</sub> in MCF-7 cells. Transcriptional expression levels of CyclinD1, Bcl-2 and Bcl-xl were measured using reverse transcription-polymerase chain reaction and  $\beta$ -actin levels were used as internal positive controls. **B)** Garcinol decreased the protein expression levels of CyclinD1, Bcl-2 and Bcl-xl which were overexpressed by E<sub>2</sub> in MCF-7 cells. Cells were lysed and immunoblotted. The blot was probed with  $\beta$ -actin antibody for normalization and with anti-CyclinD1, anti-Bcl-2 and anti-Bcl-xl antibodies. Each blot represents three independent experiments. The y-axis represents the relative protein expression level (ratio of protein/ $\beta$ -actin) \*\* $p$ <0.01 vs Con or E<sub>2</sub>+Garcinol, \* $p$ <0.05 vs Con or E<sub>2</sub>+Garcinol

compared with Con, which were suppressed ( $p$ <0.01) by 35  $\mu$ mol/L Garcinol.

#### Garcinol inhibited the expression of CyclinD1, Bcl-2 and Bcl-xl in MCF-7 cells treated with E<sub>2</sub>.

As shown in Figure 5, in MCF-7 cells, treatment with 100nmol/L E<sub>2</sub> for 48h significantly increased CyclinD1, Bcl-2, Bcl-xl in transcription ( $p$ <0.05) and translation ( $p$ <0.01) levels compared with Con, and the expression tendency was supported by the results of Western blot (Figure 3) and immunofluorescence (Figure 4), which were suppressed by 35  $\mu$ mol/L Garcinol ( $p$ <0.05).

## Discussion

Acetylation is the best characterized modification of chromatin structure and is well recognized as an epigenetic feature in cancer development (Wang et al., 2007). Lysine residues are the principal targets of acetylation (Lee et al., 1993). In general, acetylation of histones H3 and H4 correlates with regulation of gene transcriptional activation (Dhalluin et al., 1999). Aberrant acetylation modification of some nonhistones such as p53, NF- $\kappa$ B/p65, CBP, p300, STAT3, tubulin, GATA factors, nuclear receptors, c-Myc, HIF-1 $\alpha$  and FoxO1, Rb et al, is also relevant with tumorigenesis (Singh et al., 2010; Khan et al., 2013). Hyperacetylation mediated by HAT is an important way to activate oncogene (Dicerbo et al., 2013). There are four major HAT classes including: Gcn5 (general control of amino acid synthesis protein 5)/PCAF, MYST (MOZ, bf2, Sas3, Sas2, Tip60/Hbo1 (human acetylase binding to ORC1), p300/CBP and Rtt109 (regulator of

Ty1 transposition 109), each of which exhibits substrate specificity of histone (Marmorstein et al., 2009).

Abnormal acetylation modification may play an important role in the estrogen-promoted breast cancer development. Existing report demonstrates that numerous nuclear receptor coactivators usually have been found to possess intrinsic HAT activity (Chen et al., 1999). With the activation of cell signaling pathway, histone acetylation levels of nuclear receptors (such as ER, GR, RAR, VDR) target genes are significantly increased, thus activating gene transcription upon hormone induction. Genomic analyses of transcription factor binding indicate a remarkable positive correlation between the recruitment of Pol II at the promoters of E<sub>2</sub>-stimulated genes and the acetylated histone levels (Kininis et al., 2007). Using a well-established ACI rat model, Kristy R et al identified morphological and epigenetic changes in the mammary gland tissue after exposure to constitutively elevated estrogen levels, and found that progressive hyperproliferative changes were paralleled by epigenetic disturbances, which demonstrates that the hyperacetylation of histone induced by estrogen has a significant role in the early stage of breast cancer development (Kutanzi et al., 2010). Chen et al have reported that p300/CBP and PCAF mediate hyperacetylation of histone H3, H4 and transcriptional activation of ER target genes induced by estrogen in MCF-7 cells (Chen et al., 1999; Jin et al., 2010; Jin et al., 2011). PCAF predominantly acetylates histone H3. Previously, we also demonstrated that E<sub>2</sub> promotes PCAF mRNA and protein expression levels in MCF-7 cells. Our results showed that treatment with E<sub>2</sub> significantly promoted cell proliferation, cell cycle

progression and inhibited cell apoptosis rate in MCF-7 cells. As shown in Figure 3, Western blot assay indicated that lower basal expression of ac-H4 compared with ac-H3 in Con, treatment with E<sub>2</sub> led to up-regulation of ac-H3, ac-H4, NF- $\kappa$ B/ac-p65 in MCF-7 cells compared with Con. Moreover, to confirm the role of acetyltransferase inhibitor Garcinol on cell proliferation and cell apoptosis, MCF-7 cells were treated with 100 nmol/L E<sub>2</sub> followed by treatment with acetyltransferase inhibitor Garcinol. Treatment with Garcinol and E<sub>2</sub> significantly inhibited cell growth, promoted cell apoptosis and resulted in a G<sub>0</sub>/G<sub>1</sub> cell cycle arrest. Correspondingly, the protein expression levels of ac-H3, NF- $\kappa$ B/ac-p65 in MCF-7 cells were decreased, but there was no significant change in ac-H4. It indicates that Garcinol inhibited the proliferation of MCF-7 cells treated with E<sub>2</sub> by suppressing acetylation. The effect of E<sub>2</sub> on promoting proliferation and inhibiting apoptosis is linked to hyperacetylation levels of histones and nonhistone NF- $\kappa$ B/p65 in MCF-7 cells.

Garcinol (C38H50O6), with a molecular weight of 602, is a polyisoprenylated benzophenone originated from *Garcinia indica* fruit rind, an established anticarcinogenic agent, by virtue of containing both phenolic hydroxyl groups and the  $\beta$ -diketone moiety (Saadat et al., 2012). Garcinol was reported to exert acetylation inhibition effect because of the -OH groups at C-13 and C-14 which play a key role in binding to the acetyl-CoA binding site of HAT (Arif et al., 2009). It is identified that Garcinol has well cell permeability, potently inhibits the acetyltransferase activity of both p300/CBP and PCAF (Balasubramanyam et al., 2004), and acts as acetyltransferase inhibitor in experimental studies. With its great affinity to PCAF, Garcinol relatively inhibits PCAF more potently as well as fast compared with p300/CBP in vitro (Balasubramanyam et al., 2004). Global acetylation of histone H4 is mainly regulated by Hbo1 (Iuzka et al., 2009), which may explain the unobvious inhibition effect of Garcinol on ac-H4 in MCF-7 cells treated with E<sub>2</sub> in our study. Depressing oncogene activation, Garcinol may play a role in anti-carcinogenesis by inhibiting HAT, which leads to reducing the level of acetylation. This is important in suppressing the estrogen-promoted cancer development.

NF- $\kappa$ B transcription factors are implicated in the process of cell proliferation and apoptosis. Recent reports indicate that NF- $\kappa$ B has a critical role in breast cancer development and progression because of its activity uncontrolled (Baumgarten et al., 2012; Jana et al., 2012; Zubair et al., 2013). In mediating estrogen action, except directly bonding with DNA sequences in ERE, ER also cooperates with NF- $\kappa$ B to regulate gene transcription activation, participating in the development of tumors. P300/CBP is a coactivator in acetylating NF- $\kappa$ B/p65, by which estrogen may enhance NF- $\kappa$ B transcription activation (Pradhan et al., 2012). Through forming a complex with NF- $\kappa$ B/p65 and bonding the distal  $\kappa$ B elements of the cyclin D1 promoter, ER controls cyclin D1 gene expression. Importantly, activation of NF- $\kappa$ B is required for cyclin D1 protein expression in estrogen-induced proliferation (Rubio et al., 2006). Cyclin D1 belongs to important Cyclin, mainly promoting cycle progression from G<sub>0</sub> into G<sub>1</sub> phase, increasing the cell

percentage in mitotic phase. In addition, the activated NF- $\kappa$ B/p65 also regulates antiapoptotic gene expressions including Bcl-2, Bcl-xl (Sung et al., 2008). NF- $\kappa$ B is indispensable for ER recruitment to ERE of antiapoptotic gene promoter (Pradhan et al., 2012). Enhancement of NF- $\kappa$ B transcriptional activity requires p300/CBP and PCAF (CBP-associated factor) but how these various coactivators are recruited to the promoter regions of NF- $\kappa$ B target genes and the mode of action in space-time cooperation with NF- $\kappa$ B transcription factors are not very clear (Kim et al., 2012). NF- $\kappa$ B-dependent transactivation is regulated by acetylation occurring at multiple levels (Quivy et al., 2004), mainly including: increased NF- $\kappa$ B-dependent histones acetylation regulating the NF- $\kappa$ B-dependent gene accessibility, such as ERE, acetylation of the NF- $\kappa$ B subunits p65 and p50 directly regulating distinct nuclear functions of NF- $\kappa$ B, acetylation of p65 correlating with transcriptional activation and DNA-binding affinity, acetylation of p50 enhancing its binding activity with DNA.

Our results clearly show that the effects of E<sub>2</sub> on promoting proliferation and inhibiting apoptosis in breast cancer MCF-7 cells are paralleled by increased acetylation levels of NF- $\kappa$ B/p65 and enhanced p65 nuclear translocation. There is a positive correlation tendency between acetylated p65 and increased levels of CyclinD1, Bcl-2, Bcl-xl in mRNA and protein expression. The effects above can be inhibited by acetyltransferase inhibitor Garcinol. Our data indicate that treatment with E<sub>2</sub> enhances transcriptional regulation ability of NF- $\kappa$ B through acetylating NF- $\kappa$ B/p65 as epigenetic modification, which may play a crucial role in facilitating CyclinD1, Bcl-2 and Bcl-xl in both gene and protein expression levels, consequently promoting MCF-7 cells proliferation and inhibiting apoptosis. Our results suggest the possible vital mechanism that Garcinol inhibited E<sub>2</sub>-induced MCF-7 cells proliferation may involve in decreasing acetylation level of NF- $\kappa$ B/p65, restraining NF- $\kappa$ B pathway activity, thereby down-regulating the expression of CyclinD1, Bcl-2 and Bcl-xl. In the epigenetic mechanism of breast cancer, the effects of aberrant acetylation modification of histones and non-histone proteins NF- $\kappa$ B are interrelated closely, both of which deserve more attention.

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