Curcumol Inhibit Breast Cancer Growth Via NCL/ERα36 And PI3K/AKT Pathway

Zhou Lu Wei
Guilin Medical University

Wang Juan
Guilin Medical University

Dou Tong
Guilin Medical University

Li Xiao Juan
Guilin Medical University

Liu Yi Sa
Guilin Medical University

Huang Fu Meng Jie
Guilin Medical University

Guan Xiao
Guilin Medical University

Liu Guo Xiang
Guilin Medical University

Han Meng Jie
Guilin Medical University

Chen Xu (chenxu@glmc.edu.cn)
Guilin Medical University

Keywords: Curcumol, Breast cancer, cycle arrest, ERα36, NCL,

Posted Date: December 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1110942/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Curcumol Inhibit Breast Cancer Growth Via NCL/ERα36 and PI3K/AKT Pathway

Zhou Lu Wei a,1, Wang Juan b,c,d,1, Dou Tong a, Li Xiao Juan a, Liu Yi Sa a, Huang Fu Meng Jie a, Guan Xiao a, Liu Guo Xiang a, Han Meng Jie a, Chen Xu a,*

aDepartment of Pharmacy, Guilin Medical University, Guilin 541199, P.R. China

bKey Laboratory of Pharmacognosy, Education Department of Guangxi Zhuang Autonomous Region, 541199, P.R. China

cGuangxi Key Laboratory of Molecular Medicine in Liver Injury and Repair, Guilin Medical University, 541001, P.R. China

dGuangxi Health Commission Key Laboratory of Basic Research in Sphingolipid Metabolism Related Diseases, the Affiliated Hospital of Guilin Medical University, Guilin, 541001, Guangxi, China

eFaculty of Basic Medicine, Guilin Medical University, No. 109, Guilin 541004, P.R. China

1 These authors contributed equally to this work.

*Corresponding author

Xu Chen, Department of Pharmacy, Guilin Medical University, Zhiyuan Road, Lingui, Guilin, Guangxi Zhuang Autonomous Region 541199 P.R. China; Key Laboratory of Pharmacognosy, Guilin Medical University, 541199, P.R. China.

Tel: +86-773-5898018/ Fax: +86-773-5898018 Email: chenxu@glm.edu.cn

Abstract

Purpose: This study is to investigate the effect and mechanism of curcumol on ERα36 positive breast cancer cells, and the relationship between curcumol’s target protein nucleolin (NCL) and ERα36.

Methods: The anti-tumor effect of curcumol were quantified via MTT assay, colony formation and cycle arrest respectively. The expression of ERα36, NCL and the proteins involved in PI3K/AKT signaling were evaluated by western blotting. The interaction between two proteins were detected using co-immunoprecipitation (Co-IP) and immunofluorescence assay. Mouse xenograft model was
established to verify the role of ERα36 in breast cancer cells and curcumol’s effect on ERα36 positive cancer cells.

**Results:** Curcumol inhibited the cell growth, caused cell cycle arrest, decreased cell cycle related-proteins and inactivated PI3K/AKT pathway in ERα36 positive breast cancer cells. There is a positive correlation between NCL and ERα36 in breast cancer cells. In addition, ERα36 bound to NCL, the two proteins were distributed in the nucleus, cytoplasm and on the plasma membrane, where their expression were obviously decreased by curcumol. Moreover, NCL silenced by NCL siRNA blocked the cell cycle progress and inhibited the activation of PI3K/AKT in MDA-MB-231 cells, while overexpressed ERα36 increased the expression of NCL, promoted cell cycle progress and enhanced the activity of PI3K/AKT in MCF-7 cells. NCL knockdown or ERα36 overexpressed all attenuated the effect of curcumol on breast cancer cells.

**Conclusion:** Curcumol reduced the proliferation of breast cancer cells by targeting NCL/ERα36 and inactivated PI3K/AKT pathway.

**Keywords:** Curcumol, Breast cancer, cycle arrest, ERα36, NCL, PI3K/AKT

**Introduction**

Breast cancer is the most common malignancy in women worldwide (https://www.who.int/publications/i/item/who-report-on-cancer-setting-priorities-investing-wisely-and-providing-care-for-all). Nearly 70% breast cancers express estrogen receptors(ERs)[1]. For ER-positive breast cancer, endocrine therapy is a common treatment for blocking estrogen signaling pathway[2]. Tamoxifen (TAM) is a classic ER-positive breast cancer treated drugs[3]. Unfortunately, it usually develops into TAM-resistance. For triple-negative breast cancer (TNBC), which always showed high metastasis, high invasiveness and poor prognosis[4]. There has no effective therapy for the lack of receptors expression. So, finding more effective therapeutic strategies for the treatment of drug-resistance and triple-negative breast cancer is urgent.

2005, Wang and his colleagues identified a ERα66 variant named ERα36[5], It is expressed in many breast cancer cells, especially highly expressed in TAM-resistant cell lines and triple-negative breast cancer[6]. Studies have shown that expression of ERα36 is related to the prognosis, metastasis and drug resistance of breast cancer[7, 8]. It also stimulates the activation of PI3K/AKT and MAPK/ERK pathway[9]. More and more researches showed that ERα36 is a novel biomarker to
overcome drug resistance and enhance the effectivity of triple negative breast cancer[10]. Therefore, novel and effective agents target of ERα36 are urgently needed.

Curcumol, an effective ingredient extracted from the volatile oil of traditional Chinese medicine *Curcuma zedoaria (Christm.)* Rosc. Studies have shown that curcumol can inhibit cell proliferation of various cancers [11-15]. For breast cancer, curcumol can induce cell apoptosis by inhibiting cancer cell metastasis and epithelial-mesenchymal transition, induce cell cycle arrest and act as an adjuvant therapy to increase the sensitivity of cells to drugs[15]. In our previous experiment, we found that NCL is a protein target of curcumol[16]. NCL is associated with poor prognosis in multitudinous cancer, it affects DNA repair, cell survival, angiogenesis, epithelial-mesenchymal transition and stemness[17]. And anti-NCL is a promising strategy for the treatment of breast cancer[18]. Recently, we found that ERα36 and NCL could interact with each other, so we want to explore whether curcumol inhibits breast cancer growth is related with the expression of NCL and ERα36, and whether the PI3K/AKT pathway is involved in the anti-breast cancer effect of curcumol.

**Materials and Methods**

1. Reagents

   Curcumol (purity > 98%) was purchased from Guizhou Dida Technology Co., Ltd (Guizhou, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Amresco, LLC (Solon, OH, USA). DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-rabbit IgG, anti-mouse IgG and antibodies against β-actin were purchased from ZSGB-BIO (Beijing, China). The anti-PI3 Kinase P110 β, anti-PI3 Kinase P85 α, anti-Nucleolin, anti-AKT, anti-phosphor(p)-AKT, anti-PARP-1, anti-P53, anti-Bcl-2 were purchased from Abcam (Cambridge, MA, USA), anti-CDK6, anti-CyclinD1, anti-CDK2 were purchased from Cell Signaling Technology (Danvers, MA, USA), anti-PCNA, anti-P21 were purchased from Wanleibio, LLC (Shenyang, China), anti-ERα36 antibody and plasmid vector were provided by Zhao Yi Wang (Creighton University Medical School).

2. Cell culture and treatment

   The breast cancer cell lines MCF-7, MDA-MB-231 and the human normal breast epithelial cell MCF10A were purchased from Shanghai Cell Bank, Conservation Genetics, the Chinese Academy of Sciences(shanghai, China). The TAM-resistant cell lines (MCF-7 TAM-R) were provided with Dr ZY Wang. All cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY,
USA) with 10% FBS (Gibco, Auckland, New Zealand), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. In addition, MCF-7 TAM-R incubated in medium containing 1μM TAM to maintain resistance. MCF10A was cultured with Mammary Epithelial Cell Growth Medium (Lonza/Clonetics, Basel, Switzerland). The cells were pre-treated with different concentrations of curcumol (0, 12.5, 25, 50, 100 μg/mL). All cells are in the logarithmic growth phase for experiments.

3. Cell viability assay

Cells were seeded in 96-well plates at 3×10^3 per well. And then treated with curcumol at indicated concentrations for 24 h, 48 h, 72 h. Then 20 μL 5 g/L MTT solution was added to each well and cells continued to incubate for 4 h at 37°C. Subsequently, the supernatant was removed and 150 μL DMSO was added to dissolve the formed formazan crystals, then measured the absorbance at 490 nm wavelength using a microplate reader (TECAN, Männedorf, Switzerland) and calculated the cell proliferation inhibition rate.

4. Cell colony forming assay

Eight hundred cells were seeded into 6-well plates and treated with curcumol. The medium was replaced with fresh medium every 3-4 days. After 12 days, the colonies were fixed with anhydrous methanol for 15 min and stained with Giemsa Reagen (solarbio, Beijing, China) at 25°C for 30 min. The images of the stained cells were taken using the Gel System Imaging System (Bio-Rad, Hercules, CA, USA).

5. Cycle arrest analysis by flow cytometry

Cells were collected after treating with curcumol for 48 h, washed twice with ice-cold PBS and immobilized with ice-cold 80% ethanol at -20°C. The next day, the supernatant was discarded after centrifuging at 1500 rpm for 10 min, then the cells were washed twice with ice PBS, incubated with RNase (100 μg/mL) at 37°C for 30 min. Afterwards, the cells were stained with propidium iodide (50 μg/mL) at 25°C for 30 min in the dark. The cells were tested by flow cytometry (Becton Dickinson, San Jose, CA, USA).

6. Western blot analysis

Cells were seeded in 100-mm Petri dishes and treated with curcumol for 48 h. cells were collected and lysed with RIPA buffer for 30 min on ice, the lysates were centrifuged at 4°C, 12000 rpm for 20
min, the protein concentrations were measured with BCA protein assay kit. Then protein was isolated by SDS/PAGE electrophoresis and transferred to the nitrocellulose membranes, these membranes were blocked with 5% skim milk for 2 h and washed three times with PBST for 10 min each time. Then incubated with indicated specific primary antibodies for overnight at 4°C. The next day, the membranes were washed three times with PBST for 10 min each time and incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG for 1 h at 25°C. Finally, the membranes were detected using western blot imaging system (Bio-Rad, Hercules, CA, USA).

7. Co-Immunoprecipitation (Co-IP) analysis

Protein A/G Magnetic Beads were incubated with anti-NCL antibody or IgG for overnight at 4°C. Beads were resuspended in the wash buffer and incubated with the supernatant which were aspirated from cell lysis solution for 3 h in 4°C. Then, the beads were eluted with eluent buffer (0.1% SDS, 1% Triton × 100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol. Subsequently using anti-ERα36 antibody as probe to detect the expression of ERα36 on SDS-polyacrylamide gel electrophoresis.

8. Immunofluorescence assay

Cells were treated with curcumol for 48 h, cells fixed with 4% paraformaldehyde for 15 min, and then permeabilized with 0.1% Triton X-100 at 25°C for 10 min, then cells were blocked with 5% BSA and incubated with primary antibody overnight. Next day cells were blocked with 5% BSA again and incubated with another primary antibody overnight at 4°C. Next day, the cells were incubated with two secondary antibodies for 2 hours at 25°C, then cells were added into DAPI (0.1μg/mL, Abcam) which was used to visualize the cell nuclei. The samples were observed with laser scanning confocal microscope (OLYMPUS OPTICAL CO., LTD, Japan).

9. Si-RNA assay

The NCL siRNA and universal negative control siRNA were synthesized by BIOLIGO Biotechnologies (Shanghai, China). The sequences are shown in the appendix. MDA-MB-231 cells were seeded in 6-well plates. Then NCL siRNA and control siRNA were transfected into cells with Lipofectamine™ 3000 Reagent following the manufacturer’s instructions. Subsequently, the cells were treated with or without 50 μg/mL curcumol for 48 h. Then we detected cell arrest using flow cytometry or relative factors by western blotting.

10. The establishment of ERα36 stable over-expression cells
Lentiviral particles were produced in 293T cells by co-transfecting the lentiviral construct and plasmid pCDH-CopGFP-puro. Plasmids (pCDH-CopGFP-puro-ERα36 and pCDH-CopGFP-puro) were generously provided by Zhaoyi Wang. MCF-7 cells were transfected with plasmids and 5 μg/mL polybrene in six-well plates. After 8 h, DMEM medium containing 10% FBS was replaced. At 48 h, 5 μg/mL Puromycin was used to stable clone selection.

11. Animal experiments

Four-weeks-old female nude mice were purchased from SJA Experimental Animal Co., Ltd. (Changsha, Hunan). All animals were randomly divided into two groups. Every mouse was inoculated 1 × 10^7 cells suspending in 0.2 mL PBS. The cell MCF-7 ERα36 and MCF-7 NC were respectively injected into the flank of the mice. When tumor volume reached 100 mm^3, the mice were randomly assigned to control group or experimental group. The experimental group received intragastric administration of 80 mg/kg curcumol every day for 3 weeks, control group treated with solvent. The growth of tumor was monitored every 3 days. Then, animals were sacrificed, the xenograft tumors were harvested. the volume of tumors was calculated according to the formula: volume = width^2 × length × 1/2.

12. Statistical analysis

The data were analyzed by SPSS version 21.0 (SPSS, Inc.). All results were presented as the mean ± standard deviation (SD). Student’s t-test and one-way ANOVA were conducted to analyze the statistical significance between different groups. P < 0.05 was considered statistically significant.

Results

**Curcumol inhibits breast cancer cells proliferation**

As shown in Figure 1 a, MCF-7 and MCF-7 TAM-R were treated with 5, 10 μM TAM simultaneously, the viability of MCF-7 cells was decreased, while the cell viability was increased in MCF-7 TAM-R cells. Our data showed that MCF-7 TAM-R really resistance to TAM. As shown in Figure 1 c, curcumol inhibited the growth of ERα-positive cells MCF-7, triple-negative breast cancers MDA-MB-231 and tamoxifen-resistant cells MCF-7 TAM-R in a concentration and time-dependent manner. In addition, to detect the cell toxicity of curcumol on normal human breast cells, we analyzed the cell growth of MCF10A after curcumol treated, the results demonstrated that there was a slight growth-promoting effect of curcumol (Figure 1c).

**Curcumol induced breast cancer cells cycle arrest**
The cell cycle distribution of curcumol intervention were examined by flow cytometry. As presented in Fig 2, MCF-7, MDA-MB-231 and MCF-7 TAM-R cells treated by curcumol were caused G1 phase arrest. Western blotting showed that cell cycle regulatory genes CDK6, CDK2, Cyclin D1 were significantly decreased by curcumol in a dose-dependent manner in cells. In MCF-7 TAM-R cells, curcumol regulated additionally the expression of P53 and P21, which two factors were also related to G1 phase arrest.

**Curcumol inhibits the growth of breast cancer cells by regulating the PI3K/AKT pathway**

Given both of the drug resistance development in breast cancer and the anti-tumor effect of curcumol were associated with PI3K/AKT[19, 20]. We detected whether curcumol’s cell cycle arrest effect was related with the inhibition of PI3K/AKT pathway. Our results revealed that curcumol reduced the protein levels of P110, P85 and P-AKT/AKT in MCF-7, MDA-MB-231 and MCF-7 TAM-R cells (Figure 3). Taken together, these results demonstrated that curcumol inhibited the activation of the PI3K/AKT pathway, thereby inhibited the cell cycle progress.

**Curcumol inhibited the expression of ERα36 was related with its protein target NCL**

As a high-malignant biomarker, NCL is highly expressed in breast cancer and is closely related to poor prognosis. Our research group have shown that the anti-cancer target of curcumol in nasopharyngeal carcinoma is NCL. So, the expression of NCL in breast cancer form The Cancer Genome Atlas (TCGA) was acquired and analyzed using xiantao Browser (https://www.xiantao.love), then found that the level of NCL was significantly higher in 1109 breast cancer tissues than 113 normal tissues ($p< 0.001$; Figure 4 d). we found the expression of ERα36 was consistent with the expression of NCL in breast cancer cells (Figure 4 a,b,c). We further investigated the interaction of NCL with ERα36 by immunoprecipitation, and the two proteins could be co-precipitated as shown in fig 4 e. Then, we verified that the effect of curcumol on breast cancer cells were associated with ERα36 and NCL. As shown in Figure 4a-c, curcumol decreased the expression of NCL and ERα36 in three breast cancer cell lines. And dual-immunofluorescence staining showed that NCL and ERα36 co-localized in MCF-7 TAM-R cells and the two proteins expression in cell membrane were decreased under curcumol’s treatment (Figure 4f).

**NCL SiRNA reduce curcumol’s inhibition on cell cycle arrest, ER-α36 expression and PI3K/AKT activation**
Then we silenced NCL in MDA-MB-231 cells. As shown in fig 5a and b, we harvested si-RNA for NCL in MDA-MB-231 cells, we found that the expression of ERα36 was decreased along with NCL’s silenced. As shown in fig 5c, the cell percentage of G1 phase was significantly increased in curcumol and NCL siRNA single treated group, compared with control group. However, there was no obvious change in the NCL siRNA and curcumol co-treated group compared with NCL siRNA single treated group. So, the effect of curcumol on the cell cycle was reduced significantly after NCL silenced. In addition, the effect of curcumol on the expression of ERα36, P110, P85, P-AKT, CDK6, Cyclin D1, and PCNA was significantly weakened when NCL was inhibited (Figure 5d). NCL down-regulation suppressed activation of ERα36 and PI3K/AKT pathway and cell cycle associated factors.

**ERα36 overexpression attenuated the inhibitory effect of curcumol**

In order to reveal the effect of ERα36 on curcumol’s anti-cancer activity, then we constructed MCF-7 cell line with stable overexpressed ERα36. The results showed that when ERα36 was over-expressed, the expression of NCL was also up-regulated. Meanwhile, the expression of them were all decreased under curcumol’s intervention (Figure 6b). Compared with MCF-7, ERα36 overexpressed reduced the proportion of G1 phase and promoted the cell cycle progress (52.6% vs 35.4%). And the cell cycle arrest effect of curcumol was inhibited in the MCF-7 ERα36-LV cells compared with MCF-7 cells (Figure 6c). Mechanistically, the expression of P110, P85, P-AKT, Cyclin D1, PCNA, CDK6 and CDK2 were slightly up-regulated in ERα36 over expressed MCF-7 cells and the effect of curcumol on them was also inhibited when ERα36 was over expressed (Figure 6d).

**Curcumol inhibition on tumor growth was reversed by ERα36 in vivo**

In order to investigate whether the expression of ERα36 affects curcumol’s inhibition on the growth of breast cancer cells in vivo, we orthotopically grafted MCF-7 and MCF-7 ERα36-LV cell lines. As figure 7 a-c showed that ERα36 markedly increased the tumor growth of MCF-7 cells in vivo. Curcumol obviously inhibited the proliferation of breast cancer whether ERα36 overexpressed or not. However, compared with MCF-7 NC, the inhibitory effect of curcumol on tumor growth was slightly attenuated when ERα36 overexpression (Figure 7 c). And the body weight of nude mice showed no obviously difference between MCF-7 NC and MCF-7 ERα36 group (Figure 7 d). In addition, ERα36 expression in MCF-7 ERα36 xenograft tissues were markedly higher than in MCF-7 NC tissues, and curcumol’s treatment decreased the expression of ERα36 more obviously in control than in ERα36 overexpressed tissues (Figure 7 e). Besides ERα36, the expression of NCL, P110, P85, P-AKT, PCNA,
CDKS, Cyclin D1 were down-regulated not so remarkably in ERα36 overexpressed than in control group. These results suggested that curcumin still has anti-cancer effect on malignant tumors with high expression of ERα36, but ERα36 slightly reduce curcumin’s sensitivity in vivo.

Discussion

In recent years, tamoxifen resistance presents a great clinical challenge for the ER-positive breast cancer therapy. Though there has been developed a number of regimens with single or comprehensive agents for the treatment of TNBC, few of them is satisfied and most of the clinical results is also disappointed[21]. So, identifying specific targets and developing more effective therapies for tamoxifen resistance and TNBC patients is urgent.

Accumulating evidence has demonstrated that ERα36 was related with tamoxifen resistance and poor prognosis[22], it promoted cisplatin resistance through nongenomic estrogen signaling[23] and promoted cancer cells metastasis[24]. And down-regulated its expression reduced the migration and invasion of MDA-MB-231 cells[25-27]. Recently, Guangliang Li found that ERα36 overexpressed was shown to be a potential mechanism for tamoxifen resistance generation. Additionally, ERα36 plays a critical role in the development of TNBC[28]. So, ERα36 has been proposed to be a candidate therapeutic target for the treatment of TNBC and tamoxifen resistance breast cancer[19, 28]. Some natural drugs such as icaritin[28], EGCG[29] and Huaier polysaccharide[21] have been demonstrated to be potential therapeutic agents for ERα36 positive breast cancer. Curcumin, a major sesquiterpenoid compound isolated from Rhizoma zedoariae, also demonstrated obvious inhibition effect on TNBC[30].

Our team member Juan Wang found that NCL is a protein target of curcumin in nasopharyngeal carcinoma cells[16]. It is high expressed most cancer cells and closely related with tumor tumorigenesis, migration and invasion[31, 32]. So, NCL is a potential target for anticancer[33, 34]. In this study, we found that curcumin caused proliferation inhibition and cell cycle arrested in MCF-7 TAM-R and MDA-MB-231 cells. Meanwhile, curcumin inhibited the expression of NCL and ERα36, and the two factors interacted with each other. Meanwhile, NCL and ERα36 were co-located in the nucleus of MCF-7 TAM-R cells after curcumin treated. In order to further study the relationship between NCL and ERα36, we silenced the expression of NCL by siRNA and over-expressed ERα36 by lentiviral transfection, and found that blocking NCL expression decreased the expression of ERα36, and ERα36 increased the expression of NCL. Silencing of the target gene of NCL attenuated the anti-cancer effect of the curcumin.
It has been confirmed that the PI3K/AKT pathway plays an important role in estrogen receptor negative and breast cancer resistance therapy[35], its activation is associated with endocrine resistance and worse prognosis in breast cancer patients[36]. Our previous studies have revealed that curcumol induced MDA-MB-231 cells apoptosis in vitro and vivo[30]. Meanwhile, other researchers found that curcumol act as a sensitizer to improve doxorubicin’s effect [37]. ERα36 high expressed is the common characteristic for TNBC and tamoxifen-resistance breast cancer. So, we focus on the effect of curcumol on the expression of ERα36 and its relationship with PI3K/AKT pathway. In the present study, over-expressed of ERα36 increased the expression of NCL and promote cell cycle progress. In addition, high expressed of ERα36 led to the expression of carcinogenic driver CDKS increased and stimulated the activation of PI3K/AKT. Moreover, ERα36 overexpression sightly attenuated the inhibition effect of curcumol on breast cancer cells in vitro and vivo. However, the effect of curcumol on the interaction of NCL and ERα36, and the mechanism of how NCL siRNA and ERα36 overexpressed affected the effect of curcumol on breast cancer need further study.

In conclusion, our current study revealed that curcumol inhibited cell growth and induced cell cycle arrest in ERα36 positive breast cancer. And the effect of curcumol on breast cancer is closely related with the expression of NCL and ERα36, and the activation of PI3K/AKT pathway. Fully understanding of the roles of traditional Chinese herbal medicines in tumor progression may provide a new perspective for the development of novel cancer treatment strategies.

**Acknowledgments and Funding Information:** This work was supported by the National Natural Science Foundation of China (grant# 81760443, 81760663, 82160768 and 82002822), the Project of Guangxi Special Fund Project for Innovation-Driven Development (grant# GuikeAA19254025), Guangxi Science and Technology and Talents Special Project (grant no. 2019AC19009), Guangxi Natural Science Foundation (grant# Guike ZD20302006). Sincerely thanks to professor Zhaoyi Wang generously provided us MCF-7 TAM cells, ERα36 antibody and ERα36 plasmids.

**References**


2. Selli C, Dixon JM, Sims AH: Accurate prediction of response to endocrine therapy in breast


**Statements and Declarations**

**Funding:** This work was supported by the National Natural Science Foundation of China (grant# 81760443, 81760663, 82160768 and 82002822), the Project of Guangxi Special Fund Project for Innovation-Driven Development (grant# GuikeAA19254025), Guangxi Science and Technology and Talents Special Project (grant no. 2019AC19009), Guangxi Natural Science Foundation (grant# Guike ZD20302006). MCF-7 TAM cells, ERα36 antibody and ERα36 plasmids were provided by professor Zhaoyi Wang (Creighton University Medical School).

**Competing Interests:** The authors declare no conflict of interest.

**Author Contributions:** Luwei Zhou performed the cell viability, Western blot analyses and wrote the manuscript. Juan Wang performed the Western blot analyses, review the manuscript and designed the experiments. Xiaojuan Li and Meng Jie Huang Fu performed flow cytometry analysis and Western blot...
analyses. Tong Dou and Yisa Liu preformed Western blot and animal experiments. Xiao Guan analyzed the data, Guoxiang Liu and Meng jie Han preformed Immunofluorescence assay. The experiments were designed by Xu Chen. All authors have read and agreed to the published version of the manuscript.

Ethics approval: Ethical approval for the use of animals was obtained prior to the start of this study from the Institutional Animal Care and Use Committee of Guilin Medical University (Guilin, China).

Consent to participate: Not applicable.

Consent to publish: Not applicable.

Figure legend

Fig 1 Curcumol inhibits breast cancer cells proliferation (a) Cell viability of different concentrations of TAM on breast cancer MCF-7 and MCF-7 TAM-R cells were detected by MTT assay at 24, 48, 72 h. (b) Cell colony forming of curcumol on MCF-7, MDA-MB-231, MCF-7 TAM-R cells were assessed by colony formation assay. (c) Cell viability of different concentrations of curcumol on breast cancer MCF-7, MDA-MB-231, MCF-7 TAM-R cells was detected by MTT assay at 24, 48 and 72 h. And MCF 10A’s cell viability was examined by MTT assay after treated with curcumol for 72 h. $^*P < 0.05$, $^{**}P < 0.01$ vs. Control group, $^{*a}P < 0.01$, $^{*b}P < 0.05$ vs. 24 h group.

Fig 2 Curcumol induced cells cycle arrest Cell cycle distribution was detected by flow cytometry following 48 h treatment with curcumol. The expression levels of cell cycle related-proteins CDK6, Cyclin D1, CDK2 were analyzed by western blotting in MCF-7(a), MDA-MB-231(b) and MCF-7 TAM-R(c). Data are presented as means ± SD (n= 3), $^*P < 0.05$, $^{**}P < 0.01$ vs. Control group.
Fig 3 Curcumol inhibits the growth of breast cancer cells by regulating the PI3K/AKT pathway
The protein expression of P110, P85, P-AKT, AKT were analyzed by western blotting in MCF-7 (a), MDA-MB-231(b), MCF-7 TAM-R(c). Data are presented as means ± SD (n= 3), *P<0.05, **P<0.01 vs. Control group.

Fig 4 Curcumol inhibited the expression of ERα36 is related with its protein target NCL
The protein expression of NCL and ERα36 were analyzed by western blotting in MCF-7(a), MDA-MB-231(b), MCF-7 TAM-R(c). (d) different expression of NCL between normal and breast cancer, the dataset from an internet analysis tool connected the database form The Cancer Genome Atlas (TCGA)(https://www.xiantao.love/products). (e) Immune co-precipitation between NCL and ERα36. (f) Dual Immunofluorescence staining for the co-localization of NCL and ERα36. Data are presented as means ± SD (n= 3), *P < 0.05, **P < 0.01 vs. Control group.

Fig 5 Si-RNA for NCL reduced the regulation of curcumol on PI3K /AKT and reduced cycle arrest
(a) MDA-MB-231 cells were transfected with NCL siRNA and negative control siRNA(siNC) for 48 h. The cells were identified by western blotting. (b) The expression of NCL and ERα36 were detected in NCL siRNA MDA-MB-231 cells with or without curcumol treatments. (c) Flow cytometry histograms indicated the cell cycle distribution patterns of MDA-MB-231 cells and NCL siRNA MDA-MB-231 cells with or without curcumol treatments. (d) The expression of P110, P85, P-AKT, AKT and cell cycle associated factors Cyclin D1, CDK6, PCNA and PARP-1 were detected by western blotting. Data are presented as means ± SD (n= 3), *P < 0.05, **P < 0.01 vs. Control group.

Fig 6 ERα36 overexpression attenuated the inhibitory effect of curcumol
(a) MCF-7 ERα36-LV cell line was identified by western blotting. (b) The expression of NCL and ERα36 were detected in MCF-7 cells and MCF-7 ERα36-LV cells with or without curcumol treatments. (c) Flow cytometry histograms showed the cell cycle distribution patterns of MCF-7 cells and MCF-7 ERα36-LV cells with or without curcumol treatments. (d) The expression of PI3K/AKT signaling pathway key factors and cell cycle associated factors were detected by western blotting. Data are presented as means ± SD (n= 3), *P < 0.05, **P < 0.01 compared with MCF-7, ***P<0.01, #P<0.05 vs. MCF-7 ERα36 group.
Fig 7 Curcumol inhibition on tumor growth was reversed by ERα36 in vivo (a and b) Images of xenograft tumors demonstrated that MCF-7 ERα36-LV markedly increased tumor growth in vivo. Curcumol treatment significantly decreased the size of tumors in MCF-7 NC and MCF-7 ERα36-LV group in vivo. (c) Statistics of tumor sizes in different groups. (d) The body weight of nude mice were recorded very 4 days. (e) The expression proteins were detected by western blotting. The intensity of bands was quantified by Image J. Data are presented as means ± SD (n= 3), *P < 0.05, **P < 0.01 compared with MCF-7 NC, ##P < 0.01, #P < 0.05 compared with MCF-7 ERα36-LV group.

Fig 8 Schematic representation of the action mechanisms of curcumol
Figures

Figure 1

Curcumol inhibits breast cancer cells proliferation (a) Cell viability of different concentrations of TAM on breast cancer MCF-7 and MCF-7 TAM-R cells were detected by MTT assay at 24, 48, 72 h. (b) Cell colony forming of curcumol on MCF-7 MDA-MB-231 MCF-7 TAM-R cells were assessed by colony formation assay. (c) Cell viability of different concentrations of curcumol on breast cancer MCF-7 MDA-MB-231 MCF-7 TAM-R cells was detected by MTT assay at 24, 48 and 72 h. And MCF 10A's cell viability was examined by MTT assay after treated with curcumol for 72 h. *P < 0.05, **P < 0.01 vs. Control group, ##P < 0.01, #P < 0.05 vs.
Curcumol induced cells cycle arrest. Cell cycle distribution was detected by flow cytometry following 48 h treatment with curcumol. The expression levels of cell cycle related-proteins CDK6, Cyclin D1, CDK2 were analyzed by western blotting in MCF-7 (a), MDA-MB-231 (b) and MCF-7 TAM-R (c). Data are presented as means ± SD (n=3), *P < 0.05, **P < 0.01 vs. Control group.
Figure 3

Curcumol inhibits the growth of breast cancer cells by regulating the PI3K/AKT pathway. The protein expression of P110, P85, P-AKT, AKT were analyzed by western blotting in MCF-7 (a), MDA-MB-231(b), MCF-7 TAM-R(c). Data are presented as means ± SD (n= 3), *P<0.05, **P<0.01 vs. Control group.
Curcumol inhibited the expression of ERα36 is related with its protein target NCL. The protein expression of NCL and ERα36 were analyzed by western blotting in MCF-7(a), MDA-MB-231(b), MCF-7 TAM-R(c). (d) different expression of NCL between normal and breast cancer, the dataset from an internet analysis tool connected the database form The Cancer Genome Atlas (TCGA)(https://www.xiantao.love/products). (e) Immune co-precipitation between NCL and ERα36. (f) Dual Immunofluorescence staining for the co-localization of NCL and ERα36. Data are presented as means ± SD (n= 3), *P < 0.05, **P < 0.01 vs. Control group.
Si-RNA for NCL reduced the regulation of curcumol on PI3K/AKT and reduced cycle arrest (a) MDA-MB-231 cells were transfected with NCL siRNA and negative control siRNA (siNC) for 48 h. The cells were identified by western blotting. (b) The expression of NCL and ERα36 were detected in NCL siRNA MDA-MB-231 cells with or without curcumol treatments. (c) Flow cytometry histograms indicated the cell cycle distribution patterns of MDA-MB-231 cells and NCL siRNA MDA-MB-231 cells with or without curcumol treatments. (d) The expression of P110, P85, P-AKT, AKT and cell cycle associated factors Cyclin D1, CDK6, PCNA and PARP-1 were detected by western blotting. Data are presented as means ± SD (n = 3), *P < 0.05, **P < 0.01 vs. Control group.
ERα36 overexpression attenuated the inhibitory effect of curcumol (a) MCF-7 ERα36-LV cell line was identified by western blotting. (b) The expression of NCL and ERα36 were detected in MCF-7 cells and MCF-7 ERα36-LV cells with or without curcumol treatments. (c) Flow cytometry histograms showed the cell cycle distribution patterns of MCF-7 cells and MCF-7 ERα36-LV cells with or without curcumol treatments. (d) The expression of PI3K/AKT signaling pathway key factors and cell cycle associated factors were detected by western blotting. Data are presented as means ± SD (n= 3), *P < 0.05, **P < 0.01 compared with MCF-7, ##P< 0.01, #P< 0.05 vs. MCF-7 ERα36 group.
Figure 7

Curcumol inhibition on tumor growth was reversed by ERα36 in vivo (a and b) Images of xenograft tumors demonstrated that MCF-7 ERα36-LV markedly increased tumor growth in vivo. Curcumol treatment significantly decreased the size of tumors in MCF-7 NC and MCF-7 ERα36-LV group in vivo. (c) Statistics of tumor sizes in different groups. (d) The body weight of nude mice were recorded very 4 days. (e) The expression proteins were detected by western blotting. The intensity of bands was quantified by
Figure 8

Schematic representation of the action mechanisms of curcumol.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- appendix.doc