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Curcumin Suppress Glioblastoma Cell Proliferation by p-AKT/mTOR Pathway and Increased the PTEN Expression

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Abstract

Background：Glioblastoma (GB) is the most common neoplasm in the brain. Curcumin，as a known polyphenolic compound extracted from turmeric，is a chemotherapeutic used in some cancer treatments in china. However, the effects of curcumin on the survivability of GB cells remain to be elucidated.

Methods：We performed a CCK8 assay to detect viability of GB cells following treatments with curcumin and examined the migration and invasion ability of these cells using the wound-healing and transwell invasion assays. The cell proliferation and apoptotic proteins were detected by Western blot analyses. We utilized a glioblastoma-xenograft mouse model to assess cell proliferation following curcumin treatment.

Results：We found that curcumin inhibited the proliferation, migration, and invasion of U251 and U87 GB cells. We detected that curcumin decreased p-AKT and p-mTOR protein expression, and promoted apoptosis of U251 and U87 GB cells. Further, we found that curcumin promoted the PTEN and p53 expression, as the tumor suppressor genes. In addition, we administered curcumin to nude mice and found that curcumin decreased the tumor volume, caused necrosis of tumor tissue, and significantly enhanced the PTEN and p53 expression *in vivo*.

Conclusions：These results indicated that curcumin inhibited proliferation by decreasing the p-AKT/p-mTOR pathway, and promoted apoptosis by increasing the PTEN and p53 expression. Our study provided the molecular mechanisms by which curcumin inhibited glioblastoma and its targeted interventions.

Key Words: Glioblastoma; Curcumin; PTEN; AKT; Mechanism

Background

Glioblastoma is a common primary neoplasm, which includes astrocytomas, oligoastrocytomas, oligodendroglioblastomas and glioblastomas[1]. Glioblastoma (GB) is one of the most aggressive solid tumors and patients with GB have a very poor prognosis. The poorly differentiated polymorphic glioblastoma cells have diverse morphologies, nuclear atypia, and undergo active nuclear division. Their histological features show the different morphology, such as necrosis, vascular proliferation and thrombosis[2] .The current treatment methods include the tumor resection, chemotherapy, radiotherapy, and anti-vascular targeted drugs. Because current therapeutic strategies are not sufficiently effective, it is important to find new treatments or better drugs.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is isolated from the spice turmeric of Curcuma，a natural polyphenolic compound in China [3, 4]. Curcumin has been used as an adjunctive therapy in some inflammatory and neurodegenerative diseases[5, 6]. Previous studies have shown that curcumin inhibited cytotoxicity by decreasing oxidative damage caused by reactive oxygen species[5, 7, 8]. Some studies have shown that curcumin has preventive effects on some neoplasms[5, 6]. These reports demonstrated that curcumin regulates several signaling pathways, including STAT3 and NF-κB transcription factor, in proliferation, angiogenesis and metastasis. However, the spectrum of possible mechanisms by which curcumin might treat GB need to be elucidated to optimize its clinical efficacy.

Our study was designed to discover the potential ability of curcumin to suppress glioblastoma *in vitro* and *in vivo*. Our results suggest that curcumin inhibits the proliferation of glioblastoma, and that this effect is associated with the inhibition of p-AKT/mTOR signaling pathway and PTEN activation.

Materials and **Methods**

Materials

Curcumin was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies. LY294002 was purchased from Cell Signaling Technology (Boston, MA, USA). Anti-AKT, Anti-p-AKT, anti-mTOR, anti-p-mTOR, anti- caspase-3, anti-beclin-1, anti-PTEN, anti-p53 and anti-GAPDH antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All secondary antibodies were obtained from Proteintech Group (USA). Trizol reagent and the enhanced chemiluminescence kit were from Beijing Applygen Technologies (Beijing, China).

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies[9]. All procedures using animals were conducted as described previously[10] and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996) and approved by the institutional animal care and use committee at the Hubei University of Science and Technology, China.

Glioblastoma cell culture

The glioblastoma cell lines, U251and U87, were cultured in Dulbecco's modified Eagle's medium (DMEM) both supplemented with 10% fetal bovine serum (FBS) as described previously[11]. The cells were incubated under humidified conditions at 37˚C and 5% CO_2. The medium was changed every 3 days. The cells were passaged at 95% confluency for the following experiments. Curcumin was dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 10 mM and was stored for 4 □C. Culture media with 0.1% of DMSO was used as a control.

Cell proliferation assay

The cell anti-proliferation assay was examined as described previously[12]. U251and U87 cells were seeded into 96-well tissue culture plates at a density of 1×10^4 cells per well in 100 µL of medium. After overnight incubation, cells were incubated with different concentrations of curcumin (0-, 10-, 20- and 40-µM) for 24-, 48-, and 72- h at 37˚C. 10 µL

CCK8 solution was added to the plate and the optical density (OD) was measured at 450 nm using an ELISA plate reader (EL 340 Microplate Reader; Winooske, USA). Untreated cells in different time were used as a negative control (100% viability). Each experiment was performed at least three times.

Scratch wound healing assays

The scratch wound-healing assays were performed by the cell migration as described prevously[13]. Cells were seeded into 6-well plates and cultured until the confluence monolayers reached 70%. A sterile 10-μl pipette tip was used to generate a scratch through each well. The width of wound closure was measured after 0- and 24-h and photographed using a (Nikon Corporation, Tokyo, Japan) microscope. Untreated cells were used as a positive control (100% wound healing rate) and the serum-free media were used as a negative control for wound-healing assay. The percentage of the wound-healed area for each cell was calculated. Three replicates were performed.

Transwell invasion assays

The invasion assay was performed using a BioCoat Invasion Chambers (BD, Bedford, USA) system as described previously[11]. Cells were plated on the top side of a polycarbonate transwell filter, coated with Matrigel for the transwell invasion assay. After the U251 and U87 cells were treated with various concentration of curcumin for 12 h，a total of 5×10^4 cells were added to the top chamber and the bottom chamber contained medium with 10% FBS as a chemoattractant. The serum-free media in top chamber was used as negative control. After being incubated for 4 h, the cells on the upper membrane surface were removed. The invaded cells were fixed with 4% paraformaldehyde and stained with 0.1% (wt/vol) crystal-violet solution for 5 min. The invasion cells was Cells were counted in four randomly selected fields (100×) using a (Nikon Corporation, Tokyo, Japan) microscope. Three replicates were performed.

Western blots

The Western blot assay was performed as described previously[12, 14]. Following dosing of 0-,10- and 20-μM curcumin treatment for 24 h, the protein was extracted with lysis buffer and obtained after centrifugation. Equal amounts of protein extracts were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were

incubated with primary antibodies at 4˚C overnight. Bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and an enhanced chemiluminescence reagent, according to the manufacturer's instructions. GAPDH was used as an internal control. Proteins were quantified by densitometric analysis.

Cell apoptosis assay

The Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI) apoptosis Kit (BioVision, Milpitas, CA USA) was used to detect apoptosis cells in accordance with manufacturer's instructions. Following dosing of 0-,10-, and 20-μM curcumin for 24 h, the cells were washed three times and suspended at a density of $3x10^6$ cells/ml in 1X Annexin V-binding buffer. Annexin V-FITC and PI buffer were added to the cells which were incubated for 15 min at room temperature. The cells were stained with Hoechst 33342 (10 μg/mL) for 20 minutes and were observed with a fluorescence microscope (Nikon Eclipse 55i, Nikon Corporation, Tokyo, Japan). The apoptotic cells were the double positive of Annexin V-FITC (green) and PI (blue). The percentage analysis of apoptosis cells was calculated as the number of apoptotic cells to the total number of cells counted as described previously[15] .Cells treated with DMSO was used as controls.

Antitumor activity assay in vivo

Antitumor activity was performed as previously described[8]. Female nude mice were housed with pathogen-free conditions. All experimental procedures conformed to the animal experiment guidelines of the Animal Care and Welfare Committee of Hubei University of Science and Technology. U87 xenografts were performed by subcutaneous injections of 2.0×10^6 U87 cells in nude mice[8]. When the tumor volume reached approximate 100 mm^3, the mice were randomly divided into two groups (n = 5) and intraperitoneal injections of curcumin (60 mg/kg) or saline was administered daily for 14 days. The tumor size and animal body weights were measured every 2 days. Tumor volume was calculated as described[8]. The mice were sacrificed and the tumors were removed and weighed at the end of the experiment.

Statistical analysis

All results were presented as mean ±SEM of at least three independent experiments. A comparison of groups was performed using paired students t-test. Multiple comparisons of

groups were analyzed using one-way ANOVA with Bonferroni's post-hoc tests. Statistical significance was set as $P < 0.05$.

Results

1. Curcumin inhibits glioblastoma cell proliferation as depend on dose and time.

We measured the anti-proliferation effect of curcumin on glioblastoma cells using the CCK8 assay method. U251 and U87 cells are treated with 0-, 10-, 20-, 30-, and 40- μM curcumin for 24-, 48-, and 72-h.The morphology of the glioblastoma cells becomes small and shows cell shrinkage due to the increasing concentrations of curcumin. The cell number increases following the curcumin concentration. Treatments with 20- and 30-μM curcumin for 48 h to U251 cell (Fig. 1A), the proliferation are 0.66±0.07 and 0.58±0.04. These results show that curcumin inhibits glioblastoma proliferation in dose-dependent manner. 40-μM curcumin exhibits toxicity to glioblastoma cells. Treatments with 20-μM curcumin for 24h and 48 h to U87 cells (Fig. 1B), the proliferation are 0.89±0.07 and 0.64±0.02. These results show that curcumin inhibits glioblastoma proliferation in time-dependent manner. Our results indicate that curcumin downregulates glioblastoma proliferation in both U251 and U87 cells.

2. Curcumin reduces the migration and invasion of glioblastoma *in vitro*

To detect the effects of curcumin on metastasis, the wound-healing assay is used. The width changes of wound closure in migrating cells are presented in Fig. 2A. Following treatment with 0-, 10-, 20-, and 40-μM of curcumin for 24 h, the migration cell rate shows the decreased tendency. Compared to the control group, the migration cells rate are 51% in U251 and 48% in U87 of the 40μM curcumin group (Fig. 2B). Curcumin reduces the migration of U251 and U87 cells.

Next, we examined the invasion ability of glioblastoma cells using the transwell assay. The invaded cells are stained as shown in Fig. 2C. Following treatment with 0-, 10-, 20-, and 40-μM of curcumin, the number of invasion cells on the surface decreases. Compared to the control group (Fig. 2B), treatments with10-, 20-, and 40-μM of curcumin significantly reduced the invasion capacities of both U251 and U87 cells. These data indicate that curcumin reduces the invasion of glioblastoma cells in a concentration-dependent manner.

3. Curcumin decreases p-PI3K/mTOR protein expression

Since curcumin can inhibit the proliferation of glioblastoma, we determine whether curcumin regulates the expression of p-AKT/mTOR in the cellular survival pathways of glioblastoma. We examined the protein expression of AKT/mTOR. As shown in Fig. 3A, no differences between the non-phosphorylated (total) protein of AKT and mTOR in U251 cells in response to curcumin were observed. As phosphorylation is the activator, we also measured the phosphorylation levels of P13K/mTOR signaling. As shown in Fig.3B, the curcumin treatment group significantly decreased the phosphorylation of AKT(p-AKT) and mTOR(p-mTOR) in U251 cells. As a signal regulator, the PI3K/AKT inhibitor LY294002 markedly decreased the p-AKT and p-mTOR protein expression compared to the control group (Fig. 3A and 3B).

4. Curcumin enhances the apoptosis of glioblastoma cells.

The apoptosis is an important pathology in the tumor growth process. We determined whether curcumin induces apoptosis in glioblastoma. The apoptosis of glioblastoma cells is showed by the co-staining with annexin V and PI. As shown in Fig. 4A, curcumin induces the apoptosis of glioblastoma cells. The analysis shows that treatment with10-, and 20-μM curcumin significantly enhanced the apoptosis in both U251 and U87 cells (Fig.4B).Compared to the control group, the apoptotic cells in 20-μM curcumin group reached 38%±8% and 44%±10% in the U251 and U87cells, respectively.

5. Curcumin increases the apoptosis protein and restores PTEN expression in glioblastoma cells.

We determined whether curcumin regulates the apoptotic protein expression. Western blot analysis shows that curcumin induced the caspase 3 and beclin-1 protein expression. As shown in Fig. 5A, curcumin enhanced significantly the caspase 3 expression in 10- and 20-μM curcumin group (3.10±0.55 fold and 3.86±0.86 fold) compared to the control group. The beclin-1 protein expression exhibits the same tendency under curcumin treatment. The beclin-1 protein expression is 4.30±0.65 fold and 5.60±1.12 fold in 10- and 20-μM curcumin group compared to the control group. Our results suggest that the caspase 3 and beclin-1 proteins expression are enhanced in glioblastoma cells following curcumin treatment.

PTEN is a tumor suppressor gene and PTEN is widely distributed in neoplasm patients, we determined whether curcumin affected the PTEN expression in glioblastoma cells. As shown in Fig. 5B, the PTEN protein expression is 2.59±0.63 fold and 2.35±0.46 fold in the 10- and 20-μM curcumin group, respectively, when compared to the control group. Our results indicate that curcumin enhanced significantly the PTEN expression in U251 cells.

Since curcumin can enhance apoptosis in glioblastoma cells and the apoptosis gene p53 also interacts with the PTEN proteins, we tested whether curcumin contributed to the restoration of the P53 expression in glioblastoma. As shown in the Fig.5B, the P53 protein expression is 2.23±0.54 fold and 3.12±0.78 fold in the 10- and 20-μM curcumin group, compared to the control group. Our results indicate that curcumin treatment increased significantly the P53 protein expression.

6. Curcumin inhibits tumor growth and increased the PTEN expression in xenografts

To detect the inhibitory effect of curcumin on tumorigenesis *in vivo*, nude mice are used to establish a xenograft tumor model. As shown in Fig. 6A, the tumor volume becomes small in curcumin treatment compared the control group. In addition, tumor weight is significantly lower in the curcumin-treated group (0.76±0.12 g), compared the control group (1.33±0.25 g) (Fig. 6B). Hematoxylin and eosin (HE) staining shows the morphological changes of tumor tissue in the xenograft tumor model. We find that the tumor tissue in the curcumin-treated group contains focal necrosis and has a reduction in angiogenesis (Fig. 6C). Further, the PTEN (2.36±0.43 fold) expression and P53 (3.56±0.65 fold） expression are significantly higher in the curcumin-treated group, compared to the control group (Fig. 6D). Therefore, these data indicate that curcumin has a significant inhibitory effect on glioblastoma growth *in vivo*, and the mechanism appears to be the promotion of PTEN and P53 expression.

Discussion

We investigated the biological effects of curcumin in glioblastoma cells and evaluated its potential efficacy *in vitro* and *in vivo*, for inhibiting GB. We found that curcumin has a dose-dependent effect on proliferation and migration of glioblastoma cells and inhibits significantly the proliferation, migration, and invasiveness of glioblastoma cells. The inhibition was associated with the proliferating activity of the p-AKT/mTOR signaling pathway under curcumin treatment. We also found that curcumin induced apoptosis and significantly regulated the expression of PTEN and P53 in glioblastoma cells. We also found that curcumin significantly inhibited tumor volume and caused tumor necrosis, while restoring PTEN expression.

Our study indicates that curcumin inhibits the proliferation of glioblastoma cells in a dose-dependent manner. We also found that curcumin decreased the migration and invasion of glioblastoma cells *in vitro* and *in vivo*. The effect of antitumor of curcumin is consistent with previous reports[16, 17].

It is known that p-AKT/mTOR activation can promote cell proliferation and has been confirmed to contribute to the biological behaviors for many tumors[18, 19]. The growth of tumors is associated with the activation of the p-AKT/mTOR pathway[17, 20]. We found that p-AKT/mTOR expression is down-regulated in glioblastoma cell after curcumin. These data indicate that curcumin inhibits the proliferation, migration and invasion of glioblastoma cells *in vitro* and was associated with the inhibition of p-AKT/mTOR activation. Our results supported exactly these previous findings of the inhibited PI3K/Akt/mTOR pathways more efficiently in glioblastoma cell treatments [21].

Many chemotherapy drugs have the ability to induce cell apoptosis[22]. Apoptosis might be mediated by intrinsic or extrinsic signaling pathways[23] The apoptosis of glioblastoma was enhanced by curcumin treatment in the present study. Furthermore, caspase 3 and beclin-1 are key regulators for intrinsic apoptotic signaling pathways[24, 25] and induce the mitochondrial release of caspase-activated cytochrome C[26]. We found that curcumin-induced apoptosis and was associated with increased caspase 3 and beclin-1. These findings suggest that curcumin can induce intrinsic apoptotic signaling involved in the

activation of apoptosis[27].

The PTEN gene is known as a negative regulator of p-AKT/mTOR signaling for tumorigenesis[28, 29]. Inhibition of PTEN is associated with invasive tumor growth, enhanced metastasis, and poor prognosis for GB patients[30]. Our study showed that curcumin inhibited p-AKT and enhanced PTEN expression. Curcumin promoted the expression of PTEN, *in vitro*. These findings are consistent with the inhibition of tumor growth following curcumin treatment *in vitro* and *in vivo* [8, 31].

The tumor suppressor protein, P53, may inhibit the growth of cancer cells by regulating various molecular mechanisms, including apoptosis and DNA repair[32]. P53 decreased proliferation, migration and invasion in many tumor cells[18]. Our study demonstrates that curcumin enhances P53 expression *in vitro* and *in vivo*.

In summary, there are many cellular mechanisms by which curcumin can exert its beneficial effects against GB. First, curcumin reduces the p-AKT/mTOR pathway and inhibits GB cell proliferation and migration. Secondly, curcumin induces apoptosis. Curcumin increases PTEN expression and reverses P53 expression. Finally, curcumin can significantly inhibit tumor growth, induce tumor apoptosis, and up-regulates PTEN expression *in vivo*.

Conclusion

Our findings show that curcumin treatment partially inhibits the proliferative activity of glioblastoma cells and suppresses the invasive behavior by down-regulating p-AKT/mTOR activity, thereby inducing apoptosis of glioblastoma cells and increasing PTEN and P53 expression. Furthermore, this positive response to curcumin is dose-dependent and was confirmed in U87-xenografts *in vivo.* These results suggest that further explorations in evaluating the potential use of curcumin for the treatment of GB and other malignant tumors are warranted.

Abbreviations:

Cur:Curcumin
GB: Glioblastoma
AKT: protein kinase B
p-AKT: phosphorylation of AKT()
mTOR: mammalian target of rapamycin,
PI3K：phosphatidylinositol 3-kinase
PTEN：phosphatase and tensin homology deleted on chromosome 10
FBS: fetal bovine serum
DMSO: dimethylsulfoxide ()
DMEM: Dulbecco's modified Eagle's medium

Declaration

Ethics approval and consent to participate Procedures on animals were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996) and approved by the institutional animal care and use committee at the Hubei University of Science and Technology, China. No ethical approval for the use of the human glioblastoma cell lines was required.

Consent for publication: Not applicable.

Availability of data and materials All data generated or analyzed during this study are included in this published article.

Competing interests The authors declare that they have no competing interests.

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Authors' contributions LM and WZ designed the study. LM, WZ and LF performed the experiment. XH analyzed the data. YL and HZ contributed to the writing the manuscript. All authors have read and approved the final manuscript.

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Figure Legends

Figure 1 The anti-proliferation effects of curcumin on glioblastoma cells. The anti-proliferative effects of varying concentrations of curcumin on U251 (A) and U87 (B) cell lines were performed by the CCK8 assay. Untreated cells at 24-,48-, and 72-h were used as a negative control (100% viability).The relative ratio of cell viability are given as mean ± S.D. of three independent experiments.

Figure 2 Curcumin inhibits the migration and invasion of U251 and U87 cells. After U251 and U87 cells were cultured as the different concentrations of curcumin, the migration ability was determined by the scratch wound-healing assay (A). The invasion of the glioblastoma was measured by the transwell assay (C). The migration (B) and invasion (D) values are given as mean ± S.E. for three independent experiments for U251 and U87 cells. *P<0.05 and **P<0.01, compared to control group. Scale bars represent 100 μm

Figure 3. Curcumin suppresses AKT/mTOR activation in U251 cells. After U251 cells were treated with 20 μM of curcumin or 25 μM of LY294002, the levels of AKT, p-AKT, mTOR, p-mTOR and GAPDH were determined by the Western blot analysis (A). The quantitative analysis of the ratios of p-AKT to AKT and of p-mTOR to mTOR was conducted (B). Values are given as mean ± S.E. for three independent experiments. *P<0.05 , compared to control group.

Figure 4. Curcumin enhances apoptosis in glioblastoma cells. After U251 cells were treated with 10-,20-, and 40-μM of curcumin for 24h, apoptotic cells were visualized by the Annexin V and PI staining (A).The quantitative analysis of apoptotic cells was performed (B). Values are given as mean ± S.E. for three independent experiments. *P<0.05 and **P<0.01, compared to control group.

Figure 5. Curcumin enhanced the apoptosis protein and the PTEN protein expression. After U251 cells were treated with 0-, 10-, and 20-μM of curcumin for 24h, the apoptotic proteins, caspase-3 and beclin-1, were measured by the Western blot analysis and were

quantified (A). The PTEN and P53 proteins were detected by the Western blotting and were quantified (B). Values are given as mean ± S.E. for three independent experiments. *P<0.05 and **P<0.01, compared to control group.

Figure 6 Curcumin effectively inhibited the tumor growth. The tumor volume was detected on different days (A). The tumor size and wet tumor weight (B) were measured at the time of dissection. The histological features of the tumors were examined by HE (C). The PTEN and P53 protein were detected by the Western blots and were quantified (D). Values are given as mean ± S.E. for three independent experiments. *P<0.05 and **P<0.01, compared to control group.

Figure

Figure1

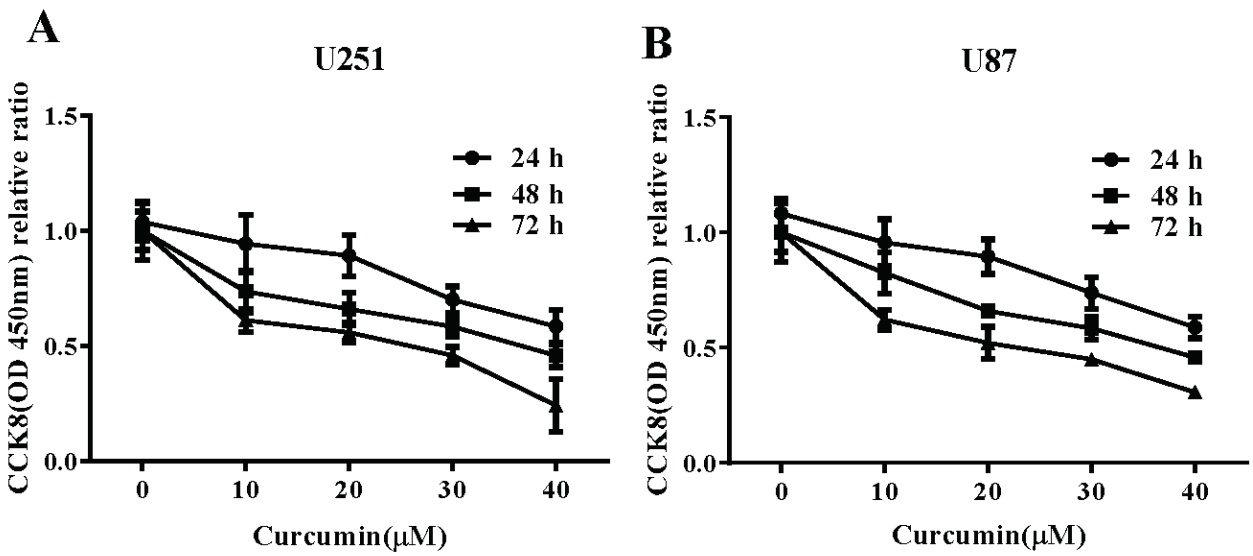
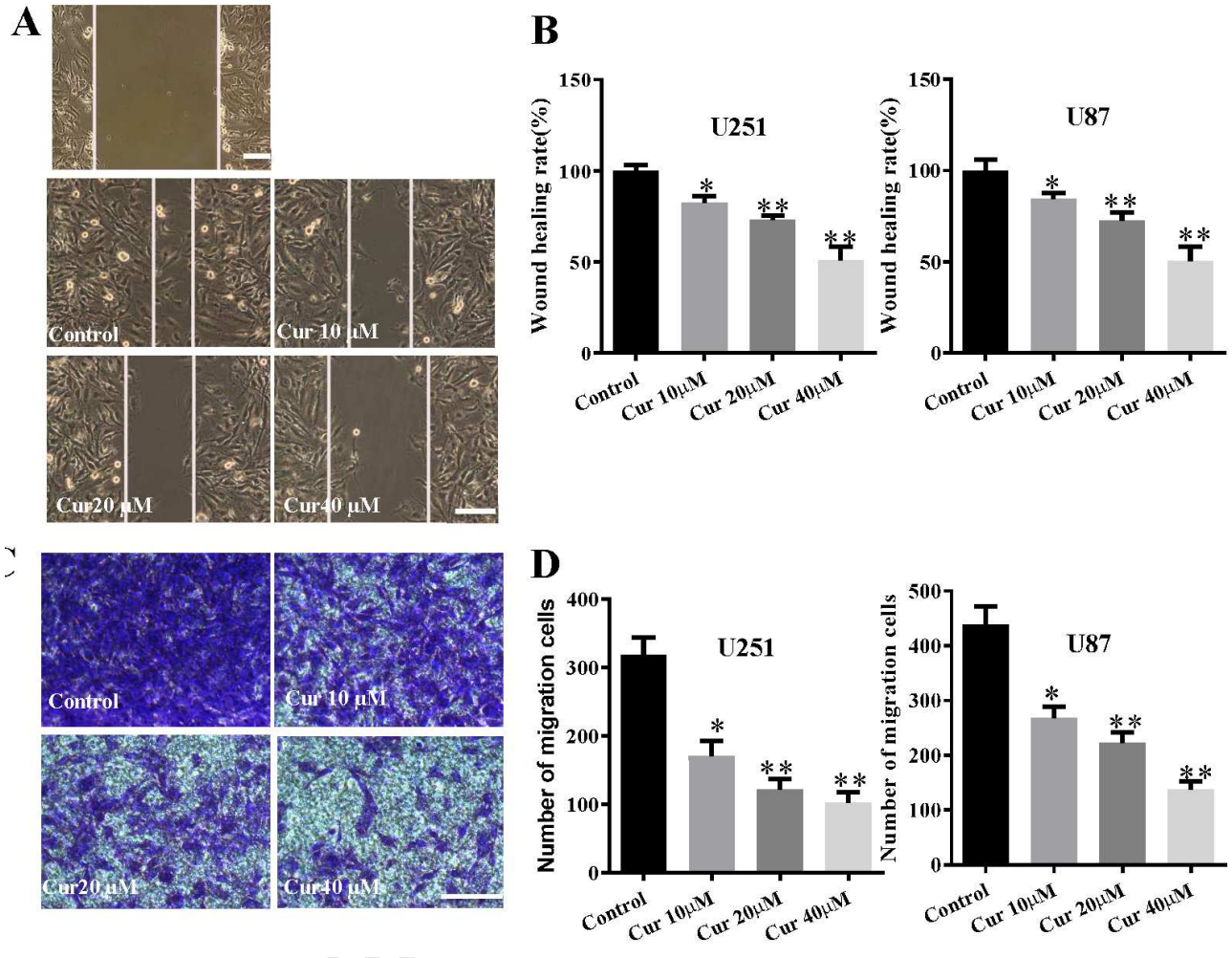
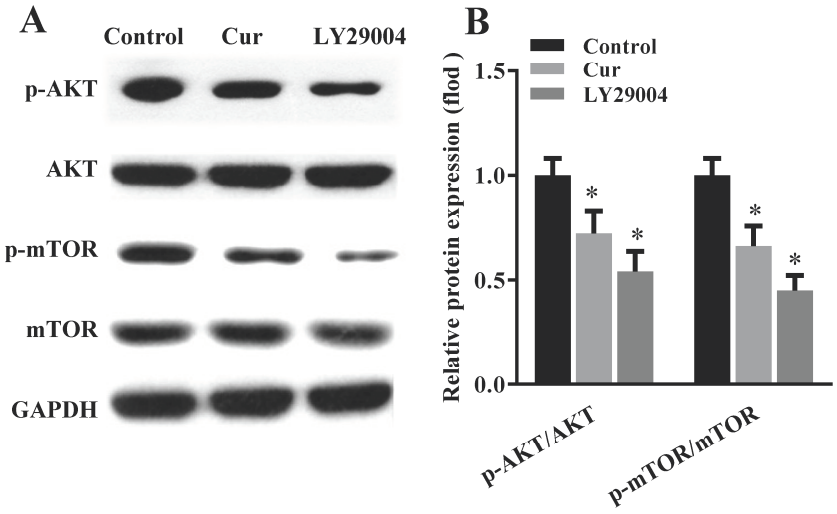


Figure2


A
Control
Cur 10 μM
Cur20 μM
Cur40 μM
B
U251
U87
Wound healing rate(%)
150
100
50
0
Control
Cur 10μM
Cur 20μM
Cur 40μM
C
Control
Cur 10 μM
Cur20 μM
Cur40 μM
D
U251
U87
Number of migration cells
400
300
200
100
0
500

1 **Figure3**

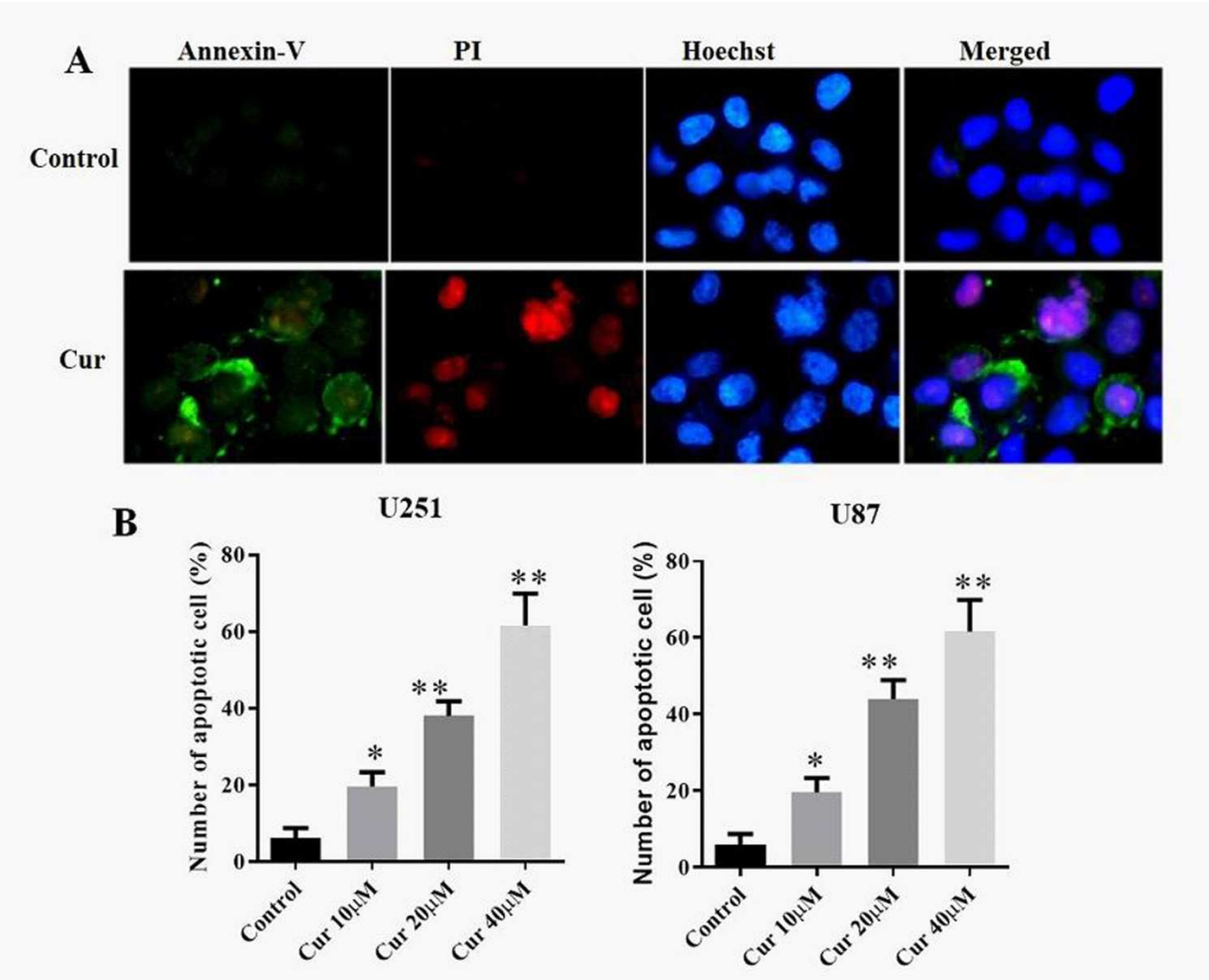


2

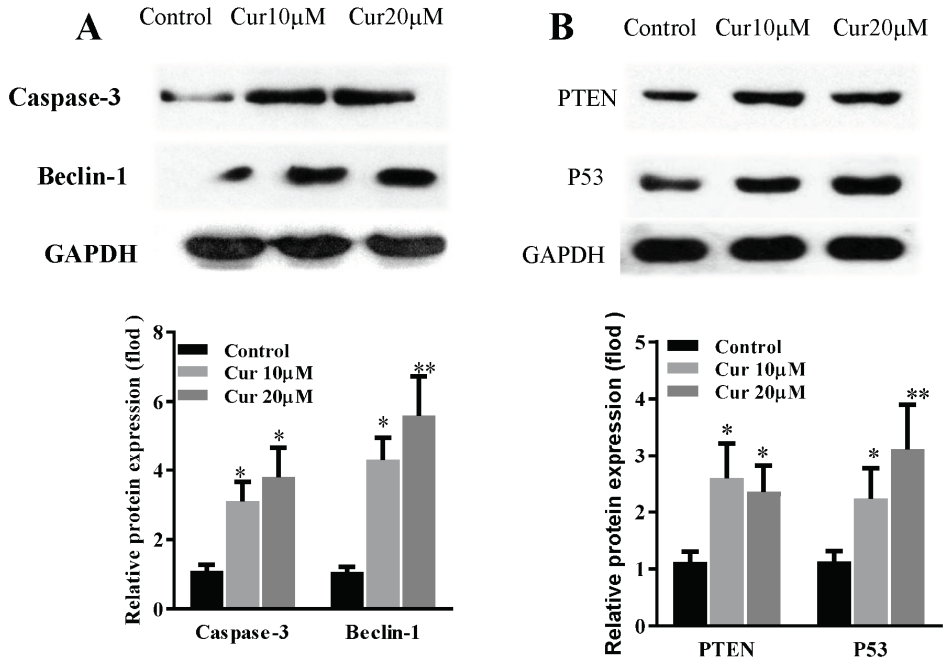
3

4

Figure4


A
Annexin-V
PI
Hoechst
Merged
Control
Cur
B
U251
U87
Number of apoptotic cell (%)
0
20
40
60
80
*
**
Control
Cur 10μM
Cur 20μM
Cur 40μM

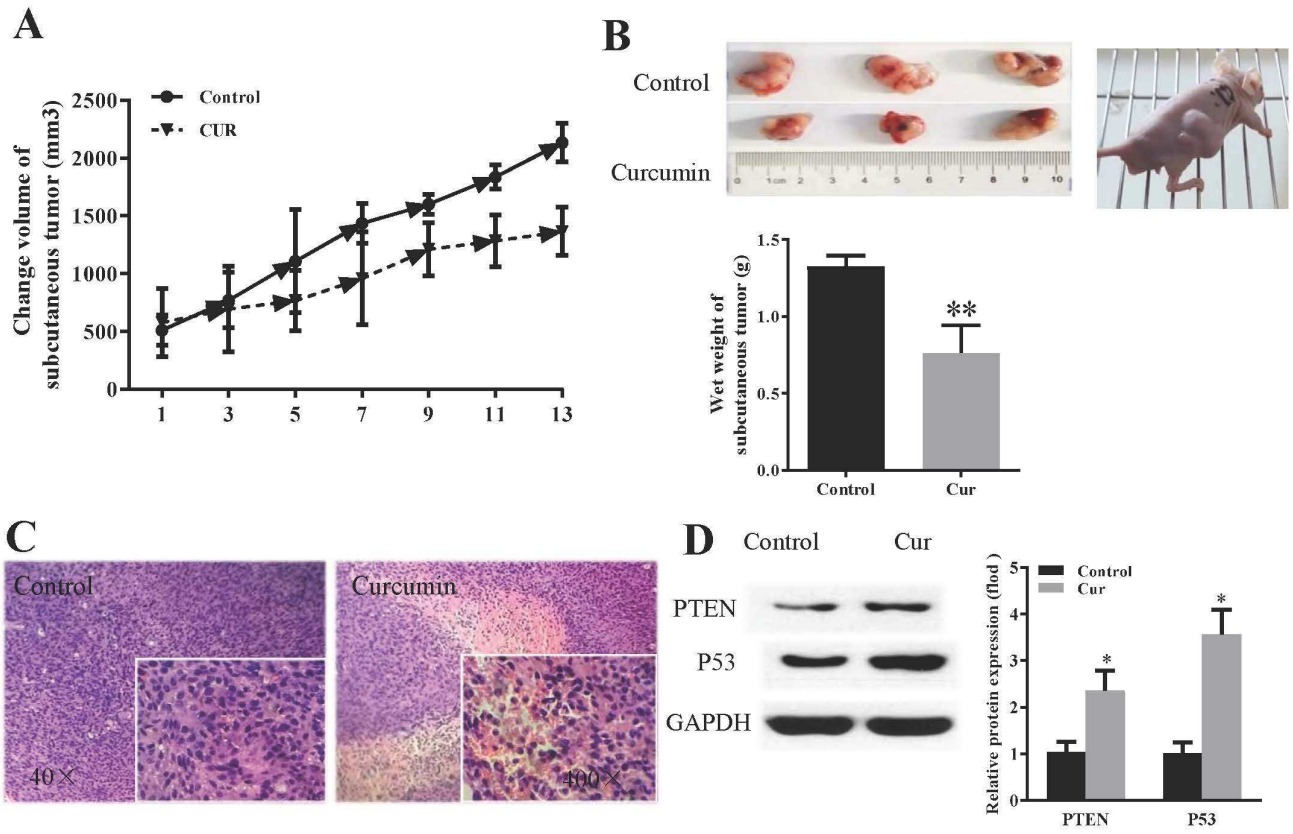
1 **Figure5**



2

3

Figure 6


A
Control
CUR
Change volume of subcutaneous tumor (mm3)
2500
2000
1500
1000
500
0
1
3
5
7
9
11
13
B
Control
Curcumin
Wet weight of subcutaneous tumor (g)
1.5
1.0
0.5
0.0
**
Control
Cur
C
Control
Curcumin
40×
400×
D
Control
Cur
PTEN
P53
GAPDH
Relative protein expression (flod)
Control
Cur
*
*
PTEN
P53